Master's thesis

The occurrence and problems of residual antibiotics during wastewater treatment

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Jätevesien ja lietteiden sisältämät haitta-ainepitoisuudet, jotka johtuvat pääosin riittämättömistä puhdistusprosesseista, ovat olleet tutkinnan alla jo vuosikymmenten ajan mahdollisten ympäristöhaittojen vuoksi. Yksi näistä huolia aiheuttavista yhdisteryhmistä on antibiootit, joita määrätään säännöllisesti bakteeri-infektioiden hoitoon, ja jotka pystyvät kulkeutumaan ruuansulatuselimistön lävitse osittain muuttumattomana ja osittain metaboliitteina, päätyen lopulta jätevesiin. Jätevesien ja lietteiden mukana ympäristöön kulkeutuneiden antibioottien tarkkaa vaikutusta ei tiedetä, sillä pitoisuudet ovat pieniä, nanogrammoja litrassa, eivätkä akuutit toksisuuskokeet anna todellista kuvaa eliöiden altistumisesta. Koska jatkuvan jätevesi- ja lietevirran mukana nämä matalat pitoisuudet voidaan laskea pysyviksi, krooniset, jopa 80 vuotta kestävät altistumistutkimukset kattaisivat paremmin todellisuuden. Tämäntapaisten tutkimusten data on kuitenkin puutteellista tai olematonta. Suurin huolenaihe koskien antibioottien matalia pitoisuuksia on antibioottiresistenssiyden kehittyminen, sillä matalissa pitoisuuksissa taudinaiheuttajat voivat selvitä hengissä sekä resistenssin kehittyneet yksilöt yleistyä. Syntynyt resisntessigeeni (antibiotic-resistance genes, ARG) voi siirtyä horisontaalisella geeninsiirrolla bakteerista toiseen (antibiotic resistant bacteria, ARB), minkä seurauksena monien sairauksien hoito hankaloituisi ja kallistuisi.

Tässä Pro Gradu –tutkielmassa kuvaillaan antibioottien käyttäytymistä ja niiden mahdollista riskiä edesauttaa antibioottiresistenttien bakteerikantojen syntymiseen jätevesijärjestelmissä aiheesta löytyvän kirjallisuuden mukaan. Lisäksi tutkittiin kolmen laajasti käytössä olevan antibiootin; siprofloksasiinin (CIP), trimetopriimin (TMP) sekä sulfametaksatsolin (SMX) pitoisuuksia Jyväskylän jätevesienpuhdistuslaitoksien jätevesistä sekä lietteistä. Tutkitut antibiootit havaittiin nestekromatografialaitteistolla, joka oli yhdistetty tandem massaspektrometriin (HPLC-MS/MS). Jätevesien pitoisuuksien perusteella CIP:sta katosi puhdistusprosessissa 80%, ja TMP kulki lähes täysin puhdistusprosessin läpi, kun taas SMX määrä oli lisääntynyt toisen laitoksen puhdistusprosessissa, mikä voi osittain johtua tämän metaboliatuotteiden muuntumisesta takaisin alkuperäiseksi antibiootiksi. CIP oli ainoa mitä havaittiin lietteessä, n. 730 µg kg^{-1}. Tulokset ovat hyvin verrattavissa Suomessa mitattuihin pitoisuuksiin.

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The concentrations of pollutants in wastewater and sludge, mainly due to inadequate cleaning processes, have been under investigation for decades due to their potential environmental damage. One of these groups of concerns is the antibiotics since they are regularly prescribed for the treatment of bacterial infections, and are able to pass through the digestive tract partly unchanged and partly as metabolites, eventually ending up in wastewater. The exact effect of antibiotics released into the environment with wastewater and sludge is unknown as the concentrations are small (ng L^{-1}), acute toxicity tests do not give a true picture of the exposure of organisms. As these low concentrations can be assumed to be persistent due to the continuous flow of wastewater and sludge, chronic, up to 80-year exposure studies would better cover the reality. However, data from this type of study is incomplete or non-existent. The greatest concern about low levels of antibiotics is the development of antibiotic resistance, because at low concentrations pathogens can survive and the developed individuals that have the resistance become more common due to natural selection. The resulting resistance gene (antibiotic-resistance genes, ARG) can be transmitted by horizontal gene transfer from one bacterium to another (antibiotic resistant Bacteria, ARB), as a result of which the treatment of many diseases would be complicated and expensive.

This Master's thesis describes the behavior of antibiotics and their potential risk of contributing to the development of antibiotic-resistant bacterial strains in wastewater systems according to literature on the subject. In addition, three widely used antibiotics were investigated; ciprofloxacin (CIP), trimethoprim (TMP) and sulfamethoxazole (SMX) concentrations in effluents and slurries from wastewater treatment plants in Jyväskylä. The investigated antibiotics were detected by liquid chromatography apparatus connected to a tandem mass spectrometer (HPLC-MS / MS). Based on wastewater concentrations, 80% of the CIP disappeared in the purification process, and TMP was almost completely passed through the purification process, while the SMX rate had increased in the second plant purification process, which may be partly due to the conversion of this metabolic product back to the original antibiotic. CIP was the only one found in the slurry, about 730 μg kg^{-1}. Results obtained in this project are similar to the ones measured in Finland.

TABLE OF CONTENT

ABBREVIATIONS

ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistance genes
CFU	Colony Forming Unit
CIP	Ciprofloxacin
DDD	Defined daily dose
HGT	Horizontal gene transfer
HPLC	High performance Liquid chromatography
IS	Internal Standard
Kd	Distribution coefficient
K_{OW}	Octanol-water partition coefficient
MIC	Minimal inhibitory concentration
MSC	Minimum selective concentration
MS/MS	Tandem-mass-spectrometry
SMX	Sulfamethoxazole
SPE	Solid phase extraction
TMP	Trimethoprim
WWTP	Waste water treatment plant

1 INTRODUCTION

Constantly growing population brings along numerous emerging problems, including environmental issues. One of the most important factors of sustaining the well-being of the population is proper medication. Antibiotics have been a life safer for many diseases for decades and are prescribed widely all over the world.

Due to increase in both consumption and population, the detection of antibiotics in wastewaters and sewage sludge is not a surprise (Golet et al., 2002; Kümmerer, 2009). Consequently, wastewater treatment plants are considered to be the major point source of antibiotic contamination in the nature (Kümmerer, 2009).

When the medication is consumed, a fraction of it is metabolized while the rest will pass through the digestion system untouched. Hence, around 30 - 90% of the intake dosage of most antibiotics is excreted via urine and feces (Golet et al. 2003; Göbel et al., 2005). These traces will eventually end up into waste water treatment plants (WWTPs), where their lack of efficient removal causes problems. Current WWTPs are built to treat high loads of organic matter. Small organic molecules like pharmaceuticals are able to pass through the waste water treatment system into the effluent water or absorb into solid matter, the sewage sludge. Along with final disposal of wastewater and sludge the compounds will eventually be released into the environment. Due to presence of antibiotics and other pharmaceuticals, the sludge cannot be utilized in productive applications. For example, sewage sludge could be used as a fertilizer for agriculture, but potential toxicity and health risk prevent it from being applied to crop farms.

Antibiotics have been considered emerging pollutants due to their continuous inflow to and persistence in the aquatic ecosystem (Giger et al., 2003). If they are not eliminated during wastewater treatment, they pass through the sewage system and eventually end up in the environment. The information available on the ecotoxicology of these compounds is still weak, and a full risk assessment is

therefore difficult to carry out. Using reclaimed wastewater for irrigation may result in an increased uptake of antibiotics by crop plants, which would lead to bioaccumulation within plant tissues and subsequently entering the food networks; potentially causing a risk for public health (Christou et al., 2017). Releasing treated wastewaters or sludge that contain traces of antibiotics may also contribute to the spread and development of antibiotic resistance genes (ARG) and antibiotic resistant bacteria (ARB) (Martinez, 2008; Devarajan et al. 2015), which carry a potential to cause a risk to human health, due to the treatment of antibiotic-treated common diseases becoming more difficult and expensive to battle. To combat this, many methods for detecting the amount of pharmaceuticals in wastewaters and ways to enhance their removal, such as using activated carbon, have been introduced and studied (Vieno et al., 2007), though many efficient methods are still highly expensive to use on such high loads of wastewater.

2 THEORECTICAL BACKGROUND

2.1 Antibiotics

Antibiotics are small organic compounds that contain special physical and chemical properties addressed to take care of human and animal health by inhibiting the growth of harmful micro-organisms, such as bacteria, fungi, or protozoa (Cizmas et al., 2015, Marzo & Bo, 1998). The classical definition of an antibiotic explains that it is a compound produced by a micro-organism which has the capacity to inhibit the growth and even to destroy bacteria and other microorganisms in low concentrations (Demain, 1999).

Antibiotic consumption is expressed as doses per day (DDD, defined daily dose, per capita), that is put into a perspective with population and time (DDD/1 000 inhabitants/day). The value reports as per mille the proportion of people who have consumed a daily dose of certain pharmaceutical. Antibiotic consumption in human medicine ranges from 8.6 to 36 in Europe (ESAC 2018). In 2015, the EU/EEA

population-weighted mean consumption of antibiotics for systemic use in the community was 22.4 and in 2016 21.9 DDD per 1 000 inhabitants and per day. In Finland, statistically significant decreasing trend has been observed in DDDs (ESAC 2018). The amount of DDDs for regularly consumed antibiotics in Finland during years 2010 – 2018 are shown in Figure 1, and the individual antibiotics are described in section 2.1.1.

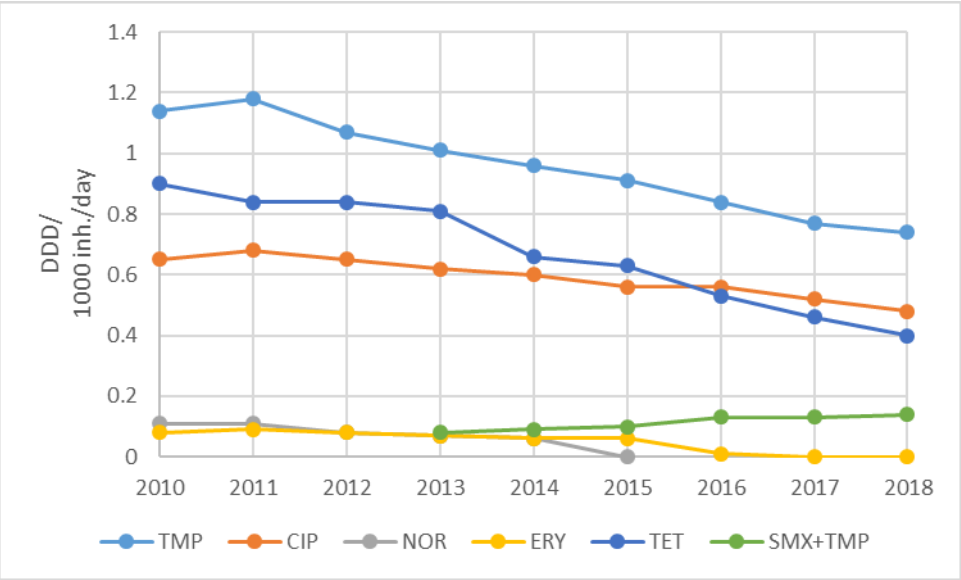

Figure 1. Defined daily doses for commonly used antibiotics in Finland (FIMEA)

In the eastern and southern countries in Europe, the antibiotic consumption varies strongly during different seasons of the year; during winter the consumption is nearly doubled compared to summer time. In Nordic countries variation has also been detected, as consumption during winter season has increased almost a quarter when compared to summer (Paakkari & Voipio, 2003).

Most antibiotics have not been detected to be biodegradable under aerobic conditions (Kümmerer et al., 2000; Ingerslev & Halling-Sørensen, 2001; Li et al., 2008). Since biodegradation by bacteria and fungi is highly ruled out due to the antimicrobial effect of antibiotics, they can persevere quite persistently through biological treatment and in environment.

2.1.1 Introduction to widely consumed antibiotics in Finland

2.1.1.1 Trimethoprim

Trimethoprim (TMP) (Figure 2) is an antibiotic that is mainly used for treating urinary infections. Occasionally TMP is used to treat other kind of infections such as acne and respiratory tract infections, and it is sometimes prescribed to prevent infections. The prescript doses for treating the infections vary often from 100 mg to 800 mg per day, depending on the type of infection, and treatment can last from 3 days to 6 months. TMP is the earliest antibacterial diaminopyrimidine that has been brought for clinical use, and it is still widely used as a sulphonamide potentiator in human and veterinary medicine. About 50% of ingested TMP is excreted unchanged in human urine (Göbel et al., 2005).

