

UNIVERSIDADE FEDERAL DO PARANÁ

GABRIELA REICHERT

THE POTENTIAL USE OF BIOFILM AS A PASSIVE SAMPLER OF EMERGING
CONTAMINANTS AND ANTIBIOTIC RESISTANCE GENES

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THE POTENTIAL USE OF BIOFILM AS A PASSIVE SAMPLER OF EMERGING
CONTAMINANTS AND ANTIBIOTIC RESISTANCE GENES

Tese apresentada como requisito parcial para obtenção do Título de Doutor no Programa de Pós-Graduação em Engenharia de Recursos Hídricos e Ambiental, Setor de Tecnologia, Universidade Federal do Paraná.

Orientador: Prof. Dr. Júlio César Rodrigues de Azevedo

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RESUMO

A poluição química, causada pelo desenvolvimento da agricultura, indústria, tecnologia e o crescimento da população, afeta profundamente os recursos hídricos, como rios e lagos próximos a centros urbanos. A alta densidade populacional e problemas na coleta e tratamento de efluentes podem causar a contaminação de rios urbanos por grandes quantidades de matéria orgânica, nutrientes e poluentes, como contaminantes emergentes. Contaminantes emergentes fármacos, produtos de cuidado pessoal, plastificantes, hormônios, filtros UV, retardantes de chama, entre outros. Esses compostos podem causar alterações importantes no meio ambiente e nos organismos aquáticos. Além disso, baixas concentrações de fármacos como antibióticos podem causar o aumento e a propagação da resistência a antibióticos no meio ambiente. Por causa dos riscos crescentes, o monitoramento ambiental é de fundamental importância para conscientização e identificação de áreas frágeis e recursos hídricos degradados. O monitoramento de contaminantes emergentes é comum em países desenvolvidos, mas em outros, como em países da América Latina, faltam informações e normalmente apenas áreas próximas a capitais e grandes centros urbanos possuem dados sobre a presença de contaminantes emergentes no meio ambiente. As ferramentas de monitoramento mais frequentemente utilizadas são as análises de amostras ambientais como água e sedimento. No entanto, outras opções, como o biofilme, podem trazer melhores resultados e respostas à constante entrada de poluentes no meio ambiente. Para a parte experimental desta pesquisa, biofilme, água e sedimento coletados de dois diferentes ecossistemas (temperado – sudoeste da Alemanha e subtropical – Sul do Brasil) foram analisados para a detecção e quantificação de plastificantes, alquilfenóis, fármacos, filtros UV e genes de resistência à antibióticos. Um amostrador e uma metodologia para a coleta de biofilme foram propostos e testados. Os resultados mostraram que a coleta de biofilme foi bem-sucedida nas duas áreas estudadas, apesar de condições extremas como baixas temperaturas e eventos de precipitação intensa, que poderiam ter afetado o crescimento do biofilme ou causado o desprendimento do biofilme do amostrador. Quanto a análise de resistência a antibióticos, grandes concentrações de genes de resistência a antibióticos foram encontradas no biofilme, indicando que este meio pode ser uma fonte de resistência a antibióticos no meio ambiente. No entanto, a presença de genes de resistência a antibióticos na água foi mais suscetível a mudanças na qualidade da água e parâmetros que mudavam sazonalmente. O biofilme também mostrou uma forte capacidade de absorver e reter vários poluentes. As concentrações no biofilme alcançaram $2700 \mu\text{g kg}^{-1}$ de DEHP, $300 \mu\text{g kg}^{-1}$ de bisfenol A e $586 \mu\text{g kg}^{-1}$ de octocrileno, enquanto que as concentrações foram menores nas amostras de água e sedimento. Os resultados são preocupantes, uma vez que o biofilme é importante para o meio ambiente e está na base da cadeia alimentar. Isso poderia causar a contaminação de outras espécies, como microinvertebrados e peixes. Nossa conclusão é de que o biofilme é uma importante ferramenta para o monitoramento ambiental, e deveria ser utilizada em conjunto a práticas tradicionais, como a análise de amostras de água e sedimento.

Palavras-chave: Monitoramento ambiental. Qualidade da água. Cromatografia líquida. Química ambiental. Bioacumulação.

ABSTRACT

Chemical pollution, caused by the development of agriculture, industry, technology and population growth, deeply affects water resources, such as rivers and lakes, mainly near urban centers. High population density and frequent problems in sewage collection and treatment systems can imply in a great load of substances entering urban rivers, such as organic matter, nutrients and a wide variety of pollutants, like emerging contaminants. Examples of emerging contaminants are pharmaceuticals, personal care products, plasticizers, hormones, UV filters, flame-retardants, and others. These compounds can cause important alterations in the environment and in the biota, including reproductive problems, mortality and antibiotic resistance. Due to the increasing risks and concerns, environmental monitoring is very important to raise awareness and identify fragile and degraded areas and water bodies. The monitoring of emerging contaminants is usual in developed countries, but in others, such as in countries from Latin America, there is a lack of information and usually only areas near capitals and big urban centers have data about emerging contaminants pollution in the environment. The countries with more information available are Brazil and Mexico. The usual monitoring tools are the sampling and analysis of water and sediment samples. However, other options, such as biofilm, could bring better results and responses to the constant input of pollutants in the environment. For the experimental part of this research, biofilm, water and sediment samples from two different ecosystems (temperate – southern Germany and subtropical – southern Brazil) were collected and a variety of pollutants was analyzed, such as plasticizers, alkylphenols, pharmaceuticals, UV filters, and antibiotic resistance. A methodology for biofilm sampling and collection, using an artificial sampler, was proposed and tested. The results show that biofilm sampling was successful in both areas, despite low temperatures and intense rain events that could harm biofilm growth or cause biofilm detachment from the samplers. Regarding antibiotic resistance, high abundances of antibiotic resistance genes were found in biofilm, indicating biofilm could act as a sink of antibiotic resistance in the environment. However, the presence of antibiotic resistance genes in water samplers was more influenced by water quality and seasonal parameters. Biofilm also had a very strong capacity of absorbing and retaining several pollutants. The concentrations in biofilm reached 2700 $\mu\text{g kg}^{-1}$ of DEHP, 300 $\mu\text{g kg}^{-1}$ of bisphenol A and 586 $\mu\text{g kg}^{-1}$ of octocrylene, while water and sediment samples presented lower concentrations, indicating bioaccumulation of these pollutants in biofilm. The high concentrations found in biofilm is worrisome, since it is an important matrix for the environment and the food web. This could cause the contamination of other species, such as microinvertebrates and fishes. Our employed for environmental monitoring, together with more traditional practices, such as water and sediment analysis.

Keywords: Environmental monitoring. Water quality. Liquid chromatography. Environmental chemistry. Bioaccumulation.

LIST OF FIGURES

Figure 2-1: Distribution of published research regarding emerging contaminants in Latin America	37
Figure 3-1: Absolute abundance of taxonomical gene markers in water samples from the Kraichbach River	52
Figure 3-2: Absolute abundance of taxonomical gene markers in sediment samples from the Kraichbach River (values in dry weight)	52
Figure 3-3: Absolute abundance of taxonomical gene markers in biofilm samples from the Kraichbach River (values in dry weight)	53
Figure 3-4: Absolute abundance of ARGs in water samples from the Kraichbach River	55
Figure 3-5: Absolute abundance of ARGs in sediment samples from the Kraichbach River	55
Figure 3-6: Absolute abundance of ARGs in biofilm samples from the Kraichbach River	56
Figure 4-1 Study area – Kraichbach River and the Hockenheim WWTP	63
Figure 4-2 Sampling and collection of biofilm	66
Figure 4-3: Mean concentrations for target compounds detected in the effluent of Hockenheim WWTP in rainy and humid weather. 4-OP: 4-tert-octylphenol, 4-NP: 4-n-nonylphenol, BP-A: bisphenol A, DEHP: di-2-ethylhexyl phthalate.	70
Figure 4-4: Concentration of target compounds in A) water, B) sediment and C) biofilm samples collected upstream and downstream of Hockenheim WWTP. 4-OP: 4-tert-octylphenol, 4-NP: 4-n-nonylphenol, BP-A: bisphenol A, DEHP: di-2-ethylhexyl phthalate. W: water, S: sediment, B: biofilm, U: upstream, D: downstream.....	72
Figure 4-5: Amount of biofilm collected from one of the glass plates in January (left) and May (right)	78
Figure 5-1: Map of the studied area and location of sampling points	82
Figure 5-2: Adapted biofilm sampler – project and.....	83
Figure 5-3: Daily precipitation in the studied area.	88

