MASTER'S THESIS

Exploring correlations between antibiotic resistance and antibiotic selective pressure in the aquatic environment

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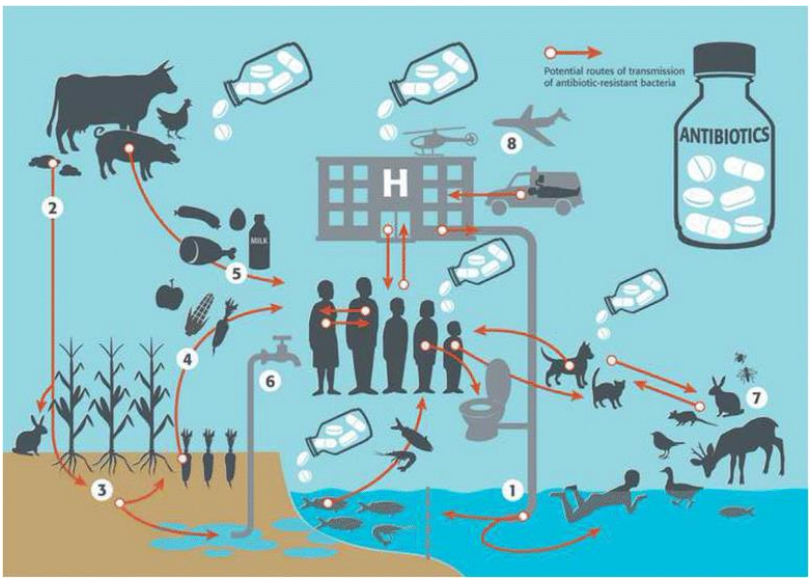


Exploring correlations between antibiotic resistance and antibiotic selective pressure in the aquatic environment

MSc Thesis Environmental Sciences NM990A

Petra Mesken

1 ABSTRACT

Antibiotics resistance (AR) in human and animal pathogens is increasingly leading to failures in treating infectious disease by means of antibiotics currently in use. Large scale antibiotics consumption in human health care and agriculture is seen as the primary driver behind this. Scientific research bears out that the environment has an important role in the emergence and spread of AR. However, a limited understanding of the mechanisms that drive AR in the environment complicates the identification of effective strategies. Many knowledge and data gaps still exist on the mechanisms that drive AR in the environment and how this eventually relates to human health risk.

This thesis aimed to investigate the correlation between antibiotic selective pressure and antibiotic resistance in the aquatic environment, by performing a meta-analysis of data retrieved from experimental research literature. Sample data extracted from 9 studies was used containing measurements of both antibiotic concentrations and antibiotic resistance genes (ARGs) abundance per 16s in surface water and sediments. Antibiotic concentrations were translated into selective pressures using the PNEC (predicted no-effect concentration) for each antibiotic type. Total selective pressure of each antibiotic class (TASP) was matched to the total resistance gene abundance (TARG) of associated gene types, resulting in 738 data points. A linear mixed effect model (LMEM) was constructed with TARG as the response and TASP as the explanatory variable. Antibiotic class (*Class*) was added as an categorical explanatory variable and environmental matrix (*Matrix*), season (*Season*), and antibiotic class nested in study (*Study/Class*) were added as covariates.

Results from data analysis showed no correlation between TARG and TASP. However, both *Class, Season* and *Study/Class* were significant factors influencing the relationship between TASP and TARG in the data set, together explaining 81% of the variance in the data. This indicates that the relationship between TASP and TARG is complex and non-linear, but temporal influences and antibiotic class might significantly affect the variance seen in the relationship between selective pressure and associated resistance gene abundance.

Additionally, Pearson correlations showed a strong and positive correlation between Tetracyclines and *tet*W in sediments. A number of relatively strong and positive correlations were seen in both matrices between antibiotic classes and unrelated gene types. Overall, selective pressure was highest from Quinolones in both matrices, followed by Cephalosporins and Tetracyclines and lowest from Sulphonamides. Highest ARG-abundances were found for mobile genetic element *intI*1 and for the resistance genes *bla*TEM and *tet*Z, conferring resistance to respectively Cephalosporins and Tetracyclines.

2 INTRODUCTION

2.1 ANTIBIOTIC RESISTANCE IS UBIQUITOUS

Infections and infectious diseases caused by (multi) drug resistant bacteria are a fast growing problem, with treatment failures exceedingly leading to increased cases of morbidity and mortality (WHO, 2014). Especially worrisome is the emergence of pan-resistant pathogens, like Mycobacterium tuberculosis, a tuberculosis-causing bacterium which is one of the deadliest bacterial infectious diseases worldwide (ASM, 2009). This trend results not only from inadequate uses of antibiotics, like underdosage, but more generally from an ever increasing antibiotics consumption driving increased AR among bacteria including pathogens (ASM, 2009; Klein *et al*, 2018). This is supported by research, where correlations were found between national antibiotics consumption and antibiotics resistance rates in humans as well as in livestock animals (Bell *et al*, 2014; EFSA, 2017; Forslund *et al*, 2014; McDonnell *et al*, 2017).

It was initially thought that AR emerges in the presence of direct selective pressures such as high antibiotics concentrations in the clinical setting. The role of hospitals and health care settings are well recognized in the development of resistant bacteria, like methicillin resistant *Staphylococcus aureus* (MSRA). But the development of AR in these settings seems to be a very specific problem, in that it concerns pathogens from a small number of bacterial phyla, like *Enterococcus, Staphylococcus, Klebsiella, Acinetobacter, Pseudomonas* and *Escherichia* (Nesme *et al*, 2015) and a relatively small number of resistance mechanisms (Pal *et al*, 2016).

From an ecological standpoint however, antibiotic resistance is ubiquitous and ancient in the environment. The chromosomally encoded multidrug efflux pumps and *ampC* beta-lactamase genes in all the strains of *Pseudomonas aeruginosa* were found to be present long before the discovery of antibiotic drugs (Martinez *et al*, 2009). Bhullar *et al* (2012) found bacterial strains resistant to 14 commercial antibiotic drugs, in a cave in New Mexico that had been isolated for millions of years. Insertion of resistance genes retrieved from a 30000 year old permafrost, into an *E.coli* host, resulted in the expression of vancomycin resistance (D'Costa *et al*, 2011 in: Nesme *et al*, 2015). They furthermore identified several resistance mechanisms that were not previously known (Bhullar *et al*, 2012). Intrinsic resistance (as opposed to acquired) is present in bacterial species originating from long before the antibiotic era (Martinez *et al*, 2009). It is now acknowledged that there is a huge reservoir and diversity of resistance genes already present in the environment. This is one reason why the environment is getting increasing attention in the fight against AR (Gaze *et al*, 2013; Nesme *et al*, 2015; Berendonk *et al*, 2015; Manaia, 2017; Bengtsson-Palme *et al*, 2018b; Larsson *et al*, 2018).

2.2 Enhancement of AR in the aquatic environment and human health risk

It is increasingly found that enhanced levels of antibiotic resistance genes (ARGs) in the environment can be related back to anthropogenic pressures (Li *et al,* 2015; Fondi *et al*, 2016; Zhu et al, 2017; Jiang *et al*, 2018a; Liu *et al*, 2018; Na *et al*, 2018; Chen *et al*, 2019a-j; Griffin *et al*, 2019; Hendriksen *et al*, 2019; Reddy *et al*, 2019, Zhao *et al*, 2018a). Antibiotic drugs, resistant bacteria and resistant genes are constantly emitted to the environment in sewage and waste water from hospitals, households, industry, agriculture and aquaculture. The aquatic environment, as a receiving body of anthropogenic waste from several sources, is indicated as an important compartment for the evolution and transportation of human-driven AR. Many studies have found significant correlations between anthropogenic activities and antibiotic resistance in surface water and sediments (Bengtsson-Palme *et al*, 2014; Bueno *et al*, 2017; Bhattacharyya *et al*, 2019; Chen *et al*, 2019e; Yi *et al*, 2019; Zeng *et al*, 2019; Sanchez-Baena *et al*, 2021), finding that ARG-abundance increased substantially from upstream to downstream (Chen *et al*, 2013) or decreased from river to open sea (Leng *et al*, 2020) indicating riverine anthropogenic pollution.

Antibiotics, antibiotic resistant bacteria (ARB) and ARGs are typically emitted into the environment together, after first being accumulated in 'hotspots' for the development of AR (Proia *et al*, 2016; Bueno *et al*, 2017; Kumar & Pal, 2018; Li *et al*, 2018; Manaia *et al*, 2018; Sanderson *et al*, 2018; Chen *et al*, 2019a-j). Characteristics of these hotspots are a combination of high microbial densities, nutrient-rich conditions and elevated concentrations and chemical diversity of compounds (Gaze *et al*, 2013). This can be in the gut of humans and animals, but also waste water treatment plants (WWTPs) provide such environments and are therefore central in many studies on environmental AR (Zhang *et al*, 2016; Guo *et al*, 2017; Jiao *et al*, 2017; Kumar & Pal, 2018; Manaia *et al*, 2018; Narciso-da-Rocha *et al*, 2018; Sabri *et al*, 2018).

WWTPs have been identified as a dominant point source in the enhancement of AR in the aquatic environment (Amos *et al*, 2015; Proia *et al*, 2016; Brown *et al*, 2019; Felis *et al*, 2020), owing to riverine emissions of both antibiotics and ARGs on surface waters in wastewater effluent causing the enrichment of the environment with both ARGs and human pathogens (Chen *et al*, 2019e; Karkman *et al*, 2019). Amos *et al* (2015) constructed a predictive model for antibiotic resistance along the river Thames, integrating environmental metadata like spatial, temporal, climatic and water-quality factors and using class-1 integron-integrase (*intI1*) prevalence as a proxy for AR. The authors found proximity, number, size and type of wastewater treatment plants as the main explanatory variables for variations in time and space of third-generation Cephalosporin resistance levels in samples taken from sediment along the river Thames. Despite treatment, much of the antibiotics and associated

resistance genes and bacteria from wastewater treatment plants are still emitted to the environment (Manaia *et al*, 2018). Removal efficiency is dependent on the technique used, but WWTP in general are currently not able to eliminate antibiotic residue, ARGs and resistant bacteria and the effects of different techniques on the fate of antibiotics, ARGs and bacteria are in many ways still a black box (Loos *et al*, 2013; Manaia *et al*, 2018; Brown *et al*, 2019; Castrignanò *et al*, 2020). Occasionally, AR is even enhanced by water treatment (Vaz-Moreira *et al*, 2014).

Hospital effluent is another important source of AR. Through metagenomic research, Oh *et al* (2018) found an even higher risk in hospital sewage compared to wastewater treatment plants, based on the cumulative risk posed by the combined presence of human pathogens, resistance genes and MGEs. However, there is generally no special treatment of hospital effluent apart from the regular treatment of community wastewater. Through hospital effluent, resistance genes that are quite specific to the clinical setting and to (multi) resistant human pathogens find their way into the environment, like *vanA* (vancomycin-resistant enterococci, VRE), *mecA* (methicillin-resistant *Staphylococcus aureus*, MRSA), and *aac(6')-Ib-cr* or *blaCTX-M15* (plasmid-encoded resistance to quinolones and beta-lactams in Gram-negative bacteria) (Manaia *et al*, 2016). Additional notable hotspots are manure lagoons, run off from soils irrigated with (un)treated wastewater or fertilized with sewage sludge (Lüneberg *et al*, 2018), industrial antibiotic and pharmaceutical pollution (Pal *et al*, 2016), leaked untreated sewage, emissions from aquaculture (Liu *et al*, 2017b; Zhao *et al*, 2018), livestock facilities and landfill leaching (Le Page *et al*, 2017; Chen *et al*, 2017; Bengtsson-Palme *et al*, 2018b).

Human related bacteria, environmental and animal related bacteria do not typically interact, because of differences in habitat. But transient interactions with potential health risk are possible, for instance in the human gut after ingesting contaminated water, interacting with wildlife or eating raw foods (Bengtsson-Palme *et al*, 2015). Other places where interactions are likely to take place are wastewater treatment plants (WWTP), agricultural settings (in livestock or fertilized soil), water bodies or the food chain (Bengtsson-Palme *et al*, 2017a). These interactions can go in all directions (i.e. human to animal and vice versa). Opportunistic human pathogens, other than commensal human pathogens, can both live in human hosts and in the general environment. This makes their role in the transfer of resistance genes from the environment to human pathogens and the dispersal of resistance genes across environmental compartments especially interesting (Bengtsson-Palme *et al*, 2018).

This is especially so for the aquatic environment, which is central to many human activities, providing water for consumption, irrigation or industrial processes and is at the same time a prime habitat for a great diversity of bacteria (Marti *et al*, 2014; Vaz-Moreira *et al*, 2014; Manaia *et al*, 2016; Gao *et al*,

2018). Throughout the water cycle there are many opportunities for the interaction between humans and resistant pathogens and the spread of antibiotics resistance. Resistant bacteria and genes travelling via surface waters can be taken up by livestock who drink the water, bringing it back into the food chain. They can also be brought back into the food chain via human consumption of drinking water or crops that have been irrigated with surface waters (Bengtsson-Palme *et al*, 2017a) or taken up by humans during swimming. Leonard *et al* (2015; 2018) found an increased risk of colonization by resistant *E.coli* in surfers from ingestion of coastal bathing waters.

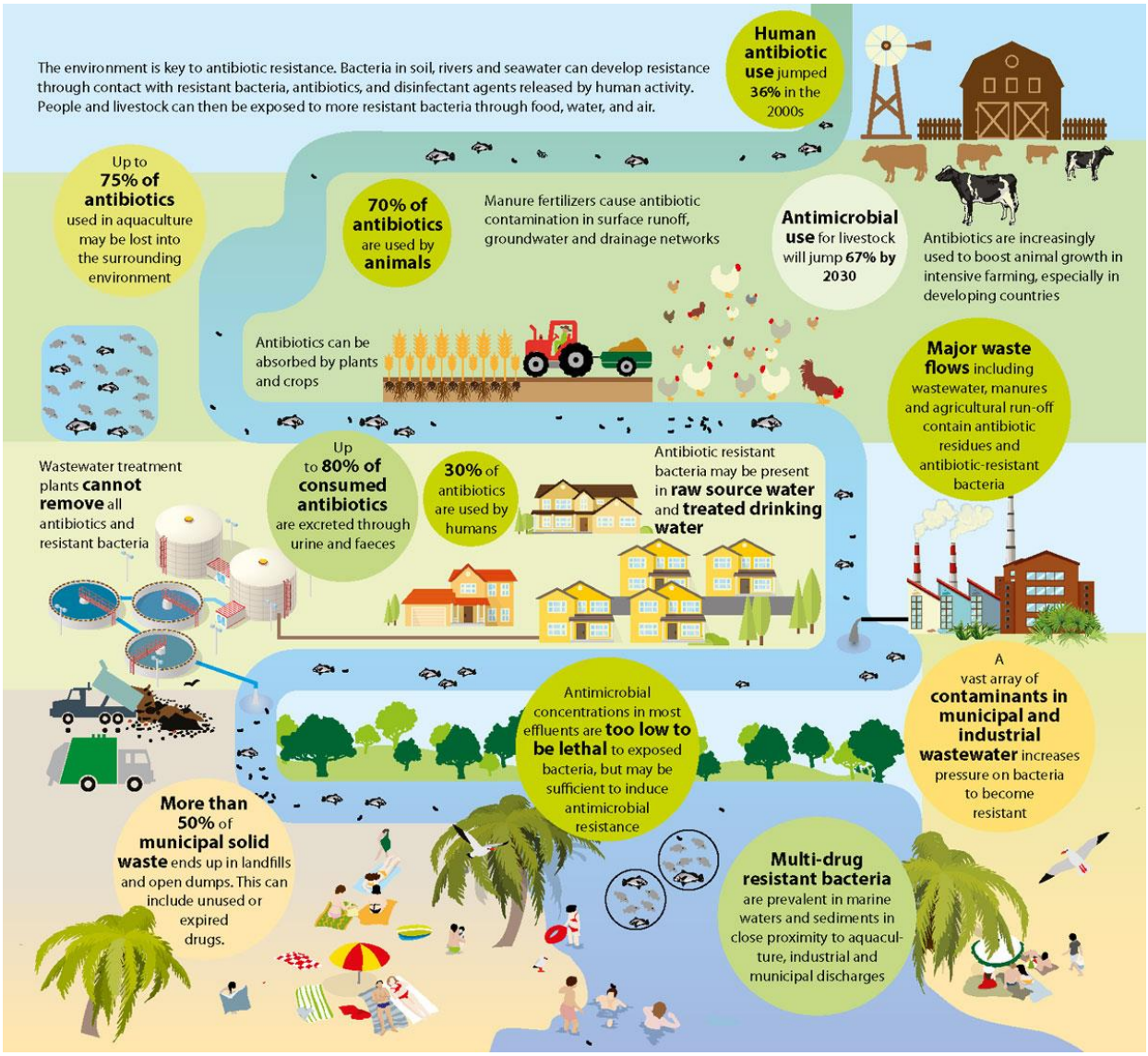


Figure 1: Infographic from UNEP on antibiotic resistance in the environment

source: https://www.unep.org/news-and-stories/story/healthy-environment-key-antibiotics-work

2.3 The effect of antibiotic pollution on AR in the aquatic environment

The effect of high antibiotic concentrations on the development of AR has been well established in the clinical environment, but contrary to what was once believed, research finds that AR can develop even at very low concentrations (Gullberg *et al*, 2011; Ashbolt *et al*, 2013; Gullberg, 2014; Bengtsson-Palme *et al*, 2018b). Gullberg et al (2011) -in an experimental set up- found that concentrations several hundred fold below the MIC (minimal inhibitory concentrations) could enhance resistance in bacteria. This indicates that antibiotics pollution can play a role as an accelerator of environmental AR at subinhibitory concentrations, potentially leading to increased incidences of antibiotic resistant pathogens (Gullberg, 2014; Bengtsson-Palme & Larsson, 2016a & b; Larsson *et al*, 2018).

Antibiotic residue is omnipresent in all environmental compartments (Jiang *et al*, 2014; Chen *et al*, 2018; Lu *et al*, 2018a and 2018b; Zhao *et al*, 2018; Li *et al*, 2020) with trace concentrations being found back in food and water (Wang *et al*, 2016; Li *et al*, 2017) and several researchers have found significant positive correlations between environmental antibiotic concentrations and ARG-abundance (Chen *et al*, 2013; Gao *et al*, 2018; Xu *et al*, 2018; Yan *et al*, 2018; Liang *et al*, 2020). This relationship seems to be stronger when high concentrations are present for instance from pharmaceutical industrial or hospital wastewaters (Gao *et al*, 2018; Karkman *et al*, 2019; Girijan *et al*, 2020) or antibiotic intensive aquaculture (Yuan *et al*, 2019) , but weaker at lower concentrations (Gao *et al*, 2018). In terms of risk assessment, however, it is not known whether a safe limit exists and there is no PNEC (predicted no effect concentration) established for the enhancement of AR within the current risk assessment framework (Bengtsson-Palme & Larsson, 2016b; Le Page, 2017).

Different environmental factors might be at play, influencing the effect of environmental antibiotics concentrations on AR. Bioavailability for instance can in some cases be enhanced owing to environmental factors that affect speciation (Zhang *et al*, 2014). Zhang *et al* (2014) found enhanced bioavailability of Tetracyclines to *E.coli* in an aqueous environment with increased concentrations of organic acids. But also pH and the presence of Cu(II) affect the speciation and thus bioavailability of Tetracyclines (Zhang *et al*, 2014). Overall, the interaction and effects of chronic sub-MIC levels of antibiotics pollution on wild bacterial communities are still poorly understood, as is the fate of antibiotics (Brandt *et al*, 2015; Carvalho & Santos, 2016; Hiltunen *et al*, 2017).

On the level of bacterial communities, selective pressures possibly trigger complex dynamics, since most bacteria live in microbiologically diverse communities (Land *et al*, 2015; EMPC, 2017; Cunha *et al*, 2018; Klumper *et al*, 2019). Sensitivity to antibiotic pressure differs between species and between strains (Le Page *et al*, 2017) and acquired resistance to one antibiotic can render strains sensitive to other classes of antibiotics (Lázár *et al*, 2013). Research on manipulated strains of *E.coli* also shows

that within bacterial communities, sensitive bacteria might to some extent be 'protected' by resistant bacteria through group-beneficial resistance mechanisms. It was shown that sensitive types could profit from the collective antibiotics degradation done by the resistant population, without expending the fitness cost associated with carrying the resistance gene (Dugatkin *et al*, 2005; Kelsic *et al*, 2015). In this way, sensitive bacteria gain an advantage from *group-benefits*. These community benefits can be very strong. Experimental research by Murray et al (2018) showed that -within a complex bacterial community- the strength of selection remained constant from low selective pressure to a 100-fold increase reaching clinically relevant concentrations. This suggests that not only mechanisms for individual survival are important, but on the system level, mechanisms that benefit resilience in the bacterial community are also at play.

Resistance can be even more increased in bacteria that live in biofilms rather than free-living. This extracellular matrix acts as an extra barrier to bacteria against direct exposure, by reducing the penetration of antibiotics (Hall *et al*, 2018). Susceptibility to stressors of bacteria living in biofilm can be reduced by a factor 10 to 1000 compared to free living bacteria (Balcázar et al, 2019). Simultaneously, biofilm formation can be induced in bacteria by stressors like antibiotics. This resistance mechanism was shown in *E. coli* and *P. aeruginosa* to occur at sub-inhibitory concentrations (Balcázar *et al*, 2015). In terms of human and environmental health, this can be positive when important environmental bacteria are protected from antibiotics pollution in the environment or negative when it allows resistant pathogens to persist (Bengtsson-Palme *et al*, 2018b).