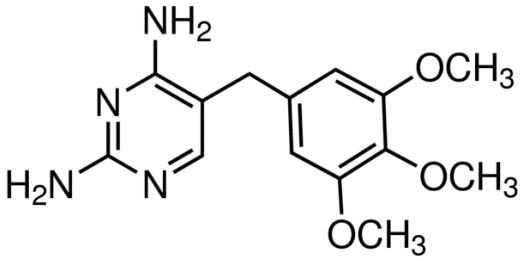

Figure 2. Chemical structure of trimethoprim ($C_{14}H_{18}N_4O_3$)

2.1.1.2 Ciprofloxacin

Ciprofloxacin (CIP, Figure 3) is a broad-spectrum, commonly used fluoroquinolone antibiotic that is effective against many Gram-positive and Gram-negative bacteria. It is mainly used for treating bladder- and gastrointestinal infections, as well as bone and joint infections and gonorrhea. Depending on the pathogen, treatment may require simultaneously some other antimicrobial pharmaceutical alongside with CIP. Treatment lasts usually 5 – 21 days, and its daily dose is about 500 mg. CIP can be taken by mouth, in eye drops, or intravenously. Around 45-62% of CIP is excreted unmetabolized via urine while 15-25% is excreted via feces (Golet et al., 2003).

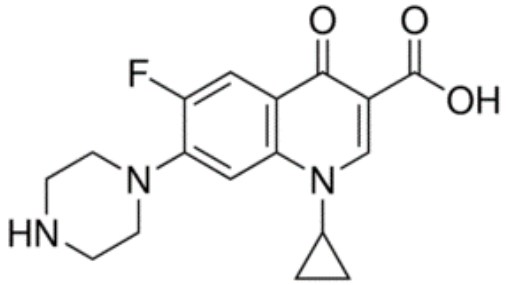

Figure 3. Chemical structure of ciprofloxacin ($C_{17}H_{18}FN_3O_3$)

2.1.1.3 Sulfamethoxazole

Sulfamethoxazole (SMX, Figure 4) is sulfonamide, bacteriostatic antibacterial agent that interferes with folic acid synthesis in susceptible bacteria. WHO has included SMX into its list of essential medicines, along with TMP and CIP (WHO, 2017). The development of resistance has been found to limit its broad spectrum of activity (Reynolds et al., 1993). SMX is usually consumed together with TMP in order to cover a wide spectrum of bacteria, both gram-positive and gram-negative (Papich 2016). It is shown by studies that when compared to either of TMP or SMX alone, bacterial resistance develops more slowly with the combination of these two drugs. This dose usually contains five parts of SMX and one part TMP, e.g. a tablet of 400 mg/80 mg SMX/TMP. Depending on the infection, treatment can last 1 – 21 days, with around 800 mg/160 mg daily dose. 10% of ingested SMX is excreted unchanged in human urine (Göbel et al., 2005).

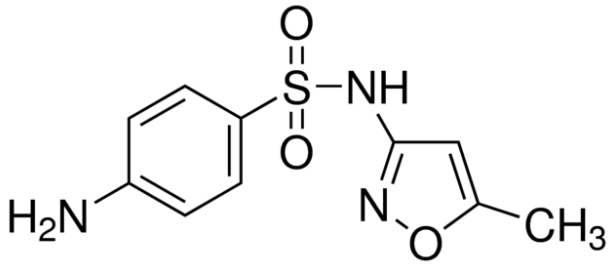

Figure 4. Chemical structure of sulfamethoxazole ($C_{10}H_{11}N_3O_3S$)

2.1.1.4 Norfloxacin

Norfloxacin (NOR, Figure 5), as well as CIP, belongs to the fluoroquinolone class. It is also used to treat urethritis and intestinal infections, though it is not as active as CIP. This medicine, as well as the fluoroquinolone class, is associated with an increased risk of tendinitis and tendon rupture in all ages, and is increased in older patients. Normal dose for adults is 400 mg twice a day, from three days to four weeks, depending of the reason for treatment. At least 30 - 40% of an oral dose of NOR is absorbed (Merck & Co., 2008).

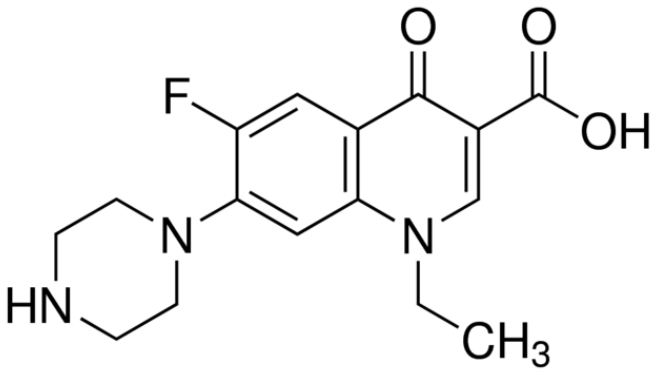

Figure 5. Chemical structure of norfloxacin ($C_{16}H_{18}N_3O_3F$)

2.1.1.5 Erythromycin

Erythromycin (ERY, Figure 6) is produced from actinobacteria *Streptomyces erythreus* that lives in soil. It is macrolide, and effective against many Gram-positive bacteria (e.g. streptococcus, staphylococcus) and works by inhibiting their protein synthesis. Regular dose for adults is 1 – 2 g, and in more serious infections, 4 g, intravascularly or as an oral solution. Treatment usually lasts around 7 to 15 days.

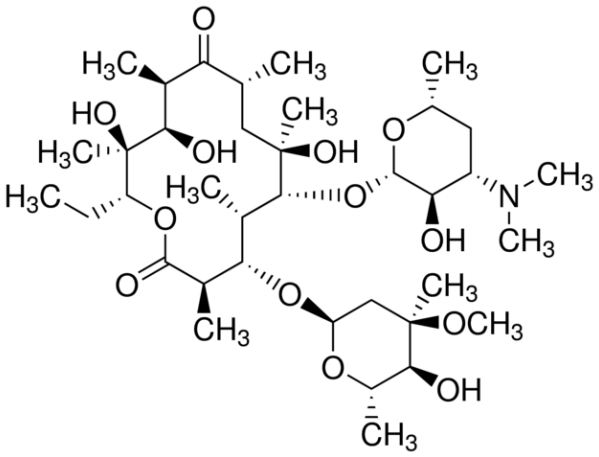

Figure 6. Chemical structure of erythromycin ($C_{37}H_{67}NO_{13}$)

2.1.1.6 Tetracycline

Tetracycline, belonging to the group of tetracycline antibiotics, inhibits the biosynthesis of bacteria. It is used for treating a number of infections, e.g. acne and respiratory infections. Daily dose contains around 500 mg 2 – 4 times a day. For treating acne, the treatment can last for 6 months.

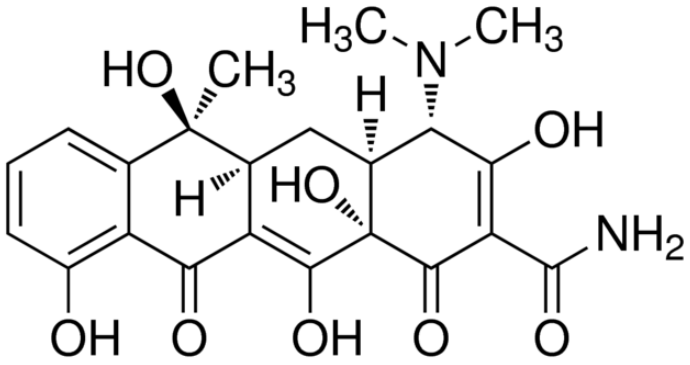

Figure 7. Chemical structure of tetracycline ($C_{22}H_{24}N_2O_8$)

2.1.2 Risks caused by antibiotics

The wide use of these antimicrobial pharmaceuticals has also a dark side: resistance against antibiotics developed by bacteria. Antibiotics should be used only to treat or prevent infections that are confirmed or strongly assumed to be caused by bacteria and in prescribed doses in consideration of reducing the probable

development of drug-resistant bacteria and sustain the effectiveness of antibacterial drugs.

Even though most pharmaceuticals are detected in natural waters in only low to very low concentrations in the developed countries, the possible risks of what these amounts of pharmaceuticals can cause for aquatic ecosystem are still greatly unknown. Antibiotics are of particular interest because it is not currently known for sure if their existence in natural waters contributes to the spread of antibiotic resistance of micro-organisms (Giger et al., 2003). As a result of resistance towards antibiotics, infection morbidity and –mortality along with health care costs will increase. For this reason, The World Health Organization (WHO, 2014) has defined antibiotic resistance as one of the major public health issues of the twenty-first century.

One example of a disease where bacteria has developed a resistance towards used antibiotic is tuberculosis. Tuberculosis (TB) is contagious and often severe airborne disease caused by a bacterial infection. Its general symptoms include chest pain, unintentional weight loss and coughing up blood. Multidrug-Resistant Tuberculosis (MDR TB) has arisen due to improper usage of the drug, especially in areas with weak TB control programs. This has led to a situation where TB bacteria can no longer be killed by the two most effective antibiotics used for treatment, isoniazid and rifampin. Drug surveillance data show that of the estimated 600 000 people developed MDT TB in 2016, 240 000 people died. In 2016, 8 000 patients with Extensively Drug-Resistant Tuberculosis (XDR TB) were reported worldwide. XDR TB is a less common form of multidrug-resistant TB in which TB bacteria have changed enough to bypass the two best antibiotics as well as most of the alternative drugs used against MDR TB. These second-line drugs include at least one of the other three injectable anti-TB drugs, and any fluoroquinolone antibiotic. It is suspected that 6.2 % of the people with MDR TB have XDR TB. TB is the tenth leading cause of death worldwide and, since 2012, the top killer among the infectious conditions, even more fatal than HIV. (WHO, 2018)

In English Surveillance Programme for Antimicrobial Utilisation and Resistance (ESPAUR, 2017) report, more than one million urinary tract infection samples were analysed, and one in three (34 %) of the samples were found to be resistant to TMP. In the report, this is assumed to have happened due to the improper use of TMP, such as taking them for mild infections that may clear-up without treatment, which is known to fuel resistance (Bengtsson-Palme et al., 2016).

Chemicals released into the environment can cause adverse ecological effects when the concentrations exceed the threshold of environmental self-purification and organism tolerance (Chen & Jiang 2011). As a result of the low and continuous concentrations of pharmaceuticals present in WWTP, WWTPs are potentially an important source of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) in aquatic environment (Martinez, 2008; Devarajan et al. 2015).

Currently there is no consensus regarding the best practices for the ecological risk assessment for pharmaceuticals. Furthermore, there is no information on is antibiotic contamination of waste- and natural waters could lead to increase in ARB and ARGs. The mixtures of different pharmaceuticals and personal care products (PPCP) may potentially also produce synergistic toxicity. In the future, the availability of usable water may be reduced due to the combination of increasing global population size and potential droughts that could become more severe due to climate change, thus increasing the concentrations of pharmaceuticals in remaining water sources (Cizmas et al., 2015). Considering the low but continuous levels of antibiotics being leaked into the environment, acute toxic effects are improbable to occur. Chronic effects arising over longer time periods are more likely, but studies to detect them may take up to decades, making laboratory-scale examinations difficult.

2.1.3 Routes to the environment

The primary sources of harmaceutical compounds carried to the aquatic environment include the incorrect disposal of drugs, bodily excretion after use, and a fraction from waste and discharges during pharmaceutical production (Figure 8). Most antibiotic compounds are not well absorbed and are poorly metabolized in

human and other animal bodies. 90 % of some compounds can be metabolized after consumption, while for others the amount can be less than 10 %. These values can explain why a high percentages of the intake dosages of different antibiotics are excreted via urine and feces either as the parent compound or as metabolites, and consequently reach a wastewater treatment plant through the sewage system (Liu et al., 2010; Zhang et al., 2014). Considering that the antibiotics are designed to be effective even in low concentrations, their existence in nature and aquatic environments lead to concerns regarding their potential unintended biological effects in different organisms (Hörsing et al., 2010).

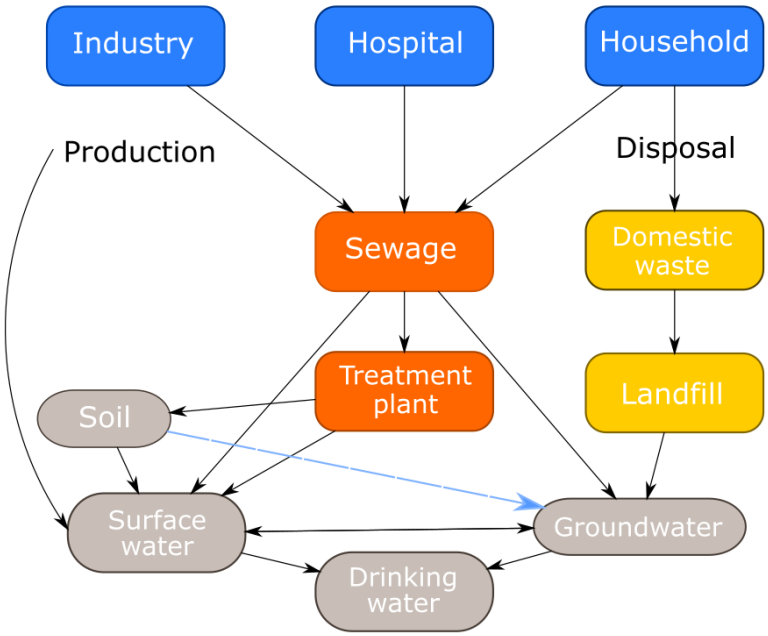

Figure 8. Exposure paths of human-use antibiotics into wastewaters and into the environment (modified from Giger et al., 2003).