LIST OF TABLES

Table 2-1: Keywords and search strings of the systematic review	20
Table 2-2: Priority list: Compounds, chemical structure and properties	41
Table 3-1: Analyzed parameters in qPCR – genes and related facultative pathogenic bacteria ...	48
Table 3-2: Analyzed parameters in qPCR – genes and related antibiotic resistance.....	48
Table 4-1 Schedule of sampling campaigns and collected samples	65
Table 4-2 Analyzed parameters on water, sediment and biofilm	67
Table 5-1 Analyzed parameters on water samples	84
Table 5-2: Physical and chemical parameters analyzed in the water samples	87
Table 5-3: Pharmaceuticals analyzed by HPLC-MS/MS – retention time, precursor and product ions, collision, and fragmentator energies.....	89
Table 5-4: Caffeine and UV filters analyzed by HPLC-MS/MS – retention time, precursor and product ions, collision, and fragmentator energies	90
Table 5-5: Linearity parameters, LOD and LOQ of the developed chromatographic methods....	91
Table 5-6: Data for intra- and inter-day precisions and recovery of the analyzed contaminants..	91
Table 5-7: Concentrations of pharmaceuticals, caffeine, and UV filters detected in water and biofilm samples.....	92

SUMMARY

DOCUMENT SECTIONS	13
1 INTRODUCTION	15
1.1 OBJECTIVES	17
1.1.1 Specific objectives.....	17
2 CHAPTER 1: EMERGING CONTAMINANTS AND ANTIBIOTIC RESISTANCE IN THE DIFFERENT ENVIRONMENTAL MATRICES OF LATIN AMERICA	18
2.1 INTRODUCTION.....	18
2.2 MATERIALS AND METHODS	19
2.3 RESULTS	20
2.3.1 Drinking Water and Potable-water Sources	20
2.3.2 Wastewater Treatment Systems	23
2.3.2.1 <i>Wastewater (Raw Sewage)</i>	23
2.3.2.2 <i>Hospital Effluent</i>	24
2.3.2.3 <i>WWTPs Effluent</i>	25
2.3.3 Freshwater Ecosystems – Surface Water and Sediment	26
2.3.4 Groundwater	28
2.3.5 Coastal Waters	29
2.3.6 Soil	30
2.3.7 Biota	31
2.3.7.1 <i>Biological Concentrations</i>	31
2.3.7.2 <i>Antibiotic Resistance</i>	32
2.4 DISCUSSION	35
2.4.1 Challenges and future perspectives	40
2.5 CONCLUSION	42
3 CHAPTER 2: DETERMINATION OF ANTIBIOTIC RESISTANCE GENES IN A WWTP-IMPACTED RIVER IN SURFACE WATER, SEDIMENT, AND BIOFILM: INFLUENCE OF SEASONALITY AND WATER QUALITY	43
3.1 INTRODUCTION.....	43
3.2 EXPERIMENTAL DESIGN	45
3.2.1 Study area	45

3.2.2	Sample collection	46
3.2.3	Physical and chemical parameters	46
3.2.4	Extraction of DNA and quantification of ARG	47
3.2.5	Antimicrobial susceptibility test.....	49
3.2.6	Statistical Analysis	50
3.3	RESULTS AND DISCUSSION	50
3.3.1	Physical and chemical parameters.....	50
3.3.2	Quantification of facultative pathogenic bacteria gene markers in water, sediment, and biofilm	51
3.3.3	Quantification of ARG in water, sediment, and biofilm	53
3.3.4	Antimicrobial susceptibility test – Multi-drug resistance	56
3.3.5	Correlations of ARGs and FPB with physical and chemical parameters	57
3.4	CONCLUSION	59

4 CHAPTER 3: BIOACCUMULATION OF ALKYLPHENOLS AND PLASTICIZERS IN BIOFILM: IMPACTS OF A SMALL WWTP IN SOUTHERN GERMANY

61

4.1	INTRODUCTION.....	61
4.2	EXPERIMENTAL DESIGN	62
4.2.1	Study area	62
4.2.2	Sampling collection.....	64
4.2.3	Physical, chemical, and chromatographic analyses	66
4.2.4	Statistical analysis	68
4.3	RESULTS	68
4.3.1	Physical and chemical analyses.....	68
4.3.2	Concentrations of DEHP, BP-A, 4-NP, and 4-OP detected in the samples	70
4.3.3	Correlation analysis.....	75
4.3.4	Biofilm growth, hydrodynamic, and other influencing parameters	77
4.4	CONCLUSION	79

5 CHAPTER 4: BIOACCUMULATION OF EMERGING CONTAMINANTS IN BIOFILM COLLECTED FROM A SUB-TROPICAL URBAN RIVER IN BRAZIL

80

5.1	INTRODUCTION.....	80
5.2	MATERIALS AND METHODS.....	81
5.2.1	Study Area	81
5.2.2	Sampling collection.....	83
5.2.3	Chemical and physical parameters	83

5.2.4	Chemicals and reagents	84
5.2.5	Extraction	84
5.2.5.1	<i>Water samples</i>	84
5.2.5.2	<i>Biofilm samples</i>	85
5.2.6	Development and optimization of the chromatographic method	85
5.2.7	Validation and quality assurance/quality control protocols	85
5.3	Results and discussion	86
5.3.1	Chemical and physical parameters	86
5.3.2	Optimization of the chromatographic methods	88
5.3.3	Method validation	90
5.3.4	Emerging contaminants detected in water and biofilm samples from the Barigui River	91
5.4	CONCLUSION	94
6	GENERAL CONCLUSION AND MAIN FINDINGS	95
7	BIBLIOGRAPHY	97
	APPENDIX	121

DOCUMENT SECTIONS

This document is divided into nine main sections. The first section, called **“Introduction - General aspects”** gives a broad explanation of the research theme and the importance of this study, and details the main objectives of the research.

This thesis has four main chapters, due to the nature of the research and the different experiences of the author, which performed experiments and analyses in two countries, Germany and Brazil.

The second section, called **“Chapter 1: Emerging contaminants and antibiotic resistance in the different environmental matrices of Latin America”** is a literature review about the occurrence of emerging contaminants and antibiotic resistance genes in environmental matrices in Latin America in published papers from 2000 until July 2019. The review helped to find research gaps in Latin America and to compile a list of priority pollutants fitted to the characteristics and needs of the countries in the area.

Sections 3 to 5 describe experimental and monitoring studies. All of them have as main goal to evaluate the use of biofilm as a monitoring tool for different kinds of pollutants. The third section is called **“Chapter 2: Determination of antibiotic resistance genes in surface water, sediment, and biofilm samples: influence of seasonality and water quality”**. This research evaluated the presence of antibiotic resistance genes in the water, sediment and biofilm samples collected from the Kraichbach River, located in Karlsruhe, Germany. The presence of a small WWTP and the influence of seasonal parameters were also evaluated as drivers and contributors of antibiotic resistance genes in the samples.

The fourth section, called **“Chapter 3: Bioaccumulation of alkylphenols and plasticizers in biofilm: impacts of a small WWTP in southern Germany”** aims to assess the occurrence of alkylphenols and plasticizers in biofilm, sediment and water samples collected from upstream and downstream of a small WWTP in southern Germany, also in the Kraichbach River. The compounds were chosen based on the priority substances list established by the European Union (Directive 2013/39/EU). The study area is the same as the previous chapter and this exact location and WWTP were chosen due to a prior project, in which these pollutants were monitored in the WWTP, giving important background information about the release of these pollutants into the environment and a possibility to compare their occurrence in different environmental matrices.