Also, antibiotics are not the only stressors that might lead to the development and spread of AR in the aquatic environment. There are indications that heavy metals (Pal *et al*, 2017; Xu *et al*, 2017; Ohore *et al*, 2019; Ebiotubo *et al*, 2020; Komaijani *et al*, 2021), biocides (Jutkina *et al*, 2018; Pal *et al*, 2015), PAHs (polyaromatic hydrocarbons) (Bhattacharyya *et al*, 2019), nutrients (Subirats, 2018), organic pollutants (Chen *et al*, 2019) and faecal pollution (Karkman *et al*, 2019) can significantly affect the abundance of ARGs found in waterbodies. In some cases it was found that other pollutants had a stronger effect on AR than antibiotic concentrations. A study by Komijani *et al* (2021) on ARG-abundance in Iranian wetlands, for instance found that heavy metals showed a stronger relationship with ARG-abundance than the antibiotics present. Bhattacharyya *et al* (2019), found that *bla*TEM in sediments strongly correlated with heavy metal and PAHs pollution in mangrove ecosystems. Reddy *et al* (2019) found significant associations between ARGs and MRGs (metal-resistance genes) in sediment and water samples from the river Ganges.

Adding to complexity, associations between antibiotics and other pollutants seem present in their effect on ARG-profiles, owing to co-selection, co-resistance and cross-resistance. Resistance to

antibiotics and other pollutants can be linked via cross-resistance when a resistance gene confers resistance to both antibiotics and other pollutants like metals (i.e. efflux-pumps) or co-resistance in case of co-location of resistance genes on the same genetic element that allows for co-selection into new bacterial cells (Li *et al*, 2017; Pal *et al*, 2017; Xu *et al*, 2017; Roberto *et al*, 2019).

Confounding factors are spatial factors like land cover and urbanization level (Amos *et al*, 2015; Sanderson *et al*, 2018; Roberto *et al*, 2019) and seasonal or temporal changes that showed significant correlations with ARG-abundance and distribution owing to hydrological conditions (flow rate) (Xu *et al*, 2018), increased precipitation (Amos *et al*, 2015; Sanderson *et al*, 2018; Di Cesare *et al*, 2020) or (temporal) variations in water quality parameters like pH, nutrients, temperatures, suspended solids (Sanderson *et al*, 2018; Roberto *et al*, 2019) or organic matter and organic carbon (Wan *et al*, 2019; Wang *et al*, 2020b). Different (seasonal) patterns were found for genes conferring resistance to Tetracyclines and Sulphonamides (Xu *et al*, 2018; Roberto *et al*, 2019). Also the interaction of spatiotemporal factors was seen to significantly impact ARGs (Xu *et al*, 2018; Roberto *et al*, 2019).

2.4 Persistence and spread of AR in the aquatic environment

A bacterial cell can acquire resistance as a consequence of selective pressures, but will the absence of a selective pressure lead to deselection? In case resistance comes at a fitness cost and reduces fitness (Bengtsson-Palme *et al*, 2018), it could be assumed that enhanced resistance would probably be undone by taking away the selective pressure. However, it is shown that fitness costs can reduce over time, possibly because coevolution of host and plasmid will lead to a better integration (Carroll & Wong, 2018; Bengtsson-Palme *et al*, 2018a). Also, some resistance genes do not seem to come at a fitness cost or even result in increased fitness. A pCT plasmid encoding an extended-spectrum-β-lactamase (ESBL) gene in certain strains of *E. coli* showed no decrease in fitness (Carroll & Wong, 2018). A sulphonamide resistance-encoding plasmid showed increased fitness in an *E.coli* host in the absence of selection (Carroll & Wong, 2018).

Fitness costs seems to differ between strains and species depending on genotype but also on the interactions with other plasmids present (Carroll & Wong, 2018). Sandegren et al (2019), warn that selection at sub-inhibitory concentrations taking place in the environment, selects for bacteria that are not only less susceptible or resistant to antibiotics, but who are overall also more fit considering the higher competition from other bacteria. This overall competitive advantage is less relevant in high selective pressure environments, where resistance is the single most important competitive advantage (Sandegren *et al*, 2019). This makes for a complex picture. Overall, the relative importance of the fitness cost of resistance genes to their persistence, in the absence of selective pressures, is not entirely understood. Even after antibiotics have degraded, been diluted or adsorbed, ARGs and

MGEs coding for resistance might be quite persistent in bacteria or as free DNA even in the absence of antibiotics pressures, despite assumed fitness costs and subsequent deselection (Martinez *et al*, 2009; Berendonk *et al*, 2015).

Once an ARG is acquired, bacteria possess several mechanisms for its exchange within and between bacterial species. AR through mutation can happen during antibiotics therapy and under these circumstance it seems to be the most important mechanism in the emergence of resistance genes in human pathogens, for instance in the human gut (Martinez *et al*, 2009). Next to intraspecies mutations, several mechanisms exist for interspecies or horizontal gene transfer (HGT). This could take the form of general transduction via virus particles (i.e. bacteriophages) and conjugation or natural transformation (fig.3) involving transfer via integrons, super-integrons, transposons or plasmids. HGT is indicated as a central mechanism in the dissemination of resistance genes in the environment at low selective pressures and across species, for instance from environmental bacteria to pathogenic ones (Martinez *et al*, 2009). Human created selective pressures like antibiotic drug pollution, but also pharmaceuticals in general, metals, disinfectants or biocides, can be the driver behind enhanced rates of HGT and thus further amplification of antibiotic resistance in bacterial communities (Gaze *et al*, 2013; Nesme *et al*, 2015; Bengtsson-Palme *et al*, 2018b; Sturød *et al*, 2018). Significant correlations have for instance been found between the class-1 integron-integrase gene *intl1,* ARG-abundance and anthropogenic pressures (Gillings *et al*, 2015; Yan *et al*, 2018; Deng *et al*, 2020; Leng *et al*, 2020; Liang *et al*, 2020)

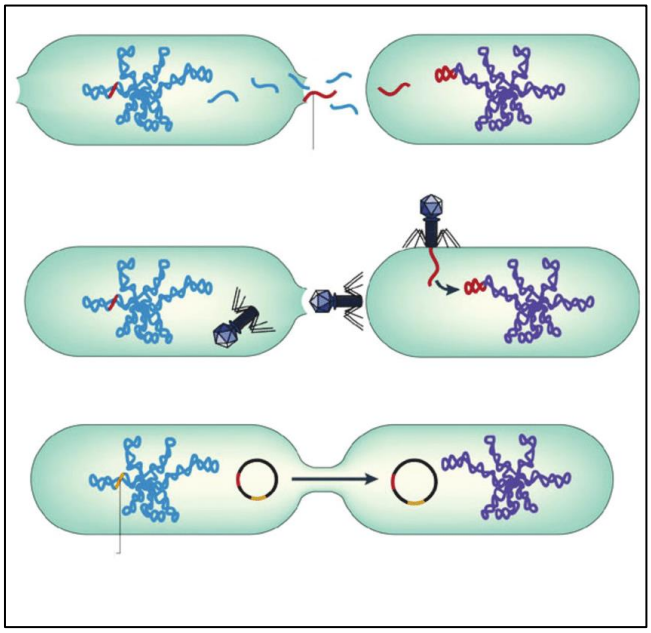


Figure 2: Horizontal gene transfer between bacteria (from: Furuya and Lowy, 2006). Top to bottom: Natural transformation (free DNA, dead cell), transduction (bacteriophage) and conjugation (integrons, plasmids, transposons).

HGT often involves the conjugation of bacterial cells, requiring close contact between cells. This is more easily achieved in denser and relatively undisturbed environments like sediments. Experiments have for instance shown that mixing can frustrate this process (Davies, 1995), which might be the case in more turbulent surface waters. Especially biofilms seem very efficient environments for the propagation of ARGs. Research shows an increased rate of HGT in biofilms due to high cell density and proximity, making it an ideal environment for the transfer of resistance genes (Balcázar *et al*, 2015). It can be formed on different kinds of surfaces, like rocks or sand on the river bed, but also on solids and organic particles floating in the surface water. Next to increased cell density, increasing temperatures can further facilitate this process (Wang *et al*, 2020). Additionally, research indicates that biofilm formation is induced by low concentrations of antibiotics (Hoffman *et al*, 2005; Salcedo *et al*, 2014). Salcedo et al (2014) found that exposure of *Escherichia coli* and *Pseudomonas aeruginosa* to low concentrations of Tetracycline and Cephradine (Cephalosporin) enhanced the level of conjugation in tandem with biofilm formation, indicating increased transfer of ARGs.

Other -non-conjugative- means of HGT between bacterial species allowing for transfer over distances, involve natural transformation by which free floating (extracellular) transposons, integrons and gene cassettes are incorporated by bacteria (Domingues *et al*, 2012; Sturød *et al*, 2018; Dong *et al*, 2019) and transduction via bacteriophages (Lekunberri *et al*, 2017; Calero-Cáceres and Blacázar, 2016 and 2019). Studies indicate that ARGs embedded on extracellular DNA or in bacteriophages might be more persistent in the environment than ARGs embedded in bacterial cells on intracellular DNA (Calero-Cáceres and Munies, 2016; Wang *et al*, 2020).

Originally it seemed that resistance genes in the environment are exchanged between bacteria more in the context of promoting genetic diversity and hence adaptability of bacterial communities, than resulting from specific selective pressures calling for protective *'survival of the fittest'* strategies (Martinez *et al*, 2009; Nesme *et al*, 2015). Research by Kelsic *et al* (2015) suggests that the interaction between antibiotics-producing, antibiotic-sensitive and antibiotic-degrading bacteria contributes to species diversity and the ecological stability of bacterial communities, despite inherent difference of growth rates between species (Kelsic *et al*, 2015). With naturally low antibiotics concentrations in the general environment, antibiotic resistance is usually not the primary function. But with rising concentrations in hotspots like wastewater treatment plants (WWTPs) to clinical settings and antibiotic therapy in animals or humans, resistance can grow out to be the primary function of these genes (Martinez *et al*, 2009).

3 PROBLEM DEFINITION AND RESEARCH QUESTION

Although antibiotic resistance is a natural and ubiquitous phenomenon in the environment, the enhancement of it through human activities is increasingly indicated as an important driver in the evolution and spread of AR, posing a potential health risk to human populations and livestock. Research indicates that emissions from hotspots correlate to increased levels of environmental AR. But scientific insight is currently not able to predict the emergence and spread of AR through the environment or assess associated health risks (Larsson *et al*, 2018).

Especially in association with human pathogens, enhanced levels of ARGs in waters are often seen as important indicators of environmental reservoirs of resistance and potential human health risk (Berendonk *et al*, 2015; Martinez *et al*, 2015; Oh *et al*, 2018). But establishing health risk posed by resistant pathogens is very difficult. There is no known infective dose for exposure to (resistant) human pathogens. Silent and cumulative colonization by resistant pathogens can take place in healthy persons, without them manifesting symptoms of infection. But in other cases, even an undetectable amount of resistant pathogens can have a devastating effect on a person's health, particularly when health is already compromised (Manaia et al, 2017). Although abundance of environmental resistance can likely pose a health risk, low abundance does not necessarily preclude it. Manaia et al (2017) suggest that without the ability to quantify health risks, it is best to apply the Precautionary Principle, focusing on the potential risks posed by human sources and practices that promote antibiotics resistance in the environment.

But also the emergence and spread of AR in the aquatic environment seems to involve many factors that interact in complex ways. There are gaps in our understanding of *how* and *under which circumstances* AR evolves and spreads in the environment (Manaia *et al*, 2016; Bengtsson-Palme *et al*, 2018b). Due to lack of an adequate mechanistic understanding, a health risk assessment is currently hard to operationalize (Ashbolt *et al*, 2013; Manaia, 2017). More insight into the contribution of environmental drivers to the modulation of AR is therefore needed for devising strategies to prevent the emergence and spread of resistance to human and animal pathogens via the environment (Amos *et al*, 2015; Bengtsson-Palme *et al*, 2018b; Larsson *et al*, 2018).

In order to add to the insight of the impact of antibiotic pollution on AR, this thesis aims to address the following question:

Can statistically significant correlations be found between the abundance of resistance genes in surface water and sediments and the selective pressure from environmental antibiotic concentrations?

To increase insight, monitoring data from the environmental resistome would be very useful, but this kind of data is still scarce (Ashbolt *et al*, 2013; Pal *et al*, 2016), since large scale monitoring efforts have so far focused primarily on clinical and health care settings (WHO, 2014; McDonnell *et al*, 2017). Much data is none-the-less generated in primary research that involves the collection and analysis of environmental samples from different compartments and matrices, simultaneously measuring the amount of resistance genes and the presence of suspected environmental drivers of AR (e.g. Knapp *et al*, 2011; Amos *et al*, 2015; Jiao *et al*, 2017; Pal *et al*, 2017). This would add to a growing understanding of the influence of environmental drivers on AR. The result from these individual studies however are context specific and do not readily provide knowledge that can be generalized or has predictive power across different contexts. Therefore, an integration of the results from the accumulated research, might allow a better understanding of AR and could be useful in the development of predictive models. Since these individual studies were not designed for the eventual comparison or integration with results from other studies, it is important to consider if and how data from individual studies can be used for this purpose.

4 DATA AND METHODS

4.1 SEARCH STRATEGY

A systematic literature review of the period 2018-2019 was performed by searching the Web of Science in November 2019 and additionally in March 2020, using the search string “antibiotic*, *water and arg$ in title, abstract and keywords. The search returned 629 articles (2018: 224, 2019: 314 and 2020: 91).

4.2 SELECTION CRITERIA

From the publications, only original research was selected with surface water and/or sediment samples from natural waters in which antibiotics concentrations were measured simultaneously with resistance genes abundance.

Furthermore, a selection was made for the DNA extraction approach, focusing on qPCR.

Different methods are in use for extracting microbial genetic material from environmental samples. Culture dependent approaches have been well established. The vast majority of microbes is however difficult or impossible to culture in the laboratory (Sharpton *et al*, 2014). Also, establishing the presence of antibiotic resistance through culture dependent methods is relatively laborious and time consuming (Forbes *et al*, 2017; Waseem *et al*, 2019). This makes culture dependent methods less suitable to study the evolution of AR in the environment. The development of *culture-independent*, molecular methods have made possible a faster, more efficient and comprehensive assessment of the microbiological communities present in the environment (Waseem *et al*, 2019). These include approaches based on polymerase chain reactions (PCR) and metagenomic sequencing approaches. Both approaches can be used to characterize and quantify bacteria and ARGs in environmental samples.

High-throughput quantitative PCR (qPCR), also known as real-time PCR, relies on the amplification of targeted DNA molecules or genomic loci, such as ARGs or 16S rRNA, using pre-designed primer sets, DNA polymerase and DNA nucleotide triphosphates (dNTPs) (Kubista *et al*, 2006). Shotgun metagenomics is a relatively newer approach to the study of microbiological communities in the environment (Sharpton *et al*, 2014). It is a culture-independent method that uses high-throughput or next-generation sequencing platforms, like Illumina or PacBio, to sequence microbial DNA directly after extraction from environmental samples. Because shotgun metagenomics does not require the use of pre-designed primers for targeting specific marker genes or genomic loci, it is considered an open-format molecular detection technology (Zhou *et al*, 2015). An advantage compared to closed formats, like qPCR, is that it makes possible the detection of all microbes in the sample, even the

ones that are currently unknown to science (Zhou *et al*, 2015; Quince *et al,* 2017; Asante *et al* , 2019). Moreover, it does not require PCR amplification. Leaving out this step, can avoid the associated bias (Sharpton *et al*, 2014), where difference in the rate and efficiency of amplification between target genes can distort the original composition of the sample (Sabina & Leamon, 2015).

Although shotgun metagenomics is a promising tool for giving an unbiased insight into the environmental resistome, it is still a relatively expensive approach and the downstream data analysis can be complex (Li *et al*, 2015; Forbes *et al*, 2017). This could be the reason that, compared to qPCR, metagenomic studies are still relatively rare and comprise fewer environmental samples in each study. Also, many of these metagenomic studies focus primarily on the abundance and types of ARGs, often in combination with characterizing the microbial communities. In these studies, less emphasis is laid on antibiotics concentrations, meaning they are either not measured or only globally and not measured in the same sample as the ARGs.

An earlier attempt to use only metagenomic studies, yielded 9 useful publications comprising a total of 52 environmental samples (appendix 1)(Bai *et al*, 2019; Chen *et al*, 2013b; Chen *et al*, 2019b; Fang *et al*, 2018; Garner *et al*, 2016; Guo *et al*, 2016; Jia *et al*, 2017; Qui *et al*, 2019; Zhang *et al*, 2018) (see the overview of studies in appendix I). Lack of comparability due to differences in the calculation of relative gene abundances further narrowed down the number of useful studies. Hence, a choice was made to abandon the meta-analysis of samples from metagenomic studies and switch to studies using qPCR.

The selection process based on qPCR-studies resulted in 19 studies meeting all the criteria (2018: 10, 2019: 6 and 2020:3).

4.3 Data extraction

The following data was extracted from the studies:

- Antibiotic concentrations
- Abundance of resistance genes
- Environmental matrix (surface water, sediment)
- Year of sampling
- Month of sampling
- Place of sampling (country, location, type of waterbody)

Data was collected from the text and tables in the articles and supplements. Data expressed in plots, was extracted using WebPlotDigitizer V.4.2 (Rohatgi, 2017). A choice was made to exclude studies

that expressed resistance gene abundance solely in heatmaps. For Guan *et al* (2018) the original data of relative gene abundance was provided by the author.

A number of studies were finally excluded, because:

- no data was provided to calculate the relative abundances of ARGs to 16s rRNA
- no antibiotic types were given to calculate selective pressures, only aggregated concentrations per class
- no month of sampling was given to infer the season of sampling

This left 9 studies with a total of 204 environmental samples (99 sediment, 105 surface water samples). A total of 28 types of antibiotics from 7 antibiotic classes were measured in the samples, along with 29 types of resistance genes and 2 mobile genetic elements. Concentrations below the detection or quantification limit were not included.

4.4 Data structure

4.4.1 ARG abundance

The abundance of antibiotic resistance genes (ARGs) was expressed relative to 16s rRNA. In most studies this was reported as such. In cases where it had not been calculated, but rRNA 16s abundance was given for samples, the following formula was applied to establish relative abundance:

$$rARG_{x,j} = ARG_x / 16s\ rRNA_j \quad \text{(eq.1)}$$

Dividing the number of resistance genes of gene (x) in sample (j), by the number of 16s rRNA genes in sample (j).

The total number of resistance genes (x) in sample (j) conferring resistance to an antibiotic class (y) was calculated with the following formula:

$$TARG_{y,j} = \sum rARG_{x,j} \quad \text{(eq.2)}$$

Where ($x \in y$), summing all antibiotic genes (x) conferring resistance to antibiotic class (y).

4.4.2 Antibiotic classification and resistance mapping

Individual antibiotics were grouped into classes according to the Anatomical Therapeutic Chemical (ATC) code. The Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al, 2020) was used to map resistance genes to the antibiotics class they confer resistance to (Table 1).

https://card.mcmaster.ca/

Table 1: Overview of antibiotic classes, with corresponding antibiotic types and resistance gene types.

ATC class	**Antibiotic**	**Antibiotic resistance gene**
Quinolones	Difloxacin, Fleroxacin, Norfloxacin, Ciprofloxacin Enrofloxacin, Ofloxacin	*qnrB, qnrS, gyrA*
Microlides Lincosamides	Aureomycin, Clindamycin Roxithromycin, Erythromycin	*ermB, ermC, erm F*
Beta-lactams/Cephalosporins	Cefazolin, Cefotaxime	*OXA, CTX, blaIMP-04, blaNMD1, blaOXY, blaSHV-01, blaTEM*
Sulfonamides	Sulfameter, Sulfadimedine, Sulfadiazine, Sulfamethazine, Sulfapyridine, Sulfamethoxazole, Sulfathiazole, Sulfamerazine, Sulfamonomethoxine, Sulfaquinoxaline	*sul1, sul2, sul3, sulA*
Amphenicols	Chloramphenicol, Thiamphenicol, Florfenicol	*catI, cmlA, floR*
Tetracyclines	Tetracycline, Oxytetracycline, Doxycyclinehyclate, Chlortetracycline	*tetA, tetB, tetC, tetD, tetE, tetM, tetO, tetW, tetZ*
All classes	All types	*intI1, intI2 (mobile genetic elements)*

Mobile genetic elements, including *intI1* and *intI2*, were also included because of their role in facilitating dissemination of antibiotic resistance (Berendonk *et al*, 2015; Martinez *et al*, 2015; Oh *et al*, 2018). These element were mapped against all classes of antibiotics in the sample.

4.4.3 Antibiotic selective pressure

Units for antibiotic concentrations were set to ng/l for surface water and ng/kg dry weight for sediment. From the antibiotic concentrations the selective pressure was derived using relevant PNEC-values (predicted no-effect concentrations), based on Bengtsson-Palme and Larsson (2016b) and the list compiled by the AMR Industry Alliance Antibiotic Discharge Targets (AMR, 2018). Both contain estimated PNEC-values for assessing risk of concentrations in surface waters, specifically taking into account risks of resistance promotion in the environment. For some types of antibiotics that were not included in either publication mentioned above, additional publications were used to derive a PNEC-value (Wang et al, 2020b, Zhang et al, 2020). For the PNEC-values and their sources, see appendix 2.