Sorption onto sewage sludge particles can represent one important pathway for pharmaceuticals into the environment if the sludge is used as fertilizer in agriculture. Areas with high population densities are likely to be of most concern, since at present there are no standards for the amounts of pharmaceuticals discharged into the environment from sewage, unlike in industrial discharges.

2.2 Wastewater treatment plants

Domestic and industrial WWTPs receive organic pollutants from several different sources. These include human excretion products, household disposals, cleaning compounds, fossil fuel spillages, lubricants, storm water runoff from highways, and urban runoff inputs that flush the compounds deposited on the ground surface from vehicles or heating systems (Shen et al., 2009). Wastewater is collected from homes, businesses, and industries with sewers and delivered to plants for treatment.

There are two main stages in the treatment of wastewater: primary and secondary. When influent is coming to WWTP, it is first screened. In screening, coarse particles and objects from a flow of wastewater that might clog pipelines or damage equipment are removed, e.g. by retention on mechanically raked bars or rotating discs. After this the wastewater is guided to primary treatment, which is a sedimentation stage where the low flow speed causes the particles that are heavier than water to settle on the bottom, eventually accumulating into sludge. Primary treatment is followed by secondary treatment that is based on natural processes. The sewage that has left the settling tank is pumped into an aeration tank, where it is mixed with air and sludge that is loaded with a diverse group of bacteria and allowed to stand for several hours. In the meantime, the bacteria break down the contaminants, in particular biodegradable organics like carbon and phosphorus, into harmless by-products. The secondary stage of wastewater treatment removes around 85% of the organic load in sewage by utilizing the bacteria in it and by attaching onto the surface of bacteria and eventually ending up into the sludge. The oxygen demand is guaranteed by pumping oxygen continuously into the wastewater. Aerated wastewater is guided to secondary sedimentation, where the sludge settles again to the bottom of the pool. The settled wastewater is then released into drainage systems.

For treatment purposes, the activated sludge process is the most commonly used biological wastewater treatment for both domestic and industrial plants worldwide, together with membrane bioreactor and moving bed biofilm bioreactor (Wei et al., 2003). Conventional wastewater treatment plants that are currently used are

designed to remove macropollutants that include chemical and biochemical oxygen demand and total suspended solids, and nutrients like phosphorous and nitrogen, through a primary and secondary treatment. For this reason, the removal of micropollutants, such as pharmaceuticals, is lacking in effectiveness, especially for polar antibiotics (Michael et al., 2013; Meinel et al., 2016).

Removal rates can be increased with tertiary treatment when high quality effluent is required, but with relatively higher cost (Luo et al., 2014). Tertiary treatment may include advanced oxidation process (e.g. ozonation), reverse osmosis, and adsorption on activated carbon (Knopp et al., 2016; Alves et al., 2018). In the wastewater treatment processes, it is assumed that the major removal pathways of antibiotics include adsorption into sludge, disinfection, biodegradation, and membrane separation. Some antibiotics can be degraded by sunlight or UV photolysis, but this kind of degradation does not have any great role at the WWTP for the reason that UV light cannot pass through the wastewater very deep due to the high amounts of suspended solids (Golet et al., 2003). Also, the vapor pressures of all antibiotics are very low ($< 5.75 \times 10^{-6}$ Pa), and thus it can be concluded that volatilization can be excluded as a significant removal route (Pérez et al., 2005; Zhang & Li, 2011). Because of the incomplete degradation in treatment plants, small amounts of antibiotics are continuously being discharged into surface waters.

2.2.1 Antibiotics in wastewater

The efficient removal of antibiotics in wastewater treatment processes alters and depends primarily on the combination of antibiotics' physicochemical properties and the operating conditions of the treatment systems used. Recorded concentrations of antibiotics in raw waste water often range from low ng L^{-1} (where 1 ng L^{-1} equates to 1 ppt, part per trillion) to low µg L^{-1} (part per billion, ppb), but can also reach concentrations up to mg L^{-1} scale (Michael et al., 2012; Ngumba et al., 2016b; Kairigo, 2019), depending on the amounts consumed, the nature of antibiotic, the treatment technology used in WWTPs and the time of the year (Michael et al., 2012). Even though these concentrations are low and difficult to detect, they are produced to be effective even in low doses and have the potential for environmental

effects. The most commonly used biological treatment methods for wastewaters are often unable to efficiently remove antibiotics from wastewaters, as was explained above.

Along with antibiotics, wastewaters may carry considerable amounts of ARB and ARGs (Rizzo et al., 2013). The existence of antibiotics in these environments is assumed to promote the selection of ARGs and ARB. The environment in biological treatment process is assumed to create a potentially suitable habitat for resistance development and spread, as a wide selection of bacteria is repeatedly mixed with antibiotics in sub-inhibitory concentrations (Auerbach et al., 2007; Davies et al., 2006; Ferreira da Silva et al., 2006).

Biological, mechanical, physical and chemical processes are some of the mechanisms that can possibly affect the fate of antibiotics as well as ARB and ARGs in various ways. This could eventually lead to the development of resistance due to diverse environments of exposure, and the spread into the natural environment. It has been measured that the final effluent discharged from WWTP can contain about 10^9–10^{12} Colony Forming Units (CFU) per day, per inhabitant equivalent (Novo & Manaia, 2010). CFUs are used as a measure of the assumed number of microorganisms that are present in or on surface of a sample and are possible to proliferate. Among the CFUs being discharged from WWTPs, at least 10^7–10^{10} is assumed to carry at least some sort of antibiotic resistance. These levels of adverse CFUs highlight the importance of WWTPs when it comes to the accumulation and spread of antibiotic resistant bacteria in the environment (Rizzo et al., 2013).

2.2.2 Sludge

During the secondary treatment in WWTPs, an excessive amount of sludge is generated, making it the largest by-product produced during the wastewater treatment process. The content of the incoming wastewater, the type of wastewater treatment processes, and the types of following treatments applied to the sludge affect the characteristics and volume of the sewage sludge generated, which is why the characteristics of the sewage sludge within an individual plant can vary when studied in different time periods, especially during different seasons.

The use of sewage sludge as an inorganic fertilizer replacement serves a potential recycling method due to its rich nutrient content, especially in phosphorus (0.5 - 0.7% of total solids) and nitrogen (2.4 - 5.0% of total solids), but it also contains potassium, sodium, and magnesium (Ahmad et al., 2004; Tyagi & Lo, 2013). However, the harmful substances that are not absolutely removed in waste water treatment will end up in agricultural soil as well. When the sewage sludge is added to the agricultural soil, a variety of toxic compounds present in sludge will be introduced to the soil as well, posing a risk to the environment (Roig et al., 2012; Petrie et al., 2014). Therefore, the appropriate chemical and biological purity of the sludge needs to be confirmed before land application in order to get rid of the environmental hazard substances.

Sludge coming from municipal WWTP often includes mixtures of different compounds whose total toxicity is not studied and remains unknown. In EU, sludge has mainly been disposed by application on agricultural soil, landfilling, and incineration, covering for nearly 90% of the total amount of produced sludge (Davis and Hall, 1997; Tyagi & Lo, 2013). By land application, the harmful substances that are less biologically degradable will end up in the natural soil. This can sooner or later lead to detrimental consequences since the surroundings will be exposed to the toxic compounds for a long period of time. If this happens in continuous rhythm, for example if the sludge is applied many times, e. g. when used as a fertilizer, the less biodegradable toxic compounds will eventually accumulate in the soil. Ciprofloxacin has been recognized to be one of these persistent compounds in sludge applied lands (Jensen et al., 2012). Because of the continuous addition of new sludge, it has a high risk of accumulating over time, and may also lead to bioaccumulation in the environment, effecting the food chain when digested by earthworms. These kind of risks caused by potentially toxic elements like pathogens, heavy metals, and persistent organic pollutants that are found in sewage sludge are the main reason why land application of sewage sludge is limited or forbidden in order to prevent health risks to human and livestock. (Wei et al., 2003).

Since it is noted that sludge constituents have these potential adverse effects, a revision of the European Union Directive 86/278/EEC concerning sewage sludge

disposal by land application has been planned to limit the amount of organic micropollutants released to the environment. According to the Directive, the sludge may be used in agriculture only if its use is regulated by the member State concerned. If the concentrations of any of the harmful substances found in sludge exceed the given limit for the compound in the soil, the use of the sludge is prohibited. However, the Directive focuses on heavy metals, and pharmaceuticals are not included. The Directive is based on the decision No 2455/2001/EC where the main focus is on aquatic environment protection against emerging pollutants that can cause a negative environmental effects. Any land application may lead to leaching of the toxics present in the applied land mass, especially in different soil pH values. Leaching makes the unfavorable compounds mobile, hence they will eventually end up in aquatic environments. Many polar compounds are resistant to degradation and are persistent in the environment. They are also less strongly adsorbed in the soil thus mobile in water, which is why they are highly likely to reach the aquifer and pollute the groundwater (Klöpffer 1996). Furthermore, accumulation in soil may lead to an uptake of the chemical by plants, leading to the spread of xenobiotic substances in living organisms and to extensive pollution (Fatta-Kassinos et al., 2011). In Finland, using sludge based fertilizers is allowed as long as it fulfills the quality standards ordered by law. The soil in Finland has lower pH than most of the EU countries, which needs to be taken into account when evaluating the possible risks for the environment. According to the Finnish Fertilizer Product Act (539/2006), utilization of byproducts suitable for fertilizing is supported as long as it is proven that they do not cause harm for humans, animals or plants, and have a positive impact on plant growth.

The distribution coefficient, K_d, describes the equilibrium concentration ratio of chemicals between soil, clay, sediment or sludge particles and water and is used in various environmental models for estimating the extent to which contaminants are sorbed to solid matter e.g. during wastewater treatment, of leaching through a soil column, and of runoff from agricultural land into adjacent waters, as examples (Wauchope et al., 2002; Shafrir & Avisar, 2012). Joss et al. (2006) reported that sorption onto secondary sludge is relevant only for compounds with K_d of more

than 300 L kg^{-1} (i.e. Log K_d >2.48). The characteristics of the chemical as well as the matrix determine the distribution of a chemical between sludge, soil or sediment and water. External factors like temperature and rainfall may additionally influence the distribution (Wauchope et al., 2002).

The level of how strongly an organic substance can absorb onto the sludge is driven by the compound's hydrophobicity, which can be predicted by the octanol-water partitioning coefficient, K_{ow} (Wijekoon et al., 2013; Rybacka and Andersson, 2016). Value for the K_{ow} is gotten from the compounds partitioning between lipid phases in the environment and water, C_O/C_W. For example, during primary sedimentation in wastewater treatment, hydrophobic chemicals in the incoming wastewater may partition to settled primary sludge solids. Considering that measured K_{ow} values can be in the millions for important environmental contaminants such as PCB's and dioxins, they are often expressed as the base 10 logarithm, Log K_{ow} (Piwoni & Keeley, 1990). Substances with high Log K_{ow} value will preferentially absorb to organic matter in soils and sediments rather than the aqueous phase (Hyland et al. 2012). The following can be used as a general guide: Log K_{ow} < 2.5 yields low sorption potential and low hydrophobic tendency, Log K_{ow} > 2.5 and < 4.0 yields medium sorption potential, and Log K_{ow} > 4.0 promotes high sorption potential and is more hydrophobic, thus is more likely to be adsorbed by the sludge (Rogers 1996). The higher the Log K_{ow} value is, the higher is also the potential to bio-concentrate to living organisms.

Since antibiotic contamination of environment has been confirmed, a concern regarding their possible contribution to the emergence of antibiotic resistance and spread of resistant, disease-causing bacteria, is growing (Issacson & Torrence, 2002; Goni-Urriza et al., 2000).

2.2.3 WWTPs as reservoirs of antibiotics, ARGs and ARB

Considering WWTPs receive sewage from multiple different sources, it also contains bacteria from different environments. WWTPs serve an optimal environment for these different bacterial species to interact with each other and encourage them to transfer genes between each other. The high bacterial densities

in waste water together with biofilms and stress caused by pollutant compounds such as disinfectants, heavy metals, antibiotics, and other pharmaceuticals can promote horizontal gene transfer (HGT) in waste water. Furthermore, HGT gives an advantage to useful genes by forming a selection pressure that favors the bacteria who can survive in certain environment, eventually being able to develop antibiotic resistance even in low concentrations. This is the main reason why waste water and WWTPs can be assumed to act as reservoirs, environmental suppliers, and sources of both antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) released into the environment (Watkinson et al., 2007; Rizzo et al., 2013; Bouki et al., 2013; Guo et al., 2017).

The concentrations of different antibiotics that can support he development of antibiotic resistance stay below therapeutic concentrations that are used in linical setting (Gullberg et al., 2011). For example, minimum selective concentration (MSC) which is considered to be the lowest concentration of antibiotic still being able to select for a given resistance mutation for ciprofloxacin have been found to be as low as 0.00001 $\mu g\ mL^{-1}$, whereas minimal inhibitory concentration (MIC) that is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation have been studied to be 0.047 $\mu g\ mL^{-1}$, meaning the pharmaceutical dose needed for treatment is over 1000 times higher (Gullberg et al., 2011). These findings suggest that resistance is very likely to develop in sub-MIC concentrations. Lower concentrations of antibiotics may indeed serve a suitable environment for the development of resistance, considering that very high concentrations are often lethal even to resistant cells (Pei et al., 2006).

