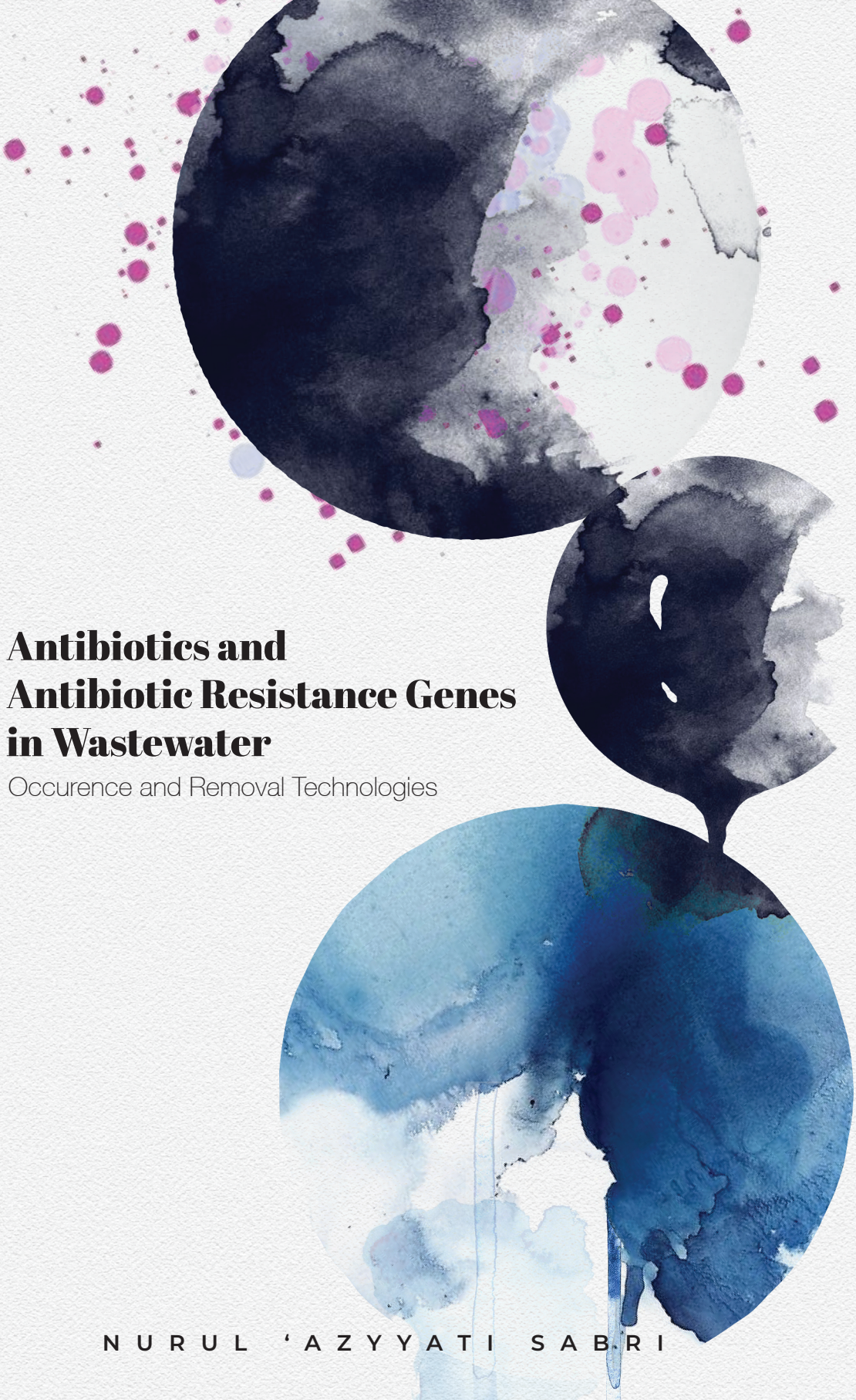

Antibiotics and Antibiotic Resistance Genes in Wastewater

Occurence and Removal Technologies

NURUL 'AZYYATI SABRI

Propositions

1. Rivers are highways for dissemination of antibiotics and antibiotic resistance genes into and throughout the environment.
   (this thesis)

2. Dreams of "one-wastewater-treatment-fits-all" conflict with bitter-sweet experiences in science and practice.
   (this thesis)

3. Market volatility and fluctuated economy during a pandemic situation are an investment opportunity in maximizing wealth. (Zhang et al., 2020, "Financial markets under the global pandemic of COVID-19" *Finance Research Letters*).

4. Transition from conventional teaching to online teaching is slowly diminishing human elements in life-long learning processes, which will affect social skills, relationships, and interaction. (Dumford and Miller, 2018, "Online learning in higher education: exploring advantages and disadvantages for engagement" *Journal of Computing in Higher Education*).

5. Having a Dutch mindset makes it challenging to survive in Asian countries.

6. Ph.D. life is like a roller coaster, becoming thrilled, intense, but lasting very shortly.

Propositions belonging to the thesis, entitled

Antibiotics and antibiotic resistance genes in wastewater: Occurrence and removal technologies

Nurul 'Azyyati binti Sabri
Wageningen, 4 September 2020

Antibiotics and antibiotic resistance genes in wastewater: Occurrence and removal technologies

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Antibiotics and antibiotic resistance genes in wastewater: Occurrence and removal technologies

Nurul 'Azyyati binti Sabri

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For my beloved family
Untuk keluarga tercinta

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CHAPTER 1

General introduction

1.1 Micropollutants

Many chemicals are widely used to improve human life. After use, these compounds can enter and are dispersed into the environment, they can have adverse effects on the natural ecosystem, food quality, or human health. In general, these compounds are referred to as pollutants, and are called micropollutants when they are present and harmful at very low concentrations. Micropollutants can be composed of pharmaceuticals (among which antibiotics, drug-related compounds and endocrine-disrupting chemicals), personal care products, pesticides, food additives, industrial chemicals, firefighting chemicals (such as perfluoroalkyl substances), and many other compounds (Kim and Zoh, 2016). It is challenging to prevent micropollutants entering the environment, since the sources of micropollutants are ubiquitous. Moreover, the use of chemicals leading to contamination of the environment with micropollutants, is increasing from year to year. The OECD (2017) reported that pharmaceutical consumption including antihypertensive, cholesterol, antidiabetic, and antidepressant drugs continue to increase, due to a higher demand in treating aging-related and chronic diseases. A similar finding has also been reported by Mohapatra et al. (2014), which stated that pharmaceutical consumption was found to increase approximately from 15 g (average per capita/year) to 50 g in industrialized countries.

The increased pollution of the environment by micropollutants, especially in water, is worrying, since the availability of clean water is increasingly becoming limited, due to increased climate change, urbanization, and growing populations in many regions around the world. Water scarcity has forced some countries to find an alternative to overcome these problems. Wastewater is considered as an excellent potential resource to be used after suitable treatment, and can provide financial and economic benefits. Therefore, some countries already assigned reused water as an important freshwater resource for general use (Angelakis et al., 2018). Jiménez Cisneros and Asano (2008) highlighted that: (1) China, Mexico, and the USA are countries with the largest volume of wastewater reuse, (2) Qatar, Israel, and Kuwait are the countries with the highest reuse per habitant, and (3) Kuwait, Israel, and Singapore are the countries with the most percentage of the total water reused. McKinsey (2009) warned that the demand for freshwater would exceed water supply by 40% within two decades.

By 2025, two out of three people will live at moderate to high water stress conditions (UNEP, 2007). WHO/UNICEF (2015) reported that approximately 340,000 children (below five years old) lack access to clean water and die every year from diarrhoeal diseases. It is stated that over 80% of the world's untreated wastewater will flow back into the ecosystem (WWAP, 2017). This situation is becoming worse if the wastewater that contains macro- and micropollutant are reused untreated. Macropollutants are often referred to as oxidizable organic compounds and nutrients, like nitrogen and phosphorous, and related to sewage or industrial wastewater. Wastewater treatment technologies and systems for macropollutants removal are widely available, though not fully implemented in many developing economy countries (Mara, 2013). For micropollutant removal, such technologies are not yet developed at a mature and

market-ready state. Therefore, an understanding how to treat wastewater to remove these micropollutants has gained attention in the last decade.

1.2 Antibiotics and Antibiotic Resistance

Antibiotics are low molecular-weight compounds that are able to inhibit or even stop the growth of microorganisms when applied at low concentrations. They are able to stimulate the die-off of a wide range of bacteria, including human and animal pathogens (Lancini et al., 1995). The discovery and use of antibiotics has been very important in the medical field. The first antibiotic, penicillin, has been discovered in 1928 and is very good in inhibiting pathogenic microorganisms (Fleming, 1929). After this discovery, an increasing variety of antibiotics has been discovered 1940 and early 2000, and the developments continue until this moment. A downside of the use of antibiotics to combat infective illnesses of people and livestock, is that bacteria can develop resistance to these antibiotics, by e.g. developing biochemical blockers, counteractive enzymes, or adjusting their cell wall architecture (Kapoor et al., 2017). Hence, development of antibiotic resistance amongst natural and pathogenic bacteria are a side effect of the widespread use of antibiotics.

Antibiotics consumption varies across countries in terms of volumes or patterns (Figure 1.1). Overprescribing of antibiotics is proposed to increase the risk of adverse effects and medicalization (Llor and Bjerrum, 2014). A few reports analyzed the antibiotics consumption around the world from different sources. Van Boeckel et al. (2014) used sales data from the IMS Health MIDAS database to review the consumption of antibiotics between 2000 and 2010 in 71 countries (in defined daily dose (DDD)/1000 inhabitants/day). These authors concluded that antibiotics consumption increased by 35% with penicillins as the most prescribed class of antibiotics. China, Brazil, India, Russia and South Africa are the leading countries responsible for this increase (Van Boeckel et al., 2014).

A recent report by Klein et al. (2018) reported antibiotics consumption from 2000 to 2015 in 76 countries by using the IQVIA MIDAS database. They found that antibiotics consumption increased by 65%, and the antibiotics consumption rate increased from 11.3 to 15.7 DDD/1000 inhabitants per day in 76 countries within 15 years. The mean antibiotics consumption rate increased from 16.4 to 20.9 DDD/1000 inhabitants per day, and the median increased from 15.5 to 19.5 DDD/1,000 inhabitants per day.

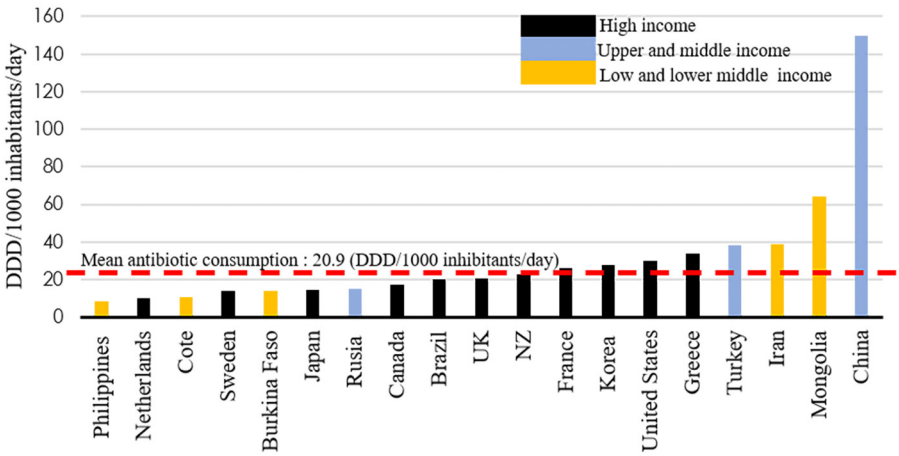

Figure 1.1: Antibiotics consumption in selected countries in the world in 2015. Adapted from (Klein et al., 2018; WHO, 2018). Mean antibiotics consumption is 20.9 DDD/1000 inhibitants/day (Klein et al., 2018). For China data was based on survey in 2013 (Ying et al., 2017).

In the same year, WHO (2018) also produced an extensive report about the consumption of antibiotics from 65 countries with a combination of different sources, such as records of import, production, wholesale, public procurement, donation, prescribing data, antibiotics dispensing, and commercial data sources. This is a continuation of the 2014 surveillance report (WHO, 2014) in response to the lack of antibiotics consumption data and standardized methodology for data collection, especially in low and middle-income countries. In general, the overall antibiotics consumption is from 4.4 (Burundi) to 64.4 DDD/1000 inhabitants per day (Mongolia).

Looking at China and the USA, the two largest economies in the world, their antibiotics consumption is unsurprisingly high. In China, this might be a result of the health system itself, which encourages doctors to prescribe antibiotics, and an extensive marketing by pharmaceutical companies (Yip and Hsiao, 2014). China is the largest manufacturer and user of antibiotics, and this is reflected in their increased antibiotics consumption from 2.3 to 4.2 billion DDDs between 2000 and 2015 (79%) (Klein et al., 2018). Meanwhile, in the USA, antibiotics use is higher than in most industrialized countries (Goossens et al., 2007), and leading in antibiotics consumers (Klein et al., 2018). This may be related to unnecessary prescription by physicians, hospital-based clinics, and emergency departments (Talkington, 2018). For example, Hicks et al. (2015) reported that healthcare providers prescribed more than 260 million of antibiotics in 2011, with penicillins and macrolides as the most common antibiotic prescribed. In addition to human health care, vast amounts of antibiotics are used in livestock farming, sometimes a factor three higher than in human health care, either as cattle growth stimulator or as pest control (O'Neill, 2014). EU countries acknowledged this livestock-related overuse as an unwanted development

and have limited the use of antibiotics for this purpose per 2006, which is followed by China per 2020.

As time goes by, the extensive use of antibiotics is becoming the main driver of antibiotic resistance, which means that various types and groups of microorganisms are slowly becoming resistant to antibiotics. Antibiotic resistance is found to develop faster than newly developed antibiotics are brought to the market, as finding new antibiotics is costly and challenging.

Antibiotic resistance bacteria (ARB) are bacteria that can withstand an attack by antibiotics. ARB harbor antibiotic resistance genes (ARGs), that survive in the presence of antibiotics and continue to multiply (Alonso et al., 2001). In recent decades, antibiotics and ARGs have been of growing concern and is the subject of research, due to the effect it has on public health and the environment (Davies and Davies, 2010). For example, ARB can be transmitted from the environment to humans via direct or indirect contact (Rodríguez et al., 2006). It leads to a direct threat to human health, as e.g. pathogenic bacteria are not responsive anymore to antibiotic treatment, or other effects such as carcinogenic reactions to humans (Yang et al., 2004). Moreover, it affects the evolution of the microbiome and their interacting biota in water ecosystems that can enter the human food provision system through e.g. fisheries or other aquatic food (Kotzerke et al., 2008). Thus, antibiotic resistance can be transferred from the ecosystem to the human interactive microbiome living on skin and in intestines. Therefore, it is projected that by 2050, antibiotic resistance will cause 10 million deaths, and a financial problem of approximately US$100 trillion in worldwide (O'Neill, 2014).

It has been proposed that antibiotic resistant bacteria and antibiotic resistance genes (ARB&Gs) might have a significant and long-term effect on the stability and rate of ecosystem functioning (Martinez et al., 2009). ARB&Gs can remain in the environment or even increase in a period of time. For example, Knapp et al. (2010) showed that ARGs in soil from all classes were able to survive and increase (more than 15 times higher) from the 1940s to 2008. In another study, ARB was detected in surface water and wastewater between 2006 and 2013 (Blaak et al., 2015), even though the use of antibiotics in agriculture and livestock was reduced by 50% from 2007 until 2012 in the Netherlands (Vandenbroucke-Grauls, 2014; Speksnijder et al., 2015). Considering this problem is alarming, WHO (2014) declared a Global Action Plan to combat antibiotic resistance by improving awareness, strengthen knowledge, reduce infections, optimize antibiotics, and ensure sustainable investment in overcoming antibiotic resistance.

1.3 Transfer of antibiotic resistance

ARGs are rapidly dispersed in the environment by horizontal genes transfer (HGT), which allows gene transfer among cells of bacterial strains of the same type or different classes (Davies and Davies, 2010; Burmeister, 2015). This can occur between the same bacterial species, such as *E. coli* that cause urinary tract infections or food poisoning, or between different bacterial species, such as *E. coli* and *Staphylococcus aureus*.

HGT also plays an important role in the development of multidrug resistance (MDR) in bacteria (Forsberg et al., 2012). For example, methicillin-resistant *Staphylococcus aureus* (MRSA), is resistant to methicillin, tetracycline, aminoglycosides, macrolides, lincosamides, chloramphenicol, and disinfectants (Nikaido, 2009). Furthermore, the group of bacteria that are part of ESKAPE and consists of both gram-negative and gram-positive species are listed as pathogens that exhibit virulence and MDR (Santajit and Indrawattana, 2016). ESKAPE is an abbreviation for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Mulani et al., 2019). MDR ESKAPE is significantly associated with a higher cost compared to susceptible organisms or those without infection (Zhen et al., 2019).

In wastewater treatment plants (WWTPs), antibiotics get in contact with bacteria when entering the treatment process, as well as with bacteria that are active in the treatment process (Ojala et al., 2014). This promotes a suitable environment for HGT. Once transferred, the genes might continue to evolve, often resulting in bacteria with a higher resistance (McCarthy et al., 2014). Figure 1.2 shows the HGT in bacteria with the three possible transfer mechanisms; transduction, transformation, or conjugation (Dröge et al., 1998). Of these three mechanisms, conjugation has the most significant influence on the dissemination of ARGs (von Wintersdorff et al., 2016).

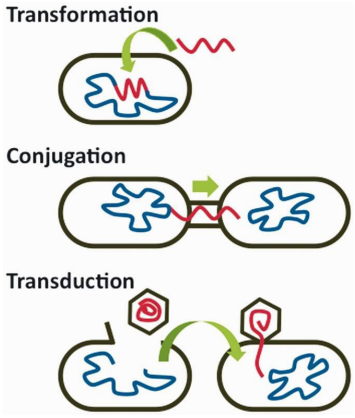

Figure 1.2: Mechanisms of bacterial horizontal gene transfer. Adapted from Burmeister (2015).

1.4 Occurrence of antibiotic resistance

A simple meta-analysis was conducted on studies that related to ARGs. A total of 3109 scientific articles has been recorded in ScienceDirect by searching for the keyword "antibiotic resistance genes" in research articles from 2018 to 2020 (up to January 12th, 2020). A total of 237 related research papers have been listed, with 127 related to ARGs. It covers a variety of studies related to ARGs in water (wastewater, drinking water), wastewater treatment, such as anaerobic digestion, manure, food, composts, sediments, soil, risk assessment, and clinical research. A heatmap of ARGs studies in 2020 is presented in Figure 1.3. Most studies related to antibiotic resistance have been conducted in China (60%) and the rest in multiple countries.

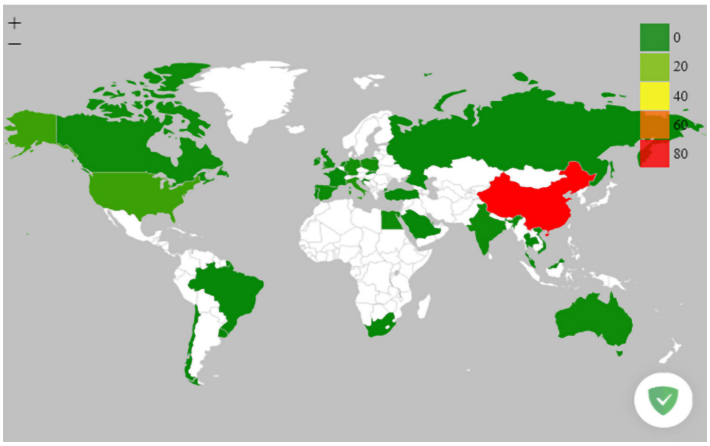

Figure 1.3: Heatmap of antibiotic resistance studies in the world in 2020 (from Science Direct). It represents the location of the principal researcher. Dark green represents 0-20 studies, light green represents 20-40 studies, yellow represents 40-60 studies, orange represents 60-80 studies, and red represents 80-100 studies.

Not only research, but there are also many reviews on antibiotic resistance and ARGs in China, for example, antibiotic resistance in the environment (Qiao et al. 2018), antibiotics removal by constructed wetlands (Guan et al., 2017), antibiotics emission in river (Luo et al., 2010; Jiang et al., 2011; Zhang et al., 2015b), and soil antibiotic resistome (Du et al., 2020).

In the USA, almost 3 million people have been infected by ARB, and more than 35,000 people died (CDC, 2019). Hampton (2013) summarized that antibiotic resistance threats in the USA are urgent, serious, and of concern. Since WHO (2014) declared antibiotic resistance as a world threat, the White House has released a national action plan to combat ARB in 2015.

As the research on antibiotics and antibiotic resistance in water and natural systems has been conducted mostly in China and developed countries, we know very little about the scale of the problem globally. Recently, a first global study by a group researchers from York University studied 14 types of antibiotics in rivers in 72 countries, representing six continents (University of York, 2019). This study has been conducted in major rivers in the world, such as the Thames

River, Mekong River, and Danube river. They reported that 65% of these 72 countries detected a high level of antibiotics that exceeded the safe level, mainly in Bangladesh, followed by Kenya, Ghana, Pakistan, and Nigeria, while Austria was ranked the highest in Europe. The pollution depends significantly on the location of e.g. WWTPs, waste dumps, and in some areas also the political chaos. Singh et al. (2019) reviewed different polluted rivers around the world in 19 countries across 5 continents. They revealed that various factors affect the pollution level in the river: (1) different classes of antibiotics, (2) slow implementation of wastewater treatment and (3) higher consumption of antibiotics especially in the developing countries. However, this research does not describe the real situation of ARB&Gs in the world. Therefore, more global research and international collaboration is needed in order to fulfill this knowledge gap.

1.5 Pathways of antibiotics and antibiotic resistance in the environment

Approximately 75% of the used antibiotics are not fully digested and converted by humans or animals, and are excreted through urine and faeces (Chee-Sanford et al., 2009). Other sources can origine from industrial areas, livestock lots, or pharmaceutical industrial wastewater, which might possess different exposures to different types of antibiotics, ARB&Gs. The pathway of antibiotics and antibiotic resistance in various compartments and their concentration range is shown in Figure 1.4.

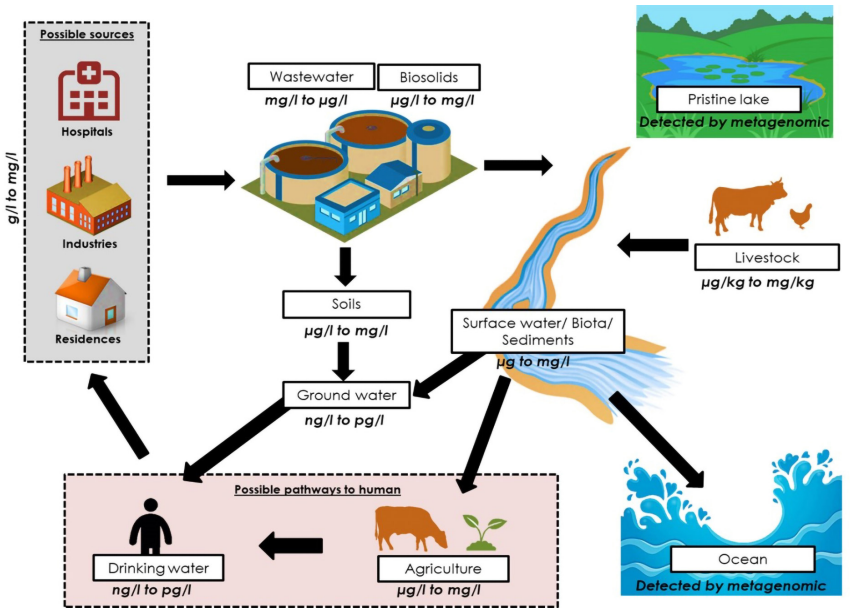

Figure 1.4: Distribution and range of concentration of antibiotics in different environments (Hatosy and Martiny, 2015; Chen et al., 2016; Kaczala and Blum, 2016; Roig and D'Aco, 2016; Zhang et al., 2017).

WWTPs are a reservoir where all varieties of organic compounds, nutrients, and metals from different sources accumulate (Naquin et al., 2015). Conventional WWTPs efficiently remove organic compounds and nutrients, but are not designed to remove these antibiotics from wastewater (Giger et al., 2003; Rowan, 2011). Therefore, WWTPs are identified as a hotspot for HGT to occur, and are suspected to propagate antibiotic resistance.

As a result, wastewater effluent can contain antibiotics, and this effluent is discharged to surface water. Wastewater effluents have been reported to comprise an abundance of ARB&Gs, most likely due to the cumulative effect of inefficient WWTPs processes and higher antibiotic concentrations inside the WWTPs (Rizzo et al., 2013). Higher antibiotic concentrations in wastewater tend to increase the concentration and overexpression of ARGs in the effluent, which may further accumulate bacterial resistomes (Rowe et al., 2017). Recently, ARB&Gs have been detected in water catchments, which were shown as a potential reservoir of ARGs (Amos et al., 2014; Czekalski et al., 2015). These compounds are present in water bodies in low concentrations, ranging from ng/l to mg/l. Via the surface water, antibiotics can enter the groundwater, and possibly even a drinking water supply (Bergeron et al., 2015). As expected, groundwater was found to be less contaminated with antibiotics (Vulliet et al., 2011).

If rivers are receiving runoff polluted antibiotics, or if wastewater effluents are used for agricultural purposes, antibiotics may be absorbed by plants through roots and leaves. Azanu et al. (2016) detected tetracycline and amoxicillin in lettuce and carrot in a range of 4-45 ng/g. Even though such concentrations are low, and hardly results in toxicity (Azanu et al., 2016), they concluded that plants could cause antibiotic resistance, when these levels are consumed. Cerqueira et al. (2019) reported that the wastewater did not contribute to the load of ARB&Gs on crops, but there is the possibility that the dissemination of ARB&Gs might occur.

The same holds for sludges from WWTPs, that can spread antibiotics and ARB&Gs to the environment (Pruden et al., 2013). Sludge application supplies organic matter and valuable nutrients such as nitrogen and phosphorus to agricultural soil and provides essential nutrients to plants (Latare et al., 2014; Lloret et al., 2016). This application on land could lead to higher soil microbial activity and biomass, but has the risk of dissemination of antibiotic resistance (Urra et al., 2019).

In addition, antibiotics can be released from agricultural lands or livestock. Livestock manure applied to land might have the potential to spread antibiotic resistance to the soil microbiome. Furthermore, antibiotics have been found in feed additives as growth promoters (such as penicillins, lincosamides, macrolides, erythromycin and tetracyclines), in spills, and in the discharge of feed or manure (Pan and Chu, 2017). The antibiotic application as a growth promoter is forbidden in European countries since 2006 (Castanon, 2007), but the application of antibiotics as a growth promoter is widely used in developing countries and China (Clement et al., 2019). China has banned the use of antibiotics for this purpose per 2020. In 2013, more

than 90,000 tonnes of antibiotics were used, with 51% being applied for livestock (Zhang et al., 2015).

ARGs can be found in the pristine natural area. The resistance mechanisms have emerged in the setting of antibiotics production itself (Wright, 2010) which has been observed in different pristine locations such as the permafrost mosaic system (Diaz et al., 2017), soils in the high arctic (McCann et al., 2019), antarctic soils (Van Goethem et al., 2018), and Tibetan lakes (Chen et al., 2016). ARGs have also been detected in the ocean (Chen et al., 2013b; Hatosy and Martiny, 2015).

As a consequence of the extensive distribution of the veterinary and human related antibiotics in the environment through different pathways, a broad range of antibiotics has been detected in municipal/industrial/pharmaceutical wastewater, soil, animal manure, sediment, surface water, groundwater and drinking water samples (Hu et al., 2010; Yang et al., 2010; Zhou et al., 2012; Zhou et al., 2013a; Awad et al., 2014; Hou et al., 2015).

1.6 Removal of antibiotics, antibiotic resistant bacteria and antibiotic resistance genes

As mentioned before, conventional WWTPs with activated sludge are designed to remove compounds such as organic compounds, nitrogen, and phosphorus. As a result, WWTPs are not a complete barrier for antibiotics and ARB&Gs to keep them from entering the environment. Therefore, establishing a good removal for antibiotics and ARB&Gs remains a challenge. Tertiary treatment technology may offer a good solution, though there are risks after such treatment, which causes changes in antibiotic resistance profiles and potentially contribute to the selection of ARB&Gs.

Various tertiary treatment technologies have been developed to remove or reduce the dissemination of antibiotics and ARB&Gs into the surface water. This tertiary treatment technology is a promising technology for enhanced wastewater treatment to improve wastewater quality andto reduce the emission of antibiotics, and ARB&Gs to surface water. This has been studied in different technologies, such as in an up-flow anaerobic sludge blanket reactor (Daud et al., 2018), anaerobic or aerobic tanks (Diehl and Lapara, 2010), constructed wetland (Yi et al., 2017; Hickey et al., 2018), chlorination (Zhuang et al., 2015) and solar disinfection (Rizzo et al., 2014; Fiorentino et al., 2015), Fenton processes (Santos et al., 2015), ozonation (Sousa et al., 2017), and filtration (Riquelme Breazeal et al., 2013). The treatment technologies might be either stand-alone or a combination of two or more treatment technologies, such as a combination of solar and H_2O_2, TiO_2-P25 (aeroxide), and graphene oxide-TiO_2 photocatalysis and photo-Fenton (Moreira et al., 2018).

In the Netherlands, limited research in full-scale tertiary treatment technologies for the removal of antibiotics and ARB&Gs has been conducted. There is a continuous concern about the risks for ecological and human health, that has resulted in monitoring ARB&Gs (NethMap-MARAN, 2015).

ARGs distribution in the Netherlands has been studied in different matrices, e.g. in pig fecal (van den Bogaard et al., 2000), birds (Veldman et al., 2013), soil (Knapp et al., 2010) and humans (Vo et al., 2006; Christian et al., 2014). In wastewater or water-related studies, limited research has been conducted. Montforts et al. (2007) observed that ARGs increased in ditches and surface waters after the application of antibiotics in a pig farm. A high percentage of ARB&Gs, which originated from human and veterinary pharmaceuticals, were found in the Meuse River, Rhine River, and New Meuse River (Blaak et al., 2011). Blaak et al. (2015) also reported that municipal wastewater contributed the highest enumeration of ARB in surface water compared to community and healthcare institutions.

Recently, there is new research reported on antibiotic resistance in the Netherlands. Paulus et al. (2020) reported ARGs profiles at eight sampling points along the Rhine river, starting from Lake Toma in Switzerland and ending at the river mouth in the Netherlands, where the water is discharged into the North Sea. Paulus et al. (2019) also reported that antibiotics and ARGs in Dutch hospital wastewater were reduced by a tertiary treatment including ozonation, a membrane bioreactor, UV irradiation and granulated activated carbon (GAC).

This thesis will also considers mitigation technologies to reduce antibiotics, ARB&Gs emissions from waste water to the environment, and the next subchapters will briefly discuss the selected treatment technologies to understand their principles.

1.6.1 Constructed wetlands

Constructed wetlands (CWs) are engineered systems comprised of water, substrate, plants, and indigenous microorganisms. CWs are currently used to treat wastewater effluents, as polishing step before the wastewater is discharged into surface water (Davis et al., 1995), and to treat trace organic compounds, such as personal care products and pharmaceuticals (Hijosa-Valsero et al., 2010). Lower costs, minimal use of mechanical/physical/chemical equipment, efficiency in removing nutrients, and environment-friendly features compared to other tertiary treatment make CWs interesting as an alternative for treating domestic, agricultural, and industrial wastewater (Vymazal, 2010).

CWs are divided into two types depending on the hydrology and flow path, which are either surface flow or subsurface flow (vertical or horizontal) (Vymazal, 2011a). Figure 1.5 shows the different configurations and flow paths of CWs (surface or subsurface flow). Commonly

used plants in CWs are *Phragmites australis, Typha latifolia, Typha angustifolia, Juncus effusus, Scirpus californicus, Scirpus lacustris,* and *Phalaris arundinacea* (Vymazal, 2013).

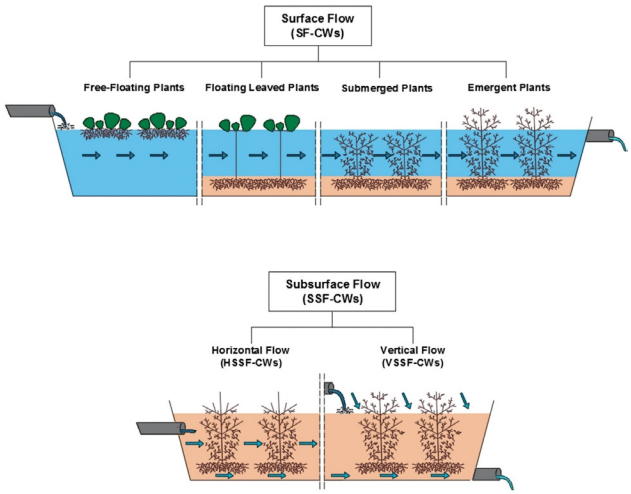

Figure 1.5: Constructed wetland configurations (surface or subsurface flow) with different types of plant growth (free-floating, floating leaves, submerged or emergent) and flow paths for subsurface flow (horizontal or vertical). Adapted from Gorito et al. (2017).

Different removal mechanisms are involved, such as physical processes (physical adsorption, filtration, and volatilization), chemical processes (ion-exchange, precipitation, and oxidation-reduction reaction) and biological processes (microbial degradation and plant uptake) (Chen et al., 2017). Apart from that, the plants growing in CWs are able to oxygenate the CWs, transform contaminants, provide a surface for periphyton attachment that can reduce the pollutants, or take up and convert the compounds in their tissues (Vymazal, 2011b).

CWs are able to control pollution and improve water quality (Vymazal, 2007). CW may remove some of the micropollutants, antibiotics and ARGs (Anderson et al., 2013; Dan et al., 2013; Fang et al., 2017), but are not able to completely remove ARB&Gs (Hijosa-Valsero et al., 2011; Berglund et al., 2014), which may contribute to the dissemination of ARB&Gs into surface water.

1.6.2 Physical treatment processes

Several studies observed the removal of antibiotics and ARB&Gs by employing physical treatment processes, such as granular media filtration, adsorption onto activated carbon (AC), and membrane filtration. AC will be discussed in more detail below.

Wastewater technologies that are based on adsorption involve binding and removing certain compounds from water through an adsorbent such as AC (Hung et al., 2005). AC is the most commonly used adsorbent, due to the affordable price and ability to adsorb a variety of organic compounds, with other adsorbents such as zeolites and polymers being less adsorbent. The powerful adsorptive properties are related to the high specific surface area (1,000 m^2/g) (Tadda et al., 2016), microporous structure and large pore volume (Choi et al., 2005). Therefore, AC has shown potential in removing disinfection by-products (Gopal et al., 2007b), micropollutants (Choi et al., 2005), antibiotics (Pachauri et al., 2009) and ARB (Ravasi et al., 2019) and ARGs (Sun et al., 2019).

AC is available in three main forms; powder, granular, and a pellet. However, the most commonly used forms are powdered and granular AC. The most common raw materials for activated carbon are carbonaceous materials like wood, sawdust, nutshells, peat, coal, coke, petroleum, bones and coconut shells (Marsh and Rodríguez-Reinoso, 2006). Production of AC involves dehydration and carbonization followed by chemical or physical activation (Tareq et al., 2019). However, to recycle and preserve the limited resources, AC needs to be regenerated. This can be done via microwave thermal treatment (Ondon et al., 2014), bioregeneration (El Gamal et al., 2018) or heat and chemical activation (Park et al., 2019).

An example of a specific set-up that uses activated carbon as a post-treatment is the 1-STEP® filter (Figure 1.6). The 1-STEP® filter is a compact fixed bed based on activated carbon, and combining four processes in one single additional treatment unit (Bechger et al., 2013; Nijhuis, 2018). The processes for removal involve adsorption and filtration by the suspended solids. Other than that, denitrification by selective carbon source for nitrogen removal and coagulation and flocculation by poly-aluminium-chloride for chemical and heavy metals removal takes place (Bechger et al., 2009; Bechger et al., 2013).

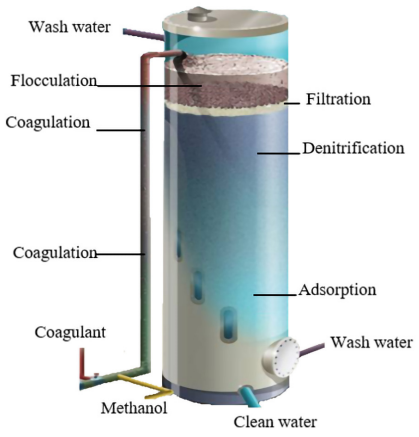

Figure 1.6: 1-STEP® filter. Adapted from Bechger et al. (2013).

1.6.3 Disinfection and oxidation processes

Disinfection is the treatment of wastewater effluent for the removal of pathogens and microbes to a safe level. A variety of treatments include chemical disinfectants, such as ozone, chlorine, chlorine dioxide, and peracetic acid. In addition to chemicals, UV irradiation has been applied for many years as well. Together with UV, hydrogen peroxide (H_2O_2) and titanium dioxide (TiO_2) are commonly combined as additional oxidants for the degradation of organic compounds and pathogens. In this subchapter, chlorination, UV irradiation and ozonation will be discussed.

a. Chlorination

Chlorination is regarded as an effective treatment to control the occurrence of waterborne pathogens, including bacteria and viruses (Gopal et al., 2007), due to its low cost and high efficiency of microbial inactivation at a minimum level of chlorine (Prasse et al., 2015). The capacity of chlorination to inactivate pathogens depends on the pH, temperature, and organic compounds in the wastewater (Russell, 2006). Chlorination offers reactive chlorine species with a high stability, such as hypochlorous acid, gaseous chlorine, and chlorine dioxide (Li et al., 2017).

After chlorine is added to wastewater, it rapidly hydrolyzes to hypochlorous acid (HOCI, electrically neutral) and hypochlorine ion (OCl−, electrically negative), which are referred to as "free available" chlorine. Since the surface of a pathogen is negatively charged, it is more attracted to uncharged HOCI compared to charged OCl− (Verma et al., 2015). This oxidation reaction by the free chlorine leads to the degradation of the organic compounds (Westerhoff et al., 2005). Although this chemical treatment is able to reduce the risk of pathogenic infection, the use of chlorination has raised concerns regarding the formation of disinfection byproducts (DBPs) as a result of a reaction with the natural organic matter (humic acid and fulvic acid) in water (Lavonen et al., 2013). Examples of DBPs are trihalomethanes, bromoform, chloroform, dibromochloromethane and bromodichloromethane, which are scrutinized due to their potential adverse human health effects (Gopal et al., 2007b; Jeong et al., 2012).

Chlorination has been proven to control nitrite in drinking water (Yang and Cheng, 2007) and has shown potential in removing micropollutants (Lee and von Gunten, 2010; Wang et al., 2019a). Chlorination is reported to inactivate ARB more efficiently compared to ARGs (Furukawa et al., 2017). However, this treatment could not eliminate the potential risk of ARGs in wastewater, since 80% of the studied ARGs persist in wastewater after chlorination (Yuan et al., 2015). Other than that, it is possible that extracellular ARGs in wastewater (Wang et al., 2019a) and antibiotic transformation products (Kennedy Neth et al., 2019) are triggered after chlorination. which may be an additional source of antibiotic resistance in wastewater. Due to this negative effect of chlorination, alternative treatments for water disinfection are needed and therefore, UV irradiation and ozonation are gaining attention.

b. UV irradiation

UV irradiation has been used as a disinfection method for water treatment since 1910 (Hijnen et al., 2006). It is applied to reduce the concentration of waterborne pathogens before discharging wastewater from WWTPs to the receiving water body (Locas et al., 2008). Concentrations of dispersed microorganisms, suspended particles or particle sizes can influence the efficiency of UV disinfection (Taghipour, 2004). Therefore, the water must have a low turbidity and is noncolored, before the water is radiated.

UV spectra (Figure 1.7) have a wavelength range of 100 nm to 400 nm. The electromagnetic spectrum of UV irradiation can be divided into three types, UVA (≈315-400 nm), UVB (≈280-315 nm), and UVC (≈100-280 nm). Commercial systems of UV operate with low to medium powered UV lamps and have a wavelength of approximately 354 nm (Russell, 2006). The UV irradiation between 200 to 300 nm, approximately 253.7 nm (UVC), demonstrates the germicidal effect, and this is dangerous for human health because they might be associated with cell skin cancer (Widel et al., 2014), bladder and rectal cancer (Morris et al., 1992), different types of cancer (Aslani et al., 2019) and reproductive outcomes (Nieuwenhuijsen et al., 2000).

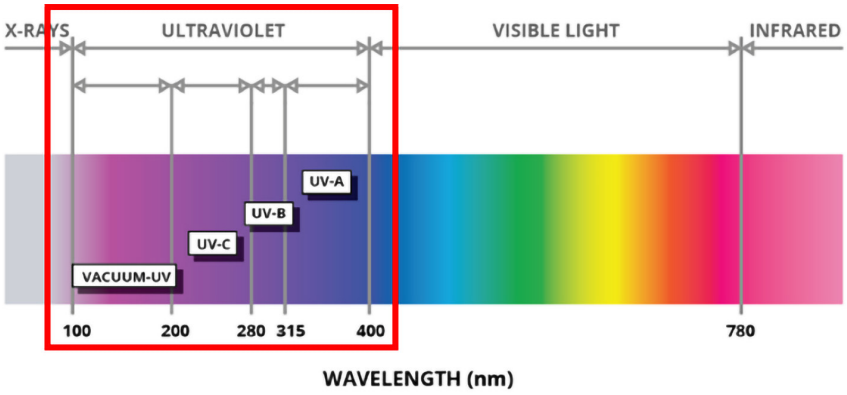

Figure 1.7: Electromagnetic spectrum of ultraviolet radiation (in the red box).

UV irradiation is safer than chlorine addition because: (1) UV produces less carcinogenic by-products while providing good removal (Hijnen et al., 2006), and (2) is directly effective against the DNA of bacteria (Samer, 2015). The nucleic acids of bacteria absorb the radiation that penetrates the cell wall and, as a result, cause the inhibition of replication and death of the cells (Hijnen et al., 2006). Occasionally, H_2O_2 or TiO_2 is added in the presence of UV irradiation to enhance oxidation due to the higher oxidation potential of hydroxyl radicals (2.8 V) compared to chlorine (1.39 V) and ozone (2.07 V).

UV has been proven in treating protozoan parasites (Hijnen, 2010; Adeyemo et al., 2019). and is increasingly used as a disinfection process to remove micropollutants, such as pharmaceuticals (Kim et al., 2009), antibiotics (Khorsandi et al., 2019) and ARB&Gs (McKinney and Pruden, 2012; Sharma et al., 2016; Guo et al., 2017). However, there isa possibility that the protozoan cell or their spores can recover, and damaged cells or spores can re-grow, which is stimulated by the inactivation of their predators and competitors (Sousa et al., 2017).

c. Ozonation

Ozone (O_3) is a powerful oxidizing agent characterized by high disinfection efficiencies and is able to oxidize inorganic and organic compounds in water (Blaney, 2014). The ozonation popularity has increased because it does not produce potential carcinogens (trihalomethanes or other chlorinated by-products related). However, potentially carcinogenic bromates and aldehydes may be produced by ozonation (Gounden and Jonnalagadda, 2019). and may have adverse health effects (Kumar, 2011; Gerba and Pepper, 2019).

O_3 is produced when oxygen (O_2) molecules are dissociated by an energy source into single oxygen atoms (O) (Collivignarelli et al., 2018). These O_2 atoms can collide to form O_3, which is an unstable gas and should therefor be produced onsite (Russell, 2006). O_3 directly oxidizes the cell wall of microorganisms, forms reactions with radical by-products, damages the DNA and RNA, and causes depolymerization (Tchobanoglous et al., 2014). The effectiveness of O_3 treatment depends on several factors, such as contact time, ozone concentration, and susceptibility of the microorganism. Ozonation is able to improve the quality of wastewater (Rosenblum et al., 2012) and drinking water (Papageorgiou et al., 2014) and can remove antibiotics, and ARGs (Zhuang et al., 2015; Collivignarelli et al., 2018).

This technology needs only short contact times (1-5 minutes), low concentrations (1-5 ppm), and results in a rapid decomposition of O_3 (Ölmez and Kretzschmar, 2009). In addition, the effectiveness of ozonation can be enhanced by adding H_2O_2 and persulfate (Oh et al., 2014; Oh et al., 2016). However, the design and technology are more complex than the other disinfection treatments (chlorination and UV irradiation), more costly, require more energy. and may produce possible carcinogenic compounds (Russell, 2006).

1.6.4 Aerobic granular sludge

Conventional activated sludge (AS) is the standard treatment technology for biological treatment of wastewater (Samer, 2015). AS requires large areas for installation of two tanks: (1) anaeration tank for the process of biological reactions such as organic carbon removal and nitrification and (2) a settling tank for separating activated sludge from the treated water by settling (Nancharaiah and Reddy, 2017). In AS, the microbial community is typically cultivated in the form of 'flocs.' These flocs are separated from the wastewater in the settling tank and can be returned to the reactor. Poor settling flocs, due to the unnecessary growth of filamentous microorganisms. may affect the properties of the sludge sedimentation.

Therefore, to overcome the drawbacks in AS, aerobic granular sludge (AGS) has been introduced (Wilén et al., 2018). AGS is known for the excellent settling ability, simultaneous removal of organic matter and nitrogen, high biomass concentration and good ability to withstand the high organic load. AGS is a granule comprised of self-immobilized cells and does not depend on material support for biofilm growth. It can retain a large number of microorganisms, at the same time permitting rapid bioconversion of many compounds and improve the performance and stability of the reactor (Bassin, 2018). In addition, AGS is a compact system (using only one tank for settling and aeration), and cost-effective wastewater treatment (Nancharaiah and Reddy, 2017). These characteristics made AGS attractive to be implemented in wastewater treatment. Some full-scale AGS have recently been installed to treat municipal and industrial wastewater (van der Roest et al., 2011; Pronk et al., 2015). As this is a relatively new technology, there are limited data, especially on the removal of antibiotics and ARB&Gs in full-scale systems (Wang et al., 2019b).

NEREDA® is a technology based on aerobic granular sludge, that has been developed in the Netherlands (van der Roest et al., 2011). The NEREDA® granules consist of different microorganisms, including phosphate accumulating organisms, nitrifiers, denitrifiers and glycogen accumulating organisms, which allows several processes at once (Giesen et al., 2013). Due to the growth in dense grains, there are different oxygen levels within the grain, and different organisms can carry out their function. Figure 1.8 shows the composition of a granule of NEREDA®.

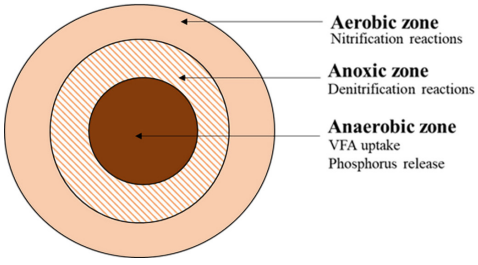

Figure 1.8: NEREDA® granules.

1.7 Aim and outline of the thesis

ARGs are recognized as a global threat that not only occur in developing countries, but worldwide (WHO, 2014). Wastewater effluent has been identified as one of the important sources in disseminating ARGs to the environment. Nowadays, ARGs can be found nearly everywhere in the environment, such as rivers (Amos et al., 2014), groundwater (Szekeres et al., 2018), manure (Xie et al., 2018), soil (Hu et al., 2010), sediment (Czekalski et al., 2014) and crops (Azanu et al., 2016). The prevalence of ARGs in the environment is evident worldwide, such as in China and the USA.

The above review of the state of the art of antibiotic and ARB&Gs dispersion by WWTP effluents into the environment indicates various knowledge gaps:

1. It is unclear what the environmental background of ARB&Gs is in rivers and their sediments to which WWTP effluents discharge.
2. Which treatments are most effective in educing the emissions and are effective in reducing environmental ARGs concentrations, specifically:
    a. Advanced biological technologies as secondary treatment, such as AGS as compared to AS
    b. Physicochemical technologies as tertiary treatment, such as UV and AC
    c. Nature based technologies as post-treatment, such as CWs.

Some full-scale studies have been studied in the Netherlands; however, these represent only a small part of case studies in the Netherlands. Hence, antibiotics and ARGs distribution in the water, and the effect of full-scale tertiary treatment technologies in removing antibiotics and ARGs in the Netherlands is still unclear. Therefore, this thesis presents the occurrence of antibiotics and ARGs in wastewater and in the environment and also the potential of full-scale treatment technology in removing antibiotics and ARGs from Dutch wastewater. Figure 1.9 presents a summary of the thesis.

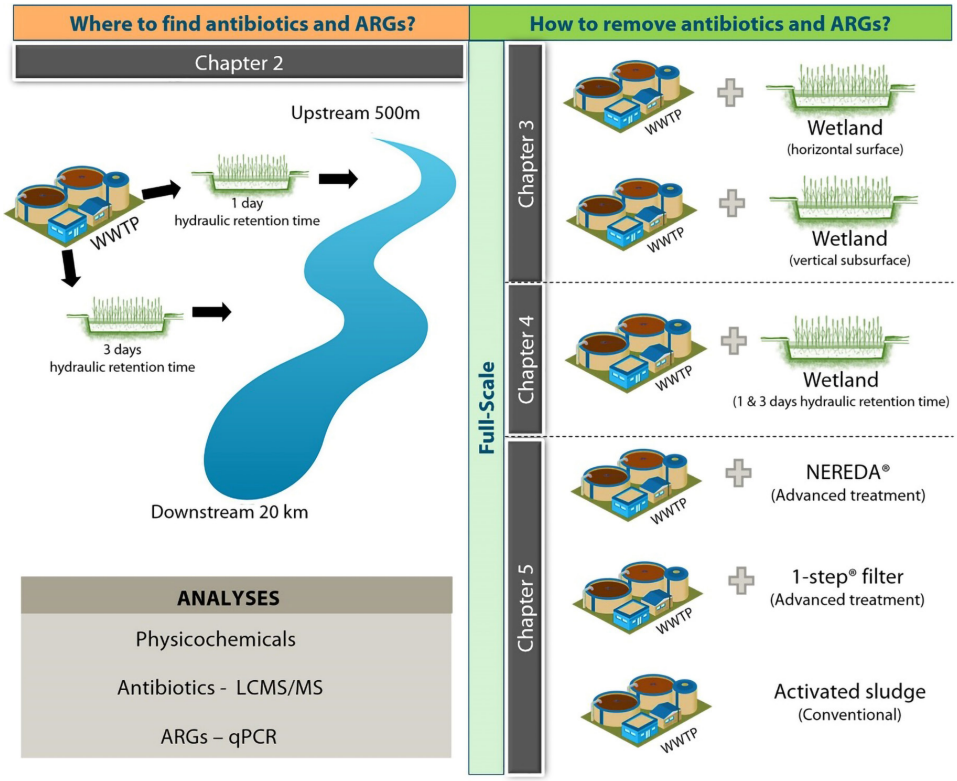

Figure 1.9: Overview of the research described in this thesis. The grey plus refers to the additional treatment added for each wastewater treatment plant.

The aim of this thesis is to investigate the occurrence and removal of antibiotics and ARGs in the Netherlands and to assess different treatment technologies in removing antibiotics and ARGs. Studies were conducted from the environment back to the lab, first to understand the pathways to, and in, the environment and second, to assess and investigate which treatment technologies are suitable to reduce antibiotics and ARB&Gs emissions from WWTP into the environment. Therefore, a one-year sampling was conducted at a Dutch WWTP with effluent receiving constructed wetlands, and the subsequent discharge in a river up to 20 km downstream to study the prevalence of antibiotics and ARGs along the river in different seasons **(Chapter 2)**. After having studied the occurrence of antibiotics and ARGs, different treatment technologies were assessed for their removal capacity of antibiotics or ARGs. The performance of full-scale constructed wetlands (horizontal or vertical flow) in removing antibiotics and ARGs was evaluated **(Chapter 3)**. In addition, two similar CWs receiving the same WWTP effluent and with different hydraulic retention times (1 or 3 days) were compared in order to assess their performance in removing antibiotics and ARGs **(Chapter 4)**. Finally, the removal performance of a conventional WWTP was compared to two advanced treatments (1-STEP® filter and NEREDA®), in order to understand the performance of these advanced treatment

technologies **(Chapter 5)**. The results of these chapters indicate the usefulness for additional advanced treatment in order to reduce the concentration of antibiotics and ARGs in WWTP effluent before being discharged into surface water. The findings from the research chapters are discussed in **Chapter 6** by summarizing the best treatment technology and the persistence of antibiotics and ARGs in the Netherlands. In addition, antibiotic resistance from the perspective of Malaysia is presented, followed by the main conclusions and recommendations for future research.

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CHAPTER 2

Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands

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Abstract

Antibiotics are being used intensively for humans and livestock worldwide and have led to the presence of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment. Wastewater treatment plants (WWTPs) have been identified as a point source for ARB&Gs, and water catchments consequently as potential receptors of ARB&Gs. The objective of this study was to investigate the occurrence of antibiotics (macrolides, sulfonamides, tetracyclines), ARGs (*ermB*, *sul1*, *sul2*, *tetW*), and class 1 integron (targeting the integrase gene) in a Dutch river, that receives WWTP effluent. Sediment and water samples along the river were collected during one year. The WWTP significantly increased the amounts of antibiotics and ARGs in the river, compared to the upstream samples, in which the antibiotics decreased once they had entered the river. ARGs were persistent in the water and sediment from the WWTP effluent discharge point, until 20 km downstream. This study provides insight in the prevalence of antibiotics and ARGs in a WWTP effluent-receiving river system in the Netherlands. Even though human antibiotic usage is low in the Netherlands, antibiotics, residues of antibiotics, and ARGs were detected in the river surface water-sediment system, which shows that a river has the potential to act as a reservoir of ARGs.

Keywords: antibiotic resistance genes, wastewater treatment plant, antibiotics, river

2.1 Introduction

For decades, antibiotics have been used to cure human or animal infectious diseases by either killing or inhibiting the growth of bacteria. Antibiotics have given a major contribution to the medical field for decades (Gould et al., 2000). As antibiotics are not fully degraded within animals or humans, and approximately 30-90% of the antibiotics used for animals are excreted through urine and faeces (Gao et al., 2012). Through the sewage system, WWTPs also receive water that can contain a number of pollutants, including nutrients, metals, antibiotics and chemicals from different sources (Naquin et al., 2015). However, our current WWTPs are not designed to remove micropollutants, such as antibiotics, antibiotic resistant bacteria and genes. Those biological components might end up in the WWTP effluent (Giger et al., 2003) as they are not fully removed by current treatment technologies (Hijosa-Valsero et al., 2011; Rowan, 2011; Rizzo et al., 2013).

WWTPs have been suggested as potential hotspots for antibiotic resistance and antibiotic resistance genes (ARGs) (Michael et al., 2013). Antibiotics, ARB and ARGs are frequently detected in WWTPs (Szczepanowski et al., 2009; Devarajan et al., 2016). Antibiotic resistance is developing faster than new antibiotics are being developed, whereas identifying new antibiotics is becoming increasingly challenging and costly (Power, 2006; Ling et al., 2015). As a result, new antibiotics are hardly introduced, and antibiotic resistance is found in hospital settings, and also in the natural environment. In addition, antibiotic resistance is also a natural phenomenon, as bacteria have evolved resistance to naturally present antibiotics (Durso et al., 2016).

As a result, WWTP effluent can contain antibiotics, ARB and ARGs, and once this effluent is discharged to the surface water, these contaminants will enter the environment. ARGs are spread into the surface water by ARB, that possibly acquired these ARGs through horizontal gene transfer. Even though the proliferation of ARGs in the environment is assumed to be low, ARB are an important contributor in transporting and spreading of antibiotic resistance in the microbial community (Larson, 2007).

Recently, dissemination of ARGs in the environments has been highlighted as an emerging problem (WHO, 2014; Berglund et al., 2015), especially if contaminated water resources are reused for cattle, irrigation, or drinking water production (Fahrenfeld et al., 2013; Su et al., 2014). Such water reuse is gaining more attention for overcoming water scarcity and achieving sustainable water management in especially arid regions (Luprano et al., 2016). World population growth and draughts are the main factors that will increase the demand of water reuse. Therefore, the need for clean and safe water, that is free of emerging wastewater contaminants, antibiotics, antibiotics residues and ARGs, are needed.

The role of WWTP as sources of antibiotics and ARGs and their dissemination in rivers is an active field of research (Pei et al., 2006; Storteboom et al., 2010b; Pruden et al., 2012; Amos et al., 2014; Amos et al., 2015; Zheng et al., 2017). An increase in concentration of antibiotics and ARGs in rivers has repeatedly been found to be caused by emissions of WWTP effluent (Storteboom et al., 2010b; Amos et al., 2015), but also by a variety of other activities, such as an increased density of population along the river, industrial operations, agricultural and/or aquacultural activities (Pei et al., 2006; Jiang et al., 2017). For example, in India, Devarajan et al. (2016) concluded that beta-lactamase genes (*blaSHV* and *blaNDM*) were identified in sediments contaminated by hospital and urban wastewaters in Cauvery River. Lekunberri et al. (2017) observed the same trend in Spain. They observed that concentrations of antibiotic and ARGs (macrolides gene (*ermB*), fluoroquinolones gene (*qnrS*) and tetracyclines gene (*tetW*) in the Ter River showed a significant difference in discharge upstream and downstream of WWTP effluent. Chen et al. (2013a) reported that tetracycline genes (*tetC*, *tetB*, *tetM*, *tetO*, and *tetW*) were detected in the Pearl River in China, which is heavily influenced by human activities. Meanwhile in China, Ling et al. (2013) found out that sulfonamide genes (*sul1* and *sul2*) and tetracycline genes (*tetG*, *tetA*, *tetO*, *tetC*, *tetX*, *tetM* and *tetQ*) were frequently observed in the Beijiang River at locations with the highest degree of urbanisation. Dissemination of ARGs in the river has not only been shown in water and sediment, but also in biofilms (Proia et al., 2016) and aquatic animal guts (Fu et al., 2017). With the dissemination of antibiotics and ARGs to rivers, ARGs finally enter marine environments, including marine water or sponge species (Chen et al., 2013a; Hatosy and Martiny, 2015; Laport et al., 2016).

This paper describes the occurrence of emerging wastewater contaminants (antibiotics and ARGs) and nutrients along a Dutch river, tracking the effect of WWTP effluent discharge. We performed the sampling in the river with only one small side stream at 2 km from 0.5 km upstream until 20 km downstream. Sediment and water samples were collected in repeated samplings during one year to identify correlations between ARGs and other environmental factors (pH, temperature, dissolved oxygen (DO), chemical oxygen analysis (COD), total phosphate (TP), ammonium (NH_4^+) and nitrate (NO_3^-)).

2.2 Material and Methods

2.2.1 Sampling site and sample collection

Field samples upstream and downstream a WWTP plant (Hapert, the Netherlands) were collected from February 2016 until January 2017. The WWTP treats 78% of domestic and 22% of industrial wastewater via a conventional system consisting of bar screens, grit removal, and an oxidation ditch. The WWTP effluent was split into two additional post treatments, namely two wetlands with a hydraulic retention time of 1 day or 3 days.

Sampling was performed in the Grote Beerze river, the Netherlands (51°22' N 5°14' E). Sediment (n=390) and water (n=390) samples were collected from 13 sampling points during one year. Sampling in the river started 0.5 km upstream (U1) and continued until 20 km downstream (D9) of the WWTP in the Grote Beerze river, and included two effluents from the two wetlands (E1 and E2), that discharged into this river (Figure 2.1). For season comparison, water temperature during sampling was used to classify the season. Temperatures above 15ºC were classified as summer season, and below 15ºC were classified as winter season. Therefore, the summer months were May, June, July and August, and the winter months were September, October, November, December, January and February.

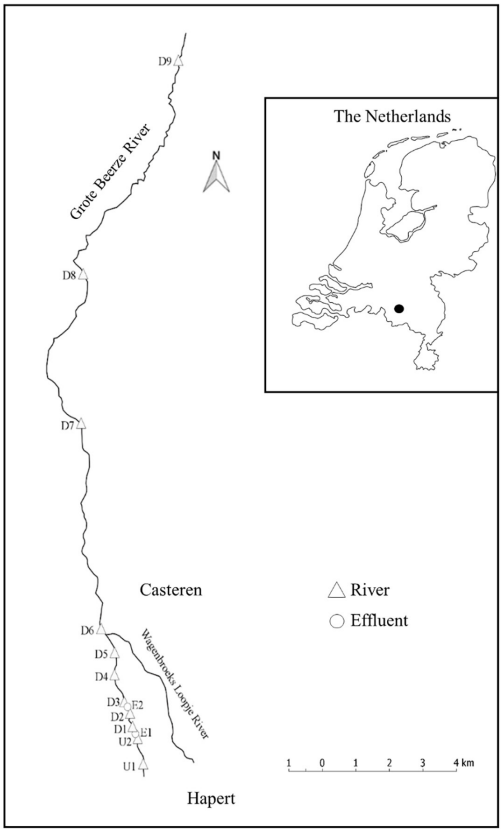

Figure 2.1: Map showing the 13 sampling points along Grote Beerze River, The Netherlands. E1 = effluent of wetland with HRT 1-day. E2= effluent of wetland with HRT 3-day. U1 = 0.5 km before E1, U2 = 0.1 km before E1, D1 = 0.1 km after E1, D2 = between E1 and E2, D3 = 0.1 km after E2, D4 = 0.5 km after E2, D5 =1 km after E2, D6 = 1.5 km after E2, D7 = 5 km after E2, D8 = 10 km after E2, D9 = 20 km after E2.

Water grab samples from each sampling point were collected into 1 L sterile green glass bottles with screw caps, and transported to the laboratory for analysis. 100 ml was stored at -20ºC for residue analysis of antibiotics and the rest of the water samples was stored at 4ºC for chemical and qPCR analysis. Chemical analyses were performed within 1-3 days of collection. Sediment samples were collected in 50 ml plastic tubes by using a grab sampler, transported to the laboratory, and stored at -20ºC for qPCR analysis. All samples were taken in triplicate.

2.2.2 General water quality and chemical analysis

General water quality parameters including pH, temperature and dissolved oxygen (DO), were measured in-situ using a pH and DO portable probe (Hach, USA). Chemical oxygen analysis (COD), total phosphate (TP), ammonium (NH_4^+) and nitrate (NO_3^-) were measured using Hach kits (USA; LCK 1414, LCK 349, LCK 304 and LCK 349, respectively) for each triplicate sample. COD and TP were measured directly in the stored samples, whereas NH_4^+ and NO_3^- were filtered using a 4-7 µm filter paper (Whatman, United Kingdom), prior to analysis. All samples were processed within 2 days after sampling.