The PNEC-sediment was derived from the PNEC in water and calculated as follows:

$$PNEC\text{-}water * 0{,}058 * Koc \quad \text{(eq.3)}$$

The EPI Suite™-Estimation Program Interface (KOCWIN-tool; EPA, 2012) was used to calculate the Koc (soil adsorption coefficient) for each antibiotic. Following Duarte et al (2019), an organic carbon content of 5.8% was assumed.

Using the PNEC-value, a measure of selective pressure from each antibiotic concentration in a sample was calculated as follows:

$$ASP_{i,j} = MEC_{i,j}/PNEC_i \text{ (eq.4)}$$

Dividing the MEC-value (measured environmental concentration) found for antibiotic type (i) in sample (j) by the PNEC for that antibiotic (i).

From this, a measure of total selective pressure per sample was calculated.

$$TASP_{y,j} = \sum ASP_{i,j} \text{ (eq.5)}$$

Where ($i \in y$), summing the selective pressure values of all antibiotic types (i) belonging to antibiotic class (y).

4.4.4 Environmental matrices

Surface water and sediment samples were collected from natural waters, including lakes, reservoirs, rivers and estuaries. Sediment samples in all studies were collected from the top layer. There were slight differences in sediment sample depth between studies, with depths ranging from several cm to approximately 20 cm.

Sampling locations included:

- Ba River in Xi'an, China (Guan *et al*, 2018 and Jia *et al*, 2018)
- Yangtze Estuary near Shanghai, China (Guo *et al*, 2018)
- Wenyu River in Beijing, China (Liu *et al*, 2019)
- Three Gorges Reservoir, China (Lu *et al*, 2018)
- Lake Taihu, China (Ohore *et al*, 2019)
- Grote Beerze River, The Netherlands (Sabri *et al*, 2018)
- Lake Honghu, China (Wang *et al*, 2020a)
- Yangtze and Jialing Rivers in Chongqing City, China (Wang *et al*, 2020b)

4.4.5 Data base

The sample database (table 2) comprised 9 studies (*Study*), 204 samples (*Sample*), 2 countries (*Country*), 2 matrices (*Matrix*), 6 antibiotic classes (*Class*), 5 sampling years (*Year*) and 4 sampling seasons (*Season*).

Table 2: An overview of the main characteristics of the environmental samples (n=205) used in this meta-analysis. Samples were assigned to a season based on the month of sampling.

Studies	**Samples**	**Countries**	**Matrices**	**Antibiotic classes**	**Sampling years**	**Sampling seasons**
Guan et al, 2018 Guo et al, 2018 Jia et al, 2018 Liu et al, 2019 Lu et al, 2018 Ohore et al, 2019 Sabri et al, 2018 Wang et al, 2020a Wang et al, 2020b	204 SW=105 SD=99	China (n=8) Netherlands (n=1)	Sediment (SD) Surface water (SW)	Quinolones (FQ) Sulphonamides (SUL) Tetracyclines (TET) Macrolides/Lincosamides (MLLS) Amphenicols (AC) Cephalosporins (CS)	2011 2015 2016 2017 2018	Spring (*March-May*) Summer (*June-Aug*) Autumn (*Sep-Nov*) Winter (*Dec-Feb*)

A database of entries for each class nested by sample and study, included 553 unique entries, matching the total selective pressure $(TASP_{y,j})_{of}$ each antibiotic class (j) in a sample (y) to the total of antibiotic resistance genes that confer resistance to that class ($TARG_{y,j}$). A total of 185 entries mobile genetic elements (*intl1, intl2*) that were mapped to all antibiotic classes, were added to the final database. The final database for data analysis comprised 738 unique entries (table 3).

Table 3: The final database (n=750) with each data point representing the total selective pressure of an antibiotic class mapped to the total relative abundance of matching resistance genes in a sample. Total relative abundance of mobile genetic elements (MGEs) were mapped against the total selective pressure of all classes in a sample.

Class / Matrix	Totals	Mobile genetic elements	Quinolones	Macrolides	Cephalosporins	Sulphonamides	Amphenicols	Tetracyclines
SW	370	97	53	45	12	100	4	59
SD	368	88	66	40	12	71	8	83
Totals	738	185	119	85	24	171	12	142

4.5 DATA EXPLORATION

4.5.1 Scatterplots and boxplots

As an initial exploration of the data, a scatterplot was made from the complete dataset (n=204) of accumulated sediment and surface water samples (fig.3).

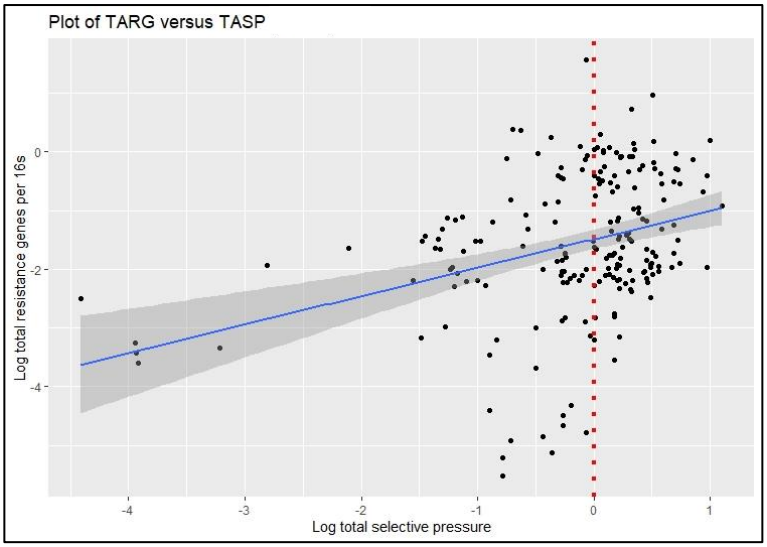


Figure 3: Scatterplot of the collective dataset of surface water and sediment samples (n=204). Data points represent sum totals of selective pressure (x-axis) and sum totals of resistance genes (y-axis) for each sample. A regression line is drawn in blue. The red dotted line indicates a selective pressure of 1.

A regression line is drawn in blue, showing a positive slope for total antibiotic resistance (TARG) values at higher total selective pressure values (TASP). Datapoints however show a lot of spread around the regression line. There does not seem to be a clear linear relationship between TASP and TARG, judging from this scatterplot.

A red dotted line is drawn in the graphs to indicate were the MEC (measured environmental concentration) divided by the PNEC (predicted no effect concentration) is 1 (10 to the power of 0). This indicates a risk quotient of 1 (RQ=1), pointing to a potential environmental risk. However, for the n=204 data set, total TASP in each sample is the sum total of selective pressures of different classes of antibiotics. The selective pressure of individual antibiotic classes within a sample might not pose a potential risk.

Also, because the number of analysed antibiotic classes differs between studies, the sum total of selective pressures is less informative. Low measures of TASP have for instance been found in the sediment samples from Three Gorges Reservoir taken by Lu *et al* (2018). But in these samples only Tetracyclines were analysed. While in Guan *et al* (2018) and Jia *et al* (2018) respectively six and seven major classes of antibiotics were analysed. The same is true for variations in TARG values for this sample set (n=204). This might very well be related to the number of ARG-types analysed in a sample rather than actual difference in TARG between samples. Overall, the n=204 sample set is not very useful for meta-analysis because of the variability between samples.

In figure 4 therefore, the total relative abundance of ARGs in samples is mapped against the antibiotic class they confer resistance to. Mobile genetic elements (MGEs) are also included and mapped against total selective pressure in the sample. Only data points were used that yielded a

value for both selective pressure (TASP) and matching ARG-types (TARG). This resulted in 738 data points.

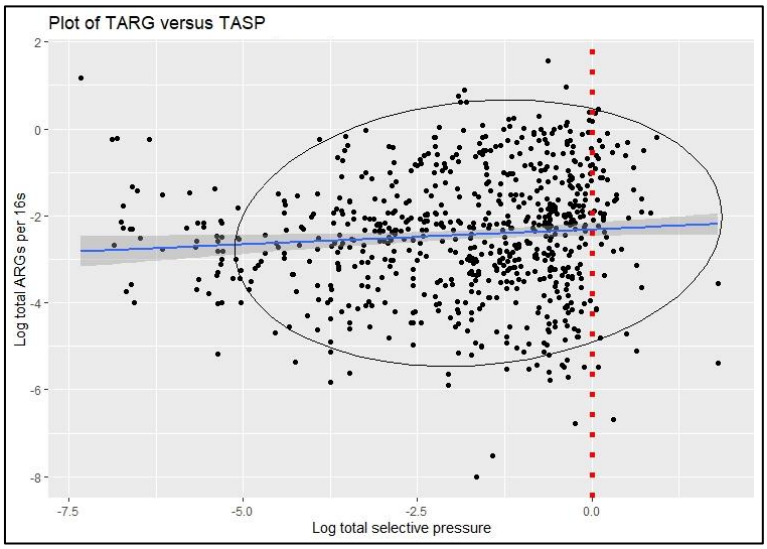


Figure 4: Scatterplot of the dataset of surface water and sediment samples. Data points represent the total selective pressure of an antibiotic class (x) in a sample (j) ($TASP_{x,j}$) on the x-axis and total resistance genes (y) in a sample (j) ($TARG_{y,j}$) on the y-axis. Mobile genetic elements (MGEs) are mapped against the sum total selective pressure of each sample. This results in 738 data points.
A 95% predictive ellipse is drawn around the dataset, indicating the area of 95% probability for new observations assuming a normal bivariate distribution. A regression line is drawn in blue. The red dotted line indicates a selective pressure of 1.

Compared to figure 3 (n=204), data points in figure 4 (n=738) seem more clustered, with some areas showing small denser clusters around the regression line, but overall the spread remains relatively high and there does not seem to be a clear linear relationship in the data. The regression line is not very steep which might indicate very weak correlation, but it still has a positive direction. Most datapoints remain (well) below a selective pressure of 1 (RQ=1, red dotted line). But a number of samples are above RQ=1 and a small number indicate a very high risk.

The two highest selective pressures were measured in the Jialing River from Quinolones and in the Yangtze River for overall selective pressure, both in the Chongqing City area (Wang *et al*, 2020b). Despite the high TASP-values in these samples, total relative abundance of matching ARGs was relatively low.

Lowest total selective pressure was found for Sulphonamides in a sample taken by Jia *et al* (2018) in the springtime in Ba River in downtown Xi'an City. However, total relative abundance of ARGs conferring resistance to Sulphonamides was very high in this sample.

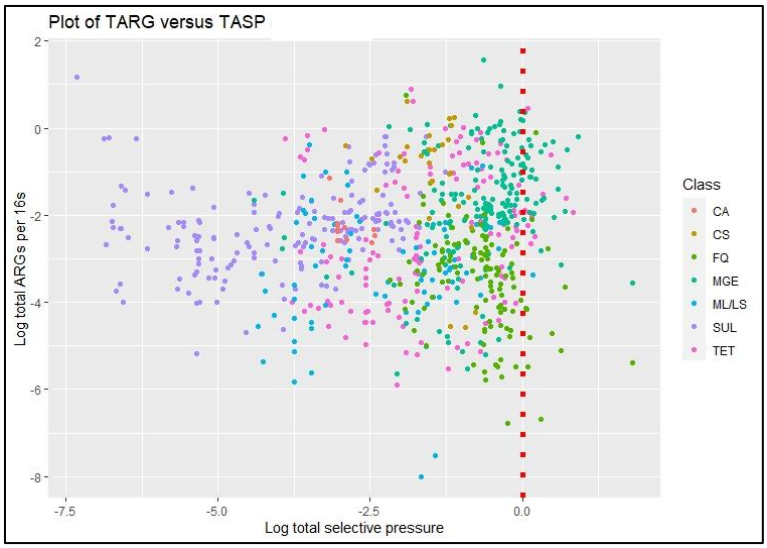


Figure 5: Scatterplot (n=738) with data sorted by class. CA=Amphenicols, CS=Cephalosporins, FQ=Quinolones, MGE=Mobile genetic elements, ML/LS=Macrolides/Lincosamides, SUL=Sulphonamides and TET=Tetracyclines.
The red dotted line indicates a selective pressure of 1.

In figure 5 antibiotic classes are made visible, showing some clustering of data points, partially from clustering along the x-axis corresponding to selective pressure. This is most visible for Sulphonamides (purple), where TASP in most samples is notably low compared to other classes. Samples with TASP higher than 1, indicating potential to very high risk, are mainly related to the Quinolones (apple green) and Tetracyclines (pink).

In the boxplot of figure 6, the spread of selective pressure values in samples for each antibiotic class is visualized. MGE are plotted against all antibiotic classes, therefore it corresponds to the combined selective pressure of all classes in the sample. The boxplot shows that highest mean and median values are found for Quinolones, being nearly on par with total selective pressures as indicated by MGEs. This implies that -of all classes- Quinolones contribute substantially to overall TASP. After that Cephalosporins and Tetracyclines show highest mean and median values. As also visible in figure 6, Sulphonamides show the lowest mean and median values for selective pressure and lowest minimum values at the low end of the whiskers, but highest spread.

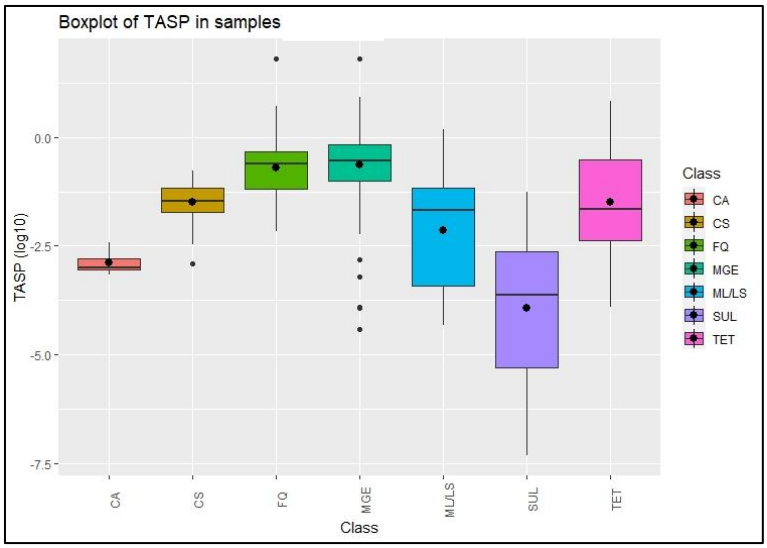


Figure 6: Boxplot indicating the spread in the data (n=738) of total selective pressure in samples for each antibiotic class. CA=Amphenicols (n=12), CS=Cephalosporins (24), FQ=Quinolones (n=119), MGE=Mobile genetic elements (n=185), ML/LS=Macrolides/Lincosamides (n=85), SUL=Sulphonamides (n=171) and TET=Tetracyclines (142).
Explanation of boxplot: The median value is indicated by the black horizontal line across the box. The coloured box indicates the upper Q1 (75th percentile) above the median line and the lower Q3 (25th percentile) below the median line. The whiskers indicate the range between minimum and maximum values. The black dot within the box indicates the mean value. The dots outside the box indicate the outliers.

But low or high selective pressure does not automatically translate into a similar profile of ARGs abundance measured in samples. Figure 7 shows substantial spread across the y-axis within the same class for most classes. Comparing the mean values and spread of TARG within the data (fig. 7) with the TASP (fig. 6), some remarkable shifts are seen especially in Quinolones and Sulphonamides.

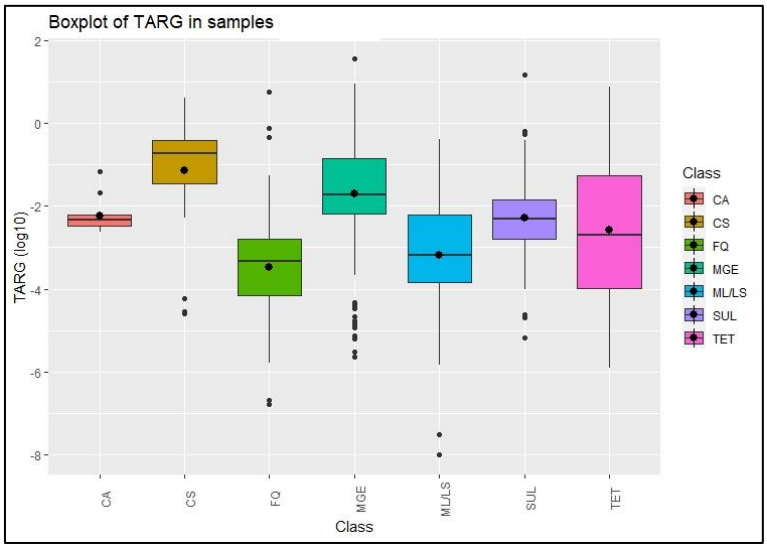


Figure 7: Boxplot indicating the spread in the data (n=738) of total ARGs in samples for corresponding antibiotic class. CA=Amphenicols (n=12), CS=Cephalosporins (24), FQ=Quinolones (n=119), MGE=Mobile genetic elements (n=185), ML/LS=Macrolides/Lincosamides (n=85), SUL=Sulphonamides (n=171) and TET=Tetracyclines (142).
Explanation of boxplot: The median value is indicated by the black horizontal line across the box. The coloured box indicates the upper Q1 (75th percentile) above the median line and the lower Q3 (25th percentile) below the median line. The whiskers indicate the range between minimum and maximum values. The black dot within the box indicates the mean value. The dots outside the box indicate the outliers.

In Quinolones, although of all classes it shows the highest mean and median values in samples for selective pressure, it is ranked lowest in median and mean values for corresponding ARG values. In Sulphonamides a somewhat reverse trend is visible, ranking lowest in selective pressures, but ranking third of all 7 classes in median and mean values of total corresponding ARGs. MGEs and ARGs conferring resistance to Cephalosporins show the highest mean and median values for ARG-abundance in samples. ARGs conferring resistance to Tetracyclines show the largest spread.

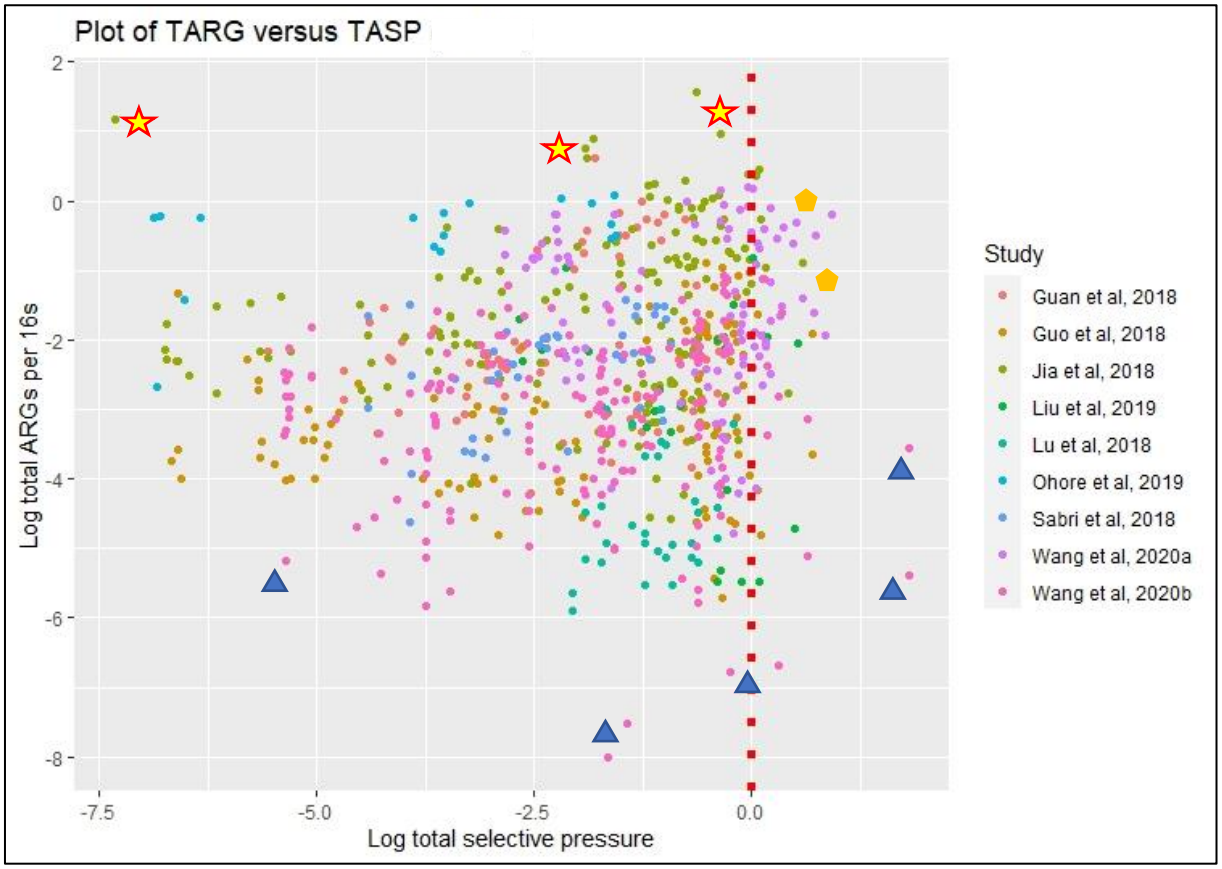
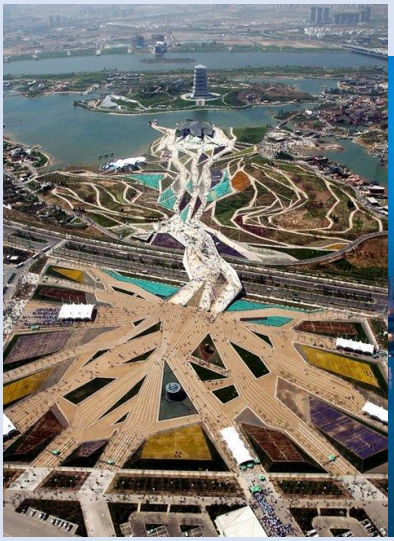


Figure 8: Scatterplot (n=738) with data sorted by study. Guan et al, 2018 (n=67), Guo et al, 2018 (n=119), Jia et al, 2018 (n=158), Liu et al, 2019 (n=28), Lu et al, 2018 (n=32), Ohore et al, 2019 (n=17), Sabri et al, 2018 (n=43), Wang et al, 2020a (n=103) and Wang et al, 2020b (n=183). The red dotted line indicates a selective pressure of 1.
Samples from studies by Jia et al, 2018 (stars), Wang et al, 2020a (diamonds) and Wang et al, 2020b (triangles) are indicated.