Gullberg et al. (2011) studied the effect of low antibiotic concentrations on the development of antibiotic resistance in bacteria (Figure 9).

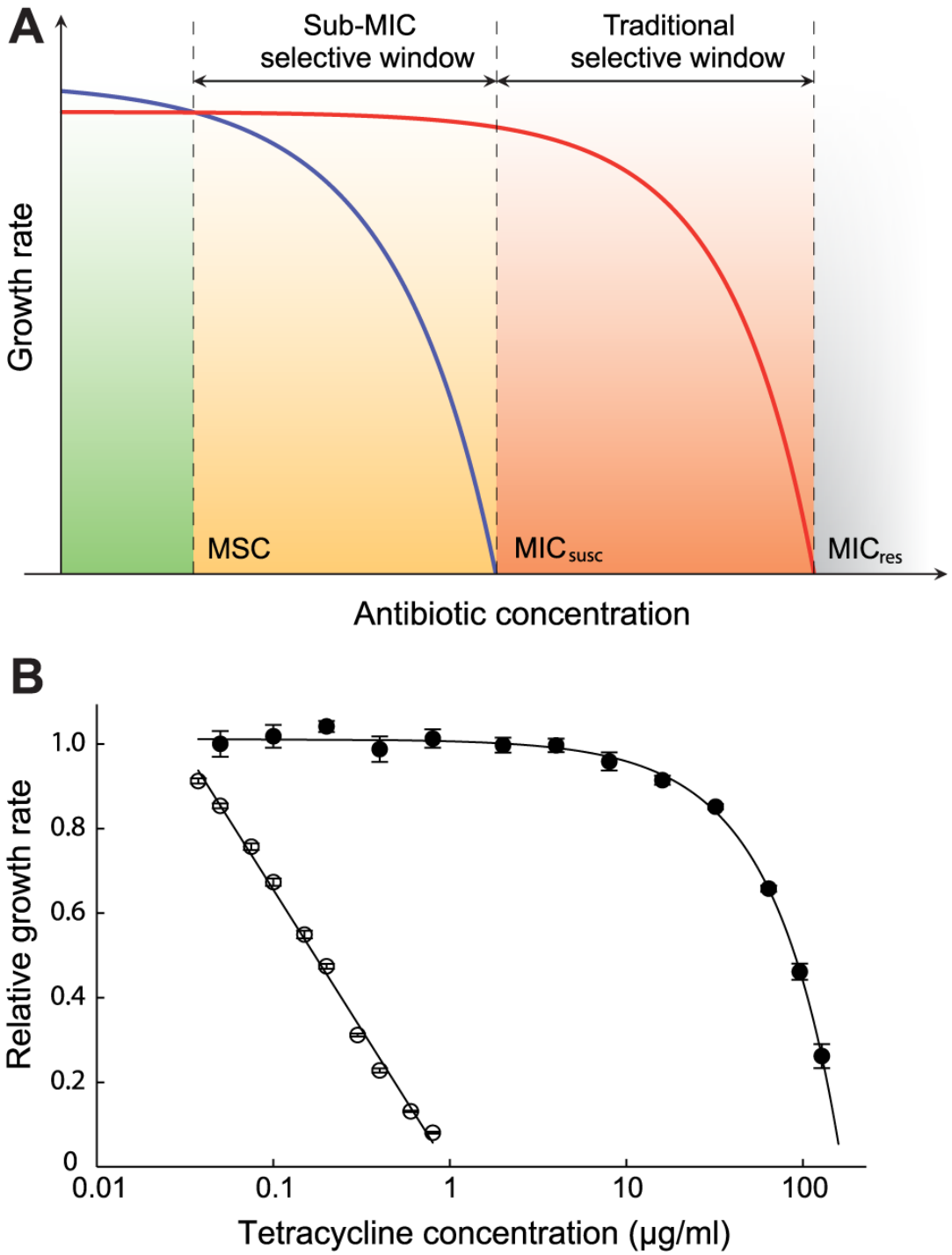

Figure 9. Growth rates of antibiotic resistance as a function of antibiotic concentrations (Gullberg et al., 2011)

In Figure 9A, green indicates the concentration range below the MSC where the bacteria can still select for a resistance mutation. In these concentrations, the susceptible strain (blue line) outcompetes the resistant strain (red line) as a result of fitness cost of resistance. Orange (sub-MIC selective window) and red (traditional

mutant selective window) express the antibiotic concentrations where the resistant strain outcompetes the susceptible strain as a result of the selectivity (MIC_{susc} = minimal inhibitory concentration of the susceptible strain; MIC_{res} = minimal inhibitory concentration of the resistant strain; MSC = minimal selective concentration.).

A comparison of the susceptible and resistant strains of S. typhimurium as a function of tetracycline concentration is shown in Figure 9B. Plot with open circles expresses the relative exponential growth rates of susceptible strain whereas plot with closed circles represent resistant strains. A relative growth rate of 1.0 corresponds to approximately 1.8 hr^{-1} (Gullberg et al., 2011).

When ARBs manage to end up in WWTPs they can spread their resistance genes to other bacteria. ARBs have been detected in WWTPs and the effluent waters, which means that they are not destroyed in the treatment (Rizzo et al., 2013). Even the advanced treatment is lacking the effectiveness on removing these genes, and on the contrary, some of these technologies may trigger the SOS response in bacteria, which escalates the mutation rate by boosting the expression of error-prone DNA-polymerases (Qin et al., 2015) and HGT of ARGs (Beaber et al., 2004). It has been shown that effluents contain different variety of bacteria and antibiotics than sewage sludge (Bengtsson-Palme et al., 2016; Yang et al., 2014) for which reason they both should be taken into account when studying their possible risks.

Sludge has been found to be the main source of tetracycline and sulfonamide resistance genes that are discharged into the aquatic environment (Munir et al., 2011). For this reason, the growing demand for reusing sludge in order to recycle the important nutrients raises a problem. The application of sewage sludge has a risk of increasing the abundance of certain ARGs already present in the soil as well as adding new ones that have not been previously present, posing a risk of distributing antibiotic resistance (Urra et al., 2018).

The most important source of drug resistance still remains unclear. The natural development of resistance in an environment with extremely low antibiotic concentrations that supports selective pressure in exposed bacterial communities is

one concern. Excretion of the organisms by patients that have undergone such antibiotic treatment, consequently transferring plasmids in the environment is another. (Jones et al., 2004)

2.3 Analytical methods for determining low antibiotic concentrations in environmental matrixes

2.3.1 Pre-treatment

When studying complex environmental samples like wastewater and sewage sludge that contain many different substances, great disturbance in the analysis may be caused by matrix effects. Matrix effects originate from the competition between the analyte and co-eluting interfering species, influencing both qualitative and quantitative analyses, and giving rise to suppression or enhancement of the signal. These possibly misleading results can be narrowed down by using internal standard (IS), and performing proper extractions and purifications as well as using correct detection conditions (Gosetti et al., 2010). A study by Snyder et al., (1997) describes that a suitable IS should have a retention time close to that of the studied compound, should not be found in the original sample, should mimic the studied compound in any sample pretreatment step, should be stable and unreactive with the sample or mobile phase, and should have a similar detector response to the studied compound for the concentration studied. Isotopically labelled ISs are often the best options for fulfilling these requirements.

In all cases, sewage sludge has challenging physical properties when developing a suitable analytical method for separating and investigating the compounds present in sludge. These challenging properties include particles with large surface areas (0.8 – 1.7 m^2 g^{-1}), negative surface charges, and available spaces in particles, all of which support strong bonding onto the particle's surface due to different charges, and promote sorption into the biomass. Furthermore, chemical additives such as lime, ferric chlorine, and cationic polyacrylamide polymers that are used in the conditioning add more challenges for analytical chemistry methods (McFarland, 2001). Best way to manage these challenges would be to overcome the strong

negative surface charges and available gaps that supply multiple active sites for charged compounds, and focus on the cleaning for the removal of unwanted co-extracted products, like fats, proteins and surfactants (Jones-Lepp & Stevens 2007).

2.3.1.1 Sonication

In order to make the analysis possible with most common analytical instruments (e. g. LC, GC), the sample must be in liquid form. Sonication with ultrasound can enhance the dissolution of the compound from solid substances like sludge into an extraction solution. This can be performed by placing the samples into a sonication bath, or by bringing the ultrasound shaker directly into a single sample. Eliminating the water content increases the extraction efficiency, and therefore samples with high water content (e.g. sewage sludge) should be dried before extraction, for example by freeze-drying.

The high frequency soundwaves shake the sample particles and stir the solution. This brings the solid matrix in touch with the organic solvent so that dissolution is much more effective. The ultrasound extraction normally takes few minutes (3 - 10 min), and often repeating the extraction increases its effectiveness. After ultrasonic extraction the desired compound is in the extraction solution, which can be separated from the solid matter by e.g. vacuum filtration or centrifugation and be collected for further treatment.

2.3.1.2 SPE

Solid phase extraction (SPE) is widely used method for pre-treating dissolved samples by cleaning them. Most environmental samples, like wastewaters, include many different compounds that can disturb the analysis, so cleaning with SPE is important. With SPE the compounds that may disturb the chromatographic analysis can be eliminated, and the studied substance can be concentrated, which is often essential when studied compounds are found in low concentrations, as in microgram scale.

In SPE, a small amount of studied sample is guided through an extraction cartridge by gravitation and suction, after which it is restrained into the sorbent and stays in the cartridge, while most impurities are flushed through. Then a small volume of a suitable solvent that will not elute the analyte is guided through the cartridge, in which the studied compounds are then dissolved. This way the studied compounds can be concentrated from higher volumes, like 1 L, into a final volume of solvent of 1 mL, while also flushing out the impurities that would other ways disturb the analysis.

2.3.2 Analysis

2.3.2.1 LC

All the chromatographic methods that include a liquid as a moving phase are called Liquid Chromatography (LC). The separation between different compounds is based on the interactions between the compounds in the sample and the stationary and liquid phase. In LC, gravitation and capillary action is replaced by a pump that pushes the liquid through a column with high pressure, therefore lowering the analysis times. Nowadays the resolution and symmetry of signals gotten from LC is excellent, so it is often called a High Performance Liquid Chromatography (HPLC).

2.3.2.2 MS/MS

Most commonly used instrument for quantifying pharmaceutical residues in wastewater is HPLC coupled with tandem mass spectrometry (MS/MS). HPLC-MS/MS (Figure 10) is an analytical chemistry technique that combines the separation techniques of liquid chromatography with the analysis capabilities of mass spectrometry, of which specificity is enhanced by two or more rounds of MS ("tandem mass spectrometry" or "MS/MS"). While MS measures the m/z (mass-to-charge ratio) of an ion, with MS/MS the m/z can be measured as in MS, followed by m/z of its constituent fragments, giving better sensitivity for detection since more than one ion can have similar m/z ratio, but not the same m/z of its daughter ions.

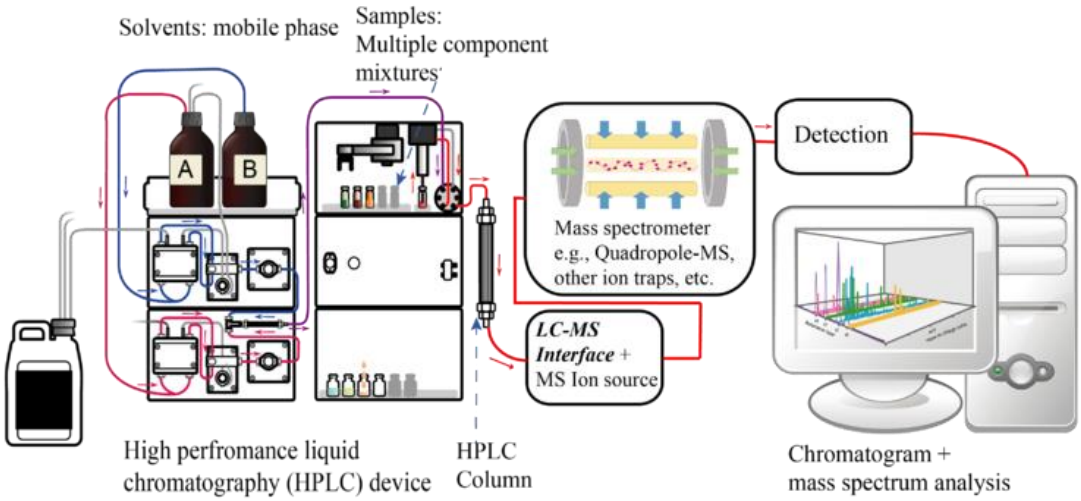

Figure 10. Diagram of liquid chromatography tandem mass spectrometry (Norena-Caro, 2017)

Mixtures of multiple components are going through a fraction before mass-spectrometry into their components by liquid chromatography. The sample is injected in liquid form into the LC and the different chemical components are separated when they move in different speeds due to different affinities for the stationary phase and mobile phase. The output of LC column is then directed into a mass spectrometer. The sample molecules are first ionised in ion source using e.g. electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), and separated according to their m/z values (mother–ions). In the second quadrupole, a collision which is guided with an inert gas occurs that causes the mother–ions to get fragmented further into daughter ions that can then be analysed in the third quadrupole. The separated ions are detected and a mass spectrum is generated. A specific peak from the mass spectrum is then chosen and isolated and collisions are caused to happen in order to force a characteristic fragmentation of the selected ion.