The fifth section, called **“Chapter 4: Bioaccumulation of emerging contaminants in biofilm collected from a sub-tropical urban river in Brazil:”** aims to develop a methodology to extract and analyze pharmaceuticals, UV filters and caffeine from biofilm using liquid chromatography coupled to

tandem mass spectrometry. The study area was the Barigui River, located in the city of Curitiba, in southern Brazil.

The sixth section, “**General conclusions and main findings**”, gathers the main findings and the conclusion of the four chapters of this thesis. Section 7 and 8 are the “**Bibliography**” and “**Appendix**”.

1 INTRODUCTION

The development of agriculture, industry, technology and consequent population growth in the last decades has caused an increase in the number and quantity of produced and consumed chemicals, both synthetic and natural, released in the environment. Many of them are ubiquitous and can be found all over the world, in all kinds of ecosystems, in rural, urban, and natural areas (Babay et al., 2014; Ebele et al., 2017).

Water resources, such as rivers and lakes, are deeply affected by chemical pollution. As an example, urban centers are usually located near important water bodies that are under constant anthropic pressure. In these areas, rivers frequently are effluent receivers and the primary causes of water quality degradation are domestic sewage and industrial effluents. High population density and frequent problems in sewage collection and treatment systems can imply in a great load of substances entering urban rivers, such as organic matter, nutrients and a wide variety of pollutants, like emerging contaminants (Ide et al., 2013; Mizukawa et al., 2018; Reichert et al., 2020).

Emerging contaminants, or contaminants of emerging concern, are substances present in the environment that were recently detected or identified as pollutants. They are usually not regulated, despite the fact that many can cause problems to the environment, animal, and human health. They comprise a group of compounds that belong to several classes: pharmaceuticals, personal care products, plasticizers, hormones, perfluorinated compounds, drugs of abuse, UV filters, flame-retardants, etc. (Agüera et al., 2013). Many of these substances can interfere in the ecosystems health and natural balance.

The presence of these compounds in the environment can also cause alterations in many organisms. Hormones and other endocrine disruptors, for example, can affect the function of the endocrine system of many species and lead to infertility, malformation, male feminization, and others (Fuhrman et al., 2015; Nowak et al., 2018; Pamplona-Silva et al., 2018). Another example of the problems caused by the presence of these substances in the environment is the detection of pharmaceuticals, especially antibiotics, in aquatic ecosystems. Antibiotics are usually detected in the environment in lower concentrations than the ones administered to patients, which can favor the appearance of antibiotic-resistant bacteria (Kraemer et al., 2019). The increase in the detection of antibiotic-resistant bacteria and the presence of harmful microorganisms in the environment is worrisome and is also considered a kind of emerging pollution.

Given the importance of this matter, there are several studies concerning the presence of emerging contaminants and antibiotic resistance in the environment. Usually, surface water and sediment samples are the matrices of choice to evaluate pollution levels. However, to assess the actual pollution status of an environment is complicated and the real, persistent effects are difficult to identify and understand because of the intricacy of biological and chemical processes (Lear and Lewis, 2009).

Another concern regarding emerging contaminants is the possibility of bioaccumulation through the food chain (Ruhí et al., 2016). Giving this concern, many studies analyzed tissues of fishes and other species. However, the process of collecting these organisms from the natural environment and sample preparation of these materials, such as muscle, liver and others, is complex and time consuming. As a possible alternative, some authors have presented their research regarding the analysis of biofilm.

Biofilm is one of the most common and successful life forms on earth (Flemming, 2002). It is a complex community of microorganisms, including bacteria, algae, fungi, and others. These microorganisms are able to attach and grow in different surfaces, mainly when exposed to humidity and water. They are assembled in a matrix produced by the organisms themselves. It is an aggregation of polysaccharides, proteins, nucleic acids, lipids and water with viscous and elastic properties known as extracellular polymeric substances (EPS). The matrix is responsible for keeping biofilm together and attached to the surface. The matrix also acts as an electron donor or acceptor, a source of food and energy and a protective barrier against toxins, antimicrobials and other predators (Flemming and Wingender, 2010; McDougald et al., 2012).

The importance of these organisms reaches several study fields, like medicine, dentistry, microbiology, ecology, and environmental sciences. They are employed in technical applications, wastewater treatment and even in bioremediation of hydrocarbons and heavy metals (Singh et al., 2006; Wuertz et al., 2004). However, their main contribution is their ability to perform important functions in the natural environment. Biofilm is an important food source for many species and they actively participate in the biological and biogeochemical fluxes, by denitrifying nitrate and emitting nitrogen into the atmosphere (Beaulieu et al., 2011; Mulholland et al., 2008) or degrading organic matter and emitting carbon within aquatic ecosystems (Raymond et al., 2013).

In rivers and streams, biofilms can be found in pebbles, tree branches, leaves, and even sediment. They are in constant contact with the nutrients, pollutants, and the microbiota present in the water, and also have the ability to absorb these substances, small solid particles and other microorganisms, incorporating them into their matrix (Battin et al., 2016; Bechtold et al., 2012).

Biofilm integrate both the abiotic and biotic compartments and it seems to be a suitable medium to evaluate the presence of emerging contaminants and antibiotic resistance bacteria within a natural ecosystem (Balcázar et al., 2015). Most of the previous research was performed with natural biofilms, collected from surfaces already incorporated in the environment, like rocks, cobbles, and others (Guo et al., 2018; Proia et al., 2016). However, biofilms grow fast on any kind of surface, which makes them good biological indicators. When associating biofilm with artificial samplers, is possible to use the biofilm as a passive sampler. Also, they could be used as potential passive samplers for a wide range of substances.

1.1 OBJECTIVES

The main goal of this research was to evaluate the use of the biofilm as a monitoring tool and possible passive sampler for different kinds of pollutants in different ecosystems, subtropical and temperate, and to establish the biofilm as a suitable, easy-to-use, affordable, and reliable monitoring matrix, besides sediment and water.

1.1.1 Specific objectives

Chapter 1: Perform a comprehensive, non-biased literature review about the occurrence of emerging contaminants in Latin America, to identify research gaps and lack of important information. Provide recommendations for future monitoring and prevention programs.

Chapter 2: Analyze the presence of antibiotic resistance in biofilm, water, and sediment samples and investigate different monitoring strategies and other influencing factors to the occurrence of antibiotic resistance in the environment.

Chapter 3: Evaluate the use of biofilm as a potential passive sampling media for alkylphenols and plasticizers in a temperate urban river.

Chapter 4: Develop a methodology to analyze pharmaceuticals, caffeine and UV filters in water and biofilm to detect emerging contaminants in biofilm from a subtropical urban river.

2 CHAPTER 1: EMERGING CONTAMINANTS AND ANTIBIOTIC RESISTANCE IN THE DIFFERENT ENVIRONMENTAL MATRICES OF LATIN AMERICA

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2.1 INTRODUCTION

A great number of substances generated through anthropic processes are considered pollutants. In order to control and prevent contamination, environmental laws, directives and watch lists are created and approved around the world to regulate their presence in environmental matrices. Examples are documents issued by institutions such as the Environment Protection Agency (EPA) and the European Commission, e.g. guidelines and watch lists for priority contaminants (EU, 2000; EPA, 2016). Although some of these documents are extremely thorough, they cannot include every pollutant. The biggest challenge remains in the high number of chemical substances discovered and created every year (Arp, 2012), employed in several human activities and partially released into the environment. Therefore, these documents should be constantly reviewed and updated.