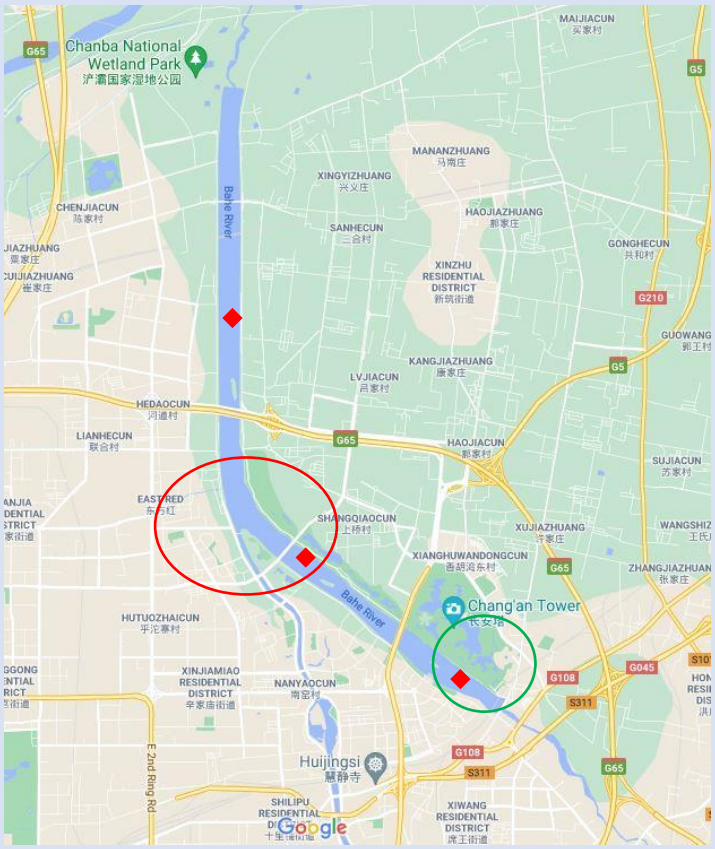
Figure 8 shows the data set sorted by study, indicating large spread of data points within studies and overlap between studies. A number of studies included more extreme measurements in samples, spanning different combinations of TASP and TARG. These measurements are indicated in figure 8 by stars (samples from Jia et al, 2018), diamonds (samples from Wang et al, 2020a) and triangles (samples from Wang et al, 2020b). Below is an impression of the three areas where these samples were taken. While extensive anthropogenic pressures are evident in all locations, quite different combinations of antibiotic selective pressure and resistance gene abundance are measured.

4.5.1.1 Extremes Xi'an: Very high ARGs with very low to moderate selective pressures.

These samples were all taken by Jia *et al*, 2018 in springtime (march, dry season) from the Ba River at downtown Xi'an (Shaanxi Provence). Xi'an is a megacity of 6.5 million people. It is an overall prosperous city, rich in cultural heritage, touristic hotspots and famous for the terracotta army of Qin Shi Huang, the first Emperor of China.



Above-left a view of Xi'an Expo Park (source: https://www.archilovers.com/projects/19597/flowing-gardens-xi-an-international-horticultural-expo.html). Above-right a view of the Ba River at downtown Xi'an Kempinski Hotel (source: tripadvisor.com)

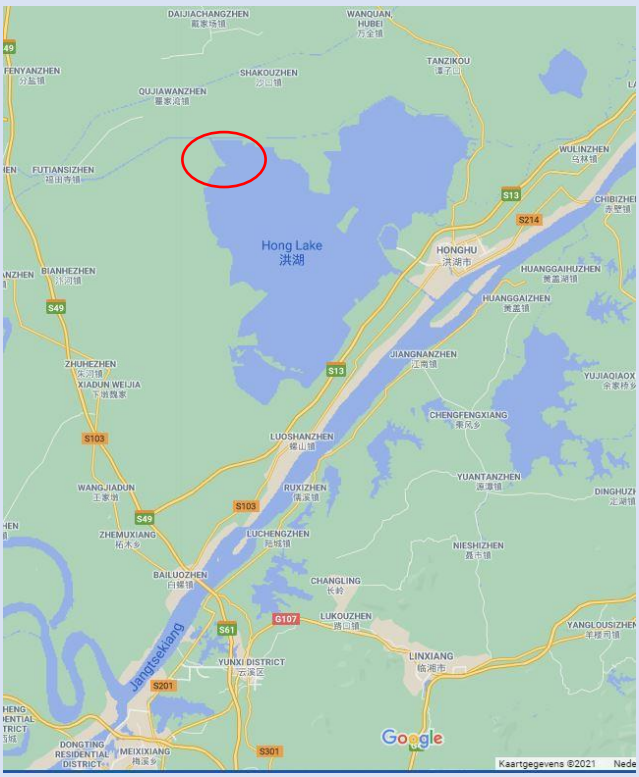


Above: Samples were taken between Chang'an Tower/Xi'an Expo Park and Chanba National Wetland park. In the red oval is the location of the picture above-right. In the green circle is the location of the Xi'an Expo Park. The red diamonds indicate the approximate locations of the very high levels of ARGs found conferring resistance to Quinolones, Sulphonamides, Tetracyclines and Cephalosporins in both surface water and sediment samples. Matching selective pressure was very low for Sulphonamides and moderate for the other classes.

4.5.1.2 Extremes Honghu Lake: Very high selective pressures with high ARGs

These samples were all from surface waters in autumn and spring, taken by Wang *et al*, 2020a from Lake Honghu in Hubei province. Honghu Lake is a shallow lake, connected to the Yangtze River and lies downstream from the Three Gorges Dam and Jingzhou, a megacity of 6.5 million people. The lake is recognized as a wetland of international importance and is an important tourist attraction, especially in summer when the lake is covered in lotus flowers in bloom. But Honghu Lake has also suffered from extensive anthropogenic pollution and overexploitation related to overfishing, surrounding agriculture, animal husbandry and aquaculture.

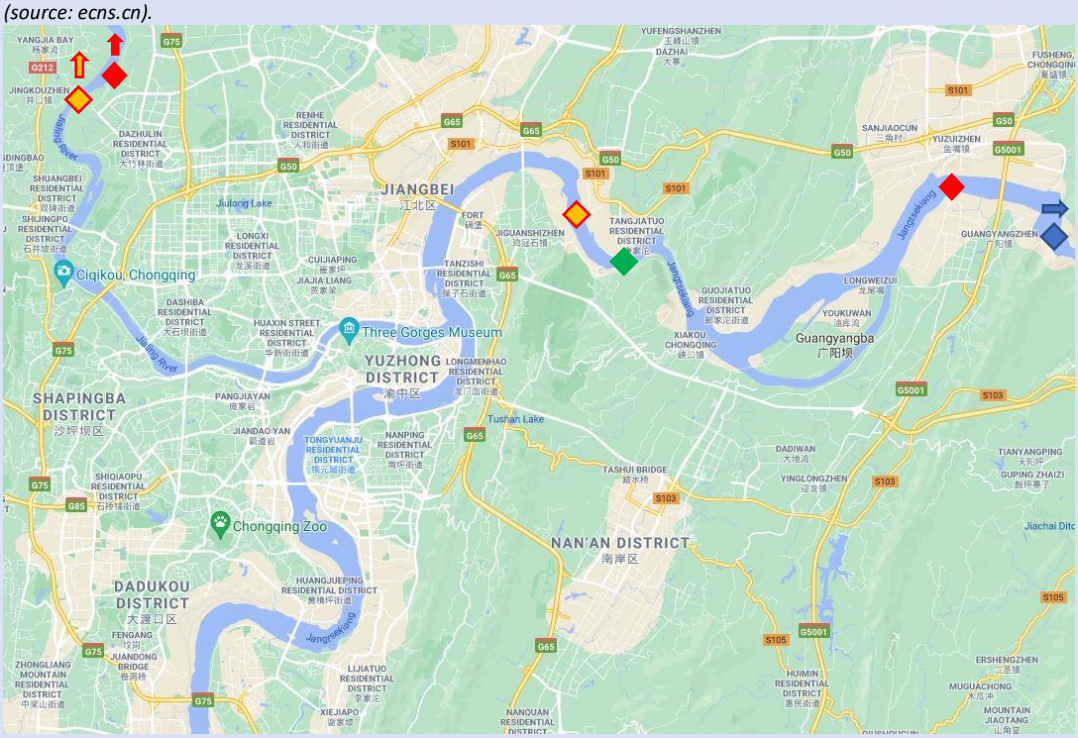
Impression from Honghu Lake. The picture above-right shows the Lantian Eco-agricultural Scenic Spot where very high selective pressures from Tetracyclines and corresponding ARGs were measured. In the map below, this area is indicated by the red oval. High selective pressure and high matching resistance genes were furthermore found for Quinolones. High MGEs related to high overall selective pressure in samples. (Sources clockwise: upper-left https://cuecc.com/chinafeature/interests/2011411219268261.htm upper-right https://www.tripadvisor.com/LocationPhotos-g1152524-Honghu_Hubei.html#164000415 lower-right https://www.globaltimes.cn/content/1116022.shtml)



4.5.1.3 Extremes Chongqing: Low to moderate ARGs with low to very high selective pressures

These samples were taken from surface waters and sediments in the Yangtze and Jialing Rivers in the Chongqing City area by Wang *et al*, 2020b. This area has around 31 million inhabitants and is a fast developing and important economic centre in China, lying in the middle reaches of the Yangtze River, upstream from Three Gorges Dam.

Above left Chongqing city at the convergence of the Yangtze and Jialing Rivers (source: http://orakxx.blogspot.com/). Above-right a view of the Bei Bei District, a hot spring tourist hotspot along Jialing River, where very high levels of Quinolones were measured in sediment samples (source: ecns.cn).



Above is a map showing part of the sampling area. In red diamonds the locations measuring very high selective pressures from Quinolones and moderately high ARGs in sediments (both in Yuzuizhen and Bei Bei district (not on map)). High selective pressure from Quinolones, but low ARGs were found in sediments both at Tong Jiaxi downstream of the Bei Bei District (not on map) and in downtown Chongqing (orange-red diamonds). Moderate selective pressure from Macrolides and very low ARGs were found in surface water samples in the Yangtze River downstream from Yuzuizhen (blue diamond, not on map). Very low selective pressure from Sulphonamides and low ARGs are found in sediment samples in downtown Chongqing (green diamond). Both downtown locations were downstream from a nearby wastewater treatment plant.

Interestingly, measures of selective pressures from Sulphonamides found in surface water samples in the only non-Chinese study (Sabri *et al*, 2018; fig.5 dark blue data points) did not seem to differentiate much from Chinese samples, although they were taken from a vastly different context in rural Netherlands along the Grote Beerze river (fig.10).

Figure 9: Left is a view of the Grote Beerze River in the Netherlands and to the right is a birds-eye view of the wastewater treatment plant between Hapert and Casteren, releasing wastewater into the Grote Beerze.

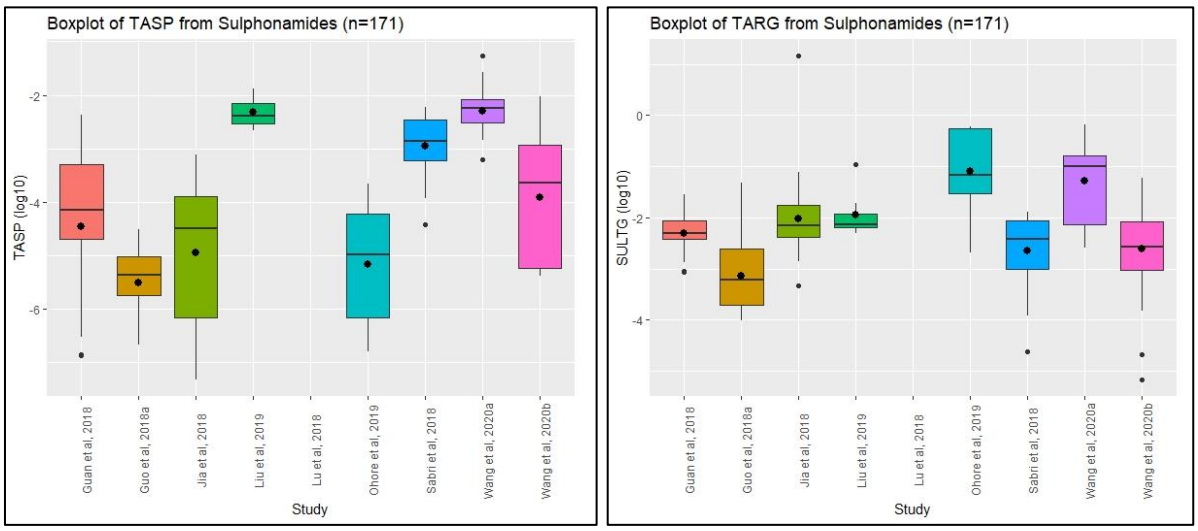
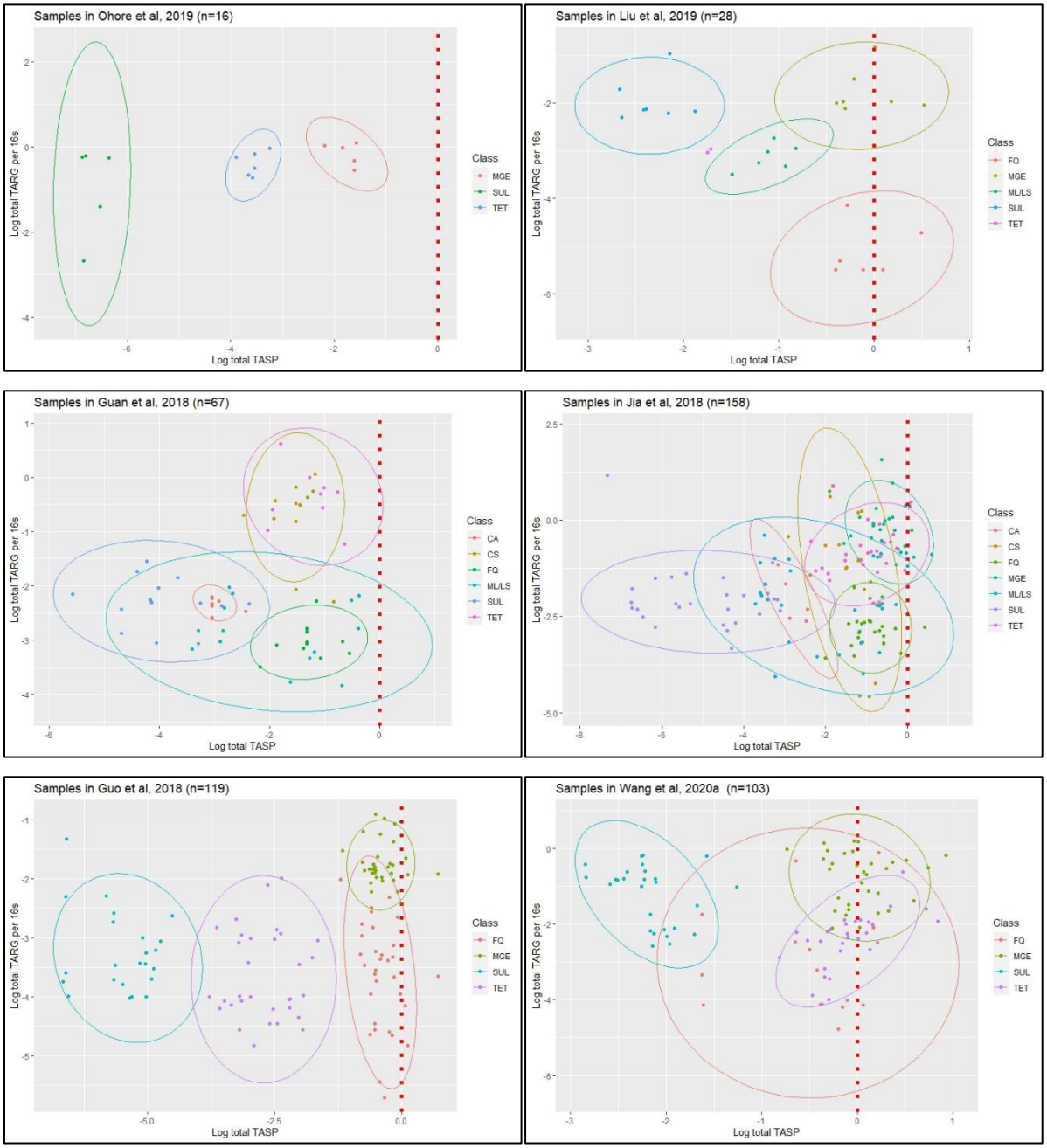


Figure 10: Boxplot with TASP (left) and TARG (right) for Sulphonamide. Showing Q1-percentile, Q2-percentile, median, mean, maximum and minimum values and outliers for each study. Guan et al, 2018 (n=13), Guo et al, 2018 (n=23), Jia et al, 2018 (n=28), Liu et al, 2019 (n=7), Ohore et al, 2019 (n=6), Sabri et al, 2018 (n=21), Wang et al, 2020a (n=31) and Wang et al, 2020b (n=42).

Median values of TASP from Sulphonamides found in the Grote Beerze were relatively high compared to most Chinese studies and only moderately below the highest mean and median values found by Liu *et al* (2018) in the Wenyu River at Beijing and by Wang et al (2020a) in Lake Honghu (fig. 10). TARG for Sulphonamides in de Grote Beerze was comparable to most Chinese studies. Trimethoprim, an antibiotic that is often administered alongside Sulfamethoxazole, was also found in the Grote Beerze River, but Tetracyclines or Macrolides were not detected.

The data from Sabri et al (2018) does not indicate notable differences between countries. But this is only based on a small data set. Non-Chinese data represents only 6% of the complete data set (43 out of 738 data points), which is inadequate for comparison between countries.

Looking closer at samples within studies, in figure 11, seven studies have been plotted with different colours indicating antibiotic classes. Not included are Sabri *et al* (2018) which only includes Sulphonamides in the dataset and Lu et al (2018) who only studied Tetracyclines. In the studies with more than one antibiotic class, there seem to be some clustering and spread patterns for different antibiotic classes. This is most apparent in Ohore *et al (*2019), Liu *et al* (2019) and Guo *et al* (2018), but more or less visible in all studies.



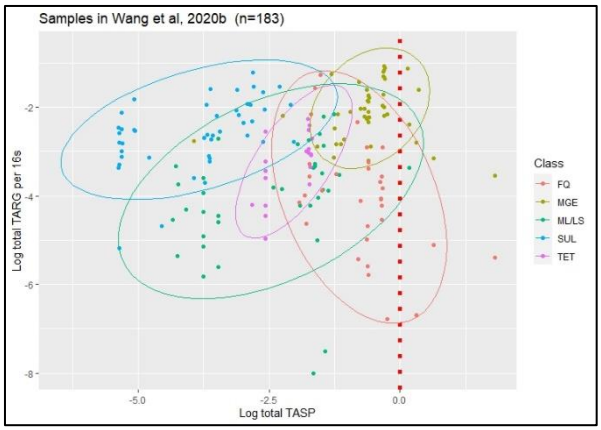


Figure 11: Scatterplots of the five studies with the largest datasets. Datapoints are coloured according to antibiotic class. CA=Amphenicols, CS=Cephalosporins, FQ=Quinolones, MGE=Mobile genetic elements, ML/LS=Macrolides/Lincosamides, SUL=Sulphonamides and TET=Tetracyclines.
A 95% predictive ellipse is drawn around datapoints to indicate the 95% probability area for new observations, assuming a normal bivariate distribution.

The data set (n=738) is further explored by plotting the data ordered by matrix and season, being two factors that might influence the relationship between TASP and TARG.