2.2.3 Sample preparation for the analysis of antibiotics

Water samples for October and November were analysed for 18 sulfonamides, trimethoprim, 6 tetracyclines, 12 quinolones and 15 macrolides. The analyses were performed in single measurement. All antibiotics are listed in Table S2.1. The antibiotics were chosen from human and veterinary antibiotics, that are frequently used and that have been detected in water (Huovinen et al., 1995; Ye et al., 2007). We also included antibiotics that correspond to the resistance gene classes analysed. Sulfonamides correspond to *sul1* and *sul2*, tetracyclines to *tetW*, and macrolides to *ermB*. Prior to chemical analysis by LC-MS/MS, the samples were concentrated by solid phase extraction (SPE). All chemicals were purchased from Sigma.

Liquid samples were taken from the freezer and thawed. Duplicate aliquots of 10 ml of each sample were transferred to two plastic tubes of 30 ml each. To both aliquots, 10 µL of a mixture of internal standard solution (500 µg/L) was added. To one aliquot, 100 µL of a mixture of all antibiotics (2.5 µg/L for the sulfonamides and 10 µg/L for the tetracyclins, quinolones and macrolides) was added. The samples were mixed and 4 mL of McIlvain buffer (0.1 M citric acid, 0.2 M phosphate buffer and Na_2EDTA; pH 4) was added. The samples were horizontally shaken for 5 minutes at 120 rpm, followed by centrifugation at 4000 g for 10 minutes.

200 mg Strata-X (Phenomenex, USA) SPE columns were washed with 5 ml methanol (MeOH), followed by 5 ml McIlvain buffer. Thereafter, the sample extract was loaded onto the column, followed by a washing step with 5 ml purified water (Milli-Q, Merck, USA). Vacuum pressure was applied to extract the liquid from the SPE columns. Hereafter, the columns were eluted with 5 ml of MeOH, and the eluting liquid was collected in clean collection glass tubes.

The collection tubes were placed in a nitrogen evaporator of 40°C to evaporate the MeOH. After complete evaporation, the samples were redissolved in 100 µl of MeOH and vortexed for 5 seconds. Finally, 400 µl of purified water was added and vortexed for another 5 seconds. The homogenized samples were transferred to LC-vials with insert. The vials were stored at -20°C until further analysis using LC-MS/MS.

2.2.4 Liquid Chromatography Mass Spectrometry (LC-MS/MS)

The samples were analysed for 18 sulfonamides, trimethoprim, 6 tetracyclines, 12 quinolones and 15 macrolides using LC-MS/MS. A standard calibration curve was prepared with levels of 0–500 ng/L for the sulfonamides and trimethoprim and 0–2000 ng/L for the tetracyclines and macrolides. The chromatographic mobile phase consisted of ammonium formate (1 M) / formic acid / water (2 / 0.16 / 1000) (V / V / V) (A) and ammonium formate (1 M) / formic acid / methanol (2 / 0.16 / 1000) (V / V / V) (B). The mobile phase gradient was ramped at a flow rate of 0.3 ml/min from 1% (A) to 25% in 2.5 min, 25% to 70% in 5.4 min, and 70% to 100% in 0.1 min, kept for 1 min, then ramped to 0% in 7.5 min and kept for 2.6 min. The LC–MS/MS consisted of an Acquity™ UPLC (Waters, USA) and AB Sciex QTrap 5500 (Applied Biosystem, USA) with a positive electrospray ionization (ESI) interface. The analytical column used was BEH C18 (Waters, 100 mm × 2.1 mm, 1.7 µm) at a temperature of 30°C. Injection volume was 10 µl and the flow rate was 0.3 ml min^{-1}. The results were recorded by a MultiQuant (Applied Biosystems, version 3.0.2). The specific instrument conditions are summarized in Table S2.2.

2.2.5 DNA extraction and quantitative PCR (qPCR)

For qPCR analyses, 100 ml water samples were filtered using 0.2 µm membrane filters (isopore filters polycarbonate, 0.2 um, 47 mm, Merck Millipore, Ireland) within 24 hours. One extraction was prepared per sampling point per month. The filter was stored at -20°C before DNA extraction. DNA extraction of water samples was conducted using a PowerWater DNA Isolation Kit (MoBio Laboratories, USA) and sediment samples with a PowerSoil DNA Isolation Kit (MoBio Laboratories, USA), according to the manufacturer's protocols. The extracted DNA was stored at -80°C until further analysis.

The 16S rRNA gene, the class 1 integrase gene (*intI1*) and four ARGs of interest, including *sul1* and *sul2* (sulfonamide resistance genes), *tetW* (tetracycline resistance genes) and *ermB* (macrolide resistance gene) were quantified by qPCR on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Canada). A reaction with a total volume of 10 µL was set up by adding 3 µL of DNA to 7 µL of master mix including IQ supermix (iTaq™ DNA Polymerase) or IQ sybrgreen supermix iTaq™ DNA polymerase (Biorad), the appropriate primers (Eurogentec, Belgium), molecular-grade water, and precision blue (Biorad, USA). Details of qPCR conditions and primers are shown in Table S2.3. All samples were run in duplicates.

Serially diluted DNA of a synthetic standard of known quantity was used as a standard and molecular-grade water was used as a negative control. The detection limit was in the range of 4.81-4.70×10^7 gene copy/µl for 16S rRNA gene, *intI1* and all ARGs. These quantifications were validated with high R^2 values (average 0.98) and high efficiencies (from 94 to 108%) (data not shown).

All samples were diluted 50 times before performing qPCR to avoid qPCR inhibition by humic acids, biological contaminants or proteins. The necessary degree of dilution had been determined in preliminary experiments with a range of dilutions of selected samples. The results were recorded by CFXManager (Biorad, version 3.0).

2.2.6 Statistical analysis

The effect of the WWTP discharge was tested in a linear model with location, season and sample type as factors in R (Version 3.4.0, USA) with Bonferroni correction for multiple testing of several genes. Linear regression was used to measure the effect between ARGs concentration (log transformed) and river distance (log transformed) from D3 until D9. Sampling months were grouped in seasons in order to prevent over-parametrisation of the model. Total load gene copies of ARGs per month were calculated by multiplying concentration of ARGs (total gene copies/ml) and water flow (m^3/day) of the sampling day. Precipitation data were provided by The Royal Netherlands Meteorological Institute (KNMI).

2.3 Results

2.3.1 WWTP discharge to the river

2.3.1.1 Fate of antibiotics

General characteristics (DO, pH, temperature, COD, NH_4^+, NO_3^-, and TP) of the effluents (E1 and E2) and along the river are summarized in Text S2.1 and Figure S2.1-S2.7. The antibiotic concentrations were measured during two sampling campaigns, October and November 2016. The fate of 52 selected antibiotics was investigated in this study. Out of the 40 target antibiotic compounds, only sulfamethoxazole (SMX), sulfapyridine (SP), and trimethoprim (TMP) were detected (range 1-150 ng/L) in the WWTP effluents and along the river (Figure 2.2). The other antibiotics were not detected. Of these three detected antibiotics, only sulfonamide was detected upstream of the WWTP (Figure 2.2). Only sulphonamides were found at the upstream of the WWTP, but at lower concentrations than in the downstream samples. Concentrations of sulphonamides were highest in the effluent of the wetlands, and remained relatively stable along the river in October and slowly decreased in concentration in November.

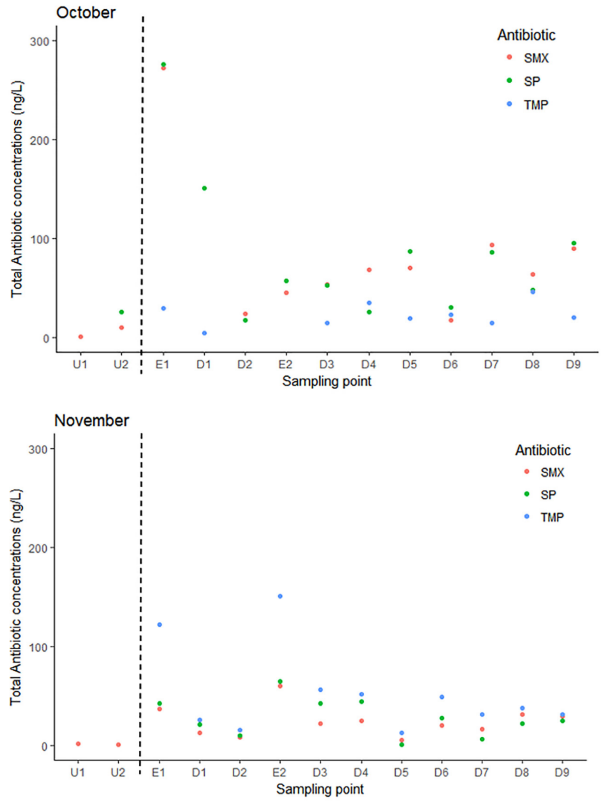

Figure 2.2: Profile of antibiotics (ng/L) upstream and downstream of a WWTP for October and November. The details of the sampling points are given in Figure 2.1. SMX=sulfamethoxazole, SP=sulfapyridine, TMP=trimethoprim. Dotted line distinguishes upstream and downstream of the WWTP.

2.3.1.2 Fate of ARGs

In this study, 4 ARGs (*ermB*, *sul1*, *sul2*, and *tetW*) as well as the 16S rRNA gene and *intI1* were quantified by qPCR in the sediment and water samples. The concentration of ARGs and *intI1* in sediment and water samples upstream and downstream of the WWTP effluent are presented in Figure 2.3. The absolute concentration of 16S rRNA gene and the relative concentrations (calculated relative to the 16S rRNA gene data) are presented in Figure S2.8-S2.13. Upstream data (U1) were compared to downstream data (D1) to determine the influence of the WWTP effluent discharge in the river. The data show elevated concentrations in the downstream locations as compared to the upstream locations. Downstream concentrations were more than two orders of magnitude higher ($2.34 \times 10^2 \pm 1.66$ gene copies from U1 to D1 ($p<0.01$) for *sul1*). The same trend was observed in all ARGs and *intI1* except for *tetW*. From U1 to D1, *ermB* increased $2.88 \times 10^1 \pm 1.48$ genes copies, $3.98 \times 10^2 \pm 0.23$ genes copies for *sul2* and $3.02 \times 10^2 \pm 0.21$ genes copies for *intI1*, all showing a statistical significant difference ($p<0.01$). The gene copy number of *tetW* did not increase significantly ($1.86 \times 10^1 \pm 1.73$ genes copies).

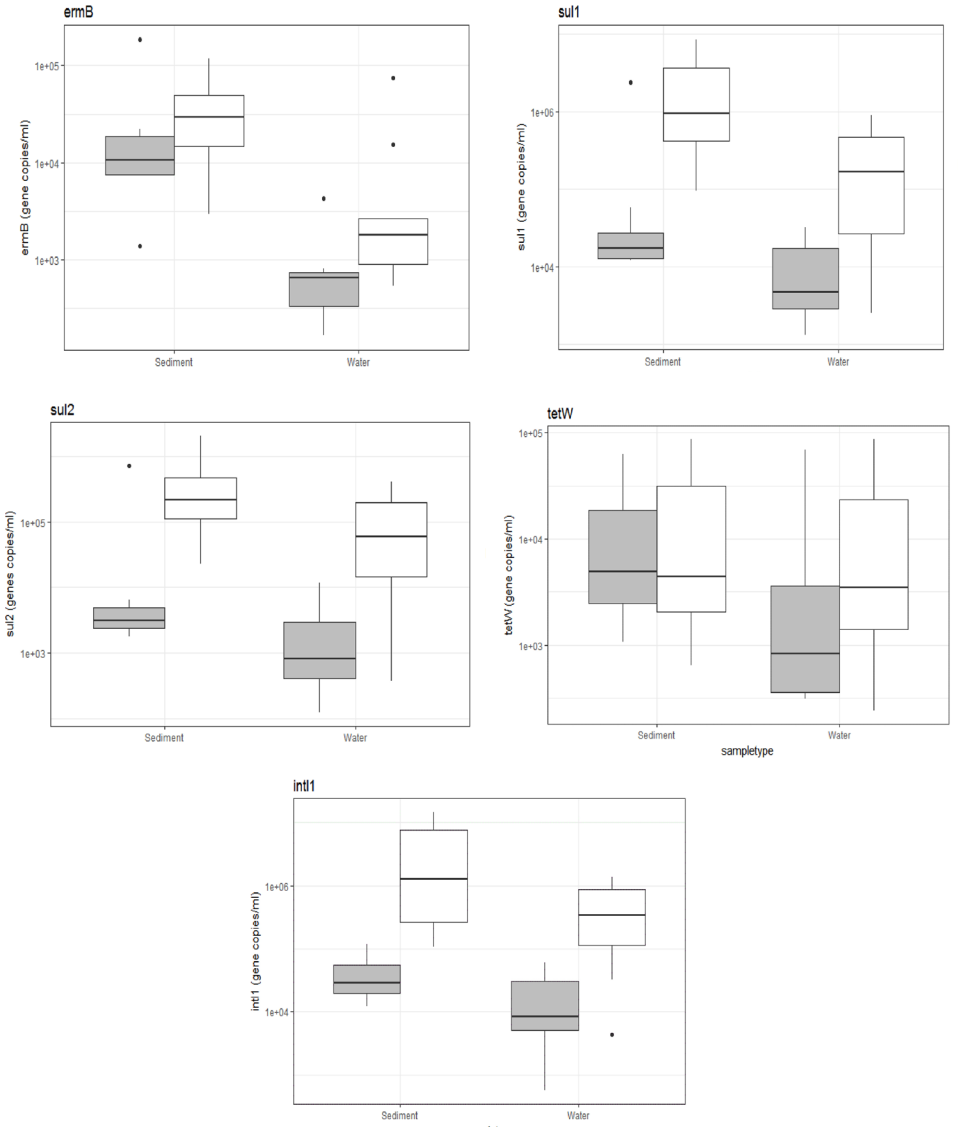

Figure 2.3: Concentration of ARGs and *intI1* in sediment and water samples upstream and downstream of the WWTP effluent. Within the box plot chart, each box plot represents maximum, upper-quartile, median (black bar), lower-quartile, and minimum values. Grey boxes represent U1 and white boxes represent D1. The black dots represent outliers, individual points of the data that do not fall within the mean value.

In general, all ARGs were detected at all sampling points in both sediment and water samples throughout the year. The fate of *sul1* along the river is presented in Figure 2.4, as a representative to show the fate and dissemination of ARGs along the river since the trend is similar for the other tested ARGs. Other ARGs and *intI1* are presented in Figure S2.15-S2.18. Significant differences in *sul1* were observed between upstream (U1 and U2) and 0.1 km

downstream from the WWTP discharge point (D1) in both sediment and water samples. *Sul1* ranged from 3.16 x 10^3 until 3.16 x 10^6 copies/g in the sediment samples, whereas *sul1* ranged from 1.00 x 10^3 until 1.00 x 10^6 copies/ml in the water samples. However, between two sampling points upstream (U1 and U2), we observed that the concentrations of ARGs and intlI1 in water already showed an increasing trend at a short distance (100 m) upstream of the effluent of wetland HRT 1 day (U2). Furthermore, once *sul1* was discharged with the WWTP effluent into the river, the concentration of *sul1* remained constant until 20 km downstream (D9).

Statistical analysis revealed that there was no significant increase or decrease of ARGs along the river passage for both water and sediment, except for *ermB* that showed a slight decrease at D8 (3.72 ± 1.58 copies/ml). Wagenbroekloopje river, a side stream located after sampling point D5, did not significantly influence concentrations of ARGs from D6 until D9.

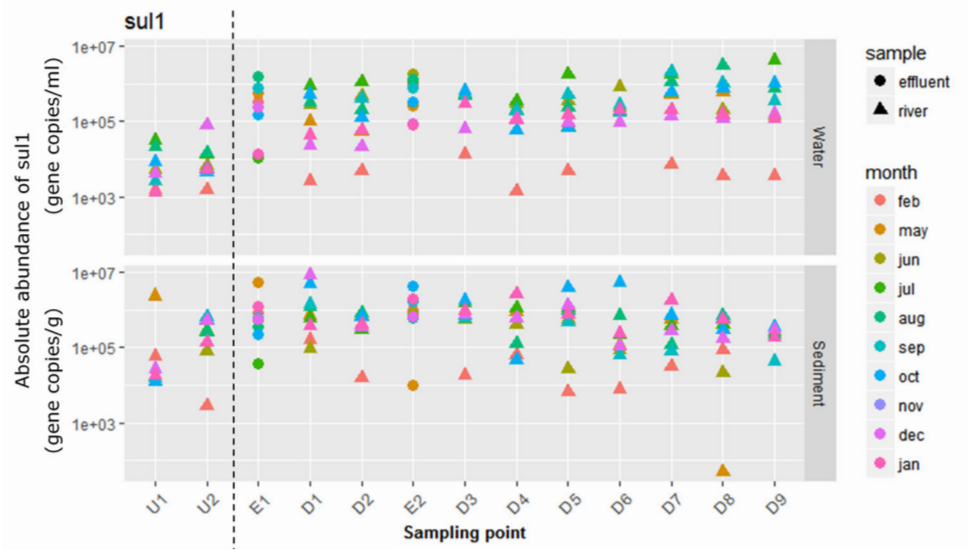

Figure 2.4: Profile of *sul1* for one year in sediment and water samples from 0.5 km upstream until 20 km downstream. The details of the sampling points are given in Figure 2.1. Dotted line distinguishes upstream and downstream of the WWTP.

2.3.2 Fate of ARGs during the seasons

Linear regression analysis was conducted to explore the correlation between ARGs and seasons. ARG concentrations of the samples during winter differed from summer (Figure S2.19-S2.23). Total load gene copies of ARGs are shown in S2.24-S2.28. This difference was most visible in February and December. Results showed that the concentration of *ermB* increased to 1.66 x 10^1 ± 1.29 gene copies/ml during winter, whereas most of the other ARGs decreased; *sul1* decreased 1.95 x 10^1 ± 1.35 gene copies/ml, *sul2* decreased 2.24 x 10^1 ± 1.35 gene copies/ml and *intI1* decreased 2.14 x 10^1 ± 1.32 gene copies/ml. The only exception was *tetW*, which remained constant.

2.3.3 Correlation between ARGs and water quality data

Multivariate analysis was conducted to explore the correlation between ARGs and water quality data (Figure 2.5). The ARGs pattern of all samples clustered together across the year, except for February, which had a lower concentration of ARGs. The genes sul1, sul2 and intI1 were highly correlated, and independent from the genes ermB and tetW. With respect to water quality parameters, a positive correlation of ermB and tetW with NH_4^+ was seen, and a negative correlation between ARGs and DO, both indicating that the influence of WWTP effluent on water quality was correlated to elevated levels of these ARGs.

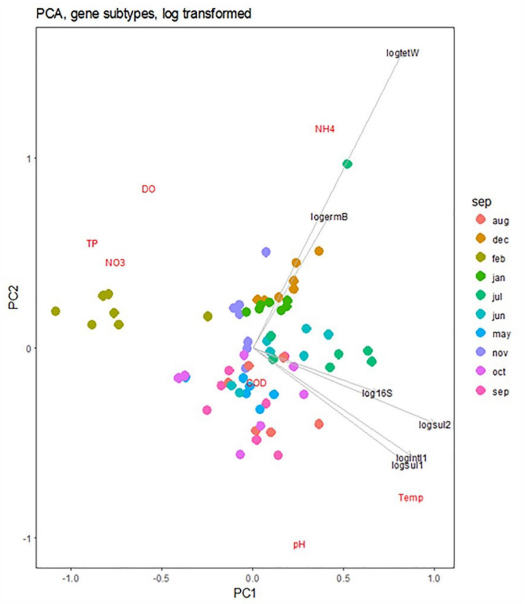

Figure 2.5: Principle component analysis between ARGs and water quality parameters. Sampling months are indicated in circles. ARGs and water quality parameters are shown as vectors.

2.4 Discussion

2.4.1 Role of WWTP effluent for presence of antibiotics along the river

As shown in Figure 2.2, antibiotics were measured in the discharged wastewater effluent and along the river at concentrations in the range of 1 ng/L up to 275 ng/L. Out of 52 antibiotics analysed, two sulfonamides and trimethoprim were detected in the water. Sulfonamides in combination with trimethoprim are widely used in veterinary practice and in human medicine (de Greeff et al., 2017). Previous studies have shown that antibiotics in treated municipal wastewater are typically present in low concentrations, in the range of ng/L (Yan et al., 2013; Chen and Zhou, 2014). In treated clinical or industrial wastewater, the concentration of antibiotics is higher, up to mg/L (Brown et al., 2006; Larson, 2007; Le et al., 2016). However, WWTP discharge is not

the only source of antibiotics. Different contamination sources such as manure fertilization might contribute directly or indirectly to the concentration of antibiotics in a river. Such sources can result in higher concentrations of antibiotics (up to 10^{10} mg/L) in the river, compared to the discharged wastewater (Pruden et al., 2006; Wang et al., 2016). As a result, the concentration of antibiotics can vary along the river. A study carried out by Chen et al. (2013a) showed that different anthropogenic activities such as aquaculture and animal farm can highly influence the concentration of antibiotics and ARGs along a trajectory from an inland river to the coast.

The river investigated in the present study was a suitable study area to investigate the fate of antibiotics from WWTP effluent discharge, and to detect antibiotic run off from the agricultural land surrounding the area. The presence of antibiotics upstream the WWTP shows that WWTP are not the only source of antibiotics in the catchment. As manure application is not allowed between September and February, alternative sources might come from the effluent of other WWTP's treating domestic and industrial wastewater, which can explain the difference between October and November. The WWTP effluents describe the role of effluent for the antibiotic concentrations. The concentration of antibiotics decreased once the antibiotics reached the river (November) or remained relatively stable (October). Other dominant sources of antibiotics or ARGs downstream the WWTP were not identified in sediment or water, making the selected river segment a good study area. Other studies have shown before that the concentration of antibiotic residues in water is strongly influenced by the type of antibiotic, water level, water quality, flow conditions, and precipitation (Kim and Carlson, 2007; Ok et al., 2011). Dilution and dissolved organic carbon in the river influences the attenuation of macrolides, quinolones, and sulfonamides (Luo et al., 2011; Zhang et al., 2011). In addition, other processes, such as photodegradation by sun irradiation, contribute to the natural removal of antibiotics in the environment. This process was shown for the antibiotics fluoroquinolone and sulfonamide (Zhang et al., 2011; Baena-Nogueras et al., 2017). The presence of dissolved organic carbon, chloride ions, nitrate and a suitable pH will enhance the reaction rate of photodegradation (Burns et al., 2012; Batchu et al., 2014; Sun et al., 2014; Bian and Zhang, 2016). This photodegradation might result in the formation of stable metabolites which can increase the sensitivity or preserve the biological activity of bacterial strains and promote ARB and ARGs proliferation (Palmer et al., 2010; Bonvin et al., 2013).

In addition to photodegradation, other factors, such as microorganisms, plants, temperature and adsorption, are an important pathway in the environment for the removal of antibiotics (Doll and Frimmel, 2003; Yi and Haibo, 2011). For example, photosynthetic and nitrifying bacteria are able to degrade antibiotics via metabolic and/or co-metabolic pathways (Barra Caracciolo et al., 2015). Plants are able to adsorb antibiotics or excrete enzymes to degrade antibiotics. However, each antibiotic will have a different removal rate, e.g. related to natural lighting conditions, and physical characteristics of the compound. Antibiotics are also able to adsorb to soil or sediment, which reduces the concentration in the water phase (Wang

and Wang, 2015). From the compounds that we detected in the WWTP effluent, sulfonamide is known to be removed from the water-phase by photodegradation and biodegradation (Li and Zhang, 2010; Zhang et al., 2011; Batchu et al., 2014), and trimethoprim by sediment adsorption (Li and Zhang, 2010). Therefore, dilution, photodegradation, adsorption and biodegradation are factors that all affect the concentration of antibiotics in a river.

A highly antibiotic contaminated environment can promote antibiotic resistance (Kristiansson et al., 2011; Guo et al., 2018). However, long term exposure to low concentrations of antibiotics and their transformation products can also promote the development and spreading of ARB and ARGs (Brown et al., 2006; Larsson, 2014). During long term exposure, the selective pressure remains present and can thus stimulate bacterial metabolism and proliferation of ARB (Szczepanowski et al., 2009; Gullberg et al., 2011). Bacteria are able to adapt to antibiotic pressure either by gene mutations or horizontal gene transfer (Munita and Arias, 2016). However, it is still unknown whether concentrations in the ng/L range, as observed in this study, are sufficient to pose a selective pressure.

2.4.2 Emission of ARGs into the river

We detected all ARGs and *intI1* in our samples, including the upstream sampling points. Similar findings were observed by Marti et al. (2013) and Lekunberri et al. (2017). We also observed that the influx of water from E1 to the river affect the river segment upstream the effluent discharge point of the WWTP (U2). Various months showed an increasing trend in ARGs before reaching E1 (Figure S2.14). For example, *sul1* concentration at U2 already showed an increasing trend before reaching E1 (Figure 2.4).

Concentrations that are detected upstream of a WWTP can have different origins, including run-off of faeces from pasture animals or wild animals, and also natural antibiotic resistance. Previous research has indeed shown that ARGs were identified in areas without exposure to contaminants and antibiotics, such as the deep terrestrial subsurface (Brown and Balkwill, 2008), pristine arctic wetland (Diaz et al., 2017), pristine river (Storteboom et al., 2010a), deep ocean sediment (Chen et al., 2013b) and pristine creek (Barkovskii et al., 2012). This implies that antibiotic resistance also exists naturally, further showing the protective nature of microorganisms themselves.

We found a significant contribution of ARGs and *intI1* in the WWTP effluent on the total concentration of ARGs and *intI1* in the river: our data showed an increasing trend for all ARGs except for *tetW* from U1 to D1 ($p<0.01$). The wastewater source originated from a municipal WWTP. Antibiotic and the corresponding gene; macrolide (*ermB*), sulphonamide (*sul1* and *sul2*) and tetracycline (*tetW*) are commonly used in livestock production, for example swine and cattle farms (Giguère, 2013; Zhou et al., 2013b). This finding shows that human activities are the major driver of the spreading of ARGs in this particular watershed, thus increasing

the prevalence of antibiotic resistance in the environment (Zhang et al., 2015b). A significant increase of ARGs and *intI1* from WWTP effluent to the receiving river was also observed by other researchers in the tested matrices, for example in water (Ling et al., 2013; Koczura et al., 2016) and sediment (Czekalski et al., 2012; Koczura et al., 2016). In a study of Koczura et al. (2016), they observed 1.2×10^4 to 2.7×10^4 gene copies/ml of *sul1*. In this study, we observed similar concentrations of *sul1*, ranging from 9.30×10^4 to 4.25×10^6. The significant increase of these ARGs can originate from resistance genes present in human faeces, and other faeces present in the sewage, such as (domestic) animals. This confirms that discharged WWTP effluents are an important route for the dissemination of ARB and ARGs into the environment as also found by other authors (Aali et al., 2014).

2.4.3 Fate of ARGs along the river

In this study, the Grote Beerze river was selected to investigate the prevalence of antibiotics and ARGs. The Grote Beerze is a suitable model for studying the occurrence of antibiotics and ARGs in a wastewater effluent receiving river, since the river has a 20 km-scale river segment, with only one small side stream at 2 km. Therefore, changes in concentration of ARGs can only be attributed to processes in the river itself, and not by dilution of water along the river. This study not only provides information on concentrations of antibiotics and ARGs in water and in sediment, but also contributes to understand the role of WWTP discharge in a river system. Macrolide, sulfonamide and tetracycline antibiotics with corresponding resistance genes were selected in this study as these antibiotics and ARGs are referred to as adequate for environmental monitoring of antibiotic resistance (Berendonk et al., 2015). This study adds a perspective of the Netherlands as a country with a low background of resistance, since human antibiotic use in the Netherlands is relatively low (OECD, 2016) and the use of antibiotics in livestock has been reduced by 50% from 2008 until 2012 (Vandenbroucke-Grauls, 2014).

Limited studies describe the impact of antibiotic resistance genes and compounds along the distance of the river without side streams (20 km) in the water and sediment across a whole year, as we have done. The presence of ARGs in a WWTP effluent-receiving river between summer and winter or in a single month has been demonstrated in many studies (Marti et al., 2013; Amos et al., 2014; LaPara et al., 2015; Xu et al., 2015; Proia et al., 2016; Lekunberri et al., 2017). However, these studies were performed either on a river with more than one discharge (WWTP, farming or mining discharges), side river inputs along the main river, or a relatively short downstream distance (0.1 km until 5 km), and focused on either sediment or the water phase.

As observed in our study, once the ARGs enter the environment, the ARGs were persistent for 20 km. We also observed that ARGs in sediment are showing similar trends as ARGs in water. For example, concentrations of *sul1*, *sul2* and *intI1* for both water and sediment samples are lower in February, compared to other months. However, the total concentration

of ARGs in sediment was higher than the total concentration of ARGs in water. A possible explanation for this is that the sediment acts as reservoir for resident organisms, antibiotics, ARB and ARGs and shield them from sunlight and other degradative inactivation (Doll and Frimmel, 2003; Haller et al., 2009; Chen et al., 2013b; Chen and Zhang, 2013; Mwanamoki et al., 2014; Calero-Cáceres et al., 2017). Moreover, extracellular and intracellular DNA in the sediment corroborates the presence of DNA in the sediment, with extracellular DNA being more stable in the sediment. This was shown by Mao et al. (2014), who observed that ARGs were present at higher concentration in extracellular DNA with most of them present in the sediment.

Other than ARGs, *intI1* has been suggested as an indicator for the spreading and disseminating of ARGs, because most ARGs are carried by mobile genetic elements such as plasmids, transposons and or integrons which are associated to mobile elements, like insertion elements (Partridge et al., 2009). Our results also revealed high concentration of *intI1*, Hence, with the consistent contribution of ARGs and *intI1* from sediment to water, they remain in the river once they have entered the water body, even though there is no input of contaminants along the river itself.

2.4.4 Fate of ARGs over the seasons

In this study, the observed concentrations of ARGs vary an order of one to three between different months. With respect to seasons, a slight decrease in concentration was found for all tested ARGs, except *tetW* during winter. This was mainly due to lower concentrations of *sul1*, *sul2*, and *intI1* in February (coinciding with higher phosphate and DO concentrations in that month). Different studies have shown ARB and ARGs profiles in different seasons. Some of them reported that summer is the optimal season for ARGs proliferation (Sui et al., 2011; Chen et al., 2013a; Mao et al., 2015), meanwhile others reported higher ARGs or *intI1* concentrations in winter (Koczura et al., 2016). Furthermore, there are also studies that report only slightly variation or inconsistent between the seasons (Chen et al., 2013a; Yuan et al., 2014). This might indicate that the effect of season depends on the source, the sampling period (variation in precipitation and temperature), antibiotic usage, geographical location, hydrodynamic conditions and disposal practice (Awad et al., 2015; Devarajan et al., 2016). In summer, Dutch rivers have a higher nutrient input and variations in precipitation that can facilitate microbial growth, but not of faecal microorganisms (Chen et al., 2013a; Franz et al., 2014). Precipitation also plays an important role in the spreading of ARGs within the microbial community of a river. In a study of Di Cesare et al. (2017), absolute concentrations of ARGs during a rain event were 8.6 times higher compared to the yearly average of rain. Storm water also has higher potential to transfer ARGs in the environment (Zhang et al., 2016). This effect was not relevant in our study, as the precipitation was 0 mm/h throughout the sampling days except in February 2016 (1.5 mm/h). One day before sampling, a small amount of precipitation (0-2 mm) was observed in February, August, September, November and December.

In this study, the water flow in the river is higher in winter (average 1000 m^3/h) than in summer (average 600 m^3/h). The ratio between the flowrate of the effluent and the flowrate of the river was different at each sampling campaign, and therefore there was not a consistent dilution of ARGs (Figure S2.29). This change in flow, changes the dilution factor, which might be the reason why the concentrations of ARGs and *intI1* decreased during winter even though the total load was very similar between months, for example in February. We also see a clear distinction in total loads between *ermB* and *sul1/sul2/intI1* (Figure S2.24-S2.28). For *ermB*, the upstream location contributes clearly to the concentration of the gene and as a result, *ermB* is independent from *sul1/sul2/intI1* in the multivariate analyses (Figure 2.5). However, E2 is the main source of ARGs (*sul1/sul2/intI1*) load in most months. This shows that other factors than just the season influences the ARGs concentration in a river. Therefore, further studies to find the correlation between variation of antibiotic resistance release in different seasons need to be performed.

2.4.5 Correlation between ARGs and water quality data

Our results show that ARGs were positively correlated to some nutrients and negatively correlated to DO, which indicates a co-occurrence of nutrients and resistance genes in effluents, which also have lower DO. A positive correlation of *ermB* and *tetW* with NH_4^+ and a negative correlation between ARGs and DO indicates a direct link between ARGs and discharged WWTP effluent. According to Novo and Manaia (2010), processes and conditions in a WWTP influences the concentration of ARGs. However, water quality data cannot be used to estimate the concentration of ARGs, as the fate of nutrients and resistance genes in water will differ, as also shown by others (Laht et al., 2014). Even so, WWTP is still a dominant source of antibiotics and ARGs in the river system. We also saw a strong correlation between *sul1*, *sul2* and *intI1*. Sul genes are commonly associated with *intI1*, since the *sul1* gene is located at 3'-conserved segments in *intI1* (Partridge et al., 2009).

2.5 Conclusion

In conclusion, our data show that WWTP effluents are a relevant source of antibiotics and ARGs, once the wastewater effluent is discharged to a river. This shows that a WWTP cannot eliminate these contaminants from the wastewater. The study gives a comprehensive profile of antibiotic resistance in a Dutch river, showing that ARGs are persistent in the water and sediment from the WWTP effluent discharge point until 20 km downstream. Wastewater indicatory effluent parameters, such as DO, nitrate and phosphate, were all below the allowed discharge levels, and showed no significant correlation with the fate of antibiotics, but did correlate to the concentrations of selected ARGs. A seasonal effect was observed for the presence of almost all ARGs in the river, except *tetW*, but could not explain all variations for all ARGs tested. The exact mechanism behind this is unclear, as ours and other studies showed different effects. Once the

antibiotics and ARGs enter the river, the concentration of antibiotics only slightly decreases, and ARGs show a persistence until 20 km downstream in the water and in the sediment. Simultaneous monitoring in water and sediment samples from the river system is therefore recommended. Our results show that the river attenuates antibiotics, but should also be considered as a reservoir of antibiotic resistant bacteria and ARGs.

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Supplementary Information

Text S2.1: Indicatory parameters of WWTP influence.

WWTP effluent quality was monitored by measuring DO, pH, temperature, COD, NH4+, NO3-, and TP. The general characteristics of the effluents (E1 and E2) and along the river are summarized in Figure S1-S7. The data show that the effluent discharged to the river meets the regulatory standards for discharge to surface waters (Council Directive 91/271/EEC). Furthermore, the WWTP effluent did hardly influence the investigated parameters downstream the WWTP. All parameters remained constant along the river for 20 km with some fluctuation in a few parameters after D6. For example, the COD concentration level at D7 - D9, was almost two times higher in February (20 mg/L), compared to all sampling points (40 mg/L).

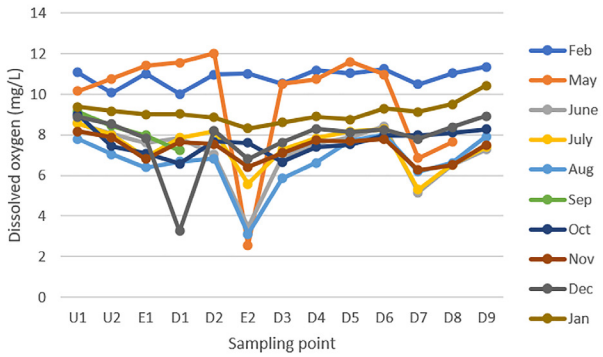


Figure S2.1: Profile of dissolved oxygen (mg/L) upstream and downstream of a WWTP. E1 = effluent of wetland with HRT 1-day. E2= effluent of wetland with HRT 3-day. U1 = 0.5 km before E1, U2 = 0.1 km before E1, D1 = 0.1 km after E1, D2 = between E1 and E2, D3 = 0.1 km after E2, D4 = 0.5 km after E2, D5 =1 km after E2, D6 = 1.5 km after E2, D7 = 5 km after E2, D8 = 10 km after E2, D9 = 20 km after E2.

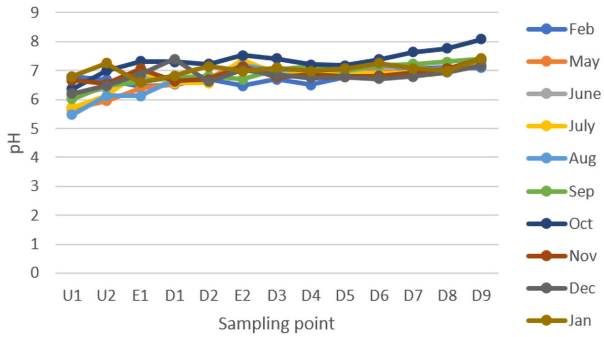


Figure S2.2: Profile of pH upstream and downstream of a WWTP. The details of the sampling points are given in Figure S2.1.

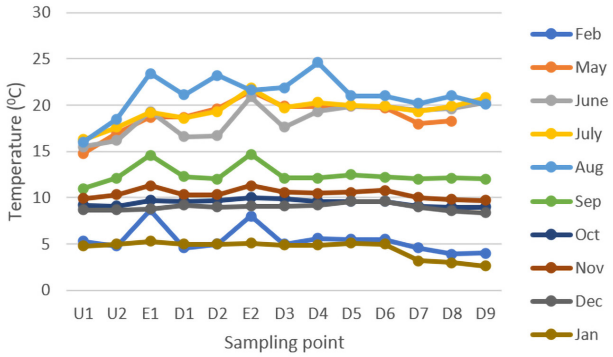


Figure S2.3: Profile of temperature (ºC) upstream and downstream of a WWTP. The details of the sampling points are given in Figure S2.1.

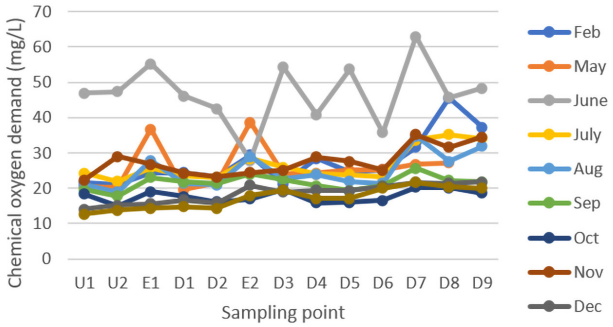


Figure S2.4: Profile of COD (mg/L) upstream and downstream of a WWTP. The details of the sampling points are given in Figure S2.1.

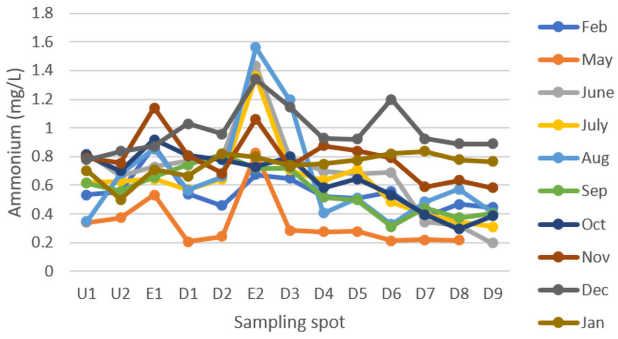


Figure S2.5: Profile of ammonium (mg/L) upstream and downstream of a WWTP. The details of the sampling points are given in Figure S2.1.

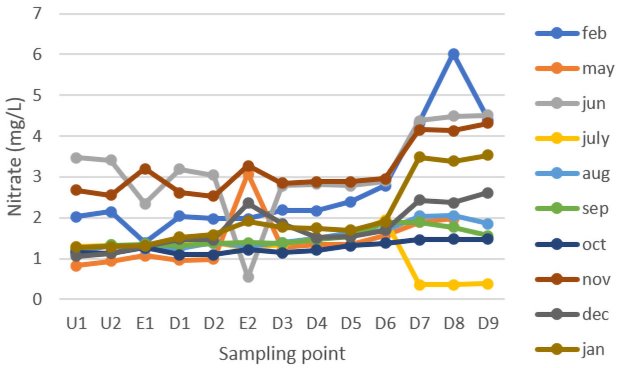


Figure S2.6: Profile of nitrate (mg/L) upstream and downstream of a WWTP. The details of the sampling points are given in Figure S2.1.

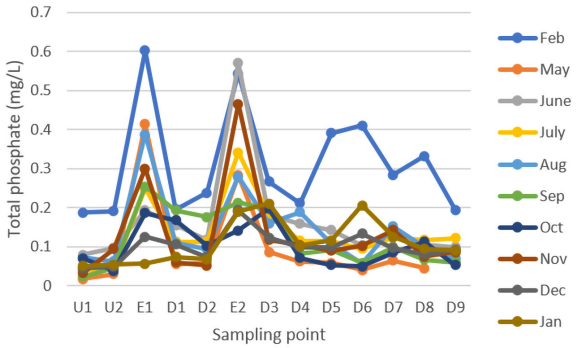
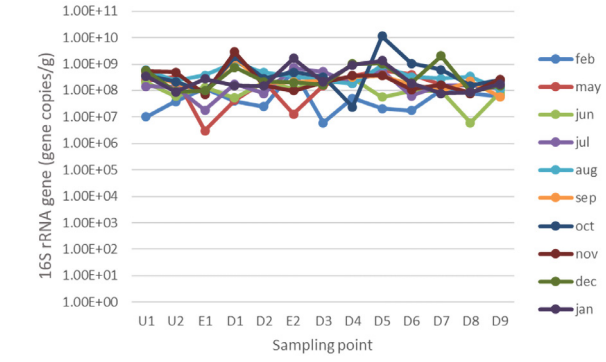
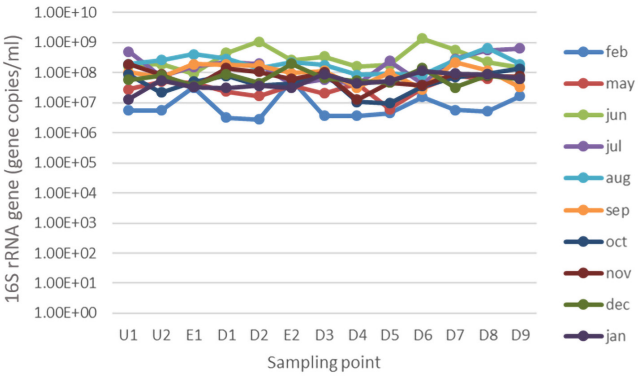


Figure S2.7: Profile of total phosphate (mg/L) upstream and downstream of a WWTP. The details of the sampling points are given in Figure S2.1.

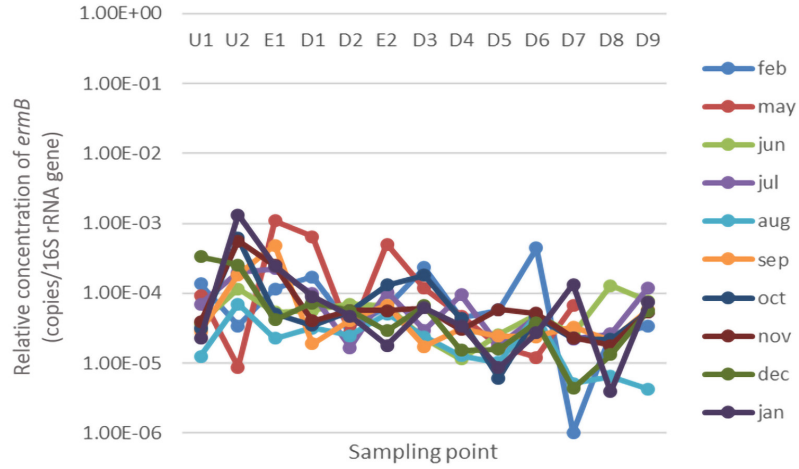


(a) Sediment samples

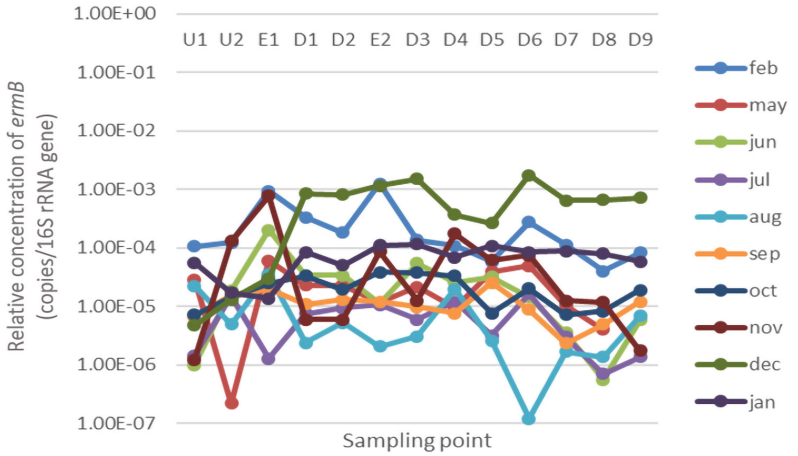


(b) Water samples

Figure S2.8: Profile of 16S rRNA gene abundance for 10 months in (a) sediment and (b) water samples from 0.5 km upstream until 20 km downstream. The details of the sampling points are given in Figure S2.1.

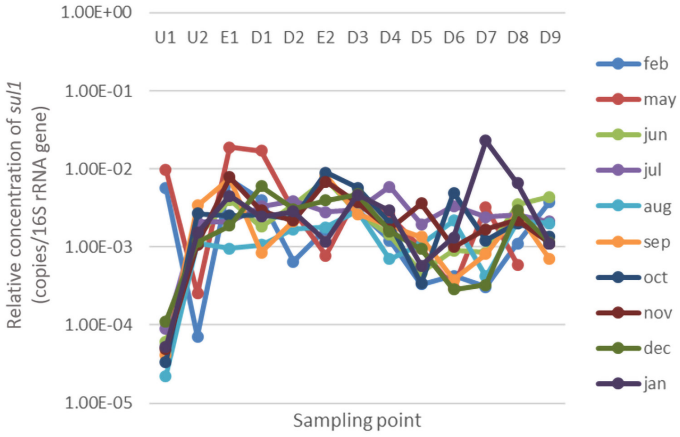


(a) Sediment samples

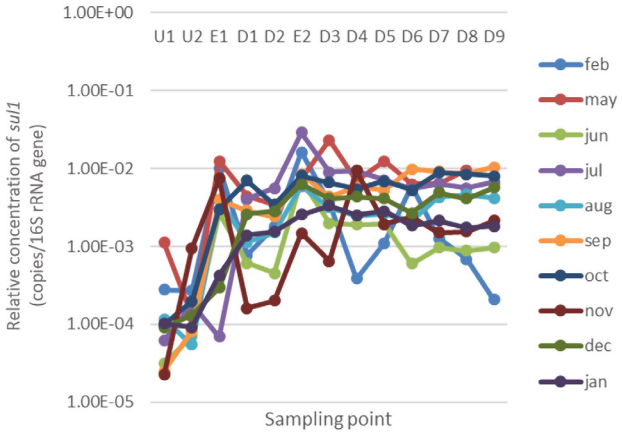


(b) Water samples

Figure S2.9: Gene copy number *ermB* normalized to 16S rRNA gene copy numbers in the sediment and water samples.

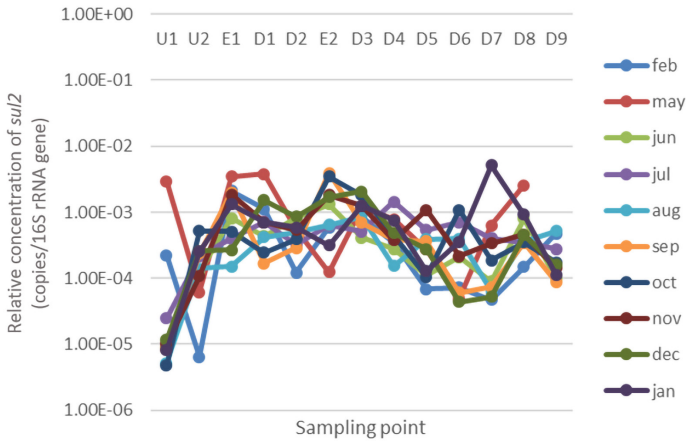


(a) Sediment samples

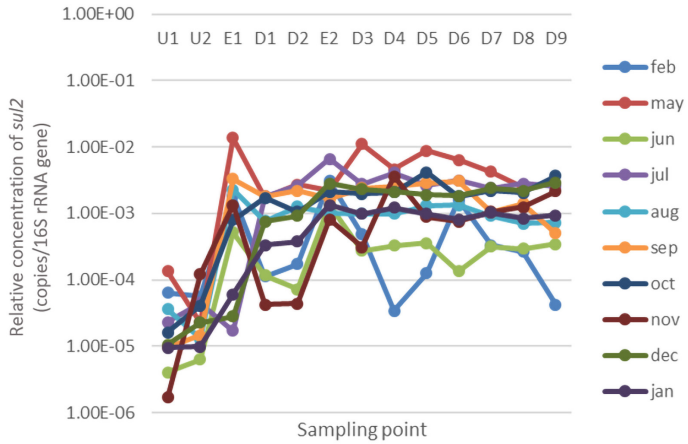


(b) Water samples

Figure S2.10: Gene copy number *sul1* normalized to 16S rRNA gene copy numbers in the sediment and water samples.

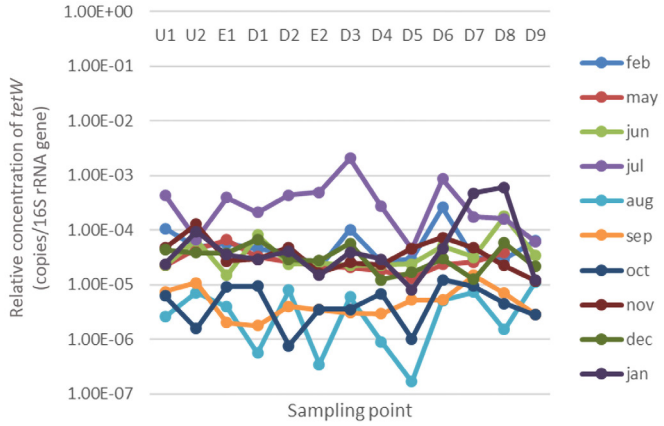


(a) Sediment samples

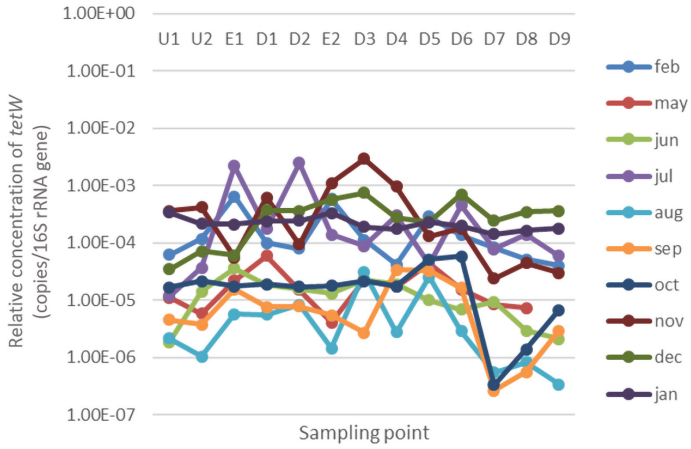


(b) Water samples

Figure S2.11: Gene copy number *sul2* normalized to 16S rRNA gene copy numbers in the sediment and water samples.

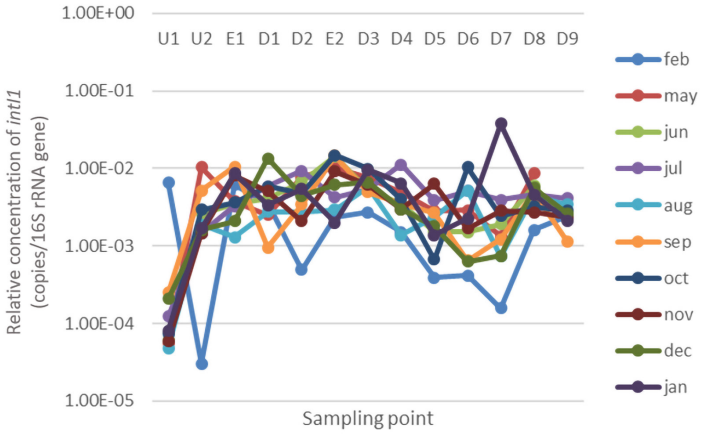


(a) Sediment samples

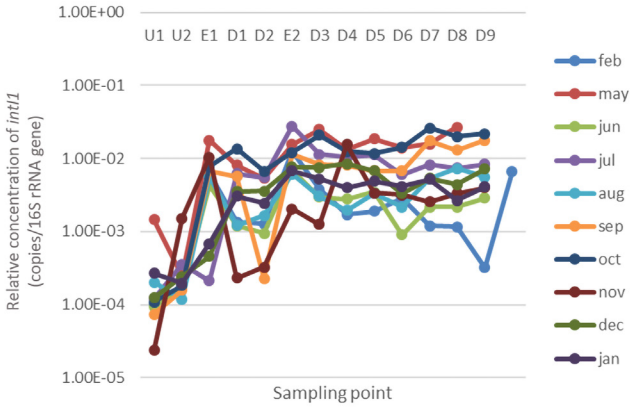


(b) Water samples

Figure S2.12: Gene copy number *tetW* normalized to 16S rRNA gene copy numbers in the sediment and water samples.



(a) Sediment samples



(b) Water samples

Figure S2.13: Gene copy number *intI1* normalized to 16S rRNA gene copy numbers in the sediment and water samples.

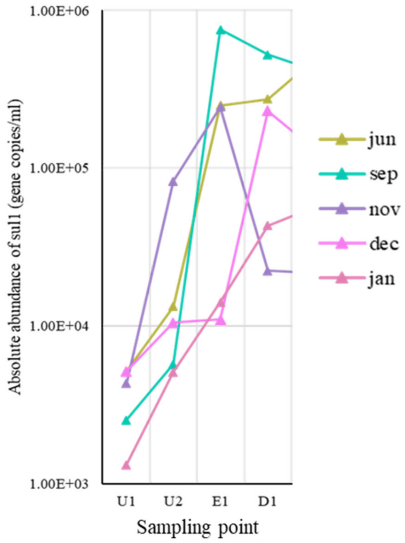


Figure S2.14: Profile of *sul1* (gene copies/ml) before reaching E1. The details of the sampling points are given in Figure S2.1.

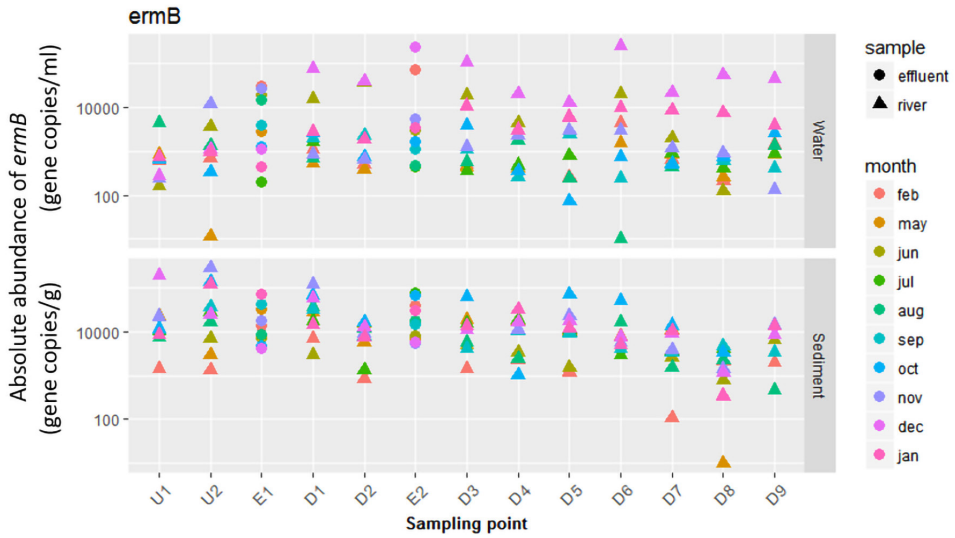


Figure S2.15: Profile of *ermB* for 10 months in sediment and water samples from 0.5 km upstream until 20 km downstream. The details of the sampling points are given in Figure S1.

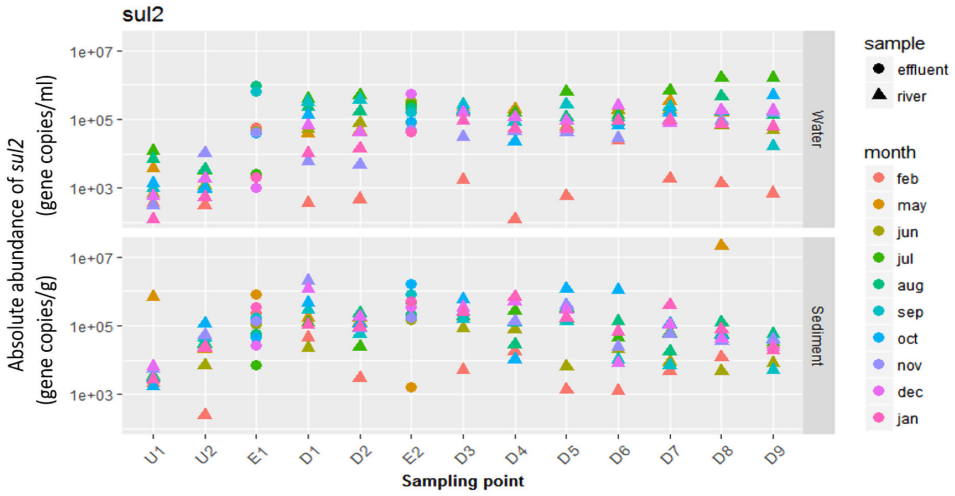


Figure S2.16: Profile of *sul2* for 10 months in sediment and water samples from 0.5 km upstream until 20 km downstream. The details of the sampling points are given in Figure S2.1.

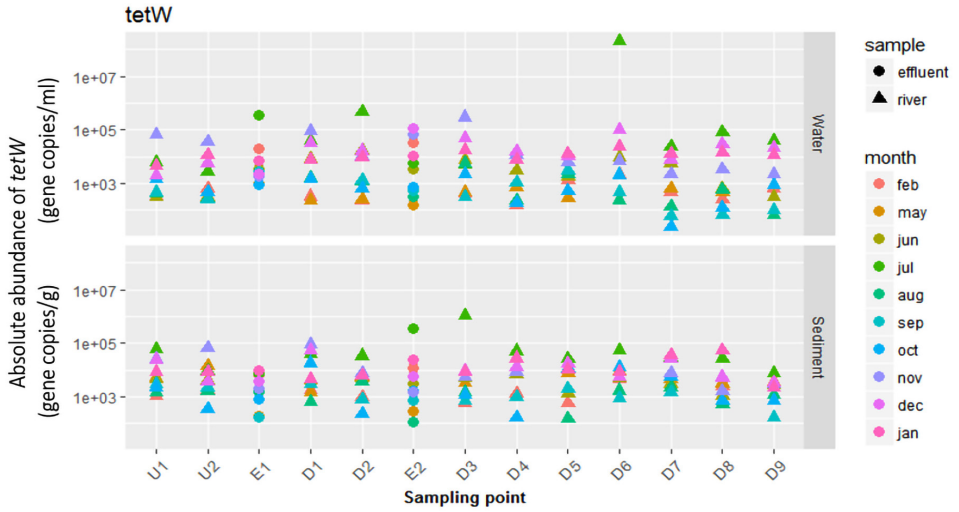


Figure S2.17: Profile of *tetW* for 10 months in sediment and water samples from 0.5 km upstream until 20 km downstream. The details of the sampling points are given in Figure S2.1.

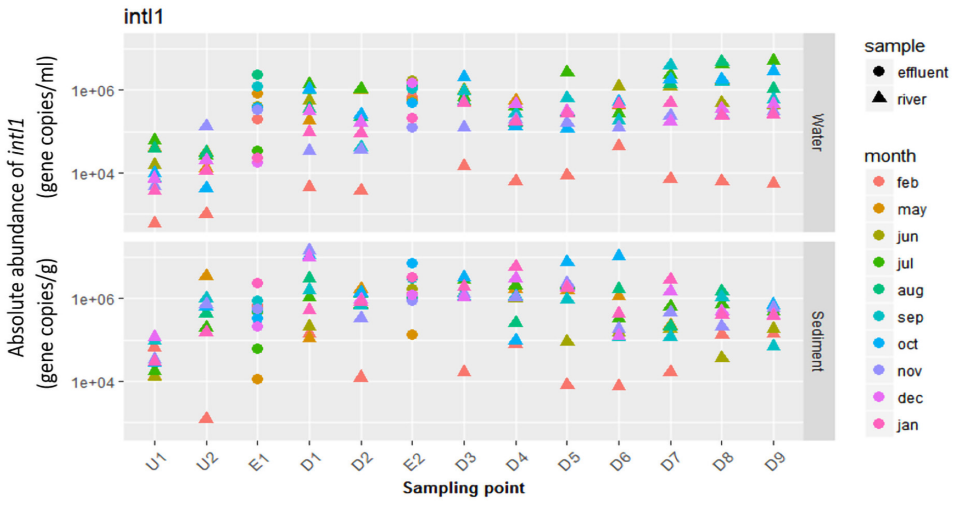


Figure S2.18: Profile of *intI1* for 10 months in sediment and water samples from 0.5 km upstream until 20 km downstream. The details of the sampling points are given in Figure S2.1.

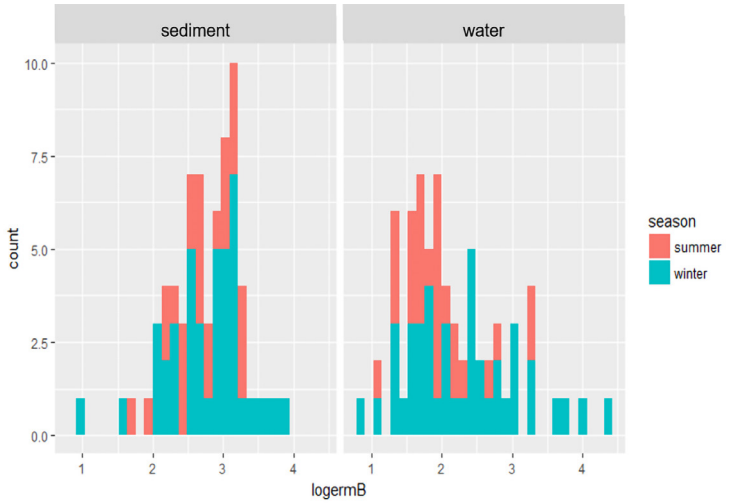


Figure S2.19: Distribution of *ermB* in summer and winter.