Examples of substances that were recently included in such documents are emerging contaminants. They are consumed daily by the overall population in products and also employed in medicine, livestock farming, veterinary sciences and industry (Babay et al., 2014; Kemper, 2008; Osorio et al., 2016) and their origin may be natural or synthetic. The comprehensive classification of emerging contaminants embodies several subgroups: pharmaceuticals, hormones, personal care products, flame retardants, perfluoroalkyl substances and others (Llorca et al., 2017). Inside the already mentioned subgroups, there are still further classifications like beta-blockers, non-steroidal anti-inflammatory drugs (NSAID), anti-depressives, UV filters, preservatives, antimicrobial agents and others. They are usually detected in low concentrations in the range of ng L^{-1} or $\mu\text{g L}^{-1}$ and their analysis and quantification were not possible until only some decades ago.

After the improvement of analytic techniques and the possible detection of these substances in low concentrations, the high number of discovered substances entering the environment daily raised concern about their safety to living organisms. The implications of emerging contaminants

in the environment are still not fully understood. Several studies indicate that wastewater treatment plants (WWTP) are the main point sources of these numerous pollutants in the environment, affecting mainly aquatic ecosystems (Haman et al., 2015). Therefore, in the last years, emerging contaminants have been frequent subjects of studies regarding their occurrence in the environment and their possible toxicity to living organisms (Dévier et al., 2013).

A great number of studies helped to improve the understanding about the dynamics and behavior of emerging contaminants, as well to characterize their presence in several ecosystems (Ebele et al., 2017; Haman et al., 2015). However, most of the monitoring efforts are focused in North America and Europe. The number of studies is also increasing in Asia, mainly in China, leaving a gap of knowledge in countries of South America and Africa (Ebele et al., 2017). In the published studies from South America, and consequently Latin America, inefficient sewage collection and treatment systems are always highlighted (Ide et al., 2017; Mizukawa et al., 2018). Also, the concentrations detected in Latin America (Ide et al., 2017; Locatelli et al., 2011; Mizukawa et al., 2017; Montagner and Jardim, 2011; Santos et al., 2016) are often higher than in other countries (Huerta et al., 2016; Li et al., 2016; Osorio et al., 2016; Patrolecco et al., 2015; Tlili et al., 2016; You et al., 2015). In contrast to Europe and other areas, many countries of Latin America do not have an established list of priority pollutants or laws controlling their production or release to the environment.

Given this background, the aim of this study is to gather and summarize information about emerging contaminants and the presence of antibiotic resistance within environmental matrices in Latin America. It also intends to identify the most commonly detected contaminants, thus contributing to the compilation of a list of priority pollutants adjusted to the needs and characteristics of Latin America. Finally, this review aims to identify research gaps and lack of important information regarding emerging contaminants in Latin America and to provide recommendations for future monitoring and prevention programs.

2.2 MATERIALS AND METHODS

In order to perform a comprehensive research and secure a representative and unbiased amount of quality data concerning emerging contaminants in Latin America, the search for articles was performed in the Scopus® database, one of the largest databases of peer-reviewed literature, containing research from several fields of study and themes.

This review considered published or in-press articles from 2000 until July of 2019. Only case studies or original articles that studied the determination and occurrence of emerging contaminants in environmental compartments of Latin America were considered. Review articles, notes, conference papers or articles that only presented the development of analytical methodologies were not included in the results.

The search strategy consisted of choosing the keywords and formulating the search string. The chosen keywords included usual technical terms, as well as usual nomenclature of frequently detected emerging contaminants in environmental compartments. The proposed keywords are listed in Table 2-1.

Table 2-1: Keywords and search strings of the systematic review

Database	Keywords and search string	Fields
Scopus®	("emerging compounds" OR "emerging contaminants" OR parabens OR triclosan OR pharmaceuticals OR "endocrine disruptors" OR NSAID OR antibiotics OR hormones OR "antibiotic resistance" OR "antibiotic resistance genes" OR "microbial contamination") AND (sediment OR wastewater OR water OR "surface water" OR soil OR periphyton OR biofilm OR determination OR occurrence)	Title Keywords

With the help of the search tools, only articles from Latin American countries were selected. The complete list of countries belonging to Latin America can be found at the UNESCO website (<http://www.unesco.org/new/en/unesco/worldwide/latin-america-and-the-caribbean>). The selected articles were analyzed and the results summarized and grouped into the following topics: "Drinking Water and Potable-water Sources", "Wastewater Treatment Systems", "Freshwater Ecosystems – Surface Water and Sediment", "Groundwater", "Coastal Water", "Soil" and "Biota".

2.3 RESULTS

2.3.1 Drinking Water and Potable-water Sources

The pollution of potable water by emerging contaminants is one of the biggest concerns in this field since there is a direct relation to human health. Therefore, many researchers evaluate the

presence of a broad range of compounds in samples from rivers, reservoirs, tap water, bottled mineral water, and inlets and outlets of water treatment plants (WTP). Detailed information about the studies is present in the Appendix A.

Ideally, the concentrations of emerging contaminants in drinking water and potable-water sources should be non-detectable or detected in low concentrations. However, rivers and reservoirs in Latin America are usually susceptible to human influence, since the surrounding areas of these resources are frequently subject to human occupation. Such strong interference may result in water contamination. This was verified by several authors reporting high concentrations in many rivers and reservoirs that provide potable water for the Latin American population. Félix-Cañedo et al. (2013), for example, analyzed 17 organic contaminants in reservoirs and distribution tanks around Mexico City. Ibuprofen (IBU), diclofenac (DIC), naproxen (NAP), salicylic acid (SA), 4-nonylphenol (4-NP) and di(2-ethylhexyl) phthalate (DEHP) were the most frequently detected (100%) substances. DEHP was the contaminant quantified with higher concentrations, reaching 2282 ng L^{-1} , but several contaminants were frequently detected below the limit of detection (LQ) or not detected at all.

DEHP was also detected with high concentrations in a reservoir in Bogotá, Colombia, where an investigation was conducted. Most of the other compounds were detected at $\mu\text{g L}^{-1}$ levels and DEHP was detected with concentrations of up to $10097000 \text{ ng L}^{-1}$ ($1009.7 \mu\text{g L}^{-1}$) (Bedoya-Ríos et al., 2017). In another reservoir in Colombia, Martinez and Peñuela (2013) analyzed triclosan (TCS) and 4-NP. Samples from tributaries and the canals that lead to the WTP were also analyzed, as well as the inlet and outlet (potable water) of the WTP. 4-NP was not detected in any sample, but TCS was detected in one of the tributaries and in the reservoir, ranging from 260 ng L^{-1} to 490 ng L^{-1} .

The quality of potable-water sources is a strong concern in Brazil, mainly in heavily urbanized southeastern states like Sao Paulo, Rio de Janeiro and Minas Gerais. The presence of emerging contaminants in the areas near capitals was reported by several authors (Campestrini and Jardim, 2017; Montagner et al., 2019; Monteiro et al., 2018; Moreira et al., 2011). Pharmaceuticals and surfactants were frequently detected in potable water sources and inlets of WTP, but in drinking water, the concentrations were lower. Also, the frequency detection of hormones is lower than that of other pharmaceuticals in most of the studies. Contaminants with higher concentrations in

Southeastern Brazil were 4-NP (1918 ng L^{-1}), estrone (E1) (600 ng L^{-1}), cefalexin (CEF) (575.5 ng L^{-1}), dibutyl phthalate (DBP) (454 ng L^{-1}), amoxicilin (AMOX) (287.5 ng L^{-1}), caffeine (CAF) (213.6 ng L^{-1}) and sulfamethoxazole (SUF) (105 ng L^{-1}) (de Sousa et al. 2014; Lopes et al. 2010; Maldaner and Jardim 2012; Monteiro et al. 2018; Moreira et al. 2009; Moreira et al. 2011; Sodré et al. 2010; Souza et al. 2012). These and other values can be found in the Appendix A. Besides pharmaceuticals, surfactants and parabens, Campestrini and Jardim (2017) analyzed illegal drugs in potable-water sources. Cocaine (COC) and its metabolite benzoylecgonine (BLGN) were detected in samples taken from reservoirs and rivers that supply over 16 million inhabitants in the most populated area in the country (Sao Paulo, Southeastern Brazil). The concentrations of COC and BLGN reached 62 and 1019 ng L^{-1} . The concentrations were similar in tap water, reaching 22 ng L^{-1} and 652 ng L^{-1} for COC and BLG.