While many other chromatographic methods need at least 10 – 30 min per sample with LC compared with MS/MS the run times can be as low as 1 - 3 min. Because of the extremely large amounts of interfering materials co-extracted with pharmaceuticals (Jones-Lepp & Stevens 2007), the wastewater and sludge analyses

are often performed using the MS/MS mode for both identification and quantification.

Considering the complexity and the low concentrations of pharmaceuticals expected in wastewater, HPLC-MS/MS is one of the most powerful analytical techniques for these analysis, because it gives extensive information on the target compounds while increasing selectivity and sensitivity.

3 EXPERIMENTAL SECTION

3.1 Background

For this thesis, the target was to investigate the concentrations of specific antibiotics found from Jyväskylä's wastewater treatment plants, Nenäinniemi and Korpilahti. Three most common antibiotics prescribed in Finland were chosen for the analyses; ciprofloxacin (CIP), trimethoprim (TMP) and sulfamethoxazole (SMX). The properties of these compounds are listed below (Table 1).

Table 1. Properties of the selected antibiotics. SMX is used together with TMP

	CIP	TMP	SMX
Empirical formula	$C_{17}H_{18}FN_3O_3$	$C_{14}H_{18}N_4O_3$	$C_{10}H_{11}N_3O_3S$
Molecular weight	331 g mol^{-1}	290 g mol^{-1}	253 g mol^{-1}
Class of antibiotics	Fluoroquinolone	Bacteriostatic	Sulfonamide
Mode of action	Inhibits DNA gyrase	Inhibits dihydrofolate reductase	Inhibits para-amino benzoic acid
Target organism	Gram-negative bacteria	Gram-negative bacteria	Gram-negative bacteria
Clinical MIC (mg/L)	Resistant: >4[1	Resistant: >8 to 152[1	
	Susceptible: <1[1	Susceptible: <2 to 38[1	
logK_d	4.3[2	2.3[2	2.05-2.60[2
logK_{OW}	0.28[3	0.91[4	0.89[4
Excreted unchanged (%)	33[2	50[2	10[5

[1)]Nagulapally et al., 2009; [2)]Golet et al., 2003; [3)]Vieno et al., 2007; [4)]Hansch et al., 1995; [5)]Göbel et al., 2005

Under investigation was the influent and effluent water from both WWTPs, and sludge from Nenäinniemi. With this experiment, the amounts of antibiotics released in the studied area were accurately measured.

3.2 Used equipment

For cleaning up and concentrating the samples, SPE was performed using Oasis hydrophilic–lipophilic balance (HLB) cartridges (6 cc/200 mg) and SPE vacuum manifold (Supelco, Bellefonte, PA, USA). For separating the studied compounds from the matrix, HPLC was used (Waters Alliance 2795 system) and detected with MS/MS (Quattro Micro triple-quadrupole).

3.3 Materials and chemicals

Ultrapure water for standards and eluents were produced from an Ultra Clear UV Plus and euRO 60 Reverse Osmosis unit (SG, Barsbuttel, Germany). Methanol and acetonitrile for SPE washing steps were of HPLC grade and purchased from Merck (Darmstadt, Germany). Formic acid (98%) that was used as an additive for HPLC was purchased from Fluka (Darmstadt, Germany). All the pharmaceutical standards (purity >95%) were from Universal Corporation Ltd, Kenya. Internal standards used were [$^2H_8$]-ciprofloxacin, [$^2H_4$]-sulfamethoxazole (both purchased from Alsachim, Illkirch, France), and [$^2H_9$]-trimethoprim (purchased from Sigma-Aldrich, Steinheim, Germany). The chemical purity of all substances exceeded 95%. All the standards had been diluted to a pooled mixed standard as a stock solution of 10 mg L^{-1} and stored in the dark at +4 °C.

For calibration, working solutions were prepared freshly before HPLC-MS/MS analysis, diluting the mixed standard with 80:20 H_2O:ACN solution.

The results obtained from HPLC-MS/MS were transferred to Excel and compared with the calibration curve in order to calculate the real concentrations. Obtaining the mass peak area from both internal standard and studied compounds is essential

for the calculations in order to estimate the amount of lost sample compound during whole sample treatment process.

3.4 Description of studied WWTPs

Samples (i.e. influent, effluent and reject wastewater, and sewage sludge) for this study were obtained from Jyväskylä's wastewater treatment plants Nenäinniemi and Korpilahti, located in central Finland. Both plants treat predominantly municipal wastewater. Nenäinniemi's wastewater treatment plant treats approximately 35 600 m^3/d of domestic sewage, serving a population equivalent of around 160.000 inhabitants. Its treatment process consists of preliminary clarification, and biological treatment, followed by secondary sedimentation.

Korpilahti is a smaller scale WWTP which treats the sewage of 3 000 inhabitants and local industrial wastewaters, with a daily volume of 525 m^3. The treatment process builds around activated sludge process.

3.5 Analysis

3.5.1 Sample preparation

Sludge contains a high amount of water, approximately 98% of its weight (UNEP, 2019). For analysis, the sludge requires dewatering process as pretreatment. One of the commonly used methods include freeze-drying (Nieto et al., 2010) which would not degrade the most heat sensitive compounds in sludge. The samples are often spiked with a standard solution as well as homogenized for example by grinding before the extraction step.

Sludge samples and influent and effluent waters from Jyväskylä's wastewater treatment plant Nenäinniemi were collected in January 2018, and later influent and effluent waters in the months of March 2018 and April 2018. Wastewaters from Korpilahti were collected in April 2018. Samples were treated separately as a single day sample, and once as a collection of a week. The water samples were collected

in 0.33 L polyethylene flasks and stored at +6 °C in the dark together with collected sludge.

3.5.2 Pre-treatment of sludge

For sludge analysis, approximately 12 g of raw sewage sludge was weighted into three polyethylene flasks, freeze-dried and stored in freezer (-20 °C). Dried sludge was crushed into smaller particles using a glass rod before weighting for analysis.

3.5.3 Sonication

Approximately 0.5 g of dried sludge was weighted into two KIMAX-tubes and 100 µL of internal standard (mixed internal standard, 10 ppm) was added. For first extraction, 4 mL of methanol was added and the samples were vortexed before sonicating with an ultrasonic device (UP 200s Ultraschall prozessor, Figure 11) for 10 minutes with amplitude of 30 and 1.0 cycle, meaning the processor was continuously switched on. The samples were then centrifuged for 5 minutes at 2500 rpm.

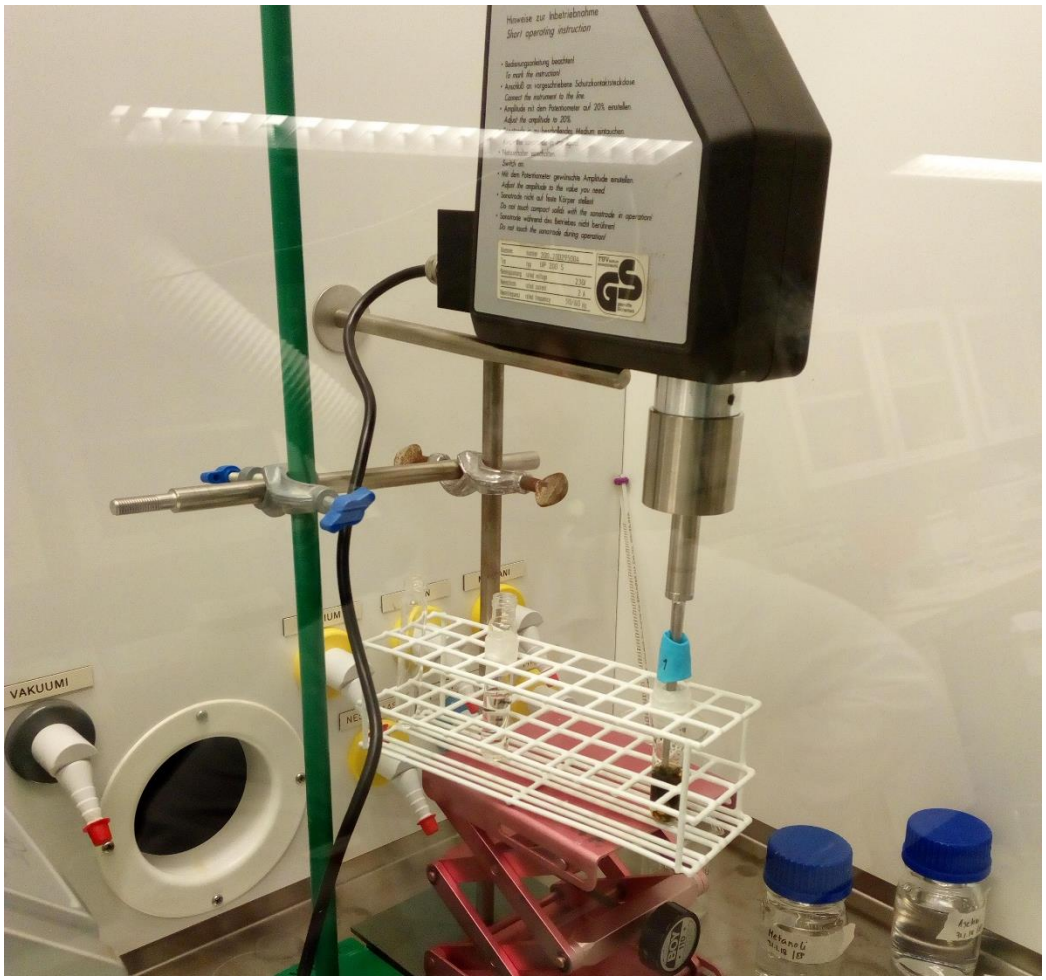

Figure 11. UP 200s Ultraschall prozessor used for ultrasonic extraction

The supernatant was then collected and transferred into a new KIMAX tube. Sonication and extraction was repeated the same way two more times, first using 2 mL of methanol and then 2 mL of acetone. The collected supernatants were then combined and evaporated with a gentle nitrogen stream in a warm water bath (around 45 °C) (Figure 12) until the volume was decreased into approximately 100 µl. The remaining solutions were diluted with 250 mL of ultrapure water in an Erlenmeyer flask (Figure 13), after which they were ready for SPE.