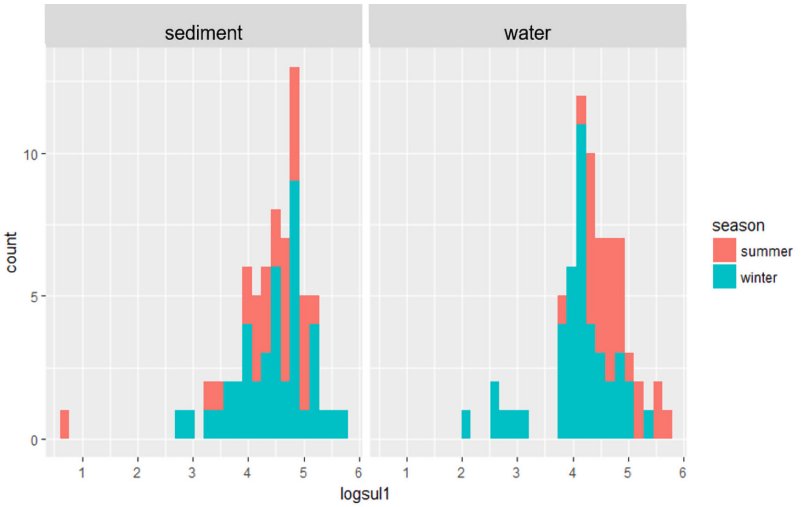


Figure S2.20: Distribution of *sul1* in summer and winter.

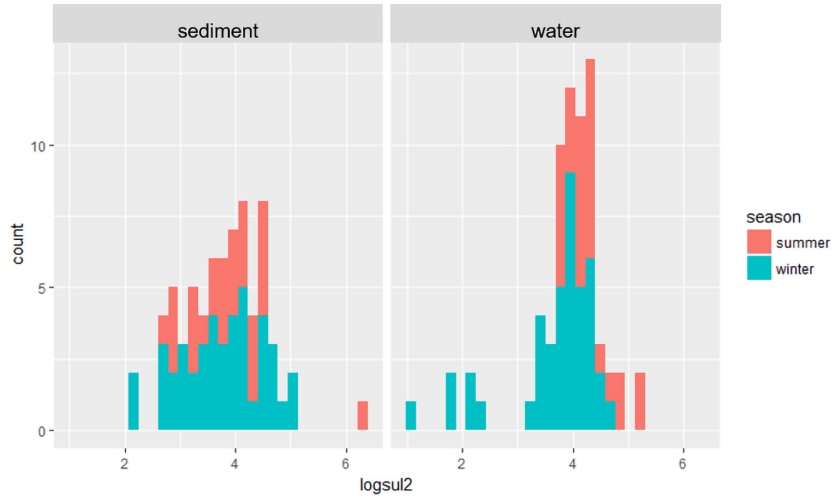


Figure S2.21: Distribution of *sul2* in summer and winter.

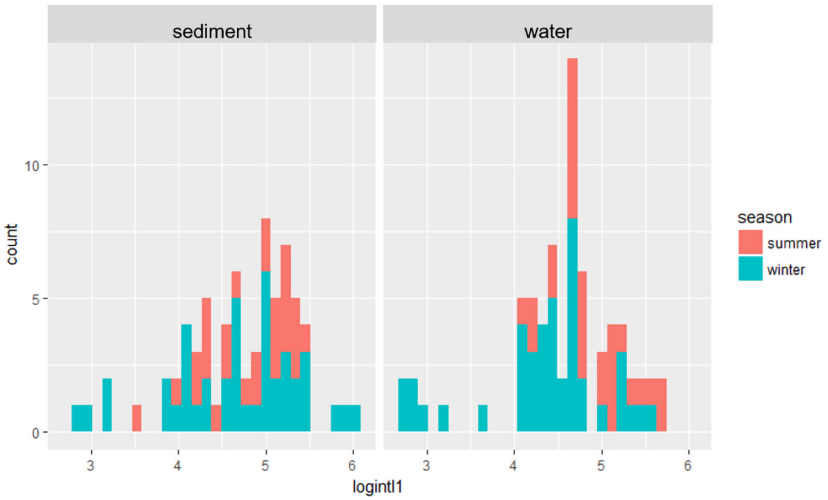


Figure S2.22: Distribution of *intI1* in summer and winter.

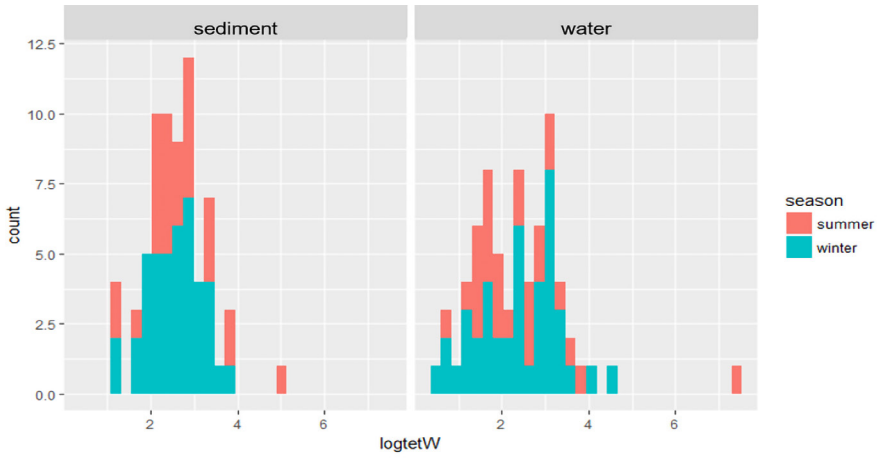


Figure S2.23: Distribution of *tetW* in summer and winter.

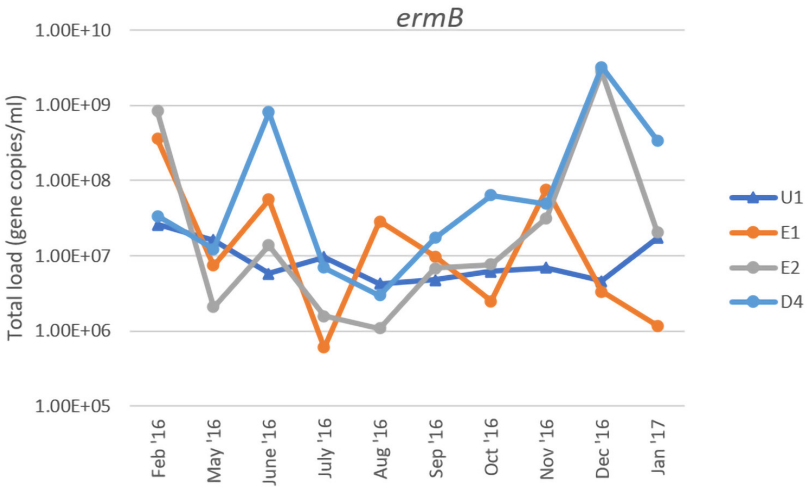


Figure S2.24: Total load of *ermB* in water samples for 10 months.

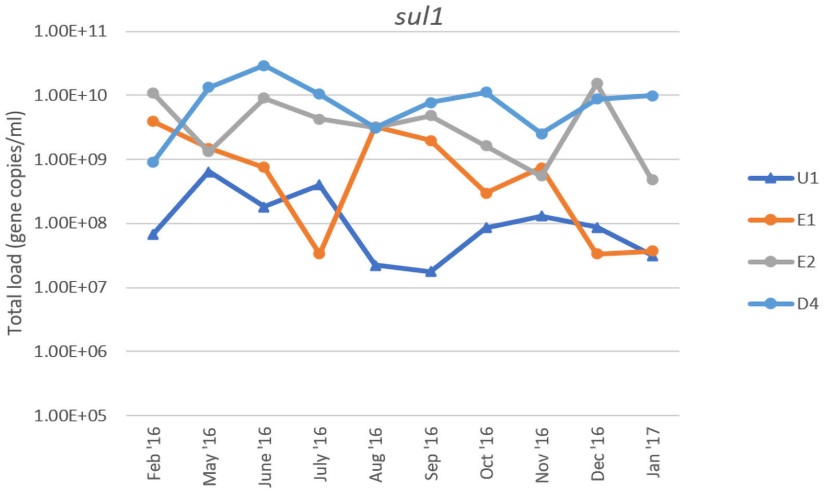


Figure S2.25: Total load of *sul1* in water samples for 10 months.

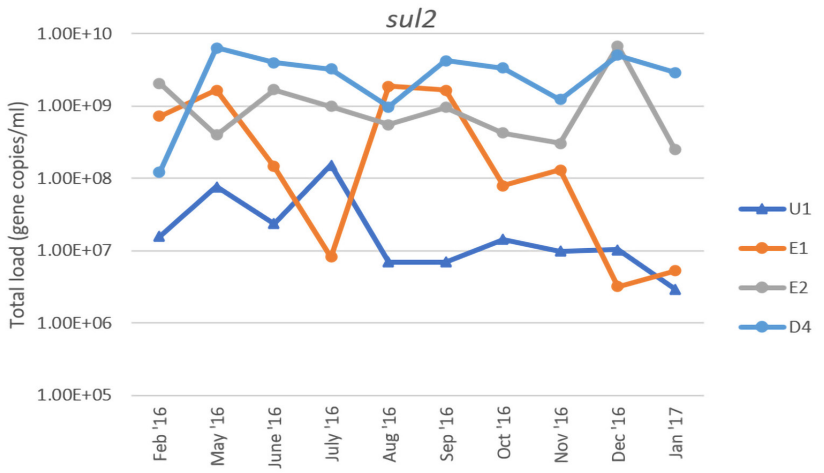


Figure S2.26: Total load of *sul2* in water samples for 10 months.

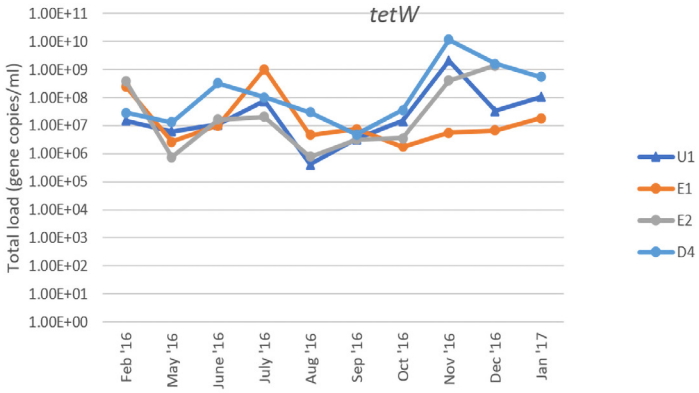


Figure S2.27: Total load of *tetW* in water samples for 10 months.

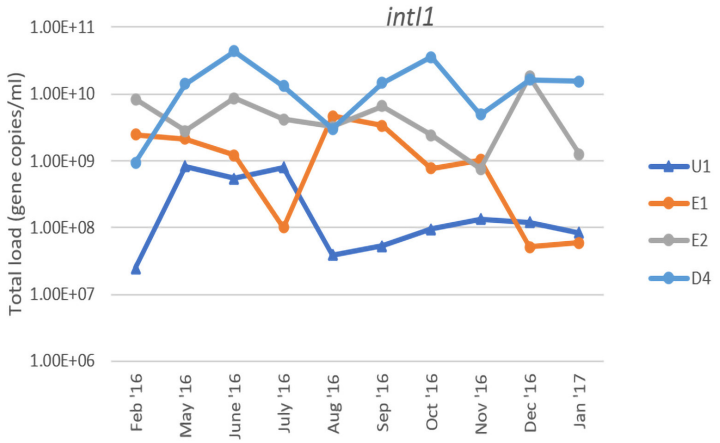


Figure S2.28: Total load of *intI1* in water samples for 10 months.

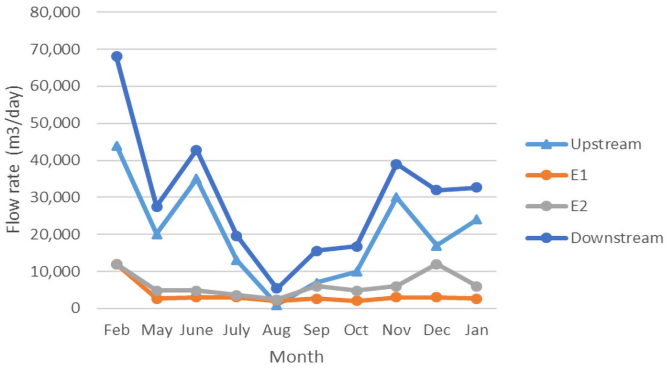


Figure S2.29: Flow rate profile of upstream, E1, E2 and downstream of Grote Beerze for one year sampling.

Table S2.1: List of analysed antibiotics.

	Antibiotic compound	Antibiotic group
1	Dapson	Sulfonamide
2	Sulfacetamide	
3	Sulfachloropyridazine	
4	Sulfadiazine	
5	Sulfadimethoxine	
6	Sulfadimidine	
7	Sulfadoxine	
8	Sulfamerazine	
9	Sulfamethizole	
10	Sulfamethoxazole	
11	Sulfamethoxypyridazine	
12	Sulfamonomethoxine	
13	Sulfamoxole	
14	Sulfaphenazole	
15	Sulfapyridine	
16	Sulfaquinoxaline	
17	Sulfathiazole	
18	Sulfisoxazole	
19	Trimethoprim	Trimethoprim
20	Chloortetracycline	Tetracycline
21	Doxycycline	
22	Methacycline	
23	Minocycline	
24	Oxytetracycline	
25	Tetracycline	
26	Ciprofloxacine	Quinolones
27	Danofloxacine	
28	Difloxacine	
29	Enrofloxacine	
30	Flumequine	
31	Marbofloxacine	
32	Nalidixinezuur	
33	Norfloxacine	
34	ofloxacin_levofloxacin	
35	Oxolinezuur	
36	Sarafloxacine	
37	trovafloxacin	

38	Tylvalosin	Macrolide
39	Erythromycine	
40	Gamithromycine	
41	Josamycine	
42	Lincomycine	
43	Natamycine	
44	Neospiramycine I	
45	Pirlimycine	
46	Spiramycine	
47	Tildipyrosine	
48	Tilmicosine	
49	Tulathromycine	
50	Tylosine	
51	Valnemulin	
52	Tiamulin	

Table S2.2: LC-MS/MS parameters (Instrument: Sciex QTRAP5500).

Ionisation mode	ESI, Positive mode
Curtain gas flow (L/Hr.)	35
Collision gas	Medium
Capillary (kV)	4.0
Desolvation temperature	450 °C
Gas flow 1 (L/Hr.)	50
Gas flow 2 (L/Hr.)	50
Entrance potential (V)	10.0

Table S2.3: Overview of primer sequencing and thermal cycling conditions for qPCR.

ARGs	Sequence (5'-3')	Thermal profile	Cycles	Detection format	Reference
16S rRNA gene	ACTCCTACGGGAGGGCAG GACTACCAGGGTATCTAATCC	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	SYBR Green	Fierer et al. (2005)
intI1	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	TaqMan	Barraud et al. (2010)
ermB	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	SYBR Green	Knapp et al. (2010)
sul1	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	TaqMan	Heuer and Smalla (2007)
sul2	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	TaqMan	Heuer et al. (2008)
tetW	CGGCAGCGCAAAGAGAAC TTTGGCGTGTTTCATTGCTT	95 °C 3 min 95 °C 15 s, 59 °C	45	TaqMan	Walsh et al. (2011)

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Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Applied and Environmental Microbiology 71 (7): 4117-4120. http://dx.doi.org/10.1128/AEM.71.7.4117-4120.2005.

Heuer, H., A. Focks, M. Lamshöft, K. Smalla, M. Matthies, and M. Spiteller. 2008. Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. Soil Biology and Biochemistry 40 (7): 1892-1900. http://dx.doi.org/10.1016/j.soilbio.2008.03.014.

Heuer, H., and K. Smalla. 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environmental Microbiology 9 (3): 657-666. http://dx.doi.org/10.1111/j.1462-2920.2006.01185.x.

Knapp, C. W., J. Dolfing, P. A. I. Ehlert, and D. W. Graham. 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environmental Science & Technology 44 (2): 580-587. http://dx.doi.org/10.1021/es901221x.

Walsh, F., A. Ingenfeld, M. Zampicolli, M. Hilber-Bodmer, J. E. Frey, and B. Duffy. 2011. Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. Journal of Microbiological Methods 86 (2): 150-155. http://dx.doi.org/10.1016/j.mimet.2011.04.011.

CHAPTER 3

Evaluation of attenuation of pharmaceuticals, toxic potency, and antibiotic resistance genes in constructed wetlands treating wastewater effluents

A modified version of this chapter is published as:

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https://doi.org/10.1016/j.scitotenv.2018.03.083

Abstract

The performance of constructed wetlands (CWs) in the removal of pharmaceutically active compounds (PhACs) is generally evaluated on the basis of chemical analysis. In this work, we used a combination of chemical, toxicological, and molecular analyses to assess the attenuation of PhACs, toxic potency and antibiotic resistance genes (ARGs) in a field study of three CWs serving as tertiary treatment of wastewater treatment plants. First, 17 PhACs were analysed chemically, of which 14 were detected and seven at concentrations greater than 0.1 μg/L. Even though some of the individual PhACs were moderately or highly removed in the CWs investigated, median removal of overall PhACs was approximately 50% in the vertical subsurface flow CW (VSF-CW) with a lower hydraulic loading rate while the removal in the other two free water surface flow CWs (SF-CWs) was negligible. Second, toxic potency of wastewater extracts was assessed in a range of bioassays. Estrogenicity was overall attenuated in CWs, while the neurotoxic potency of wastewater extracts did not decrease after treatment in the two CWs investigated. Third, the VSF-CW and one of the SF-CW showed a positive removal of an integrase gene and three ARGs tested. The increased concentrations of ARGs in the other SF-CW, as well as the increase of total bacteria in all CWs, may relate to regrowth of resistance-carrying bacteria. Finally, multivariate analysis shows that most PhACs are positively correlated to the observed toxic potency. Additionally, low removal of organics and nutrients seems to parallel with low removal of PhACs. ARGs positively correlated with organics, nutrients and some PhACs, and the integrase gene but not to the respective antibiotics. The insufficient removal of PhACs, toxic potency, and ARGs indicates the need of an optimal design of CWs as tertiary treatment facilities.

Keywords: domestic wastewater; tertiary treatment; micropollutants; bioanalyses; ARGs; multivariate analysis

3.1 Introduction

The occurrence of pharmaceutically active compounds (PhACs) in the environment is a growing concern due to their potential threat to the aquatic environment and human health. The term PhACs encompasses a diverse group of compounds, such as antibiotics, hormones, analgesic and anti-inflammatory drugs, β-blockers, lipids regulator agents, and antiepileptic drugs (Liu and Wong, 2013). Tons of PhACs are consumed on a global scale (Sebastine and Wakeman, 2003). For example, although annual consumption in the European Union is generally lower than other areas, nonetheless approximately 15,000 tons of human antibiotics are released yearly into the EU environment (Van Boeckel et al., 2014). Due to manufacturing processes, improper disposal and metabolic excretion, PhACs are continuously released into the aquatic environment and as a result exhibit a pseudo-persistent behaviour (Hernando et al., 2006).

PhACs are originally made to elicit a biological effect in target organisms (Henschel et al., 1997). However, with the continuous release of PhACs in the aqueous environment, non-target organisms are and have been exposed over many species generations, which is raising concern towards adverse developmental effects in aquatic ecosystems (Fatta-Kassinos et al., 2011). Among the diverse pool of PhACs, antibiotics are of particular concern (Hernando et al., 2006) as they may accelerate the development of antibiotic resistance genes (ARGs) in microorganisms. This may compromise the effectiveness of antibiotics in curing diseases in humans and live-stock (Kemper, 2008).

Wastewater treatment plants (WWTPs) are the key barrier against the release of PhACs and ARGs to the aquatic environment. However, the conventional treatment processes in WWTPs are not specifically designed for removing PhACs and ARGs, and they are not readily or fully removed (Munir et al., 2011; Verlicchi et al., 2012). To further remove PhACs and ARGs from wastewater effluents, tertiary treatment processes are required. From an economic and environmental perspective, a constructed wetland (CW) could be a promising tertiary treatment technique for removing PhACs and ARGs, as various studies have shown at lab-scale (Matamoros et al., 2008; Hussain et al., 2012; Zhang et al., 2012; Liu et al., 2013), and at field-scale (Conkle et al., 2008; Matamoros et al., 2009; Hijosa-Valsero et al., 2011; Chen et al., 2015b).

To date, the performance of CWs related to PhAC removal is in most studies only evaluated on the basis of chemical analysis. However, it is nearly impossible to monitor all PhACs and candidate intermediates in all treatment situations, which is further complicated by complex effects of wastewater matrices on analytics of concentrations in the nano- or microgram per litre level. Furthermore, chemical analysis alone gives limited information to understand the potential effects of PhACs and intermediates on the aquatic environment (Välitalo et al., 2016). Bioanalyses employing different test organisms can directly characterize the toxic potency of known and unknown components in a mixed wastewater matrix (Yu et al., 2014). To date, such combined chemical and toxicological studies for CWs are limited and no clear correlation has yet

been established between those two analyses.

The emergence of ARGs in humans and animals have been confirmed to correlate significantly with antibiotic use (Wu et al., 2015). However, correlations between the antibiotic abundance and the ARGs levels in the environment are uncertain (Wu et al., 2015; Xu et al., 2016). Additionally, antibiotic resistance selection might not only occur through antibiotic selective pressure but also through other chemical pollution such as biocides, heavy metals and detergents (Alonso et al., 2001; Martínez, 2008). Therefore, correlations between ARG levels and the abundance of PhACs including antibiotics in the environment need to be further investigated.

In the work reported here, we conducted a single sampling campaign and assessed the attenuation performance of CWs on 17 PhACs, toxic potency, and three ARGs based on a combination of chemical, toxicological, and molecular analyses. Three full-scale operating CWs were selected, including two free water surface flow CWs (SF-CWs) and one vertical subsurface flow CW (VSF-CW). Bioanalyses were conducted by employing different varieties of receptors, varying from enzymes to algae. The objectives of this study are to (1) identify the level of PhACs, toxic potency and presence of ARGs in wastewater effluents and their attenuation in different types of CWs, and (2) explore the correlations between levels of PhACs and toxic potency, and between levels of PhACs and ARGs.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Target PhACs were selected from different categories (Table S3.1 in the supplementary materials), including ketoprofen, diclofenac, ibuprofen, naproxen, erythromycin, lincomycin, sulfamethoxazole, propranolol, metoprolol, clofibric acid, carbamazepine, caffeine, bisphenol A, estrone, 17β-estradiol, ethinylestradiol and estriol, which were purchased from Sigma Aldrich Chemie B.V (the Netherlands). Properties of the target PhACs are shown in Table S3.1. Other chemicals and reagents used are described in the Text S3.1.

3.2.2 Sampling

Wastewater samples were collected in July 2015 from three CWs acting as tertiary treatment process of three WWTPs: Land van Cuijk (L), Hapert (H), and Kaatsheuvel (K) in the Netherlands. Detailed background information of related WWTPs and CWs are shown in Table 3.1. July was selected as the sampling month due to its low precipitation among the warm months (Figure S3.1). CW-L and CW-H are the SF-CWs while CW-K is a VSF-CW.

Table 3.1: Overview and operational parameters of target WWTPs and their CWs.

Parameters	Land van Cuijk (CW-L)	Hapert (CW-H)	Kaatsheuvel (CW-K)
Capacity (inhabitant equivalent)	175,000	71,000	57,300
Wastewater source	43% domestic, 57% industrial[a] & hospital[b]	78% domestic, 22% industrial[c]	Domestic
Biological treatment	Activated sludge with sand filter	Oxidation ditch	Oxidation ditch with sand filter
Flow rate of WWTPs (m^3/h)	2,500	718	2,200
Effluent treated by CWs	Approx. 25%	Approx. 15%	Approx.10%
Type of CWs	Surface flow, since 1999	Surface flow, since 2001	Vertical subsurface flow, since 1997
Area of CWs (m^2)	20,000	7,009	7,800
Flow rate of CWs (m^3/h)	360	300	58
Hydraulic retention time of CWs (d)	4	0.82	1.7
Hydraulic loading rate of CWs (cm/d)	43.3	102.7	17.6
Plant species	*Phragmites australis*	*Phragmites australis* (reed bed); trees (swamp)	*Phragmites australis*
Receiving water	River Maas	River Grote Beerze	Lake Ven west

a. Sources of industrial wastewater are industrial process water, organic biodegradable wastewater, and paper manufacturing wastewater. b. Two small hospitals are connected to the sewage system of WWTP Land van Cuijk. The exact individual percentage of industries and hospitals is unknown; c. Main sources of industrial wastewater are from meat processing industries, metal industries, and food industries.

In order to evaluate the attenuation of PhACs, toxic potency, and ARGs in CWs and the discharge effect of residual PhACs to the following aquatic system, duplicate grab samples were collected at locations indicated in Figure 3.1. The attenuation performance of three CWs were calculated based on the difference between CW influent and effluent which is represented by L1-L2, H2-H4, and K1-K2, respectively. It should be noted that the single sampling campaign executed in this study may limit the evaluation of CW attenuation. Possible dilution or concentration of compounds can have occurred depending on the amount of rain water received. Water samples were collected in 500 ml brown glass bottles, transferred to the laboratory the same day, and stored at 4 °C. Glass bottles for PhAC analysis were pre-washed with ethanol and deionized water, and air dried. For ARGs analysis, glass bottles were further autoclaved at 121 °C for 20 min and capped until sampling. Pre-treatment of samples (as described in 3.2.3) was completed within 48 hours before being analysed. Wastewater characteristics were detected: dissolved oxygen (DO), pH, and temperature were analysed using a multi-parameter digital meter

(Hach HQ40d, USA); chemical oxygen demand (COD), ammonium (NH_4-N), nitrate (NO_3-N), and total phosphate (TP) were analysed by using commercial test kits (Dr. Lange, Hach Lange GmbH, Germany) on a Hach DR/3900 spectrophotometer.

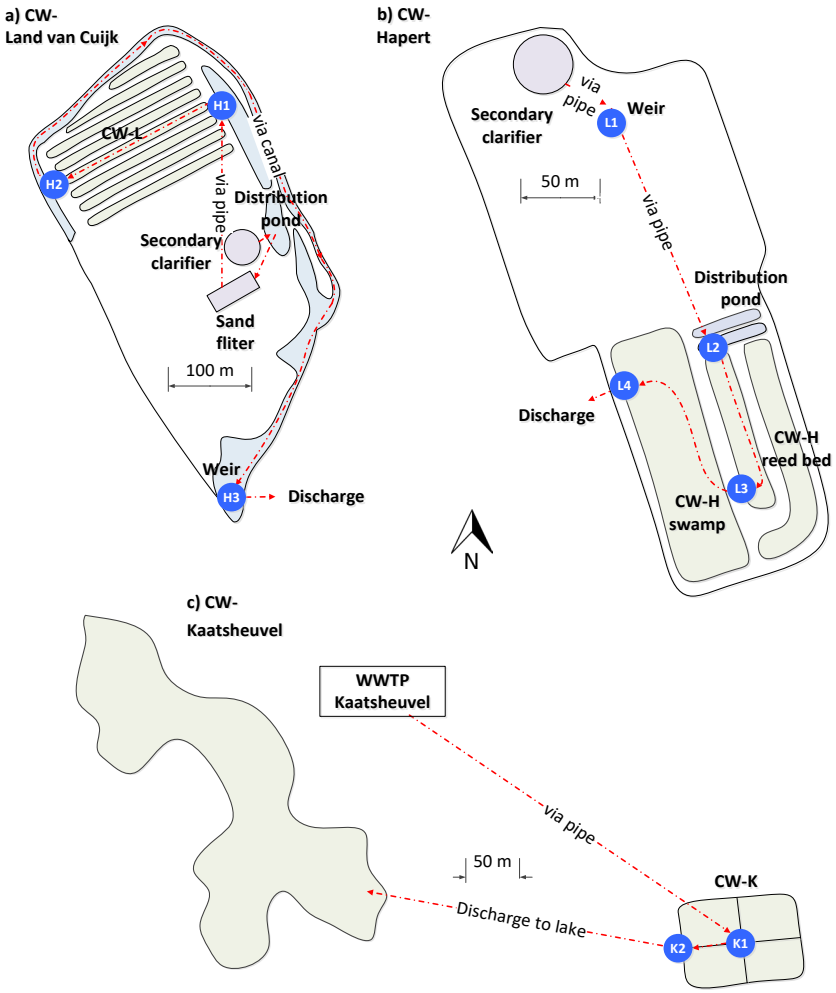

Figure 3.1: Sampling points in three constructed wetlands (CWs) (a) Land van Cuijk (L), L1: inlet of CW; L2: outlet of CW; L3: discharge point; (b) Hapert (H); H1: effluent of secondary clarifier; H2: inlet of CW (after distribution pond); H3: after reed bed treatment; H4: discharge point to the surface water (after swamp treatment); (c) Kaatsheuvel (K). K1: effluent of sand filter/inlet of CW; K2: outlet of CW. Dash lines only indicate the flow direction rather than the real flow paths. The figure was drawn based on the geographical map.

3.2.3 Sample pre-treatment

For chemical and toxicological analyses, samples were pre-treated by filtration (0.7 μm glass filters, GF/F, Whatman, USA) and solid phase extraction (SPE), as previously described (de Wilt et al., 2018). The pre-treatment procedure is described in detail in Text S3.2 and Figure S3.2. In general, 400 ml of samples was loaded on the SPE cartridges to obtain 12 ml elute, of which 3 ml elute was evaporated to achieve a final 500 μl extract with 10% methanol for chemical analysis while 9 ml elute to 500 μl dimethyl sulfoxide (DMSO) for bioanalysis. During SPE, 17β-estradiol-d3 and 10,11-dihydrocarbamazepine were added as the internal standards for gas chromatography tandem mass spectrometry (GC-MS/MS) and ultra-high-performance liquid chromatography (UHPLC)-MS/MS analysis, respectively. Recovery of individual PhACs were tested by spiking PhACs in two different matrices, deionized water and wastewater effluent collected from Bennekom WWTP, the Netherlands. Recovery rates are summarized in Table S3.2. As recovery of diclofenac was low from wastewater, concentration of diclofenac was determined by direct injection on UHPLC-MS/MS after being pre-treated by centrifugation at 10,000 rpm for 10 min (Microlite, Thermo IEC, USA).

For ARGs analysis, 500 ml water samples were filtered using 0.2 μm membrane filter (Merck Milipore, Ireland) and the filter was placed in centrifuge tubes. Those tubes were stored at -20°C before DNA extraction.

3.2.4 Chemical and bioanalyses

Hormones were analysed on a GC-MS/MS and quantification of other PhACs was performed by a UHPLC-MS/MS. Detailed analytical methods are shown in Text S3.3, Table S3.3, and Table S3.4.

Bioanalyses to quantify the toxic potency were performed by using 96-wells plates and detected by a plate reader (Tecan infinite M200 PRO, Switzerland). Different receptors, including yeast, green algae, acetylcholinesterase (AchE) and luminescence bacteria were exposed to the wastewater extracts to determine their acute and chronic toxic potency (Table S3.5). The AchE assay quantifies the potency of the compounds present to inhibit the acetylcholine esterase enzyme, a measure of neurotoxic potency. The REA (RIKILT Estrogen Assay) with the human estrogen receptor α (hERα), comparable to the yeast estrogen screening assay, was performed to quantify the estrogenicity specifically for the hERα. The microtiter Microtox assay provides measure of general toxic and the microtiter algal growth inhibition assay (AGIA) is a measure of the phytotoxicity of the hydrophilic compounds present in the wastewater extracts. Methods were validated by reference compounds (Figure S3.3) and the responses expressed relative to that of assay-specific standards. DMSO was used as the blank control. The emission and excitation spectra of wastewater extract were scanned (Figure S3.4) to make sure that the background of wastewater did not overlap with selected measurement wavelengths in Table S3.4. The

wastewater extracts were tested in quintuplicate in microtiter AGIA and in triplicate for the other bioanalyses. More information about the bioanalysis protocols are shown in Text S3.4.

For all the conducted bioanalyses, results were reported in inhibition percentage as well as toxic equivalence concentrations for that assay (TEQs, equations 3.1-3.3), which is the concentration of a reference compound used to elicit the same response as the unknown and undefined mixture of compounds actually present (Macova et al., 2010).

$$TEQ_s = \frac{EC_{50}(reference\ compound)}{EC_{50}(sample)} = \frac{C_{reference\ compound} \times dilution\ factor_{reference\ compoud}}{enrichment\ factor_{SPE} \times dilution\ factor_{sample}} \quad \text{(eq 3.1)}$$

$$Dilution\ factor_{reference\ compound} = \frac{volume\ of\ reference\ compound\ added\ to\ the\ bioassay}{total\ volume\ of\ the\ bioassay} \quad \text{(eq 3.2)}$$

$$Enrichment\ factor_{SPE} = \frac{volume\ of\ sample\ loading\ in\ SPE}{volume\ of\ extract} \quad \text{(eq 3.3)}$$

$C_{reference\ compound}$ is the 50% effective concentration (EC_{50}) of reference compound added to the wells. In principle, any effect level can be used to derive TEQs other than EC_{50}, provided that the concentration-effect curves are reliable (Villeneuve et al., 2000). In this study, dilution factor of reference compound was the same with that of wastewater extracts in each bioanalysis. Therefore, the TEQs of the extracts ($TEQ_{extract}$) are the ratios of $C_{reference\ compound}$ and $Enrichment\ factor_{SPE}$ (600 times).

3.2.5 DNA extraction and ARGs quantification

DNA filters of water samples were processed using a PowerWater DNA Isolation Kit (MoBio Laboratories, USA), according the manufacturer's protocols. The extracted DNA was stored at -80°C until further analysis.

Quantitative PCR (qPCR) was used to quantify the abundances of 16S rRNA gene, the integrase gene *intI1* and three ARGs, including *sul1* and *sul2* (sulfonamide resistance genes), and *ermB* (macrolide resistance gene). These genes have been recommended for environmental monitoring of antibiotic resistance (Berendonk et al., 2015; Gillings et al., 2015) and have been detected in wastewater at high prevalence and concentrations (Chen et al., 2015c; Rodriguez-Mozaz et al., 2015), making them suitable for analysis of attenuation efficiencies. A synthetic standard with a known quantity was used as the standard for each gene and DNase free water was used as the blank control. Samples, standards and blanks were run using the same procedure in duplicate. Samples were diluted 50 times to avoid qPCR inhibition by humic acids, biological contaminants or proteins. qPCR assays were conducted using the iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, USA) and iQ™ Supermix (Bio-Rad Laboratories, USA) for the SYBR Green reactions and TaqMan reactions, respectively. The reaction mixture of 10 µl consisted of our sample, master mix, primers (Eurogentec, Belgium), precision blue (Biorad, USA),

DNase- and RNase-free water. Details of qPCR conditions and primers are shown in Table S3.6. qPCR assays were conducted on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Canada). Abundances of genes were normalized to 16S rRNA gene values to represent the resistance genes per total bacteria. Detection limit is in the range of 4.81×10^{0} -4.70×10^{7} copies/ml for 16S rRNA gene, *intI1* and all ARGs.

3.2.6 Statistical analysis

In order to investigate the correlation between removal of PhACs and their physicochemical properties, principal component analysis in section 3.3.2 was conducted by using SIMCA 13.0. In addition, multivariate analysis in section 3.3.5 was conducted using the CANOCO 5 software package (Biometrics, the Netherlands) to analyze the correlation (1) among wastewater characteristics, concentration of PhACs and related toxic potency, and (2) among wastewater characteristics, the abundance of PhACs and ARGs. Response variables in multivariate analysis were centralized and standardized to achieve zero average and unit variance. ARGs data was processed by BioRad CFX Manager 3.1 (Biorad, USA).

3.3 Results and discussion

3.3.1 Wastewater characteristics

The wastewater macro-chemical characteristics DO, pH, temperature, COD, NH_4-N, NO_3-N, and TP, as well as removal efficiencies of these by the CWs are presented in Table S3.7. Discharged wastewater of all three CWs complied with the urban wastewater treatment standards (Council Directive 91/271/EEC). CWs showed a capacity to remove nutrients, but this removal was incomplete: CW-L removed 66% of NO_3-N and 21% of TP; CW-H removed 23% of NH_4-N; CW-K removed 26% of COD and 37% of NO_3-N (Table S3.7). Levels of NO_3-N and TP in the wastewater stream further decreased with 66% and 21% after passing the canal and ponds (L2 to L3 in CW-L), indicating that open waters could attenuate nutrients as well.

3.3.2 PhACs

Of the 17 target PhACs, only the lipid regulator clofibric acid and the estrogenic compounds ethinylestradiol and estriol, were not detected in the WWTP effluent before entering the CWs, and estrone and 17β-estradiol only in a few samples (Table 3.2). It cannot be excluded that this was because the samples were not deconjugated before analysis, as (synthetic) hormones are excreted as conjugates (Legler et al., 2002) and as such not detected by the chemical analysis. Ketoprofen only was detected in CW-H. Seven out of 14 detected PhACs discharged to the surface water were at concentrations higher than the guidance value of 0.1 μg/l adopted by toxicologists for drinking water (Verliefde et al., 2007). No regulations exist yet for PhACs discharged from WWTPs to the surface water. Concentrations of ibuprofen (5.0-6.5 μg/l)

and bisphenol A (0.9-3.7 µg/l) even reached the microgram per liter level. The abundance of ibuprofen was also shown by the study of Verlicchi and Zambello (2014), in which ibuprofen was found to be the most abundant PhAC out of 137 PhACs in 136 different CW systems with an average of 32 µg/l and 9.8 µg/l in the influent and effluent of horizontal subsurface flow CWs (HSF-CWs), respectively. Bisphenol A levels were higher in CW-L and CW-H than in CW-K. The latter only receives wastewater from households while the other two CWs also receive wastewater from industries as well (Table 3.1). We assumed that part of the detected bisphenol A in CW-L and CW-H might come from the industries, considering bisphenol A is an industrial compound manufactured in large quantities as a monomer for producing polycarbonate and epoxy resins (Im and Löffler, 2016).

Table 3.2: Concentrations of target pharmaceutically active compounds (PhACs) in different sampling points (average ± std, n=2).

Category	PhACs	Concentration (ng/l)								
		Land van Cuijk (CW-L)			Hapert (CW-H)				Kaatsheuvel (CW-K)	
		L1	L2	L3	H1	H2	H3	H4	K1	K2
Anti-inflammatory drugs	Ketoprofen	< 7	< 7	< 7	16±11	17±8	17±8	18±5	< 7	< 7
	Diclofenac	38±13	25±4	22±1	82±8	71±6	78±1	65±5	46±2	21±5
	Ibuprofen	5319±346	5905±1132	3691±702	6492±261	4325±295	5266±510	5770±292	4971±130	4584±486
	Naproxen	355±5	211±25	307±40	180±17	181±14	174±0	173±3	174±4	74±1
Antibiotics	Erythromycin	19±0	20±1	21±2	42±0	45±4	47±9	42±4	45±4	3±1
	Lincomycin	4±0	5±0	5±0	7±0	7±1	7±0	42±1	4±0	3±0
	Sulfamethoxazole	74±4	70±4	55±3	362±8	378±74	447±9	330±4	179±4	43±2
Beta-blockers	Propranolol	41±2	26±14	13±0	58±10	67±27	79±7	68±1	61±1	11±6
	Metoprolol	434±15	536±23	123±0	1426±35	1407±16	1454±1	1334±8	1114±3	168±12
Lipid regulators	Clofibric acid	<10								
Psychiatric drugs	Carbamazepine	230±11	226±7	227±5	204±7	211±0	218±1	216±4	248±1	316±1
Stimulants	Caffeine	123±14	142±13	70±6	118±22	130±2	141±7	132±2	133±10	207±48
Estrogenic compounds	Bisphenol A	1232±1107	1593±69	1995±1215	3682±1847	2033±728	2722±1020	3828±806	870±319	1441±63
	Estrone	ND	3±2	3±0	3±0	ND	ND	ND	ND	ND
	17β-Estradiol	ND	ND	ND	ND	45±4	53±15	76±4	ND	ND
	Ethinylestradiol	<10								
	Estriol	<10								

Some PhACs were detected at higher levels in the subsequent sampling point compared to the upstream point closer to the WTTP effluent discharge point, which might have originated from the single sampling campaign or caused by deconjugation. A number of compounds are excreted in the conjugated form. It has been reported that many PhACs metabolized as glucuronides or other conjugated metabolites can be converted back to the parent compound by enzymatic processes (Sun et al., 2008; Breitholtz et al., 2012). Deconjugation has been observed in WWTPs occasionally for antibiotics, hormones, and psychiatric drugs (Vieno et al., 2007; Gros et al., 2010; He et al., 2013).

Among the detected 14 PhACs, erythromycin, sulfamethoxazole, propranolol, and metoprolol could be highly removed (>75%) in the compartments of the CWs; diclofenac, naproxen, and lincomycin were removed moderately (30%-60%); while there was almost no removal or even an increase for the other compounds such as bisphenol A (Table S3.8). Fate of PhACs was reported to be correlated with their physicochemical properties (Lee et al., 2011). In our study, principal component analysis was conducted to investigate the relationships between removal efficiencies of PhACs and combination of their properties including the dissociation constant pK_a, the octanol–water partition coefficient $\log K_{ow}$ and $\log D_{ow}$. $\log D_{ow}$ is the apparent partitioning coefficient considering the combined effect of pK_a and $\log K_{ow}$. The results showed no correlation between PhACs removal and those properties (Figure S3.5). The non-correlation with pK_a and $\log K_{ow}$ is also in line with previous research, which proved that PhACs behavior in the biological treatment processes cannot be determined by a certain chemical parameter alone (Park et al., 2009; Breitholtz et al., 2012; Zhang et al., 2012). The absence of correlation with a single or a combination of physical parameters indicates that more removal mechanism (e.g. phytoremediation) might be involved other than biodegradation and adsorption in our case.

Removal efficiencies of 14 detected PhACs were evaluated in three CWs and open waters. Strikingly, the median removal of PhACs in CW-K was approximately 50% while that in CW-L and CW-H (both reed bed and swamp compartment) was negligible (Figure 3.2). The removal efficiencies we observed are relatively low compared with previous studies where removal of most investigated PhACs in CWs were higher than 50% (Matamoros et al., 2009; Hijosa-Valsero et al., 2011). The better PhAC removal performance of CW-K might be caused by its vertical configuration and operational parameters such as hydraulic loading rate (HLR). On the one hand, CW-K is a VSF-CW while the other two CWs are SF-CWs. Compared with SF-CWs, VSF-CWs usually achieve a better oxygenation and possess a superior rhizosphere effect in rhizodegradation as well as adsorption (Matamoros et al., 2007; Zhang et al., 2014). In fact, Matamoros et al. (Matamoros et al., 2009) reported that more than 50% of the studied PhACs were better and more consistently removed in the VSF-CWs as compared with other technologies such as compact bio-filters and biological sand filters. On the other hand, a lower HLR in CWs was reported to result in a higher removal of PhACs due to longer contact and interaction among nutrients, substrate and roots (Zhang et al., 2012; Ávila et al., 2014). In our study, CW-K with a lower HLR (Table 3.1) indeed showed higher removal for PhACs.

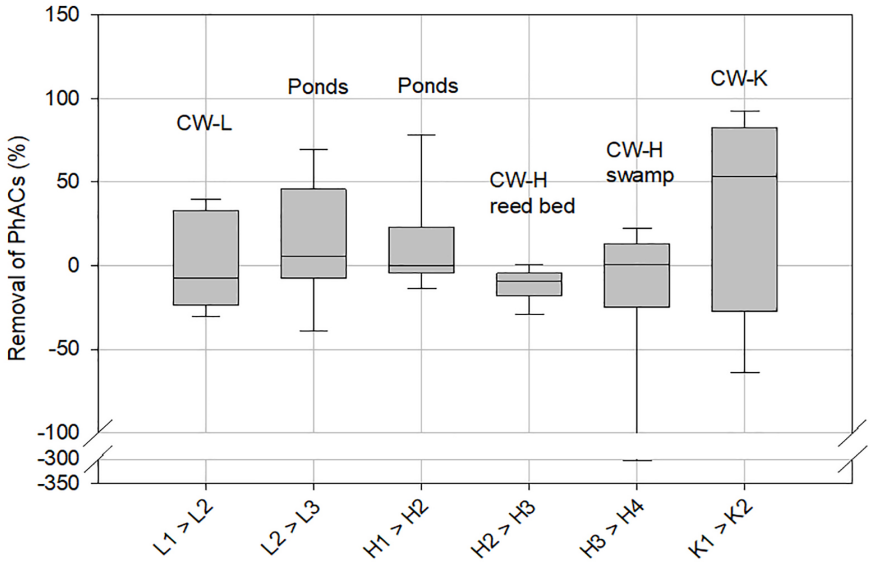

Figure 3.2: Removal efficiencies of 14 detected pharmaceutically active compounds (PhACs) in constructed wetlands (CWs) and open waters. The box plot shows the values in maximum, third quartile, median, first quartile, and minimum.

PhACs could be attenuated in open waters (L2 to L3, H1 to H2 in Figure 3.2), in which PhACs are directly exposed to the sunlight. In fact, PhAC removal has been verified in ponds, either as polishing ponds followed by CWs or as tertiary treatment units in WWTPs, in which photodegradation might play an important role (Hijosa-Valsero et al., 2010; Matamoros and Salvadó, 2012; Rühmland et al., 2015). Therefore, CWs are suggested to include shallow open water compartments to enhance photodegradation of PhACs. But still, CWs with plants are useful as they are rich in biomass and thus are less affected by seasonal changes for removing biodegradable PhACs compared with ponds (Matamoros and Salvadó, 2012).

3.3.3 Toxic potency

In the present study, toxic potency of wastewater extracts was assessed by five bioanalyses based on different receptors. Results are expressed as inhibition or response to receptors and relative to that of the reference standard (toxic equivalence concentrations, TEQs for that assay). In the microtiter AGIA and Microtox assays no toxicity was observed (Figure S3.6). Interestingly, the vitality of the algae and bacteria was even enhanced when wastewater extracts were added, possibly because the extracts contained nutrients. It was confirmed that this was not due to background color or fluorescence from the extracts. The same enhancement phenomenon was also found in previous studies (Lundström et al., 2010; He et al., 2016).

In the YTA, toxic potency of wastewater extracts was notably attenuated in CW-H but not in the other two CWs (Figure 3.3a). The retained toxic potency reflected from TEQ YTA is of 0.85-3.33 µg tributyltin-EQ/l. The AchE inhibiting potency decreased in CW-L, while the other two CWs had no capacity to attenuate this toxic potency (Figure 3.3b). The TEQAChE was in the range of 6.4-11.8 µg dichlorvos-EQ/l (DEQ) for surface water discharging points. This level of dichlorvos is exceeding the maximum permissible concentration of 0.7 ng/l in Dutch surface waters (Crommentuijn et al., 2000) and the 50% lethal concentration (LC50) of 190 ng/l for Daphnia (Hamers et al., 2001). This shows that the potential aquatic risks in the wastewater are not negligible.

The estrogenic potency of the sample extracts, as analysed in the REA, decreased overall in sequential operational compartments, from point L1 to L3, H1 to H2, and K1 to K2 (Figure 3.3c). Only in H3 and H4 the potency increased, which happens to be the only locations in which 17β-estradiol was found with the chemical analysis. The observed 17β-estradiol may originate from additional sources such as hormones from the massive inhabitant birds that we visually observed. The same assumption was also previously reported for CW-H in the work of Foekema (2012). In our study, the level of estradiol equivalents (EEQ) was 0.4-1.6 pmol/l (Figure 3.3c), which is of the same magnitude as the EEQ detected in Dutch wastewater effluent in previous studies: 0.9-2.5 pmol/l (Foekema, 2012) and 0-2.1 pmol/l (Vethaak et al., 2002). Even though the estrogenicity decreased in operational compartments before discharging to surface water bodies, still the observed estrogeniticity level is of concern. A previous study showed that 1.3 pmol/l EEQ could affect immature male rainbow trout to produce estrogen biomarker vitellogenin after 28 weeks of dosing (Sheahan et al., 1994).

In summary, wastewater extracts showed in general toxicity in YTA and specific toxicity in REA and AChE assays. The DEQ levels as determined in the AChE assay are above the LC50 for Daphnia and above the maximum permissible concentration for dichlorvos, and therefore are of environmental concern. No attenuation of the toxic potency in the AchE assay was observed after treatment in two CWs investigated.

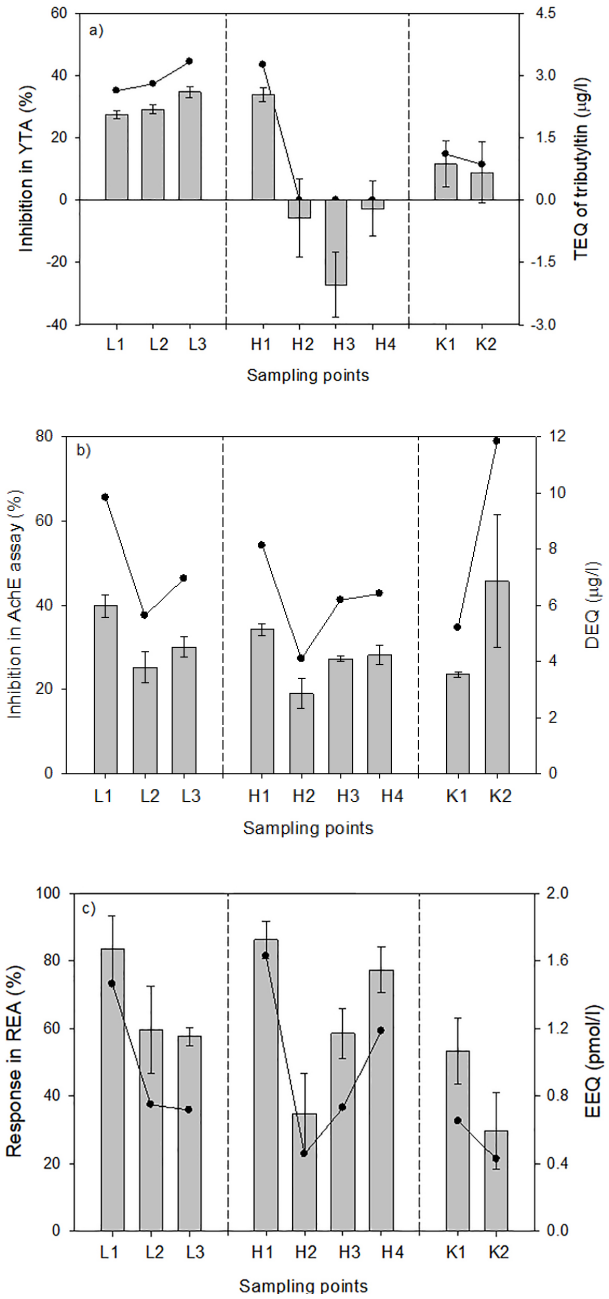

Figure 3.3: Toxic potency and toxic equivalence concentrations (TEQs) of wastewater extracts in various compartment positions in the CW for three different bioanalyses: (a) yeast toxicity assay (YTA); (b) acetylcholinesterase (AChE) assay; (c) RIKILT Estrogen Assay (REA). The left axis corresponds to data in bars; the right axis corresponds to data in dots. Toxicity data are mean value ± standard error (n = 6).

3.3.4 Antibiotic resistance genes

In the present study, an integrase gene (*intI1*) and three ARGs (*sul1*, *sul2*, *and ermB*) were investigated in the wastewater. All ARGs were detected in the wastewater samples except that *ermB* was under detection limit at K2 (Figure 3.4a). Overall, the class 1 integron gene *intI1* had the highest concentrations. Among ARGs, the abundance of *sul1* was highest followed by *sul2* and *ermB,* in terms of both absolute concentrations and concentrations relative to the total bacterial community. The detected ARG concentrations varied from 4.9 copies/mL (*ermB*, L2) to 1.7×10^5 copies/mL (*sul1,* H4) in wastewater samples (Figure 3.4a). The findings in this study are in line with previous studies in which *sul1* and *sul2* genes were the most abundant ARGs in CWs (Chen et al., 2015c; Chen et al., 2016), rivers (Chen et al., 2015a; LaPara et al., 2015; Proia et al., 2016), and marine environments (Suzuki et al., 2013).

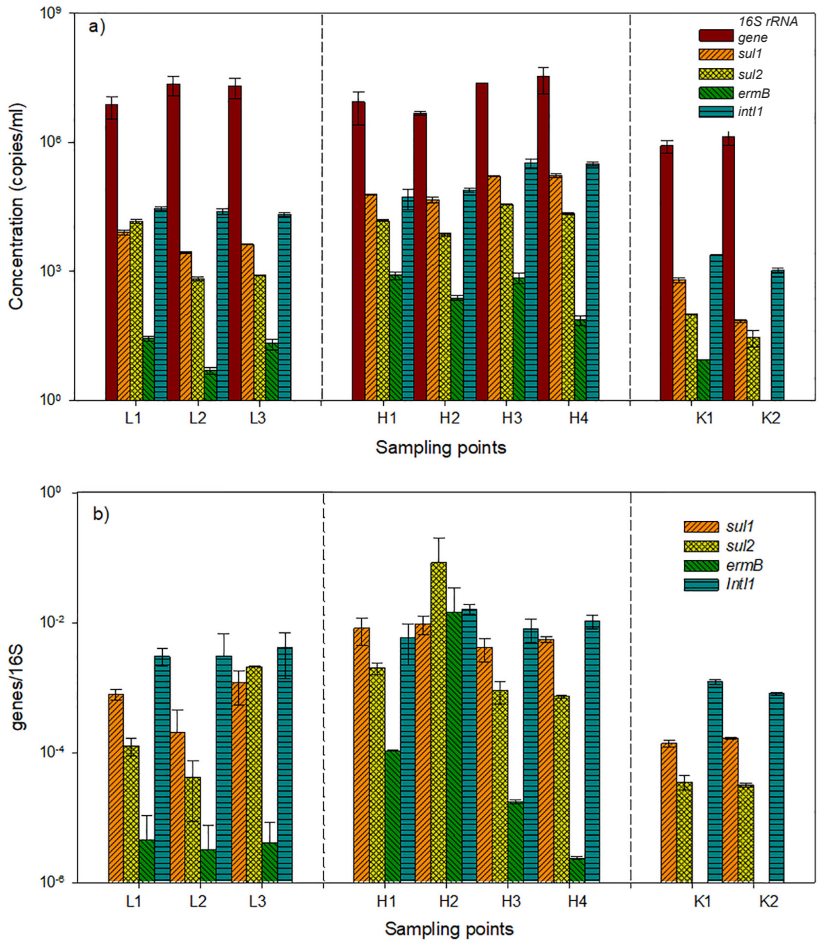

Figure 3.4: The (a) absolute concentrations and (b) normalized concentrations of genes in three constructed wetlands CW-L, CW-H and CW-K. Results are mean value ± standard deviation (n = 2).

CW-L and CW-K showed positive removal of the absolute concentrations of all ARGs in the range of 14% (*intI1*) to 95% (*sul2*), and 57% (*intI1*) to almost 100% (*ermB*) (Table S3.9). Meanwhile, CW-H showed negative removal (i.e. increase) of all ARGs except for *ermB* (70%). Notably, the total bacteria increased in all the investigated CWs (Table S3.9). Relative to the total bacterial, most resistance genes remained stable or showed a decrease after CW treatment (L1-L2, H2-H4, K1-K2, Figure 3.4b). Some of the previous researches concluded that CWs are able to reduce the concentration of ARGs (Auerbach et al., 2007; Chen et al., 2016; Fang et al., 2017), while some also observed a significant increase in antibiotic resistance after CW treatment, either in absolute concentrations (Nõlvak et al., 2013) or relative concentrations (Liu et al., 2013; Huang et al., 2015). Suitable conditions can promote regrowth of microorganisms after the treatment (Zhang et al., 2016), which might lead to the observed absolute increases of ARGs. In addition, selective pressures might be present that also promote regrowth of resistance-carrying bacteria, including antibiotic selective pressure which might be present even at low concentrations (Gullberg et al., 2011; Tello et al., 2012), interaction mediated by antibiotics or non-antibiotic metabolites (Bernier and Surette, 2013), or heavy metal selective pressure (Baker-Austin et al., 2006).

3.3.5 Multivariate analysis of PhACs, toxic potency, and ARGs data

Multivariate analysis was conducted to explore chemical, toxicological and molecular outcomes, and their correlation was investigated through projections onto the ordinations obtained. The detected toxic potency is positively correlated to wastewater characteristics and PhACs (Figure 3.5a). Various researchers have positively correlated organics (COD) and nutrients to the toxic potency of wastewater (Bayo et al., 2009; Yu et al., 2014; Ma et al., 2016). In contrast, PhACs have rarely been correlated with toxic potency in real wastewater matrixes. In our study, toxic potency described in REA and YTA seemed to be positively correlated with wastewater characteristics and PhACs. Especially REA positively correlated to organics, nutrients (NH_4-N, NO_3-N, TP) as well as most of the PhACs. In comparison, AchE correlated less with environmental variables, indicating that the neurotoxic potency of wastewater extracts might be related to other pollutants than the PhACs we tested.

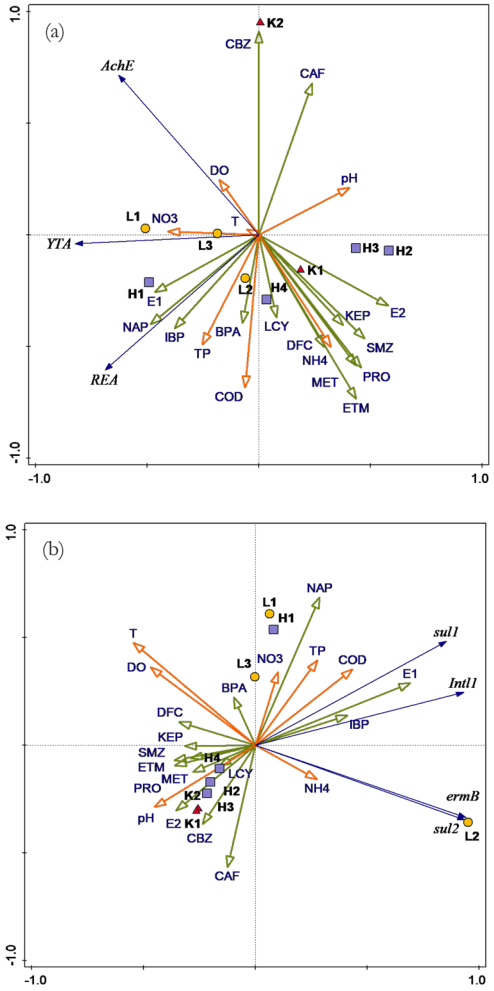

Figure 3.5: Multivariate analysis of the correlation (a) among toxic potency, wastewater characteristics and PhACs; (b) among ARGs (normalized), wastewater characteristics and PhACs. Sampling points are indicated in circles for CW-L, rectangles for CW-H, and triangles for CW-K, respectively. Environmental variables are shown in arrows. The eigenvalues of the first and second canonical axis are 0.51 and 0.29 in (a), 0.84 and 0.13 in (b).

ARGs levels are not correlated to the abundance of related antibiotics but rather to organics, nutrients, and some PhACs (Figure 3.5b). Higher concentrations of SMZ and ETM did not correlate with higher concentrations of *sul1/2* and *ermB*, respectively. This lack of correlation may result from three reasons: (1) wastewater already contains high amounts of resistance genes, which are not necessary related to the actual wastewater antibiotic content; (2) abundance of resistance gene in the CWs result from survival (or even growth) of wastewater bacteria carrying these genes, or selection of resistant bacteria in situ. These processes are in turn

possibly partly, but not exclusively mediated by PhACs or other selective pressures; (3) mobile genetic element such as plasmids, integrases, and transposases are able to assist the spread of ARGs without antibiotics being present (Zhu et al., 2013). Thus, antibiotics and resistance genes do not necessarily have to be correlated, as also shown in previous research (Pruden et al., 2006; Anderson et al., 2013; Wu et al., 2015). However, a positive correlation was found between concentrations of ARGs and concentrations of organics, nutrients and some PhACs. This might indicate that organics and nutrients stimulate growth of resistant bacteria in CWs, and that processes removing these pollutants also reduce resistance genes to a similar extent.

Sul1, *sul2* and *ermB* show strong correlations with *intI1* (Figure 3.5b), indicating that removal or regrowth of bacteria harboring these genes in general co-occurs. The *intI1* gene has been found to be correlated with the dissemination of both types of *sul* genes in the environment (Chen et al., 2015b). The *sul1* gene is normally found in class 1 integrons *intl1* (Sköld, 2000), whereas *sul2* is usually located on small non-conjugative plasmids (Enne et al., 2001) or large transmissible, multi-resistance plasmids (Heuer and Smalla, 2007). In a study of Antunes et al. (2005), they observed *intI1* presence in almost 98% of *sul1* isolates. Shehabi et al. (2006) also found that 62% of *sul1*/*sul2* was positively associated with *intI1*. With this correlation, Muziasari et al. (2014) suggested that *intI1* may play a role in the prevalence of *sul1* through horizontal gene transfer.

As indicated in Figure 3.5a and 3.5b, the observed positive correlation between concentrations of organics and nutrients and concentrations of PhACs shows that conditions that remove organics and nutrients most likely remove PhACs as well. Similar results were found in the study of Matamoros et al. (2007), in which the authors positively linked the high removal of most targeted PhACs with the high removal of BOD, total suspended solid (TSS), and NH_4-N in a VSF-CW and sand filter systems. Therefore, the low removal of organics and nutrients might explain the low attenuation of PhACs in this study.

Although the single sampling campaign in this work may limit the evaluation of CW attenuation performance, the multivariate analysis implemented in this study provides more insight into the presence and removal of PhACs as well as their associated environmental hazards (i.e. toxic potency and ARGs). These results overall show a snapshot of limited and variable attenuation of PhACs, toxic potency and ARGs in the three CWs. The findings might indicate many removal processes in the CWs are sub-optimal and more knowledge generation on the attenuation mechanisms under varying CWs operational conditions is essential. More repeated measurement should be conducted in the future to confirm this indication.