In Southern Brazil, many studies were performed in WTPs. Caldas et al. (2019) evaluated the occurrence of 51 pesticides pharmaceuticals and PCPs in WTP over a 4 year period. Eight pharmaceuticals and PCPs were detected, and MeP and NIM were detected in more than 50% of the samples. Silveira et al. (2013) analyzed pharmaceutical and personal care products (PCPs) in the inlet and outlet of a WTP. In the non-treated water, haloperidol (HAL), methylparaben (MeP) and nimesulide (NIM) were detected with 0.1 ng L^{-1} , $7.6\text{-}29.8 \text{ ng L}^{-1}$ and 0.05 ng L^{-1} , respectively. The only detected compound in treated water was MeP, but the concentration level was always below the LQ. The removal efficiency of six other WTP in Southeastern Brazil was also evaluated for UV filters, another kind of PCPs, but only benzophenone-3 (BP-3) and ethylhexyl methoxycinnamate (EHMC) were detected above the LQ. During winter time, none of the UV filters were detected. Nonetheless, during summer, the concentrations of BP-3 reached 44 ng L^{-1} in non-treated water and 105 ng L^{-1} in treated water. EHMC was detected with 101 ng L^{-1} in non-treated water and 75 ng L^{-1} in treated water (da Silva et al. 2015). The presence of PCPs was also evaluated in another WTP in Southern Brazil and in bottled mineral water. MeP was detected below the LQ in the water samples from the WTP, but was quantified with 10 ng g^{-1} in one of the WTP sludge samples, indicating that the sludge may be acting as a sink for MeP. The concentrations reported in mineral water samples were higher than in the WTP ($< \text{LQ-}242 \text{ ng L}^{-1}$), which suggests contamination may have occurred either in the mineral water source or in the process of water bottling (Marta-Sanchez et al., 2018).

In Northeastern Brazil, Melo and Brito (2013) analyzed the presence of bisphenol-A (BP-A) and ethinylestradiol (EE2) in surface waters, tap water and underground water in Sao Luiz, Maranhao. In surface water, none of the contaminants were detected. On the other hand, BP-A was detected with concentrations of up to 3610 ng L⁻¹ and EE2 was detected below the LQ in tap water. Also in Maranhao, Verbinnen et al. (2010) analyzed estrogens (E1, estradiol (E2), EE2, and estriol (E3)) in water samples from the outlet of two WTP, a distribution reservoir and tap water. Once again, none of the hormones was detected in any sample. The absence of estrogens (hormones) in water samples may be the consequence of their hydrophobic characteristics (Froehner et al. 2011A).

In Chile, the presence of atrazine (ATZ), CAF and TCS in tap water and WTP was reported, with concentrations of up to 95 ng L⁻¹ (Rozas et al., 2016). In another study, Becerra-Herrera et al. (2018) analyzed parabens in tap water samples from Santiago, and no compound was detected.

2.3.2 Wastewater Treatment Systems

Several authors reported that WWTPs are the major point sources of emerging contaminants in the environment due to low removal efficiencies of emerging contaminants (Ide et al., 2017; Proia et al., 2016; Ruhí et al., 2016; Ryu et al., 2011). In addition, raw sewage is also directly applied in agricultural areas of Latin America. Therefore, it is important to investigate the occurrence of emerging contaminants in raw sewage and treated wastewater that will, eventually, reach the environment. Since the concentrations found in wastewater and WWTP effluents are higher than in other matrices, the concentrations of emerging contaminants in this topic will be presented in µg L⁻¹ and detailed information about the studies can be found in Appendix B.

2.3.2.1 Wastewater (Raw Sewage)

To determine the concentration levels of emerging contaminants in raw sewage in Latin America is especially important, since raw sewage is used for irrigation in many arid agricultural areas, like in the Mezquital Valley in Mexico, one of the oldest examples of untreated wastewater irrigation in the world. The valley receives the wastewater from Mexico City through a complex system formed by dams and irrigation canals, which maintain a productive agricultural area. Due to concerns regarding human safety and contamination, water from dams and canals was analyzed and tested for 218 compounds of different classes. Of the 218 compounds, 14 volatile and semi-

volatile organic compounds, 1 polycyclic aromatic carbon (naphthalene), 65 pharmaceuticals and 3 hormones were detected in untreated wastewater. The highest concentration detected was $107 \mu\text{g L}^{-1}$ of MET, showing that the wastewater irrigation could be harmful to human health (Lesser et al., 2018). In a previous research, three hormones were detected in the sewage canals in the metropolitan area of Mexico, which contain the sewage of Mexico City. E1, E2 and EE2 were detected, and their concentration reached $0.093 \mu\text{g L}^{-1}$ (Estrada-Arriaga et al., 2013a).

In Tulla Valley, another example of wastewater irrigation in Mexico, raw sewage samples were also tested for several compounds. Some of the compounds were always detected below the LQ, nonetheless, high concentrations of NAP, BP-A and DEHP were detected in all samples (Gibson et al., 2010, 2007). In Cuernavaca (state of Morelos, in Mexico), 35 pharmaceuticals were analyzed in the influent of a WWTP. The three most detected compounds were naproxen (NAP), paracetamol (PARA) and diclofenac (DIC), with concentrations up to $14.9 \mu\text{g L}^{-1}$ (Rivera-Jaimes et al., 2018a).

In Bogotá, Colombia, Bedoya-Ríos et al. (2017) reported the presence of endocrine disruptors in wetlands. The list of compounds included pharmaceuticals, phthalates and organophosphorus compounds. BP-A, carbamazepine (CBZ), TRI, primidone (PRI) and phthalates were detected frequently in wastewater. DEHP and diphenyl phthalate (DPP) were detected with $309.6 \mu\text{g L}^{-1}$, while BP-A was detected with $22.73 \mu\text{g L}^{-1}$ and CBZ with $15.15 \mu\text{g L}^{-1}$.

2.3.2.2 Hospital Effluent

Hospital effluents are also a concern, since their effluent might contain even higher concentration of pharmaceuticals than domestic sewage. According to the search, hospital effluent was only analyzed in Brazil. In Sao Paulo, antiparasitic drugs were detected in concentrations up to $3.81 \mu\text{g L}^{-1}$ (Porto et al., 2019). In Southern Brazil, Chiarello et al. (2016) analyzed four pharmaceuticals in hospital sewage samples. The concentrations found were: $2 \mu\text{g L}^{-1}$ of MET, $7.5 \mu\text{g L}^{-1}$ of PARA and $1 \mu\text{g L}^{-1}$ of enalapril (ENA). Tetracycline (TC) was also analyzed but it was not detected in any sample. In another hospital in Southern Brazil, also in Rio Grande do Sul, Minetto et al. (2012) quantified DIC in raw sewage samples, and the concentration range was from $0.83\text{-}3.59 \mu\text{g L}^{-1}$.

2.3.2.3 WWTPs Effluent

Studies regarding WWTP usually contain information about WWTP influent, sludge and effluent. Since the main focus of this research is the presence of emerging contaminants in environmental matrices, only concentrations detected in the final effluent, which could reach the environment, will be described and discussed.

Robledo-Zacarias et al. (2017) reported the presence of several emerging contaminants in the effluent of a WWTP in Michoacán, Mexico. Similarly, as in the case of the influent, the highest concentrations were those of polyethylene glycol ($64.6\text{--}564.2\ \mu\text{g L}^{-1}$) and TC ($2.7\text{--}119.4\ \mu\text{g L}^{-1}$). Also in México, Estrada-Arriaga et al. (2016) assessed the presence of 65 emerging contaminants in the effluent of two biological nutrient removal WWTPs. In WWTP 1, the removal efficiency was not high and the concentration range of the final effluent was from $<\text{LQ}$ to $3.77\ \mu\text{g L}^{-1}$. The concentrations were higher during the dry season. In WWTP 2, many of the detected compounds in the influent were partially or totally removed from the final effluent. The concentrations found in the effluent during the monitoring campaign were below $0.21\ \mu\text{g L}^{-1}$. In Cuernavaca (state of Morelos, in Mexico), the effluent of a WWTP was analyzed and the three most detected compounds were NAP, sulfamethoxazole (SUF) and DIC, with concentrations up to $2.18\ \mu\text{g L}^{-1}$ (Rivera-Jaimes et al., 2018a).