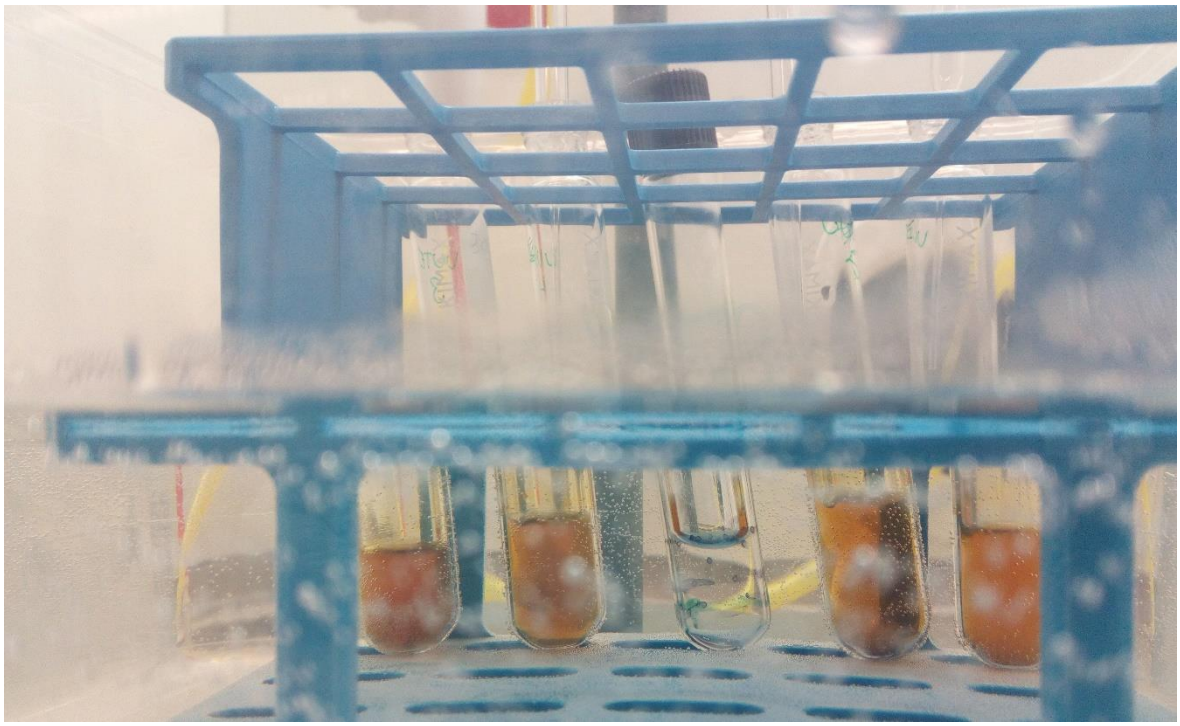

Figure 12. Drying the collected supernatants with nitrogen gas in water bath

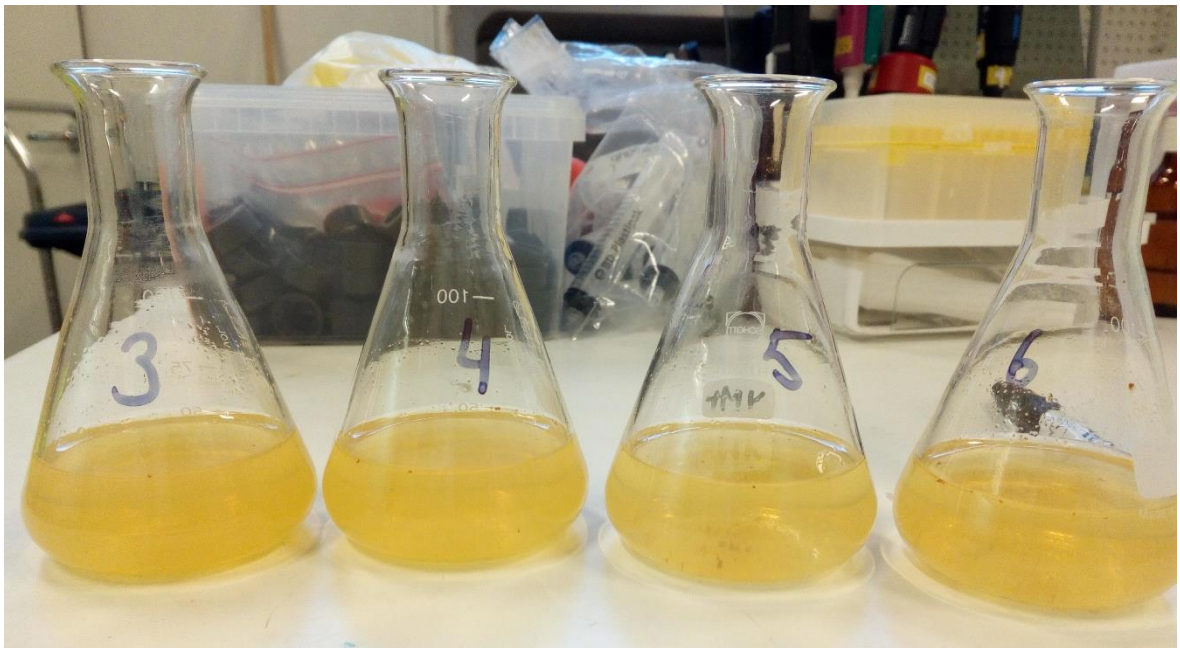

Figure 13. Dried and diluted samples

3.5.4 Water samples for LC-MS/MS analysis

200 ml of each collected wastewater sample was first filtrated through GF/D Glass microfiber filters (Ø 47 mm, pore size 2.7 µm) followed by filtration through GF/F

(pore size 0.7 µm) filter. In each sample, 40 µL of IS (mixed IS, 10 ppm) was added, followed by SPE procedure as described below.

3.5.5 Solid-phase extraction

Because of the low concentrations of pharmaceuticals in wastewater media, it is essential for the detection to concentrate the studied compounds. This was done by solid phase extraction (SPE).

Before extraction, the cartridges were first preconditioned in order to prepare the cartridge to accept sample by using with 3 mL of methanol followed by 3 mL of ultrapure water and allowed to dry in the vacuum manifold. The cartridges were then loaded with studied compounds by pouring the samples into 60 ml syringes which were attached into the manifold and then letting them flow through the cartridges in a flow rate of approximately 6 mL per minute (Figure 14).

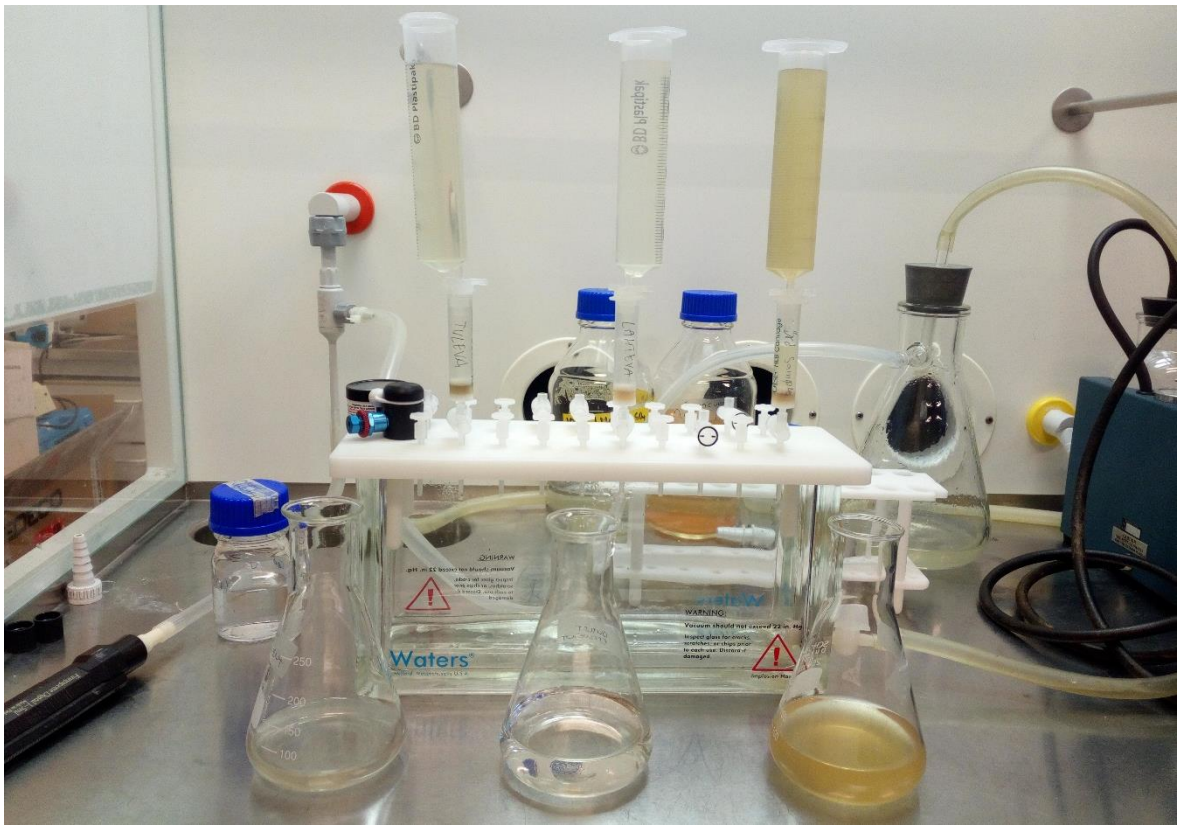

Figure 14. Syringes containing the samples attached to the SPE manifold

After loading the cartridges were dried for 5 minutes under vacuum and washed by adding 5 mL of ultrapure water and let dry for another 5 minutes. The samples were then concentrated via elution with 4 mL of methanol:acetonitrile (1:1) into KIMAX tubes (Figure 15).

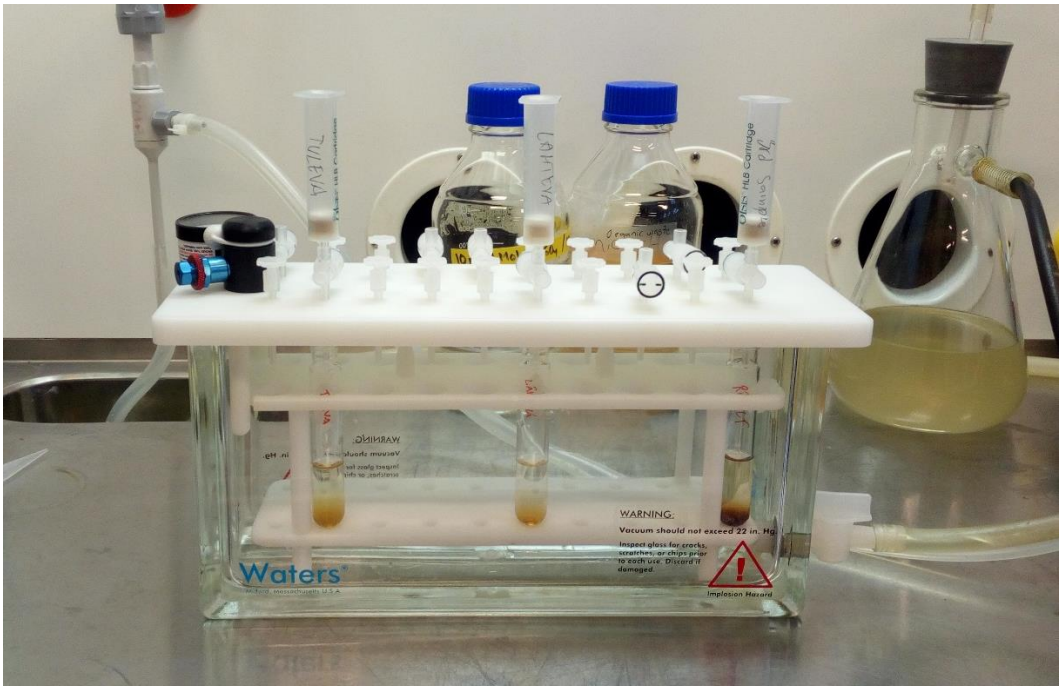

Figure 15. Samples concentrated into KIMAX tubes

The eluted extracts were then evaporated under a nitrogen stream and reconstituted into 1 mL of 20% ACN in ultrapure water. These samples were then filtrated through 0.2 µm sterile syringe filter (Cellulose Acetate, VWR) into an HPLC preparation vial (1.5 mL glass vials) (Figure 16) and were now ready for analysis by LC-MS/MS.

Figure 16. Preparation flask with a sample, ready for HPLC-MS/MS.

3.5.6 Liquid Chromatography – mass spectrometry

The analytical method used in this study was already developed by Ngumba (2016a). The analysis was carried out with a Waters Alliance 2795 HPLC coupled with Quattro Micro triple-quadrupole mass spectrometer, equipped with an ESI source, where analysis was performed in positive ion mode (ESI^+). Nitrogen was used as the desolvation gas with a flow-rate of 8 L min^{-1} and nebulizing gas with a flow-rate of 0.8 L min^{-1} while argon was used as the collision gas at a pressure of 2.8×10^{-4} mBar. The desolvation and the source had temperatures of 200°C and 100°C.

3.5.7 Quantification

Co-extractives in wastewater and sludge samples cause matrix-induced effects that influence the sensitivity of LC-ESI-MS/MS detection of antibiotics in sample extracts. In order to put the real concentrations of analyzed antibiotics into perspective, a calibration needs to be performed. By adding an internal standard (IS) before any step of analysis, the possible loss during the extraction and clean up steps can be taken into account.

Isotopically labelled internal standards used in the quantification of the studied pharmaceuticals were [$^2H_8$]-ciprofloxacin, [$^2H_4$]-sulfamethoxazole, and [$^2H_9$]-trimethoprim. The IS method of calibration incorporating isotopically labelled standards is usually the most preferred since it is simple, quick and efficient (Ngumba et al., 2016a). The standard addition was performed by adding 100 µl of 10 ppm mixed IS for all antibiotics and into every sample.

For the quantification, a 5-point calibration curve with a range from 0.2 to 1.0 ppm of standard stock solution in eluent (80:20, H_2O:ACN) with mixed Internal Standards was used. When compared to the calibration curve, the concentration of the studied compound is plotted versus the ratio of the response of the studied compound and the response of the internal standard, in which way the real concentrations can be calculated.

4 RESULTS

4.1 Liquid chromatography and mass spectrometry

Considering the predicted and formerly detected low concentrations of antibiotics in wastewater matrixes, a preconcentration step using SPE was necessary before measurement. When analyzing multiple different compounds simultaneously, their separation needs to be effective. Peak areas given by the mass spectrometer had been classified for each studied antibiotic compound. In order to determine the real concentrations of the studied compounds, their mass peaks should match the MS/MS parameters.

In WWTP, the overall removal refers to the losses of all the parent compounds during different chemical and physical transformation mechanisms, which is mainly influenced by the treatment technology used, wastewater characteristics, and current operational conditions. These transformation mechanisms mainly include biodegradation and sorption to solid matter.

4.2 Antibiotics in WWTP influent and effluent

All of the studied antibiotics were detected in all the analyzed influent samples and in most of the effluent water samples as well. In the first analysis, three water samples from different sources were collected in the beginning of January; influent that comes to the treatment plant, effluent that leaves the treatment plant, and reject water that was squeezed from the sludge, and their antibiotic concentrations were analyzed (Table 2).

Table 2 Concentrations of the selected antibiotic from 1.1.2018 collected wastewaters

	CIP (ng L^{-1})	TMP (ng L^{-1})	SMX (ng L^{-1})
Influent	930	390	20
Effluent	200	390	*loq*
Reject water	720	*loq*	*loq*

According to the results above, the reject water had a great amount of CIP. This could indicate that CIP would be found from the sludge itself, too, since only around 21% from the influent concentration was passed into the effluent water. TMP did not seem to bind into sewage sludge or to be removed in the wastewater treatment system, since it had passed through the treatment completely. SMX was detected to some extent in influent, but not in either of the other samples. This could be explained partly by the treatment plant having managed to remove it from the system, though the concentration was relatively low to begin with.