3.4 Conclusions

In this study, performance of CWs to attenuate PhACs, toxic potency, and ARGs has been assessed. Furthermore, correlations between toxic potency, PhACs, ARGs, and water characteristics were explored. The main findings are: (1) Several PhACs discharged to the surface water were at concentrations higher than 0.1 μg/l, especially for bisphenol A and ibuprofen. Even though some of the PhACs were moderately or highly removed, the median removal of PhACs in CWs was approximate 50% in CW-K and negligible in other two CWs. (2) Wastewater extracts showed general toxicity in YTA and specific toxicity in REA and AChE assays. The DEQ levels are above safe levels and therefore are of environmental concern. In addition, the DEQ levels did not show attenuation in two of the CWs investigated. (3) Positive ARG removal was observed in CW-L and CW-K in terms of both absolute and relative concentrations. The increased absolute concentrations of *sul1*, *sul2*, and *intI1* in CW-H as wells as the increase of total bacteria in all CWs may link to regrowth of microorganisms mediated by suitable growth conditions and/or selective pressures. (4) Most PhACs were positively correlated to the toxic potency, either indicating a potential hazard of these compounds to the environment or indicating co-occurrence of PhAC with other substances in the wastewater causing toxic potency. Concentrations of organics, nutrients, and some PhACs were positively correlated to ARG concentrations while no concrete pattern of ARGs can be predicted from the concentration of the antibiotics analysed.

Considering the insufficient removal of PhACs, toxic potency and ARGs in CWs, enhancement of CW performance is desirable, where optimal construction (e.g. vertical configuration, constructed with open waters) and operational parameters (e.g. HLR) can be considered. Multivariate analysis in this study offers a great potential to comprehensively evaluate the performance of CWs by associating chemical, toxicological, and molecular analyses.

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Supplementary Information

Text S3.1: Chemicals and reagents.
Methanol, formic acid, ammonium formate and water (Actu-All Chemicals, the Netherlands) used for ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis was of UPLC grade. The derivatization reagent MSTFA++ was a mixture of N-methyl-N-trimethylsilyl-trifluoroacetamide from Alltech (Anaconda, MT, US), ammonium iodide from Fluka (Zwijndrecht, The Netherlands), and dithioerythreitol from AnalaR (1000:2:4, v/w/w). All other chemicals and reagents used were of analytical grade or higher. Deionized water from a Milli-Q system (Millipore, USA) was used to prepare solutions. DMSO used in bioassays was purchased from Sigma (≥ 99.5%, USA).

Text S3.2: Pre-treatment of water samples for chemical and toxicological analyses.
For chemical and bioanalysis, samples were filtered through 0.7 µm glass filters (GF/F, Whatman, USA) prior to solid phase extraction (SPE). Oasis HLB cartridges (6 cc/60 mg, Waters, USA) were used for the SPE. Firstly, pH of water samples was adjusted by adding 4 ml buffer (pH=10, Merck, Germany). The cartridge was pre-conditioned with 5 ml methanol and then equilibrated with 5 ml deionized water. Next, 400 ml of samples passed through SPE cartridges with a flow of 5-10 ml/min controlled by a vacuum pump (Buchi V-700, Switzerland). After loading samples, cartridges were washed with 12 ml deionized water and eluted with 12 ml of 25% NH_4OH: methanol (8/92, v/v). The eluent of each 400 ml sample was divided into 3 ml and 9 ml for chemical and bioanalysis, respectively (Figure S3.2). The 3 ml eluent was evaporated to 200 µl at 35 °C under a gentle stream of nitrogen (VLM evaporator, Germany). Then, 250 µl Milli-Q water was added and the remaining methanol (200 µl) was evaporated. As a final step, the extract was adjusted with water:methanol (80:20, v/v) by weight to achieve a final 500 µl extract with 10% methanol. The 9 ml eluent was evaporated to dryness and replaced with 500 µl DMSO by weight.

Text S3.3: Chemical analysis of pharmaceutically active compounds (PhACs).
Hormones were analysed on a gas chromatography (GC)-MS/MS. The processing method was based on the protocol used in RIKILT, Wageningen University & Research. Samples were derivatized prior to chemical analysis. The 10% methanol extract was evaporated at 60 °C to dryness under a gentle nitrogen flow. The dry residue was derivatized by adding 25 µl $MSTFA^{++}$ followed by incubation at 60 °C for 1 h. The derivatized extract was evaporated at 60 °C to dryness and reconstituted with 25 µl of iso-octane. Afterwards, the extract was sonicated for 2 min and mixed by vortexing. GC-MS/MS analysis was performed on a Varian 1200 triple quadrupole MS system (Varian, USA) comprised of a Varian CP 3800 GC and a Varian CP 8400 auto-sampler. The GC was equipped with a J&M GC column (30 m × 0.25 mm, 0.25 µm, Agilent, USA). 2 µl of samples were injected in splitless mode to the GC column by a pulsed pressure of 30 psi for 1.2 min. The injector was kept at 250 °C for 1 min. The oven temperature program was as follows: 110 °C (held for 1 min), ramped at 20 °C/min to 250 °C (held for 2

min), then ramped at 5 °C/min to 280 °C, and finally ramped at 25 °C/min to 330 °C (held for 2 min). Helium was used as the carrier gas and the flow was kept at 1 ml/min. Selected ion monitoring (SIM) mode was conducted to analyse E3 while the other hormones were measured in multiple reaction monitoring (MRM) mode. Detailed transitions and collision energies are listed in Table S3.3. Quantification of GC-MS/MS detection results was calibrated based on internal and external standards.

Quantification of other PhACs was performed by using a Waters UHPLC Acquity system coupled to a Waters Xevo TQ MS. 10 µl extract was injected on a Atlantis HILIC Silica T3 column (3.0 × 100 mm, 3 µm) (Waters, USA) and was separated using gradient elution with a stable flow of 0.4 ml/min. The solvent used were A: water/ammonium formate/formic acid (1000/2/0.16) and B: methanol/ammonium formate/formic acid (1000/2/0.16). The gradient was set as: 0-0.5 min 10% B; 0.5-6 min linear increased to 70% B; 6-7 linear increased to 100% B and hold 1 min; 8-8.1 decreased to 10% B and hold until 10 min. Column temperature was maintained at 60 °C. Waters Xevo TQ was operated in MRM mode using electrospray ionization. The instrument conditions for positive mode were: capillary voltage 2.2 kV, cone voltage 40 V, desolvation gas flow 800 L/h at 600 °C, con gas flow 150 L/h, collision gas flow 0.18 ml/min. While in negative mode, capillary voltage was 1.5 kV and other parameters were the same with positive mode. Product ions were chosen for confirmation, in which the most intensive product ion was selected for the quantification. MS parameters including optimized collision energy were summarized in Table S3.4. Peak identification and quantification was performed using MassLynx software version 4.2. Quantification of UHPLC-MS/MS detection results was calibrated based on external standards.

Text S3.4: Procedure of five bioanalyses. Percentages of DMSO in wells were 0.5%, 1.0%, 1.7%, 0.5%, and 0.45% in the following five bioanalyses respectively.

YTA. The Yeast Toxicity Assay was adjusted based on Fai and Grant (2009). Toxic potency of wastewater samples was quantified relative to the lethality of yeast cells induced by tributyltin. *Saccharomyces cerevisiae*, a regular baking and brewing yeast, was purchased from the local supermarket. Briefly, 0.15-0.18 g yeast was activated by adding into 100 ml of the mixture of phosphate-buffered saline (PBS) and glucose and incubating for 1 h at 30 °C. The mixture was prepared by mixing 1 pill of PBS and 2 g glucose in 100 ml deionized water. Next, 5 ml of tributyltin or samples were diluted with 495 ml of the PBS/glucose mixture. 100 ml of the diluted tributyltin or samples were added to the plate in triplicate. Afterwards, 70 ml yeast suspension and 30 ml redox dye resazurin were added to each well as substrate. Fluorescence of resorufin was analysed after 1 h of cultivation at 30 °C.

REA. RIKILT Estrogen Assay with the human estrogen receptor α (hERα) was performed based on the study of Bovee et al. (2005). Yeast used was genetically modified to express human estrogen receptor-beta by RIKILT, Wageningen University and Research, the Netherlands.

AchE assay. Acetylcholinesterase (AchE) assay was performed based on previous studies (Hamers et al., 2000; Hamers et al., 2003). AchE is a key enzyme for stopping signal transmission in humans and animals after excitation. The neurotoxic potency of xenobiotics is quantified based on their ability to block esterases, thus inhibiting the hydrolysis of the neurotransmitter acetylcholine by AChE (Hamers et al., 2000; Hamers et al., 2003). AchE was extracted from honey bees heads collected from a clean area in Renkum, The Netherlands and prepared according to Hamers et al. (Hamers et al., 2000).

Microtiter Microtox Assay and microtiter AGIA. Luminescence bacteria *Vibrio fischeri* was purchased from Microlan (the Netherlands). Green algae *Pseudokirchneriella subcapitata* was obtained from Department of Aquatic Ecology and Water Quality Management, Wageningen University. The microtiter Microtox assay was based on the method developed by Hamers et al. (2001) with some modifications as described in He et al. (He et al., 2016). The microtitier algal growth inhibition assay (AGIA) was performed as described previously (Blaise and Férard, 2005; Jonker et al., 2006).

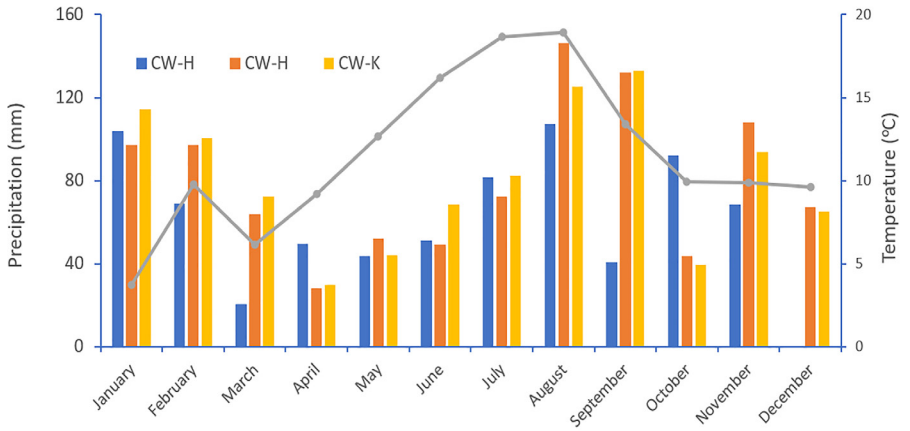


Figure S3.1: Precipitation and temperature of CW locations through the year 2015 (Data source: Royal Netherlands Meteorological Institute). July was selected as the sampling month due to its low precipitation among the warm months.

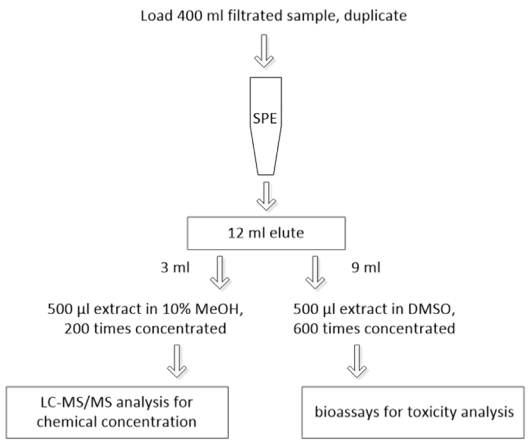


Figure S3.2: Pre-treatment of wastewater samples for chemical and bioanalyses.

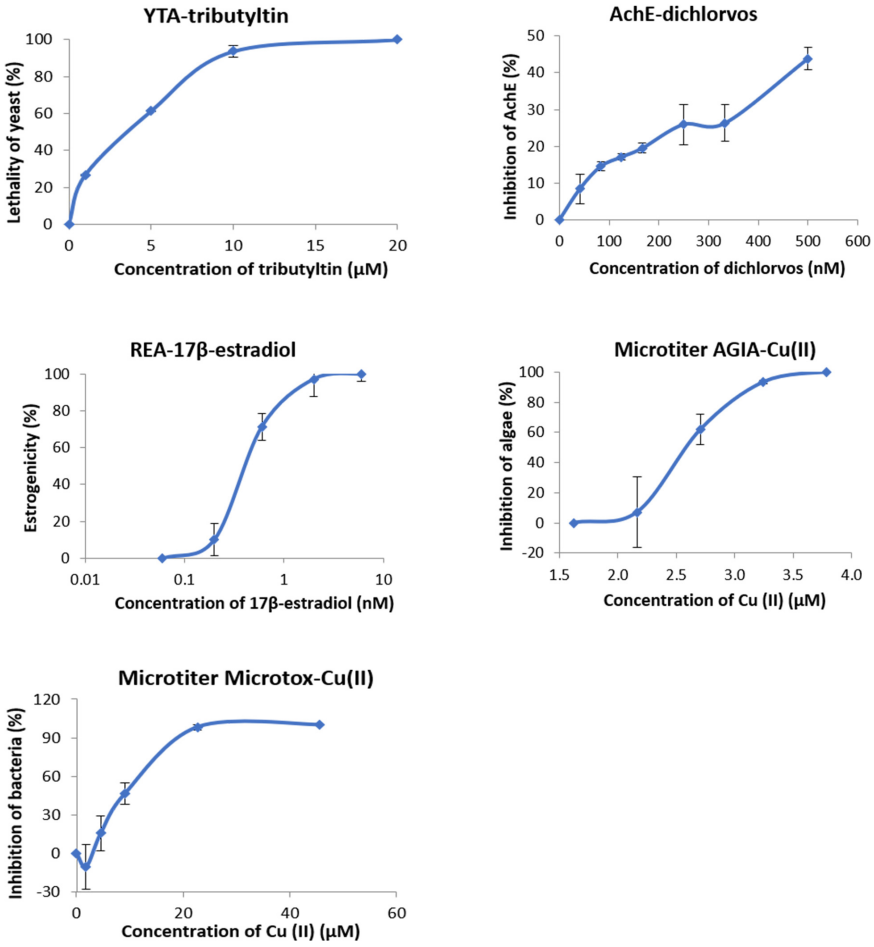


Figure S3.3: Standard curves of reference compounds in five in vitro bioassays. Values of Axis x are the concentrations of standard compounds in the wells. Results are shown as average and standard error (n = 5 in microtiter AGIA; n = 3 in the other bioanalyses).

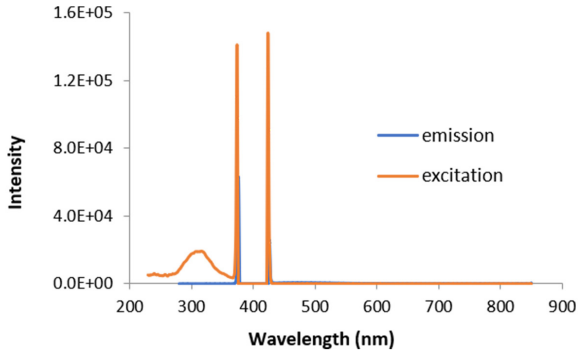


Figure S3.4: Fluorescence scans of the wastewater extract (taking one extract sample as an example). Results showed that maximum fluorescence-related wavelength did not overlap with the measured wavelengths in bioanalyses as shown in Table S3.5.

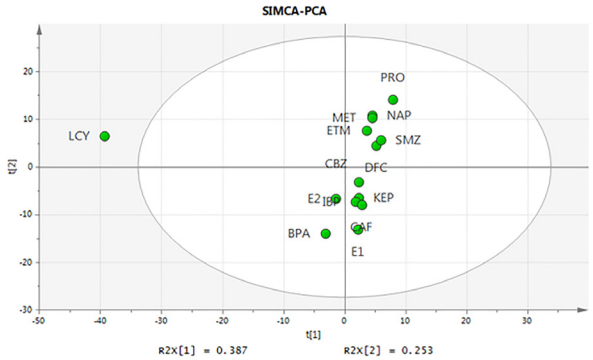


Figure S3.5: Principle component analysis of the relationship between removal efficiency of PhACs and their physicochemical properties, including pKa, log K_{ow} and log D_{ow} (Table S3.1). No clear correlation was found because PhACs with similar properties did not cluster.

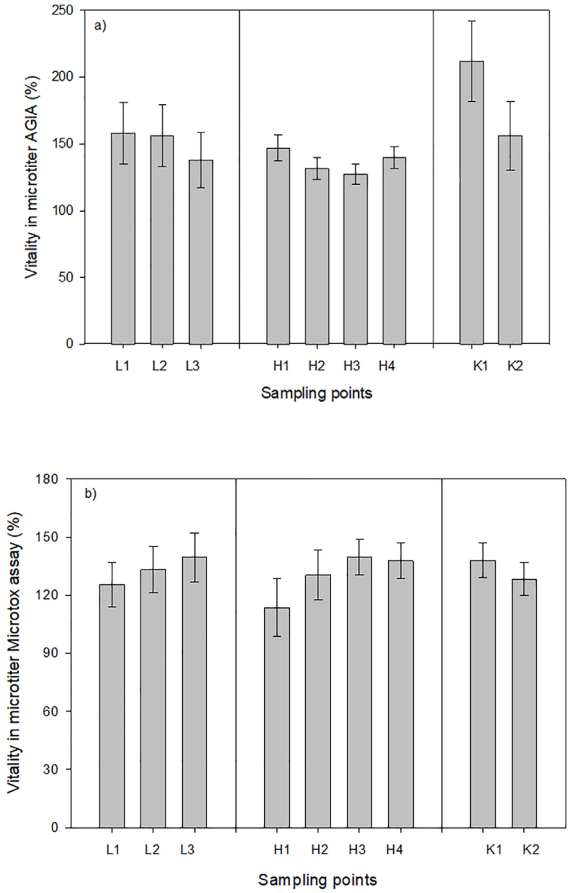
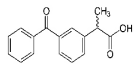
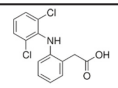
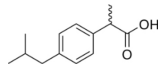
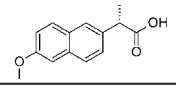
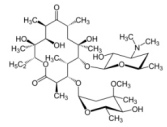
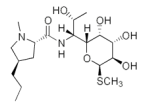
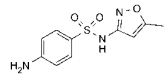
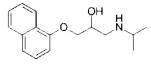
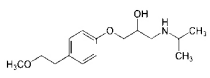
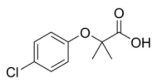
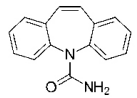
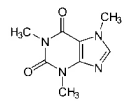
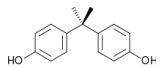
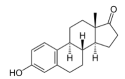
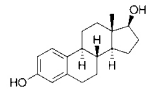
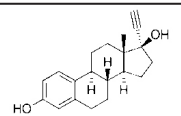
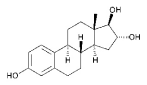


Figure S3.6: Toxic potency of sample extracts in different sampling points in two different bioanalyses: a) Microtiter algal growth inhibition assay (AGIA); b) Microtiter Microtox assay. Toxicity data are mean value ± standard error (n = 6).

Table S3.1: Chemical structures and physicochemical properties of 17 target PhACs.

Category	No.	PhACs	Abbr.	Formula	Structure	Sw[a] (mg/l)	pKa[b]	log K_{ow}[c]	log D_{ow}[d]
Anti-inflammatory drugs	1	Ketoprofen	KEP	$C_{16}H_{14}O_3$		51	4.45	3.12	0.57
	2	Diclofenac	DFC	$C_{14}H_{11}Cl_2NO_2$		2.4	4.15	4.51	1.66
	3	Ibuprofen	IBP	$C_{13}H_{18}O_2$		21	4.91	3.97	1.88
	4	Naproxen	NAP	$C_{14}H_{14}O_3$		16	4.15	3.18	0.33
Antibiotics	5	Erythromycin	ETM	$C_{37}H_{65}NO_{12}$		1.4	8.88	3.06	1.17
	6	Lincomycin	LCY	$C_{18}H_{34}N_2O_6S$		927	7.97	0.56	-0.45
	7	Sulfamethoxazole	SMZ	$C_{10}H_{11}N_3O_3S$		3942	6.16	0.89	-0.01
Beta-blockers	8	Propranolol	PRO	$C_{16}H_{21}NO_2$		61.7	9.42	3.48	1.06
	9	Metoprolol	MET	$C_{15}H_{25}NO_3$		17000	9.67	1.88	-0.79

Lipid regulators	10	Clofibric acid	CFC	$C_{10}H_{11}ClO_3$		582.5	3.2	2.57	-1.23
Psychiatric drugs	11	Carbamazepine	CBZ	$C_{15}H_{12}N_2O$		17.7	13.9	2.45	-4.45
Simulants	12	Caffeine	CAF	$C_8H_{10}N_4O_2$		22000	10.4	-0.07	-3.47
Estrogenic compounds	13	Bisphenol A	BPA	$C_{15}H_{16}O_2$		300	9.6	3.32	0.72
	14	Estrone	E1	$C_{18}H_{22}O_2$		30	10.33	3.13	-0.20
	15	17β-estradiol	E2	$C_{18}H_{24}O_2$		3.9	10.33	4.01	0.68
	16	Ethinylestradiol	EE2	$C_{20}H_{24}O_2$		11.3	10.33	3.67	0.34
	17	Estriol	E3	$C_{18}H_{24}O_3$		27.34	10.54	2.45	-1.09

a. Sw: solubility at 25 °C (mg/l) in water; b. pKa: acid dissociation constant; c. log K_{ow}: octanol-water partition coefficient; d. log D_{ow} was calculated at pH=7. Data source: Hazardous substance data bank (https://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm); DrugBank (http://www.drugbank.ca/).

Table S3.2: Recovery rate of individual PhACs in deionized water and wastewater effluent (average ± std, n = 2).

Category	Compounds	Recovery (%)	
		Deionized water	Wastewater
Anti-inflammatory drugs	KEP	128.5±0.7	112±4.2
	DFC[a]	109.5±9.2	1.5±0.7
	IBP	156±2.8	124±11.3
	NAP	172±18.4	110±2.8
Antibiotics	ETM	25.5±17.7	168±18.4
	LCY	91±0	83±0
	SMZ	54.5±2.1	93±0
Beta-blockers	PRO	6.5±4.9	85.5±20.5
	MET	91±11.3	145.5±2.1
Lipid regulators	CFC	96.5±3.5	16±1.4
Psychiatric drugs	CBZ	87.5±9.2	87±2.8
Simulants	CAF	116±1.4	130.5±0.7
Internal standard	Dihy-CBZ[b]	19	17
Estrogenic compounds	BPA	79±1.4	58±18.4
	E1	105.5±6.4	48.5±0.7
	E2	95±1.4	61.5±0.7
	EE2	97.5±2.1	53±15.6
	E3	98±11.3	48±5.7
Internal standard	E2-d3[b]	95±56.6	78.5±34.6

a. The recovery of DFC by direct injection is ≥ 98%; b.Dihydrocarbamazepine, the internal standard for UHPLC-MS/MS analysis. b. The internal standard for GC-MS/MS analysis.

Table S3.3: Operational parameters of PhACs analysis on GC-MS/MS.

Segment	Compounds	Retention time (min)	Parent and product ion (from high to low)			
			1	CE (v)[a]	2	CE (v)
1	BPA	9.616	372.3>357.3	15	372.3>191.1	32
4	E1	14.656	414.3>155.3	16.5	414.3>296.5	7
3	E2	14.748	416.3>285.3	7	416.3>326.3	6
4	EE2	16.228	425.3>193.2	14	425.3>231.2	15
5	E3[b]	16.456	504.4	414.3	386.3	311.2
6	E2-d3	14.707	419.3>285.3	7	/	/

a. CE = collision energy. b. Estriol was analysed in selected ion monitoring (SIM) mode.

Table S3.4: Operational parameters of PhACs analysis on UHPLC-MS/MS.

Compounds	Mode	Rentition time (min)	Parent ion	CE (v)	product ion-1	product ion-2	product ion-3
KEP	-	7.5	253	10	209	197	**105**[a]
DFC	-	8.24	294	10	**250**	214	
IBP	-	1.33	205	10	**161**	159	
NAP	-	7.63	229	10	**185**	169	
ETM	+	6.86	734	20	558	576	**158**
LCY	+	3.81	407	29	**359**	126	
SMZ	+	4.51	254	15	**188**	156	
PRO	+	5.72	260	20	**183**	116	
MET	+	4.73	268	20	**159**	133	
CFC	-	7.5	213	10	**127**	85	
CBZ	+	6.95	237	20	194	**192**	
CAF	+	4.31	195	20	138	**110**	
Dihy-CBZ	+	7.15	239	20	195	**180**	

a. Product ion in bold was selected for the quantification.

Table S3.5: Description of bioassays applied for bioanalysis of the toxic potency of wastewater extracts.

Toxicity	Bioassays	Receptors	Exposure time	Measurement	Reference compound
Acute	YTA	*Saccharomyces cerevisiae*	1 h	Fluorescence $\lambda_{emission}$=590 nm, $\lambda_{excitation}$=530 nm	Tributyltin
	AchE Assay	Acetylcholinesterase	14 min	Absorbance λ=412 nm	Dichlorvos
	REA	Genetically modified baker's yeast	24 h	Fluorescence $\lambda_{emission}$=530 nm, $\lambda_{excitation}$=485 nm	17 β-estradiol
	Microtiter Microtox Assay	*Vibrio fischeri*	15 min	Luminescence	$CuSO_4$
Chronic	Microtiter AGIA	*Pseudokirchneriella subcapitata*	72 h	Fluorescence $\lambda_{emission}$=680 nm, $\lambda_{excitation}$=435 nm	$CuSO_4$

Table S3.6: Overview of primer sequencing and thermal cycling conditions for qPCR.

ARGs	Sequence (5'-3')	Thermal profile	Cycles	Detection format	References
16S rRNA gene	ACTCCTACGGGAGGGCAG GACTACCAGGGTATCTAATCC	95 °C 3 min 95 °C 15 s, 60 °C 45 s	1 40	SYBR Green	Fierer et al. (2005)
intI1	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	1 40	TaqMan	(Barraud et al., 2010)
sul1	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	1 40	TaqMan	(Heuer and Smalla, 2007)
sul2	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	1 40	TaqMan	(Heuer et al., 2008)
ermB	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	1 40	SYBR Green	(Knapp et al., 2010)

Table S3.7: Physicochemical analysis of collected wastewater samples and removal efficiencies in CWs (average ± std, n = 2).

WWTPs	Sites	DO (mg/l)	pH	T (°C)	COD (mg/l)	NH_4-N (mg/l)	NO_3-N (mg/l)	TP (mg/l)
CW-L	L1	8.1	6.4	22.6	29.4±0.28	0.05	2.69	0.38±0.01
	L2	1.2	6.5	20.3	32.70±0.85 (-11) [a]	0.64±0.01 (-1180)	0.91±0.01 (66)	0.30±0.03 (21)
	L3	16.2	7.1	24.6	35.35±0.78	0.02	0.28±0.02	0.13±0.01
CW-H	H1	5.7	6.5	22.5	28.65±1.48	0.70±0.01	0.4	0.38±0.17
	H2	5.7	6.7	22.4	31.70±1.27	0.7	0.54±0.05	0.30±0.03
	H3	5.9	6.8	22.9	29.95±0.07	0.70±0.01	0.52±0.01	0.28
	H4	5.5	6.7	21.6	30.15±2.05 (5)	0.54±0.01 (23)	0.78±0.01 (-44)	0.40±0.04 (-33)
CW-K	K1	9.4	6.8	22.7	19.85±0.92	0.02	1.18±0.01	0.08±0.01
	K2	8.4	6.8	21.9	14.70±4.10 (26)	0.02 (0)	0.74±0.01 (37)	0.12 (-50)
EU standard (Council Directive, 1991)	/	/	/	/	< 125	Total nitrogen (TN) < 15 (10,000-100,000 p.e.) < 10 (> 100,000 p.e.)		< 2 (10,000-100,000 p.e.) < 1 (> 100,000 p.e.)

a. Removal efficiencies in three CWs are displayed in brackets (%) and were calculated based on L1 to L2, H2-H4, and K1-K2, respectively.

Table S3.8: Removal of PhACs in three CWs (average ± std, n = 2). Negative numbers mean the levels of the compounds increased over the CW.

PhACs	Removal efficiency (%)		
	CW-L	CW-H	CW-K
KEP	ND	-7.3±57.9	ND
DFC	32.8±24.8	8.4±10.5	53.1±11
IBP	-11.0±22.5	-33.4±11.3	7.8±10.1
NAP	40.6±7.1	4.4±7.6	57.7±1.1
ETM	-7.2±5.3	6.3±12.2	94.3±2.3
LCY	-31.0±0	-534.1±86.9	41.2±0
SMZ	5.6±7.4	12.7±17.1	76.3±1.2
PRO	36.3±34.3	-0.9±40.9	82.8±9.8
MET	-23.6±6.8	5.2±1.2	84.9±1.1
CFC	ND	ND	ND
CBZ	1.5±5.6	-2.8±1.9	-27.4±0.7
CAF	-15.3±16.9	-2±2.2	-56.4±37.9
BPA	-29.2±116.3	-88.3±78.2	-65.7±61.2
E1	ND	ND	ND
E2	ND	-70.2±17.4	ND
EE2	ND	ND	ND
E3	ND	ND	ND

Notes: Ellipse frames represent removal higher than 75%; rectangle frames represent removal higher between 30-60%. ND = not determined because levels were below the limit of detection (Table 2.1).

Table S3.9: Removal detected genes in the three CWs.

CWs	Genes	Influent (copies/mL)	Effluent (copies/mL)	Removal (%)
CW-L	*16S rRNA gene*	7.5×10^{6}	2.3×10^{7}	-207.7
	sul1	8.2×10^{3}	2.7×10^{3}	66.7
	sul2	1.5×10^{4}	6.9×10^{2}	95.4
	ermB	2.8×10^{1}	4.9×10^{0}	82.3
	intI1	2.9×10^{4}	2.5×10^{4}	13.8
CW-H	*16S rRNA gene*	4.7×10^{6}	3.4×10^{7}	-619.8
	sul1	4.6×10^{4}	1.7×10^{5}	-261.5
	sul2	7.3×10^{3}	2.2×10^{4}	-206.5
	ermB	2.4×10^{2}	7.3×10^{1}	69.8
	intI1	7.8×10^{4}	3.2×10^{5}	-308.9
CW-K	*16S rRNA gene*	8.4×10^{5}	1.3×10^{6}	-60.0
	sul1	6.4×10^{2}	7.0×10^{1}	89.1
	sul2	1.0×10^{2}	3.0×10^{1}	70.5
	ermB	9.0×10^{0}	ND[b]	Almost 100
	intI1	2.4×10^{3}	1.1×10^{3}	56.7

a. ND = not determined because levels were below the limit of detection 4.81 copies/ml.

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CHAPTER 4

Performance of full scale constructed wetlands in removing antibiotics and antibiotic resistance genes

A modified version of this chapter has been submitted as:

Sabri, N.A., Schmitt, H., van der Zaan, B.M., Gerritsen, H.W., Rijnaarts H.H.M., Langenhoff, A.A.M. Performance of full scale constructed wetlands in removing antibiotics and antibiotic resistance genes.

Abstract

Additional treatment of wastewater, such as constructed wetlands (CWs), is a possible solution to reduce the discharge of antibiotics and antibiotic resistance genes (ARGs) from households and industry to the environment. This study aims to investigate the occurrence in and removal of antibiotics and ARGs by two full scale CWs operated at different hydraulic retention times (HRT), namely 1 day and 3 days. Both CWs were receiving the same WWTP effluent. Temporally and spatially distributed sampling of water and sediment was conducted for one year and samples were analysed for antibiotics and ARGs by using LC-MS/MS and qPCR, respectively. Results showed that both CWs removed antibiotics significantly with a comparable overall removal of 28% - 100%, depending on the type of antibiotics. Meanwhile, ARGs were removed from the wastewater by 0.8 to 1.5 log by the CW treatment but tended to accumulate in its sediment. The HRT did not influence the removal of either the antibiotics or the ARGs. In general, higher concentrations of antibiotics and ARGs were found during winter compared to summer. A strong correlation was found between sul genes and *intI1*. The results also revealed a positive and a negative relationship: a positive relation between abundance of antibiotics, ARGs, and of NO_3–N, NH_4–N, TP, COD and a negative relation between antibiotics, ARGs, and temperature. The ability of CWs to reduce the input of micropollutants into the environment makes CWs an ideal post treatment to WWTP to reduce antibiotics, and most likely, also other micropollutants.

Keywords: antibiotics, antibiotic resistance genes, wastewater treatment plant, constructed wetlands, *Phragmites australis*, full-scale

4.1 Introduction

Antibiotics are widely used to treat and prevent diseases in humans and for livestock but has gained attention since antibiotic resistance is becoming a serious threat to global public health and the environment (Chee-Sanford et al., 2009; WHO, 2014). Approximately 30-70% of antibiotics are partially or not metabolized by the human body, and consequently, antibiotics enter wastewater treatment plant (WWTP) via the sewage system (Kümmerer, 2004; Bouki et al., 2013). The use of antibiotics and possibly also their presence in wastewater might promote the proliferation of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), which, as a consequence, can enter the environment.

WWTPs are designed to treat wastewater to meet quality requirements before being discharged into the environment, by reducing the concentration of chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen (TN) and total phosphate (TP). However, WWTPs are not sufficiently tailored to remove micropollutants such as pharmaceuticals, personal care products, and antibiotics (Margot et al., 2015). For example, azithromycin was removed less than 25% after WWTP treatment (Ghosh et al., 2016); however, in some cases, there was even an increase in antibiotic concentrations, for example, lincomycin was found increased by 11% after WWTP treatment (Behera et al., 2011). Watkinson et al. (2007) reported that WWTPs significantly reduced antibiotic concentrations, with an average removal percentage of 92% from the water phase. Radjenović et al. (2009) also demonstrated that pharmaceuticals and antibiotics were removed with an average of 20-90% by activated sludge. Even though these micropollutants are removed in a WWTP, they are still detected in the effluents in the range of ng/L to mg/L. Therefore, additional treatment is needed to ensure the high quality of wastewater effluent before it is released to the surface water.

A possible solution to remove antibiotics and ARGs is the addition of tertiary treatment steps to existing WWTPs. Tertiary treatments, such as advanced oxidation technologies (ozonation, UV irradiation) and membrane technologies, have shown to remove antibiotics and ARGs (Radjenović et al., 2009; Zheng et al., 2010; Zhang et al., 2016). However, such treatment processes have high installation, operation, and maintenance costs and require much energy (Hijosa-Valsero et al., 2011; Li et al., 2014). This is a drawback, especially for developing countries that are looking for an alternative to remove antibiotics and ARGs (Gruchlik et al., 2018), but also in developed countries where treatment plant managers want to reduce their ecological footprint. Hence, alternative sustainable technologies are desired for all economic contexts.

Constructed wetlands (CWs) are designed to imitate natural processes using plants and soil to treat wastewater in a controlled environment (Verhoeven et al., 2006). Compared to other tertiary treatments such as ozonation, CWs are affordable and sustainable, and therefore, CWs have been extensively investigated as a tertiary treatment (Kadlec and Wallace, 2009; Verlicchi and

Zambello, 2014). CWs have a high rate of biological activity compared with other ecosystems, and they have the potential to transform several common pollutants into harmless by-products (Fernandes, 2014). For example, CWs can reduce pollutants that are present in wastewater, such as organic matter, nutrients, and metals (Meers et al., 2005; Meers et al., 2008). Other than that, CWs also showed potential in removing micropollutants such as diclofenac, ibuprofen, caffeine enrofloxacin, and ceftiofur (Matamoros et al., 2008; Sochacki et al., 2018; Santos et al., 2019) and even micropollutants which are known as recalcitrant (i.e., carbamazepine) (Chen et al., 2018).

CWs have proven to be effective in reducing antibiotic concentrations at different scales, flow configurations, or plant types. For example, sulfamethoxazole and trimethoprim were removed 60 to 95% in various combinations of flow configuration in mesocosm systems planted with *Typha angustifolia* or *Phragmites australis* (Hijosa-Valsero et al., 2011). Oxytetracycline, ciprofloxacin, and sulfamethazine were significantly attenuated (68 to 95%) at the mesocosm scale in vertical flow wetlands planted with hybrid *Pennisetum* (Liu et al., 2013). In other studies with the same flow configuration and planted with *Phragmites australis,* oxytetracycline and difloxacin were removed more than 90%, and tetracycline resistance genes and integrase genes were removed up to 99% (Huang et al., 2015; Huang et al., 2017).

In addition, CWs have also demonstrated ARGs removal regardless of their scales, dependent upon the operating conditions, type of media, and the presence of plants (Vacca et al., 2005; Liu et al., 2013). In a study by Chen and Zhang (2013a), they reported that mesocosm-scale CWs removed 1−3 log of ARGs (*tetM*, *tetO*, *tetQ*, *tetW*, *sul1*, and *sul2*). Chen et al. (2015) found that the mesocosm-scale CWs could remove more than 50% of ARGs compared to 80% removal of various ARGs in an integrated CW.

The published studies on the removal capacities of CWs for antibiotics and ARGs at the mesocosm scale are limited, and there are only a few full scale studies on antibiotics and ARGs. Hence, data to measure the effect of attenuation of antibiotics and ARGs in full scale CW are needed, including a better understanding of the relation between retention time and removal efficiency. To our knowledge, no studies have compared CWs with different retention hydraulic times (HRTs) that receive wastewater from the same WWTP. Therefore, the objective of this study was to investigate the occurrence and removal of antibiotics and ARGs in two full scale CWs that have been in operation for 15 years. The investigations aimed for identifying temporal and spatial trends. In addition, correlations between antibiotics, ARGs and general water qualities were analyzed to determine the relation between generic water qualities with the distribution of antibiotics and ARGs in both CWs.

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4.2 Material and Methods

4.2.1 Sampling site and collection of samples

Sampling was performed at two different surface-flow CWs at Hapert WWTP (the Netherlands). This WWTP treats a mixture of domestic (78%) and industrial (22%) wastewater via a conventional system consisting of bar screens, grit removal, and an oxidation ditch. Effluent from the WWTP is channeled to two CWs, each with a different hydraulic retention time (HRT) located south and north of the WWTP, 1 day (HRT-1) and 3 days (HRT-3), respectively. The total surface area of the CW with HRT-1 is 7,010 m^3 and the size of the CW with HRT-3 is 17,820 m^3. The average water flow of the CWs is 718 m^3/hour during dry weather and 2543 m^3/hour during the raining season. The average CW water flow during our sampling is presented in Figure S4.1. The CWs have been serving as a WWTP post-treatment for 15 years. Each wetland consists of reed beds (*Phragmites australis* (*P. australis*)) and swamps (trees) (Figure 4.1). The depth of the reed beds is 133 cm, and the depth of the swamp is 25 cm. For seasonal comparison, the average air temperature during sampling was used to classify the season. The data of air temperature was extracted from The Royal Netherlands Meteorological Institute (KNMI, the Netherlands), respectively. Temperatures above 15ºC are classified as summer season, and below 15ºC are classified as winter season. Therefore, the summer months in this study are May, June, July, August and September, and the winter months October, November, December, January and February.

The influent of the CW (sampling point 1) was sampled only in the CW with HRT-1 since the effluent from the WWTP is from the same source (Figure 4.1). Within each CW, three sampling points were determined. Sampling point 2 represents 30% of the reed bed, sampling point 3 represents the influent of swamp, and sampling point 4 represents 30% of swamp. 30% indicated the travel time of the water in the reed bed or swamp. The effluent of each CW (sampling point 5) was taken at the effluent of the CW before the water entered the river part. Samples were taken from February 2016 until January 2017. Water (n=270) and sediment samples (n=270) were collected from 9 sampling points.

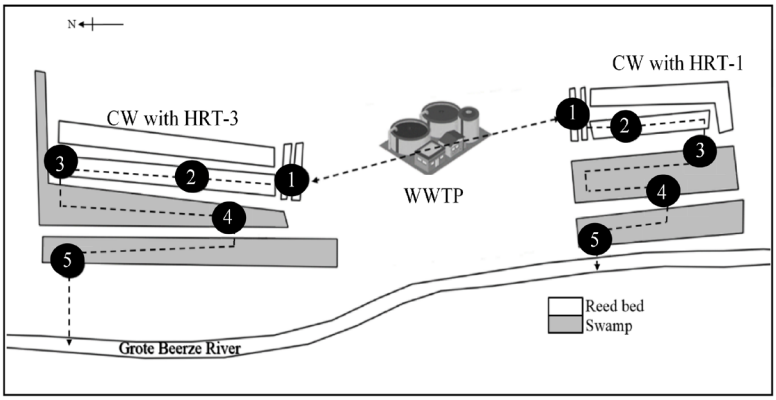


Figure 4.1: Sampling point for CW with HRT-1 and CW with HRT-3. 1 = influent of both CWs. 2 = 30% of reed bed. 3 = influent of swamp. 4 = 30% of swamp. 5 = effluent of the CW.

In total, triplicate samples were taken at five points in the CW with HRT-1 and four in the CW with HRT-3 and were transported back to the lab. Samples for chemical analyses and DNA isolation were stored at 4°C and processed within 24-48 hours after sampling. The water and sediment samples for antibiotic analysis were stored at -20°C until the samples were processed.

4.2.2 Physicochemical analysis

In the field, the pH, temperature, and dissolved oxygen (DO) were directly measured in the water using a pH and DO portable probe (Hach, USA). Groundwater samples were analyzed in the lab for chemical oxygen demand (COD), total phosphate (TP), ammonium (NH_4^+), nitrate (NO_3^-) using Hach kits (USA; LCK 1414, LCK 349, LCK 349 and LCK 304, respectively). All water samples were manually mixed by shaking the sample bottles before analyses.

4.2.3 Antibiotics analysis

All water and sediment samples were extracted and concentrated by solid phase extraction (SPE) before performing LC-MS/MS analyses. The first samples were measured in triplicate after which all samples were measured in a single measurement, as no significant difference was found in the triplicate (data not shown). Samples and standards were prepared as previously described (Sabri et al., 2020). For sediment samples, matrix-matched standard samples were prepared in blank pot soil and spiked with sulphonamides and trimethoprim (0-50 µg/kg) and tetracyclines, quinolones, and macrolides (0-200 µg/kg). Sediment samples (2 g) were weighed in 50 ml tubes in duplicate. 80 µl of a mix-standard solution was added to duplicate samples for standard addition quantification. 20 µl of internal standard (Jansen et al., 2017) (5000 µg/kg) was added to all samples. After 20 minutes, 4 ml of pure acetonitrile (with freshly added 0.125% of

trifluoroacetic acid) was added and shaken manually for 30 seconds, followed by the addition of 4 ml of McIIvain (0.1M; pH 4.0). All samples were casted on a tube rotator for 15 mins before adding 2 ml of lead acetate (200 g/L). The tubes were shaken vigorously before centrifuging for 10 mins at 3500 g. The supernatant was transferred in a glass tube, and 4 ml of acetonitrile was vaporised at 40ºC under nitrogen gas. After adding 13 ml of 0.2 M EDTA, the samples were ready for SPE purification as previously described by Sabri et al. (2020).

All samples were analysed by LC-MS/MS on 18 sulfonamides, trimethoprim, 17 macrolides, 12 quinolones, and 6 tetracyclines (Table S4.1). The details of the method have been described in our previous study (Sabri et al., 2020). Briefly, antibiotics were analysed using Acquity™ UPLC (Waters, USA) and AB Sciex QTrap 6500 (Applied Biosystem, USA) with electrospray ionization (ESI). The mobile phase was composed of eluent A (ammonium formate (1 M): formic acid: water (2V: 0.16V: 1000V) and eluent B (ammonium formate (1 M): formic acid: methanol (2V: 0.16V: 1000V)). The antibiotics separation was acquired by a Waters C18 column (100 mm × 2.1 mm, 1.7 μm) at a flow rate of 0.3 ml/min. The limit of quantification (LOQ) of the target compounds in water was 5 ng/L for sulfonamides, 500 ng/L for tetracyclines, 25 ng/L for quinolones, and 50 ng/L for macrolides. Meanwhile, the LOQ of the target compounds in sediment was 2.5 μg/kg for sulfonamides and 10 μg/kg for tetracyclines, quinolones and macrolides. For quality control, a known amount (100 μg/L) and internal standard of each compound was spiked to every sample. The recovery percentages of compounds spiked to the sample ranged from 70 to 120%.

4.2.4 DNA extraction and ARGs detection

Water samples were filtered by vacuum filtering through 0.2 μm membrane filters (isopore filters polycarbonate, 0.2 um, 47 mm, Merck Millipore, Ireland), and stored at -20ºC until extraction. Sediment samples were used directly in the DNA isolation process. Total genomic DNA was extracted from water filters using the PowerWater DNA Isolation Kit (MoBio Laboratories, USA) and from sediment using the PowerSoil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions. The extracted DNA was stored at -80°C until further analysis. Absolute quantification of genes was performed with quantitative PCR (qPCR) assays for the detection of 16S rRNA gene, the class 1 integrase gene (*intI1*) and four ARGs, including *sul1* and *sul2* (sulfonamide resistance genes), *tetW* (tetracycline resistance genes) and *ermB* (macrolide resistance gene), as previously described (Sabri et al., 2020). qPCR was carried out on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Canada) and was recorded by CFXManager (Biorad, version 3.0). The results were expressed as genes per ml for water and genes per gram dry weight (DW) after adjusting for the dry weight of the sediment.

4.2.5 Statistical analysis

The removal percentage was calculated by comparing mean concentrations of antibiotics (ng/L) in sampling point 1 (influent of the CW) and sampling point 5 (effluent of the CW), according to Equation 4.1.

$$Removal\ percentage\ = \left(\frac{Sampling\ no\ 1\ concenration}{Sampling\ no\ 5\ concentration}\right) \times 100\% \qquad \text{(eq 4.1)}$$

Statistical analyses were based on linear models, Pearson correlation and principal component analysis (PCA) on the R (Version 3.5.2). Antibiotic concentrations (ng/L and μg/kg) and ARGs abundance values (copies/ml and copies/g (DW)) were log-transformed prior to the linear model analysis. Statistical significance was determined at the 95% confidence level. The p-values for multiple testing were corrected using Bonferroni correction.

4.3 Results

4.3.1 General operational parameters

Measured concentrations of COD, DO, pH, water temperature, NH_4^+, NO_3^- and TP in all sampling points for the monitoring period are shown in Figure S4.2 – S4.8. The reduction of each parameter is presented in Figure S4.9. Temperature and precipitation on the sampling day are presented in Figure S4.10. Generally, the effluent quality of both CWs was in a similar range. However, there was more variation and fluctuation within the sampling points in the CW with HRT-1 compared to the CW with HRT-3 for COD and TP. Also, NH_4^+, NO_3^- and TP values increased with 300% in both CWs in November and December, compared to other months. The COD and nutrients were reduced in the reed bed and further reduced in the swamp part.

4.3.2 Occurrence and removal of antibiotics in the constructed wetlands

The performance of CWs in removing antibiotics in two HRTs was evaluated. LC-MS/MS analysis revealed that 14 out of 54 analyzed antibiotics were detected in the water samples. Figure 4.2a and 4.2b show the measured antibiotics in water during one year in both CWs. Figure S4.11a and S4.11b shows an enlarged segment of Figure 4.2a and 4.2b from May to November with the antibiotics oxytetracycline (OTC), sulfadiazine (SF), sulfadimidine (SDM), sulfadoxine (SFX), sulfamethoxazole (SMX), sulfapyridine (SP), trimethoprim (TRI), azithromycin (AZI), clarithromycin (CLA), lincomycin (LIN), tiamulin (TIA), tylosin (TYL), ciprofloxacin (CIP) and flumequine (FLU).

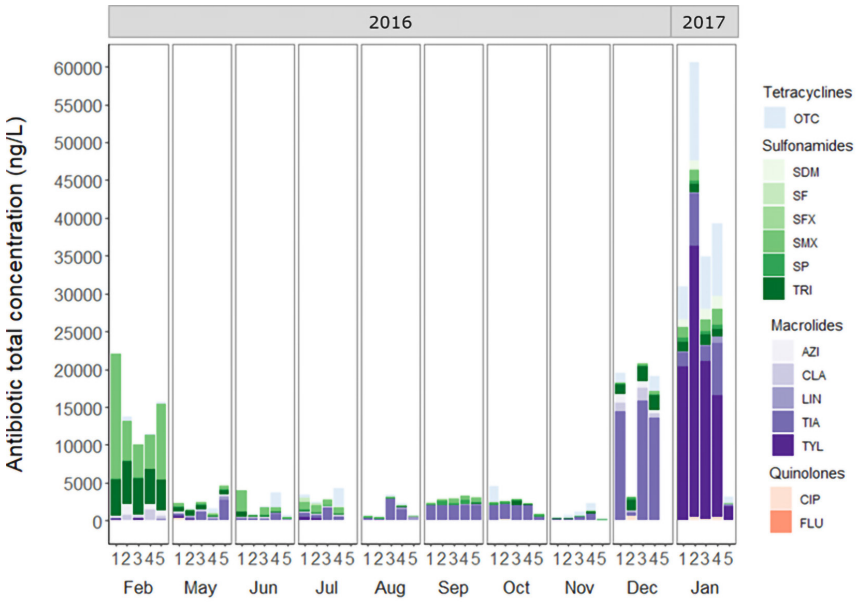
The total antibiotic concentrations in the influent of the CW per month was below 5000 ng/L except in February, December, and January. These three months showed higher concentrations than the rest of the months, with a total concentration up to 20000 ng/L. Concentration levels of the antibiotics ranged from 200 to 20000 ng/L in the influent of the CW, with tiamulin being the most abundant compound in the influent of the CW. After treatment by the CWs, the detected antibiotics decreased to only seven antibiotics, detected up to 16000 ng/L in the effluent of the CW, with again tiamulin as the most abundant compound. The overall removal percentage per month for the CW with HRT-1 was from -97% to 100%. Meanwhile, for the CW with HRT-3, the removal percentage was between -46% to 89%. The removal of each group of antibiotics in both CW effluents is illustrated in Figure S4.12. The performance of the CWs in reducing antibiotics after the reed bed or swamp part is presented in Figure S4.13. Macrolides and sulfonamides remained the same after the reed bed and swamp. Meanwhile, quinolones and tetracyclines showed a decreasing trend after the reed bed and the swamp.

Figure 4.3a and 4.3b show the antibiotics detected in the sediment. Among 54 antibiotics tested, 21 out of 54 antibiotics were detected; OTC, chlortetracycline (CTC), tetracycline (TET), doxycycline (DC), SF, sulfapyridine (SP), SDM, SFX, SMX, TRI, sulfadimethoxine (SMT), AZI, CLA, LIN, TIA, TYL, CIP, FLU, norfloxacin (NOR), enrofloxacin (ENRO), and levofloxacin (LEV).

The sum of the concentrations of antibiotics found per sample point was below 4000 μg/kg in each month. Concentration levels of the individual antibiotics ranged from 275 to 1220 μg/kg in the influent of the CW. In the sediments, mainly tetracyclines (OTC, CTC, TC, and DC) were detected. The total concentration of antibiotics in the sediment fluctuated within the CW, regardless of the CW with HRT-1 or the CW with HRT-3. The total concentration of antibiotics at sampling points 2, 3, and 4 were higher than the concentration in influent of the CW, whereas it decreased again at the effluent for both CWs. This trend is almost similar throughout the year. In the effluent of the CW, the number of detected antibiotics was decreased to only seven with concentrations ranging from <LOQ to 198 μg/kg. The removal percentage for the individual antibiotics was 86% to 100% for the CW with HRT-1 and 66% to 99% for the CW with HRT-3. The removal of each antibiotic group is illustrated in Figure S4.14.

The performance of the CWs in reducing antibiotics in the sediment after the reed bed and swamp part is presented in Figure S4.15. After the reed bed, quinolones and tetracyclines showed an increase followed by macrolides and sulfonamides. All antibiotics showed a decrease after the swamp. Lastly, antibiotics did not show a significant accumulation or reduction in the sampling points within the CWs (Table S4.2), except in the sediment (sampling point 2).

(a) CW with HRT-1



(b) CW with HRT-3

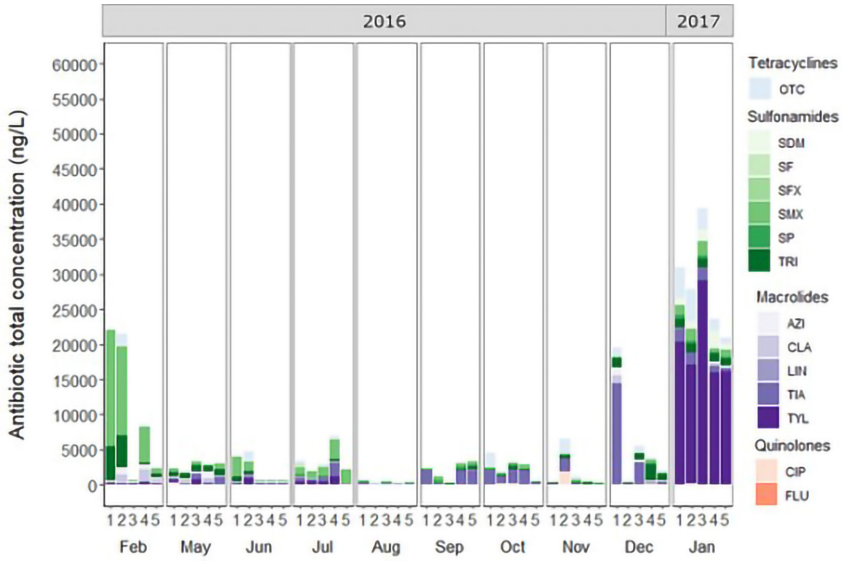
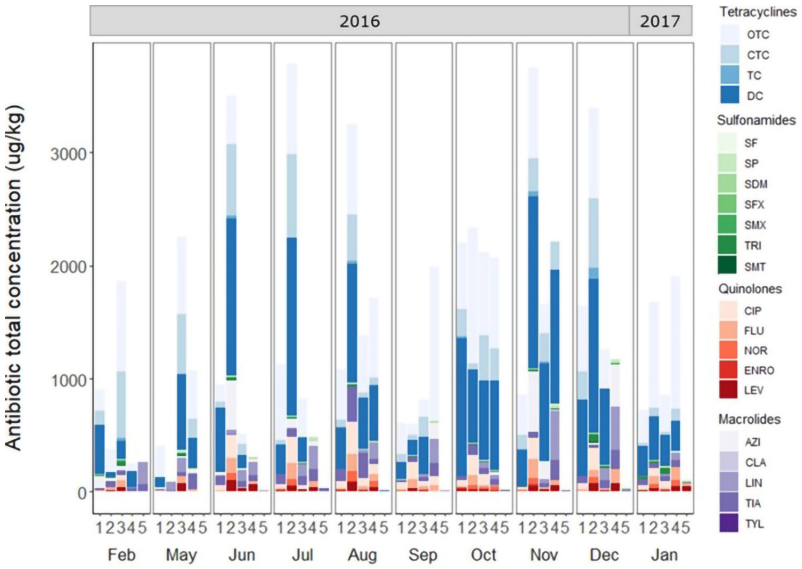


Figure 4.2: Concentrations of antibiotics (ng/L) in water in a CW with (a) HRT-1 and (b) HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

(a) CW with HRT-1



(b) CW with HRT-3

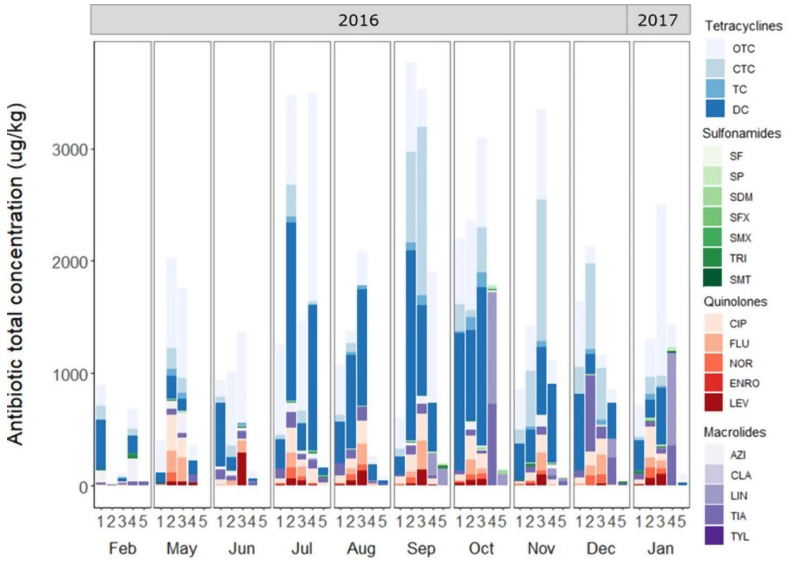


Figure 4.3: Concentrations of antibiotics (µg/kg) in sediment in a CW with (a) HRT-1 and (b) HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

4.3.3 Occurrence and removal of ARGs in the constructed wetlands

16S rRNA gene, integrase genes (*intI1*), and four ARGs (*ermB*, *sul1*, *sul2*, and *tetW*) were detected in both CWs in all water and sediment samples. Figure 4.4a and 4.4b show the gene abundance for sul1. The other genes in water are presented in Figure S4.16(a-e) and in sediment are presented in Figure S4.17(a-e).

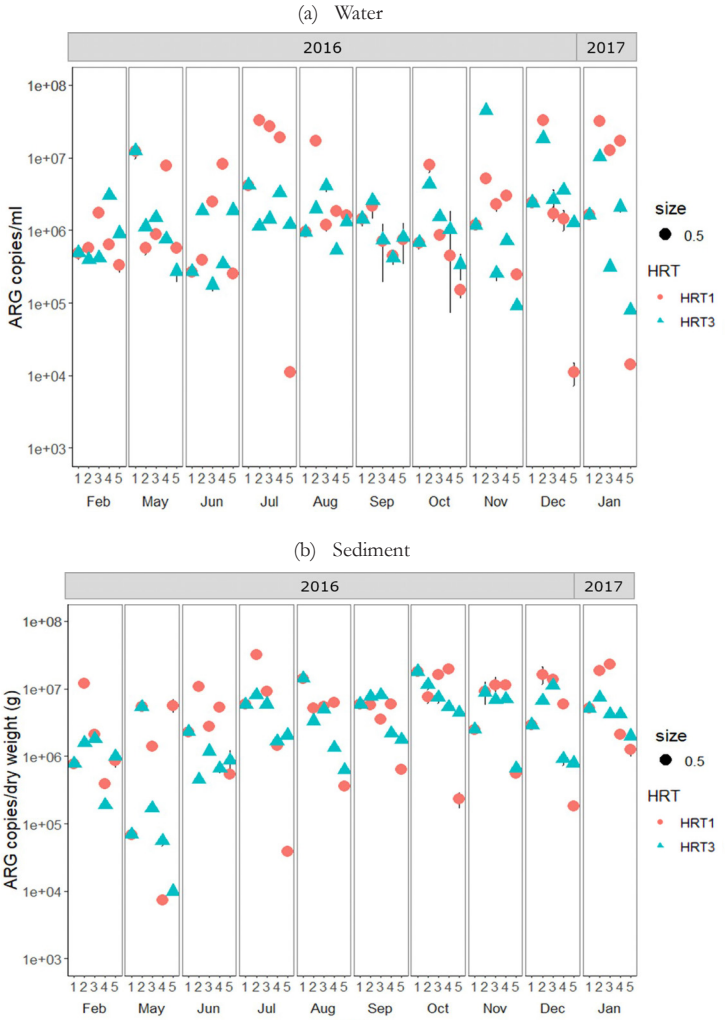


Figure 4.4: Concentrations of *sul1* detected in the CWs for one year in (a) water and (b) sediment. Numbers 1, 2, 3, 4, 5 refer to sampling points.

At the influent of the CWs, the concentrations of ARGs in water varied from 4.9×10^2 copies/ml to 1.4×10^7 copies/ml. The concentration of ARGs ranged from 1×10^1 copies/ml to 2.4×10^8 copies/ml in the CW with HRT-1 and ranged from 4.2×10^1 copies/ml to 9.5×10^7 copies/ml in the CW with HRT-3. *intI1* was the most abundant in both CWs, followed by *sul1*, *sul2*, *tetW*, and *ermB*. Higher variability of ARGs was observed in the water samples compared to sediment samples.

Meanwhile, at the sampling no 1 of the CW, the concentrations of ARGs in sediment, varied from 5.5×10^3 copies/ml to 4.2×10^7 copies/g DW. The concentration of ARGs ranged from 1.2×10^2 copies/g DW to 4.9×10^7 copies/g DW in the CW with HRT-1 and ranged from 1.2×10^2 copies/g DW to 4.2×10^7 copies/g DW in the CW with HRT-3. *intI1* was the most abundant gene in both CWs, followed by *sul1*, *sul2*, *tetW*, and *ermB*. All four ARGs were significantly reduced in the CWs by 0.2–3 orders of magnitude ($p<0.05$).

Nevertheless, concentrations within the CWs varied. For example, at the CW with HRT-1 in February, *sul1* showed no significant difference from the sampling point 1 to sampling point 2, slightly increased at sampling point 3, then decreased at sampling point 4 and 5. Meanwhile, in June, *sul1* increased up to 1 log at sampling point 2, decreased at sampling point 3 and increased again at sampling point 4 and effluent of the CW with HRT-1.

The performance of the CWs in removing ARGs is presented in Figure 4.5. The overall removal of the CW with HRT-1 (water) was 0.84 log and the overall removal of the CW with HRT-3 (water) 0.53 log. Meanwhile, the overall removal of the CW with HRT-1 (sediment) was 0.8 log, and the overall removal of the CW with HRT-3 (sediment) was 0.5 log. However, relative abundances (ARGs/16S rRNA gene) of *ermB*, *sul1*, *sul2*, and *tetW* were not significantly lower in the effluent of the CWs compared to in the influent of the CWs (Figure S4.18).

The performance of the CWs after the reed bed (i.e., sampling point 1 until sampling point 3) and swamp (sampling point 3 until sampling point 5) for both water and sediment are presented in Figure S4.19 (CW with HRT-1) and Figure S4.20 (CW with HRT-3). After the reed bed, ARGs showed a decrease (e*rmB*), remained similar (*sul1, intI1, tetW*), or increased (*sul2*), with the exception of *sul2* (no changes) and *tetW* (decrease) in the CW with HRT-3. Meanwhile, in the sediment samples, ARGs remained the same or increased (*ermB, sul1, sul2, intI1*) or decreased (*tetW*) after the reed bed in the CW with HRT-1, with the exception of decreased *ermB* in the CW with HRT-3. Lastly, similar to antibiotics, ARGs did not show a significant accumulation or reduction in the sampling points within the CWs (Table S4.3), except at the sampling point 2 in the sediment.