In Southern Brazil, Froehner et al. (2011A) analyzed the removal efficiency of five emerging contaminants in three WWTPs. The higher removal efficiencies were detected in the activated sludge system. The highest concentration detected in the effluent was $0.76\ \mu\text{g L}^{-1}$ of E2. In Northeastern Brazil, Pessoa et al. (2014) analyzed the removal of four estrogens in the effluent of five WWTPs. Their occurrence and concentrations were lower than in the influent. The lowest detection frequency was of E2 (12%) and the highest of E1 (48%). The highest concentration measured in the effluent was also of E1 ($2.08\ \mu\text{g L}^{-1}$). The removal of each estrogen had a great variation in each WWTP, but the average removal for every compound was higher than 63%.

Rozas et al. (2016) determined ATZ, CAF, DIC and TCS in the effluent of two WWTPs in Chile. Comparing to the influent, some compounds like CAF and TCS were efficiently removed from the wastewater ($0.06\text{--}0.85\ \mu\text{g L}^{-1}$ and $0.04\text{--}0.1\ \mu\text{g L}^{-1}$, respectively). Others, like ATZ and DIC, were still detected in high concentrations, if compared to the influent ($0.7\text{--}0.15\ \mu\text{g L}^{-1}$ and $0.3\text{--}0.4\ \mu\text{g L}^{-1}$).

2.3.3 Freshwater Ecosystems – Surface Water and Sediment

Monitoring emerging contaminants in freshwater sources is especially important due to the big ecological diversity within these environments. Latin America has the greatest water availability per capita in the world, holding 31% of global freshwater sources (Wilkinson, 2010). Examples of these environments are lakes, ponds, reservoirs, rivers, streams and wetlands. The frequent human contact with these ecosystems, and their influence in human life is the major motivation of assessing freshwater quality. Since the reported concentration ranges are very broad, concentrations in this topic will be described in ng L^{-1} for water samples and ng g^{-1} for sediment samples. A complete description of the studies is presented in Appendix C.

Despite the abundance of water resources in Latin America, many areas in Mexico face water scarcity. This is one of the reasons why untreated wastewater is used for irrigation in the country, as previously discussed. However, these practices may endanger surface water resources. Some researchers aimed to assess this kind of pollution. For example, Lesser et al. (2018) and Gibson et al. (2007) analyzed springs near the Mezquital Valley and Tulla Valley, respectively. Both are agricultural areas which are irrigated with untreated wastewater. But while the highest concentration detected in the springs of Tulla Valley was 25 ng L^{-1} of DEHP, the concentrations in Mezquital valley reached 5280 ng L^{-1} of the insect repellent N,N-diethyl-m-toluamide (DEET). In another agricultural area near Mexico City, water samples from Xochimilco wetland were also tested for emerging contaminants. This kind of wetland, which is called Chinampa, consists of artificial rectangular floating plots. However, in the last years, the wetlands have been receiving semi-treated wastewater from urban areas, and have also been influenced by traditional agriculture and livestock areas located nearby. In water samples, BP-A and TCS were detected with the highest concentrations, with $240000000 \text{ ng L}^{-1}$ and $110000000 \text{ ng L}^{-1}$, respectively. Other compounds were also detected in the same concentration levels, showing that the environment is highly degraded and contaminated by anthropic activities (Díaz-Torres et al., 2013; Hernández-Quiroz et al., 2019).

In another area in Mexico, near Hidalgo, Díaz and Peña-Alvarez (2017) analyzed water and sediment samples from the Tula River in Mexico, which is considered one of the most degraded rivers in the country. The area is highly urbanized and industrialized. The highest concentration detected in water samples was 250 ng L^{-1} of NAP. In sediment samples, TCS, NAP and IBU concentrations stayed below 5 ng g^{-1} . In Cuernavaca, the capital of the state of Morelos, surface

water was analyzed for 35 pharmaceuticals. Of those, 12 were quantified and the highest concentrations were of NAP, PARA and DIC. Concentrations reached values higher than 4000 ng L⁻¹ (Rivera-Jaimes et al., 2018a).

Of the 31% of global freshwater resources in Latin America, Brazil itself holds 18% (Wilkinson, 2010). Therefore, freshwater ecosystems monitoring in Brazil is especially valuable, and areas with high demographic densities like Southeastern Brazil should be prioritized. Several rivers in the state of Sao Paulo were monitored and tested for several compounds. The detected contaminants included propylparaben (PrP) (55600 ng L⁻¹), CAF (129580 ng L⁻¹), atenol (ATEN) (1180 ng L⁻¹) and PARA (30420 ng L⁻¹) (Campanha et al., 2015; de Sousa et al., 2014; Galinaro et al., 2015; Sodré et al., 2010b; Torres et al., 2017). River sediments were also analyzed in the area and (De Sousa et al., 2015) determined different classes of compounds in sediments from Jundiaí River Basin. TCS had the highest detected concentration (176 ng g⁻¹) and detection frequency (100%), followed by CAF (41 ng g⁻¹) propranolol (PROP) (28.5 ng g⁻¹) and ATEN (13.8 ng g⁻¹).

In Southern Brazil, Caldas et al. (2016) analyzed 32 pesticides and 26 pharmaceuticals and personal care products (PPCP) in surface water samples from Rio Grande do Sul. Bentazone had the highest concentration (6700 ng L⁻¹), followed by avobenzone (UV filter, 5930 ng L⁻¹), BP-A (4420 ng L⁻¹) and SA (1670 ng L⁻¹). BP-A was also detected in concentrations of up to 517 ng L⁻¹ in the same area, as well as CAF, reaching 28000 ng L⁻¹ (Petteffi et al., 2019). PCPs were also detected in the Upper Iguassu River Basin, also in Southern Brazil. TCS and Parabens were detected with concentrations of up to 10300 ng L⁻¹ (Mizukawa et al., 2018). In the same area, water samples were tested for CAF, BP-A and musk xylene (XYL). The highest concentrations detected were of CAF, ranging from 174 ng L⁻¹ to 123450 ng L⁻¹. BP-A and XYL had concentrations in the range of nd-12610 ng L⁻¹ and 40-560 ng L⁻¹ (Froehner et al. 2011B). The results show that the river suffers a strong influence of the urban area. In another study, Froehner et al. (2011C) analyzed sexual hormones (estrogens) in the sediment of a mangrove inside an urban area at the coast of Santa Catarina. The most detected estrogen was E2, found in five out of eight sampling points. The concentration range was from nd-39.77 ng g⁻¹. However, the highest concentration detected was 129.75 ng g⁻¹ of EE2.

In Central-West Brazil, the presence of emerging contaminants was reported in two different areas. Annuniação et al. (2017) analyzed polybrominated diphenyl ethers (PBDEs) in

sediments of Paranoá Lake, Brasília (the capital of Brazil). Only two PBDE were detected: 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47) and 2,2',4,4'-Tetrabromodiphenyl ether (BDE-47). All concentrations were below 3 ng g^{-1} . Also, da Rocha et al. (2015) determined CBZ and thiamethoxam (THIA) in surface water samples from an urban river in Mato Grosso do Sul. Agriculture is the main economic activity of the studied area. CBZ was not detected in any sample, but THIA, an insecticide, was detected in a range of $1230\text{-}1580 \text{ ng L}^{-1}$.