Another study period was within a week in March, where influent and effluent waters from each day were collected and analyzed separately and as a combination mixture, in order to give better integrated sampling results. Also few samples were collected from April and May so that more information about the seasonal variation of the concentrations would be gathered. In April and May, also a smaller scale WWTP, Korpilahti, was included in the analysis for comparison. Results from Nenäinniemi WWTP can be found in Figure 17 and from Korpilahti in Figure 18.

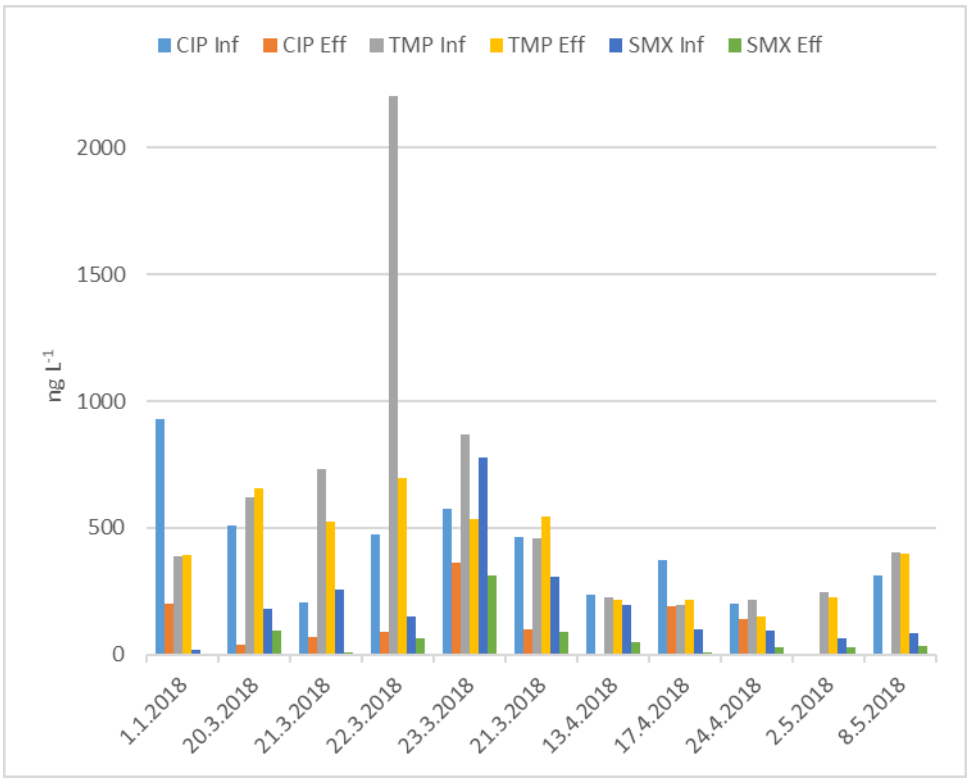

Figure 17. Antibiotic concentrations in influent and effluent waters in Nenäinniemi's wastewater

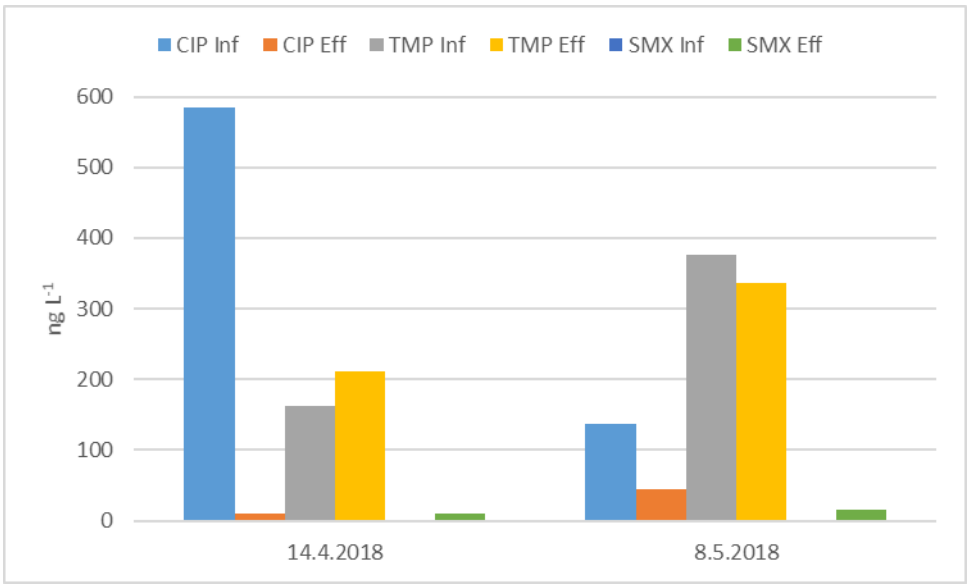

Figure 18. Antibiotic concentrations in influent and effluent waters in Korpilahti's wastewater.

In the influent waters CIP was present in highest amount in both wastewater treatment plants, ranging from below the detection limit to 930 ng L^{-1} in Nenäinniemi and from below the quantification limit (< 10 ng L^{-1}, Kosunen 2015) to

1170 ng L^{-1} in Korpilahti. Ranges for TMP were 190 – 2260 ng L^{-1} & 90-380 ng L^{-1} and for SMX 20 – 780 ng L^{-1} & <10 ng L^{-1}. In effluent waters, TMP was present in highest concentrations when compared to the other compounds, in average of 450 ng L^{-1} in Nenäinniemi and 250 ng L^{-1} in Korpilahti, followed by CIP (110 ng L^{-1} & 20 ng L^{-1}) and SMX (80 ng L^{-1} & 10 ng L^{-1}). TMP did not seem to be removed efficiently in either of the plants except on March, as can be seen in the graphs (Figure 17 & Figure 18).

Table 3 Average amount of antibiotic removed during WWTP process (%) in studied plants

	CIP	TMP	SMX
Nenäinniemi	72	36	67
Korpilahti	95	-8	-680

This shows the removal efficiency for TMP to be very low or non-existent, which is supported by the earlier results from similar studies (Lindberg et al., 2005). For Korpilahti treatment plant, there did not seem to be any removal efficiency for SMX, instead the effluent concentrations were higher, though the concentrations were very low to begin with, especially in studied influent (<10 ng L^{-1}).

CIP did get removed from the studied wastewaters to great extent. The removal efficiency for ciprofloxacin have been found to be mainly in the range of 37–90% by sorption transfer to sewage sludge (Lindberg et al., 2005, Giger et al., 2003).

4.3 Extractions from sludge

According to the results in Table 2, it would be predicted that at least CIP would be present in sewage sludge. A few modifications to the method modelled from Carballa (2005) were performed separately when trying to manage the extraction of antibiotics in the most efficient way.

All the results from each extraction are shown in Table 4. The first extraction with the method modelled from Carballa (2005) did not give any values for the studied compounds (sludge samples 1 & 2). Also the internal standard was not well recovered either during the extraction process, which would suggest that the extraction method was not efficient enough.

One assumption for the poor recoveries would be that the ultrasonic waves in the extraction step with Ultraschakkprozessor up200s are so powerful that they break the bonds of the studied molecules, since it also managed to heat up the solvent to 45 °C, for which reason they would not be detectable in their parent compound form. Due to this assumption, the experiment was repeated in a sonication bath where the intensity would be a bit lower, but still have the extraction effect from sonic waves (sludge samples 3 & 4). In addition, the liquid volume of the supernatant was evaporated to 2 mL (sludge samples 5 & 6) and 0.75 mL (7 & 8) instead of 100 µL.

These variations in experimental procedures did not result in satisfactory recoveries. Usable results were obtained for 5. sludge (CIP) and 3. & 6. sludge samples for SMX. However, both values for SMX were below the detection limit. Quantification of Ciprofloxacin from sample number 5 indicated a concentration of 0.73 mg kg^{-1} in the sludge. Performing this experiment with plain water with internal standard using this ultrasonic extraction step would enable us to assess the probability of ultrasonic treatment affecting the studied compounds. More value could be added to the results if a parallel sample with no ultrasonication step was included.

Table 4. Results obtained from LC-MS/MS for Area & IS Area, for all extracted sludge samples

Sample	CIP Area	CIP IS Area	TMP Area	TMP IS Area	SMX Area	SMX IS Area
1. sludge	-	-	-	4016.61	-	900.63
2. sludge	-	-	93.10	3317.82	-	1088.65
3. sludge	-	10.34	44.89	-	11,55	435.74
4. sludge	-	-	-	-	-	461.80
5. sludge	39.53	28.95	2.76	-	-	389.23
6. sludge	-	-	-	-	1,86	693.41
7. sludge	-	2.87	-	2484.88	-	1196.08
8. sludge	-	40.20	-	2575.49	10,21	1080.02
9. sludge	4.00	-	-	-	-	948.72
10. sludge	32.71	-	-	-	-	989.18
11. sludge	2.18	2.74	-	3460.68	-	703.95
12. sludge	29.64	-	-	-	-	1241.66
13. sludge	55.34	-	-	3370.84	-	1251.86
14. sludge	-	-	-	3389.43	-	927.88

To rule out all possible detection losses that could be caused by sonication, the ultrasonic extraction step was replaced trough vortexing (sludge samples 9 & 10).

Another problem was detected with all the samples when the centrifuged supernatants were dried with nitrogen gas and resuspended into ultrapure water. Thin solid fragments remained in the water after resuspending as seen in Figure 19, which would be expected to cause some detection loss and/or clogging in the later phases.

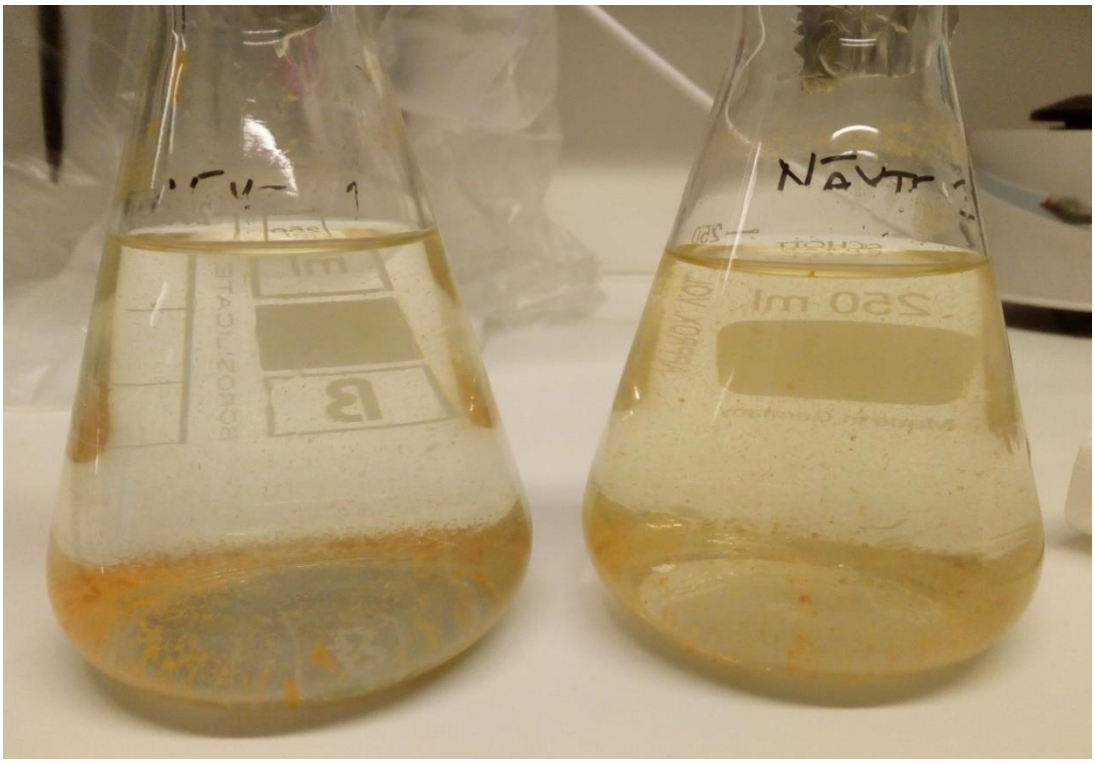

Figure 19. Dried and diluted samples. Solid pieces are clear for naked eye.

In one extraction experiment an attempt was made to solve this problem by treating the resuspended samples in an ultrasonic bath for 10 minutes (sludges 11. & 12.) in order to break the pieces by the force of ultrasonic sound.

For samples 13. & 14. an additional repetition of acetone extraction was added to the protocol. This method performed reasonably well for the recovery of TMP internal standard, but no traces of TMP was detected. Sludge sample no. 11 suggests that the concentration of CIP in the studied sludge was 0.69 mg kg^{-1}.

Overall the sonication step that took place right before SPE did not have a significant positive influence on the results. Instead, it ended up partly clogging the SPE cartridges and stronger suction had to be used for these samples.

5 DISCUSSION

While the concentrations found from influent can give information about the compound's consumption rates, the effluent and sludge concentrations are important in the environmental point of view, since they are the ones to be discharged into the receiving waterways or be disposed for example to agricultural land. The amount of the compound found in effluent or sludge depend on the physiochemical properties of the compounds and/or the removal efficiency of WWTP.