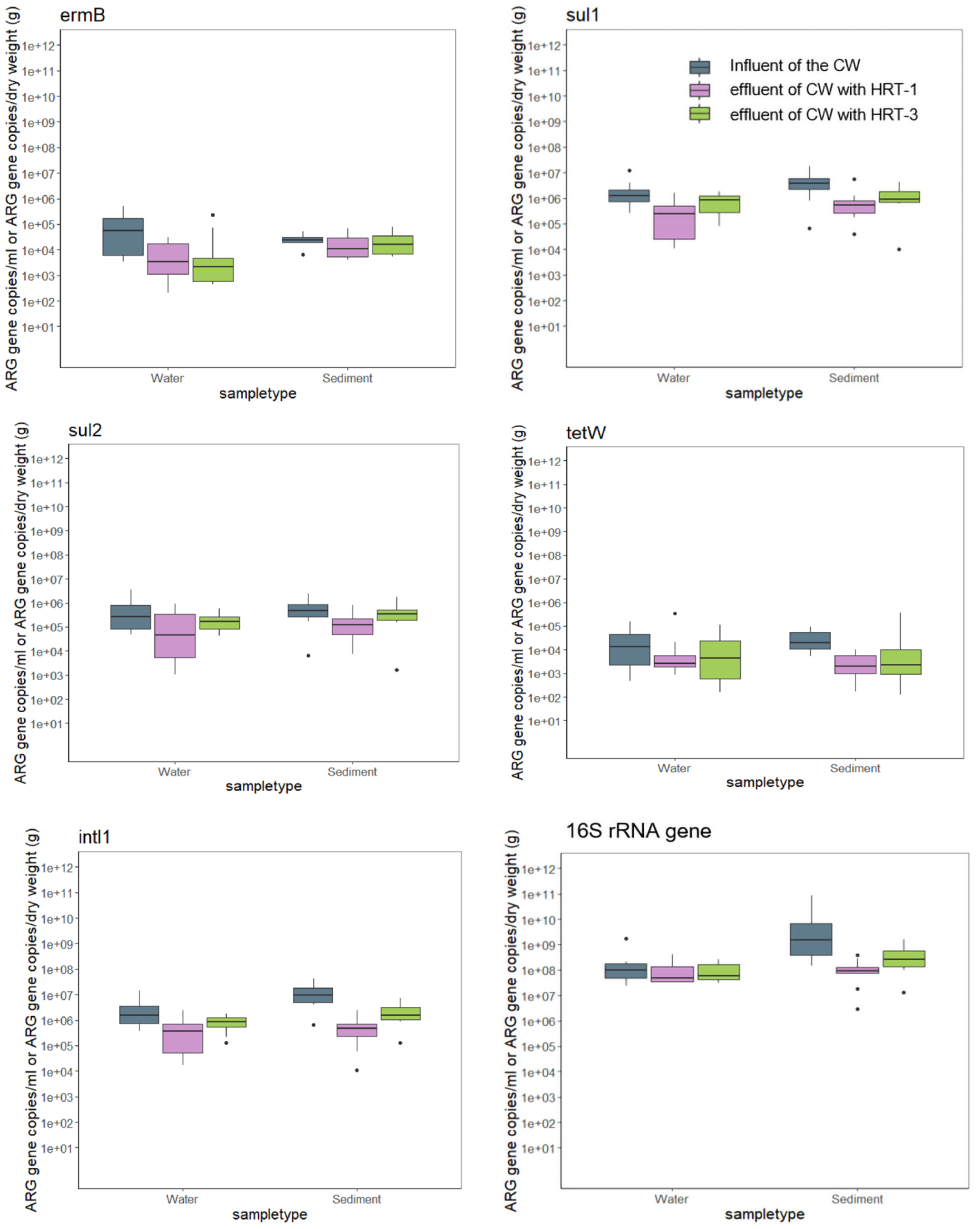


Figure 4.5: Gene abundance for all ARGs in water and sediment samples measured in the constructed wetlands for one year.

4.3.4 Temporal distribution of antibiotics and ARGs

Temporal variations of antibiotics and ARGs in water and sediment are shown in Figure S4.21 and Figure S4.22, respectively. Some antibiotics and ARGs showed an increase in winter compared to summer. All antibiotics showed significant temporal variation ($p<0.05$) in water samples. Meanwhile, insignificant differences were found for antibiotic concentrations in sediment samples during summer and winter, except for sulfonamides ($p<0.05$). Among the ARGs, *ermB*, *sul2* and *tetW* increased significantly in winter ($p<0.05$) in water and sediment samples, whereas *sul1* demonstrated a significant temporal variation only for the sediment. In addition, *intI1* was not affected by the season throughout the year of study.

4.3.5 Correlation between antibiotics, ARGs and general water qualities

The antibiotics, ARGs and general water qualities at different sampling points within the CWs demonstrated a broader variation in winter compared to summer, as shown in an ordination of antibiotics, ARGs, and general water qualities (Figure 4.6).

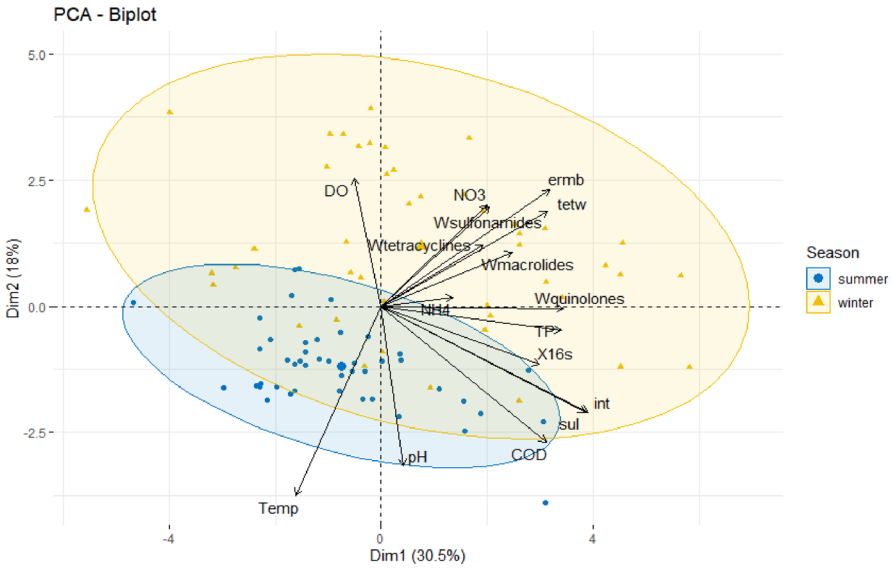


Figure 4.6: Principal component analysis of antibiotics (log-transformed), ARGs (log-transformed), and general water qualities in water in both CWs. The first two PCs explain 50.2 % of the variations of total antibiotics, ARGs abundance, and general water qualities. PC1 explains 32.5 % of the variation.

Furthermore, our study shows that significant correlations exist between the abundances of antibiotics and ARGs (Figure 4.6). The correlation between antibiotics and ARGs are presented in Table S4.4. The total concentration of antibiotics (macrolides, sulfonamides, tetracyclines and quinolones) were correlated to their corresponding and non-corresponding genes ($p<0.05$), with a stronger correlation between antibiotics and non-corresponding ARGs. Second, correlations between antibiotics, ARGs and general water qualities were revealed. A

significant temporal variation among different general water qualities was observed. The total concentration of all antibiotic groups (except sulfonamides) was significantly associated with concentrations of nutrients, such as NO_3-N, NH_4-N, COD, and TP ($p<0.05$), and the positive or negative orientation of the correlation depends on the type of nutrient (Table S4.5). The ARGs (*sul1*, *sul2,* and *intI1*) were most strongly associated with TP, followed by COD ($p<0.001$) (Table S4.6). Finally, temperature correlated negatively to antibiotics and ARGs, i.e. indicating higher temperatures made both less persistent.

4.4 Discussion

4.4.1 General performance of CWs

The two studied CWs with a different HRT showed no significant difference in overall performance, and both showed high variability in nutrient removal per compound. Both CWs removed COD and nutrients up to 70%, showing that the CWs performed well to meet the EU effluent requirement by (Council Directive, 1991). Van de Moortel et al. (2010) also reported average removal efficiencies of their CW for NH_4-N, total nitrogen, TP, and COD in the range of 22% - 53%.

Regardless of the HRT, the COD concentration at the effluent of the CW with HRT-1 and the CW with HRT-3 was in a similar range. This indicated that the retention time in the CW with HRT-1 appeared to be sufficient for nutrient removal, and that additional retention time had no added value to improve the performance of the CW. The DO level also affected by the degradation of excessive organic matters, and resulting DO level was lower in summer compared to winter (Zhou et al., 2019).

The removal of nutrients in the CW is brought about by ammonification, nitrification, denitrification, plant uptake, volatilization, biomass assimilation (Vymazal, 2002), and substrate adsorption (Santos et al., 2019). In our study, the best performance of the CWs was observed during the summer. This can be due to the elevated temperature enhancing microbial activity resulting in better removal. The nitrate loss rates were positively and significantly correlated ($p<0.05$) with water temperature and negatively with DO, and therefore, it is highly seasonal, generally peaking in the summer months (June–August) (Beutel et al., 2009). Other than that, *P. australis* has also been shown to be able to provide more oxygen and remove more nitrogen due to the larger root mass and deeper root growth compared to other plant species (Liu et al., 2011).

A few events of relatively high nutrient concentration were observed. For example, an NH_4-N peak in November. Apart from lower biological activity within the CW compared to the summer months, this higher concentration can be related to rain events (Di Cesare et al., 2017). The same observation was recorded by (Van de Moortel et al., 2010). This may be due to wash-off plant materials from the reed beds that might contain nutrients, or the presence of

ammonium in the rainwater (Vieira et al., 2013). Data in KNMI shows that there was rainfall (4-6 mm/day) before and on the sampling day in November (Figure S4.10). Occasionally, effluent NH_4-N concentrations are exceeding influent of the CW values, attributed to the complexities of nitrogen detention times in wetlands (O'Luanaigh et al., 2010).

Other than that, shorter HRT generates higher redox potentials and leads to more significant nutrient removal. In longer HRT, the oxygenation is low due to the decreased oxygen diffusion into the CWs (Armengol, 2015). That explains better DO in the CW with HRT-1 ($p<0.05$) even though we did not see any significant difference between the HRTs for the other parameters.

4.4.2 Removal of antibiotics in CWs

The performance of two CWs receiving wastewater from the same WWTP was evaluated during one year. Both CWs removed antibiotics significantly, and the two different HRTs showed a comparable overall removal. This indicates that the CWs can attenuate these compounds, as demonstrated before (Dordio et al., 2010; Hijosa-Valsero et al., 2011; Zhang et al., 2016). However, in some cases, either at certain months or some locations within the CW, negative removal of specific antibiotics were observed. This might indicate either the release of antibiotics sorbed onto the sediments or the presence of deconjugates in the influent of the CW interfering with the biological transformation of the deconjugated compounds, i.e. resulting in an apparent production of the compound in the wetland (Verlicchi and Zambello, 2014).

When looking at the antibiotic concentrations in the water, and the removal within the CWs, the total concentration of antibiotics fluctuated at the different sampling points for both CWs. This may be explained by the different processes, such as biological and chemical processes occurring within the CWs. Biodegradation, plant uptake, and substrate adsorption are biological processes that all contribute to the total removal of antibiotics (Krzeminski et al., 2019). Plants in CWs are essential to assuring the substrate's hydraulic conductivity, contribute to the uptake of nutrients, and promote microbial assemblages within their roots (Webb et al., 2012). Among these processes, biodegradation might act as the main removal process in the CWs (Chen et al., 2016). In this study, we did not distinguish to which content the individual removal processes contributed to the total removal of antibiotics.

Other than that, the biological reaction rate is positively associated with higher temperatures, and enzyme-catalyzed reactions are most active within the range of optimal temperatures (Bruce and Perry, 2001). Sunlight photodegradation and temperature can also be involved in the removal of antibiotics in the CWs under open water spaces (Kim and Carlson, 2007; Choi et al., 2016; Jiang et al., 2018). Data from KNMI (Royal Netherlands Meteorological Institute) showed that solar irradiation is less than 500 Joule/cm^2 (average 1

hour) in winter compared to 7000 Joule/cm2 (average 10 hours) in summer, thus confirming less photobiodegradation potential in winter. As photodegradation rates are different per antibiotic, differences in removal were observed. For example, sulfathiazole degrades relatively quickly, followed by sulfisoxazole, sulfamethizole, and SMX (Boreen et al., 2004), whereas TRI was not susceptible to photodegradation (Nguyen Dang Giang et al., 2015). We also observed that concentration of SMX and TRI remained almost the same within the CW, in February (less photodegradation), and in August (most photodegradation). That explains lower antibiotics concentration in warmer months as we also can observe less total antibiotics were measured (average less than 1000 ng/L) in August. Another explanation for different concentration concentrations in summer compared to winter months is variation in antibiotics use over the year. Hence, the combination of slow photodegradation rates, a shorter time of sunlight in winter (average of 1 hour) in winter, and high consumption of the antibiotics most likely contribute to a high concentration of sulfonamides in February.

Surprisingly, macrolides were detected at high concentrations in the water phase, especially in January. Tylosin, which was found at the highest concentrations, is a veterinary antibiotic. These compounds are used for treatment and prophylaxis of dysentery, pneumonia, and mycoplasmal infections in pigs and poultry (Lewicki, 2006; Islam et al., 2009). As manure application is prohibited between October and February, surface run-off from agricultural fields is not likely in winter months. These findings might be related to farming cleaning activities, resulting in the release of manure into the sewer system.

In the sediments, we observed a more stable trend of the total antibiotic concentrations throughout the year. However, within the CW, the total concentration of antibiotics fluctuated, especially at the sampling point 2. This can be explained by the difference in biological processes within the wetland as described before, but also relates to the properties of the antibiotics and sediment. Sediment properties such as organic carbon content, pH, ionic strength, clay content, and texture affect the extent of adsorption of organic compounds, such as antibiotics (Rabølle and Spliid, 2000), thus reducing the mobility, reactivity, bioavailability for microbial degradation and their presence in the water phase (Liu et al., 2013; Almeida et al., 2016; Choi et al., 2016; Pan and Chu, 2016).

Our data showed that sulfonamides were mostly found in the water phase, whereas tetracyclines were mostly found in the sediment phase. This is in accordance to the nature of the chemicals, reflected by the water distribution coefficient (K_d) and organic carbon normalized partition coefficient (K_{oc}) of these compounds (Tolls, 2001). Hence, the sulfonamides are hardly adsorbed to sediment and are usually detected in surface water, groundwater, and drinking water (Wegst-Uhrich et al., 2014). The presence of lower concentration sulfonamides in the sediment suggests that degradation in the water phase may play a more dominant role than substrate adsorption and plant uptake, like has been demonstrated by Chen et al. (2016). For example, SMX is expected to have high mobility based on the estimated K_{oc} of 72 (National Center for

Biotechnology Information, 2020c). In contrast, tetracyclines and quinolones have higher K_{oc} tend to adsorb in the sediments due to their ability to form complexes with doubly charged cations (Tolls, 2001; Kümmerer, 2009). K_{oc} of oxytetracycline is ranging from 195 to 93317, and ciprofloxacin is 6100, are expected to have moderate to no mobility in the sediment (National Center for Biotechnology Information, 2020b, 2020a). Similar observations have been reported by previous research, who showed a low concentration of sulfonamides (8.67 μg/kg) in the sludge compared to macrolides (438.85 μg/kg) (Hu et al., 2018).

As a result, sediment acts as a potential sink, as demonstrated by our data and in line with prior studies. Kerrigan et al. (2018) reported that the history of ten types of antibiotics in sediment cores for 100 years is increased rapidly since the 1950s with an accumulation rate of up to 20.5 ng cm^{-2}/year. Tetracyclines were detected up to 2.08×10^3 μg/kg in sediment samples in the China river (Jia et al., 2018). Meanwhile, Li et al. (2019) observed that the concentration of 60 types of antibiotics in the sediment of different wetlands was up to 118 ng/g.

In addition, we also observed that the concentration of the antibiotics indicated accumulation after the reed bed compared to the swamp. This is maybe due to the difference of the sediment materials, loamy soil (after the reed bed), and sandy loam soil (after the swamp/effluent of the CW). The availability and mobility of antibiotics in the sediment depend on conditions that prevail in the soil, such as soil type, pH and temperature (Sarmah et al., 2006). We did not further study the effect of the sediment type however, this merits more research in the future to understand the role of different sediment/soil type in ARGs removal.

4.4.3 Removal of ARGs in CWs

In this study, we found that the analyzed genes, 16S rRNA gene, *intI1*, and 4 ARGs were reduced by 0.2 to 3 log in the water phase from the influent of the CW to the effluent of the CW, even though some of ARGs were increased in aqueous concentrations in the influent of the CW just for a few months. Like antibiotics, a variety of mechanisms is involved in ARGs removal in the CWs, such as biological, physical and chemical processes (Vacca et al., 2005; Dordio et al., 2010). The biological processes include plant uptake, and die-off of bacterial hosts (Diehl and Lapara, 2010). The presence of plants in the CW also can remove a higher number of bacteria (García García and Becares, 1997; Vacca et al., 2005; García et al., 2008; Sidrach-Cardona and Bécares, 2013). The physical processes include sorption to sediment or organic matter, mechanical filtration, or sedimentation. Of these mechanisms, biological processes such as plant uptake and sorption to organic material could be the main mechanisms for ARGs elimination (Toet et al., 2005; Chen et al., 2016).

The removal efficiency of ARGs we found in our study is in agreement with previous research. Chen and Zhang (2013a) reported that mesocosm-scale CWs remove 1−3 log removal of ARGs (*tetM, tetO, tetQ, tetW, sul1,* and *sul2*). Chen et al. (2019) also reported approximately 1

log removal of various ARGs in different combinations of hybrid CWs. Liu et al. (2013) showed a reduction of 0.5 to 1 log genes (*tetW*, *tetM*, and *tetO*), depending on the type of wetland media. Chen et al. (2015) found that the mesocosm-scale CWs could remove more than 0.5 log of ARGs with an even better reduction of relative abundance. In contrast, other studies report no removal of ARGs by CW treatment and suggest that the bacterial population in the CW may have genes that posses antibiotic resistance (Anderson et al., 2013).

The ARGs abundance in both CWs suggests that a CW can act as sinks for ARGs. ARGs still can persist in sediments when associated with organic substances and clay particles. Sediments adsorb DNAses that hydrolyze free DNA, including ARGs, and as a result, the sediment contains more ARB and ARGs (Luo et al., 2010). In addition, the source of the extracellular ARGs can be derived from the secretion from live cells or the lysis of dead cells and also deposits in the sediments (Pietramellara et al., 2009). Mao et al. (2014) demonstrated the high persistence and concentration of ARGs in river sediments than the river water. They also reported that indigenous bacteria able to assimilate with the present extracellular ARGs in the river sediments.

We observed high concentrations of *intI1* and *sul1* in the water and sediment. *sul1* and *intI1* (r=0.92) and *sul2* and *intI1* (r= 0.89) were strongly correlated ($p < 0.001$) in water. A similar observation was seen in the sediment, for *sul1* and *intI1* (r= 0.67, $p<0.001$) and *sul2* and *intI1* (r= 0.60, $p<0.001$). *Sul1* was measured at relatively high concentrations (10^6-10^7) compared to other ARGs in other studies CW (Anderson et al., 2013; Chen et al., 2015). The relatively high concentration of the genes in the CW can be explained by the continuous input of the genes into the CW and resulted in the low variability of ARGs in sediment. As a result, sediments may act as a reservoir where the inflowing ARGs are immobilized and maintained. At the same time, new ARGs may emerge, spread, and new and stored ARGs may be mobilized by resuspension depending on the flow and weather conditions (Heß et al., 2018).

Apart from that, ARGs removal efficiency is also related to operating conditions such as HRT. Similar to the antibiotics, a different HRT did not affect the removal of the ARGs ($p > 0.05$). Although a longer HRT was expected to have a positive effect on the removal of ARGs, this was not shown in our study. CW with HRT-3 may contribute to higher flux of all substrates and promote the adsorption and degradation of antibiotics or ARGs (Huang et al., 2017). This might relate to the unknown history of both CW streets, differences in organic material, and complex biological activity between the CWs, which can not only be explained by the difference in HRT.

Even though the concentration for some ARGs in the effluent of the CWs was significantly higher than in the surface water of river Beerze that received the treated wastewater after the passage of the CW as we showed in our previous work (Sabri et al., 2020) and other work (Choi et al., 2016), CW improved the removal efficiency of ARGs compared to treatment

by conventional WWTPs only (Chen and Zhang, 2013b; Zhou et al., 2013). This shows that CW can be an effective additional treatment in removing antibiotics and ARGs from domestic sewage treatment plant effluents.

4.4.4 Temporal and spatial distributions of antibiotics and ARGs

We observed a significantly higher concentration of antibiotics in winter in water compared to sediment. this higher concentration in the water is likely related to the higher consumption of antibiotics by humans and excretion of antibiotics in winter. In addition, antibiotics could persist for a longer time duri ng lower temperatures in winter (Yang et al., 2011). Furthermore, adsorption of antibiotics in the sediment may be an important mechanism of antibiotics removal from water, as shown by the significant occurrence of antibiotics in the sediment during winter and summer in our study. This has also been demonstrated by other water-sediment investigations (Liu et al., 2019).

The ARGs *ermB*, *sul2* and *tetW* showed increased concentrations (up to 1 log) in winter ($p<0.05$) in water and sediment. The higher concentration of ARGs in winter can be due to a higher concentration of antibiotics, as demonstrated by their correlation in this study, and others (Caucci et al., 2016). However, other studies reported that most of the ARGs in river sediments were higher in summer than in winter, especially *sul1* (Guo et al., 2018). The findings of lower ARG's in water in summer in this study may be related to elevated temperatures, providing a suitable condition for microorganisms and predators to proliferate and degrade ARGs and antibiotics.

In general, both CWs showed a reduction in antibiotics from sampling point 1 to sampling point 5. Both antibiotics and ARGs did not show significant upwards or downwards fluctuations in the sampling points within the CWs except for sampling point 2 in the sediment. Sampling point 2 is located in the reedbed, which indicates that the reedbed area is a potential area to accumulate antibiotics and ARGs, through particle sedimentation and adsorption to the sediment matrix. The plants and soil types in the reedbed play a role in this, as a higher concentration of antibiotics accumulated in the soil than in the media and vegetation as also observed by others (Liu et al., 2013).

4.4.5 Relationship between antibiotics, ARGs and general water qualities

A low concentration of antibiotics may promote antibiotic resistance and can therefore indirectly correlate with their corresponding ARGs (Bouki et al., 2013). In this study, we found correlation between the total concentration of antibiotics (sulfonamides, macrolides, quinolones, tetracyclines) and their corresponding or non-corresponding ARGs (*sul1*, *sul2*, *ermB, tetW*). A stronger correlation between the concentration of antibiotics and non-corresponding ARGs was found, compared to antibiotics and their corresponding ARGs. However, such a

generic correlation is inconsistent with previous studies: i.e. Gao et al. (2012) observed a negative correlation between tetracyclines and tet genes but a significant correlation for sulfonamides and sul genes. No correlation was found between antibiotics and their corresponding genes once the wastewater reached the surface water (Guo et al., 2018; Yang et al., 2019). Therefore, our results suggest that the distribution of antibiotics, and their corresponding ARGs over the aquatic environmental compartments depends on many factors, such as their co-release with feces, and most importantly, differences in their fate. This can lead to a lack of correlation in surface waters, in contrast to correlations in sediments.

In this study, nutrients (NO_3-N, NH_4-N, TP and COD) correlated positively and temperature negatively with the occurrence of the antibiotic groups (except sulfonamides). Furthermore, TP and COD positively correlated to ARGs. These findings are in line with previous studies that observed a similar relationship between the concentration of antibiotics or ARGs with some of the general water qualities (Novo and Manaia, 2010; Nõlvak et al., 2013; Zhu et al., 2020). These results support the hypothesis that (at least a part of) the nutrients undergo similar changes as antibiotics and ARGs along the CWs. In this sense, improvements in general water qualities by WWTP or CW can go hand in hand with removal of antibiotics and ARGs (Yuan et al., 2018).

4.5 Conclusion

The present study evaluated the performance of two full scale CWs, receiving the same wastewater for the removal of antibiotics and ARGs from wastewater. The results of this study demonstrate that the CWs reduced the concentration of targeted antibiotics and ARGs from wastewater. Antibiotics were removed from 28% to 100% in both CWs, depending on the type of antibiotics. Macrolides were most abundantly found in the effluent of the CWs, with strongly fluctuating concentrations in time. ARGs were removed 1 to 3 log by the CWs, with *intI1* as most abundantly found in the effluent of the CWs. Both sul genes were strongly correlated to *intI1*. The antibiotics removal may be attributed to microbial degradation, substrate adsorption and plant uptake. ARGs removal was more complex and variable, as ARGs mass and composition also depend on the biological activities in the CWs.

Results showed that different HRTs (1 day or 3 days) of the CWs did not affect the removal of the antibiotics or ARGs. A higher concentration of antibiotics and ARGs were found during winter compared to summer. No significant accumulation of antibiotics and ARGs was found within the CWs. The total concentration of antibiotics (sulfonamides, macrolides, tetracyclines) correlated to the concentration of ARGs (*sul1*, *sul2*, *ermB*, and *tetW*). Our results also show a relationship between concentrations of antibiotics and ARGs with general water qualities, antibiotics correlated with NO_3-N, NH_4-N, and TP, and ARGs correlated with TP and COD.

In general, CWs are easy to operate and to maintain, show a good removal of antibiotics and moderate removal of ARGs, making CWs an ideal post treatment to WWTP to reduce antibiotics, and most likely also other micropollutants. CWs reduce the input of micropollutants into the environment and prevent the spreading of antibiotic resistance. However, CWs can accumulate antibiotics and ARGs in the sediment. A better understanding of the elimination processes of ARGs within a CW might result in adjusted designs for CWs, leading to higher removal efficiencies for antibiotics and ARGs in the future.

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Zhou, L. J., G. G. Ying, S. Liu, R. Q. Zhang, H. J. Lai, Z. F. Chen, and C. G. Pan. 2013. Excretion masses and environmental occurrence of antibiotics in typical swine and dairy cattle farms in China. *Science of The Total Environment* 444 (Supplement C): 183-195. https://doi.org/10.1016/j.scitotenv.2012.11.087.

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Zhu, N., H. Jin, X. Ye, W. Liu, D. Li, G. M. Shah, and Y. Zhu. 2020. Fate and driving factors of antibiotic resistance genes in an integrated swine wastewater treatment system: From wastewater to soil. *Science of The Total Environment* 721: 137654. https://doi.org/10.1016/j.scitotenv.2020.137654.

Supplementary Information

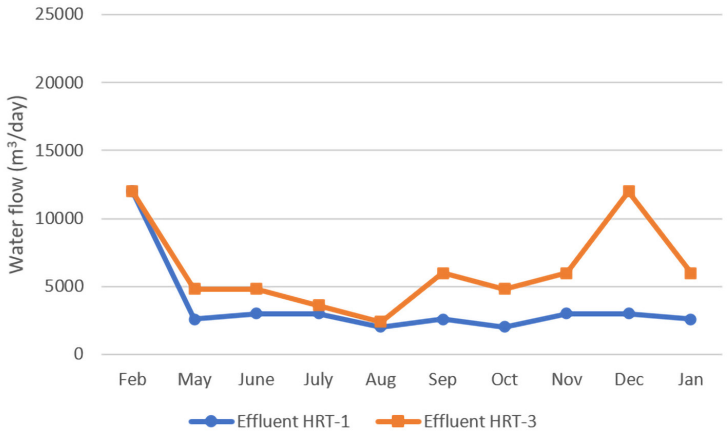


Figure S4.1: Flow rate profile of effluent of the HRT-1 and HRT-3 for one year sampling.

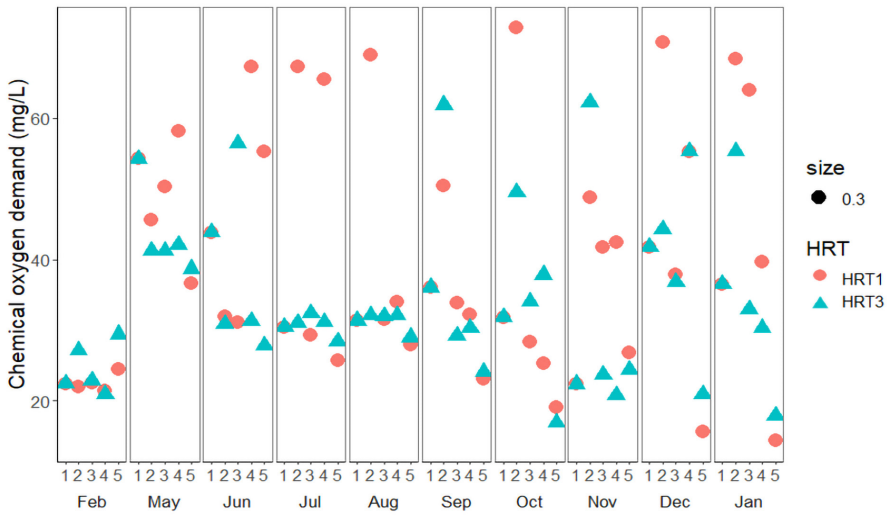


Figure S4.2: COD profile of CW with HRT-1 and HRT-3. 1 = influent of both CWs. 2 = 30% of reed bed. 3 = influent of the swamp. 4 = 30% of swamp. 5= effluent of the CW.

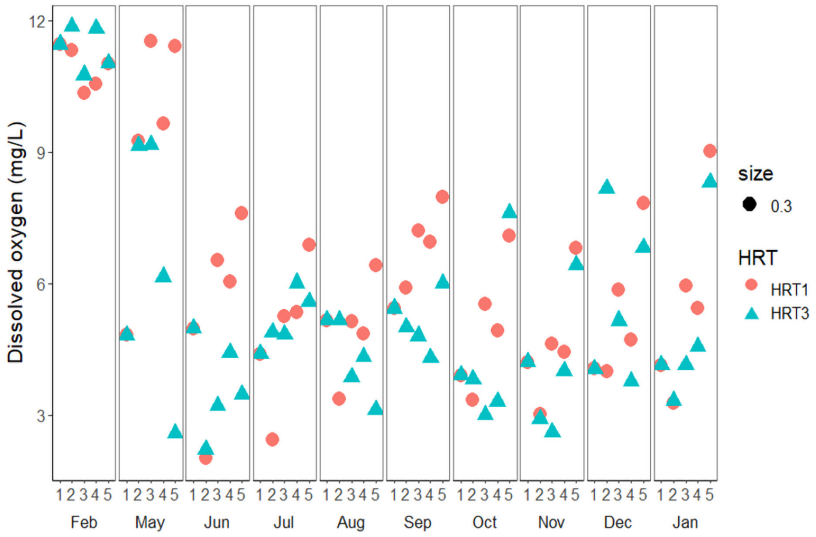


Figure S4.3: DO profile of CW with HRT-1 and HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

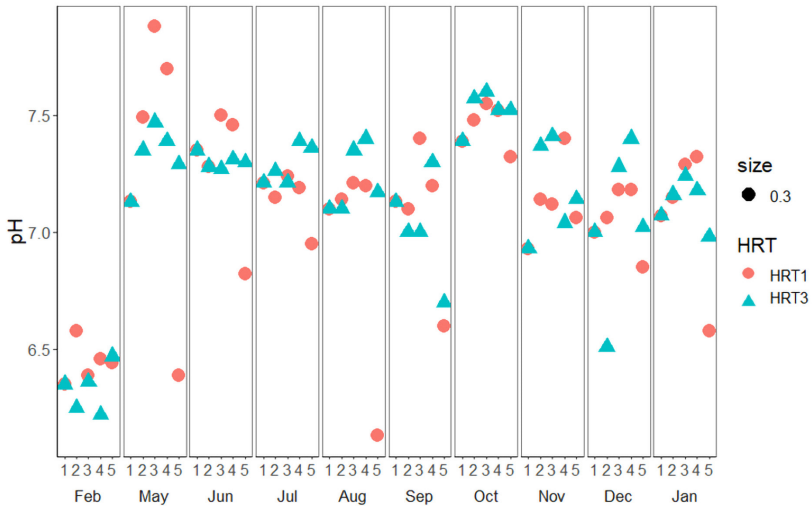


Figure S4.4: pH profile of CW with HRT-1 and HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

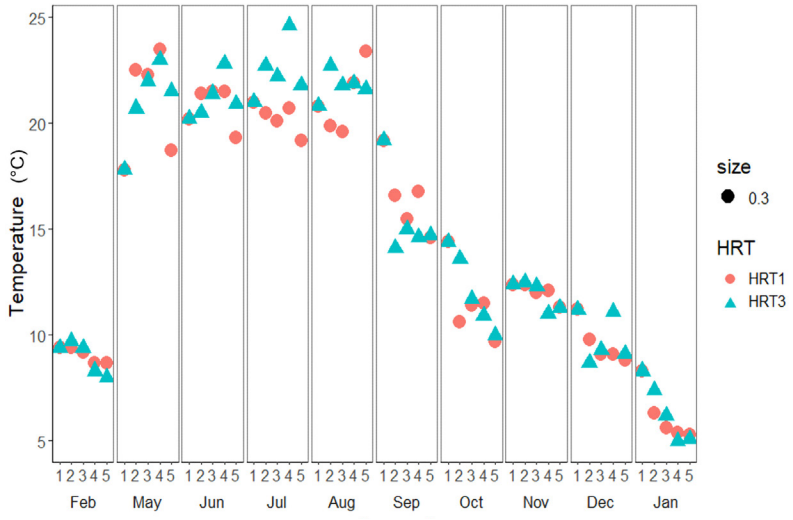


Figure S4.5: Water temperature profile of CW with HRT-1 and HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

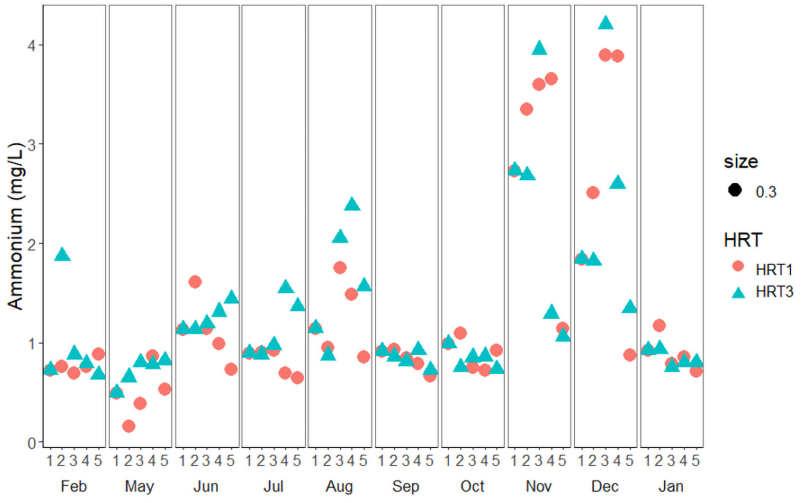


Figure S4.6: NH_4-N profile of CW with HRT-1 and HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

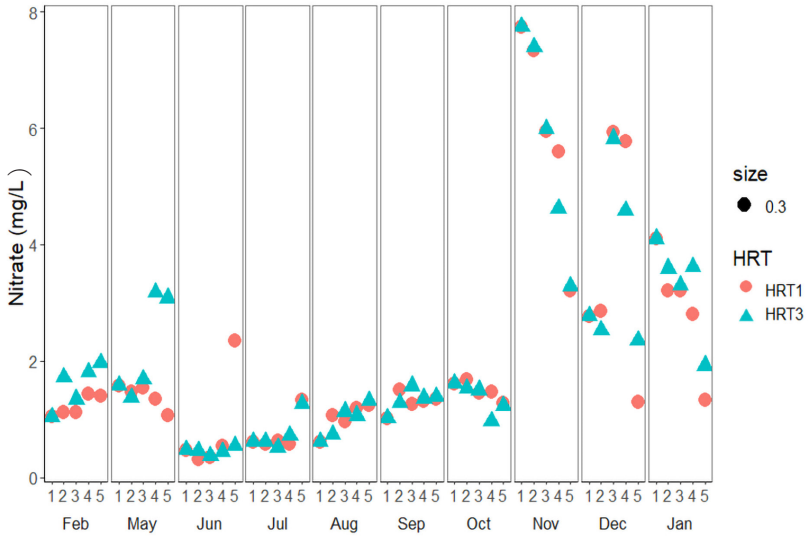


Figure S4.7: NO_3-N profile of CW with HRT-1 and HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

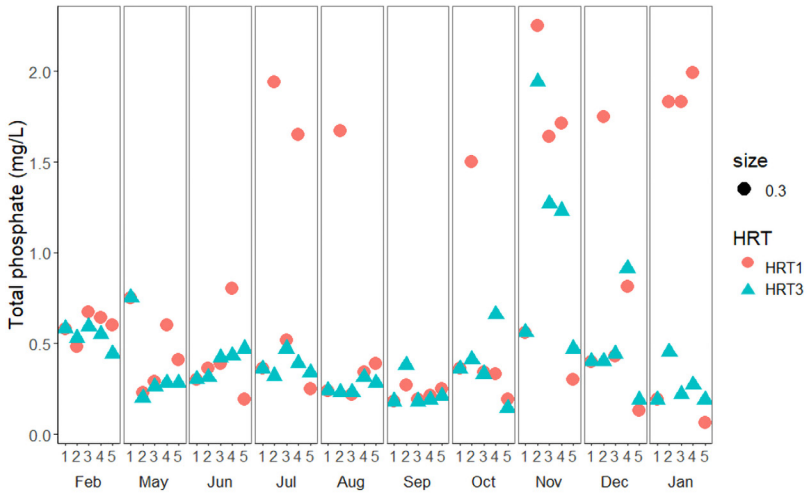


Figure S4.8: TP profile of CW with HRT-1 and HRT-3. Numbers 1, 2, 3, 4, 5 refer to sampling points.

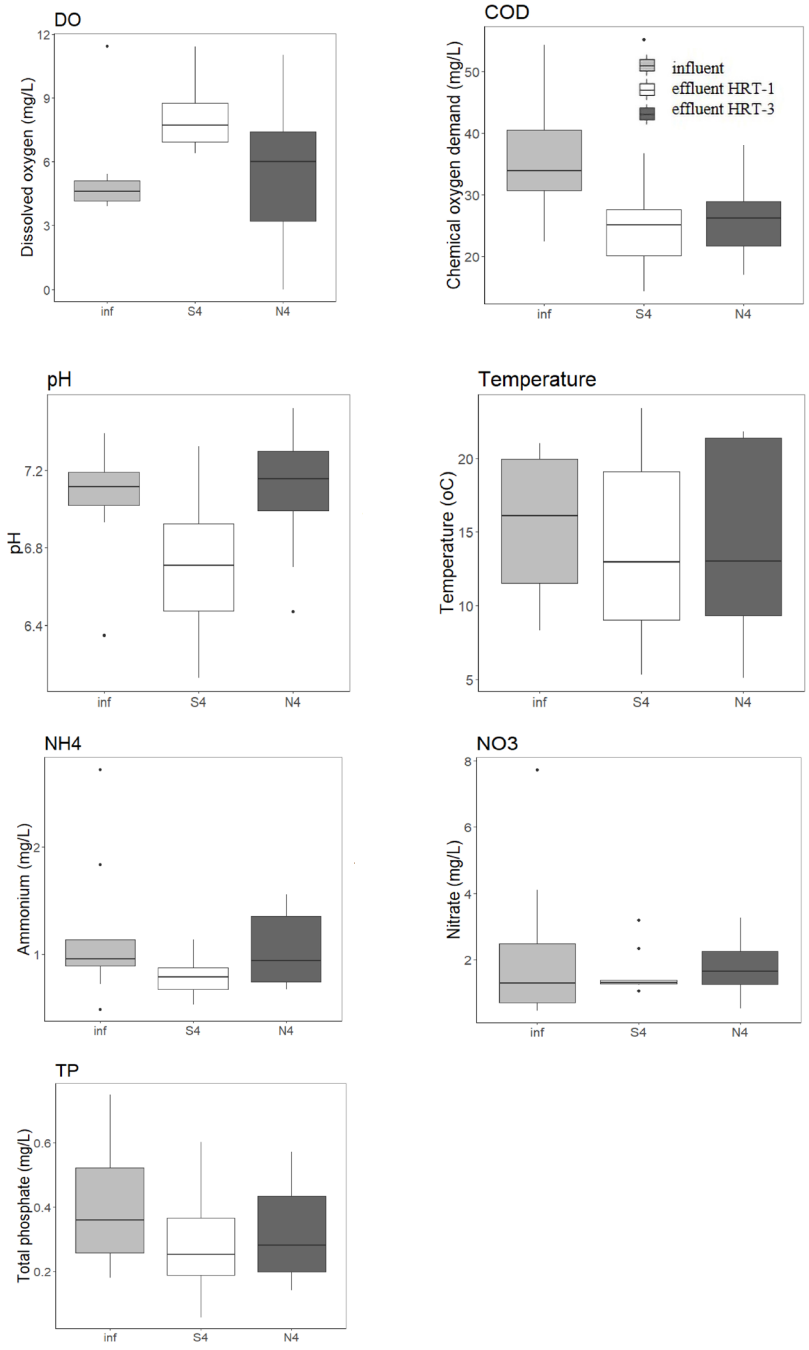


Figure S4.9: COD, DO and nutrients measured at the influent and both effluents of the CWs for one year. The black dots represent outliers of the respective data set.

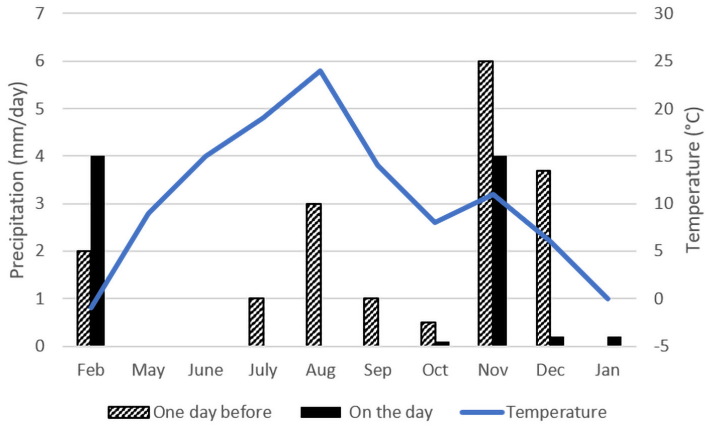
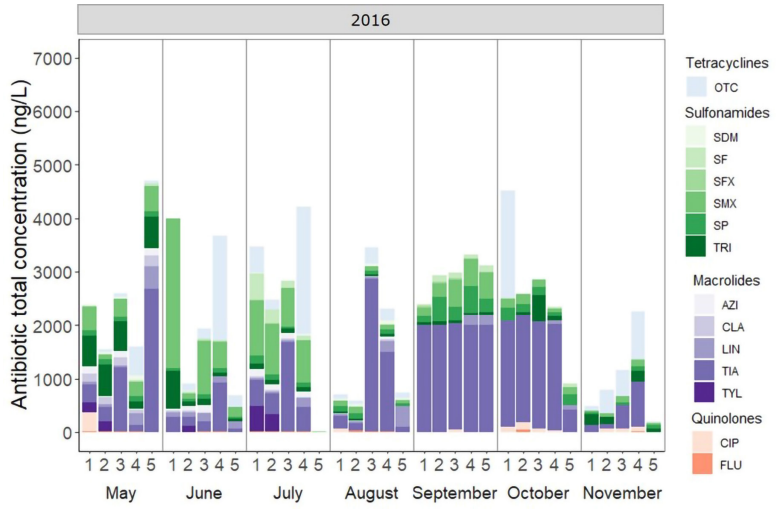


Figure S4.10: Precipitation and temperature on the sampling days. The bar chart represents the precipitation (mm/day) one day before and on the day of the sampling. The blue line represents the air temperature on the sampling day.

(a) CW with HRT-1



(b) CW with HRT-3

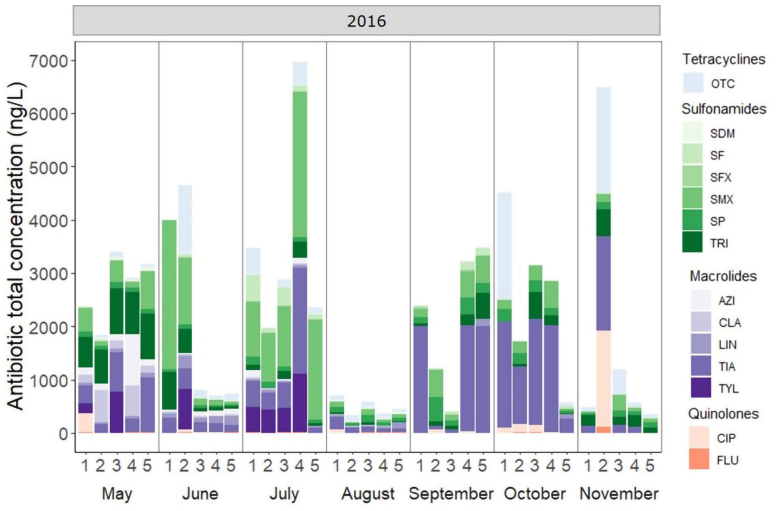


Figure S4.11: Antibiotic concentration in water phase in CW with (a) HRT-1 and (b) HRT-3 from May to November.

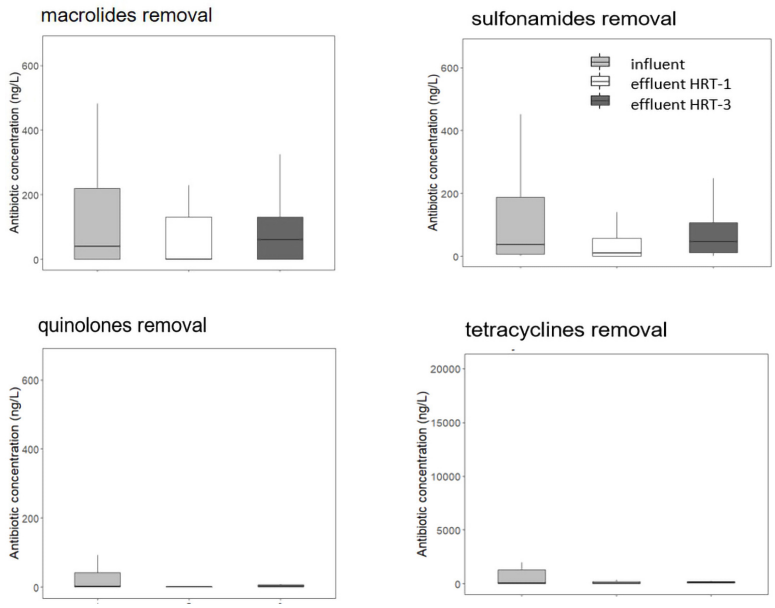


Figure S4.12: Antibiotic concentrations in water samples measured in the influent and the effluent in CW with HRT-1 and HRT-3. Outliers have been removed for the readability of the figures.

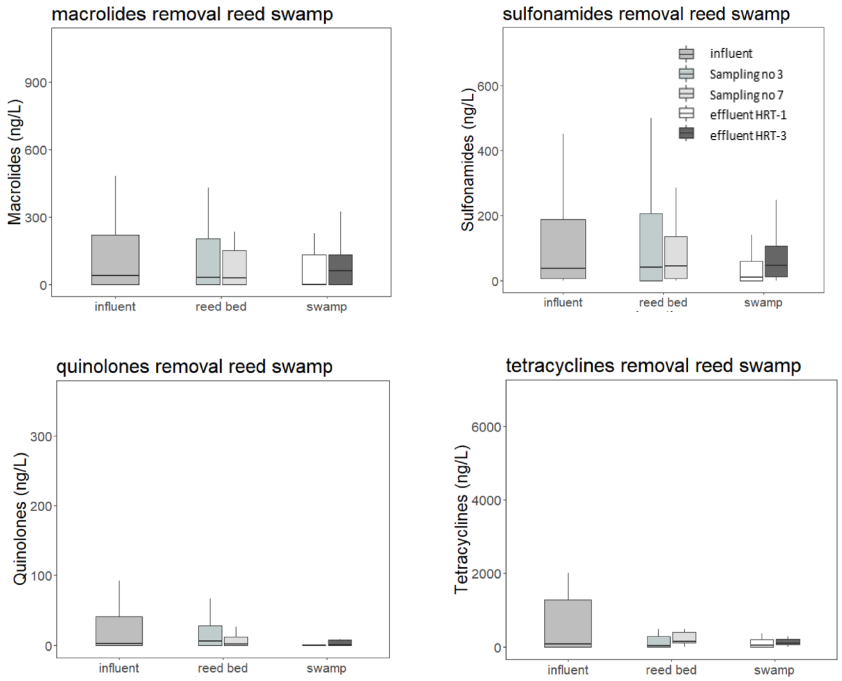


Figure S4.13: Antibiotic concentrations in water samples measured in the reed bed and swamp in CW with HRT-1 and HRT-3 for one year. Outliers have been removed for the readability of the figures.

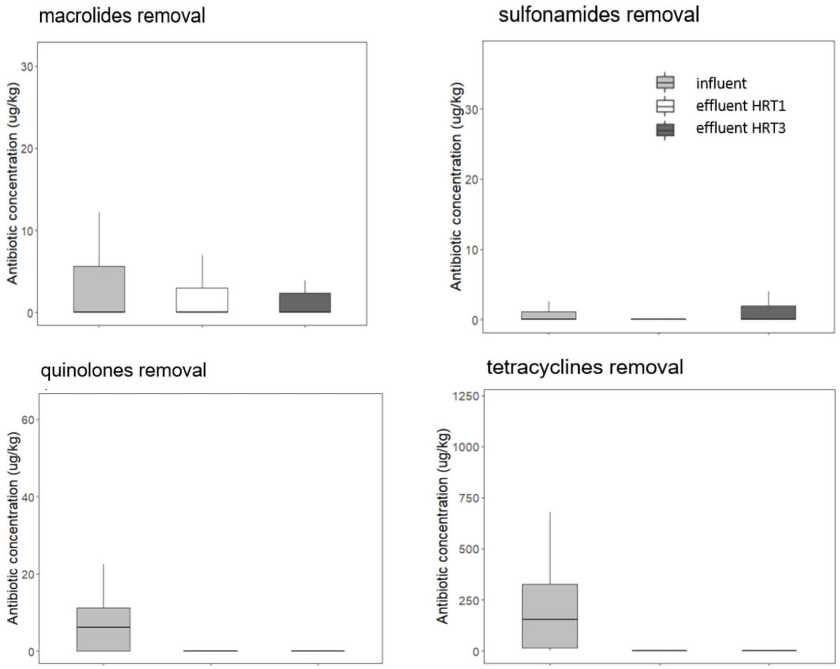


Figure S4.14: Antibiotic concentrations in sediment measured in the influent and the effluent in CW with HRT-1 and HRT-3. Outliers have been removed for the readability of the figures.

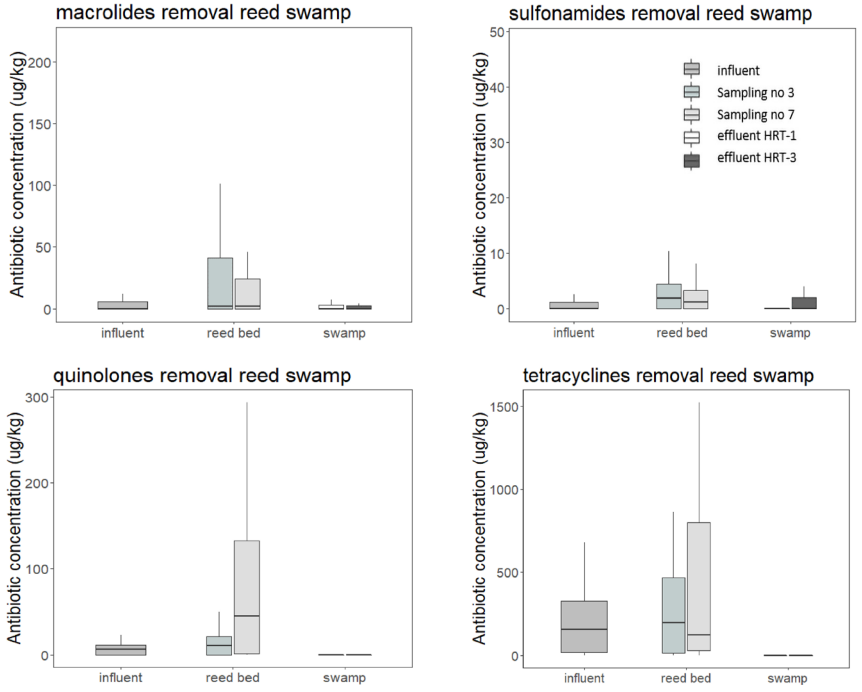
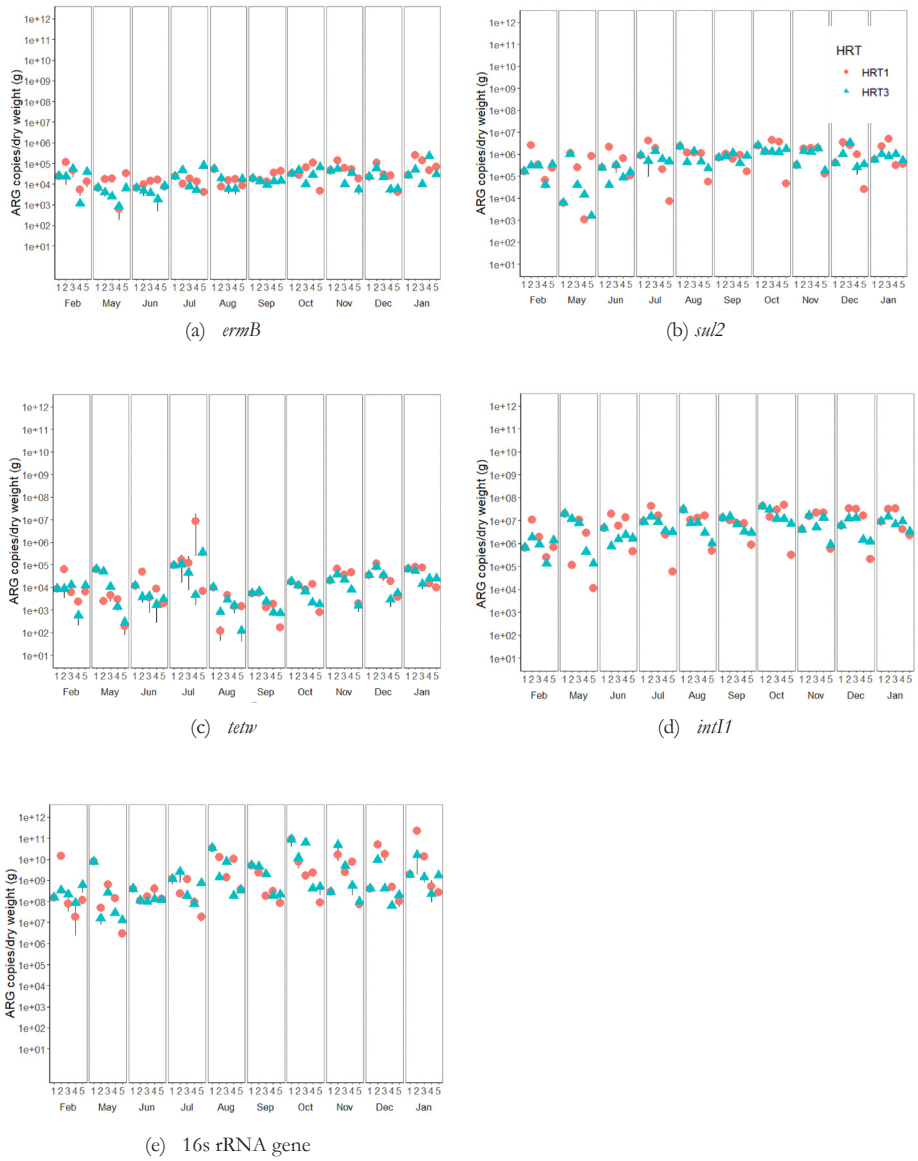


Figure S4.15: Antibiotic concentrations in sediment samples measured in the reed bed and swamp in CW with HRT-1 and HRT-3 for one year. Outliers have been removed for the readability of the figures.

Figure S4.16: One year profile of different ARGs in water for one year (a) *ermB* (b) *sul2* (c) *tetW* (d) *intI1* and (e)16s rRNA gene.



(a) *ermB*

(b) *sul2*

(c) *tetw*

(d) *intI1*

(e) 16s rRNA gene

Figure S4.17: One year profile of different ARG in sediment for one year (a) *ermB* (b) *sul2* (c) *tetW* (d) *intI1* and (e)16s rRNA gene.

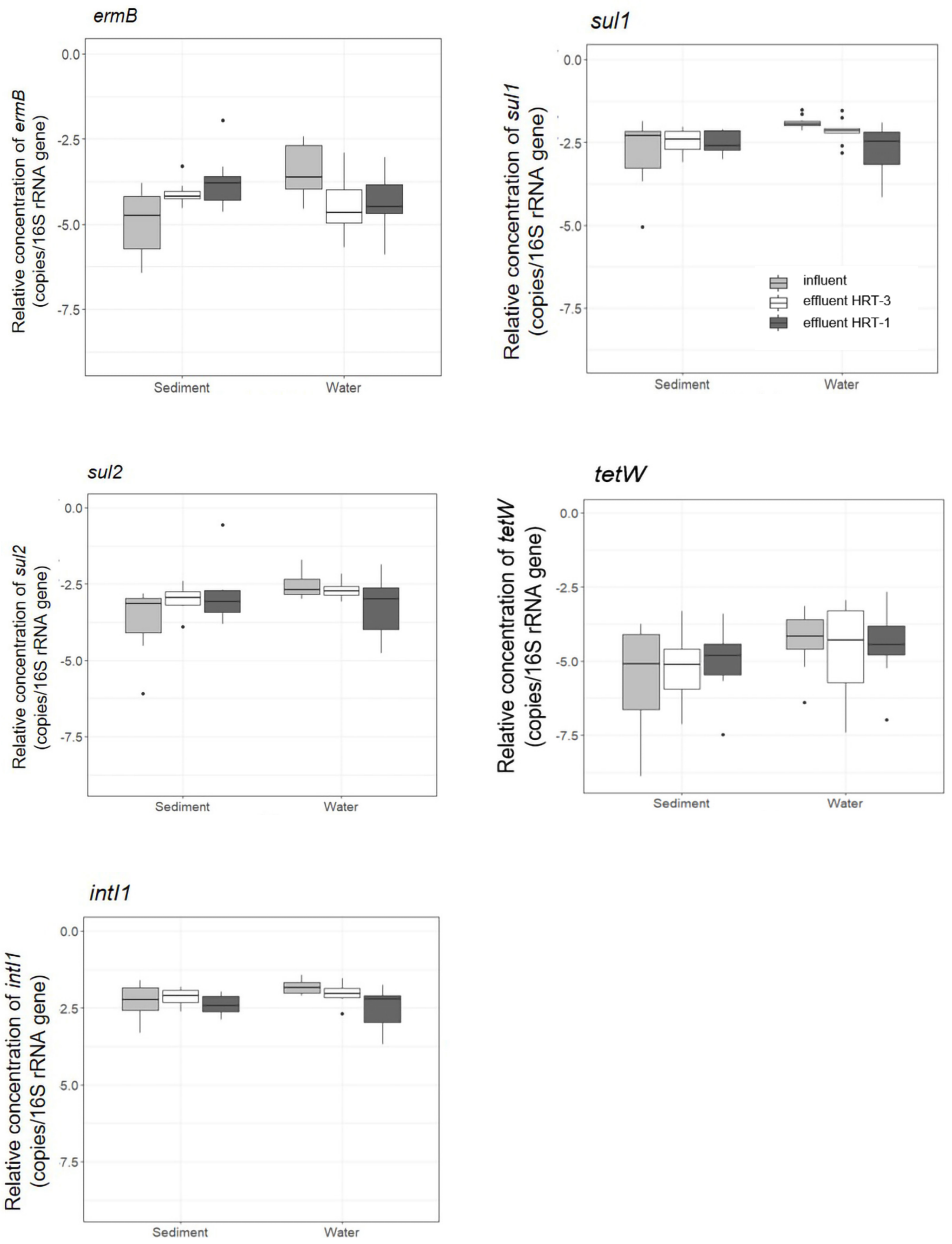


Figure S4.18: Relative abundance of targeted genes (copies/16S rRNA gene) in each sample collected from both CWs. The black dots represent the outlier of the respective data set.

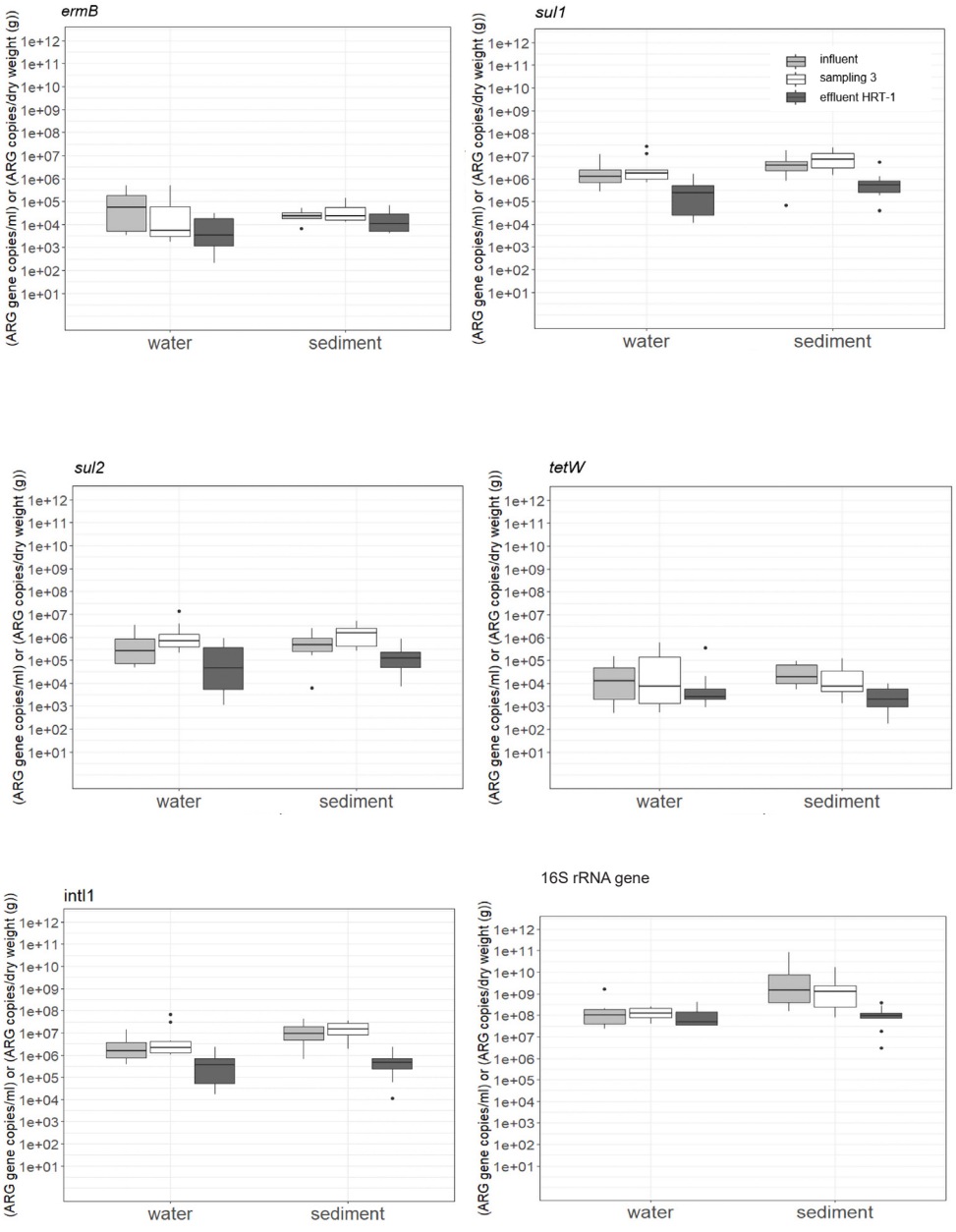


Figure S4.19: Gene abundance for all ARGs in sediment and water samples measured in the reed bed and swamp in CW with HRT-1 for one year. The black dots represent outliers of the respective data set.