In Chile, Suazo et al. (2017) analyzed pharmaceuticals and personal care products in rivers and lakes. All compounds (CBZ, E2, IBU, 2,4-dihydroxybenzophenone, 3- (4-methylbenzylidene) camphor) were detected below LQ. Also in Chile, water samples from the Lonquimay River were tested for several compounds, like ATZ, CAF, DIC and TCS. The detected concentrations were low and the medium concentrations were 22 ng L^{-1} of ATZ, 8 ng L^{-1} of CAF, 15 ng L^{-1} of DIC and 25 ng L^{-1} of TCS (Rozas et al., 2016).

Bedoya-Ríos et al. (2017) studied the occurrence of endocrine disruptors in Bogotá, Colombia. Samples from the Bogotá River and its tributaries were taken. The list of compounds included pharmaceuticals, phthalates and organophosphorus compounds. Among the other contaminants, BP-A had a 38.6% detection frequency, with a maximum concentration of 76820 ng L^{-1} , while CBZ was reported in 17.6% of the samples with concentrations of up to 36920 ng L^{-1} .

Babay et al. (2014) determined the occurrence of nonylphenols in surface water and sediment samples from 13 sampling points in the city of Buenos Aires in Argentina. Three nonylphenols were analyzed: nonylphenol, nonylphenol mono-ethoxylate (NP1EO) and nonylphenol di-ethoxylate (NP2EO). In water samples, the highest concentration detected was 8600 ng L^{-1} of NP1EO. The highest concentration detected in sediment was of nonylphenol (6359 ng g^{-1}). The other samples also presented high concentrations of nonylphenol when compared to the other compounds. The highest detected concentrations of NP1EO and NP2EO were 3357 ng g^{-1} and 437 ng g^{-1} , respectively.

2.3.4 Groundwater

The real or potential use of groundwater for human consumption causes concern regarding the water quality, since treatment and recovery of groundwater sources are challenging. However, the degradation of this resource is frequent due to anthropic activities and the improper disposal of

effluents and waste (Essien and Bassey, 2012). Therefore, monitoring and reporting water quality in groundwater sources is important for prevention and recovery of degraded environments. Despite the importance of monitoring groundwater, only a few works regarding contamination by emerging compounds in Latin America were found in this search. The concentrations presented in the following paragraphs will be described in ng L^{-1} . Further information can be visualized in Appendix D.

Besides wastewater and spring waters, Lesser et al. (2018) analyzed groundwater samples from wells in the Mezquital Valley in Mexico. Some of the most frequently detected compounds in groundwater were SUF, DEET, CBZ and BLGN (cocaine metabolite). However, the highest concentrations detected were 698 ng L^{-1} of TCS and 756 ng L^{-1} of DEET. Also in México, Félix-Cañedo et al. (2013) determined the occurrence of several emerging contaminants in groundwater near Mexico City. Several compounds were not detected, but SA and BP-A had detection frequencies of 87% and 63%. The higher concentrations were 460 ng L^{-1} of SA and 340 ng L^{-1} of TCS.

In Northeastern Brazil, Melo and Brito (2013) also analyzed the presence of BP-A and EE2 in groundwater in Sao Luiz, Maranhao. The sample was collected from a residential well. BP-A was detected with a concentration of 1110 ng L^{-1} . In addition, UV filters were determined in Southeastern Brazil in groundwater samples in Sao Paulo. Ethylhexyl salicylate (ES) and BP-3 were only detected below LQ, and Octocrylene (OC) was not detected. The only UV filter detected above the LQ was EHMC, reaching 137 ng L^{-1} (da Silva et al. 2015).

2.3.5 Coastal Waters

Coastal and marine environments are among the most man-influenced areas in the world, housing a high percentage of the global population. Also, coastal areas offer several resources, recreational activities and easy access to transportation and marine trade (Neumann et al., 2015), increasing the number of tourists and ports. Since several important Latin American cities are located on the shoreline, coastal and marine areas are important both from the environmental and socioeconomic perspective (de Andres and Barragan, 2015). In the paragraphs below, the concentrations will be described in ng L^{-1} for water samples and in ng g^{-1} for sediments. Further information can be visualized in Appendix E.

Of all countries in Latin America, México and Brazil have the largest shorelines. However, no research concerning emerging contaminants in the coastal zones of México was found. In Brazil, three areas were monitored, two in Southeastern Brazil. Pereira et al. (2016) analyzed 33 emerging contaminants in the coastal zone of Santos Bay. Nine compounds were detected in the seawater (ATEN, PARA, CAF, COC, BLGN (COC metabolite), IBU, DIC, losartan and valsartan). The highest concentration detected was 2094.4 ng L⁻¹ of IBU. COC and CAF also presented high concentrations, reaching 400.5 ng L⁻¹ and 648.9 ng L⁻¹, respectively. Salgado et al. (2019) analyzed 20 pharmaceuticals and PCPs in the coastal area of Cananeia Island, in Sao Paulo. Only three compounds were quantified, Propranolol (PROP), Metoprolol (MTL) and Fenoprofen (FEN), with concentrations varying from >LD to 66470 ng g⁻¹. In Northeastern Brazil, Lisboa et al. (2013) developed a methodology to analyze eight endocrine disruptors in seawater. The method was applied to seawater samples from ten sampling sites around Todos os Santos Bay, in Bahia. Most of the compounds were detected below the LQ in all sampling points. The only compound detected in all sampling sites above the LQ concentration was 4-Octylphenol (4-OP), with a maximum concentration of 134 ng L⁻¹.

In the Argentinian Atlantic coast, De Waisbaum et al. (2010) chose 12 sampling sites to monitor tributyltin (TBT). Water and sediment samples were taken. In water, the concentration range was 160-6700 ng L⁻¹ (100% frequency), while in sediment it was from 60-5200 ng g⁻¹, but TBT was not detected in every sediment sample.

2.3.6 Soil

Soil pollution by emerging contaminants is becoming frequent, since the disposal of sludge and effluent of WWTPs is also frequent. However, the soil has a delicate balance between soil composition and microorganisms, which could be affected by pollutants and other compounds. In this search, only two articles regarding emerging contaminants in soil samples were found, both from México. The concentrations will be described in ng g⁻¹, and other information can be found in Appendix F.

Gibson et al. (2010) analyzed pharmaceutical and endocrine disruptors in soils irrigated with wastewater in the Tulla Valley in Mexico. The highest concentration detected was 299 ng g⁻¹ of 4-NP. In a previous work in Tulla Valley, Durán-Alvarez et al. (2009) analyzed several emerging contaminants, and eight out of seventeen compounds were not detected in any samples. However,

DEHP had a maximum concentration of 2079 ng g⁻¹. The experiment shows that most of the compounds can suffer degradation processes within soils and can act as an effective barrier, protecting groundwater from contaminants.

2.3.7 Biota

The continuous input of emerging contaminants into the environment can affect several organisms, mainly microorganisms present in freshwater and aquatic biota in general. Bioaccumulation, biomagnification and the development of bacterial resistance genes, which could also affect antibiotics' effectiveness and have the potential to spread in the environment, are among the main concerns in this context. For this reason, reporting and monitoring the bioaccumulation of emerging contaminants and the development of resistance genes are necessary in degraded environments such as those found in Latin America. This topic is divided into two subtopics, "Biological Concentrations", where emerging contaminants found within biota are reported in ng g⁻¹, and "Resistance Genes", where the presence of bacterial resistance genes in Latin America is discussed. Further information can be visualized in Appendix G.

2.3.7.1 *Biological Concentrations*

According to the results of this review, only two countries in Latin America reported a contamination of biota by emerging contaminants. In Brazil, most of the studies were concerned about the bioaccumulation of such substances within edible fishes and the direct relation with human health. Guidi et al. (2018) analyzed quinolones and tetracyclines in muscles of Nile tilapias and rainbow trouts but enrofloxacin (ENRO) was the only antibiotic detected above the LQ. Its presence in only four out of 29 samples may indicate good practices regarding the application of antibiotics in the studied fish farms. However, another study performed in a fish farm in the border between Southeastern and Central-West Brazil detected oxytetracycline (OXTC), florfenicol (FFC) and (TC) above LQ in Nile tilapia tissues. Samples from younger fishes presented higher concentrations than those from old ones. The highest concentration detected in a sample was 1298.7 ng g⁻¹ of OXTC. Despite high concentrations found in younger fishes, considering that only older fishes are consumed by humans, concentration levels were below the security limits established by laws (Monteiro et al. 2016). Munaretto et al. (2013) analyzed several endocrine disruptors in fish fillets ready for consumption. BP-A, chlorpyrifos and bifenthrin were detected above LQ: BP-A

had a concentration range of 20.7-14.5 ng g⁻¹, but chlorpyrifos was detected with the highest concentrations of 34.7 ng g⁻¹.