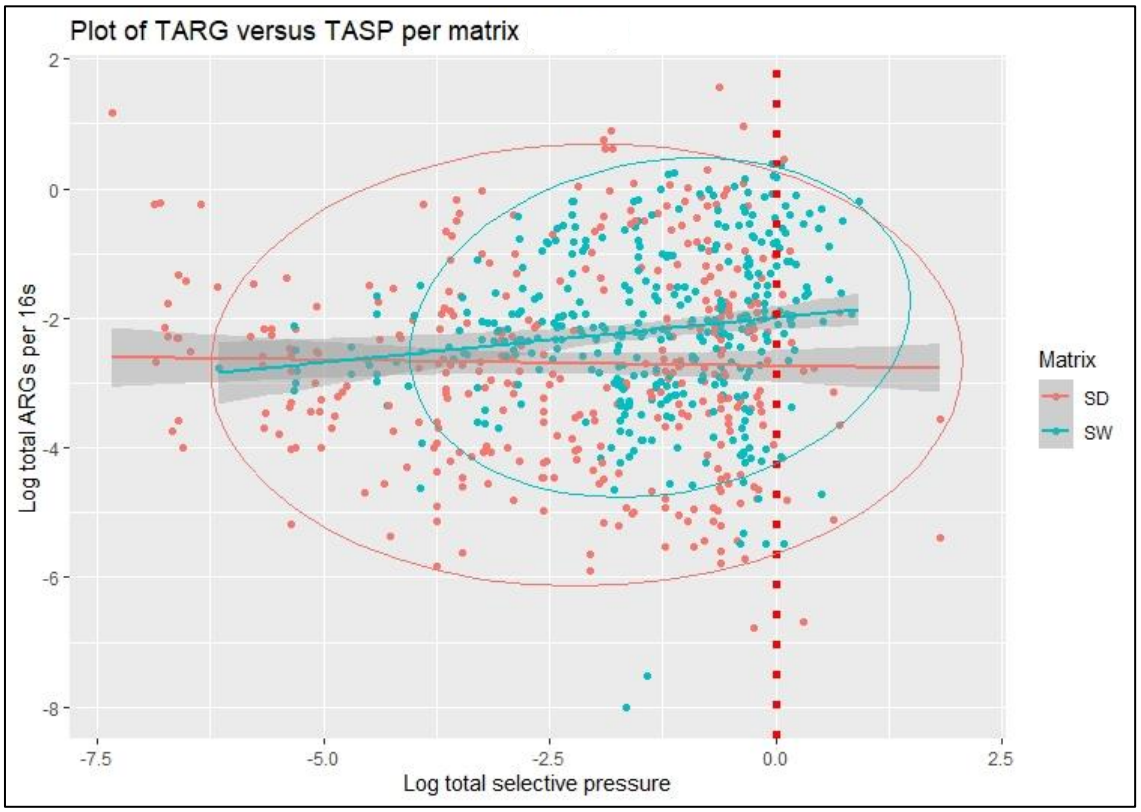


Figure 12: Scatterplot of the data set (n=738) ordered by matrix. SD=sediments (n=368) and SW=surface waters (n=370). A 95% predictive ellipse is drawn around datapoints to indicate the 95% probability area for new observations, assuming a normal bivariate distribution.

The scatterplot in figure 12 -dividing data according to environmental matrix -, shows much overlap between surface water and sediment samples, but a larger spread in sediment samples especially along the x-axis, indicating more variation in selective pressure values. Highest values for TASP as well as TARG were found in sediments. Lowest value for TARG was found in surface water.

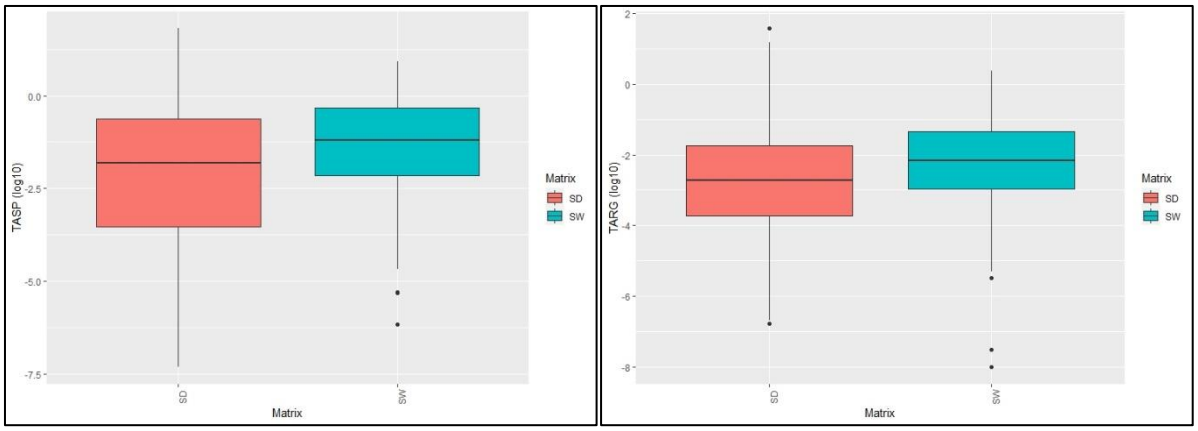


Figure 13a: Boxplots showing mean, median, Q1-Q3-percentile, maximum and minimum values and outliers of (left) total selective pressures (TASP) in both matrices and (right) total resistance (TARG).

Spread and median values in both matrices in figure 13a, show that in sediments spread is higher for both TASP and TARG. Median values are higher in surface waters for TASP and TARG.

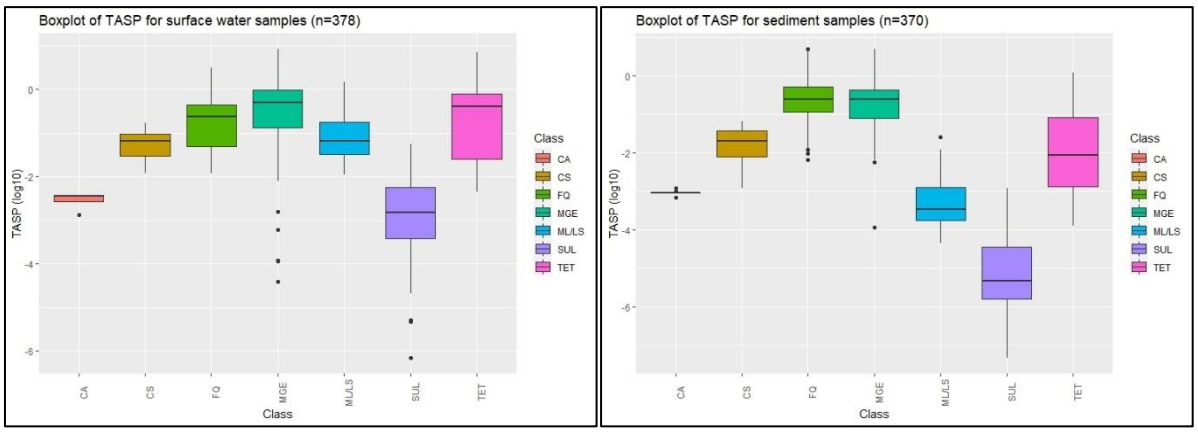


Figure 13b: Boxplots showing mean, median, Q1-Q3-percentile, maximum and minimum values and outliers of total selective pressures (TASP) in surface water (left) and sediment samples (right) for each antibiotic class. MGE is mapped to all classes and thus TASP is equal to selective pressures from alle classes.
CA (Amphenicols, SW=4, SD=8), CS (Cephalosporins, SW=12, SD=12), FQ (Quinolones, SW=53, SD=66), MGEs (eq. to total TASP, SW=97, SD=88), ML/LS (Macrolides/Lincosamides, SW=45, SD=40), SUL (Sulphonamides, SW=100, SD=71) and TET (Tetracyclines, SW=59, SD=83).

The boxplots in figure 13b show some differences between surface water and sediment samples for selective pressure from different antibiotic classes. In surface waters Tetracyclines together with Quinolones have the highest median values in samples which is nearly on par with MGE (indicating total TASP from all classes). This indicates that Tetracyclines and Quinolones contribute substantially to TASP in surface water, followed closely by Macrolides and Cephalosporins. Sulphonamides have the lowest median in surface water and overall lower values compared to other classes.

In sediments Quinolones have the highest median -almost on par with MGE- and with little spread, indicating that most selective pressure values from Quinolones are relatively high in sediments, with some indicating potential risk (TASP>1). Cephalosporins values tend to be lower in sediments than in

surface waters, but the sample set is relatively small so chance might play a bigger role in this. Tetracyclines and even more so Macrolides and Sulphonamides tend to be lower in sediments than in surface waters.

The two highest values for TASP were found in sediments. These values were indicated as outliers. For the purpose of having fairly similar scales for better visual comparison between boxplots these were left out (n=368-2).

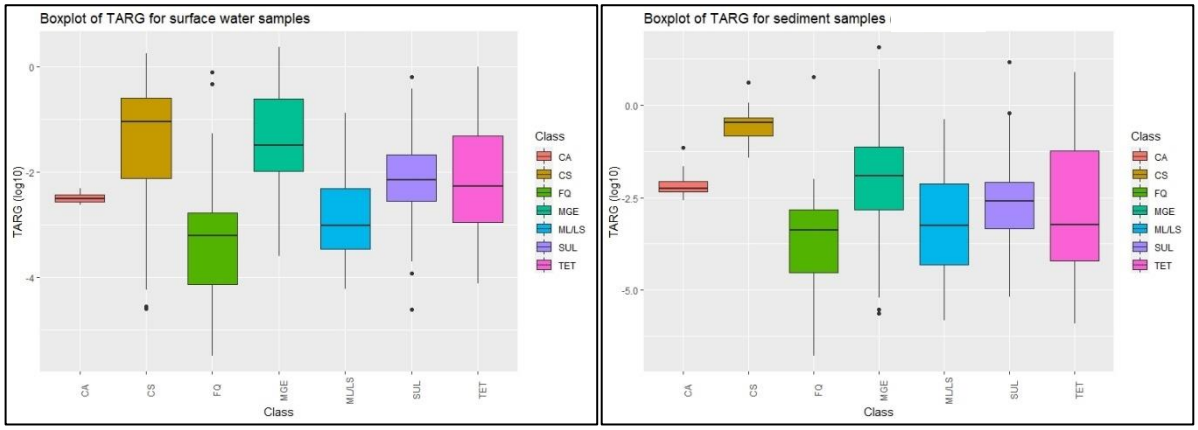


Figure 13c: Boxplots showing median, Q1-Q3-percentile, maximum and minimum values and outliers of total resistance gene abundance (TARG) in surface water (left) and sediment samples (right) for each antibiotic class. CA (Amphenicols, SW=4, SD=8), CS (Cephalosporins, SW=12, SD=12), FQ (Quinolones, SW=53, SD=66), MGEs (eq. to total TASP, SW=97, SD=88), ML/LS (Macrolides/Lincosamides, SW=43, SD=40), SUL (Sulphonamides, SW=100, SD=71) and TET (Tetracyclines, SW=59, SD=83).

For TARG (fig. 13c) surface waters and sediments do not show very different trends across classes. Somewhat higher values are seen for Cephalosporins in sediments and little spread compared to other classes, but the sample set is relatively small (n=12). TARG values for Quinolones are in the lower end. This is in contrast with their comparatively high contribution to selective pressure (fig.13b). All classes however show considerable spread in both matrices, indicating much overlap of the values found for antibiotic resistance.

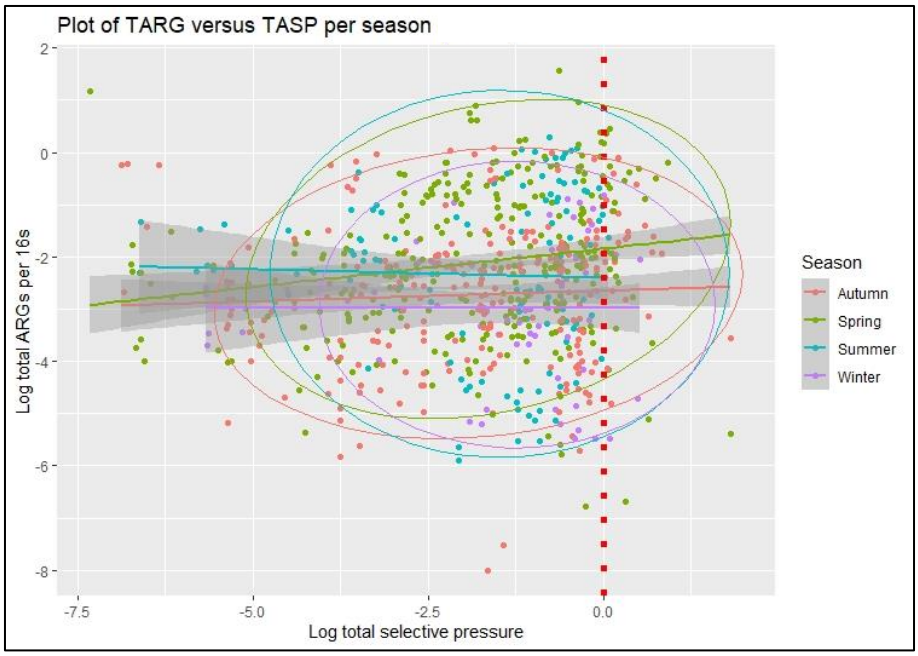


Figure 14: Scatterplot of the dataset (n=738) ordered by season. Spring = 305, Summer = 117, Autumn = 245, Winter = 71). A 95% predictive ellipse is drawn around datapoints to indicate the 95% probability area for new observations, assuming a normal bivariate distribution.

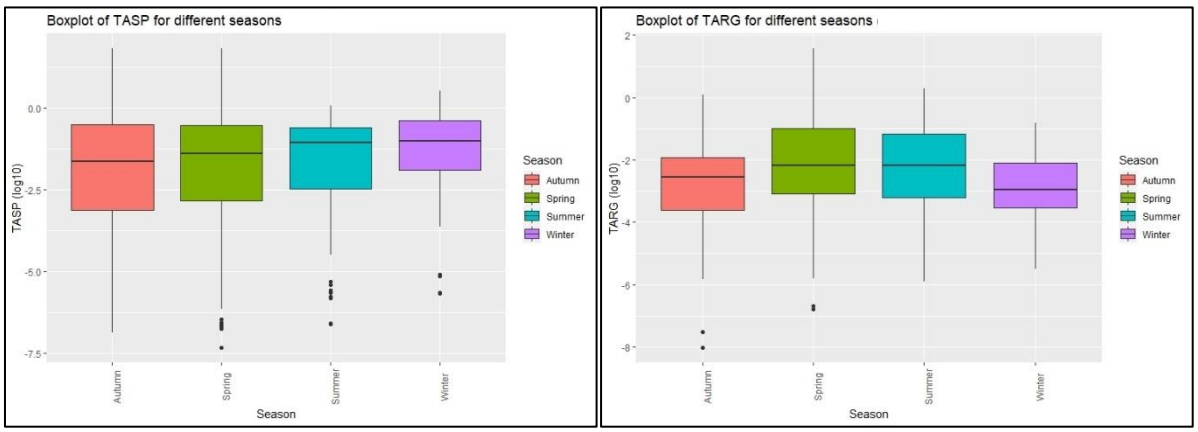


Figure 15: Boxplots showing median, Q1-Q3-percentile, maximum, minimum values and outliers of total selective pressure (TASP)(left) and total resistance gene abundance (TARG)(right) for different seasons.

Figures 14 and 15 show the data set ordered by season. The scatterplot in figure 14 indicates a lot of overlap between seasons. Regression lines are almost horizontal in summer, autumn and winter, but some positive correlation is seen for spring. Looking at the boxplots in figure 15, there seem to be some small differences in median values both in TASP and TARG. There are somewhat higher values in winter for TASP, but autumn and spring showing a very large spread. TARG shows higher values in spring and autumn, with maximum values highest in spring.

It should be noted however, that there are large differences in the size of the dataset per season, with spring containing 307 data points and winter only 74.

4.5.2 Pearson correlations

A Pearson correlation between TASP and TARG across different antibiotic classes is visualized in figure 16 for surface waters (left) and sediments (right). Only significant correlations are shown ($p < 0,05$).

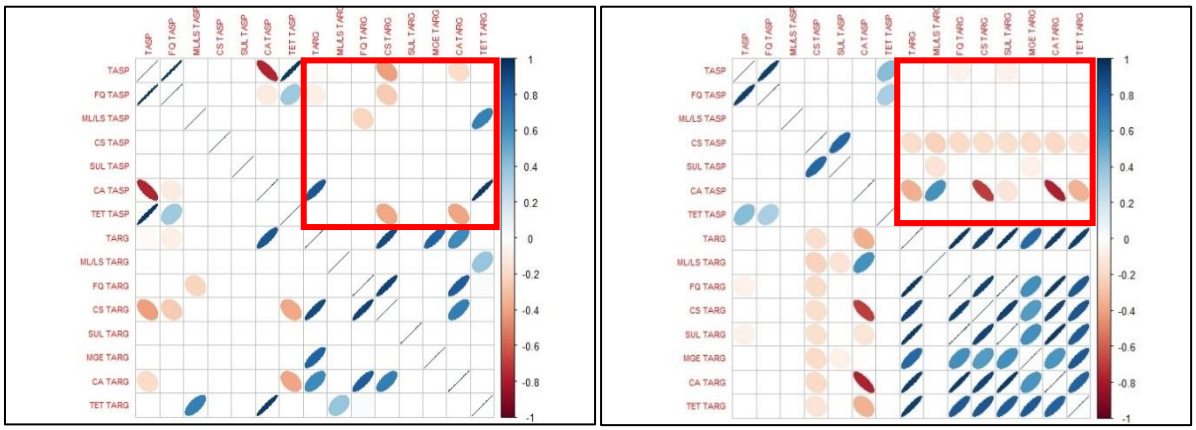


Figure 16: Pearson correlation plots between total selective pressures (TASP) and total relative gene abundance (TARG) for surface water samples (left) and sediment samples (right). Only significant correlations are shown (p<0,05). In the red rectangle the correlations between TARG and TASP are shown. A blue ellipse indicates a positive correlation. A red ellipse indicates a negative correlation. The sharpness of the ellipse is indicative of the strength of the correlation, with perfect correlation indicated by a diagonal line.
Sample sizes: TASP (SW=105, SD=99), CA (Amphenicols, SW=4, SD=8), CS (Cephalosporins, SW=12, SD=12), FQ (Quinolones, SW=53, SD=66), ML/LS (Macrolides/Lincosamides, SW=45, SD=40), MGE (SW=97, SD=88), SUL (Sulphonamides, SW=100, SD=71) and TET (Tetracyclines, SW=59, SD=83).

The correlation plots indicate no significant correlations between overall TASP and TARG in either surface waters or sediment. Also, no correlations are present between TASP and matching TARG for individual antibiotic classes. Correlations between overall TARG and MGEs are moderately strong in both matrices. There is a moderately strong positive correlations in surface waters between.

Macrolide selective pressure and Tetracycline resistance genes. Other correlations are mostly with Cephalosporins or Amphenicols, but these are based on a relatively small data set.

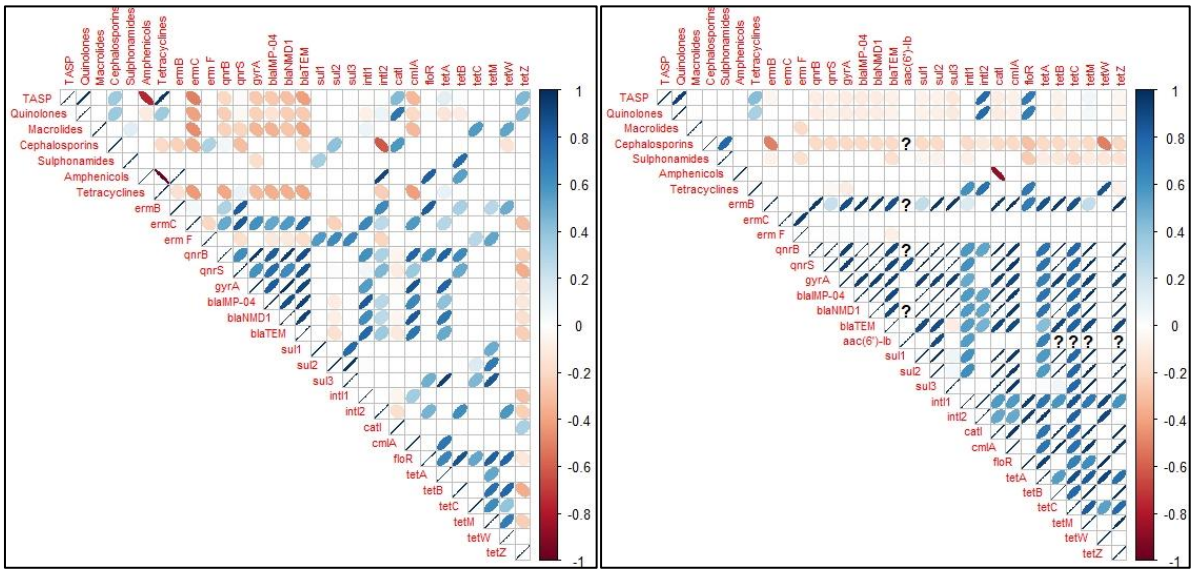


Figure 17: Correlation plots showing only significant (p<0,05) Pearson correlations between gene types and between gene types and TASP overall and for antibiotic classes in surface waters (left) and sediments (right). A blue ellipse indicates a positive correlation. The correlations between resistance genes and intI1 and intI2 are highlighted in the red rectangle. A red ellipse indicates a negative correlation. The sharpness of the ellipse is indicative of the strength of the correlation, with perfect correlation indicated by a diagonal line. Some gene types were left out because of a very limited number of data points.

In figure 17 correlations are shown between gene types in surface water (left) and sediment (right) and between gene types and selective pressure from antibiotic classes. Only significant correlations are shown (*p<0,05*).

In surface waters, selective pressure by Amphenicols is strongly correlated with the associated floR-gene and to *intI*2, but this is only based on a very small sample set (n=4). No other correlations are present between antibiotic classes and corresponding resistance genes. Cephalosporins (n=12) show strong negative correlations with *intI*2 in surface water.

In sediments a very strong negative correlation is found for Amphenicols and its corresponding resistance gene *cat*1. This is based on a small sample set (n=8). Another very strong and positive correlation is seen for Tetracyclines (n=99) and the associated resistance gene *tet*W. Tetracyclines also show relatively strong positive correlations with *intI*1 and *intI*2. Quinolones are positively correlated with *intI*2 in sediments.