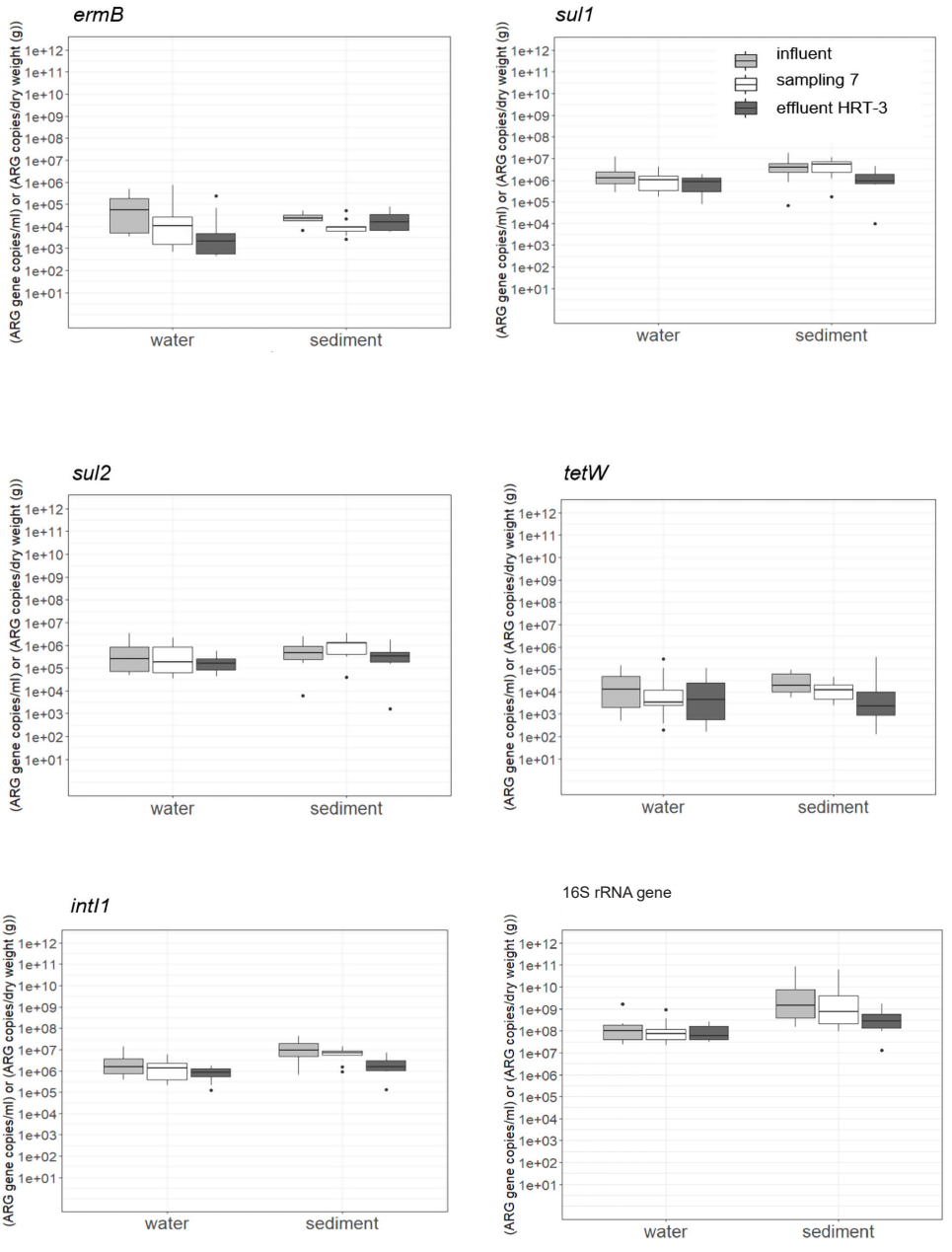
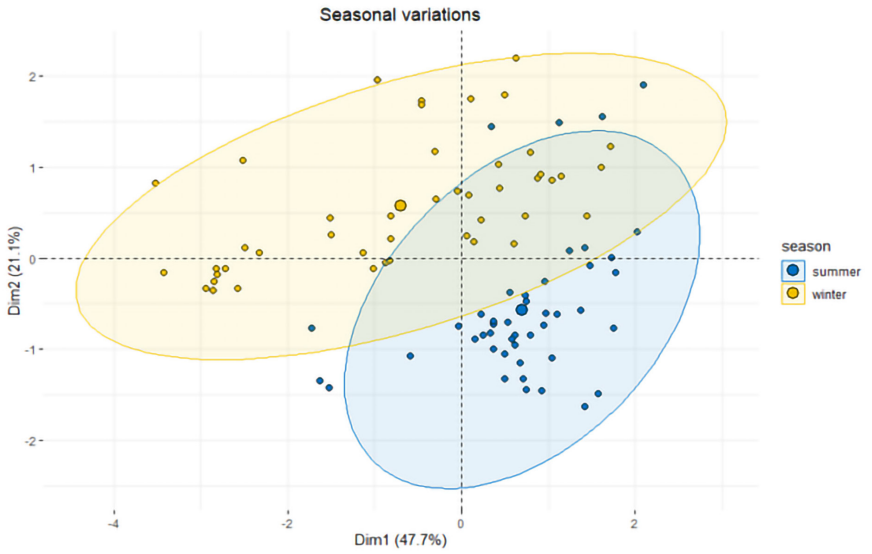


Figure S4.20: Gene abundance for all ARGs in sediment and water samples measured in the reed bed and swamp in CW with HRT-3 for one year. The black dots represent outliers of the respective data set.

(a) Water



(b) Sediment

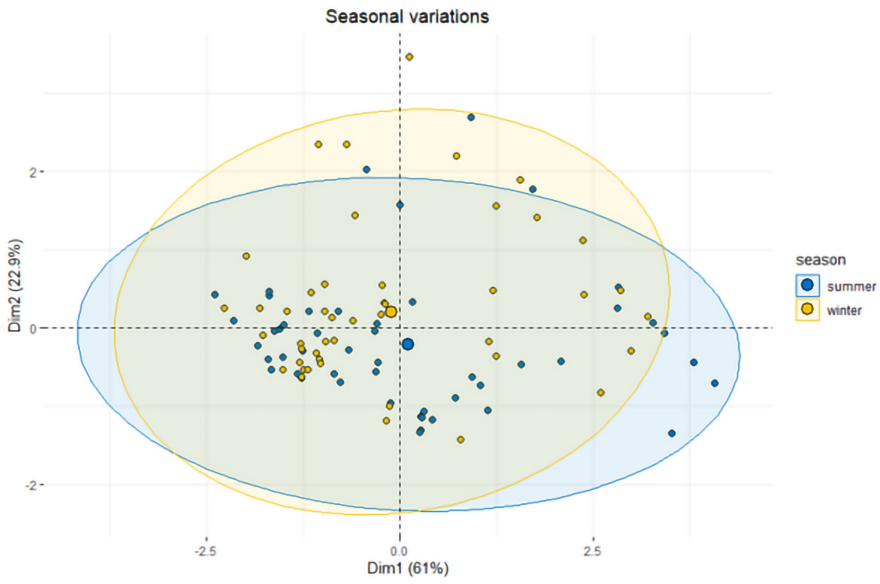
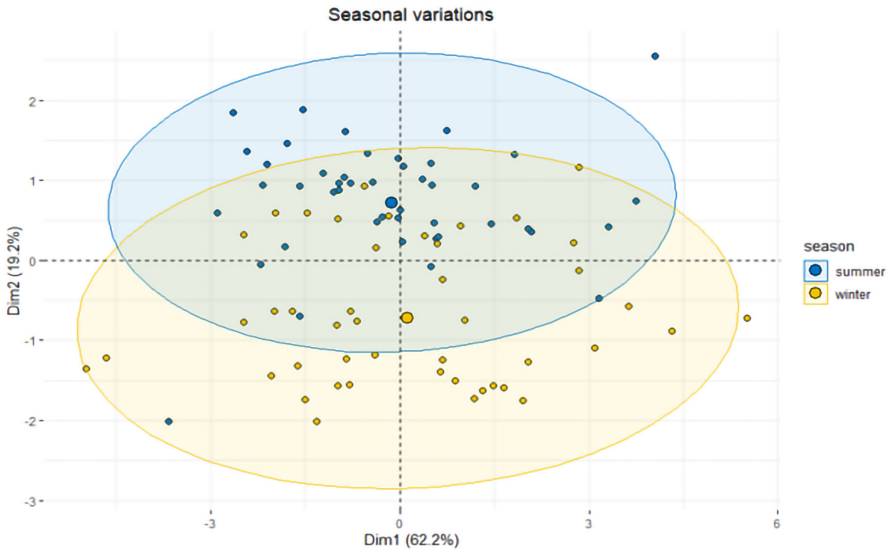


Figure S4.21: Seasonal variations of antibiotics (log-transformed) in (a) water and (b) sediment.

(a) Water



(b) Sediment

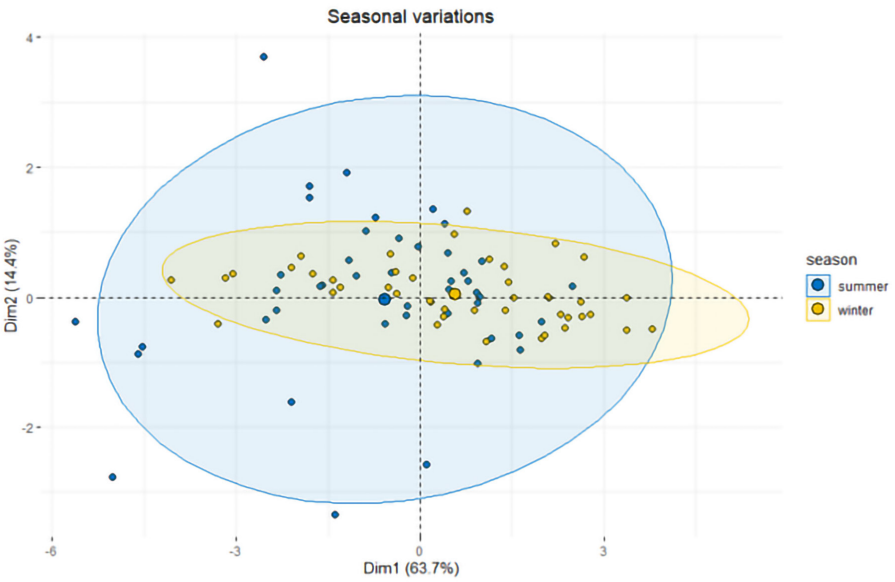
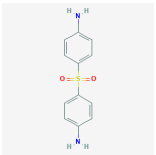
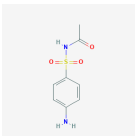
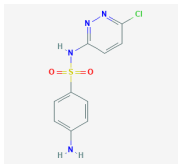
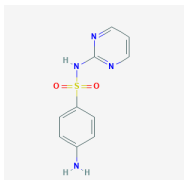
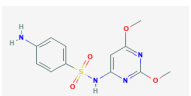
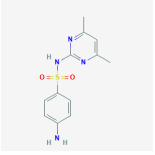
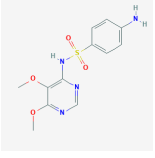
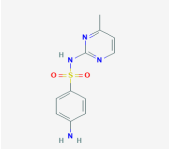
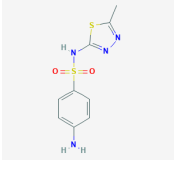
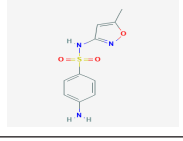


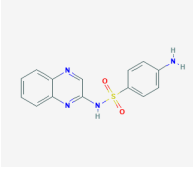
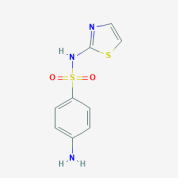
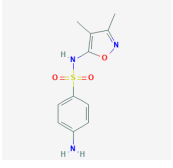
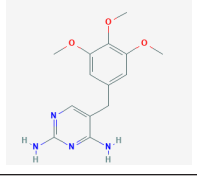
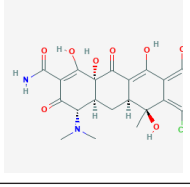
Figure 4.22: Seasonal variations of ARGs (log-transformed) in (a) water and (b) sediment.

Table S4.1: List of targeted antibiotics and their chemical properties.

	Antibiotics	Molecular formula	Molecular weight (g/mol)	pK_a	Log K_{ow}	CAS	Chemical structure	Antibiotic group
1	Dapson	$C_{12}H_{12}N_2O_2S$	248.3	2.41	0.97	80-08-0		Sulfonamides
2	Sulfacetamide	$C_8H_{10}N_2O_3S$	214.24	-	-	144-80-9		
3	Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	284.72	-	-	80-32-0		
4	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	250.28	6.36	-	68-35-9		
5	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	310.33	-	-	122-11-2		

6	Sulfadimidine	$C_{12}H_{14}N_4O_2S$	278.33	7.59	0.14	57-68-1		Sulfonamides
7	Sulfadoxine	$C_{12}H_{14}N_4O_4S$	310.33	-	-	2447-57-6		
8	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	264.31	-	-	127-79-7		
9	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	270.3	Pka1: 2.1 pka2: 5.3	0.54	144-82-1		
10	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28	pKa1 = 1.6; pKa2 = 5.7	0.89	723-46-6		

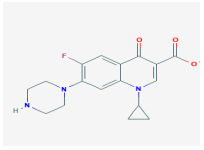
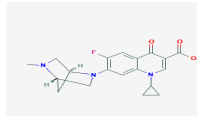
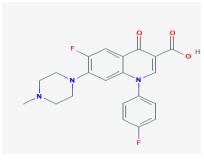
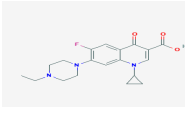
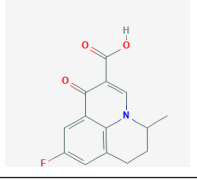
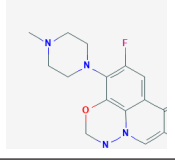
							Sulfonamides
11	Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	280.31	-	-	80-35-3	
12	Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	280.31	-	-	1220-83-3	
13	Sulfamoxole	$C_{11}H_{13}N_3O_3S$	267.31	-	-	729-99-7	
14	Sulfaphenazole	$C_{15}H_{14}N_4O_2S$	314.4	-	-	526-08-9	
15	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	249.29	8.43	-	144-83-2	

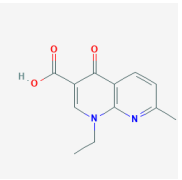
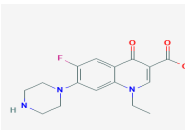
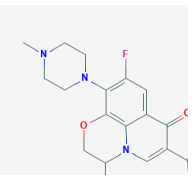
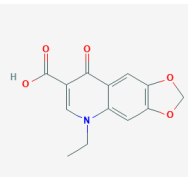
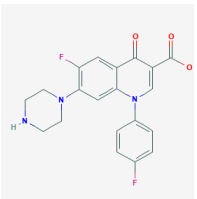
16	Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	300.34	5.1	1.68	59-40-5		Sulfonamides
17	Sulfathiazole	$C_9H_9N_3O_2S_2$	255.3	pKa1 = 2.2; pKa2= 7.24	0.05	72-14-0		
18	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	267.31	2.2	0.05	127-69-5		
19	Trimethoprim	$C_{14}H_{18}N_4O_3$	290.32	7.12 (at 20°C)	0.91	738-70-5		Trimethoprim
20	Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.9	-	-	57-62-5		Tetracyclines

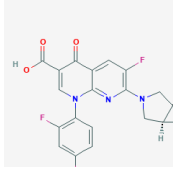
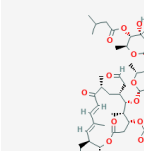
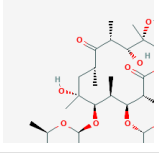
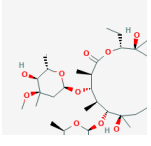
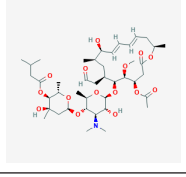
							Tetracyclines
21	Doxycycline	$C_{22}H_{24}N_2O_8$	444.4	3.09	-	564-25-0	
22	Methacyclin	$C_{22}H_{22}N_2O_8$	442.4	-	-	914-00-1	

23	Minocycline	$C_{23}H_{27}N_3O_7$	457.5	pKa1= 2.8;
				pKa2= 5.0;
				pKa3= 7.8;
				pKa4= 9.3
					0.05	10118-90-8	

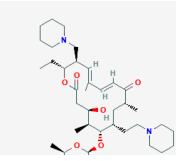
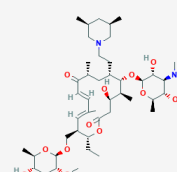
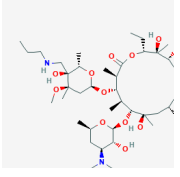
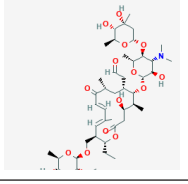
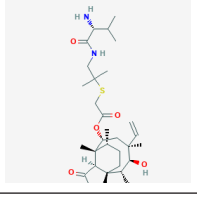
24	Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.4	3.27	-0.90	79-57-2	
25	Tetracycline	$C_{22}H_{24}N_2O_8$	444.4	3.3 At 25°C	-1.37	60-54-8	

26	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.34	6.09	0.28 (non-ionized)	85721-33-1		Quinolones
27	Danofloxacin	$C_{19}H_{20}FN_3O_3$	357.4	-	-	112398-08-0		
28	Difloxacin	$C_{21}H_{19}F_2N_3O_3$	399.4	-	-	98106-17-3		
29	Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.4	-	-	93106-60-6		
30	Flumequine	$C_{14}H_{12}FNO_3$	261.25	6.5	1.6	42835-25-6		
31	Marbofloxacin	$C_{17}H_{19}FN_4O_4$	362.4	-	-	115550-35-1		

32	Nalidixic acid	$C_{12}H_{12}N_2O_3$	232.23	8.6	1.41	389-08-2		Quinolones
33	Norfloxacin	$C_{16}H_{18}FN_3O_3$	319.33	pKa1 = 6.34; pKa2 = 8.75	0.46	70458-96-7		
34	ofloxacin_levofloxacin	$C_{18}H_{20}FN_3O_4$	361.4	pKa1 = 5.97 (carboxylic acid); pKa2 = 9.28 (piperizinyl ring)	-0.39	82419-36-1		
35	Oxolinic acid	$C_{13}H_{11}NO_5$	261.23	-	-	14698-29-4		
36	Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	385.4	pKa1= 5.6; pKa2 = 8.2	1.07 (est)	98105-99-8		

37	Trovafloxacin	$C_{20}H_{15}F_3N_4O_3$	416.4	-	-	147059-72-1		Quinolones
38	Tylvalosin	$C_{53}H_{87}NO_{19}$	1042.3	-	-	63409-12-1		Macrolides
39	Erythromycin	$C_{37}H_{67}NO_{13}$	733.9	8.9	3.06	114-07-8		
40	Gamithromycin	$C_{40}H_{76}N_2O_{12}$	777	-	-	145435-72-9		
41	Josamycin	$C_{42}H_{69}NO_{15}$	828	-	-	16846-24-5		

								Macrolides
42	Lincomycin	$C_{18}H_{34}N_2O_6S$	406.5	7.6	0.20	154-21-2		
43	Natamycin	$C_{33}H_{47}O_{13}N$ or $C_{33}H_{47}NO_{13}$	665.7	-	-	7681-93-8		
44	Neospiramycin I	$C_{36}H_{62}N_2O_{11}$	698.9	-	-	70253-62-2		
45	Pirlimycin	$C_{17}H_{31}ClN_2O_5S$	411	-	-	79548-73-5		
46	Spiramycin	$C_{43}H_{74}N_2O_{14}$	843.1	pKa1 = 7.88; pKa2 = 9.28 (est)	1.87 (est)	8025-81-8		

47	Tildipirosin	$C_{41}H_{71}N_3O_8$	734	-	-	328898-40-4		Macrolides
48	Tilmicosin	$C_{46}H_{80}N_2O_{13}$	869.1	8.18 (tertiary amine)	3.80	108050-54-0		
49	Tulathromycin	$C_{41}H_{79}N_3O_{12}$	806.1	-	-	217500-96-4		
50	Tylosin	$C_{46}H_{77}NO_{17}$	916.1	7.73	1.63	1401-69-0		
51	Valnemulin	$C_{31}H_{52}N_2O_5S$	564.8	-	-	101312-92-9		

							Macrolides
52	Tiamulin	$C_{28}H_{47}NO_4S$	493.7	9.51 (est)	4.75 (est)	55297-95-5	
53	Clarithromycin	$C_{38}H_{69}NO_{13}$	748	8.99	3.16	81103-11-9	
54	Azithromycin	$C_{38}H_{72}N_2O_{12}$	749	8.5	4.02	83905-01-5	

Table S4.2: Linear regression analysis of each antibiotic group concentration (ng/L) at every sampling point (from sampling point 1) within the CWs.

Antibiotics	Sample type	Sampling point			
		2	3	4	5
Macrolides	Water	570	690	340	-3700
	Sediment	130	83	258*	-28
Sulfonamides	Water	-1400***	-1300	-1400	-1700
	Sediment	25***	21**	19**	-2
Tetracyclines	Water	600	-40	940	-670
	Sediment	800*	170	-63	-916**
Quinolones	Water	67	-26	-4	-70
	Sediment	270***	32	56	-51

* Significant p <0.05 ** Significant p <0.01 ***Significant p <0.001

Table S4.3: Linear regression analysis of each ARGs (genes copies/ml) at every sampling point (from sampling point 1) within the CWs.

ARGs	Sample type	Sampling point			
		2	3	4	5
ermB	Water	-2.2×10^{4}	-4.5×10^{4}	-4.0×10^{4}	$-1.1 \times 10^{5**}$
	Sediment	$4.3 \times 10^{4**}$	1.5×10^{4}	6.3×10^{3}	-5.4×10^{3}
sul1	Water	$1.1 \times 10^{7**}$	2.6×10^{6}	3.5×10^{6}	-2.2×10^{6}
	Sediment	$6.5 \times 10^{6**}$	3.1×10^{6}	5.1×10^{4}	$-4.7 \times 10^{6*}$
sul2	Water	$3.1 \times 10^{6**}$	1.3×10^{6}	1.4×10^{6}	-7.0×10^{5}
	Sediment	$1.3 \times 10^{6***}$	$1.7 \times 10^{6**}$	1.9×10^{5}	-6.1×10^{5}
tetW	Water	$8.2 \times 10^{4*}$	7.8×10^{4}	1.4×10^{4}	2.1×10^{3}
	Sediment	2.3×10^{4}	-3.8×10^{3}	8.5×10^{5}	-3.0×10^{4}
intI1	Water	$5.0 \times 10^{7***}$	8.3×10^{6}	9.6×10^{6}	-2.6×10^{6}
	Sediment	5.3×10^{6}	3.6×10^{6}	-3.8×10^{5}	$-1.3 \times 10^{7***}$

* Significant p <0.05 ** Significant p <0.01 ***Significant p <0.001

Table S4.4: Correlation analysis between the antibiotics and ARGs.

	Macrolides	Sulfonamides	Quinolones	Tetracyclines
ermB	0.30**	0.35***	0.41***	0.27**
sul1	0.34***	0.23*	0.52***	0.17
sul2	0.35***	0.19	0.38***	0.08
tetW	0.25*	0.40***	0.37***	0.26**

* Significant p <0.05 ** Significant p <0.01 ***Significant p <0.001

Table S4.5: Correlation analysis between the total antibiotics concentrations and general water quality.

	TP	NO_3-N	NH_4-N	COD	pH	Temp	DO
Macrolides	0.05	-0.21*	-0.05	0.21*	0.04	-0.36**	0.02
Sulfonamides	0.20	0.07	-0.13	0.08	-0.21*	-0.36***	0.17
Quinolones	0.45***	0.21*	0.13	0.43***	0.16	-0.34***	-0.17
Tetracyclines	0.25*	0.30**	0.25*	0.13	0.00	-0.24*	-0.17

* Significant p <0.05 ** Significant p <0.01 ***Significant p <0.001

Table S4.6: Correlation analysis between the abundance of ARGs and general water quality.

	TP	NO_3-N	NH_4-N	COD	pH	Temp	DO
ermB	0.38***	0.57***	0.31**	0.20	-0.25*	-0.50***	0.19
sul1	0.62***	0.10	0.18	0.66***	0.18	0.06	-0.22*
sul2	0.48***	0.03	0.03	0.66***	0.27*	0.19	-0.16
tetW	0.40***	0.21*	0.10	0.11	-0.26*	-0.46***	0.26*
intI1	0.57***	0.13	0.04	0.69***	0.29*	0.07	-0.21*

* Significant p <0.05 ** Significant p <0.01 ***Significant p <0.001

CHAPTER 5

Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants

A modified version of this chapter is published as:

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Abstract

Information on the removal of antibiotics and ARGs in full-scale WWTPs (with or without additional treatment technology) is limited. However, it is important to understand the efficiency of full-scale treatment technologies in removing antibiotics and ARGs under a variety of conditions relevant for practice to reduce their environmental spreading. Therefore, this study was performed to evaluate the removal of antibiotics and ARGs in a conventional wastewater treatment plant (WWTP A) and two full-scale combined with additional treatment technologies. WWTP B, a conventional activated sludge treatment followed by an activated carbon filtration step (1-STEP® filter) as a final treatment step. WWTP C, a treatment plant using aerobic granular sludge (NEREDA®) as an alternative to activated sludge treatment. Water and sludge were collected and analyzed for 52 antibiotics from four target antibiotic groups (macrolides, sulfonamides, quinolones, tetracyclines) and four target ARGs (*ermB*, *sul 1*, *sul 2* and *tetW*) and integrase gene class 1 (*intI1*). Despite the high removal percentages (79-88%) of the total load of antibiotics in all WWTPs, some antibiotics were detected in the various effluents. Additional treatment technology (WWTP C) showed antibiotics removal up to 99% (tetracyclines). For ARGs, WWTP C reduced 2.3 log followed by WWTP A with 2.0 log, and WWTP B with 1.3 log. This shows that full-scale WWTP with an additional treatment technology are promising solutions for reducing emissions of antibiotics and ARGs from wastewater treatment plants. However, total removal of the antibiotics and ARGs cannot be achieved for all types of antibiotics and ARGs. In addition, the ARGs were more abundant in the sludge compared to the wastewater effluent suggesting that sludge is an important reservoir representing a source for later ARGs emissions upon reuse, i.e. as fertilizer in agriculture or as resource for bioplastics or bioflocculants. These aspects require further research.

Keywords: full-scale WWTP, antibiotics, antibiotic resistance genes, treatment technology, tertiary treatment

5.1 Introduction

Antibiotics inhibit the growth of microorganisms and have been used widely since the 1930s to combat infectious diseases (Gallo et al., 1995; Chee-Sanford et al., 2009). Only a fraction of these antibiotics are completely metabolized within bodies of humans and animals, and 20% to 90% is generally excreted as parent compound or metabolite through urine and feces (Jelic et al., 2015).

As a result, antibiotics are widely present in our domestic sewage waters and enter WWTPs that have only limited capacities to remove these compounds, thus they end up in the environment. Even though antibiotics are detected at low concentrations (ng/L to µg/L scale) in the environment, these antibiotics can persist there for a long time (Kolář et al., 2001). This persistence depends not only on the characteristic of the respective antibiotics, but also on environmental conditions such as oxygen and other electron acceptors and donors, and light. As a result, some antibiotics are readily degraded, while others are not. Their presence in the environment can lead to the emergence and prevalence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Andersson and Hughes, 2012). Antibiotic resistance develops naturally. However, use, misuse and inappropriate antibiotic prescriptions has accelerated the occurrence of ARB and ARGs in the environment (Kraemer et al., 2019), especially in wastewater (Sharma et al., 2016; Karkman et al., 2018; Pazda et al., 2019). WHO has listed antibiotic resistance as one of the world threats since 2014 (WHO, 2014).

Conventional WWTPs are designed to remove high concentrations of total organic carbon, and nutrients such as nitrates, and phosphates (de Kreuk et al., 2010; Pronk et al., 2015) but not specifically designed to remove micropollutants, including antibiotics and ARGs (Novo et al., 2013; Pal et al., 2015). In a conventional WWTP, activated sludge is the most common treatment technology for the biological treatment of wastewater (Samer, 2015). Two tanks are needed for activated sludge; one for aeration of biological reactions and for settling of the sludge.

The removal of antibiotics and ARGs varies (1 -2 log) in conventional WWTPs (Pallares-Vega et al., 2019). Some studies showed an increased relative concentration of antibiotics and some ARGs after WWTP treatment (Pärnänen et al., 2019). Others reported a decrease in the prevalence of antibiotics and relative concentrations of ARGs after WWTP treatment (Czekalski et al., 2012; Gao et al., 2012b; Liao et al., 2016; Kulkarni et al., 2017; Pärnänen et al., 2019). Even when a significant removal in the wastewater effluent was found, antibiotics were still detected in the receiving (surface) water body, ranging from 2 ng/L to 25 mg/L (Singh et al., 2019).

Therefore, additional treatment technologies may provide a promising contribution to reduce antibiotics and ARGs before effluent is discharged to the environment. Various additional treatment technologies have shown to remove antibiotics and/or ARGs from wastewater, e.g.,

physical treatment processes (Sun et al., 2019), disinfection (Khorsandi et al., 2019; Shen et al., 2020), advanced oxidation processes (Zhuang et al., 2015; Collivignarelli et al., 2018), as well as aerobic granular sludge (AGS) (Mihciokur and Oguz, 2016).

Activated carbon (AC) is a treatment technology that is based on adsorption and filter material, and can act as a carrier matrix for biomass. It is known to remove dissolved compounds, suspended matters, nitrogen, and phosphates (Hung et al., 2005). AC is a form of an amorphous carbonaceous material showing a high specific surface area (1,000 m^2/g) (Tadda et al., 2016) microporous structure, and large pore volume (Choi et al., 2005). Therefore, due to its high adsorptive capacities, AC has shown potential in removing color/odor/taste (Matsui et al., 2015; Huang et al., 2019b), disinfection by-products (Gopal et al., 2007a), micropollutants (Choi et al., 2005; Kårelid et al., 2017), antibiotics (Pachauri et al., 2009; Yu et al., 2016) and ARB (Ravasi et al., 2019) and ARGs (Sun et al., 2019). AC was also applied as a post-treatment to solar photo fenton treatment to remove antibiotics from wastewater (Michael et al., 2019).

AGS has been introduced to overcome the drawbacks in activated sludge, (Wilén et al., 2018). AGS is known for the excellent settling ability, simultaneous removal of organic matter and nitrogen, high biomass concentration, and a good ability to withstand the high organic load. AGS contain granules comprised of self-immobilized cells and does not depend on material support for biofilm growth. It retains a large number of microorganisms, at the same time permitting rapid bioconversion of many compounds and improving the performance and stability of the reactor (Bassin, 2018). In addition, AGS is a compact (using only one tank for settling and aeration) and cost-effective wastewater treatment (Nancharaiah and Reddy, 2017). Due to these characteristics, AGS gain more attention to be implemented in the wastewater treatment. Some full-scale AGS have recently been implemented to treat municipal and industrial wastewater (van der Roest et al., 2011; Pronk et al., 2015). As this is a relatively new technology, limited data are available on the removal of antibiotics in full-scale plants (Wang et al., 2019b) and ARB&Gs.

We hypothesize that additional treatment technologies have the potential to improve the removal of antibiotics and ARGs at current WWTPs. Most studies that compare different treatment technologies have been carried out at lab-scale (Guo et al., 2017; Sousa et al., 2017; Zheng et al., 2017b; Karaolia et al., 2018). However, limited research on antibiotics and ARGs removal has been performed in full-scale WWTPs, and studies so far focused on the comparison between conventional and additional treatment technology, e.g. membrane nanofiltration and reverse osmosis in China (Lan et al., 2019), parallel membrane bioreactors in China (Li et al., 2019a) and four separate treatments based on UV irradiation in Spain (Rodríguez-Chueca et al., 2019).

Therefore, this study evaluates the removal of antibiotics and ARGs in full-scale WWTPs, with and without additional treatment technologies. A conventional WWTP operated with activated sludge is chosen as a control, receiving domestic wastewater for comparison

purposes. In addition, two full-scale WWTPs with different additional treatment technologies were studies, a 1-STEP® filter (based on AC), and a NEREDA® technology (based on AGS). Grab samples were collected for two months and analysed for 52 antibiotics from 4 groups (macrolides, sulfonamides, quinolones, tetracyclines), 4 ARGs (*ermB*, *sul 1*, *sul 2* and *tetW*) and integrase gene class 1 (*intI1*).

5.2 Material and Methods

5.2.1 WWTPs

Three Dutch WWTPs were selected to study the removal of antibiotics, and ARGs; one conventional WWTP (WWTP A) and two WWTPs with additional treatment technologies (WWTP B and WWTP C). WWTP A employs conventional steps, such as a primary treatment (grit removal), a reactor with activated sludge, a sludge settling tank, and finally sand filtration.

WWTP B consists of a conventional activated sludge treatment followed by an AC filtration step (1-STEP® filter). The 1-STEP® filter is in operation since August 2012 and consists of a vertical, compact fixed bed activated carbon filter, combining filtration, denitrification, coagulation, flocculation, and adsorption in one single treatment unit (Bechger et al., 2009; Bechger et al., 2013). It is in use as final treatment step (as a tertiary treatment) to improve the nitrogen and phosphorus removal and the final effluent quality meets the requirements of the European Water Framework Directive 2000/60/EC (Council Directive, 1991).

WWTP C employs a NEREDA® technology, which is based on AGS as an alternative to activated sludge (as a secondary treatment). The NEREDA® technology is in operation since 2011 and developed in the Netherlands (van der Roest et al., 2011). The granules have a robust structure of aerobic granular biomass, with dense, compact, large particles (0.2 - 2 mm) with a high specific gravity (van der Roest et al., 2011). The granules consist of different microorganisms, including phosphate accumulating organisms, nitrifiers, denitrifiers, and glycogen accumulating organisms, which allows several processes simultaneously (Giesen et al., 2013). Due to the growth in dense grains, there are different oxygen levels within the grain, allowing different organisms to be active.

The selected treatment technologies have been reported in different parameters such as chemical oxygen demand (COD), phosphorus in the 1-STEP® filter (Scherrenberg et al., 2012) aerobic granulation (Li et al., 2014a; Pronk et al., 2015), and microorganisms analysis (Liu et al., 2016a; Świątczak and Cydzik-Kwiatkowska, 2018) in AGS.

Process flow diagrams and sampling points of each WWTP are shown in Figure 5.1. WWTP A has the capacity to treat 22,000 person equivalents (p.e.) with an average treated volume of 2,860 m^3/d, WWTP B treats 200,000 p.e. with an average treated volume of 26,000

m^3/d and WWTP C is designed for 59,000 p.e and an average treated volume of 8,000 m^3/d. Details for the three WWTPs are shown in Table S5.1.

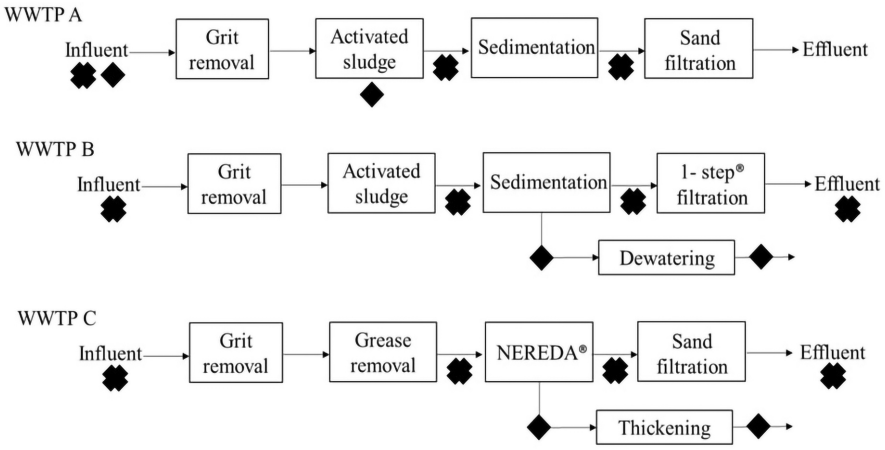


Figure 5.1: Schematic flow diagram of the WWTPs, with the sampling points for three wastewater treatment plants. ✸ Water sample point. ◆ Sludge sample point.

5.2.2 Sample collection and pre-treatment

Collection of grab samples (water and sludge samples) of the WWTPs was carried out during the winter season (February and March 2017) at 3 sampling points (WWTP A) and 4 sampling points (WWTP B and WWTP C), which are shown in Figure 5.1. The sampling was performed between 9:00 – 12:00 (after the daily morning peak hour), two times each month in duplicate at all sampling points. Average air temperatures during sampling were between 8°C and 11°C (data from the WWTPs), respectively.

Water samples (n=12 per WWTP A, n=16 per WWTP B and C) were taken at the outfall of the tank. A bucket was dipped into a tank for water sample collection, and the samples were stored in 1 L sterile glass bottles. On the sampling day, the water was measured for pH, water temperature and dissolved oxygen (DO) by using an HQ40D portable meter (Hach, Germany). The water samples were divided into two parts at different storage: for physicochemical and DNA filtration, the samples were stored at 4°C and processed within 48 hours. For antibiotics analyses, the samples were stored at −20°C until processed. All sludge samples (n=8 per WWTP) were collected in duplicate. For sludge collection, a bucket was dipped at the influent and at the sludge return flow (WWTP A) or via a sludge tap (WWTP B and C). The sludge samples stored in a 50 ml tube at -20°C before DNA extraction.

5.2.3 Chemical Analysis

5.2.3.1 Physicochemical parameters

During sampling, temperature, pH, and DO were measured using a probe (Hach, USA). Conductivity (in µS/cm) was measured in the laboratory by a conductivity probe (Hach, USA). Other analyses, total phosphate (TP), nitrogen ((ammonium (NH_4-N), nitrate (NO_3-N) nitrite (NO_2-N)), and COD, were determined using Hach kits in laboratory (USA; LCK 349, LCK 304, LCK 339, LCK 342, and LCK 1414 respectively). Total suspended solids (TSS) analyses were performed by filtering 200 mL of raw samples through a glass filter (Whatman, England) and placed at 105°C overnight. After weighing, the same samples were incubated at 550 °C for two hours for volatile suspended solids (VSS). All procedures of analyzing nutrients and COD were performed according to the international standards of the American Public Health Association (APHA, 2005).

5.2.3.2 Solid phase extraction (SPE) and LC-MS/MS

SPE purification and concentration, and LC-MS/MS procedures were performed as previously described by Sabri et al. (2020). This analysis was performed for only water samples and not for sludge samples. The target antibiotic groups (macrolides, sulfonamides, quinolones, tetracyclines) and their chemical characteristics are shown in Table S5.2. No significant difference was found after some samples at influent and effluent were tested for triplicate (data not shown). Therefore, the analyses were performed in a single measurement. In short, 10 mL of water sample of each sampling point was processed by using a Strata X Polymeric Reversed Phase SPE column (Phenomenex, USA). First, the SPE column was equilibrated by washing with 5 mL MeOH, followed by 5 mL EDTA-McIlvain buffer 0.1 M; pH 4.0. Then, the sample was loaded onto the SPE column and washed with 5 mL Milli-Q water. After this, the sample was eluted with 5 mL MeOH and dried under a nitrogen evaporator with a temperature of 40 °C. Finally, the dried sample extracts were dissolved in 500 µl MeOH: Milli-Q water (20:80). The extracts were analysed by LC-MS/MS (Sciex, USA, QTRAP 6500), equipped with a BEH C18 Waters Acquity column (Waters Corporation, USA, 100* 2.1 mm), at a column temperature of 40 °C. Data were analyzed by using MultiQuant software (Sciex, version 3.0.2). The limit of detection ranged from 5 to >1000 ng/L (depending on the antibiotics). Details for each compound is presented in Table S5.3. For quality control, a known amount (100 µg/L) and internal standard of each compound were spiked to every sample. The recovery percentage of the spiked compound in the sample ranged from 70 to 120%.

5.2.4 DNA extraction and quantitative PCR (qPCR)

Molecular analyses were performed as previously described by Sabri et al. (2020). Briefly, the DNA extraction of 100 ml samples was filtered onto a membrane filter with a 0.2 μm pore size (Millipore, USA) and stored at -20 °C until further use. DNA was extracted by using the PowerWater kit (MoBio Laboratories, USA) for water samples and PowerSoil kit (MoBio Laboratories, USA) for sludge samples, following the manufacturer's protocols. Four ARGs of interest were selected: *ermB*, *sul1*, *sul2* and *tetW*, as well as 16S rRNA gene and *intI1*. These ARGs were chosen based on antibiotics that have been frequently used and detected in water (Ye et al., 2007). Each ARG corresponding to the respective antibiotic such as macrolides are corresponding to *ermB*, sulfonamides are corresponding to *sul1* and *sul2*, and tetracyclines are corresponding to *tetW*. Meanwhile, *intI1* was proposed by Gillings et al. (2015) as a good marker for environmental pollution since it is commonly linked to genes conferring resistance to antibiotics and rapid change in response to environmental pressures.

A PCR master mix was prepared, depending on the gene of interest. The master mix contained, as described in detail in Sabri et al. (2020), water, precision blue (Biorad, USA), a probe (if needed), the forward and reverse primer (Eurogentec, Belgium), and super mix (SYBR-Green or IQ (Bio-Rad, USA)). The primers are presented in Table S5.4. Negative control and a calibration curve were also included. The data were processed by Bio-Rad-CFX manager (Version 3.1). The results were expressed as genes per ml for water and genes per gram dry weight (DW) after adjusting for the dry weight of the sediment.

5.2.5 Calculations and statistical analysis

Linear regression, T-test and correlation analyses were conducted to examine the influence of specific parameters on the performance of the WWTPs in removing antibiotics and ARGs. All statistical analyses were performed on the R platform (Version 3.5.2). The difference was considered statistically significant at $p < 0.05$.-

The removal percentage of antibiotics and ARGs was calculated by comparing the total concentrations between influent and effluent, or before and after the respective additional treatment technology. The calculations for antibiotics (Equation 5.1) and ARGs (Equation 5.2) were used as below:

$$Removal\ percentage = \left(\frac{Influent\ concentration}{Effluent\ concentration}\right) \times 100\% \quad \text{(eq 5.1)}$$

$$Log\ removal = Log_{ARGs}\ before\ treatment\ technology - Log_{ARGs}\ after\ treatment\ technology \quad \text{(eq 5.2)}$$

5.3 Results and Discussions

5.3.1 Performance of the studied WWTPs

The general performance of the studied WWTPs was identified by measuring removal of suspended solids, COD and nutrients. All measured parameters (DO, pH, water temperature, conductivity, TP, NH_4-N, NO_3-N, NO_2-N, COD, TSS, and VSS) met the effluent regulatory targets of the European Water Framework Directive (Council Directive, 1991). The increase of NO_3-N is the result of production through nitrification and associated with the decrease of NH_4-N. The removal percentages are summarized in Table 5.1. Detailed data for each parameter is given in Table S5.5a and Table S5.5b. More information is given in the Text S5.1.

Table 5.1: Removal (%) for selected parameters in three studied WWTPs. Data are mean value ± standard deviation (n=12 per WWTP A, n=16 per WWTP B and C).

	COD (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)	TP (mg/L)	TSS (g/L)	VSS (g/L)
WWTP A	74 ± 31	97 ± 0	-511 ± 359	96 ± 0	100 ± 0	99 ± 3
WWTP B	73 ± 3	100 ± 0	-5 ± 5	99 ± 1	100 ± 0	99 ± 1
WWTP C	55 ± 27	98 ± 2	-525 ± 139	97 ± 1	97 ± 3	95 ± 4

Dissolved organic compounds, nutrients, and WWTP operating parameters can affect the concentration of antibiotics and ARGs in a WWTP and its effluent. In our study, we found a good correlation (Pearson correlation, r= 0.4 to 0.8, $p<0.05$) between NH_4-N and all antibiotics groups (only in February) and ARGs (in both sampling months) (Figure S5.1). Huang et al. (2019c) observed that *sul2* was positively correlated with COD and NH_4-N, while other ARGs (*ermB*, *intI1*, *sul1* and *sul2*) were positively correlated with pH. However, in this study we did not observe significant correlation between ARGs and water temperature, DO and COD. Literature also described that WWTP operating parameters such as hydraulic retention time could enhance the antibiotics removal ability, e.g. a better removal of antibiotics was observed at a higher longer hydraulic retention time with AGS (Liao et al., 2019). We did not study the correlation between dissolved organic compounds, nutrients, and WWTP operating parameters in-depth, due to the limited number of WWTPs involved. Therefore, this warrants more research to study this further.

5.3.2 Occurrence and distribution of antibiotics in three WWTPs

The presence of antibiotics in the influent and effluent of the WWTPs in conventional and in additional treatment technologies WWTPs was investigated. In general, 8 out of 52 antibiotics were detected in the influent of all 3 WWTPs in our sampling campaigns of February and March 2017 (Figure 5.2). These antibiotics are norfloxacin (NOR), ciprofloxacin (CIP), levofloxacin/ofloxacin (LEV), sulfamethoxazole (SMX), trimethoprim (TRI), sulfadiazine (SF), sulfapyridine (SP) and tetracycline (TET). The antibiotics chlortetracycline (CTC), doxycycline (DC),

flumequine (FLU), oxytetracycline (OTC), and lincomycin (LIN) were found occasionally in the WWTPs. The other analysed antibiotics (Table S5.6) were all below the quantification limit.

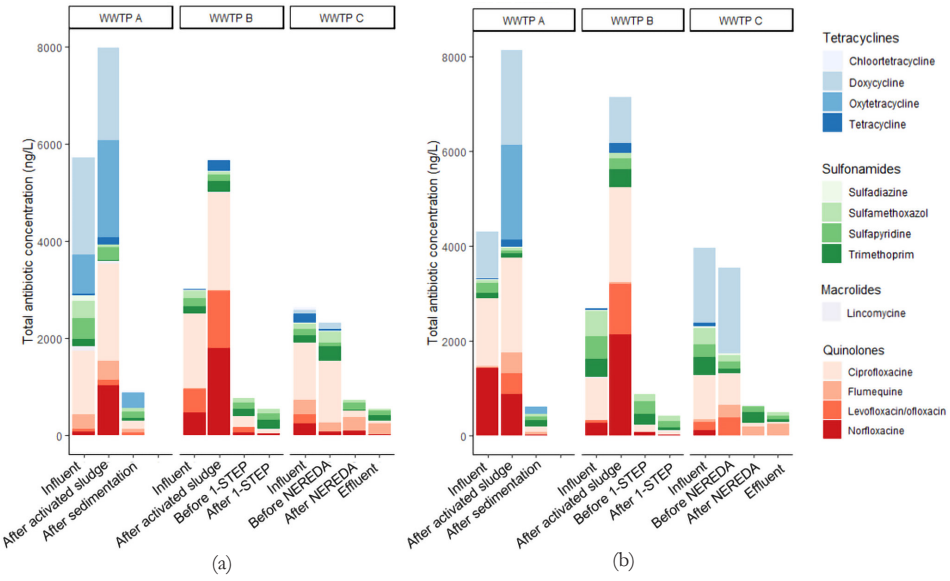


Figure 5.2: Antibiotic concentrations (ng/L) in the water of the WWTPs in (a) February 2017 (b) March 2017.

The total antibiotics concentration ranged from 3000 ng/L to 6000 ng/L in the influent of all WWTPs for both months. The quinolones were the most abundant antibiotics found in all influents, with an average concentration of 2300 ng/L (WWTP A), 1900 ng/L (WWTP B) and 1600 ng/L (WWTP C). Two antibiotics showed a high variation between the sampling moments in two months; Norfloxacin was measured 18 times lower in February (80 ng/L) compared to March (1400 ng/L) in WWTP A, and doxycycline was measured 20 times higher in February (80 ng/L) compared to March (1500 ng/L) in WWTP C.

An average of 3000 ng/L antibiotics was detected in the influent of all WWTPs, with the quinolones as most abundant antibiotics. This is in the same range as reported in winter time from other studies, e.g., 1000 ng/L (Huang et al., 2019a) to 3300 ng/L (Zheng et al., 2019) in Chinese wastewater influent, and can reach up to 6000 ng/L in Czech Republic (Golovko et al., 2014). It is known that more antibiotics are prescribed during winter periods, as a result of increased respiratory tract infections (Ferech et al., 2006; Werner et al., 2011). This is also in line with a study of Diwan et al. (2013) who showed that higher concentrations of quinolones were measured in the winter. Van Boeckel et al. (2014) reported that the consumption of antibiotics is highest between January and March in the northern hemisphere. For example, antibiotics prescription in the USA is 24.5% higher in the winter than in the summer (Suda et al., 2014), and 32% in Israel (Dagan et al., 2008). This high consumption of quinolones in winter also is shown

in various countries in Europe (Adriaenssens et al., 2011). This elevated use in winter might explain the relatively high concentrations detected in the wastewater influent since our data were collected in the winter with maximum levels of antibiotics. As a consequence, this made it possible to study the ir removal in different treatments in a WWTP.

Along the WWTP treatments, a similar decreasing trend from influent to effluent was observed for all WWTPs in February and March. The only exception was the elevated concentration in the activated sludge tank in WWTP A and WWTP B, where higher concentrations of quinolones (LEV, CIP, and NOR) and of oxytetracycline were detected.

For the WWTP effluent concentrations, 5 of the 52 analysed antibiotics were detected in all WWTP effluents. These detected antibiotics are CIP, SMX, SP, SF, and TRI in which sulfonamides (SMX, SF, SP) and TRI were consistently detected in all WWTPs for both sampling months. Average concentrations of the sum of these sulfonamides and TRI were 230 ng/L (WWTP A), 370 ng/L (WWTP B), and 260 ng/L (WWTP C). A few antibiotics (TET, LEV, NOR, FLU, LIN) were found occasionally in only one of the WWTPs or in only one sampling campaign. The removal efficiencies of the total of antibiotics were 83-85% (WWTP A), 82 - 84% (WWTP B), and 82 - 88% (WWTP C), based on concentrations of the detected antibiotics.

Sulfonamides and quinolones were detected in the effluent of all studied WWTPs. Poor removal of sulfonamides in WWTPs was also observed by Marx et al. (2015) and Bengtsson-Palme and Larsson (2016). Sulfonamides group are not easy to degrade, have a low potential to volatilize, are very hydrophilic (K_{ow} <1) and are highly mobile in the sand and groundwater infiltration systems (log sorption-distribution coefficients (K_d) < 2) (Kolpin et al., 2002; Wegst-Uhrich et al., 2014a). Therefore, sulfonamides are easily transferred into the aquatic environment, which can explain their high reported occurrence in the water phase (Xu et al., 2007; Jiang et al., 2018).

5.3.3 Antibiotics removal within conventional and additional treatment technologies

The efficiency of the removal of antibiotics in the WWTPs with additional treatment technologies (WWTP B and C) and without (WWTP A) was evaluated by comparing the water before and after the individual treatment processes. All the treatments in the respective WWTPs showed removal in the total load of antibiotics, regardless of conventional or additional treatment technology, although differences within the treatment steps in WWTPs were observed.

First, two conventional treatment steps were evaluated; activated sludge in WWTP A and WWTP B were compared. Concentrations of some antibiotics were significantly increased in concentration in the water phase after the activated sludge process, while others decreased in concentration ($p < 0.05$). For example, WWTP A showed a 177% increase of tetracyclines and 66% of quinolones, whereas 55% of sulfonamides and 74% of macrolides were removed.

In WWTP B, the tetracyclines increased with 1650%, quinolones with 200%, whereas 29% of sulfonamides were removed. No removal of macrolides was observed.

In the activated sludge treatment steps of WWTP A and WWTP B, the concentration of quinolones and tetracyclines increased. The increase of both antibiotics in water are most likely due to quinolones and tetracylines being released from hydrolyzed organic waste fractions that enter the activated sludge and water phase with the influent. This is followed by a redistribution over the water and sludge phase by sorption processes, as also shown by another study Jia et al. (2012). We observed that the TSS at this treatment step was higher than in the other sampling points within the WWTP (Table S5.5), indicating that the amount of antibiotics attached to the particles in the wastewater could have been higher ($p<0.05$). This is supported by the high correlation between TSS content and antibiotic concentration (Pearson correlation 0.95 in February and 0.96 in March). This indicates that the higher the particle concentration in the wastewater, the higher concentration of the antibiotics. CIP and TET are multivalent zwitterions with strong dipole and exhibited significant sorption capacity onto suspended solids and sludge in previous research (Polesel et al., 2015). Quinolone sorption is high ($\log K_d > 3$) and it adsorbs to sludge surfaces through electrostatic interactions (Golet et al., 2003). Concentrations up to 18.4 mg/kg have been measured in sludge (Jia et al., 2012) and up to 2.4 mg/kg of dry weight (Golet et al., 2003).

Other treatment steps of conventional treatment technology, such as the settling tank and subsequent sand filtration, were present in WWTP A and WWTP C. In the sedimentation tank, average removal fractions were respectively 94% for tetracyclines, 5% for sulphonamides and 94% for quinolones. In the sand filtration, the average removal of tetracyclines was 100%, 20% for sulphonamides, and 16% for quinolones. Macrolide concentrations increased after both the sedimentation tank and sand filtration.

The main removal mechanism in the sedimentation tank is the sorption of antibiotics on the colloidal matter, followed by removal in the coagulation/flocculation/sedimentation process (Adams et al., 2002; Shah, 2008). Xing and Sun (2009) showed that this resulted in 87% antibiotics removal after sedimentation and suggested this as an effective removal step to treat wastewater of antibiotics and pharmaceutical manufacturers. Low removal percentage in the sand filtration in WWTP C, which only removed 0.3% (quinolones) to 0.4% (sulfonamides), showed that antibiotics are largely unaffected by sand filtration. This is consistent with Rooklidge (2004), who also indicated that sand filtration removed less than 4% of sulfonamide and demonstrated limited mobility of lincomycin, trimethoprim, and tylosin within the sand filter. This is indicating that sand filter possesses low sorption properties and has a high persistence of microorganisms not adapted to biodegradation of these specific antibiotics (Ternes et al., 2002).

For the additional treatment technologies studied, the 1-STEP® filter removed 19% of sulfonamides, and 65% of quinolones, and produced concentrations of macrolides increased with

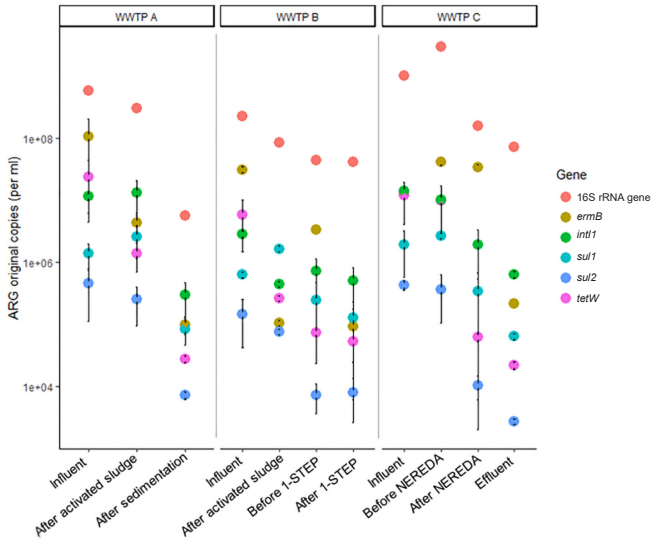
113%. No tetracyclines were detected before 1-STEP®. The present activated carbon removes antibiotics by physico-chemical adsorption onto the activated carbon and by the biofilm on the activated carbon (Ahmed, 2017; Östman et al., 2019). Activated carbon removes effectively hydrophobic compounds with a log K_{ow} > 4, for example tetracyclines, and quinolones (Raevsky et al., 2009; Grandclément et al., 2017; NCBI, 2018). The removal observed in this study is lower than reported in other studies, although those were lab-scale studies (Choi et al., 2008; Zhang et al., 2016f). In a full-scale WWTP, the lifetime of the AC, the saturation level of the AC with other organic compounds, the water flow, hydraulic retention times, and the concentration of antibiotics in the influent will influence the removal percentage.

Meanwhile, NEREDA® removed 100% of tetracyclines, 36% of sulfonamides, 84% of macrolides, and 74% of quinolones. No increase of antibiotics was observed within the treatment, indicating little accumulation and consequent desorption within the treatment. This can be explained by the relatively high microbial activity of aerobic granules (Wang et al., 2019b). The bacteria produce compact granules compared to flocs in conventional activated sludge and these granules settle faster in the wastewater (Forster, 2015). The aerobic granules are formed by bacteria that produce extracellular polymeric substances (EPS) and are stabilized by slow growing microorganisms (Świątczak and Cydzik-Kwiatkowska, 2018). EPS influence the surface properties of biomass and increase the sorption of organic pollutants (Schmidt et al., 2012; Kang et al., 2018). Xu et al. (2013) reported that protein in EPS interact and bind with sulfamethazine by hydrophobic interaction, contributed to the stability of the complex, and improve the efficient removal of sulfamethazine by harvesting the sludge EPS. This is also supported by Pi et al. (2019), and these authors reported that the chemisorption and hydrophobic interaction of tryptophan and tyrosine during the binding process to EPS and sulfonamides played an important role in adsorption capacity. The removal percentage in our NEREDA® technology is in the same range as other studies of AGS (Kang et al., 2018; Wang et al., 2018b; Wang et al., 2019b). Not only AGS has a promising step to remove antibiotics from wastewater, but also anaerobic granular and flocculent sludge showed a high removal of micropollutants (Butkovskyi et al., 2017).

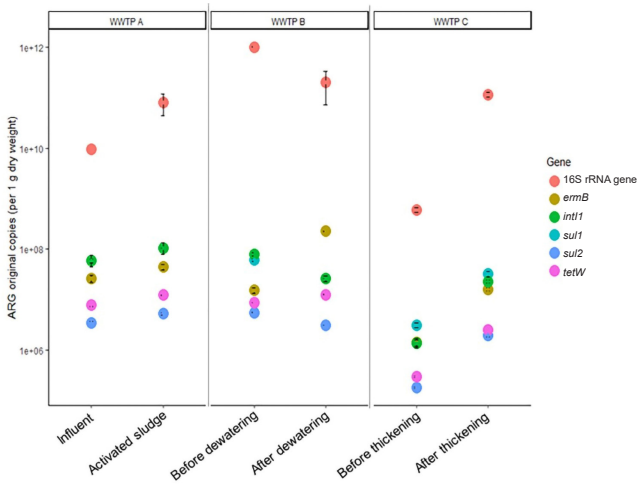
When we compare 1-STEP® filter and NEREDA®, 1-STEP® filter removed 30% - 52%, meanwhile NEREDA® removed from 68% - 82%. The location of the treatment step may contribute to the removal difference. However, we did not observe any significant difference between the locations ($p > 0.05$). The 1-STEP® filter is located after activated sludge and acts as a polishing step of the treatment. This contributes to a low load of antibiotics in the system, when compared to NEREDA® which is located after the primary treatment. This indicates that the location of the treatment technology (either in the secondary treatment or as a polishing treatment) did not affect the removal percentage of antibiotics. The difference of the antibiotic concentration can be the results of other factors, such as concentration of the influent and WWTP operating parameters.

5.3.4 Occurrence and distribution of ARGs over the treatment phases in the three WWTPs

In this study, the 16S rRNA gene, the *intI1* gene, and four ARGs (*ermB*, *sul1*, *sul2*, and *tetW*) were detected at all sampling points in all WWTPs (Figure 5.3 and Table S5.7), in water and in sludge samples. This illustrates the prevalence of ARGs along the phases of WWTPs in water and sludge.



(a)



(b)

Figure 5.3: Concentrations of antibiotic resistance genes in the (a) water and (b) sludge at different sampling points in three wastewater treatment plants. Error bars indicate the standard deviation of the respective gene data set (duplicate).

Generally, the absolute abundance of ARGs in the influent ranged from 4.6×10^5 to 1.1×10^8 copies/ml in WWTP A, 1.5×10^5 to 3.1×10^7 copies/ml in WWTP B and 4.3×10^5 to 1.4×10^7 copies/ml in WWTP C respectively. The most abundant ARG in the influent of all WWTPs was *ermB* with a range of 3.7×10^7 to 2.5×10^8 copies/ml in WWTP A, 2.1×10^5 to 7.9×10^7 copies/ml in WWTP B and 5.6×10^7 to 2.3×10^8 copies/ml in WWTP C. The second most abundant ARG was *intI1*, followed by *tetW*, *sul1*, and *sul2*. The relative abundance of the ARGs to the 16S rRNA gene shows a decreasing trend for all ARGs, except for *intI1*, which is stable in the treatment transect from the influent to the effluent (Figure S5.2).