Simultaneously analyzing the pharmaceutical substances from complex matrixes is a challenging job since there is also a great quantity of compounds with similar properties such as solubility, polarity, and partitioning coefficients.

For fluoroquinolones like CIP, even though they are considered as quite hydrophilic compounds, sorption to the sludge particles is found to be the main elimination mechanism in the WWTPs. The sorption of fluoroquinolones could be driven by electrostatic interactions with the interactions between the antibiotic and the cell membranes of the micro-organisms present in sewage sludge.

5.1 Wastewater

The higher concentrations of SMX in this study compared to the yearly DDD values (Figure 1) of each studied antibiotic could be explained by the flu season, when respiratory infections and ear infections bloom, and TMP together with SMX is more often prescribed for the treatment of these infections.

Comparing the two plants, Nenäinniemi offers better removal for TMP and SMX, but Korpilahti had a higher removal for CIP. Nenäinniemi's activated sludge plant featured by a tertiary treatment might improve the removal of these compounds when compared to Korpilahti plant, which mainly relies on activated sludge process.

The amount of SMX was higher in effluent than influent water in all samples from Korpilahti. The act of disposing pharmaceuticals does not happen in a stable flow but instead in unpredictable amounts and time intervals, which could easily give out false removal rates when samples have been collected as grab samples from WWTP. This is one reason why negative removal rates can occur. The negative removal could also be explained by the formation of human metabolism and transformation products that cannot be yet detected in influent water, and when they pass through the plant they could convert back to the parent compounds. The complex metabolic processes in human body as well as biochemical processes during wastewater treatment may promote transformation from parent compound to metabolites and derivatives, and vice versa. Similar findings have been reported in other studies as well, such as the ones conducted by Bhandari et al. (2008), Sinthuchai et al. (2015) and Nyamukamba et al. (2019).

Göbel et al. (2004 & 2005), as well as Hilton and Thomas (2003), have detected N^4-acetylsulfamethoxazole, which is a metabolite of SMX, in WWTPs. It has been reported that N^4-acetylsulfamethoxazole, as well as other SMX metabolites e.g. N^4-acetylated sulfonamides, could be re-transformed back to their active parent compound (Göbel et al., 2005a; Göbel et al., 2005b; Polesel et al., 2016), which would partly explain why the concentrations of SMX in effluent waters were higher than in influent water. These negative removal values could also be explained by the fact that the analyzed samples were grab samples that only show the amount present at the sampling time, which has limitations in reflecting the overall treatment processes, especially when the concentrations were so small (< 100 ng L^{-1}).

The difference of K_d could partly explain why fluoroquinolone concentrations in sludge are found in higher concentrations than those of sulfonamides, macrolides, and others. CIP has logK_d value of 4.3, TMP 2.3, and SMX 2.05 - 2.6 (Golet et al., 2003), which would suggest CIP to adsorb onto sludge more efficiently than TMP or SMX.

The concentrations found in influent and effluent vary greatly in similar experiments made from the plant Nenäinniemi (Table 5). The removal efficiencies

also differed greatly. Kosunen (2015) noted that in every effluent, the concentration of TMP was higher than in influent. Possible explanations for these observations include deconjugation of conjugated metabolites back to the parent compound due to the biological activity in wastewater treatment process (Miao et al., 2002) or a change in the adsorption behavior of the analytes to particles during treatment processes, influencing the ratio between influent and effluent water (Lindberg et al., 2005).

Table 5 Results from similar studies in Jyväskylä's WWTP (ng L^{-1}) and this experiment

	Kosunen, 2015		Kairigo, 2019		Current study	
Compound	Influent(1	Effluent(1	Influent(2	Effluent(2	Influent	Effluent
CIP	300	nd	1 800	2 500	410	110
TMP	170	230	500	400	710	450
SMX	150	nd	700	600	230	80

Vieno et al. (2007) detected CIP around 600 ng L^{-1} in Finnish influents and around 60 ng L^{-1} in effluents. As Polesel et al. (2016) assessed especially for CIP and SMX, retransformation via deconjugation of certain excreted metabolites can potentially occur anywhere after excretion, both in sewers upstream of the WWTP and in the WWTP itself. Therefore, as a result of a comparably higher effluent concentrations can be observed, which could partially explain the results from the study conducted in early 2019, with higher CIP effluent concentrations.

The strong variation for the concentration results may be present and caused by the strong matrix effect in wastewater matrixes. Also interactions that may produce false identifications and thus incorrect concentration values that supports variation as well (Jelic et al., 2011). Co-eluted metabolites, impurities and degradation products may cause this matrix effect because they have influence on the ionization of the target compound (Chambers et al. 2007). A high variation between removal efficiencies in different experiments can be expected since it is influenced by many

factors, like chemical and biological properties of the compound, influent characteristics, operational conditions, and treatment technology used.

In literature, the concentrations of CIP detected from influent water vary around 90–300 ng L^{-1} and effluent <6-60 ng L^{-1} (Lindberg et al., 2005). Sulfamethoxazole has been observed in WWTP effluents with concentrations up to 200 ng L^{-1} (Hirsch et al., 1999).

The removal efficiency for ciprofloxacin from wastewater have been found to be mainly in the range of 37–90% (Lindberg et al., 2005), whilst TMP has been shown to withstand sewage water treatment, with almost 100% of the environmental load transferred to the final effluent (Lindberg et al., 2005). These literature values agree with the findings of WWTP efficiencies examined in this study.

5.2 Sludge

Extraction effectiveness in this set up turned out to be poor as seen from the lack of the recovery of internal standard, as opposed to expectations based on the concentrations detected in influent and effluent waters. The main focus was on CIP, since it was present in the analyzed reject water (see table Table 2), and it is often found in sewage sludge. Since the IS area for SMX was detectable in each experiment, it can be assumed to not bind as tightly onto the sludge matter as the other studied antibiotics.

The reported levels of pharmaceuticals for sludge are usually ranging from microgram per kilogram to milligram per kilogram of dry weight (Giger et al., 2003). Especially fluoroquinolones ciprofloxacin and norfloxacin have been shown to be substantially eliminated by sorption transfer to sewage sludge (80 – 90%) during wastewater treatment process (Giger et al. 2003).

Göbel et al. (2005) investigated two different extraction methodologies for the extraction and determination of various antibiotics, such as sulfamethoxazole and trimethoprim; ultrasonic solvent extraction (USE) and a pressurized liquid extraction (PLE). PLE was found to be the better extraction methodology.

Recoveries of studied antibiotics from activated and digested sewage sludge ranged from not detected (nd) to 113 µg kg^{-1} for SMX and 133 µg kg^{-1} for trimethoprim (Table 6).

Table 6 Literature values of studied antibiotics found from sewage sludge (dw µg/kg)

	CIP	TMP	SMX
Literature	2,300-2,400 (Golet et al. 2002)	nd-133 (Göbel et al. 2005)	nd-113 (Göbel et al. 2005)
Thesis	690-740	nd	nd

Pharmaceuticals which have a high solid-water distribution coefficient (K_d > 2) such as fluoroquinolones are known to have specific electrostatic interactions which could support the adsorption into the sludge (Golet et. al., 2003), although the hydrophobicity is not applicable for the interaction between the sludge and fluoroquinolones due to the low K_{OW} values (e.g for CIP, log K_{OW} = 0.28; log K_d (sludge) = 4.3) (Vieno et al., 2007; Van Doorslaer et al. 2014).

The majority of the elimination of the fluoroquinolones like CIP in wastewater treatment has been shown to occur as a result of adsorption via electrostatic interactions, which is caused by the positively charged amino groups of the compound and negatively charged surface of the micro-organism (Golet et al., 2003), although it is possible that hydrophobic forces are also involved (Holten Lützhøft et al., 2000). CIP has been found to be present in a cationic form in an environment with pH value below 6.1 (Ma et al., 2015), which may cause it to be attracted to negatively charged sludge. In 6.1 - 8.7 pH range CIP is present in zwitterionic form, which suggests that the adsorption onto activated sludge is for the most part affected by the electrostatic repulsion rather than hydrophobic interaction (Genç et al., 2013). The extraction of anionic CIP has been noted to be easier at alkaline pH (pH > 8.8) since it keeps the CIP in anionic form thus increases the solubility of an antibiotic in the aqueous solution (Yu et al., 1994; Goulas el. al.,

2016). Due to the sensitiveness of different pH conditions, when identifying solid-water distribution coefficient value, experiments with a strict control over pH need to be performed. This helps to evaluate the convenient sorption properties of CIP.

CIP has been found to accumulate in soils where CIP-bearing biosolids like sludge has been applied (Golet et al., 2003). CIP can adsorb on sewage sludge with concentrations up to 2.42 mg kg^{-1}, and CIP-bearing biosolids that have been applied on land as fertilizers has been noted to cause accumulation of CIP in the soil (Golet et al., 2003; Li et al., 2011). Even though these concentrations are relatively low, as a persistent polar compound CIP could still cause toxic effects on some microorganisms. For this reason, environmental risk assessment considers CIP as an environmental hazard (Wu et al. 2010). Absorbing into soil can weaken CIP's effectiveness, but it can stay biologically active for long periods of time in sediment and then be released again into the surrounding water, for which reason its potential effects cannot be ruled out (Girardi et al., 2011). The fact that fluoroquinolones are found in the environment has a great contribution on the increase of fluoroquinolone resistance in the microorganisms. Their presence can cause toxic effect on aquatic organisms which produces a critical threat to the biota and human health (Taylor et al. 2008, Ebert et al. 2011).

Similar findings have been conducted by Osemwengie et al. (2006) who found that USE gave out lower recoveries when compared to PLE, thus PLE would be more plausible method for these kind of extractions. Furthermore, Hirsch et al. (1998) noticed that ultrapure water was not the most useful standard solvent as it showed decreasing recoveries for macrolide antibiotics due to its low to nonexistent salt content. Instead, mountain spring water which is free of any anthropogenic organic contaminants was found to perform better.

Differences in recoveries in this experiment and literature may be due to different compositions of studied sludge samples; e.g. varying levels of lipids, pH, chemical stabilizers or additives, de-watering processes, and large and positively charged molecules tend to bound into the negatively charged sewage sludge, which makes the extraction more difficult. For this thesis's experiment, sludge matter was only

loosely crushed using a glass rod in order to homogenize the sample in preparation phase. Homogenizing the sample matter properly, for example by grinding the solids using a pestle to turn it into a fine powder before weighting would increase the sludge matter particle surface, thus improve the effectiveness of extraction

6 CONCLUSIONS

In this thesis, antibiotics in wastewater treatment system were studied and three commonly used antibiotics in Finland were analyzed from Jyväskylä's wastewater treatment plants. The antibiotics were isolated and concentrated by using SPE, analyzed with HPLC and selectively detected with MS/MS, which gave results for CIP, TMP and SMX in ng L^{-1} levels. The studied antibiotics were found in influent samples in concentrations from <10 ng L^{-1} to 2260 ng L^{-1}, and in effluent samples the measured concentrations varied from 10 ng L^{-1} to 450 ng L^{-1}. The elimination effectiveness for each antibiotic observed was in agreement with previous literature, showing that CIP was the most efficiently removed from the water matrix (around 84%) while TMP managed to pass through the system almost completely, and SMX were either removed in some extent or its amount had increased during the wastewater treatment process. This could be due to difference in composition of incoming wastewater and effluent at the sampling time. Furthermore, metabolites present in the influent could have reverted back to original form during treatment. When these compounds are not removed in WWTPs, they will be released to the environment with wastewater discharge. There is a possibility for adverse effects for aquatic organisms already at these low ng L^{-1} concentrations of pharmaceuticals.

According to the measured influent, effluent and reject water concentrations in first experiment, CIP was assumed to adsorb into the sewage sludge since it was found in the reject water that had been squeezed from the sludge. The performed extractions did not succeed in enriching the studied compounds, and due to lack of recovery of even the internal standards, the obtained results were deemed unreliable. Thus theoretically the sludge still contains the analyzed compounds.

Nutrients are the principal content of fertilizers, and since sewage sludge has a high nutrient content, it could be classified as a valuable fertilizer and should be used as such. Regardless, sewage sludge disposed in landfills or used in agriculture may still be an important route of pharmaceuticals to the terrestrial environment and water ways, and may encourage the spread of antibiotic resistance. According to the results obtained in this study, introduction of the sludge as a fertilizer could potentially be a source of CIP in farmlands. Pharmaceutical compounds are produced to be biologically active even in low concentrations, which poses a risk if they are unintentionally released into the environment.

However, the environmental behavior of most pharmaceuticals in soils is still unclear. It is plausible that crop plants may take up antibiotics from the soil pore water that is present in bioavailable fraction as a consequence of raw waste water irrigation. This uptake by plants may result to bioaccumulation within plant tissue and the compounds could eventually enter the food web, potentially posing a threat to animal and human health.

Environmental dispersal of antibiotic resistant bacteria from municipal WWTPs can be hypothesized to be able to spread the resistance gene among environmental bacteria. The bacteria bearing matter (effluent, sludge) is usually discharged or transported into different environments, where they can be in close contact with other micro-organisms, supporting the spread of genes. The focus of environmental risk assessment of pharmaceuticals should be on the chronic, long term exposure effects that pharmaceuticals and their mixtures can cause to the natural organisms, as well as the resistance gene development.

7 REMARKS

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