There are numerous significant and moderate to very strong correlations between gene types in both matrices. Most notable in surface waters are Quinolone and Cephalosporin resistance genes who seem to correlate strongly amongst each other and with several other gene types including *intI*1,

*cml*A and *tet*A. Moderately strong and strong positive correlations are indicated between the MGEs *intl1* and *intl*2 with *erm*B, *erm*C, *qnr*B, *gyr*A, *bla*IMP-04, *bla*NMD1 and *bla*TEM*, tet*B *and tet*W.

Interestingly, in sediments correlations are even stronger among genes, with many near perfect correlations. MGEs *intl*1 and *intl*2 show moderate to strong positive correlations with all gene types except the genes conferring resistance to Macrolides (*erm*B, *erm*C and *erm*F). Macrolide resistance genes show overall the least correlation with other gene types in sediments.

4.5.3 Prevalence of gene types

In figure 18 the relative abundance of resistance genes in all samples were added together and sum totals are shown in the bar plot below. It would seem that five resistance gene types stand out as most prevalent. However, data sets differ substantially between gene types, because in each study different choices were made regarding the gene types to analyse. Some gene types were analysed more than others, influencing sum totals (see also appendix III). The sum total TARG of *bla*TEM is based on 49 detections, while *sul*1 is based on 189 and *intl*1 on 187 detections. Detection rates were high for all three genes. The bar plot is therefore partially an indication of research bias and accounting for sample size yields a different picture.

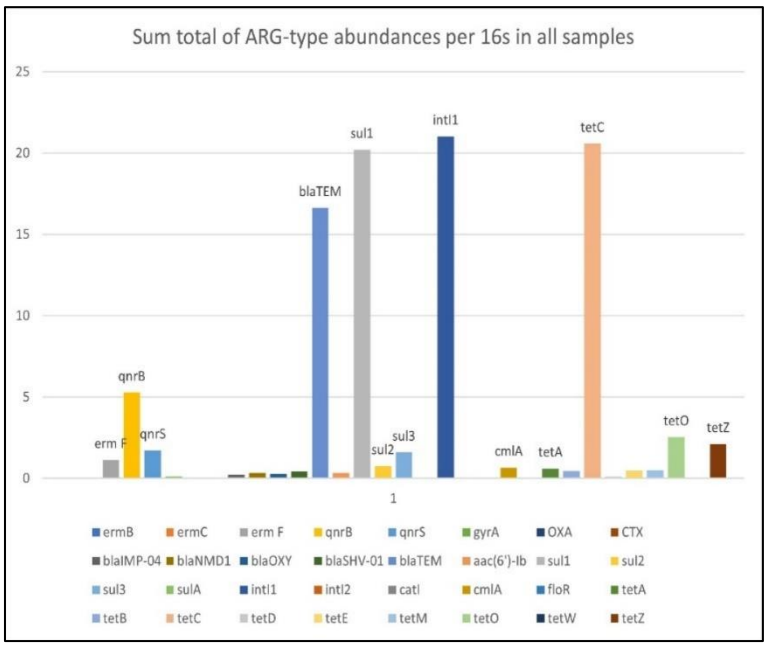


Figure 18: Sum totals for all gene types that were detected in the samples. Several gene types stand out, but sample sizes differ per gene type. Sample sizes: ermB=99, ermF=42, qnrB=41, qnrS=129, blaTEM=49, sul1=189, sul2=137, sul3=42, intl1=187, cmlA=42, tetA=127, tetB=80, tetC=88, tetM=109, tetO=7, tetW=99, tetZ=42 (see also appendix III).

Mean and median values shown in the boxplot below (fig.19) indicates that *tet*O is highest of all gene types. This is based however on only 7 values which were measured by Ohore *et al* (2019) in sediments from Taihu Lake in China. Highest values following *tet*O are three Cephalosporin (or β-

lactam) resistance genes resp. *bla*OXY, *bla*TEM and *bla*SHV-01. These values were based on only 7 detections for both *bla*OXY and *bla*SHV-01 , but on 49 detections for *bla*TEM.

Next, *tet*Z (n=42) and *intI*1 (n=187) are the runners up. *tet*E (n=7) and *tet*C (88) have moderately high median values compared to other genes, but have a very large spread. Maximum values -indicated by the high end of the whiskers- of tetC are highest of all gene types.

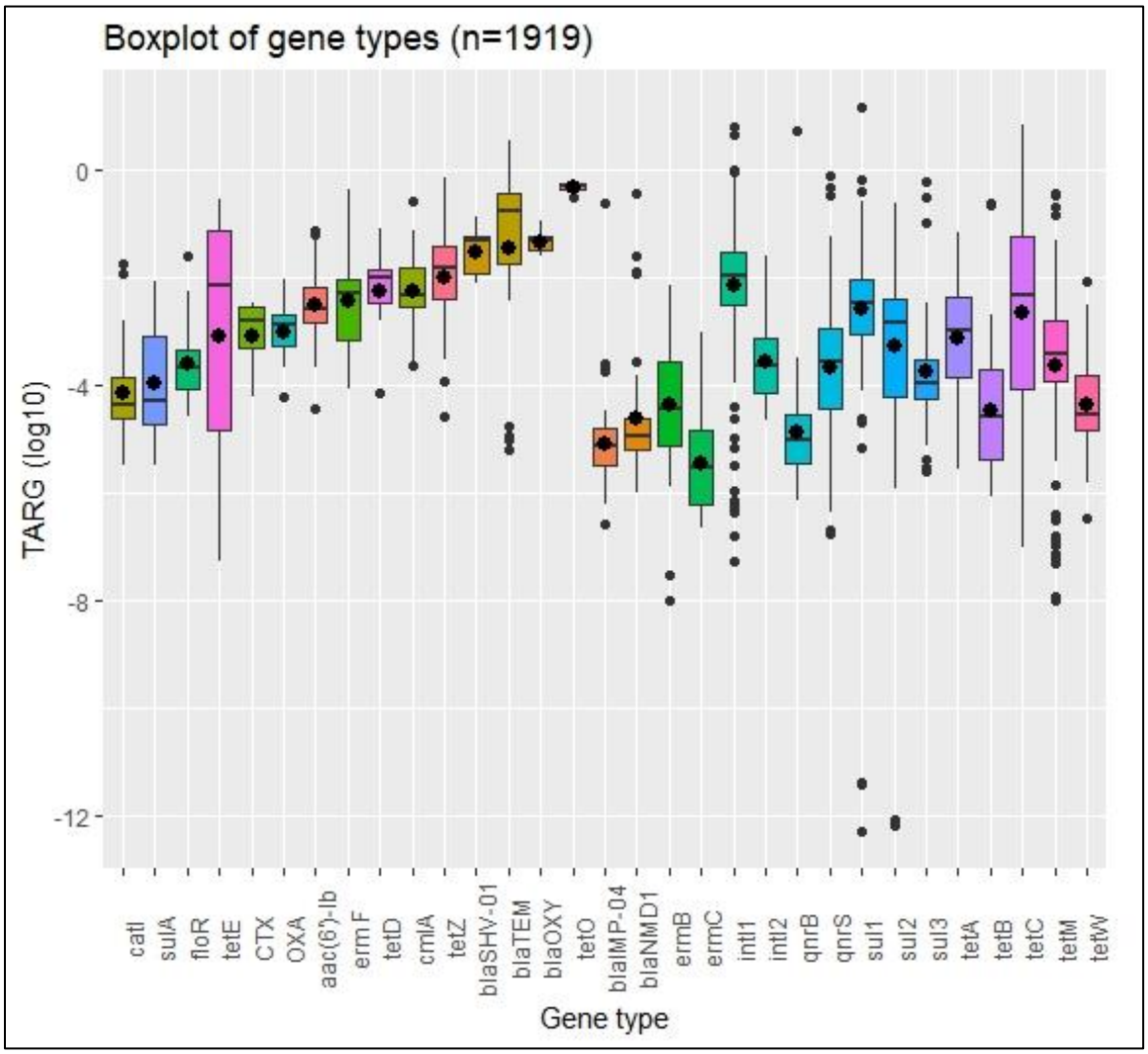


Figure 19: Boxplot showing median, mean, Q1-Q3-percentile, maximum, minimum values and outliers for resistance gene types.

4.5.4 Highlights from the data exploration

From the data exploration, no clear indication was found for a relationship between TASP and TARG.

However, relative to other antibiotic classes, Sulphonamides and Quinolones seemed to show an interesting trend. While TASP from Sulphonamides was comparatively low, TARG from Sulphonamides was comparable to other classes. For Quinolones it was the reverse. Comparatively high TASP values were observed for Quinolones, but relatively low TARG.

From the limited non-Chinese data, there were no indications of difference between countries.

Some clustering of TASP-values seems to be present for different antibiotic classes. This is especially apparent in the datasets within the studies.

Some differences were found when zooming in on the different matrices. TASP-values from Quinolones were highest in sediments, while values for Tetracyclines together with Quinolones were highest in surface waters. TASP from Sulphonamides was lowest compared to other classes in both matrices.

For TARG-values, there was much spread within the classes in both matrices. TARG from Cephalosporins were high in both matrices compared to other classes, especially in sediments. TARG from Quinolones was relatively low in both matrices.

Overall, the spread of TASP and TARG was highest in sediment samples.

Differences between seasons seemed minor. Somewhat higher values were measured in winter for TASP, but with autumn and spring showing a very large spread. TARG seemed overall somewhat higher values in spring and autumn, with maximum values highest in spring. But samples sizes differed substantially between seasons.

Correlation plots show no significant correlations between TASP and TARG, not overall or for individual antibiotic classes.

The only significant correlation between selective pressure and corresponding gene types (based on an ample sample size) was found in sediments for Tetracyclines and *tet*W. Several positive correlations in both matrices were present between antibiotic classes and unrelated gene types.

A strong negative correlation was found between Cephalosporin selective pressure and *intI*2 in surface waters. Strong positive correlations were seen between Quinolones and Tetracycline selective pressures and the MGEs in sediments.

Strong positive correlations were present amongst several gene types in both matrices. Correlations among gene types were comparably stronger in sediments.

There was a moderately strong and positive correlation for TARG and MGEs in both matrices. In surface waters MGEs correlate positively with *erm*B, *erm*C, *qnr*B, *gyr*A, *bla*IMP-04, *bla*NMD1 and *bla*TEM*, tet*B *and tet*C. In sediments MGEs correlate positively with all gene types except those conferring resistance to Macrolides.

From the resistance gene types that were analysed in sufficient numbers in studies (n>40), *bla*TEM, *intI*1 and *tet*Z showed highest abundance values. *tet*C did not show very high median values compared to other gene types, but has a very large spread, showing the highest maximum values of all gene types.

4.6 DATA ANALYSIS

4.6.1 Model architecture

For the analysis of the data, a linear mixed effects models (LMEM) is used. As indicated in the introduction, antibiotic resistance in the environment can result from several factors that interact in complex ways. Compared to traditional linear regressions models, LMEM allow for the incorporation of a combination of explanatory variables as either fixed or random factors, where random factors make possible a hierarchical modelling of the data in cases of non-independence or nested data (Zuur *et al*, 2009; Harrison *et al*, 2018). LMEM thus allows for a more complex modelling of the data than simple linear regression models, which makes it suitable for the analysis of antibiotic resistance. Also, because data is collected from different studies, greater similarity of data within studies, can also be modelled.

It is hypothesized that antibiotic selective pressure will have an effect on the relative abundance of antibiotic resistance genes in the aquatic environment. TARG is therefore taken as the response variable and TASP as the explanatory variable or fixed factor. *Class* is added as an additional explanatory or fixed factor, assuming a difference in effect of each class on TARG owing to differences in physicochemical behaviour in the environment for instance influencing bioavailability and owing to differences in the mechanism of action of each class, influencing resistance responses in bacterial communities. It is further assumed that resulting TARG will be more similar within individual seasons due to to more similar temperatures and hydrological conditions. Therefore, *Season* is added as random factor. Next to season, *Matrix* is added as a random factor, assuming more similarity in resistance response within the same matrix, with sediment providing a more compact and stable matrix for gene transfer processes, than the more dynamic surface water. From data exploration, there was indication that antibiotic classes within studies show stronger clustering. So, as a third random factor *Class* nested in *Study* was added. This resulted in the following full model (eq.6).

Full model:

$$TARG = TASP + Class + (1|Season) + (1|Matrix) + (1|Study/Class) + \varepsilon \quad \text{(eq.6)}$$

Where ε is the random error.

For data analysis and visualization the packages ‘lme4’, ‘AICcmodavg’, ‘pbkrtest’, ‘MuMIn’, ‘ggplot2’, ‘corrplot’ and ‘ggcorrplot’ were used with the statistical software RStudio (Version 1.2.5001).

5 RESULTS AND DISCUSSION

5.1 RESULTS

In this research the correlation between antibiotic selective pressure and antibiotic resistance was investigated based on the sample data (n=204) obtained from 9 studies. Data analysis was performed using a linear mixed effect model (LMEM).

To find the best fit model for the data, different candidate models were created from different combinations of the random effects. These were fitted using restricted maximum likelihood (REML) estimations. The corrected Akaike Information Criterion (AICc) was used to evaluate the random structure (table 4).

Random Structure	*k*	AIC_c	ΔAIC_c	*w*	*L*
(1|Season) + (1|Matrix) + (1|Study/Class)	12	1348,481	1,471805	3,239008e-01	-661,9233
(1|Season) + (1|Matrix)	10	1599,629	252,620207	9,423873e-56	-789,5919
(1|Season) + (1|Study/Class)	**11**	**1347,009**	**0,00000**	**6,760992e-01**	**-662,2367**
(1|Matrix) + (1|Study/Class)	11	1384,881	37,871965	4,038477e-09	-681,1727
(1|Season)	9	1603,667	256,658221	1,251370e-56	-792,6517
(1|Matrix)	9	1637,163	290,154434	6,664623e-64	-809,3998
(1|Study/Class)	10	1637,163	39,018723	2,276158e-09	-682,7911

***Table 4**: The random structure with lowest AICc is marked in bold, k=number of estimated parameters, w=Akaike weight, L= restricted log-likelihood.*

For establishing the significance of the fixed effect, F tests with Kenward-Roger approximations were used (table 5).

Fixed effects	*F-statistic*	*df*	*ddf*	*p-value*
TASP + Class (41-42)	3,4892	6	19,2057	0,01673*
Class (42-43)	**4,039**	**5**	**15,070**	**0,01591***
TASP (42-44)	0,6505	1	493,4411	0,4203
TASP x Class (42-45)	1,6964	5	480,2231	0,1339

***Table 5**: F-test with Kenward-Rogers approximations for the fixed effects, with significant fixed effects marked in bold.*

The uncertainty of the fixed and random terms was computed using a bootstrap method performing 1000 iterations. Uncertainty was expressed in 95% confidence intervals. As a final step, marginal and conditional coefficients were calculated for the best fitting model (table 6 and 7).

Fixed Effects	Coefficients	SE	*t*-value	LCI_{95}	UCI_{95}
Intercept	-2,8604	0,8025	-3,564	-4,3938064	-1,20915
ClassCS	1,6452	0,7484	2,198	0,1000056	3,1269241
ClassFQ	-0,5974	0,6521	-0,916	-1,9420499	0,6779273
ClassML/LS	0,2243	0,6728	-0,333	-1,6285494	1,1360693
ClassSUL	0,6795	0,6577	1,033	-0,6565748	1,9020862
ClassTET	0,6684	0,6501	1,028	-0,6574808	1,9572323
R2-marginal 0.109					

***Table 6**: Coefficients, standard error (SE), t-value and 95% lower and upper confidence intervals (LCI95 and UCI95) for the fixed effects using bootstrap method performing 1000 simulations.*

Random Effects	Variance	Sd	LCI_{95}	UCI_{95}
Class : Study	0,4705	0,686	0,3954592	0,9813888
Study	1,6703	1,292	0,4887540	2,0279089
Season	0,4058	0,637	0,1113378	1,1829833
Residual	0,6707	0,819	0.7987441	0,8726545
R2-conditional 0.814				

***Table 7**: Variance, standard deviation (Sd) and 95% lower and upper confidence intervals (LCI95 and UCI95) for the random effects using bootstrap method performing 1000 simulations*

No indication of a linear relation was found between TASP and TARG. Data analysis bore out that the continuous fixed factor of TASP did not significantly explain TARG. This result differs from comparable studies, where a significant relationship between TASP and TARG was found (Duarte *et al,* 2019) or where correlations were found between total antibiotics concentrations and ARG-abundance (Chen *et al*, 2013; Gao *et al*, 2018; Xu *et al*, 2018; Yan *et al*, 2018; Liang *et al*, 2020).

The best fit fixed structure found to describe the data, showed that antibiotic class (*Class*), not total selective pressure (TASP), correlated significantly with total resistance genes (TARG) (Table 5). Class accounted for close to 11% of the variance (Table 6). This relatively low percentage corresponds with the findings that ARG-abundance in the natural environment is the result of complex interactions between microbial communities, biotic and abiotic environmental factors and anthropogenic

pressures (Bai *et al*, 2019; Bengtsson-Palme *et al*, 2018b; Smalla *et al*, 2018). So it is not expected that one factor should contribute to a very high degree.

Class in the LME model is a categorical fixed factor. Therefor its significance does not imply any linear relationship with TARG. Rather the differences in deviations from the TARG-mean were significantly explained by antibiotic class. This could indicate that different antibiotic classes affect total antibiotic resistance in different ways. Or more neutrally, that the relationship between antibiotic selective pressure and the abundance of corresponding resistance genes differs per class.

Including the random effects explained 81.4% of the variance in the data (Table 7). The most parsimonious random structure included season (*Season*) and class nested in study (*Study/Class*), but not matrix (*Matrix*)(Table 4). Seasonal or temporal factors influencing AR have been found in a number of studies (Amos *et al*, 2015; Sanderson *et al*, 2018; Xu *et al*, 2018; Roberto *et al*, 2019; Wan *et al*, 2019; Di Cesare *et al*, 2020; Wang *et al*, 2020b), including different patterns for ARGs conferring resistance to different antibiotic classes (Xu *et al*, 2018; Roberto *et al*, 2019).

Selective pressure in samples was highest from Quinolones in both matrices, followed by Cephalosporins and Tetracyclines and lowest for Sulphonamides in both matrices. Values above TASP=1 were measured for both Quinolones and Tetracyclines in surface water and for Quinolones in sediment, indicating a potential risk. Interestingly, while TASP from Sulphonamides was comparatively low, TARG from Sulphonamides was comparable to other classes. For Quinolones it was the reverse. Comparatively high TASP values were observed for Quinolones, but TARG-values were relatively lowest of all classes. TARG from Cephalosporins were high in both matrices compared to other classes, especially in sediments. Overall, there was much spread of TARG-values within the classes in both matrices.

Additionally, Pearson correlations showed that the only correlation between TASP and the corresponding resistance genes was found in sediments for Tetracyclines and *tet*W. But several positive correlations between TASP from antibiotic classes and unrelated resistance genes were present, for instance a relatively strong positive correlations ($p<0.05$) between selective pressure from Macrolides and Tetracycline resistance gene abundance in surface water samples. Regarding the mobile genetic elements *intI*1 and *intI*2, Cephalosporins correlated negatively with *intI*2 in surface waters, but Quinolones and Tetracyclines correlated positively and strong with MGEs in sediments.

Amongst gene types, several strong positive correlations were present in both matrices, but correlations were comparably stronger in sediments, which might be explained by the denser and more stable environment that is provided in sediments offering better circumstances for HGT.

Overall, there was a moderately strong and positive correlation for TARG and MGEs in both matrices. In surface waters MGEs correlate positively with *erm*B, *erm*C, *qnr*B, *gyr*A, *bla*IMP-04, *bla*NMD1, *bla*TEM*, tet*B *and tet*C. In sediments MGEs correlate positively with all gene types except those conferring resistance to Macrolides. This is in line with other findings of significant correlations between MGEs -particularly *intl1*- with ARGs (Yan *et al*, 2018; Deng *et al*, 2020; Leng *et al*, 2020; Liang *et al*, 2020) pointing to the important role of MGEs in the transfer of ARGs (Gillings *et al*, 2015 and 2017).

Highest ARG-abundances were found for mobile genetic element *intl*1 and for the resistance genes *bla*TEM and *tet*Z, conferring resistance to respectively Cephalosporins and Tetracyclines. *tet*C did not show very high median values compared to other gene types, but had a very large spread, showing the highest maximum values of all gene types.

5.2 Discussion

In this study, data extracted from environmental samples measuring antibiotic concentrations and resistance gene abundance, showed much spread and scatter between selective pressure and ARG-abundance. No significant relationship was found between antibiotic selective pressure and antibiotic resistance genes in surface water and sediments. But both antibiotic class and temporal factors could explain 81% of the variance. Pearson correlations did find strong associations amongst many resistance genes in both matrices. *intl1* -indicated for its important role in HGT- ranked among the most abundant genes found and showed correlation with selective pressures from Quinolones and Tetracyclines in sediment, but showed no correlation with TASP. These results are in line with the growing body of research indicating that many factors influence the evolution of AR in the environment and the relationship between TASP from antibiotic pollution and TARG is complex and possibly non-linear.

In part this could be due to processes determining the fate and behaviour of antibiotic residue in the environment. Chronic sub-MIC levels of antibiotics in the environment can steer bacterial evolution towards resistance (Hiltunen *et al*, 2017) and many antibiotic drugs are persistent in the environment (water, soil, sediment), meaning they can exert their influence long after being excreted from humans or farm animals (Gaze *et al*, 2013). But speciation, stability and mobility of antibiotics and their metabolites are difficult to predict under complex environmental circumstances and knowledge is particularly scarce. Antibiotics are usually metabolized in humans and animals before they reach the environment. Upon entry to the environment, the parent compound and its metabolites undergo further structural changes from biotic and abiotic processes, for instance during waste water treatment, resulting in changed physicochemical and pharmaceutical properties (Carvalho & Santos, 2016).