In sludge samples, ARGs were detected before, and after dewatering at 3.5×10^6 to 8.3×10^{10} copies/g DW in WWTP A, 3.1×10^6 to 1.0×10^{12} copies/g DW in WWTP B, and 1.8×10^5 to 1.2×10^{11} copies /DW in WWTP C. *Sul1* and *intI1* were the most abundant in each treatment unit of all WWTPs, followed by *ermB*, *tetW* and *sul2*. ARGs concentration increased in WWTP A and C when compared before and after the dewatering system.

Unlike the antibiotics, the total amount of ARGs did not accumulate in the water phase in the activated sludge tank in WWTP A. We observed an average removal of 0.44 log from the water phase and -in parallel- we observed a slight increase (0.34 log) in the sludge phase. This was expected, as the sludge or sediments are known as hot spots of high bacterial density, activities and biofilm formations (Heß et al., 2018a). This accumulated ARGs from the water phase to attach to the sludge since the majority of bacteria are known to live in association with surfaces (Davey and O'Toole, 2000). It has been shown that reduction of microbial biomass might correlate with the reduction of ARGs in the water phase and lead to an equivalent accumulation in the sludge phase (Zhang et al., 2018b). The precise mechanism behind this is not fully clear. As a result, ARGs will accumulate in the sludge and also in the sediment and soil (Peng et al., 2018; Chen et al., 2019a).

Along the WWTP, all ARGs except *sul1* and *intI1* showed a decreasing trend. *Sul1* and *intI1* increased slightly but not significantly after some steps. *Sul1* increased with 0.26 ± 0.20 log in WWTP A after grit removal and activated sludge treatment, and 0.12 ± 0.22 log after activated sludge in WWTP B. *intI1* increased 0.20 ± 0.14 log at the activated sludge in WWTP A.

ARGs were present in the effluent ranging from 2.7×10^3 (*sul2*) to 6.3×10^5 (*intI1*). *intI1* was the most abundant gene in the effluents of all WWTPs. This is followed by *ermB*, *sul1*, *tetW*, and *sul2* in WWTP A, *sul1*, *ermB*, *tetW*, and *sul2* in WWTP B and *ermB*, *sul1*, *tetW* and *sul2* in WWTP C. Since a major amount of the ARGs ends up in the sludge phase, lower ARGs concentrations were detected at the effluent as compared to the influent. All WWTPs significantly ($P < 0.05$) reduced the total ARGs (copies/ml) from the influent to the effluent. A similar range of reduction about 1-3 log removal was observed in China (Chen and Zhang, 2013a; Lee et al., 2017), 2.4 to 4.6 log removal in Michigan (Munir et al., 2011) and less than 2 log removal in Italy (Fiorentino et al., 2019). Our study showed that *ermB* was the most removed

gene in all three WWTPs, as also reported by Rafraf et al. (2016). *ErmB* genes have been found mainly in gram-positive bacteria (Gupta et al., 2003), and it has been shown before that gram-positive bacteria were removed from influent to effluent (Forster et al., 2002).

In the effluent, *intI1* was the most detected gene in all three WWTPs. This was also observed by Narciso-da-Rocha et al. (2014), who suggested that *intI1* is stable in wastewater. Furthermore, ARGs and the *intI1* gene were not efficiently reduced during wastewater treatment (Rafraf et al., 2016). We also observed low log removal of *sul1* (0.60 - 1.63 log) in the three WWTPs, and a similar log removal (0.9-1.9 log) was observed by Chen and Zhang (2013a). The limited removal of both *sul1* and *intI1* ($r = 0.81$) and *sul2* and *intI* ($r = 0.93$) were strongly correlated ($p < 0.05$), as shown by others, as *sul1* is one of the backbone genes of the 39-conserved segments in *intI1* (Partridge et al., 2002; Muziasari et al., 2014).

Through the sorption of ARG-carrying bacteria, sludge has the potential to act as ARGs reservoir and mitigate the spread of antibiotic resistance in the environment through effluents (Munir et al., 2011). This situation can also increase the exposure risks, especially in countries applying WWTP sludge for agricultural purposes, or producing and using products made from WWTP sludge materials.

In our study, the antibiotics and ARGs showed different patterns of reduction in the investigated treatments. In WWTP A, the total amount of antibiotics increased after the activated sludge treatment, while the concentration of ARGs decreased. The ARGs were removed in all treatment steps in the WWTP. There are inconsistencies in the literature in determining the correlation between antibiotics and ARGs. Some studies reported there is a correlation between presence and removal of antibiotics and ARGs (Wu et al., 2010; Rodriguez-Mozaz et al., 2015), and some studies showed no or partial correlations (Gao et al., 2012b; Xu et al., 2015). In this study, we did not find such correlations either. This is maybe due to the different environments and pollution levels associated to the three different full-scale wastewater treatment systems. Therefore, further and more extensive studies for a multitude of full-scale WWTPs should be performed in order to provide a better insight into the absence or presence of generic correlations between the removal from effluents and the accumulations into sludges of antibiotics, ARB, and ARGs.

Overall, all WWTPs reduced ARGs significantly ($P < 0.05$), with respectively 2.0, 1.3, and 2.3 log ARGs for WWTP A, B, and C (Figure 5.4 and Table S5.8). The highest removal was found for *ermB*, respectively 2.92, 2.22, and 3.11 log for WWTP A, B, and C. Finally, *sul1* and *intI1* were least removed in all WWTPs, with less than 1.4 log removal.

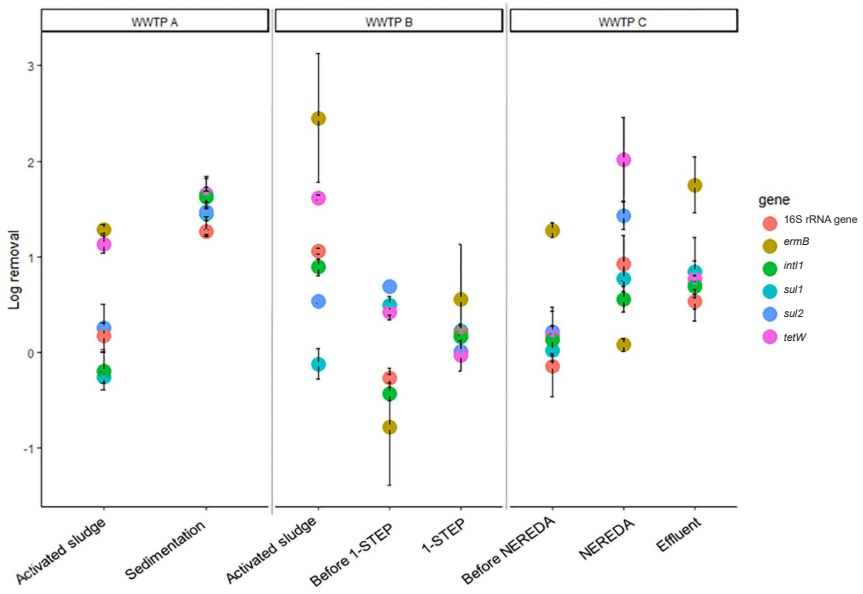


Figure 5.4: Log removal of ARGs in the water at different sampling points in the three studied WWTPs Error bars indicate the standard error of the respective gene data set (duplicate).

5.3.5 ARGs removal in conventional and additional treatment technologies

The efficiency of conventional and additional treatment technologies in removing ARGs was evaluated by comparing their presence in the water and sludge at different stages. First, two conventional treatments were evaluated, activated sludge in WWTP A and WWTP B.

Unlike antibiotics, ARGs did decrease significantly after the activated sludge process. In WWTP A, all ARGs decreased (except *sul1* and *intI1*), ranging from 0.26 log (*sul2*) to 1.29 log (*ermB*). *Sul1* increased 0.26 log while *intI1* increased 0.20 log. In WWTP B, conventional treatment removed 0.53 log *sul2* to 2.45 *ermB*, whereas *sul1* increased 0.12 log.

In the sedimentation tank, the average removal for *ermB* was 1.63 log, 1.45 log for *sul1*, 1.47 log for *sul2*, 1.66 log for *tetW*, and 1.26 log for *intI1*. For sand filtration, average removal for *ermB* was 1.75 log, 0.84 log for *sul1*, 0.71 log for *sul2*, 0.77 log for *tetW*, and 0.53 log for *intI1*. Interestingly, the sludge settling (sedimentation) tank of WWTP A showed the highest log removal in the water phase among the studied treatments. This implies that the sedimented sludge contains a large amount of the ARGs, as also observed by others (Lee et al., 2017; Nnadozie et al., 2017; Su et al., 2018). The final treatment in WWTP A, the sand filtration, also decreased the concentrations of ARGs. Similar removal in conventional WWTPs was shown by Hu et al. (2018), e.g. *sul1*, *sul2*, and *intI1* were removed in the flocculation, sedimentation, and sand filtration tank.

The 1-STEP®-filter removed -0.03 log (*tetW*) to 0.55 log (*ermB*). Overall, 1-STEP® showed the least removal of ARGs. Its activated carbon filter is known for removing organic contaminants, natural organic matter, humic and fulvic acids, and biodegradable compounds. However, poor removal in this study indicated that the ARGs did not adsorb onto the activated carbon may be due to the majority of genes being present in viable bacterial and non-adhering cells, that were not removed from the water phase during filter passage.

The additional treatment technology NEREDA® removed 0.08 log (*ermB*) to 2.02 log (*tetW*) and showed the second highest log removal of ARGs. The granules in the NEREDA® retain organic waste fractions and bacteria in close proximity to each other, thus allowing interactions to occur, including cell-cell communication, and the formation of synergistic microbial consortia (Flemming and Wingender, 2010). Furthermore, the excellent settling properties result in high biomass concentrations (Liu et al., 2003). These properties result in the accumulation of the ARGs within the NEREDA® granules, those granules sink at the bottom and reduce the concentration ARGs in the water. The capture mechanism of ARGs either in solution, bound to suspended solids, or as present of in free bacterial cells by NEREDA granules, is yet to be defined.

The total ARGs in our study were increased in the sludge with an average of 1.26 log in WWTP C. The NEREDA® granules consist of EPS-producing bacteria such as bacteria belonging to the order Xanthomonadales, Sphingomonadales, and family of Rhizobiales (Hyphomicrobiaceae) (Świątczak and Cydzik-Kwiatkowska, 2018). EPS also has the potential to control the lateral transfer of ARGs. This may result in an accumulation of ARGs and indicates that sludge can represent a sink for resistant bacteria and might become an important reservoir for the ARGs (Zhang et al., 2016a).

This study, however, is subject to two critical points; the sampling method (grab sampling) and the limited duration of the sampling campaign (two months). Grab sampling may help in determining the presence of the compounds of interests; however, it captures the concentration of antibiotics and ARGs at a specific time. Furthermore, the data only represent two months in winter, and the result might differ in different seasons throughout the year. However, our approach provides data and insights on the performance of WWTPs with additional treatment technologies (in this study, 1-STEP® and NEREDA®) in removing antibiotics and ARGs. Future research could, for instance, perform studies during a longer time (e.g., 1 year) by using composite sampling. Such research could contribute to better understanding the performance of WWTPs with additional treatment technology over time. Improvement or upgrading of the treatment technology can then be more specifically proposed. Furthermore, only 1 WWTP with advanced treatment options per type of treatment was available for this study, basically because the number of full-scale installations with these additional treatments in the Netherlands is limited, due to their innovative character. As a result, the results might be affected by local sewage parameters. Results from additional WWTPs with similar treatments are

therefore needed.

5.3.6 Implications on public health, water industry, and regulations

Clean water as a source for drinking water is increasingly becoming limited due to climate change, urbanization, and growing populations in the world. Therefore, wastewater reuse is considered as an alternative to tackle this problem (Angelakis et al., 2018). However, the increasing presence of antibiotics, ARB, and their associated ARGs in water are of concern(Hong et al., 2013). Water pollution has been listed as one of the top three concerns in water industry, together with climate change and political instability, from a survey conducted by American Water Works Association (AWWA, 2019). However, there are currently no legal regulations or guidelines that define the permitted levels of antibiotics or antibiotic resistance determinants that are allowed into the environment (Pazda et al., 2019).

This study shows that antibiotics and ARGs are present in WWTP effluent, even with additional treatment technologies. Such technologies can induce the mitigation of antibiotic and ARGs emissions to a limited extent. Here, we show that the removal efficiency of additional activated carbon and AGS differs. Therefore, techniques for advanced treatment should be chosen carefully, depending on the micropollutants targeted in a specific situation. For example, AC is not recommended when there is recreational water downstream and ARGs removal is needed. If limited human exposure to ARG is intended, AC only modestly increases ARG removal according to our results, and is thus insufficient. The wastewater macro- and micropollutants (antibiotics and ARGs included) have a high impact on public health if the removal is insufficient and discharged effluents are directly or indirectly reused water for irrigation, washing and drinking water preparation (Helmecke et al., 2020).

5.4 Conclusion

In this study, the removal of antibiotics and ARGs were studied in water and sludge of three WWTPs with (1-STEP® filter and NEREDA®) and without additional treatment technologies. Total concentrations of 3000 ng/L antibiotics were found in the influent and decreased over the different treatment steps to less than 1000 ng/L in the effluent. Tetracyclines and quinolones concentrations were elevated in the water after the activated sludge treatment step. This shows that these compounds were able to adsorb and desorb in the sludge, with the activated sludge acting as a reservoir for quinolones. Generally, good removal (79-88%) of total antibiotics were observed at all WWTPs. However, sulfonamides and quinolones were still present in the effluent in all three WWTPs, with or without additional treatment technologies.

All WWTPs showed 1-2 log removal for the analyzed ARGs from influent to effluent. Of the measured ARGs, *ermB* was most abundant in the influent, and the most removed ARG in the three WWTPs. WWTPs with or without additional treatment technologies were able to reduce antibiotics with a similar efficiency, although ARGs were best removed in NEREDA®, followed by conventional treatment. This is the first study of removing antibiotics and ARGs in NEREDA®. The 1-STEP® filter decreased the concentrations of ARGs with up to 0.5 log extra on top of the reduction in the conventional part of the plant. When looking at specific treatments, the sedimentation tank showed the highest log removal of ARGs. In the activated sludge, a relatively higher concentration of ARGs was detected compared to other treatment steps, suggesting that sludge is an important reservoir and transmission point for the ARGs. This study demonstrates that in most cases, WWTP with additional treatment technologies have the potential to provide a higher removal of both antibiotics and ARGs compared to conventional WWTP. Further research is needed to identify and optimize the most suitable treatment technology, and further reduce spreading of antibiotics and ARGs via WWTPs into the environment.

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Supplementary information

Text S5.1: Detailed information on the used WWTP.
Three WWTPs with different treatment technologies were evaluated; a conventional treatment with activated sludge, sedimentation and sand filtration (WWTP A), and two advanced treatment systems; 1-STEP® filter (WWTP B) and NEREDA® (WWTP C). Previous research has shown that WWTPs with or without advance treatments effectively remove both COD and nutrients to produce clean effluent (Radjenovic et al., 2007; Nourmohammadi et al., 2013; Ekama, 2015; Liu et al., 2016b; Ma et al., 2018; McConnell et al., 2018; Wang et al., 2018b).

The 1-STEP® filter in WWTP B combines a few different technologies: filtration, denitrification, coagulation, flocculation, and adsorption. Its activated sludge compartment showed elevated COD and TP concentrations in the water phase, which might be related to the degradation of organic compounds by aerobic microorganisms (Ma et al., 2018). The 1-STEP® filter also contains activated carbon, which has been widely used to remove TOC and nutrients from wastewater because of its high adsorption capacity (Putra et al., 2009). Similarly, the aerobic granular sludge in the NEREDA® plant allows the removal of carbon, nitrogen, and phosphorus in a single sludge system (de Kreuk et al., 2005; Nancharaiah and Reddy, 2018). The layered structure of the aerobic granules, which consist of the aerobic outer layer and an anaerobic/anoxic core, enable nitrifying bacteria to accumulate outside the granule and denitrifiers inside the granule core (Winkler et al., 2012). (Świątczak and Cydzik-Kwiatkowska, 2018) also observed that organic and nutrient in aerobic granular sludge might be removed by the abundances of Betaproteobacteria, Deltaproteobacteria, Flavobacteria, and Cytophagia.

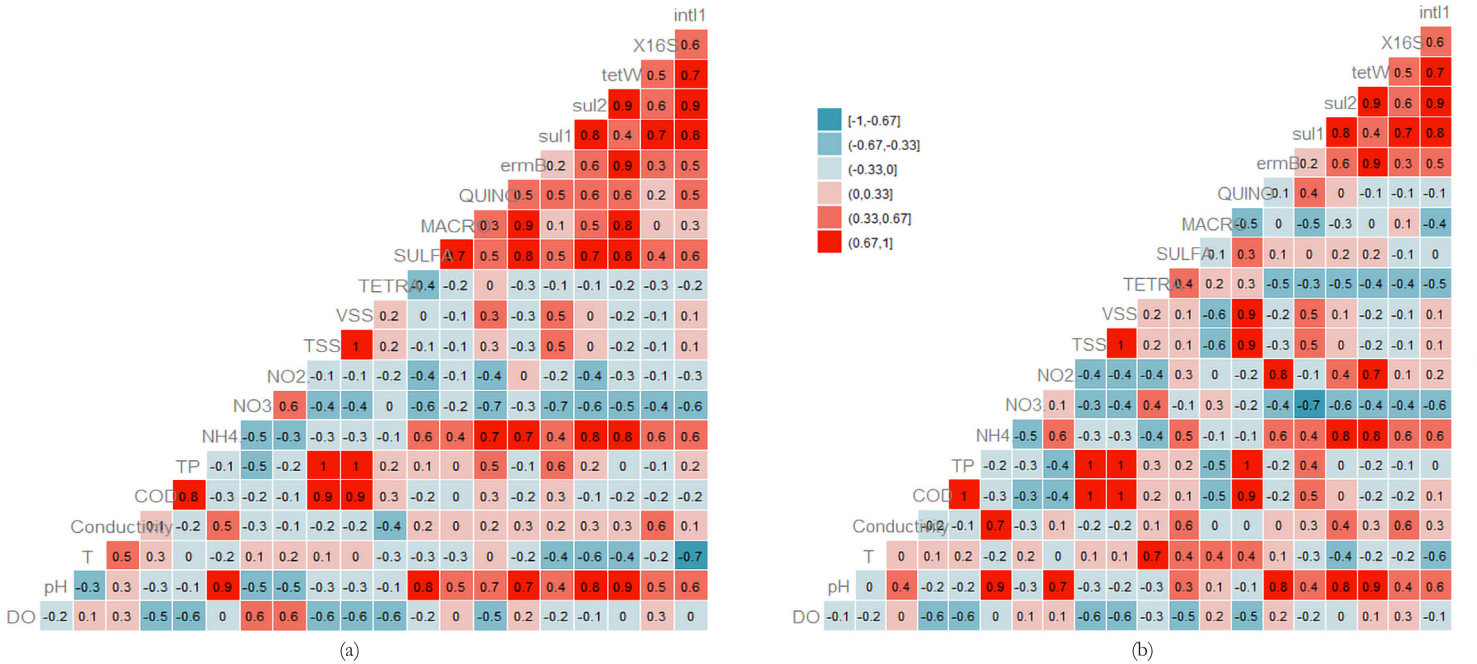


Figure S5.1: Correlation between the concentration of antibiotics, ARGs, and nutrients and operational parameters in (a) February and (b) March.

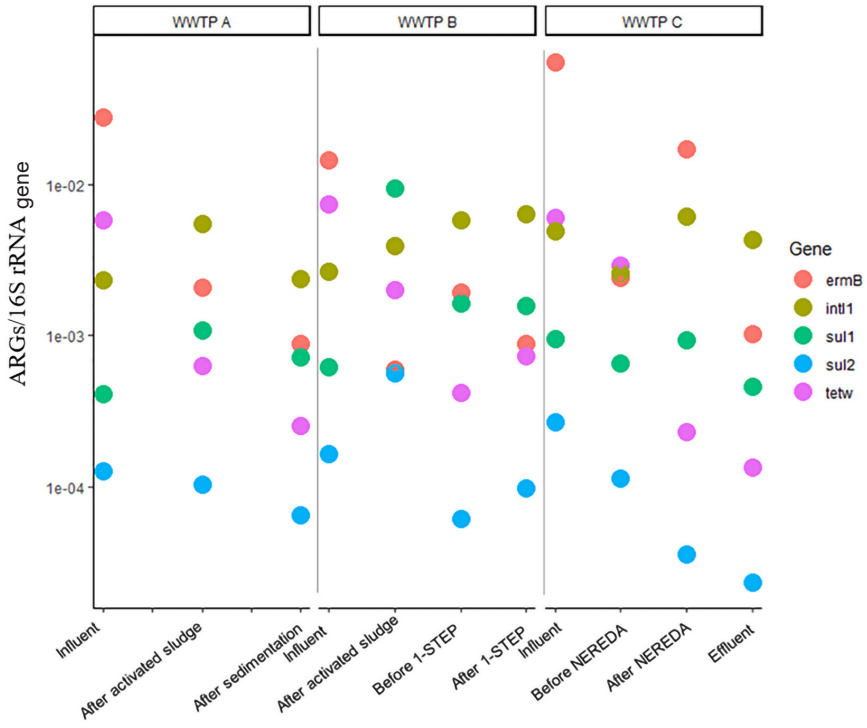
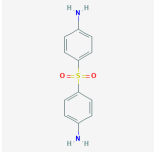
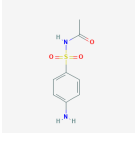
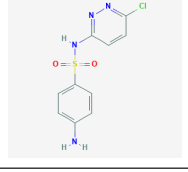
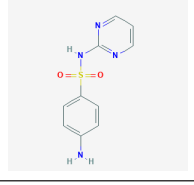
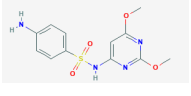


Figure S5.2: Relative abundance of the ARGs to 16S rRNA gene in the water at different sampling points in three wastewater treatment plants.

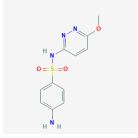
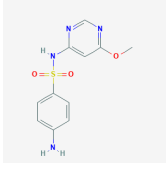
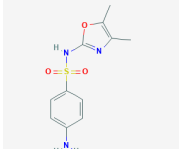
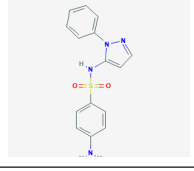
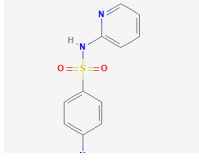
Table S5.1: Information of WWTPs and the treatment technology.

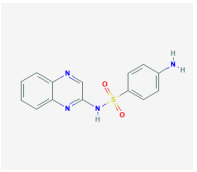
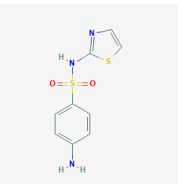
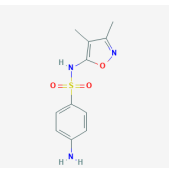
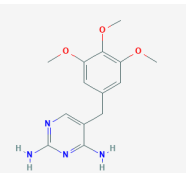
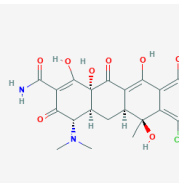
WWTP	WWTP A	WWTP B	WWTP C
Technology	Activated sludge	1-STEP® filter	NEREDA® granular activated sludge
Influent Composition	Domestic wastewater, and rainwater	Domestic wastewater, and rainwater	15% water from slaughter houses, 85% domestic wastewater and rain water
Average volume treated / day	2860 m^3/day	25,000 m^3/day	8000 m^3/day
Person Equivalent (p.e)	22,000 p.e	150,000 p.e.	59,000 p.e.
Peak flow	1000 m^3/h	5000 m^3/h	1500 m^3/h
Hydraulic retention time	2 days	1.6 days	3 days
Suspended solids	3 g/L	4 g/L	2 g/L
Biological oxygen demand (influent/effluent)	268/1 mgO_2/L	235/2.2 mgO_2/L	263/2 mgO_2/L
Solids retention time	12 days	22 days	21 days
Effluent limits	TN< 5 mg/L and TP< 0.3 mg/L	TN< 5 mg/L and TP< 0.5 mg/L	TN< 5 mg/L and TP< 0.3 mg/L

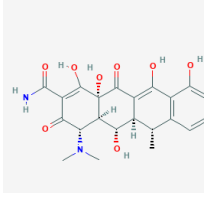
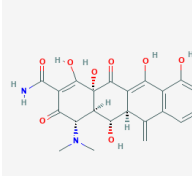
Table S4.1: List of targeted antibiotics and their chemical properties.

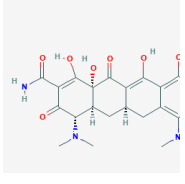
	Antibiotics	Molecular formula	Molecular weight (g/mol)	pK_a	Log K_{ow}	CAS	Chemical structure	Antibiotic group
1	Dapson	$C_{12}H_{12}N_2O_2S$	248.3	2.41	0.97	80-08-0		Sulfonamides
2	Sulfacetamide	$C_8H_{10}N_2O_3S$	214.24	-	-	144-80-9		
3	Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	284.72	-	-	80-32-0		
4	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	250.28	6.36	-	68-35-9		
5	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	310.33	-	-	122-11-2		

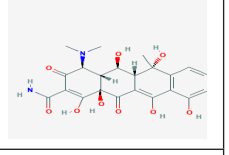
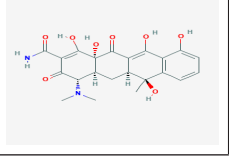
							Sulfonamides
6	Sulfadimidine	$C_{12}H_{14}N_4O_2S$	278.33	7.59	0.14	57-68-1	
7	Sulfadoxine	$C_{12}H_{14}N_4O_4S$	310.33	-	-	2447-57-6	
8	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	264.31	-	-	127-79-7	
9	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	270.3	Pka1: 2.1 pka2: 5.3	0.54	144-82-1	
10	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28	pKa1 = 1.6; pKa2 = 5.7	0.89	723-46-6	

11	Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	280.31	-	-	80-35-3		Sulfonamides
12	Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	280.31	-	-	1220-83-3		
13	Sulfamoxole	$C_{11}H_{13}N_3O_3S$	267.31	-	-	729-99-7		
14	Sulfaphenazole	$C_{15}H_{14}N_4O_2S$	314.4	-	-	526-08-9		
15	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	249.29	8.43	-	144-83-2		

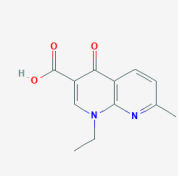
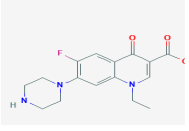
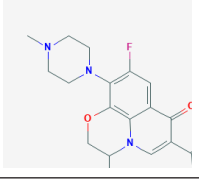
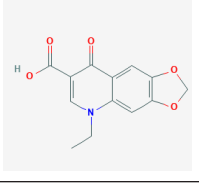
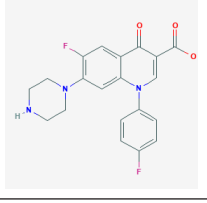
16	Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	300.34	5.1	1.68	59-40-5		Sulfonamides
17	Sulfathiazole	$C_9H_9N_3O_2S_2$	255.3	pKa1 = 2.2; pKa2= 7.24	0.05	72-14-0		
18	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	267.31	2.2	0.05	127-69-5		
19	Trimethoprim	$C_{14}H_{18}N_4O_3$	290.32	7.12 (at 20°C)	0.91	738-70-5		Trimethoprim
20	Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	478.9	-	-	57-62-5		Tetracyclines

21	Doxycycline	$C_{22}H_{24}N_2O_8$	444.4	3.09	-	564-25-0		Tetracyclines
22	Methacyclin	$C_{22}H_{22}N_2O_8$	442.4	-	-	914-00-1		

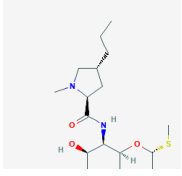
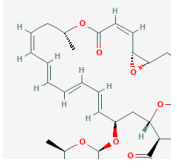
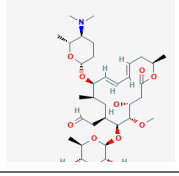
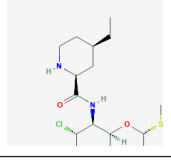
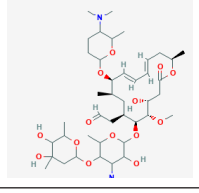
23	Minocycline	$C_{23}H_{27}N_3O_7$	457.5	pKa1= 2.8;
				pKa2= 5.0;
				pKa3= 7.8;
				pKa4= 9.3
					0.05	10118-90-8		

24	Oxytetracycline	$C_{22}H_{24}N_2O_9$	460.4	3.27	-0.90	79-57-2		
25	Tetracycline	$C_{22}H_{24}N_2O_8$	444.4	3.3 At 25°C	-1.37	60-54-8		

							Quinolones
26	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	331.34	6.09	0.28 (non-ionized)	85721-33-1	
27	Danofloxacin	$C_{19}H_{20}FN_3O_3$	357.4	-	-	112398-08-0	
28	Difloxacin	$C_{21}H_{19}F_2N_3O_3$	399.4	-	-	98106-17-3	
29	Enrofloxacin	$C_{19}H_{22}FN_3O_3$	359.4	-	-	93106-60-6	
30	Flumequine	$C_{14}H_{12}FNO_3$	261.25	6.5	1.6	42835-25-6	
31	Marbofloxacin	$C_{17}H_{19}FN_4O_4$	362.4	-	-	115550-35-1	

32	Nalidixic acid	$C_{12}H_{12}N_2O_3$	232.23	8.6	1.41	389-08-2		Quinolones
33	Norfloxacin	$C_{16}H_{18}FN_3O_3$	319.33	pKa1 = 6.34; pKa2 = 8.75	0.46	70458-96-7		
34	ofloxacin_levofloxacin	$C_{18}H_{20}FN_3O_4$	361.4	pKa1 = 5.97 (carboxylic acid); pKa2 = 9.28 (piperizinyl ring)	-0.39	82419-36-1		
35	Oxolinic acid	$C_{13}H_{11}NO_5$	261.23	-	-	14698-29-4		
36	Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	385.4	pKa1= 5.6; pKa2 = 8.2	1.07 (est)	98105-99-8		

37	Trovafloxacin	$C_{20}H_{15}F_3N_4O_3$	416.4	-	-	147059-72-1	
							Quinolones
38	Tylvalosin	$C_{53}H_{87}NO_{19}$	1042.3	-	-	63409-12-1	
39	Erythromycin	$C_{37}H_{67}NO_{13}$	733.9	8.9	3.06	114-07-8	
40	Gamithromycin	$C_{40}H_{76}N_2O_{12}$	777	-	-	145435-72-9	
41	Josamycin	$C_{42}H_{69}NO_{15}$	828	-	-	16846-24-5	
							Macrolides

42	Lincomycin	$C_{18}H_{34}N_2O_6S$	406.5	7.6	0.20	154-21-2		Macrolides
43	Natamycin	$C_{33}H_{47}O_{13}N$ or $C_{33}H_{47}NO_{13}$	665.7	-	-	7681-93-8		
44	Neospiramycin I	$C_{36}H_{62}N_2O_{11}$	698.9	-	-	70253-62-2		
45	Pirlimycin	$C_{17}H_{31}ClN_2O_5S$	411	-	-	79548-73-5		
46	Spiramycin	$C_{43}H_{74}N_2O_{14}$	843.1	pKa1 = 7.88; pKa2 = 9.28 (est)	1.87 (est)	8025-81-8		

							Macrolides
47	Tildipirosin	$C_{41}H_{71}N_3O_8$	734	-	-	328898-40-4	
48	Tilmicosin	$C_{46}H_{80}N_2O_{13}$	869.1	8.18 (tertiary amine)	3.80	108050-54-0	
49	Tulathromycin	$C_{41}H_{79}N_3O_{12}$	806.1	-	-	217500-96-4	
50	Tylosin	$C_{46}H_{77}NO_{17}$	916.1	7.73	1.63	1401-69-0	
51	Valnemulin	$C_{31}H_{52}N_2O_5S$	564.8	-	-	101312-92-9	

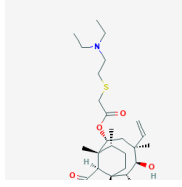
52	Tiamulin	$C_{28}H_{47}NO_4S$	493.7	9.51 (est)	4.75 (est)	55297-95-5		Macrolides

Table S5.3: Limit of quantification for each antibiotic (ng/L).

		Antibiotics	Limit of quantification
1	Sulfonamides	Dapson	5
2		Sulfacetamide	5
3		Sulfachloropyridazine	5
4		Sulfadiazine	5
5		Sulfadimethoxine	5
6		Sulfadimidine	5
7		Sulfadoxine	5
8		Sulfamerazine	5
9		Sulfamethizole	5
10		Sulfamethoxazole	5
11		Sulfamethoxypyridazine	5
12		Sulfamonomethoxine	5
13		Sulfamoxole	5
14		Sulfaphenazole	5
15		Sulfapyridine	5
16		Sulfaquinoxaline	5
17		Sulfathiazole	5
18		Sulfisoxazole	5
19	Trimethoprim	Trimethoprim	5
20	Tetracyclines	Chlortetracycline	500
21		Doxycycline	500
22		Methacyclin	500
23		Minocycline	>1000
24		Oxytetracycline	100
25		Tetracycline	50
26	Quinolones	Ciprofloxacin	25
27		Danofloxacin	25
28		Difloxacin	25
29		Enrofloxacin	25
30		Flumequine	25
31		Marbofloxacin	25
32		Nalidixic acid	25
33		Norfloxacin	25
34		ofloxacin_levofloxacin	25
35		Oxolinic acid	25
36		Sarafloxacin	25
37		Trovafloxacin	25

38	Macrolides	Tylvalosin	500
39		Erythromycin	25
40		Gamithromycin	>1000
41		Josamycin	50
42		Lincomycin	5
43		Natamycin	25
44		Neospiramycin I	>1000
45		Pirlimycin	5
46		Spiramycin	5
47		Tildipirosin	>1000
48		Tilmicosin	>1000
49		Tulathromycin	>1000
50		Tylosin	50
51		Valnemulin	50
52		Tiamulin	5

Table S5.4: Overview of primer sequencing and thermal cycling conditions for qPCR.

ARGs	Sequence (5'-3')	Thermal profile	Cycles	Detection format	Reference
16S rRNA gene	ACTCCTACGGGAGGGCAG GACTACCAGGGTATCTAATCC	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	SYBR Green	Fierer et al. (2005)
intI1	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	TaqMan	Barraud et al. (2010)
ermB	AAAACTTACCCGCCATACCA TTTGGCGTGTTTCATTGCTT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	SYBR Green	Knapp et al. (2010)
sul1	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	TaqMan	Heuer and Smalla (2007)
sul2	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT	95 °C 3 min 95 °C 15 s, 60 °C 45 s	40	TaqMan	Heuer et al. (2008)
tetW	CGGCAGCGCAAAGAGAAC TTTGGCGTGTTTCATTGCTT	95 °C 3 min 95 °C 15 s, 59 °C	45	TaqMan	Walsh et al. (2011)

Table S5.5: Physicochemical and nutrient for each WWTP. The values are average of two sampling days per month for (a) February (b) March.

(a)

		DO (mg/L)	pH	T (water) (°C)	Conductivity (µS/cm)	COD* (mg/L)	TP (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	TSS (g/L)	VSS (g/L)
WWTP A	**Influent**	4.0 ± 0.6	8.0 ± 0.4	7.6 ± 3.5	986.5 ± 166.2	61.0 ± 1.8	10.3 ± 2.8	48.7 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
	After activated sludge	2.1 ± 0.7	7.0 ± 0.1	7.2 ± 1.3	486.0 ± 49.5	334.0 ± 0.0	29.4 ± 0.1	1.4 ± 1.5	0.5 ± 0.0	0.2 ± 0.1	2.5 ± 0.1	2.1 ± 0.1
	After sedimentation	2.6 ± 0.5	7.1 ± 0.1	7.3 ± 1.5	479.5 ± 55.9	29.1 ± 2.2	0.4 ± 0.2	1.5 ± 1.4	5.8 ± 3.8	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
WWTP B	**Influent**	1.3 ± 0.1	7.6 ± 0.1	11.4 ± 0.3	1067.5 ± 10.6	110.4 ± 70.9	8.8 ± 0.0	47.6 ± 3.4	0.5 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	After activated sludge	0.3 ± 0.2	6.8 ± 0.1	10.9 ± 0.4	1064.5 ± 108.2	2366.0 ± 0.0	39.2 ± 4.1	0.2 ± 0.2	0.8 ± 0.3	0.1 ± 0.2	3.9 ± 0.0	2.9 ± 0.0
	Before 1-STEP®	7.1 ± 0.3	7.1 ± 0.1	10.4 ± 0.5	1089.5 ± 119.5	74.1 ± 5.5	0.3 ± 0.1	0.3 ± 0.2	7.6 ± 0.3	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	After 1-STEP®	2.3 ± 1.3	7.1 ± 0.1	10.6 ± 0.7	1081.5 ± 132.2	32.0 ± 2.1	0.2 ± 0.1	0.1 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
WWTP C	**Influent**	3.6 ± 3.7	7.6 ± 0.2	6.5 ± 1.3	1146.5 ± 679.5	57.2 ± 0.7	6.4 ± 2.2	40.1 ± 29.6	0.6 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1
	Before NEREDA®	7.3 ± 0.4	7.5 ± 0.2	9.5 ± 2.3	1399.0 ± 782.1	61.0 ± 0.7	6.1 ± 2.6	40.7 ± 29.6	0.7 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	After NEREDA®	7.9 ± 0.7	6.7 ± 0.0	9.9 ± 0.1	961.5 ± 75.7	56.7 ± 7.7	0.5 ± 0.0	3.1 ± 0.5	4.2 ± 1.1	0.4 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
	Effluent	7.5 ± 0.7	6.8 ± 0.0	10.0 ± 0.6	922.0 ± 49.5	36.7 ± 8.6	0.2 ± 0.1	1.2 ± 1.2	5.5 ± 0.2	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0

* COD value slightly affected by the sedimentation in stored samples.

(b)

		DO (mg/L)	pH	T (water) (°C)	Conductivity (µS/cm)	COD* (mg/L)	TP (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	TSS (g/L)	VSS (g/L)
WWTP A	**Influent**	4.9 ± 0.7	8.4 ± 0.1	11.4 ± 0.4	493.7 ± 689.1	567.3 ± 553.3	10.2 ± 4.4	59.1 ± 16.1	0.8 ± 0.4	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
	After activated sludge	1.1 ± 0.6	7.0 ± 0.1	8.9 ± 0.3	345.0 ± 97.6	3366.3 ± 1225.1	81.8 ± 2.5	0.6 ± 0.8	0.3 ± 0.1	0.0 ± 0.0	2.6 ± 0.1	2.1 ± 0.1
	After sedimentation	3.4 ± 0.3	7.0 ± 0.1	9.0 ± 0.6	328.5 ± 75.7	24.5 ± 5.4	0.4 ± 0.1	1.6 ± 0.7	2.9 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
WWTP B	**Influent**	1.4 ± 0.1	7.5 ± 0.0	13.1 ± 0.6	1232.5 ± 176.1	153.3 ± 8.1	12.5 ± 3.1	57.4 ± 0.1	0.9 ± 0.5	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	After activated sludge	1.5 ± 1.3	6.9 ± 0.0	13.1 ± 1.1	755.5 ± 112.4	4518.0 ± 82.0	177.8 ± 9.5	0.8 ± 0.8	0.9 ± 0.6	0.0 ± 0.0	3.8 ± 0.2	2.8 ± 0.0
	Before 1-STEP®	4.5 ± 2.7	7.2 ± 0.0	12.9 ± 0.8	751.5 ± 85.6	59.6 ± 5.4	0.2 ± 0.1	0.0 ± 0.0	3.7 ± 0.3	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
	After 1-STEP®	3.8 ± 3.9	7.2 ± 0.1	13.0 ± 0.4	745.0 ± 90.5	37.1 ± 3.0	0.1 ± 0.0	0.0 ± 0.1	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
WWTP C	**Influent**	4.9 ± 0.3	7.7 ± 0.1	7.1 ± 0.1	1423.5 ± 256.7	103.1 ± 73.5	8.8 ± 1.9	64.8 ± 20.6	0.6 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
	Before NEREDA®	6.6 ± 0.1	7.6 ± 0.1	11.3 ± 1.2	1223.0 ± 168.3	113.5 ± 72.8	7.3 ± 0.5	47.5 ± 3.2	0.5 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
	After NEREDA®	8.2 ± 0.0	6.7 ± 0.1	10.5 ± 0.1	568.0 ± 65.1	47.7 ± 3.3	0.3 ± 0.1	0.2 ± 0.1	1.0 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
	Effluent	8.3 ± 0.0	6.8 ± 0.0	10.6 ± 0.5	544.0 ± 49.5	27.0 ± 2.4	0.1 ± 0.0	0.1 ± 0.1	2.0 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

* COD value slightly affected by the sedimentation in stored samples.

Table S5.6: List of antibiotics below the detection limit.

	Antibiotic compound	Antibiotic group
1	Dapson	Sulfonamides
2	Sulfacetamide	
3	Sulfachloropyridazine	
4	Sulfadimethoxine	
5	Sulfadimidine	
6	Sulfadoxine	
7	Sulfamerazine	
8	Sulfamethizole	
9	Sulfamethoxypyridazine	
10	Sulfamonomethoxine	
11	Sulfamoxole	
12	Sulfaphenazole	
13	Sulfaquinoxaline	
14	Sulfathiazole	
15	Sulfisoxazole	
16	Methacycline	Tetracyclines
17	Minocycline	
18	Oxytetracycline	
19	Danofloxacine	Quinolones
20	Difloxacine	
21	Enrofloxacine	
22	Marbofloxacine	
23	Nalidixinezuur	
24	Oxolinezuur	
25	Sarafloxacine	
26	trovafloxacin	
27	Tylvalosin	Macrolides
28	Erythromycine	
29	Gamithromycine	
30	Josamycine	
31	Natamycine	
32	Neospiramycine I	
33	Pirlimycine	
34	Spiramycine	
35	Tildipyrosine	
36	Tilmicosine	
37	Tulathromycine	
38	Tylosine	
39	Valnemulin	
40	Tiamulin	

Table S5.7: Concentrations of antibiotic resistance genes in (a) water (copies/ml) and (b) sludge (copies/dry weight (g)) at different sampling points in three wastewater treatment plants. Data are mean value ± standard deviation.

(a)

WWTP	Sampling	*ermB*	*sul1*	*sul2*	*tetW*	*intI1*	16S rRNA gene
WWTP A	Influent	1.05E+08 ± 9.88E+07	1.37E+06 ± 6.17E+05	4.60E+05 ± 3.47E+05	2.40E+07 ± 1.95E+07	1.16E+07 ± 1.31E+06	5.89E+08 ± 1.09E+07
WWTP A	After activated sludge	4.40E+06 ± 1.89E+06	2.55E+06 ± 1.42E+06	2.51E+05 ± 1.55E+05	1.39E+06 ± 6.84E+05	1.31E+07 ± 7.53E+06	3.07E+08 ± 6.35E+06
WWTP A	After sedimentation	1.20E+05 ± 3.00E+04	8.36E+04 ± 3.74E+04	7.12E+03 ± 1.05E+03	2.77E+04 ± 3.24E+03	2.96E+05 ± 1.69E+05	5.71E+06 ± 5.70E+04
WWTP B	Influent	3.13E+07 ± 3.78E+06	6.28E+05 ± 6.98E+04	1.47E+05 ± 1.05E+05	5.79E+06 ± 4.29E+06	2.85E+06 ± 3.51E+05	2.27E+08 ± 3.85E+06
WWTP B	After activated sludge	1.05E+05 ± 1.04E+04	1.65E+06 ± 1.79E+05	7.66E+04 ± 7.92E+03	2.64E+05 ± 2.74E+04	4.43E+05 ± 4.47E+04	8.59E+07 ± 2.01E+06
WWTP B	Before 1-STEP®	3.32E+06 ± 1.05E+05	2.45E+05 ± 2.22E+05	7.25E+03 ± 3.66E+03	7.27E+04 ± 7.40E+03	7.10E+05 ± 4.41E+05	4.38E+07 ± 7.68E+05
WWTP B	After 1-STEP®	1.63E+05 ± 8.66E+04	1.27E+05 ± 1.03E+05	7.96E+03 ± 5.32E+03	9.66E+04 ± 2.89E+04	5.99E+05 ± 3.22E+05	4.16E+07 ± 9.59E+05
WWTP C	Influent	1.25E+07 ± 8.41E+05	1.90E+06 ± 1.32E+06	4.32E+05 ± 7.62E+04	1.18E+07 ± 7.73E+06	1.40E+07 ± 1.62E+06	1.01E+09 ± 2.36E+07
WWTP C	Before NEREDA	4.08E+07 ± 3.95E+06	2.65E+06 ± 2.48E+05	3.64E+05 ± 2.59E+05	9.82E+06 ± 7.44E+06	1.03E+07 ± 1.37E+06	2.95E+09 ± 1.06E+08
WWTP C	After NEREDA	3.45E+07 ± 3.14E+06	3.38E+05 ± 3.36E+05	1.04E+04 ± 4.35E+03	6.26E+04 ± 9.33E+03	1.94E+06 ± 1.41E+06	1.59E+08 ± 3.87E+06
WWTP C	Effluent	7.51E+04 ± 1.48E+04	6.35E+04± 7.29E+03	2.71E+03 ± 2.82E+02	1.10E+04 ± 3.01E+03	6.33E+05 ± 8.61E+04	7.31E+07 ± 2.11E+06

(b)

WWTP	Sampling	*ermB*	*sul1*	*sul2*	*tetW*	*intI1*	16S rRNA gene
WWTP A	Influent	2.59E+07 ± 4.15E+06	5.83E+07 ± 4.50E+06	3.47E+06 ± 2.21E+05	7.67E+06 ± 4.59E+05	5.99E+07 ± 1.54E+07	9.76E+09 ± 1.58E+08
WWTP A	Activated sludge	4.37E+07 ± 5.33E+06	1.03E+08 ± 1.22E+05	5.20E+06 ± 3.22E+05	1.22E+07 ± 2.71E+05	1.06E+08 ± 2.65E+07	8.26E+10 ± 3.79E+10
WWTP B	Before dewatering	1.50E+07 ± 1.77E+06	6.04E+07 ± 2.99E+06	5.48E+06 ± 1.40E+05	8.64E+06 ± 7.14E+04	7.78E+07 ± 5.10E+06	1.02E+12 ± 1.84E+10
WWTP B	After dewatering	2.25E+08 ± 7.11E+06	2.57E+07 ± 9.74E+04	3.10E+06 ± 1.12E+03	1.25E+07 ± 1.50E+05	2.56E+07 ± 3.14E+06	2.08E+11 ± 1.34E+11
WWTP C	Before thickening	1.40E+06 ± 3.02E+05	3.12E+06 ± 3.39E+05	1.77E+05 ± 5.30E+03	2.94E+05 ± 7.25E+03	1.35E+06 ± 1.90E+05	6.04E+08 ± 6.64E+07
WWTP C	After thickening	1.58E+07 ± 1.16E+06	3.21E+07 ± 4.37E+06	1.92E+06 ± 8.97E+04	2.47E+06 ± 9.02E+03	2.24E+07 ± 1.54E+06	1.17E+11 ± 1.13E+10

Table S5.8: Log removal of ARGs in the water at different sampling points in the three studied WWTPs. Data are mean value ± standard error.

WWTP	Sampling	*ermB*	*sul1*	*sul2*	*tetW*	*intI1*	16S rRNA gene
WWTP A	Activated sludge	1.29 ± 0.05	-0.26 ± 0.06	0.26 ± 0.25	1.13 ± 0.10	-0.20 ± 0.19	0.17 ± 0.14
WWTP A	Sedimentation	1.63 ± 0.06	1.45 ± 0.07	1.47 ± 0.26	1.66 ± 0.16	1.63 ± 0.21	1.26 ± 0.03
WWTP B	Activated sludge	2.45 ± 0.67	-0.12 ± 0.16	0.53 ± 0.02	1.62 ± 0.03	0.89 ± 0.09	1.06 ± 0.03
WWTP B	Before 1-STEP®	-0.78 ± 0.61	0.49 ± 0.10	0.69 ± 0.00	0.42 ± 0.08	-0.43 ± 0.07	-0.27 ± 0.05
WWTP B	1-STEP®	0.55 ± 0.58	0.23 ± 0.04	0.01 ± 0.21	-0.03 ± 0.01	0.16 ± 0.03	0.21 ± 0.09
WWTP C	Before NEREDA®	1.28 ± 0.08	0.02 ± 0.17	0.22 ± 0.25	0.17 ± 0.26	0.13 ± 0.14	-0.15 ± 0.32
WWTP C	NEREDA®	0.08 ± 0.07	0.77 ± 0.01	1.43 ± 0.15	2.02 ± 0.44	0.55 ± 0.13	0.93 ± 0.29
WWTP C	Effluent	1.75 ± 0.29	0.84 ± 0.12	0.71 ± 0.05	0.77 ± 0.44	0.69 ± 0.11	0.53 ± 0.08

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CHAPTER 6

General discussion

6.1 Introduction

Micropollutants such as pharmaceuticals, personal care products, household chemicals, industrial agents, fragrances, and flame retardants are extensively used in our daily life. Antibiotics, as part of the micropollutants, are applied to inhibit or stop the growth of microorganisms. However, up to 90% of these ingested antibiotics can pass a body (human or animals) without being metabolized and are thus excreted via faeces and urine. Even though antibiotics are present at low concentrations (ranging from ng/l to occasionally mg/l) in the environment, they can affect bacterial metabolism and possibly contribute to resistance features (antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs)) (Jutkina et al., 2018). Through wastewater, ARB&Gs may also accumulate in a wastewater treatment plant (WWTP), and as a result, a WWTP is recognized as a hotspot of antibiotics and ARB&Gs. However, a WWTP is designed to remove bulk organic compounds and nutrients, and not to remove antibiotics and ARB&Gs. Consequently, most of the antibiotics and ARB&Gs are not removed in conventional WWTPs. Hence, additional treatment technologies that can be combined with the current treatment are needed to ensure that antibiotics and ARB&Gs are removed before entering the water system and environment, such as aerobic granular sludge, activated carbon, or constructed wetlands (CWs).

In this thesis, we have measured antibiotics and ARGs in the surface water up to 20 km downstream of the point of discharge of a WWTP with post-treatment (Chapter 2). We saw a significant level of antibiotics and ARGs in the aquatic environment. Therefore, we evaluated the role of the type of treatment technology and explored solutions for reducing antibiotics and ARGs by using natural systems (Chapter 3 and 4), physicochemical technology, namely granular activated carbon (Chapter 5), and advanced biological technologies (Chapter 5). To better understand the fate of and treatment options for antibiotics and ARGs, we have measured organic carbon and nutrients with probes and test kits by colorimetric methods, antibiotic concentrations by using LCMS/MS, and ARGs by using qPCR, in selected WWTPs with and without treatment technology. Figure 6.1 shows an overview of the investigated environments and treatment technologies.

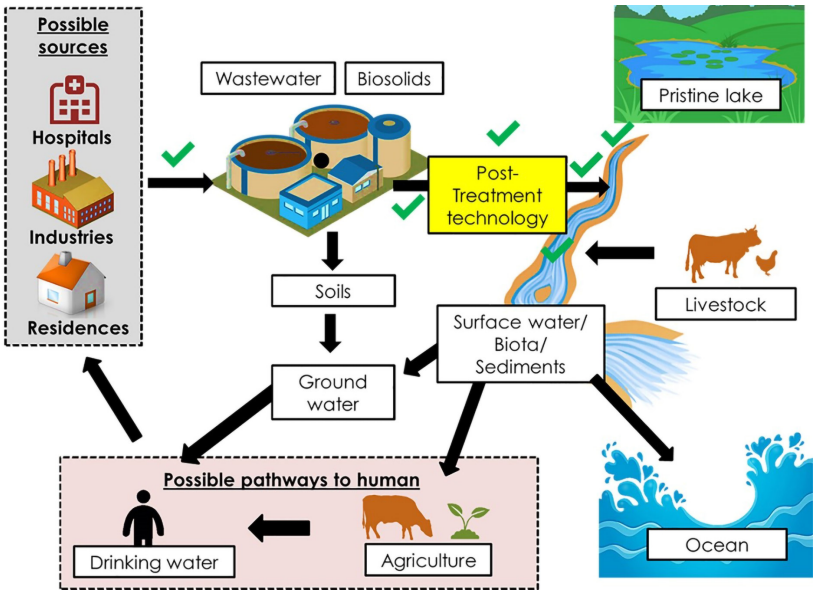

Figure 6.1: Overview of the measured environments and treatment technologies. ✔ represents the locations where antibiotics and antibiotic resistance genes were detected.

In this concluding chapter, three topics are discussed, related to (1) the occurrence of antibiotics and ARGs in the environment, (2) optimal treatment technologies for removing antibiotics and ARGs from wastewater, (3) the fate of antibiotics and ARGs - within and after wastewater treatment, and in the environment. These discussions are based on a case study in the Netherlands as a developed country (high-income country). Additionally, the state of the art of antibiotic resistance occurrence and removal in Malaysia (the author's home country) is presented as a perspective on a developing country (middle-income country).

6.2 The occurrence of antibiotic resistance

Different types and ranges of concentrations of antibiotics and ARGs are detected in rivers around the world, as reported by this study (Chapter 2) and others (Singh et al., 2019). Antibiotics are water-soluble, and are not completely metabolized in human or animal bodies. Antibiotics and ARGs enter surface water mainly via the following discharge pathways: (1) effluent from WWTPs; (2) chemical manufacturing plants; (3) livestock and (4) aquaculture (Singer et al., 2016).

We measured and detected antibiotics and ARGs in a river up to 20 km downstream of the discharge point of a WWTP in the Netherlands (Chapter 2). We observed that the studied WWTP effluent significantly increases antibiotic and ARG (up to 2 log) concentrations in the river when we compared downstream and upstream data. The antibiotics and ARGs showed to be persistent (with minimal effect by dilution) along the river throughout the year. This shows

that (1) WWTPs are a significant anthropogenic source of antibiotics entering surface water, (2) WWTPs are the main source of the antibiotics and ARGs entering this particular surface water and have a role in their spreading to various environmental compartments (water, sediment, biota), (3) antibiotics and ARGs are not easily degraded or removed in the environment after being discharged from WWTP into the surface water. WWTPs as a hotspot for antibiotics and ARGs are also confirmed by Wang et al. (2018).

Since antibiotics and ARGs were detected in the surface water, and originate from the effluent of WWTPs (Chapter 2), we studied six WWTPs to assess the occurrence of the antibiotics and ARGs in the WWTP and their contribution to the emissions of these pollutants (Figure 6.2). The studied WWTPs are located in the centre and the south of the Netherlands. Different additional treatment technologies were employed in full-scale in these WWTPs (Table 6.1). Samples were taken from the influent, the WWTP reactors, the effluent of the WWTPs, and the effluent of additional post-treatment technologies (Chapter 5). In other chapters, samples were taken only from the effluent of the WWTPs, and within and after the post-treatment technologies (Chapter 2, 3, and 4).

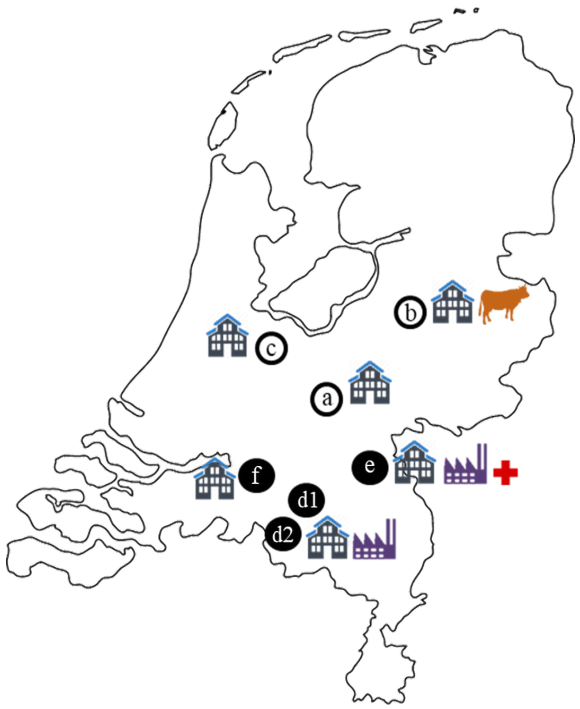

Figure 6.2: Location of the studied WWTPs in the Netherlands. Wastewater treatment plants are labeled as a, b, c, d, e, f. Symbols represent wastewater from slaughterhouse ( ), industrial ( ), hospital ( ) and domestic ( ). ● indicates sampling of effluent of WWTPs and additional treatment technology only. ○ indicates sampling of influent, effluent of WWTPs and additional treatment technology.

Table 6.1: Overview of the WWTPs with and without additional treatment technologies.

	WWTP	Biological treatment	Additional technologies	Function of the additional treatment technologies	Label for treatment technology	Chapter
1	WWTP a	Activated sludge	X	X	AS	5
2	WWTP b	X	Aerobic granular sludge (NEREDA®)	Secondary treatment	NEREDA®	5
3	WWTP c	Activated sludge	Granular Activated Carbon (1-STEP® filter)	Tertiary treatment	1-STEP®	5
4	WWTP d1	Oxidation ditch	Constructed wetland horizontal with HRT of 1 day	Post-treatment	CW1	2,3,4*
	WWTP d2		Constructed wetland horizontal with HRT of 3 days	Post-treatment	CW3	2,4
5	WWTP e	Activated sludge with sand filter	Constructed wetland horizontal with HRT 4 of days	Post-treatment	CW4	3
6	WWTP f	Oxidation ditch with sand filter	Constructed wetland vertical with HRT of 1.7 days	Post-treatment	CWV	3

X indicates no respective treatment at the WWTP. HRT= hydraulic retention time.

As expected, antibiotics and ARGs were detected in the influent of the three tested WWTPs (Chapter 5) treating wastewater from slaughterhouses (15%) and domestic sources (85-100%). The influent characteristics were similar with influent wastewater sources from other studies (Luo et al., 2014). We observed that the number of inhabitant equivalents did not influence the total concentration of antibiotics and ARGs. In other studies, the concentration and types of antibiotics varies and depend heavily on the sources, population, and size of the WWTPs (Pazda et al., 2019). Possibly, these domestic sources were the most significant contributors of antibiotics and ARGs to our tested WWTPs (Chapter 5).

After the treatment, antibiotics and ARGs were still present in the effluent of the WWTPs and also in the effluent of the additional treatment technologies, in this thesis and other studies (Zheng et al., 2017; Song et al., 2018). Sulfonamides, trimethoprim, macrolides, and fluoroquinolones were the most common antibiotics found in WWTPs effluent worldwide and their concentrations, correlated to the consumption of each country (Tran et al., 2018). Moreover, the antibiotic concentration and their removal percentages, as well as the occurrence and log removal of ARGs in WWTPs, also depended on: dynamics of inputs through sewage; ARG bacterial hosts during treatment (Yang et al., 2014; Narciso-da-Rocha et al., 2018); operational conditions (e.g., temperature, hydraulic retention time); microbial composition; and reactor size (Pärnänen et al., 2019).

Given the number of parameters influencing occurrence and removal of antibiotics and ARGs in WWTP, the types of antibiotics and ARGs detected in the effluent of different WWTPs and their concentrations varies greatly. There are different patterns in types and concentrations of antibiotics and ARGs in the effluent of different WWTPs. Lindberg et al. (2005) detected sulfamethoxazole, trimethoprim, fluoroquinolones, ciprofloxacin, ofloxacin, and doxycycline concentrations, ranging between 7 and 900 ng/L, in the effluent of five conventional WWTPs in Sweden. Karthikeyan and Meyer (2006) showed tetracycline, trimethoprim, sulfamethoxazole, macrolides, and fluoroquinolones were present in a range of 50–300 ng/L in the effluent of seven conventional WWTPs in the USA. Leung et al. (2012) confirmed that fluoroquinolones, norfloxacin, β-lactams, macrolides, sulfamethoxazole, and trimethoprim were observed in a range of 170 – 7900 ng/L in the effluent of seven conventional WWTPs in Hong Kong. Du et al. (2014) detected ARG concentrations up to copies/ml after treatment WWTP with anaerobic/anoxic/aerobic-membrane bioreactor.

Effluent can be released to the environment only after meeting specific effluent discharge standards. The purpose of these standards, such as the Urban Waste Water Directive (91/271/EEC) approved and adopted by Europe (Council Directive, 1991), is to reduce the load of organic compounds and nutrients entering the environment. Not only countries in Europe, but also countries outside of Europe have established standards, such as the National Pollutant Discharge Elimination System in the USA (EPA, 2016), and the Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant (GB 18918–2002) in China (Zhang et

al., 2016). These standards are specifically set for organic compounds and nutrients to ensure that water quality is suitable for aquatic organisms and for water reclamation purposes. However, no specific discharge standards for antibiotics or ARGs have been established due to limited knowledge on which environmental concentrations exert a selection for resistant bacteria among environmental, human and livestock microbiomes (Bengtsson-Palme and Larsson, 2016; Pazda et al., 2019). The current legislation at the European level does not define an environmental quality standard for antibiotic concentrations (European Commission, 2017), although some regulations are available for limited compounds such as pesticides (Code of Federal Regulation (40 CFR Part 455)) (Code of Federal Regulations, 1978). Overall, our observations prove that antibiotics and ARGs are abundantly present in our environment, particularly in the influent of WWTPs, within the WWTPs, and in their effluent,as well as in the surface water where WWTP discharge to.

6.3 Optimal treatment technologies

To ensure that effluent from WWTPs meet the earlier mentioned standards, treatment technology plays an important role. Treatment processes in a WWTP typically consist of primary treatment, secondary treatment and additional treatment, if needed (Figure 6.3).

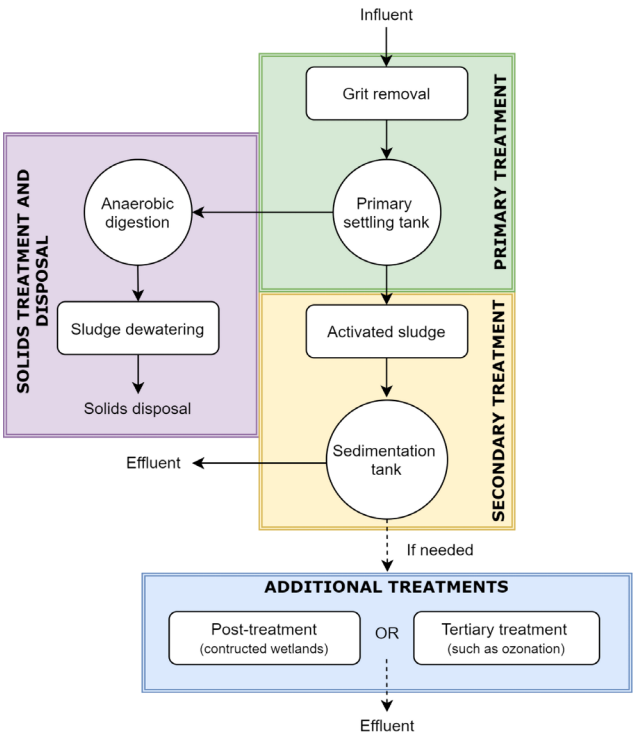

Figure 6.3: Wastewater treatment processes.

Wastewater treatment is needed to remove contaminants from wastewater (Samer, 2015) and ensure the treated water can be recycled back to the water body with minimum impact on the environment and human health (Peirce et al., 1998). A typical WWTP employs various biological and physicochemical processes to remove organic carbon and nutrients (TP, NH_4-N, NO_3-N, NO_2-N) before discharge into the surface water (Wagner and Loy, 2002). Such expected nutrient removal was observed in Chapter 2, 3, 4, 5 for all the studied WWTPs (with or without additional treatment technology) and these were complying to the regulatory targets of the European Water Framework Directive 91/271/EEC (Council Directive, 1991).

WWTPs are able to remove organic carbon and nutrients, but the removal is limited for the antibiotics and ARGs. A conventional WWTP containing activated sludge (AS) with dense flocs, followed by a sedimentation tank is the most commonly used process in WWTPs worldwide (Rajapaksha et al., 2019). AS is comprised of a complex biological community of bacteria, fungi, viruses, protozoa, algae, and metazoa and plays an important part in wastewater treatment around the globe (Miura et al., 2007). Three WWTPs with AS and sedimentation tank (WWTP a, WWTP c (Chapter 5), and WWTP e (Chapter 3)) in Figure 6.2, showed the presence of antibiotics and ARGs in the effluent. This effluent was the influent of the studied constructed wetlands (CWs) as post-treatments. WWTPs appear to be the main source of the dissemination of antibiotics and ARGs towards aquatic ecosystems and connected environmental compartments (Karkman et al., 2018).

In this study, AS did not entirely remove antibiotics and ARGs (Chapter 5), as also demonstrated by others (Zhang et al., 2015b). Additional treatments are needed to enhance the removal of the antibiotics and ARGs. Various studies related to lab-scale tertiary treatments showed promising results. Stange et al. (2019) showed that UV and chlorination reduced up to 5.6 log of the bacteria and up to 2.8 log of ARGs. Zheng et al. (2017) reported that ozonation reduced ARGs by up to 2.5 log. Iakovides et al. (2019) demonstrated that the ozonation degraded eight antibiotic compounds and reduced 2 to 3 log of ARGs.

Despite these positive results, there are also drawbacks of these treatments. Selection of ARB might occur during or after treatment (Zhang et al., 2017), which might lead to a negligible reduction on ARGs (Stange et al., 2019; Wang et al., 2019). However, the translation from lab-scale to pilot/full scale is challenging, and therefore data from full-scale treatment systems is needed.

Different full-scale technologies were compared in this thesis: aerobic granular sludge (technology NEREDA®; Chapter 5), granular activated carbon (using 1-STEP® filter; Chapter 5), a CW with two types of water flow, horizontal surface flow (Chapter 3 and 4) and vertical flow (Chapter 3). These additional treatment technologies showed potential in removing selected antibiotics (Table 6.2) and ARGs (Table 6.3). The performance of the WWTPs with additional treatment technologies improved the removal of antibiotics (Table 6.4 for removal along the

whole WWTP including the additional treatment) and ARGs (Table 6.5). Each treatment technology is discussed in the next subchapter.

Table 6.2: Overview of the removal percentage of selected antibiotics for additional treatment technologies.

	This study							Others studies		
	AS	NEREDA®	1-STEP®	CW				Wang et al. (2019)	Östman et al. (2019)	
				CW1	CW3	CW4	CWV	Aerobic granular sludge	O_3	GAC
SMX								NA	NA	NA
TRI						NA	NA	NA		
CIP						NA	NA		NA	NA
AZI	ND	ND	ND			NA	NA	NA	NA	NA
CLA	ND	ND	ND			NA	NA	NA	NA	NA
LIN								NA	NA	NA

NA = Not available; ND=not detectable, = increase = 0 - <20% removal, = 20 - 50% removal, = 50 - <80% removal, = >80% removal. SMX=sulfamethoxazole, TRI=trimethoprim, CIP=ciprofloxacin, AZI=azithromycin, CLA=clarithromycin, LIN=lincomycin

- The percentage of antibiotic removal was calculated before and after the additional treatment technologies.
- Removal efficiency for antibiotics is assessed in four categories: none/increase concentration after the additional treatment technologies, low (0 – <20%), moderate (20 – <50%), good (50 - <80%) or excellent (>80%) removal efficiency.
- AS= activated sludge and sedimentation (Chapter 5), CW1 = a horizontal surface flow (HSF) CW with HRT of 1 day (Chapter 3 and 4), CW3 = HSFCW with HRT- of 3 days (Chapter 3), CW4 = HSFCW with HRT of 4 days (Chapter 3), CWV = vertical subsurface flow CW (Chapter 3), O3 = ozonation, GAC = granular activated carbon.

Table 6.3: Overview of the log removal of *intI1* and antibiotic resistance genes (gene copies/ml) for additional treatment technologies.

	This study							Other studies	
	AS	NEREDA®	1-STEP®	CW				Iakovides et al. (2019)	Jun et al. (2014)
				CW1	CW3	CW4	CWV	O_3	CW
ermB	2.9	0.1	0.6	1.0	1.1	0.2	1	NA	<2
sul1	1.2	0.8	0.2	1.0	0.4	0.3	0.3	<2	<1
sul2	1.7	1.4	0.0	0.9	0.3	0.4	0.2	NA	<1
tetW	2.8	2	0.0	0.4	0.4	NA	NA	NA	NA
intI1	1.4	0.6	0.2	0.9	0.4	0.1	0.2	<3	<1

NA = Not available, = increase = <1 log = <2 log = <3 log =<4 log

- The log removal of ARGs was calculated from the ARG concentration before and after the additional treatment technologies (in gene copies/ml).
- Log removal for ARGs is assessed in four categories; none/increase concentration after the additional treatment technologies, low (0 – 0.99 log), moderate (1 – 1.99 log), good (2 – 2.99 log) or excellent (3 – 3.99 log) removal efficiency.
- AS= activated sludge and sedimentation (Chapter 5), CW1 = a horizontal surface flow (HSF) CW with HRT of 1 day (Chapter 3 and 4), CW3 = HSFCW with HRT- of 3 days (Chapter 3), CW4 = HSFCW with HRT of 4 days (Chapter 3), CWV = vertical subsurface flow CW (Chapter 3), O3 = ozonation, GAC = granular activated carbon.

Table 6.4: Overview of the removal percentage of selected antibiotics in wastewater treatment plants with additional treatment technology.

	This study			Other studies		
	WWTP a	**WWTP b**	**WWTP c**	**Göbel et al. (2005)**	**Anderson et al. (2013)**	**Rodríguez-Chueca et al. (2019)**
	AS	**NEREDA®**	**1-STEP®**	**AS**	**CW**	**UV**
SMX						
TRI					NA	
CIP				NA	NA	
AZI	ND	ND	ND		NA	
CLA	ND	ND	ND		NA	
LIN				NA	NA	NA

inf=influent, eff=effluent, NA=Not available; ND=not detectable, = increase, = 0 - <20% removal, = 20 - 50% removal, =50 - <80% removal, = >80% removal. SMX=sulfamethoxazole, TRI=trimethoprim, CIP=ciprofloxacin, AZI=azithromycin, CLA=clarithromycin, LIN=lincomycin

- The percentage of antibiotic removal was calculated from the influent of the WWTPs to the effluent of the additional treatment technologies (for WWTP a, WWTP b and WWTP c).
- AS= activated sludge and sedimentation, CW = Constructed wetlands, UV = ultraviolet irradiation.

Table 6.5: Overview of the log removal of *intI1* and antibiotic resistance genes (in gene copies/ml) in wastewater treatment plants with additional treatment technology.

	This study			Other studies			
	WWTP a	**WWTP b**	**WWTP c**	**Di Cesare et al. (2016)**	**Lamori et al. (2019)**	**Di Cesare et al. (2016)**	**Czekalski et al. (2016)**
	AS	**NEREDA®**	**1-STEP®**	**AS**	**CW**	**UV**	**O_3**
ermB	2.9	3.11	2.22	2.4	NA	3.3	NA
sul1	1.2	1.63	0.60	NA	NA	NA	<1
sul2	1.7	2.37	1.24	1.8	NA	1.8	NA
tetW	2.8	2.97	2.01	NA	NA	NA	NA
intI1	1.4	1.37	0.62	2.2	<2	2.0	NA

NA = Not available, = increase = <1 log = <2 log = <3 log =<4 log

- The log removal of ARGs was calculated from 1) the ARG concentration influent of the WWTPs (in gene copies/ml) to the concentration in effluent of the additional treatment technologies (for WWTP a, WWTP b and WWTP c).
- AS= activated sludge and sedimentation, CW = constructed wetlands, UV = ultraviolet irradiation, O_3 = ozonation.

6.3.1 Advanced biological technologies as secondary treatment by using aerobic granular sludge

Aerobic granular sludge is based on fast sedimentation of granules, which is the result of the autoaggregation capacity of the biomass in a system with a high selection pressure (Rollemberg et al., 2019). This technology improves possible operational problems of AS, such as sludge bulking and can reduce space needed for multiple tanks in continuous flow systems (He et al., 2018a). Aerobic granular sludge improves the removal of organic carbon and nutrients (Pronk et al., 2015) and has shown potential for removing antibiotics (Wang et al., 2019) and ARGs.

In this thesis, NEREDA®, which is based on the technology of aerobic granular sludge (Chapter 5), removed antibiotics, ranging from 36% (sulfonamides) to 99% (tetracyclines) and 2.3 log of ARGs, demonstrating reduction of antibiotics and ARGs within the treatment. This may be due to the high microbial activity of aerobic granules (Wang et al., 2019). The aerobic granules are formed by bacteria that produced extracellular polymeric substances (EPS) and the granules are stabilized by slow-growing bacteria (Świątczak and Cydzik-Kwiatkowska, 2018). In addition, EPS influences the surface properties of biomass and increases the sorption of organic pollutants (Schmidt et al., 2012; Kang et al., 2018). This is in accordance with our data, high removal that may related to adsorption to the granules, showed that NEREDA® was able to remove up to 99% of antibiotics. Nonetheless, the removal was not complete for all antibiotics and ARGs.

The optimum performance of the studied additional treatment technologies in removing antibiotics was in the order of CW1>NEREDA®>1-STEP®>AS and for ARGs was AS>NEREDA®>CW1>1-STEP®. The optimum performance of the WWTP combined with the additional treatment technologies in removing antibiotics was in the order of NEREDA®/1-STEP®>AS and for the order for ARGs was NEREDA®/AS >1-STEP®. This ranking is based on the removal percentage of antibiotics and the log removal of ARGs for the additional treatment technology and the combination of WWTP with the additional treatment technology. It should be noted that the removal of antibiotics and ARGs for AS, NEREDA and 1-STEP are based on the full-scale WWTP and CW are only based on the additional treatment technology.

The difference in the performance of removing antibiotics and ARGs between the tested additional treatment technologies might relate to chemical compounds of antibiotics and ARGs. Even though the additional treatment technology was implemented in the WWTPs, the complete removal of all antibiotics and ARGs could not be achieved. However, we can see the improvement in removing the antibiotics and ARGs by implementing the additional treatment technology in the WWTPs. Still, the removal is insufficient and therefore more research needs to be done in order to achieve a complete removal of antibiotics and ARGs.

6.3.2 Physicochemical technologies as tertiary treatment by using granular activated carbon

Physicochemical treatments, such as sorption-based techniques, are used because of the ease of operation, simple design, and low initial cost (Xu et al., 2017). Granular activated carbon (GAC) is playing an important role in water treatment (Rajapaksha et al., 2019). Adsorption is both physical and chemical processes are involved by accumulating a compound by a sorption redistribution from liquid to solids phases. GAC has a strong attraction for organic compounds and adsorbs them onto the carbon surface by van der Waals forces. The highly porous material characteristics provide a large specific surface area (1,000 m^2/g) that makes GAC a potential treatment for removing taste, odor, organic and inorganic compounds (Cecen and Aktaş, 2011) as well as antibiotics (Kårelid et al., 2017; Fan et al., 2019) and ARGs (Yang et al., 2019).

GAC was used as the main component in the 1-STEP® filter (Chapter 5). The GAC can remove antibiotics not only by physicochemical adsorption but also by the biofilm on the activated carbon (Ahmed, 2017; Östman et al., 2019). The removal percentage in this filter was lower (19% (sulfonamides) to -113% (macrolides) than reported values at lab-scale >80% for trimethoprim and macrolides (Kårelid et al., 2017) and tetracyclines (Choi et al., 2008), which might be affected by the lifetime of the activated carbon, the saturation level of the GAC with other organic compounds, the water flow, hydraulic retention time, and the concentration of antibiotics in the influent. This reflects that data from the lab-scale may not or only partially be translated to full-scale conditions of water treatment. The conditions in the lab-scale reactor are more controlled than for the full-scale system. All related variables such as wastewater sources (real or wastewater), starting concentrations of antibiotics, operational variables (flow rate, size of the column, contact time), and physicochemical (pH, temperature) can be controlled.

6.3.3 Nature-based treatment as a post-treatment by using constructed wetlands

Nature-based treatment by using CWs has been designed and constructed to mimic and stimulate a range of processes in a plant mediated system such as physical, chemical, and biological processes (Verhoeven et al., 2006). Different removal mechanisms are involved, mainly biodegradation influenced by plants and adsorption to substrates in the CW (Ju et al., 2014; Choi et al., 2016). Plants are capable of absorbing nutrients and allowing the growth of bacteria in the rhizosphere. The substrates serve as support media for plant growth, and, microbial attachment depending on the material characteristics.Substrates can also adsorb micropollutants, and nutrients (Kadlec and Wallace, 2009; Huang et al., 2017). Multiple mechanisms of sorption are involved, such as cation exchange, hydrophobic partitioning, van der Waals interaction, and surface complexation (Liu et al., 2013). CWs are able to remove dissolved organics and suspended solids effectively (Gajewska et al., 2020). Combining these properties, CWs showed also highpotential in removing antibiotics and ARGs (as described in Chapter 3 and 4), and the degree of removal depends on the type of CWs.

Two types of CWs were evaluated in this thesis; horizontal surface flow CWs (HSF-CWs) (Chapter 3 and 4) and vertical subsurface flow (VSSF-CW) (Chapter 3). When comparing HSF-CW and VSSF-CW, a better removal was observed in the VSSF-CW (Chapter 3). The high oxygen transfer rates from air to the water phase in the plant's rhizosphere in the VSSF-CW (Perdana et al., 2018) contributes to the better nitrification of NH_4-N (García-Ávila et al., 2019). Furthermore, VSSF-CW is more efficient in filtering out bacteria than the HSF-CW and indirectly contributes to the reduction of ARGs that are commonly carried by faecal microorganisms in wastewater (Huang et al., 2017). Meanwhile, anoxic conditions prevail in an HSF-CW (Nowrotek et al., 2016), making the HSF-CW more efficient in removing COD, BOD, and suspended solids (Marzec et al., 2018). Despite the different types of CWs, both type of CW showed potential in removing the antibiotics and ARGs (Chapter 3 and 4).

6.4 Fate of antibiotics and antibiotic resistance genes

This thesis demonstrated different removal processes determining the fate of antibiotics and ARGs within the treatments, after the treatments (with or without the additional treatment technology), and in natural surface water. Factors that were demonstrated to affect the removal and fate of antibiotics and ARGs are discussed below.

6.4.1 Physicochemical properties of antibiotics

The environmental behaviour of antibiotics depends partially on the physicochemical properties of the antibiotic, such as water solubility, volatility, lipophilicity, and partition potential (Treadgold et al., 2012). Partition potential such as octanol-water partition coefficient (K_{ow}) and organic carbon-water partition coefficient (K_{oc}) influence the accumulative and dispersive character of the compounds. Lower log K_{ow} values (from 1.5) indicate a hydrophilic character, and high log K_{ow} values (up to 4 or higher) indicate a hydrophobic character (Ying et al., 2013; Cumming and Rücker, 2017). With these values, K_{ow} predicts processes in the environment, such as bioaccumulation, bioavailability, toxicity, sorption to soils, and sediment, and bioconcentration (Hermens et al., 2013). Higher K_{oc} values suggest that the antibiotics are less mobile (Wegst-Uhrich et al., 2014).

Sulfonamides were detected consistently in all water samples tested. Sulfonamides are widely used both for humans and livestock (Peixoto et al., 2016). These compounds are often combined with trimethoprim to increase antibiotic activity (Eliopoulos and Huovinen, 2001), and also trimethoprim is found consistently in the water phase (Chapter 2, 4, and 5). Sulfonamides are resistant to degradation (Jiang and Wang, 2017), hydrophilic and stable chemical property, represented by low log K_{ow} and K_{oc} (Chapter 4 and 5). Due to these properties, the sulfonamides are more transportable in the aquatic environment compared to the sediment phase (Chapter 4 and 5). This will lead to greater risks of surface water contamination.

Meanwhile, macrolides, tetracyclines, and quinolones show a different pattern. A significant concentration of macrolides, tetracyclines, and quinolones was observed in the sediment (Chapter 4). The sum of the concentrations of these antibiotics in sediment could reach values of 4000 μg/kg in each month. Macrolides are used to treat respiratory tract infections and soft-tissue infections (Kaneko et al., 2007). In the Netherlands, tetracyclines are used extensively as a livestock feed additive and to treat diseases (Granados-Chinchilla and Rodríguez, 2017) but not as growth promotor which has been prohibited in the Netherlands since 2001. Quinolones are broad-spectrum antibiotics that are active against both gram-positive and gram-negative bacteria (Pham et al., 2019). Compared to sulfonamides, the group of tetracyclines, macrolides, and quinolones possess high log K_{ow} and K_{oc} (Chapter 4). As a result, these compounds tend to immobilize in and adsorb onto the sediment particles (Chapter 4) due to their hydrophobicity and their ability to form complexes with doubly charged cations, Ca^{2+} and Mg^{2+} (Tolls, 2001; Kümmerer, 2009; Luo et al., 2011). This strong sorption delays their degradation (Zhang et al., 2015a). The concentrations of antibiotics in the sediment are not as dynamic as the concentrations of antibiotics in the water phase, the latter appears to be more affected by season and water flows. This suggests that the sediment acts as a more stabile memory of past exposures and thus can provide a way to assess long term antibiotic levels in the system.

6.4.2 Type of ARGs

TetW (corresponding to tetracycline) and *ermB* (corresponding to macrolides) showed better removal compared to *sul* genes (Chapter 4 and 5). The presence of *tetW* might also indicate fecal contamination from humans and/or animals (Pei et al., 2006). *ErmB* was mainly hosted by gram-positive bacteria (Di Cesare et al., 2016) in which gram-positive bacteria were efficiently removed after wastewater treatment (Forster et al., 2002). This might explain the lower concentration of *ermB* and *tetW* detected in our water samples, which is consistent with previous studies (Aydin et al., 2015; Rodriguez-Mozaz et al., 2015; Aali, 2016; Lee et al., 2017; Li et al., 2018).

sul1 and *sul2* (ranging from 8.34 to 2.7) were found abundantly at the influent of a WWTP (Chapter 5), within the treatment in a WWTP, at the effluent of WWTPs and inside the water in additional treatment technology (Chapter 3, 4, 5), and their effluents (Chapter 2, 3, 4), in the river sediment (Chapter 4), and in the sludge leaving WWTP's (Chapter 5). *Sul* genes showed their persistence during primary and AS stages (Hiller et al., 2019). The dominance of *sul* genes is probably related to the location of *sul* genes in transposons and self-transferable or mobilizable plasmids with a broad host range (Phuong Hoa et al., 2008; Wang et al., 2014). It is known from the literature that these genes are often carried by various environmental bacteria and have high genetic stability (Gao et al., 2012).

The integrase gene coding for a class 1 integron (*intI1*), was strongly correlated with *sul1* and *sul2* (Chapter 2 and 3), suggesting a high potential mobility of this gene between bacteria.

Sul genes were located on the efficient microbial biomolecular vehicles commonly found to be involved in gene dissemination and detected mainly in fragments carrying *intI1* (Sköld, 2001). Co-location of *intI1* on the same fragment with *sul* genes may contribute to the persistence and dissemination of *sul* genes (Jiang et al., 2019). It has been shown before that the *intI1* gene is correlated with the dissemination of both types of *sul* genes in the environment (Chen et al., 2015). *Sul1* is typically found in *intI1* (Sköld, 2000), whereas *sul2* is usually located on small non-conjugative plasmids (Enne et al., 2001) or large transmissible, multi-resistance plasmids (Heuer and Smalla, 2007). Like *tetW*, *intI1* indicates the faecal pollution levels in the wastewater (Karkman et al., 2019) and has been suggested as a proxy of environmental pollution (Gillings et al., 2015) but is not recommended as indicator for overall anthropogenic ARGs pollution (Paulus et al., 2020).

6.4.3 Sludge/sediment as a reservoir

River or CW sediments (Chapter 2 and 4), or sludge in AS (Chapter 5) are regarded as hot spots of bacteria and bacterial activity. This possibly promotes horizontal gene transfer and can indirectly lead to proliferation of antibiotic resistance. The sediment in the river (Chapter 2) received inputs from the CWs (Chapter 3 and 4) or effluents of the WWTPs (Chapter 5). The sediment in the CWs received inputs from the WWTPs (Chapter 3 and 4), and a portion of the sludge that did not settle in the WWTP (Chapter 5). As such, it shows the history of accumulation over time.

The sludge/sediment appears to serve as a reservoir for antibiotic resistance (Calero-Cáceres et al., 2014; Chen et al., 2020) due to high bacterial densities and biofilm formation (Heß et al., 2018) and makes ARGs are less variable than the water phase. As a result, the sludge in the WWTPs and sediment floors of the CWs and rivers act as reservoirs of antibiotics and ARGs, which can be, under more extreme weather conditions, remobilized by resuspension (Heß et al., 2018). After deposition of the sediments and sludge particle at a new place downstream of the river under relatively quiescent conditions, antibiotics and ARGs might subsequently be released from these sludge and sediment layers into the water column (Luo et al., 2010). Moreover AB and ARG spread from sludge is a concern, especially for countries that apply sludge as an amendment to land, in which sludge is used to provide nutrients for crops (Kominko et al., 2017).

6.4.4 Seasonal variations

A seasonal effect was observed for the occurrence of the ARGs in the river, except *tetW* (Chapter 2). This points to the relevance of factors such as type of influent, sampling period (variation in precipitation and temperature), antibiotic consumption and disposal practice, geographical location, and hydrodynamic conditions. For example, the river flow is higher in winter (average

1000 m^3/h) than in summer (average 600 m^3/h), and this might lower the concentrations of ARGs and intI1 during winter even though the total load was very similar between months.

A seasonal effect was observed for the consumption of antibiotics (Chapter 4) but not visible in Chapter 3 and 5 due to limited sampling campaigns. (Li et al., 2014) performed the sampling campaign only in April and did not report any seasonal effect. The sampling campaign was performed only one-time in July 2015 (Chapter 3) and two months (Chapter 5). We observed that the concentration of the antibiotics in the influent of a WWTP fits with the antibiotic consumption in winter. The same trend was shown in various countries in Europe (Van Boeckel et al., 2014). Previous reports revealed that the level of antibiotics in the influent is more significant in winter than the other seasons (Sui et al., 2011; Zhang et al., 2015b). Antibiotics are inter alia used to cure infections of the respiratory tract, which happens more in winter than in summer (Werner et al., 2011). Lower concentration of antibiotics (Zhang et al., 2015b) and higher bacterial numbers (Pärnänen et al., 2019) were observed at higher temperatures since the bacteria's optimal growth temperature is above 30°C (Garrity et al., 2005). A similar observation was done in August in which the lowest concentration of antibiotics was detected when the water temperature was the highest during the sampling (25°C) (Chapter 4).

6.4.5 Nutrients in the wastewater

Some nutrients (NH_4^+-N and/or COD) were found positively correlated with ARGs (Chapter 2 and 5). Similar positive observations were observed between concentrations of organic carbon, nutrients, and pharmaceuticals (included antibiotics) (Chapter 3). This indicates that the nutrients might display a co-occurrence with antibiotics and ARGs in the wastewater, which means if certain WWTP operating conditions can remove organic carbon and nutrients, the same conditions will most likely contribute to removing antibiotics and ARGs as well.

6.5 The state of antibiotics and antibiotic resistance in Malaysia

The discussions in the previous subchapters are based on the results of our study in the Netherlands, as a developed and high-income country. In this subchapter, the perspective from Malaysia is discussed as a developing and middle-income country in terms of (1) general information of the country, wastewater treatment, medicine prescribing culture in society and antibiotic consumption (2) research on antibiotic resistance in Malaysia and (3) policy in combating antibiotic resistance in Malaysia.

Malaysia is a developing country located in South East Asia, with a population of over 30 million (Department of Statistic Malaysia, 2019). Malaysia is the third-largest economy in Southeast Asia after Indonesia and Thailand, and the 35th largest economy in the world (International Monetary Fund, 2019). Even though Malaysia is one of the developing countries,

the development of wastewater and sewage treatment is less developed than its drinking water treatment.

In general, a WWTP is important to reduce the emission of organic carbon and nutrients to ensure the water is less polluted, according to the effluent limit, when discharged to the receiving water body. Malaysian WWTPs also rely on primary and secondary treatment, but compared to the Netherlands, Malaysia does not have a wide implementation of well-developed treatment technologies (ITA, 2016). In Malaysia, only 56% of the population is served by a WWTP, which is concentrated in the big cities, whereas suburban areas, small towns and villages are relying on septic tanks and pour-flush systems. However, wastewater treatment in Malaysia has shown some improvement in the most recent years. In 2018, the government announced to replace 165 small WWTPs in Kuantan, in the east coast region, with an integrated regional WWTP, which would benefit almost half a million residents (Bernama, 2018). The project is predicted to be accomplished in 2022. Recently in January 2020, advanced moving bed bioreactor technologies were implemented in Langkawi Island and this will continue as a pilot project until 2022 (Awang, 2020). This technology will be introduced to all WWTPs across the country if the pilot project produces a better quality of wastewater than the existing WWTPs. Apart from that, sewage sludge is treated by anaerobic digestion (Kumaran et al., 2016; Hanum et al., 2019). Meanwhile, in the Netherlands, 100% of the population is connected to public WWTP (OECD, 2020). Apart from that, a few additional treatment technologies are implemented in their WWTP, such as Anammox (anaerobic ammonium oxidation), NEREDA® (aerobic granular sludge), and 1-STEP® (granular activated carbon). In addition, Pharmafilter (a wide set of technologies such as anaerobic digestion, membrane bioreactor, ozonization, UV disinfection, and filtration of activated carbon) is used to treat hospital waste.

As for the medicine prescribing culture in Malaysia, the separation of dispensing from prescribing has not yet taken place in private clinics (Mak and Hassali, 2015). The doctors in private clinics were found to prescribe 87% higher than the primary care clinics (Ab Rahman et al., 2016) and 7 times higher than the public clinics (Teng et al., 2004) as well as 1.6 times higher than university-based clinics (Teng et al., 2004). The separation of dispensing is important to avoid overprescribing, overuse, and irrational selection of medicines. Malaysia is one of the few countries where physician dispensing practices are still allowed. Most of the countries that used to have similar practices (such as Korea, Taiwan, Japan) have already separated prescribing and dispensing (Shafie et al., 2011). In the Netherlands, this separation is common practice for years, as one needs a prescription for medicines from a general practitioner, and these are dispensed by pharmacists.

Surprisingly, even though the unseparated medicine prescription is still practiced in Malaysia, the antibiotics consumption in Malaysia in 2015 of 12 defined daily dose (DDDs) per 1000 inhabitants per day is almost similar to the Netherlands (11 DDDs per 1000 inhabitants per day) (Klein et al., 2018). Penicillins (~31%), cephalosporins (~24%), and macrolides (~16%)

are the most commonly prescribed antibiotics in Malaysia. Broad-spectrum antibiotics such as quinolones and azithromycin are frequently prescribed in private clinics (Ab Rahman et al., 2016). In the Netherlands, macrolides are predominantly prescribed (~66%), followed by amoxicillin/clavulanate and fluoroquinolones (van den Broek d'Obrenan et al., 2014).

The occurrence and prevalence of antibiotics and ARGs in Malaysia have been studied in the catfish (Budiati et al., 2013) and chicken (Makkar et al., 2005; Ho et al., 2014). Apart from that, limited research was conducted in the environment, which confirmed that antibiotic resistance is present in the Malaysian environment. Malintan and Mohd (2006) reported traces of sulfonamides detected in swine wastewater, Jalal et al. (2010) observed ARB in mangrove soil and Al-Odaini et al. (2013) studied 18 pharmaceutical compounds in surface waters.

Realizing that antibiotic resistance is becoming a global problem, Malaysia has taken an initiative to combat this problem by declaring a Malaysian Action Plan on Antimicrobial Resistance (MyAP-AMR) 2017-2021 (Ministry of Health Malaysia, 2017). As reported by Hsu et al. (2017), Malaysia is one of the Southeast Asian countries that has established a national antimicrobial surveillance programs, like Singapore, Thailand, and the Philippines. Malaysia is also part of the Global Antimicrobial Resistance Surveillance System (GLASS) by WHO.

To conclude, some initiatives and approaches have been done to understand and overcome the antibiotic resistance prevalence in Malaysia. It is important to: (1) ensure appropriate use of antibiotics, (2) understand the state of antibiotic resistance occurrence by establishing more research, surveillance, and monitoring, (3) improve infection prevention and control, and (4) increase more awareness and public knowledge in understanding of antibiotic resistance. This shows that antibiotic resistance is a worldwide issue, as seen in developed countries, but also valid for others, for example, in Malaysia. Therefore, comprehensive and collaborative action needs to be taken by national leaders, scientists, veterinarians, industry leaders, farmers, and all related stakeholders to reduce the spread of antibiotic resistance.

6.6 Concluding remarks

This thesis focussed on the occurrence of antibiotics and ARGs in the environment and the effectiveness of selected treatment technologies in removing them. This study showed that antibiotics and ARGs were detected along a 20 km river transect throughout the year. A trace back approach was followed, from river to treatment technologies at WWTP facilities, to study the efficiency of various treatment steps in removing antibiotics and ARGs. The combination of WWTPs and additional treatment technologies removed some antibiotics and ARGs, but not completely. Antibiotics and ARGs were found to be present in the influent and effluent of the WWTPs, as well as within and in the effluent of the additional treatment technologies tested. The antibiotics showed a consistent trait based on the physicochemical characteristic, in which

some antibiotics were more favourably removed in the water phase compared to the sediment/sludge phase. Meanwhile, some ARGs were more persistent, and some ARGs were more susceptible to treatment in the WWTP. Due to the complex nature of antibiotics and ARGs, this thesis shows that it will be very difficult to remove them completely via a combination of a conventional WWTP and commonly used and even advanced additional treatment technologies. It takes different strategies to remove chemical compounds such as antibiotics, which is a first step in ARG abatement. However, this is not the same for ARGs because if the ARGs are not completely removed, these can easily rebound to sludge and sediment and thus maintain a long persistence. Worst, through sludge and sediment remobilisation, it can disseminate more. Therefore, more comprehensive research on the improvement of treatment technologies is needed to increase the removal percentage of antibiotics and ARGs.

6.7 Recommendation for future research

This thesis describes the occurrence of antibiotics and ARGs, as well as the potential of existing treatment technologies for their removal. Further research can be performed to complement and extend the conclusions from this thesis.

First, predictive models using fieldwork data are useful for future risk assessment. In this thesis, one-year sampling was performed along the river to observe the occurrence of antibiotics and ARGs (Chapter 2). The river is not influenced by any small river, in a transect up to 20 km. Therefore, the data may fit into predictive models to predict the trend and the fate of physical, biological, and chemical characteristic and removal of antibiotics and ARGs. Mass balances can be applied to different parameters, such as suspended particles, and general water quality. The model also can be validated by comparing laboratory and field measurements. Other than that, a model to predict the effect of human activities in disseminating ARGs is also important as also proposed by Vikesland et al. (2017). Other than that, a model of evolution, mobilization, transfer and dissemination of ARGs in the environment is also significant as recommended by Bengtsson-Palme et al. (2017). Past research used a model to predict the prevalence of ARGs in the river. For example, Ikuma and Rehmann (2020) developed a model to predict the fate of intracellular DNA and the extracellular DNA of ARGs in wastewater downstream in the river Thames. They concluded that the extracellular DNA fraction of ARGs in WWTP discharges is important in improving the risk assessment of antibiotic resistance. Also, Amos et al. (2015) generated three models for rivers in the United Kingdom and revealed that the WWTPs, type of land, temporal factors, and rainfall affected the prevalence of ARGs and influenced the environmental resistome.

Second, sampling campaigns over a longer time frame (1 year or longer) can give a full perspective of treatment technology in terms of trends, the removal percentage of antibiotics, and the log removal of ARGs. Longer time frames can generate seasonal, temporal, or spatial

patterns within the treatment technology. These patterns are important to understand the transportation, removal, or other involved processes of antibiotics and ARGs and the effect of seasonal differences in influencing mass transport rates of ARGs in the treatment technology. Pei et al. (2019) concluded that season played an important in determining the concentration of ARGs in their CWs. Knapp et al. (2012) confirmed that dry-season indicates the impact of individual waste inputs to surface water, and wet-season indicates the transportation of ARGs to downstream parts of the surface water. One-year observation was performed in the CWs (Chapter 4), therefore, a similar time frame (1 year or longer) can be applied for investigating the additional treatment technology (NEREDA® or 1-STEP®) in WWTPs (Chapter 5) to get the overview of performance in removing the antibiotics and ARGs.

Third, the sampling method is important in designing an experiment. Composite sampling can be performed instead of grab sampling (Chapter 2). Grab sampling may capture a specific event and give wrong interpretation about the antibiotics or ARGs quantification. This is due to the variation of antibiotic concentration and volume in the WWTP over time, which leads to inaccurate load calculations. Composite sampling is a mixture of grab samples collected over a specific period; for example, 24 hours for a daily composite. Therefore, composite sampling can be performed to overcome this problem. It can provide a detailed picture of sampling events to identify the real-time occurrence of the antibiotics and ARGs and improve comparability (Pärnänen et al., 2019).

Fourth, newly built CWs treatment has better bed material, and available adsorption sites, which makes the antibiotics and ARGs adsorb better and improves the removal percentage. The CWs with 1 day and 3 days of hydraulic retention time (Chapter 4) were operated for more than 15 years. These CWs were rebuilt in 2017 to improve the performance of the CWs, and the newly rebuilt CWs are in operation since 2019. A new sampling campaign at the new rebuilt CWs is proposed to compare the performance with the "old" CWs (Chapter 4). Apart from that, the treatment technology of 1-STEP® with ozonation is also in progress, and a similar sampling campaign is proposed at this new treatment technology. With this, we can gain more insights into new CWs and additional treatment technology in removing antibiotics and ARGs.

Fifth, a single unit treatment technology may not be able to completely remove the antibiotics and ARGs. Therefore, a combination of more treatments may be needed to remove antibiotics and ARGs thoroughly. There is potential in a single unit of the treatment technology, which can be seen from the removal percentage of antibiotics and log removal of ARGs in various different treatments (Chapter 3, 4, and 5). We observed that both NEREDA® and CW removed antibiotics and ARGs better compared to others. Therefore, a combination of NEREDA® and CW instead of a combination of AS and CW may enhance the removal of antibiotics and ARGs. A lab-scale study is needed to optimize the operational parameters in order to achieve good removal. A different combination of treatment technologies has been studied by other researchers. Liu et al. (2014) proved that the combination of UV and ozonation

further eliminated antibiotics after nanofiltration treatment. Meanwhile, Guo et al. (2017) reported that a combination of UV and titanium dioxide was able to reduce more than 5.5 log of ARB&Gs. Possibly, the CWs can be modified with immobilized titanium dioxide or combined with UV disinfection (using solar or artificial UV disinfection). This has been studied in a lab/pilot-scale and showed potential in removing nutrients (Yang et al., 2018), pharmaceutical active compounds (He et al., 2018b) and bacteria (Mishra et al., 2018).

Sixth, the sediment type may affect variations and the dissemination of antibiotics and ARGs in the environment. As we observed, the concentration of antibiotics and ARGs fluctuated in the sediment in the reed bed and the swamp part (Chapter 4). Sediment contains cumulatively adsorbed antibiotics and exogenous microorganisms such as feces carrying ARB&Gs (Devarajan et al., 2015; Marathe et al., 2017) and this favours horizontal gene transfer that may contribute to ARGs dissemination (Nesme and Simonet, 2015). Soil type contributes to the fate and the evolution of antibiotics (Sarmah et al., 2006) as well as ARGs (Zhang et al., 2018). Therefore, detailed lab experiments may help to understand this phenomenon further.

Seventh, plants and bacteria in the CWs may contribute to the reduction of the antibiotics and ARGs (Chapter 3 and 4). However, we did not study the mechanisms behind it. Further research to understand the uptake, metabolism, and degradation of the antibiotics or ARGs by the plants is needed, like mechanisms involved in the reduction of ibuprofen by *Phragmites australis* that were demonstrated by He et al. (2017). Apart from that, the plants in CWs usually live in symbiosis with bacteria in the rhizosphere. Nowrotek et al. (2017) observed that the bacteria in the CWs showed the ability to degrade sulfamethoxazole by detecting four sulfamethoxazole transformation products. To understand plants/bacteria interactions in removing antibiotics and ARGs, we need to study (1) the detailed characterization of plant physiology and bacteria community, and (2) the behavior of antibiotics to undergo photodegradation and biodegradation (Nowrotek et al., 2017).

Last but not least, antibiotic transformation products with antibiotic resistance properties may be produced after a treatment technology. This concern might be significant in treatment technologies that include disinfection. For example, Kennedy Neth et al. (2019) observed that fluoroquinolone formed transformation products after chlorine disinfection, which could be a source of antibiotic resistance in the environment. In addition, this risk of producing transformation products is also applicable to AS (Terzic et al., 2018). Therefore, further investigation of their role is needed.

To conclude, the continuity of the existing research on antibiotic and ARG dispersion and removal is important to ensure that ultimately the problem can be solved. This requires a lot of effort from different teams, peoples, and perspectives to achieve the desired objective: the complete removal of antibiotics and ARGs from waste streams discharged to natural environments.

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Summary and samenvatting

Summary

Antibiotics are used to treat bacterial infection in humans and animals. Since most of the antibiotics are poorly absorbed or metabolized by humans or animals, approximately 30–90% are excreted unaltered or as metabolites via feces and urine. As a result, the largest portion of antibiotics consumed end up in our wastewater and subsequently in wastewater treatment plants (WWTPs). WWTPs are designed to treat high loads of organic carbon and nutrients, but not to adequately remove antibiotics, which finally end up in significant quantities in the surface waters to which the WWTP effluents are continuously discharged. WWTPs are identified as a point source for antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), which can end up in the different environmental matrices, and lead to human exposure to resistant bacteria and/or resistance genes through contact with water. There are potentially two ways to mitigate antibiotic resistance. Firstly, the prevention and reduction of use of antibiotics, and secondly, the enhanced removal in or after WWTPs. This thesis focusses on additional treatment technologies to ensure the sufficient removal of antibiotics and ARB&Gs after the WWTPs. A variety of additional treatments at full-scale were studied and reported in this thesis, as more extensive studies were performed at lab-scale in previous research.

This thesis, "**Antibiotics and antibiotic resistance genes in wastewater: Occurrence and removal technologies",** explores the occurrence of antibiotics and ARGs in wastewater and in the environment and the potential of full-scale treatment technology in removing antibiotics and ARGs from wastewater. The following subsequential WWTP treatment steps were distinguished: primary treatment (removing solids generally by filtration and sedimentation), secondary treatment (organic and nutrient removal generally by biological processes), tertiary treatment (removing pollutant residuals by various -often physico-chemical- techniques), and quaternary treatment or post-treatment (for removing residual pollutants, micropollutants and/or pathogens). These treatment technologies were selected from (1) advanced biological technologies (for secondary treatment) such as aerobic granular sludge; (2) physicochemical technologies (for tertiary treatment) such as activated carbon, and (3) nature-based technologies (for post-treatment) such as constructed wetlands (CWs).

Chapter 2 describes the occurrence of 52 antibiotics (macrolides, sulfonamides, tetracyclines), 4 ARGs (*ermB*, *sul1*, *sul2*, *tetW*), and a class 1 integron (*intI1*) in a Dutch river to which WWTP effluent is and has been discharged in the past decades. The WWTP effluent was channeled to CWs with a hydraulic retention time of 1 day or 3 days. A monthly sampling campaign was performed during 1 year. Sediment and water samples were taken from WWTP effluent and the CWs, and from the receiving river, from 500 m upstream to 20 km downstream. We observed that the WWTP significantly increased the concentration of antibiotics and ARGs in the river when compared to the upstream samples. However, the antibiotic concentrations decreased downstream the WWTPs. Meanwhile, ARGs were persistent in both water and sediment samples from the WWTP effluent discharge point onwards until 20 km downstream,

with minimal effect of dilutions of the river water by other water sources. These findings indicate that antibiotics and ARGs are persistent in a wastewater effluent-receiving river, and a river has, through water-sediment interactions the potential to act as a reservoir for ARGs.

Since a WWTP had shown to contribute significant concentrations of antibiotics and ARGs in the receiving river, the performance of different treatment technologies in removing antibiotics or ARGs was assessed in subsequent chapters.

In **Chapter 3**, the performance of full-scale constructed wetlands (CWs) (horizontal surface flow or vertical subsurface flow) were evaluated in removing antibiotics, pharmaceutical compounds, and ARGs by using chemical (GCMS/MS and UHPLC-MS/MS), toxicological (bioassays), and molecular methods (qPCR). The CWs served as a post-treatment of a WWTP. 17 pharmaceutical compounds were analyzed, that comprise various medical use categories such as antibiotics, anti-inflammatory drugs, lipid regulators, estrogenic compounds, psychiatric drugs, stimulants, and β-blockers. The concentration in the influent of the CWs was higher than 0.1 μg/l for 14 out of 17 studied compounds, with a median removal of 50% in the vertical subsurface flow CW. The removal of the 17 pharmaceutical compounds in two horizontal surface flow CWs was insignificant, but difficult to assess in light of high variations in influent and effluent concentrations creating high uncertainties. In addition, the CWs removed *intI1* and ARGs, in which the log removal depended on the CW type. However, some ARGs had increased levels in the effluent, which indicates that resistance-carrying bacteria grow in the CW and are released from the CW sediment. Although the CWs showed potential in removing antibiotics, pharmaceutical compounds, and ARGs, optimization of the CWs is still needed since insufficient removal was observed for certain compounds.

In **Chapter 4,** two full-scale horizontal surface flow CWs were compared to assess their removal of antibiotics and ARGs. The CWs received the same WWTP effluent and were operated with different hydraulic retention times (1 or 3 days). Water and sediment from the CWs were sampled for one year. Antibiotics and ARGs were measured using LC-MS/MS and qPCR, respectively. Both CWs removed antibiotics with a range of 28% - 100%, depending on the type of antibiotic. In addition, ARGs were removed from the water by 0.8 to 1.5 log by CW treatment, but the ARGs tended to accumulate in sediment. A longer hydraulic retention time did not result in better removal of antibiotics but did show better removal of ARGs. The result of this study shows the potential of CWs in reducing the input of antibiotics into the environment, which makes CWs an ideal post-treatment technology for WWTP.

In **Chapter 5,** the performance of three WWTPs, with and without the full-scale of additional technology, were evaluated in removing antibiotics and ARGs. The conventional WWTP A operated with activated sludge as secondary treatment. The WWTP B operated with activated sludge followed by an activated carbon filtration step that serves as a final treatment step (1-STEP® filter, tertiary treatment). The WWTP C was a treatment plant consisting of

aerobic granular sludge (NEREDA®) as an alternative to activated sludge treatment (advanced secondary treatment). Water and sludge were collected and analyzed for antibiotics and ARGs. All WWTPs removed 79-88% of the total load of antibiotics present in the influent. Despite the high removal percentages, some antibiotics were detected in the various effluents. Higher removal efficiency of antibiotics were observed in WWTP C that the other treatments. For ARGs, WWTP C reduced their concentration by 2.3 log, WWTP A with 2.0 log and WWTP B with 1.3 log. The ARGs were more abundant in the sludge phase compared to the water phase suggesting that sludge is an important reservoir and might be a future source for ARG emissions upon reuse. This chapter indicates the potential and usefulness of additional treatment technologies to reduce the concentration of antibiotics and ARGs in WWTP effluent before discharging wastewater to surface water.

Lastly, in **Chapter 6**, the occurrence and fate of antibiotics and ARGs are summarized, as well as the optimum treatment technology in removing antibiotics and ARGs is accessed. The thesis shows that antibiotics and ARGs are abundant in the influent of a WWTP, the effluent of a WWTP, and in a river to which the WWTP discharges. The combination of a WWTP and an additional treatment technology has the potential to remove some of the antibiotics and ARGs, but the treatment technologies could not completely remove them all. Therefore, some traces of antibiotics and ARGs were still detected after the treatment in the WWTPs, and also detected in the river. More studies to improve the treatment technology are needed to improve the removal of the antibiotics and ARGs from wastewater. Finally, the fate of antibiotics and antibiotic resistance in the author's home country (Malaysia) is presented. To complement and extend the conclusions from this thesis, a few recommendations for future studies are presented as follows; (1) build predictive models using present fieldwork data to predict future risk assessment; (2) perform sampling campaigns over a longer time frame (1 year or longer) to give a full perspective of treatment technology in terms of trends, the removal percentage of antibiotics, and the log removal of ARGs; (3) perform composite sampling to provide a detailed picture of sampling events to identify the real-time occurrence of the antibiotics and ARGs and improve comparability; (4) perform a new sampling campaign at a new rebuilt CW to gain more insights on the additional treatment technology; (5) combine one or more treatment technologies to enhance the removal of antibiotics and ARGs; (6) study the effect of sediments on the occurrence and removal of antibiotics and ARGs; (7) study the mechanisms of plants/bacteria interactions in removing antibiotics and ARGs to understand the uptake, metabolism, and degradation of the antibiotics or ARGs by the plants; and (8) study the possible transformation products with antibiotic resistance properties may be produced after a treatment technology.

The research presented in this thesis assessed the occurrence of antibiotics and ARGs in the environment and measured the effectiveness of selected treatment technologies in removing them. Due to the complex nature of antibiotics and ARGs, this thesis presents that conventional WWTP do not effectively remove antibiotics and ARB&G's from wastewater.

Moreover, this thesis shows that by adding commonly used and even advanced additional treatment technologies complete removal is still not achieved. CW post-treatment was shown to contribute significantly to the removal of ARG&B's but is also unable to reach full removal of antibiotics and ARG&B's. Hence, more comprehensive research on the improvement of treatment technologies is still needed to increase the removal efficiency of antibiotics and significant log removals of ARB&Gs.

Samenvatting

Antibiotica worden gebruikt om bacteriële infecties bij mensen en dieren te behandelen. De meeste antibiotica worden slechts gedeeltelijk opgenomen en omgezet in het menselijk lichaam en zo'n 30-90% verlaat het lichaam via de ontlasting en urine in de originele vorm of als transformatieproduct. Hierdoor belandt het grootste gedeelte van geconsumeerde antibiotica in ons afvalwater en vervolgens in een rioolwaterzuiveringsinstallatie (RWZI). RWZI's verwijderen hoge belastingen aan organisch materiaal en nutriënten en zijn niet ontworpen om antibiotica adequaat te verwijderen. Hierdoor komen de antibiotica uiteindelijk via lozing van het RWZI effluent in het oppervlaktewater terecht. RWZI effluent is zodoende een bron van antibiotica, antibiotica resistente bacteriën (ARBs) en antibiotica resistente genen (ARGs) voor verspreiding in het milieu. Via deze route kunnen mensen vervolgens in contact komen met resistente bacteriën en/of resistente genen. Twee mogelijke manieren om antibiotica resistentie te verminderen zijn (1) het voorkomen of verminderen van het antibiotica gebruik, en (2) een betere verwijdering van antibiotica in RWZI's. Deze thesis focust zich op aanvullende technologieën voor verwijdering van antibiotica, ARBs en ARGs in RWZI effluent. In deze thesis worden een aantal technologieën beschreven en bestudeerd die bij RWZI's operationeel zijn. Daaraast zijn intensievere studies uitgevoerd op laboratorium-schaal.

Deze thesis **'Antibiotica en antibiotica resistente genen in afvalwater: Verspreiding en verwijderingstechnologieën'** beschrijft de verspreiding van antibiotica en ARGs in het afvalwater en in het milieu, en de verwijderingscapaciteit van verschillende full-scale technologieën. Onderscheid is gemaakt tussen de volgende RWZI onderdelen: primaire behandeling (verwijdering van vaste deeltjes door middel van filtratie en sedimentatie), secundaire behandeling (verwijdering van organisch materiaal en nutriënten door middel van biologische processen), tertiaire behandeling (verwijdering van verontreinigingen door middel van fysisch-chemische technieken) en quaternaire en/of nabehandeling (verwijdering van microverontreinigingen en pathogenen). De volgende full-scale technologieën zijn geselecteerd: (1) geavanceerde biologische technologieën (voor secundaire behandeling) zoals aeroob korrelslib; (2) fysisch-chemische technologieën (voor tertiaire behandeling) zoals actief kool en (3) natuurlijke behandelingstechnologieën (voor nabehandeling) zoals helofytenfilters.

Hoofdstuk 2 beschrijft de aanwezigheid van 52 verschillende antibiotica (macroliden, sulfonamiden, tetracyclines), 4 ARGs (*ermB, sull, sul2, tetW*) en een klasse 1 integron (*intI1*) in een Nederlandse rivier, afkomstig vanuit lozing van RWZI effluent gedurende de laatste decennia. Voordat het RWZI effluent in de rivier wordt geloosd, wordt dit eerst door een helofytenfilter met een verblijftijd van 1 of 3 dagen geleid. Gedurende een jaar zijn maandelijks sediment- en watermonsters genomen van het RWZI effluent, de helofytenfilters en de ontvangende rivier van 500 meter bovenstrooms tot 20 kilometer benedenstrooms. De lozing vanaf de RWZI veroorzaakte een significante verhoging van de concentraties aan antibiotica en ARGs in de rivier ten opzichte van de bovenstroomse concentraties. Benedenstrooms van de RWZI namen

de antibiotica concentraties af. ARGs waren persistent in zowel de water- als bodemmonsters vanaf het lozingspunt van de RWZI tot 20 kilometer benedenstrooms, met minimale verdunningseffecten door de rivier of andere waterstromen. Deze resultaten laten zien dat antibiotica en ARGs persistent zijn in een door RWZI effluent gevoede rivier, en dat de rivier een reservoir is van ARGs, als gevolg van water-sediment interactie. Dit toont aan dat de RWZI voor significant verhoogde concentraties van antibiotica en ARGs in de ontvangende rivier zorgde. Vervolgens is het verwijderingsrendement van verschillende behandelingstechnologieën voor de verwijdering van antibiotica en ARGs onderzocht.

Hoofdstuk 3 beschrijft het verwijderingsrendement van helofytenfilters (horizontaal of verticaal doorstroomd) voor antibiotica, medicijnen en ARGs, aangetoond met behulp van chemische (GC-MS/MS en UHPLC-MS/MS), toxicologische (bioassays) en moleculaire methodes (qPCR). De helofytenfilters fungeerden hierbij als een nabehandeling van RWZI effluent. De 17 onderzochte medicijnen behoorden tot verschillende groepen, zoals ontstekingsremmers, lipide-regulatoren, estrogene stoffen, psychiatrische medicijnen, stimulanten en β-blokkers. De influent concentraties van deze medicijnen in de helofytenfilters was meer dan 0.1 μg/L voor 14 van de 17 bestudeerde medicijnenresten, met een gemiddelde verwijdering van 50% in het verticaal doorstroomde helofytenfilter. De verwijdering van de 17 medicijnen in de twee horizontaal doorstroomde helofytenfilters was verwaarloosbaar, maar ook lastig te bepalen door hoge variaties in de influent- en effluent concentraties. De helofytenfilters verwijderden ook *intI1* en ARGs, waarbij de logaritmische verwijdering afhankelijk was van het type helofytenfilter. Echter, de concentratie van sommige ARGs was hoger in het effluent dan in het influent, wat aantoont dat antibiotica resistente bacteriën in het helofytenfilter groeien en vervolgens vanuit het sediment van het helofytenfilter kunnen uitspoelen. Alhoewel de helofytenfilters de potentie hebben antibiotica, medicijnen en ARGs te verwijderen, is optimalisatie van de helofytenfilters nodig, omdat een aantal componenten onvoldoende verwijderd werden.

In **hoofdstuk 4** worden twee full-scale horizontaal doorstroomde helofytenfilters met elkaar vergeleken voor de verwijdering van antibiotica en ARGs. De helofytenfilters ontvangen hetzelfde RWZI effluent en hebben verschillende hydraulische verblijftijden (1 of 3 dagen). Water- en sedimentmonsters zijn genomen gedurende één jaar en antibiotica en ARGs zijn gemeten met respectievelijk LC-MS/MS en qPCR. In beide helofytenfilters werd 28-100% antibiotica-verwijdering behaald, afhankelijk van het type antibiotica. Daarnaast vond een 0.8-1.5 log reductie plaats van ARGs in water van het helofytenfilter, maar accumuleerde ARGs in het sediment van het helofytenfilter. Een langere hydraulische verblijftijd resulteerde niet in een hoger antibiotica-verwijderingsrendement, maar wel in meer verwijdering van ARGs. Dit onderzoek toont aan dat helofytenfilters potentie hebben om de verspreiding van antibiotica in het milieu te verminderen, en daardoor een ideale methode zijn als nabehandeling-technologie na een conventionele RWZI.

In **hoofdstuk 5** wordt het verwijderingsrendement van drie RWZI's met en zonder nabehandeling voor antibiotica en ARGs onderzocht. RWZI A gebruikt actief slib als secundaire behandelingsstap. RWZI B bevat actief slib, gevolgd door een laatste behandelingsstap door middel van actief kool filtratie (1-STEP® filter, tertiaire behandelingsstap). RWZI C gebruikt aeroob korrelslib (NEREDA®) als een alternatief voor actief slib (geavanceerde secundaire behandelingsstap). Water- en slibmonsters uit deze RWZI's zijn verzameld en geanalyseerd op antibiotica en ARGs. Alle drie de RWZI's verwijderden 79-88% van de antibiotica, die aanwezig waren in het influent. Ondanks dit hoge verwijderingsrendement waren een aantal antibiotica nog steeds aanwezig in het effluent. RWZI C had het hoogste verwijderingsrendement vergeleken met de andere RWZI's. RWZI C resulteerde in een 2.3 log reductie in ARGs, terwijl RWZI A een 2.0 log en RWZI B een 1.3 log reductie behaalden. ARGs waren meer aanwezig in het slib dan in het water van de RWZI's, wat aantoont dat het slib een belangrijk reservoir en mogelijke toekomstige bron van ARG emissie is bij hergebruik hiervan. Dit hoofdstuk demonstreert de potentie en de waarde van aanvullende behandelingstechnologieën voor het verwijderen van antibiotica en ARGs uit RWZI effluent.

Tenslotte wordt in **hoofdstuk 6** de aanwezigheid en verspreiding van antibiotica en ARGs samengevat, en de optimale behandelingstechnologie voor de verwijdering van antibiotica en ARGs bediscussieerd. Deze thesis laat zien dat antibiotica en ARGs wijd verspreid zijn in RWZI influent en effluent, en de rivier waarop een RWZI loost. De combinatie van conventionele zuivering en aanvullende behandelingstechnologieën heeft de potentie om een selectie van, maar niet alle, antibiotica en ARGs te verwijderen. Als gevolg daarvan werden sommige antibiotica en ARGs gedetecteerd na de RWZI en in de rivier. Verder onderzoek is nodig om het verwijderingsrendement van de zuiveringstechnologieën voor antibiotica en ARGs te verhogen. Ten slotte wordt de verpreiding van antibiotica en antibioticaresistentie in het thuisland van de auteur (Maleisië) gepresenteerd. Aanbevelingen aan de hand van de conclusie van het onderzoek in deze thesis zijn als volgt; (1) ontwikkel voorspellende modellen die velddata gebruiken om toekomstige risico's te adresseren; (2) verricht monstername-campagnes over langere periodes (1 jaar of langer) voor een beter overzicht van de trend in verwijdering voor antibiotica en ARGs bij verschillende behandelingstechnologieën; (3) verricht composiet-monstername om een gedetailleerd beeld van de real-time verspreiding van antibiotica en ARGs te kunnen schetsen voor een betere vergelijkbaarheid; (4) verricht nieuwe monstername-campagnes bij de herbouw van een helofytenfilter om meer inzicht te krijgen in aanvullende behandelingstechnologieën; (5) combineer één of meerdere behandelingstechnologieën om de verwijdering van antibiotica en ARGs te verhogen; (6) bestudeer het effect van sediment op de verspreiding van antibiotica en ARGs; (7) bestudeer de mechanismes van plant/bacterie interactie voor de verwijdering van antibiotica en ARGs om meer inzicht te krijgen in de opname, het metabolisme en de degradatie van antibiotica en ARGs in planten en (8) bestudeer mogelijke transformatieproducten met antibioticaresistente eigenschappen die ontstaan bij een behandelingstechnologie.

Deze thesis beschrijft het onderzoek naar de verspreiding van antibiotica en ARGs in het milieu en de verwijdering hervan in een aantal behandelingstechnologieën. Deze thesis demonstreert dat antibiotica en ARGs onvoldoende uit afvalwater worden verwijderd in conventionele RWZI's als gevolg van de complexe karakteristieken van antibiotica en ARGs. Daarnaast maakt deze thesis duidelijk dat de toevoeging van gangbare en geavanceerde behandelingstechnologieën niet leiden tot volledige verwijdering. Het gebruik van een helofytenfilter resulteerde in significante verwijdering van ARG&Bs, maar resulteerde niet in complete verwijdering van antibiotica en ARG&Bs. Verder onderzoek is nodig om de verwijdering van antibiotica en ARB&Gs in verschillende additionele behandelingstechnologieën te verhogen.

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p/s: if you are reading this and not yet completing your Ph.D. I suggest to start your name list for the acknowledgements earlier because it will take some time to think about to whom you should put here without forgetting someone.

Love
Azie

About the author

Nurul 'Azyyati Sabri (Azie) was born on 19th July 1983 in Pekan, Pahang, Malaysia. She received her bachelor's degree in applied biology (major in Biotechnology) from Universiti Sains Malaysia, Malaysia (2005). During her final year project, she worked on heavy metal uptake and identification of heavy metal genes induced by cobalt in *Pistia stratiotes* L. She later pursued her master's degree in Universiti Teknologi Malaysia, Malaysia with a project entitled "Production and evaluation of performances of locally produced effective microbes on okra cultivation and composting." She continued her Ph.D. at Wageningen University & Research in the Netherlands in 2015. Her project focused on antibiotics and antibiotic resistance genes in wastewater. Her research interest is in micropollutants, specifically in antibiotic resistance in the environment and wastewater treatment to reduce the micropollutant emission to the environment. Other than that, she is also working on the microbial inoculant for agriculture application. Azie is now working as a lecturer in the Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Malaysia.

List of publications

Sabri, N.A, Schmitt, H., Van der Zaan, B., Gerritsen, H.W., Zuidema, T., Rijnaarts, H.H.M., Langenhoff A.A.M.(2020). Prevalence of antibiotics and antibiotic resistance genes in a wastewater effluent-receiving river in the Netherlands. *Journal of Environmental Chemical Engineering. 8 (1): 102245.* https://doi.org/10.1016/j.jece.2018.03.004

He, Y., Nurul, S., Schmitt, H., Sutton, N.B., Murk, T.A.J., Blokland, M. H., Rijnaarts, H.H.M., Langenhoff, A.A.M. (2018). Evaluation of attenuation of pharmaceuticals, toxic potency, and antibiotic resistance genes in constructed wetlands treating wastewater effluents. *Science of the Total Environment*. 631-632: 1572-1581. https://doi.org/10.1016/j.scitotenv.2018.03.083

Sabri, N.A., Schmitt, H., van der Zaan, B.M., Gerritsen, H.W., Rijnaarts H.H.M., Langenhoff, A.A.M. Performance of full scale constructed wetlands in removing antibiotics and antibiotic resistance genes. Submitted.

Sabri, N.A., van Holst, S., Schmitt, H., van der Zaan, B.M., Gerritsen, H.W., Rijnaarts H.H.M., Langenhoff, A.A.M. (2020). Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants. *Science of The Total Environment* 140199. https://doi.org/10.1016/j.scitotenv.2020.140199

Sabri, N.A., Schmitt, H., van der Zaan, B.M., Gerritsen, H.W., Rijnaarts H.H.M., Langenhoff, A.A.M. UV disinfection reduces bacteria and proliferates antibiotic resistance genes. In preparation.

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- Research in context activity: 'Designing and creating two communication videos on 'Antibiotic resistance genes' and on how to 'Reduce the spread of antibiotic resistance genes' (both made available on Youtube)' (2019)
- Basic statistics (2016)

Other PhD and Advanced MSc Courses

- Methods for detecting and quantifying antibiotic-resistant bacteria and antibiotic resistance genes in the environment, Nereus Cost Action/ IDAEA-CSIC (2016)
- Project and time management, Wageningen Graduate Schools (2016)
- Introduction to R for Statistical Analysis, Wageningen Graduate Schools (2016)
- Techniques writing and presentation for scientific, Wageningen Graduate Schools (2016)
- Scientific artwork with Photoshop & Illustrator, Wageningen Graduate Schools (2016)
- Adobe InDesign Essential Training, Wageningen Graduate Schools (2016)
- Teaching and supervising Thesis students, Wageningen Graduate Schools (2016)
- Brain training, Wageningen Graduate Schools (2017)
- Data management planning, Wageningen Graduate Schools (2017)
- Systematic approaches to reviewing literature, WASS Graduate School (2017)
- Reviewing a scientific paper, Wageningen Graduate Schools (2017)
- Communication with the media and the general public, Wageningen Graduate Schools (2017)
- Environmental and human health risk assessment of antibiotics, KWR Watercycle Research Institute (2018)

Management and Didactic Skills Training

- Organize weekly colloquium in Environmental Technology (2015-2016)
- Supervising three MSc students with thesis entitled (2016-2018)

Oral Presentations

- *Evaluation of full scale constructed wetlands in removing antibiotics and antibiotic resistance genes.* XENOWAC II, 10-12 Oct 2018, Limassol, Cyprus

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