Also other explanations of the evolution of AR in the environment are possible. Although several studies found indications of a relationship between TASP and TARG, many have not. These studies rather point to the importance of other factors, like the relevance of co-emission of ARGs and resistant bacteria from anthropogenic sources (Brown *et al*, 2019; Karkman *et al*, 2019) or found a more relevant relationship between bacterial community structure and ARG-abundance (Huerta *et al*, 2013; Zhou *et al*, 2017). In Karkman et al (2019) fecal pollution could explain antibiotic resistance in most environments, with the exception of environments highly polluted with antibiotics. In other studies the prominent role of continuous wastewater discharges, including organic matter, solid particles, bacteria and ARGs, in the spread of ARGs was emphasized above environmental selection (Kumar & Pal, 2018; Brown *et al*, 2019).

Especially metagenomic studies found that the characteristics of bacterial communities could better explain ARG presence and patterns than environmental antibiotic concentrations (Novo *et al*, 2013; Huerta *et al*, 2013; Zhou *et al*, 2017; Gao *et al*, 2018; Brown *et al*, 2019; Reddy *et al*, 2019; Zheng *et al*, 2019; Deng *et al*, 2020, Leng *et al*, 2020). Fondi *et al* (2016) found evidence that ecology predominantly shaped the resistance gene pools in these bacterial communities, identifying similarities in communities in similar ecological niches like seawater, freshwater, soil, gut and air, with relatively little overlap and exchange between niches. Each of the environmental compartments seems to have a distinct group of bacteria that are the primary host of ARGs (Zeng *et al*, 2019). But although these niches remain relatively separate, fresh water seems to connect a number of these ecological niches, acting as a bridge for the exchange of some highly mobile ARGs between different compartments (Fondi *et al*, 2016).

These findings indicate that the direct effect of antibiotics on promoting AR is obscured or might be trumped by other more systemic processes. But this does not rule out the influence of antibiotics. There is evidence that antibiotics are equally able to exert influence on the composition, diversity and functioning of bacterial communities as well (Huerta *et al*, 2013; Balcázar *et al*, 2015; Roose-Amsaleg en Laverman, 2016; Zhou *et al*, 2017; Chen *et al*, 2019e; Deng *et al*, 2019; Roberto *et al*, 2019). Roose-Amsaleg en Laverman (2016) found influence of antibiotics on microbial functioning and their role in biogeochemical cycles. Zheng *et al* (2019) showed influence of antibiotics on the (reduced) functioning of bacterial communities in the biodegradation of perchlorate. This points to a more indirect influence of antibiotic pressures (Deng *et al*, 2020) on AR by influencing bacterial communities together with other environmental stressors, like heavy metals and water quality parameters (Zhou *et al*, 2017). Resistance might be more related to forces that shape and influence microbial communities, nonetheless antibiotics seem to be one of those forces.

To further enhance insight into the relationship between AR and selective pressure, future research is suggested to combine metagenomic research into the changes in functioning, diversity and composition of bacterial communities in different environmental compartments with the analysis of environmental pressures from antibiotics by different classes and heavy metals in the same samples.

It is further suggested to incorporate additional parameters pertaining to water quality (e.g. pH, organic carbon, solids, nutrients, temperature, metal concentrations), hydrological conditions (flow rate, water depth) and spatiotemporal data (land use, land cover, precipitation trends, seasonal trends).

5.3 LIMITATIONS

This meta-analysis has a number of limitations. Because many studies -for different reasons- did not qualify for incorporation in this study, data from a only a limited number of studies (n=9) were extracted for data analysis. Additional sample data will increase the robustness of statistical findings and will allow for a deeper analysis of the data, for instance by incorporating additional meta-data in the analysis.

Also, research using qPCR involves the preselection of resistance gene markers, which could bias analysis in favour of the most investigated or clinically relevant genes, underexposing possibly relevant but less researched genes. Moreover, it is not possible to find new genes that might be relevant to AR-enhancement in the environment. A similar bias can be involved in the choice for the analysis of antibiotics.

Because antibiotic concentrations in themselves are less telling of selective pressure, an indication of selective pressure was calculated using the PNEC. The development of a PNEC that is tailored to bacteria living in complex environmental conditions and often in complex microbial communities, is however still in its infancy. It is still debated which species is most useful for the calibration of the PNEC and which environmental endpoints and protection goals should be central (Bengtsson-Palme & Larsson, 2016b and 2018c; Le Page *et al*, 2017). Also, research data is (very) scant on many antibiotics for establishing a PNEC. Most PNECs used in this meta-analysis were suggested by Bengtsson-Palme & Larsson (2016b) based on a similar methodology that they developed, but for some antibiotic types that were not included in their research, other sources were found (Wang *et al*, 2020b; Zhang *et al*, 2020). The PNECs from these additional sources were established using different methodologies. These combined factors yield uncertainty in the establishment of selective pressures.

Finally, linear mixed effects models were applied to the data, making possible a more complex modelling of the data and the incorporation of non-independence. These models however involve a subjective choices that can influence outcomes, most importantly the choice for fixed and random effects and whether data is nested or not. Also, statistical results of LMEM are not always easy to interpret. Results from statistical analysis should therefore not be taken as an indication of causation, but only serve as an aid in the interpretation of complex data.

6 APPENDICES

6.1 APPENDIX 1: OVERVIEW OF METAGENOMIC STUDIES

Appendix 1: Overview of metagenomic studies researching the correlation between antibiotics concentrations and resistance genes abundance in surface water and sediments (Blue header = data extracted, Pink header = data not (yet) extracted)

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
1. Bai et al, 2019	**China** 12 surface water samples were taken from drinking water sources in the upper and middle branch of Huaihe River Basin. It was reported that the antibiotic emission into Huaihe River basin is over 3000 tons per year, and is listed as one of the highest sites of antibiotic emission in surface waters across China (Zhang et al., 2015). Date: 2017-November	Enrofloxacin (FQ) Norfloxacin (FQ) Ciprofloxacin (FQ) Ofloxacin (FQ) Fleroxacin (FQ) Tetracycline Chlortetracycline (TET) Oxytetracycline (TET) Metacycline (TET) Doxycycline (TET) Sulfadiazine (SUL) Sulfamethoxazole (SUL) Sulfathiazole (SUL) Sulfamerazine (SUL) Sulfadoxine (SUL) Sulfadimethoxine (SUL) Sulfamethoxidiazine (SUL) Sulfamethazine (SUL)	Rationale not explicitly stated. *Highest concentrations found*: 1. Metacycline 2. Doxycycline 3. Oxytetracycline 4. Chlortetracycline 5. Sulfamethoxazole 6. Tetracycline 7. Sulfamethoxidiazine	Tetracyclines (TET) Sulphonamides (SUL) Bacitracin (PEP) CAMP (PEP) Vancomycin (PEP) Beta-lactams (BeLa) Aminoglycosides (AG) Lincosamides (ML) Multidrug Trimethoprim (DHFR) Fosmidomycin Chloramphenicol Others	Most abundant: 1. Beta lactamase 2. Others 3. Multidrug 4. Bacitracin 5. Tetracyclines Least abundant: 1. Chloramphenicol 2. Trimethoprim 3. Fosmidomycin 4. Sulphonamides	PPM ARG-like reads per million reads (not assembled) Range: From 657 ppm in sample 5 To 1023 ppm in samples 1 and 9	No

Full citing
Bai, Y., Ruan, X., Xie, X., & Yan, Z. (2019). Antibiotic resistome profile based on metagenomics in raw surface drinking water source and the influence of environmental factor: A case study in Huaihe River Basin, China. *Environmental Pollution*, *248*, 438–447. https://doi.org/10.1016/j.envpol.2019.02.057

Correlations found within this study
*Water quality (antibiotics, COD, TP, TN, NH4) seems to contribute little to the ARGs abundance.
*Number of livestock, health facility and agricultural areas in the watershed showed a strong influence on the total ARG abundance in drinking water sources, among which land use type was the dominant factor.
*The specific biogeographic distribution pattern of ARGs demonstrated that total ARG abundance was also greatly impacted by the natural condition of the drinking water resources.
*In the present study, the limited correlation between ARG and microbial community composition, as well as the absence of class 1 integron (intI1) in sampled drinking water sources confirmed the low HGT potential.
*No pathogen was identified in the sampled drinking water sources. While *Polynucleobacter* was an abundant ARGs host in the microbial community, and was significantly related to the ARG profile.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
2. Chen et al, 2013	**China** 2 sediment samples were taken from the Pearl River Estuary In total 9 samples were taken in this study, but only two also provided antibiotics concentrations that were above the LOQ. Date: 2011-June	Enrofloxacin (FQ) Norfloxacin (FQ) Ofloxacin (FQ) Tetracycline (TET) Sulfadiazine (SUL) Sulfamethoxazole (SUL) Sulfamethazine (SUL) Erythromycin (ML) Roxithromycin (ML)	*Rationale*: Common antibiotics, widely used in the surrounding area *Highest concentrations found*: Norfloxacin (FQ) (found in both samples) Other antibiotics were found in low concentrations or were not detected in both or one sample	Fluoroquinolone (FQ) Sulphonamides (SUL) Polypeptide (PEP) Beta-lactam (BeLa) Aminoglycoside (AG) Macrolide (ML) Erythromycin (ML) Multidrug Fosmidomycin Chloramphenicol	*Most abundant*: 1. Sulphonamides 2. Fluoroquinolones 3. Aminoglycoside *Least abundant*: 1. Erythromycin 2. Beta-lactamase 3. Tetracycline *Main mechanisms of resistance:* efflux pump Antibiotic inactivation	PPM ARG-like reads per million reads (not assembled) *Range*: From 9,54 ppm in sample A8 To 3,39 ppm in sample B2	No

Full citing
Chen, B., Yang, Y., Liang, X., Yu, K., Zhang, T., & Li, X. (2013). Metagenomic Profiles of Antibiotic Resistance Genes (ARGs) between Human Impacted Estuary and Deep Ocean Sediments. *Environmental Science & Technology*, *47*(22), 12753–12760. https://doi.org/10.1021/es403818e

Correlations found within this study
* The abundance of ARGs significantly correlated with the abundance of the two MGEs (e.g., integrons and plasmids) on the level of $p < 0.01$ in the PRE and SCS sediments, and a relationship of significance was also observed for the diversity of ARGs and MGEs in sediments ($p < 0.01$). These results strongly suggest that MGEs play an important role in the dissemination of ARGs in the aquatic environment.
*Antibiotic concentrations are generally low in the environments; even near sources of pollution. Such subinhibitory levels of antibiotics could not be expected to exert a significant stress for selecting ARB and ARGs in the ambient environments. Alternatively, it is considered that the release of bacteria from the human and/or farmed animal flora is the predominant reason for the wide dissemination of ARGs in the human impacted environments.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
3. Chen et al, 2019	**China** 4 sediment samples taken from Chaobai River. The river not only is the main source of drinking water for Beijing, but also provides the important water resource for agricultural irrigation within the basin drainage area. Date: 2017-December	Sulfadiazine (SUL) Sulfachlorpyridazine (SUL) Sulfamethoxazole (SUL) Sulfamethazine (SUL) Chlortetracycline (TET) Oxytetracycline (TET) Tetracycline (TET) Ciprofloxacin (FQ) Enrofloxacin (FQ) Lomefloxacin (FQ) Norfloxacin (FQ) Ofloxacin (FQ) Erythromycin (ML) Roxithromycin (ML) Tylosin (ML) Trimethoprim (TMP)	A total of 16 specific antibiotics frequently detected in the Haihe River system (Chen et al., 2018) were analysed in this study. *Highest concentrations found*: pm *Lowest concentrations found*: pm	Trimethoprim Fosmidomycin Chloramphenicol Sulphonamides Quinolone Fluoroquinolones CAMP (PEPTIDES) Aminoglycoside Vancomycin Tetracycline Bacitracin Multidrug Fosfomycin Beta lactam Peptide Aminocoumarin Rifampin Polymyxin MLS (macrolides) Triclosan Mupirocin Kasugamycin Glycopeptide Bleomycin	Most abundant: 1. Multidrug 2. Bacitracin 3. MLS 4. Sulphonamides 5. Quinolones	In Chen et al, 2019b: Coverage x/Gb (assembled contigs) In Chen et al, 2019a: Copies per 16s is used (also taking gene length into account). Results in this article are not assembled. PPM could be derived from the occurrence of ARGs in each sample divided by the number of million clean reads per sample. *Range*:	No

						From 80,01 ppm to 191,61 ppm	

Full citings

Chen, H., Bai, X., Li, Y., Jing, L., Chen, R., & Teng, Y. (2019a). Characterization and source-tracking of antibiotic resistomes in the sediments of a peri-urban river. *Science of The Total Environment*, *679*, 88–96. https://doi.org/10.1016/j.scitotenv.2019.05.063

Chen, H., Bai, X., Jing, L., Chen, R., & Teng, Y. (2019b). Characterization of antibiotic resistance genes in the sediments of an urban river revealed by comparative metagenomics analysis. *Science of The Total Environment*, *653*, 1513–1521. https://doi.org/10.1016/j.scitotenv.2018.11.052

Chen, H., Chen, R., Jing, L., Bai, X., & Teng, Y. (2019c). A metagenomic analysis framework for characterization of antibiotic resistomes in river environment: Application to an urban river in Beijing. *Environmental Pollution*, *245*, 398–407. https://doi.org/10.1016/j.envpol.2018.11.024

Correlations found within this study

*Host tracking analysis identified *Dechloromonas, Pseudoxanthomonas, Arenimonas, Lysobacter* and *Pseudomonas* as the major hosts of ARGs.
*More importantly, the co-occurrence analysis via ACCs showed a strong association of ARGs with B/MRGs and MGEs, indicating high potential of co-selection and active horizontal transmission for ARGs in the river environment, likely driven by the frequent impact of anthropogenic activities in that area.
*It can be seen most of the identified genera exhibited high correlation with multidrug resistance genes.
*The class 1 integron was positively correlated with most of the targeted ARGs, while no significant correlations were observed between the ARGs and selective pressure factors, including antibiotics and metals.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
4.Fang et al, 2018	**China** 6 samples (3 sediment and 3 surface water) were taken upstream and downstream from a pigfeed lot in Cixi, Zhejiang. Date: 2016-July 15	Enrofloxacin (FQ) Norfloxacin (FQ) Ciprofloxacin (FQ) Tetracycline (TET) Chlortetracycline (TET) Oxytetracycline (TET) Sulfadiazine (SUL) Sulfamethoxazole (SUL) Erythromycin (ML) Chloramphenicol	Eleven representative antibiotics were selected in this pig feedlot, and their average annual usage over five years (from 2013 to 2017) is summarized. *Highest concentrations found*: 1. Ciprofloxacin (FQ) mostly in SD 2. Norfloxacin (FQ) mostly in SD 3. Enrofloxacin (FQ) mostly in SD 4. Erythromycin (ML) only in SD *Lowest concentrations found*: 1. Sulfamethoxazole (SUL) only in SD 2. Chloramphenicol (only in SW)	Fluoroquinolones Tetracyclines Sulphonamides Beta-lactams Aminoglycosides Lincosamides Macrolides MLS Multidrug Chloramphenicol Acridines Others	The most abundant ARG type in stream sediments was: 1. Tetracycline (tetM, tetA(P), tetA408, and tetA(G)) 2. Sulphonamides (sul1), AGR (aac and aac6) 3. Macrolide (MLR) 4. Multidrug (mexF). The most abundant ARG type in stream water was: 1. Multidrug (mexF, OprB, and NodT) 2. Tetracycline (tetA(G), tetM, tetX2, tetA408, tetA(P), tetX, tetA(33), and tetW) 3. Aminoglycosides (aac, aadA, aac6, and aadB) 4. Sulphonamides (sul1) 5. Chloramphenicol 6. Beta lactam (CARB-8, CARB-5, and OXA).	ARG-like iTags per million iTags (assembled; so not quite the same as ppm) *Range*: From 22,2 iTags per million to 160,6 iTags per million.	Yes, partial

Full citing

Fang, H., Han, L., Zhang, H., Long, Z., Cai, L., & Yu, Y. (2018). Dissemination of antibiotic resistance genes and human pathogenic bacteria from a pig feedlot to the surrounding stream and agricultural soils. *Journal of Hazardous Materials*, *357*, 53–62. https://doi.org/10.1016/j.jhazmat.2018.05.066

Correlations found within this study

*It is mentioned that multidrug resistance seems to be correlated to manure sources: "It is noteworthy that the much higher abundance of MDR genes was found in DSW and NEW compared to USW and FPM, indicating that the MDR genes could mainly be disseminated into the downstream and estuary water of the stream along the flow of stream from the discharge site of pig sewage."
*Pearson's bivariate correlation analysis showed significant ($P \leq 0.05$) positive correlations on ARGs between pig manures and other samples.
*Furthermore, significant ($P \leq 0.05$) positive correlations were observed between antibiotic residues and ARGs in FPM, CPM, DOW, DOS, **DSW**, **NES**, or NGS samples (Table S8), and significant ($P \leq 0.05$) positive correlations were also found between residues of sulphonamides, tetracyclines, fluoroquinolones, macrolides, chloramphenicol's, and the corresponding abundance of ARG types among all samples.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
5.Garner et al, 2016	**USA** Samples were taken from Poudre River in Colorado at several sites and several moments in time, 12 months before a flood and 3 and 10 months after. Surface water samples from 2 sites were used for metagenomic analysis (n=6). Antibiotics concentrations were only analysed for the post-flood samples (n=4) Date: 2013-December and 2014-July	Sulfamethoxazole (SUL) Sulfamethazine (SUL) Erythromycin (ML) Azithromycin (ML) Clarithromycin (ML)	*Highest concentrations*: Sulfamethoxazole Erythromycin	Fluoroquinolones Tetracycline Peptide Polymyxin Glycopeptide Beta-lactams Aminoglycosides MLS Multidrug Aminocoumarin Trimethoprim Fosfomycin Rifampin	1. Trimethoprim (39%) 2. Multidrug (30%) 3. Polymyxin (11%) 4. Aminocoumarin (4%) 5. Peptide (4%) 6. Tetracycline (3%) *Most common mechanism of resistance was:* efflux (46%), followed by antibiotic target replacement (39%), cell wall charge alteration (8%), antibiotic inactivation (5%), and molecular bypass (2%)	ARGs per 16s, taking gene length differences into account. *Range*:	Yes

Full citing

Garner, E., Wallace, J. S., Argoty, G. A., Wilkinson, C., Fahrenfeld, N., Heath, L. S., … Pruden, A. (2016). Metagenomic profiling of historic Colorado Front Range flood impact on distribution of riverine antibiotic resistance genes. *Scientific Reports*, *6*(1), 38432. https://doi.org/10.1038/srep38432

Correlations found within this study

*Bulk water bacterial phylogeny did not correlate with ARG profiles while sediment phylogeny varied along the river's anthropogenic gradient.
*The potential role of antibiotics as selective agents influencing the re-establishment of ARGs during post-flood recovery was investigated by examining correlations between sulfonamide (sul1, sul2), tetracycline (tet(O), tet(W)), and macrolide (ermF) ARGs in bed sediment and bulk water, quantified **using qPCR** (Fig. S3), and 23 antibiotics (Table S2) in bulk water at all sites (Fig. 4). Analysis of antibiotics was limited to the bulk water.
*All ARGs demonstrated significant Spearman's rank correlations with at least one antibiotic against which they conferred resistance.
*All ARGs identified were also found to significantly correlate with certain antibiotics against which they do not confer resistance indicating potential for co-selection. It is challenging to determine whether observed correlations are truly indicative of selective pressure or simply co-transport of antibiotics and ARGs from the same source.
***Based on metagenomic data**, positive correlations were observed between MLS, rifampin, and fosfomycin ARGs and the antibiotics sulfamethazine ($\rho = 0.8452, 0.8452, 0.8262$, $p = 0.0341, 0.0341, 0.0427$) and clarithromycin ($\rho = 0.8452, 0.8452, 0.8262$, $p = 0.0341, 0.0341, 0.0427$).
*Although we could not precisely quantify the extent to which horizontal gene transfer shaped the resistome based on the present study, the numerous associations of plasmids and prophages with ARGs were striking, suggesting that it is a significant phenomenon in the riverine environment.
*Interestingly, the overall bulk water phylogeny was not correlated with ARG profiles (2STAGE, weighted UniFrac: Spearman's $\rho = -0.1$) indicating that phylogeny alone may not be the most important factor controlling the profile of ARGs.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
6.Guo et al, 2016	**China** 12 sediment samples were taken at different sites following a pollution gradient along the marine coast of Hong Kong. Date: 2012-June 5-8	Ofloxacin (FQ) Ciprofloxacin (FQ) Sulfadiazine (SUL) Sulfomethoxazole (SUL) Sulfamethazine (SUL) Cefalexin (BeLa) Erythromycin (ML) Roxithromycin (ML)	Rationale not explicitly given. *Highest concentration of antibiotics found*: Roxithromycin Sulfadiazine Sulfamethoxazole *Lowest concentrations found*: All the quinolones	Fluoroquinolone Tetracycline Sulfanomides Bacitracin Polymixin Vancomycin Beta-lactams Aminoglycoside MLS Multidrug Trimethroprim Fosmidomycin Chloramphenicol Fosfomycin others	The abundant ARG types were genes encoding resistance to *multidrug* (3.2 × 10−3 in average), *bacitracin* (1.2 × 10−3 in average) and *sulphonamide* (1.4 × 10−3 in average).	In the article number of ARG-reads normalized by 16s reads are used. ppm-numbers were calculated by multiplying by the 16s counts (referred to in the article as the bacterial smallest sub-unit (SSU)) and dividing by the number of million clean reads. *Range*: From 6,63 ppm to 172,43 ppm	No

Full citing
Guo, F., Li, B., Yang, Y., Deng, Y., Qiu, J.-W., Li, X., … Zhang, T. (2016). Impacts of human activities on distribution of sulfate-reducing prokaryotes and antibiotic resistance genes in marine coastal sediments of Hong Kong. *FEMS Microbiology Ecology*, *92*(9), fiw128. https://doi.org/10.1093/femsec/fiw128

Correlations found within this study
*Although total ARGs were enriched in sediments from the polluted sites, distribution of single major ARG types could be explained neither by individual sediment parameters nor by corresponding concentration of antibiotics. It supports the hypothesis that the persistence of ARGs in sediments may not need the selection of antibiotics.
*Correlation analyses (both Spearman correlation and Kendall correlation were tested since the data of antibiotic concentrations were not normally distributed) were performed to examine the potential implications between antibiotics and ARGs. However, the statistics showed no significant correlation (P 0.1 in all cases, bootstrap N>1000). This result suggested that the occurrence of ARGs could not be explained by the local distribution of the two major antibiotics classes.
*Although the total ARGs abundance seemed to be positively influenced by human impact, the distribution of a few major ARG types only could be well explained by the general factors, such as COD and Zn, instead of the corresponding antibiotics.
*Our results support the idea that the occurrence and persistence of ARGs in the marine sediments may not be directly associated with the in situ stress of the antibiotic residues in sediment. They could be derived from the direct continuous input of biomass or non-selective effect.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
7.Jia et al, 2017	**China** 5 surface water samples were (1 upstream of a pig farm and the rest at greater distances downstream) in Changzhou along Hongqi river, Jongan river and Taige river. Samples in this study were taken at 7 sites in May, August and November. The November samples were used for metagenomic analysis. Two locations were not included here, because they were located at the swine wastewater discharge, leaving 5 samples. Date: 2013-November	Ofloxacin (FQ) Tetracycline (TET) Chlortetracycline (TET) Oxytetracycline (TET) Sulfadiazine (SUL) Sulfamethoxazole (SUL) Sulfamethazine (SUL) Cefalexin (BeLa) Erythromycin (ML) Roxithromycin (ML) Trimethoprim (Among the 21 antibiotics tested, 11 ones were detectable in the wastewater or river water)	Rationale not explicitly given. *Highest concentrations found:* 1. Sulfamethazine 2. Roxithromycin 3. Oxytetracycline 4. Sulfomethoxazole	Tetracyclines Sulphonamides Bacitracin Beta-lactam Aminoglycosides MLS Multidrug Chloramphenicol	*Most abundant*: Tetracycline and sulphonamides resistance genes.	ppm (assembly was performed), ppm was determined as the portion of the hits of one type or subtype of ARG in the total metagenome reads). *Range*: From 4,72 ppm (upstream) to 209,83 ppm (downstream closest to the pig farm)	Yes

Full citing
Jia, S., Zhang, X.-X., Miao, Y., Zhao, Y., Ye, L., Li, B., & Zhang, T. (2017). Fate of antibiotic resistance genes and their associations with bacterial community in livestock breeding wastewater and its receiving river water. *Water Research*, *124*, 259–268. https://doi.org/10.1016/j.watres.2017.07.061

Correlations found within this study
* This study showed that swine wastewater contained a broad range of antibiotics covering different families, and the results agreed with previous studies indicating that tetracyclines and sulphonamides were the main antibiotics in swine manure and wastewater (Chen et al., 2012). The reason may be that tetracyclines and sulphonamides are commonly used as growth promoter and disease preventer in animal farming (Zhang et al., 2015).
*Correlation analysis and host analysis consistently showed that the changes in the abundances of several key genera like *Prevotella* and *Treponema* were significantly and positively correlated with the antibiotic resistome alteration.
*Wastewater discharge evidently elevated the total abundance of ARGs in the receiving water, which then showed decreasing treads along the river flow, but the decreasing rate seemed higher in August than in May and November.
*Among all the types of the detectable ARGs, tetracycline and aminoglycoside resistance genes dominated in the wastewater and the severely contaminated downstream river water (Fig. S5A), occupying 69.55e80.73% of the total abundance.
*In addition, the relative abundance of MGEs including integrons, plasmids and ISs varied greatly among the wastewater and river water samples.
*Interestingly, network analysis based on the co-occurrence patterns between ARG subtypes revealed the incidences of non-random co-occurrence of ARGs within the same types or among different types in the wastewater and river water. This study showed that co-occurrence was evident for the ARGs within the same type (including aminoglycoside and beta-lactam), which is supported by Sun et al. (2013) revealing the positive selection on ARGs posed by antibiotics in manure-polluted aquatic environment.
*The results of this study show that: "High levels of nutrients and antibiotics in wastewater drive bacterial community shift in the wastewater-receiving river water, which is mainly responsible for the resistome alteration."

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
8.Qui et al, 2019	**China** 4 sediment samples were taken from Maozhou river – one of the Shenzhen rivers in the Pearl River Delta. Maozhou river is the most complex water	Ofloxacin (FQ) Norfloxacin (FQ) Pefloxacin (FQ) Tetracycline (TET) Sulfadiazine (SUL) Sulfathiazole (SUL) Sulfadimoxine (SUL) Sulfamethiadiazole (SUL)	The occurrences of total 20 antibiotics which were selected for analysis based on the known types used in China.	TET: Tet(G) SUL: Sul1, Sul2, Sul3 ML: AAC(6')IIA ereA2 (confers resistance to erythromycin) ereA (confers resistance to erythromycin)	In all four selected sediment samples, the *sul1 gene* has the highest level of relative abundance compared with other ARGs annotated using the CARD database, and its relative abundance value was as high as 0.006 in theMZ8 sample. While the	Data was assembled. Relative abundance calculated from formula, $G_i = \frac{r_i}{L_i} \times \frac{1}{\sum_i^n \frac{r_i}{L_i}}$	Yes

	system in Shenzhen. It flows through different industrial areas and its black waters and unpleasant odour indicate that this river has become severely contaminated. Date: not stated	Sulfamethazine (SUL) Sulfamethoxazole (SUL) Sulfafurazole (SUL) Sulfamethoxine (SUL) Penicillin (BeLa) Ampicillin (BeLa) Tylosin (ML) Clarithromycin (ML) Roxithromycin (ML) Lincomycin (ML) Clindamycin (ML) Trimethoprim	*Highest concentrations found*: 1. Sulfathiazole 2. Sulfadiazine		abundance patterns of other ARGs differed among the different sediment samples.	where G_i is the relative abundance of gene *i*. r_i is the reads quantity when gene *i* compared with gene database. L_i is the length of gene *i*. *n* is the gene quantity of gene catalogue of the selected sample.	

Full citing
Qiu, W., Sun, J., Fang, M., Luo, S., Tian, Y., Dong, P., … Zheng, C. (2019). Occurrence of antibiotics in the main rivers of Shenzhen, China: Association with antibiotic resistance genes and microbial community. *Science of The Total Environment*, *653*, 334–341. https://doi.org/10.1016/j.scitotenv.2018.10.398

Correlations found within this study
* Several antibiotics were correlated with corresponding ARGs at the p < 0.05 level, indicating that exposure to antibiotics would lead to a selective pressure for certain ARGs. Still, high concentration of sulfadiazine and a high abundance of the sul1 gene (encoding resistance to sulfonamides) were quantified in the sediment samples, while no significant correlation was observed between them.
*Statistical analysis figured out the relations among antibiotics, ARGs and microbial community. Sulfamethazine was significantly correlated with both the bla_d gene (r = 0.969, p b 0.05) and Fusobacteria (r = 0.954, p b 0.05), and a significant correlation was also observed between the bla_d gene and Fusobacteria (r =0.965, p b 0.05).
* The results of this study indicate that antibiotics introduced into natural water systems may serve as a major selective pressure that promotes the proliferation of antibiotic-resistant bacteria, and thereby significantly alters the structure of bacterial communities.

Study	Samples	Antibiotics analysed	Rationale for AB choice and concentrations ranking	ARGs detected	Abundance ranking ARGs	Unit for relative abundance	Significant correlations found in this study between antibiotics and ARGs?
9.Zhang et al, 2018	**China** 3 sediment samples were taken from the Pearl River. Samples were taken 100 meters apart. The Pearl River samples represent a typical level of anthropogenic antibiotic pollution. In this study samples were also taken from glacial soil and permafrost, but these were not included here. Date: 2016-April 30	Enrofloxacin (FQ) Norfloxacin (FQ) Ciprofloxacin (FQ) Ofloxacin (FQ) Tetracycline (TET) Sulfadiazine (SUL) Sulfamethoxazole (SUL) Sulfamethazine (SUL)	8 commonly used antibiotics were analysed. *Highest concentrations found*: 1.Ofloxacin 2.Norfloxacin	Tetracyclines Sulphonamides Bacitracin Polymyxin Cephalosporin Penicillin Streptomycin Lincomycin (=Lincosamide) Macrolides Erythromycin Multidrug Trimethroprim Chloramphenicol Fosfomycin	Highest abundance of ARGs: 1. Bacitracin 2. Tetracycline 3. Sulphonamides	ppm Range: From 401,36 ppm to 418,39 ppm	Yes, for some ARGs with corresponding antibiotics.

Full citing
Zhang, S., Yang, G., Hou, S., Zhang, T., Li, Z., & Liang, F. (2018). Distribution of ARGs and MGEs among glacial soil, permafrost, and sediment using metagenomic analysis. *Environmental Pollution*, *234*, 339–346. https://doi.org/10.1016/j.envpol.2017.11.031

Correlations found within this study
*The diversity of MGEs was significantly correlated with the abundance and diversity of ARGs. The diversity of MGEs was better correlated with the abundance and diversity of ARGs than with the abundance of MGEs.
* The significant positive correlations that we found between sulfonamides and genes for sulfonamide-resistance, and between tetracycline and genes for tetracycline-resistance suggested that anthropogenic use of sulfonamides and tetracycline influence the distribution of the related ARGs in different environments.
The most common ARGs found across all three environments (sediment, glacial soil and permafrost) and primarily encoded resistance to bacitracin.

6.2 Appendix 2: Overview of PNEC used

PNEC-values used to calculate the selective pressure potential of each antibiotic in surface water and sediment.		
Antibiotic	**PNEC-water from literature ng/L**	**PNEC-sediment, calculated from PNEC-water ng/Kg dw**
Cefalexin (BeLa)	*4000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*24000*
Cefazolin (BeLa)	*1000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*3220*
Trimethoprim	*500 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*21000*
Enrofloxacin (FQ)	*60 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*55*
Norfloxacin (FQ)	*500 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*550*
Ciprofloxacin (FQ)	*60 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*37*
Ofloxacin (FQ)	*500 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*355*
Fleroxacin (FQ)	*1000 (lowest MIC-derived, Zhang et al, 2020)*	*3250*
Tetracycline (TET)	*1000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*2550*
Chlortetracycline (TET)	*2000 (lowest MIC-derived, Zhang et al, 2020)*	*8350*
Oxytetracycline (TET)	*500 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*2135*
Doxycycline (TET)	*2000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*5725*
Sulfadiazine (SUL)	*70 (Ecotox derived, ref. Lemna minor (plant), Wang et al, 2020b)*	*300*

Sulfamethoxazole (SUL)	*16000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*240000*
Sulfathiazole (SUL)	*4890 (Ecotox-derived, ref. Lemna minor (plant), Wang et al, 2020b)*	*46000*
Sulfamerazine (SUL)	*680 (Ecotox-derived, ref. Lemna minor (plant), Wang et al, 2020b)*	*4700*
Sulfadoxine (SUL)	*600 (Ecotox-derived, ref. algae, AMR alliance)*	*1114*
Sulfadimethoxine (SUL)	*2300 (Ecotox-derived, ref. algae, Wang et al, 2020b)*	*4070*
Sulfamethoxidiazine syn. Sulfameter (SUL)	*250 (lowest MIC-derived, Zhang et al, 2020)*	*700*
Sulfamethazine (SUL)	*1740 (Ecotox-derived, ref. algae, Wang et al, 2020b)*	*14150*
Erythromycin (ML)	*1000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*32880*
Roxithromycin (ML)	*1000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*559000*
Clarithromycin (ML)	*250 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*2166*
Azithromycin (ML)	*250 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*46000*
Chloramphenicol (CA)	*8000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*6050*
Florfenicol (CA)	*2000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*6750*
Thiamphenicol	*1000 (lowest MIC-derived, Bengtsson-Palme et al, 2016)*	*580*

Method for calculating PNEC-sediment from PNEC-water

The EPI Suite™-Estimation Program Interface (KOCWIN-tool) was used to calculate the Koc (soil adsorption coefficient) for each antibiotic. Following Duarte et al (2019), an organic carbon content of 5,8% was assumed. The PNEC-sediment was calculated as follows: PNEC-water*0,058*Koc

References PNEC:

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6.3 APPENDIX 4: R SCRIPT USED FOR DATA EXPLORATION AND ANALYSIS

```
#Transforming the data to log scale and factors

Clean_DATA_2$TARG <- log10(Clean_DATA_2$TARG)

Clean_DATA_2$TASP <- log10(Clean_DATA_2$TASP)

Clean_DATA_2$Class <- as.factor(Clean_DATA_2$Class)

Clean_DATA_2$Study <- as.factor(Clean_DATA_2$Study)

Clean_DATA_2$Matrix <- as.factor(Clean_DATA_2$Matrix)

Clean_DATA_2$Year <- as.factor(Clean_DATA_2$Year)

Clean_DATA_2$Season <- as.factor(Clean_DATA_2$Season)

Clean_DATA_2$Sample <- as.factor(Clean_DATA_2$Sample)

#LME models for different fixed and random effect combinations

fullmodel <- lmer(TARG ~ TASP + Class + (1|Season) + (1|Matrix) +  (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod1 <- lmer(TARG ~ TASP + Class + (1|Season) + (1|Matrix), data = Clean_DATA_2, REML = TRUE)

candmod2 <- lmer(TARG ~ TASP + Class + (1|Season) + (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod3 <- lmer(TARG ~ TASP + Class + (1|Matrix) +  (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod4 <- lmer(TARG ~ TASP + Class + (1|Season), data = Clean_DATA_2, REML = TRUE)

candmod5 <- lmer(TARG ~ TASP + Class + (1|Matrix), data = Clean_DATA_2, REML = TRUE)

candmod6 <- lmer(TARG ~ TASP + Class + (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

# Corrected Akaike Information Criterion

cAIC <- aictab(as.list(c(fullmodel, candmod1, candmod2, candmod3, candmod4, candmod5, candmod6)))

# Model selection (fixed structure)

candmod41 <- lmer(TARG ~ 1 + (1|Season) + (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod42 <- lmer(TARG ~ TASP + Class + (1|Season) +  (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod43 <- lmer(TARG ~ TASP + (1|Season) + (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod44 <- lmer(TARG ~ 1 + Class + (1|Season) + (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

candmod45 <- lmer(TARG ~ TASP * Class + (1|Season) + (1|Study/Class), data = Clean_DATA_2, REML = TRUE)

# F-test (Kenward-Roger approach)

KRmodcomp(candmod41, candmod42)

KRmodcomp(candmod42, candmod43)

KRmodcomp(candmod42, candmod44)

KRmodcomp(candmod42, candmod45)

# Boostrap confidence intervals of model estimates

confint.merMod(candmod44, method = "boot", level = 0.95, nsim = 1000, oldNames = FALSE)

# R2-marginal and R2-conditional

r.squaredGLMM(candmod44)
```

6.4 APPENDIX 5: DATA ANALYSIS RESULTS RUN IN R

	Modnames	K	AICc	Delta_AICc	ModelLik	AICcWt	Res.LL	Cum.Wt
3	Mod3	11	1347.009	0.000000	1.000000e+00	6.760992e-01	-662.2367	0.6760992
1	Mod1	12	1348.481	1.471805	4.790729e-01	3.239008e-01	-661.9233	1.0000000
4	Mod4	11	1384.881	37.871965	5.973202e-09	4.038477e-09	-681.1727	1.0000000
7	Mod7	10	1386.028	39.018723	3.366604e-09	2.276158e-09	-682.7911	1.0000000
2	Mod2	10	1599.629	252.620207	1.393859e-55	9.423873e-56	-789.5919	1.0000000
5	Mod5	9	1603.667	256.658221	1.850868e-56	1.251370e-56	-792.6517	1.0000000
6	Mod6	9	1637.163	290.154434	9.857463e-64	6.664623e-64	-809.3998	1.0000000

Showing 1 to 7 of 7 entries, 8 total columns

```
> KRmodcomp(candmod41, candmod42)
F-test with Kenward-Roger approximation; time: 1.81 sec
large : TARG ~ TASP + Class + (1 | Season) + (1 | Study/Class)
small : TARG ~ 1 + (1 | Season) + (1 | Study/Class)
        stat     ndf     ddf F.scaling p.value
Ftest  3.4892  6.0000 19.2057    0.9896 0.01673 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> KRmodcomp(candmod42, candmod43)
F-test with Kenward-Roger approximation; time: 1.47 sec
large : TARG ~ TASP + Class + (1 | Season) + (1 | Study/Class)
small : TARG ~ TASP + (1 | Season) + (1 | Study/Class)
       stat    ndf    ddf F.scaling p.value
Ftest  4.039  5.000 15.070   0.99974 0.01591 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> KRmodcomp(candmod42, candmod44)
F-test with Kenward-Roger approximation; time: 0.97 sec
large : TARG ~ TASP + Class + (1 | Season) + (1 | Study/Class)
small : TARG ~ 1 + Class + (1 | Season) + (1 | Study/Class)
        stat      ndf      ddf F.scaling p.value
Ftest   0.6505   1.0000 493.4411         1  0.4203
> KRmodcomp(candmod42, candmod45)
F-test with Kenward-Roger approximation; time: 1.05 sec
large : TARG ~ TASP * Class + (1 | Season) + (1 | Study/Class)
small : TARG ~ TASP + Class + (1 | Season) + (1 | Study/Class)
        stat      ndf      ddf F.scaling p.value
Ftest   1.6964   5.0000 480.2231   0.99992  0.1339
```

```
> confint.merMod(candmod44, method = "boot", level = 0.95, nsim = 1000, ol
dNames = FALSE)
Computing bootstrap confidence intervals ...

16 message(s): boundary (singular) fit: see ?isSingular
3 warning(s): Model failed to converge with max|grad| = 0.00200838 (tol =
0.002, component 1) (and others)

                                  2.5 %     97.5 %
sd_(Intercept)|Class:Study  0.3954592  0.9813888
sd_(Intercept)|Study        0.4887540  2.0279089
sd_(Intercept)|Season       0.1113378  1.1829833
sigma                       0.7687441  0.8726545
(Intercept)                -4.3938064 -1.2091509
ClassCS                     0.1000056  3.1269241
ClassFQ                    -1.9420499  0.6779273
ClassMLLS                  -1.6285494  1.1360693
ClassSUL                   -0.6565748  1.9020862
ClassTET                   -0.6574808  1.9572323


>
```

```
> r.squaredGLMM(candmod44)
          R2m       R2c
[1,] 0.1093788 0.8143296
```

```
> candmod44
Linear mixed model fit by REML ['lmerMod']
Formula: TARG ~ 1 + Class + (1 | Season) + (1 | Study/Class)
   Data: Clean_DATA_2
REML criterion at convergence: 1321.315
Random effects:
 Groups      Name        Std.Dev.
 Class:Study (Intercept) 0.686
 Study       (Intercept) 1.292
 Season      (Intercept) 0.637
 Residual                0.819
Number of obs: 505, groups:  Class:Study, 28; Study, 9; Season, 4
Fixed Effects:
(Intercept)      ClassCS      ClassFQ    ClassMLLS     ClassSUL     ClassT
ET
    -2.8604       1.6452      -0.5974      -0.2243       0.6795       0.66
84
> summary (candmod44)
Linear mixed model fit by REML ['lmerMod']
Formula: TARG ~ 1 + Class + (1 | Season) + (1 | Study/Class)
   Data: Clean_DATA_2

REML criterion at convergence: 1321.3

Scaled residuals:
    Min      1Q  Median      3Q     Max
-4.5258 -0.5199  0.0138  0.4546  4.3482

Random effects:
 Groups      Name        Variance Std.Dev.
 Class:Study (Intercept) 0.4705   0.686
 Study       (Intercept) 1.6703   1.292
 Season      (Intercept) 0.4058   0.637
 Residual                0.6707   0.819
Number of obs: 505, groups:  Class:Study, 28; Study, 9; Season, 4

Fixed effects:
```

```
            Estimate Std. Error t value
(Intercept)  -2.8604     0.8025  -3.564
ClassCS       1.6452     0.7484   2.198
ClassFQ      -0.5974     0.6521  -0.916
ClassMLLS    -0.2243     0.6728  -0.333
ClassSUL      0.6795     0.6577   1.033
ClassTET      0.6684     0.6501   1.028

Correlation of Fixed Effects:
          (Intr) ClssCS ClssFQ ClMLLS ClsSUL
ClassCS   -0.489
ClassFQ   -0.645  0.603
ClassMLLS -0.597  0.585  0.736
ClassSUL  -0.647  0.599  0.774  0.732
ClassTET  -0.676  0.605  0.798  0.735  0.777


>
```

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