Accumulation of emerging contaminants was also reported in wild animals. Tissues from Franciscana dolphins collected in coastal areas from Southeastern and Southern Brazil were tested for organochlorine compounds. Hexachlorobenzene (HCB) was the organochlorine detected with lower concentrations (10-61 ng g⁻¹), while polychlorinated biphenyls (PCB) and dichlorodiphenyltrichloroethane (DDT) had similar concentration ranges of 909-5849 ng g⁻¹ and 264-5811 ng g⁻¹, respectively. Such high concentrations indicate that coastal areas are strongly affected by human contamination (Lailson-Brito et al., 2011). This hypothesis is endorsed by the results found by (Rossato et al., 2016), who determined the occurrence of organotins (TBT, dibutyltin, monobutyltin and triphenyltin) in tissues samples from the muricid *Stramonita haemastoma*. Five sampling sites were chosen in Baía da Babitonga in the Southern Brazilian coast. Muricid tissues from four sampling points were contaminated by organotins. All contaminants had the same detection frequency (80%) and the highest concentration detected was 335.29 ng g⁻¹ of monobutyltin.

The other country that reported contamination of emerging contaminants in biota tissues was Argentina. In the northeastern area of the country, Stoker et al. (2011) evaluated the presence of organochlorine compound (PCBs and pesticides) residues in eggs of *Caiman latirostris*. Detectable concentrations of the compounds were found in all sampled clutches. PCBs reached 136.6 ng g⁻¹ lipid, and pesticides (sum) reached 189 ng g⁻¹ lipid. *Caiman latirostris* is a non-migratory species and has a long lifetime, therefore, accumulation of such contaminants in eggs of *Caiman latirostris* indicates the presence in the environment. Also, these results show that the species is suitable for biomonitoring.

2.3.7.2 Antibiotic Resistance

The presence of resistance genes was widely reported in Southeastern and Southern Brazil, but also in Mexico and Argentina. Resistance genes and resistant bacteria were found in several areas and types of environments, showing that contamination may come from different sources and could spread to other areas. Detailed information about the published studies can be found Appendix H.

One of the main believed sources of antibiotic resistance is hospital sewage, due to the extensive and frequent use of such compounds by a wide range of patients, but only few Latin American studies regarding antibiotic resistance had hospital sewage or sludge as objects of study. Fuentefria et al. (2011) analyzed antibiotic resistance genes in *Pseudomonas aeruginosa* in raw sewage from two hospitals and their receiving water bodies in Southern Brazil, Rio Grande do Sul. Resistance to all eleven antibiotics was detected at all sampling points (both raw sewage and surface water) with a frequency higher than 68.8%. The only exception was sampling point S3 (hospital sewage) where only 2.2% of *P. aeruginosa* colonies presented resistance to all tested antibiotics. However, multi-antibiotic resistance was still detected, and 44% of the colonies from S3 were resistant to at least seven antibiotics. The antibiotic with more resistant colonies was imipenem (IMP). The presence of antibiotic resistance in aquatic ecosystems that receive hospital sewage was also investigated in Southeastern Brazil by Martins et al. (2014). Two different isolates were obtained and presented resistance only to TC. The results of both studies indicate that there is a high possibility of antibiotic resistance transfer from hospital sewage to surface waters.

Domestic sewage is also considered a source of antibiotic resistant bacteria, and the transfer of antibiotic resistance from domestic WWTP sewage to receiving water bodies is of concern. In Southern Brazil, Lopes et al. (2016) assessed the occurrence of antibiotic-resistant *E. coli* isolates in the effluent of a WWTP and in the sediments of the receiving river. The antibiotics with higher resistance were TC, cephalothin (CPT), streptomycin (STM) and AMOX. In Southeastern Brazil, Paiva et al. (2017) identified resistance genes in raw sewage and activated sludge from a WWTP. The resistance to quinolones and aminoglycosides ranged from 62.5-100% and 25.4-86.2%, respectively. Multiresistance to at least three antimicrobials was high in raw sewage. Also in Southeastern Brazil, *P. aeruginosa* strains from a WWTP were collected. The frequency of bacteria resistance to antibiotics was higher in raw sewage than in the effluent of the WWTP, showing that the treatment may have decreased the concentration of resistant bacteria. IMP and Meropenem (MER) had the highest resistance rates among the antibiotics (Oliveira et al., 2017). In a particular case, Heck et al. (2015) detected antimicrobial resistance in sewage sludge samples from a composting process at a recycling plant in Southern Brazil. Of the 344 bacterial isolates, Gram-negative bacteria were prevalent over Gram-positive bacteria. Of these isolates, 89.5% showed resistance to at least one antibiotic and 60% of them showed a multi-resistance profile. The antibiotic with the highest number of resistant strains was ampicillin (AMP).

But domestic and hospital sewage are not the only potential sources of antibiotic resistance genes in the environment. Agricultural wastewater originated mainly from animal husbandry is also a concern, since antibiotics are frequently applied to the animals and are even consumed daily to prevent diseases. Gambero et al. (2018) studied the presence of antibiotic resistance in *E. coli* in an area of animal husbandry in Argentina. Surface and groundwater were analyzed and it was reported that the antibiotics with more resistance were AMP and TC. However, more strains presented antibiotics resistance in groundwater than in surface water. In Brazil, water samples from different areas were investigated for antibiotic resistance patterns in *E. coli* in Southeastern Brazil, in Rio de Janeiro, and the environments where antibiotic resistance was more frequent, were in agricultural effluents and recreational waters (Rebello and Regua-Mangia, 2014). The investigation of antibiotic resistance in Brazilian agricultural areas was also performed by Furlan and Stehling (2018), who detected the presence of β -lactamases encoding genes in feces, soil and water from a Brazilian swine farm. Resistant strains of *E. coli* were also detected in swine farms in feces, water and soil samples. The highest resistance rates were obtained for Sulfamethoxazole + Trimethoprim, Colistin and Enrofloxacin. Also, 37% of the isolates were resistant to at least three antimicrobials (Brisola et al., 2019a). Palhares et al. (2014) detected Salmonella resistant strains in agricultural areas in Southern Brazil. Of the collected Salmonella isolates, 49.5% were resistant to at least one antimicrobial and 18% were resistant to two or more antimicrobials. The antimicrobials with highest resistance percentages were kanamycin (KAN) (21.1%), TC (13.8%), doxycycline (13.3%) and neomycin (12.2%).

In urban areas, the occurrence of antibiotic-resistant bacteria and antibiotic resistance-encoding genes in surface water was also frequently reported. Sanchez et al. (2018) assessed the presence of β -lactamase-encoding genes and plasmid-mediated quinolone resistance in surface water samples from Sao Paulo State, Southeastern Brazil. Samples collected near urban areas, which received domestic sewage, presented the greater number of resistance genes. High resistance rates to TC, aztreonam, ticarcilin-tazobactam, STM, AMOX, IMP, AMP, cefotaxime, ceftriaxone and ceftazidime were also reported in surface water, lakes, wells and even in potable-water reservoirs (Bortoloti et al. 2018; Da Silva et al. 2011; Giowanella et al. 2015; Nascimento et al. 2017; Paiva et al. 2017; Zanetti et al. 2013). In the coastal area of Brazil, Andrade et al. (2015) detected antimicrobial resistance of *Escherichia coli* isolated from water and sand samples in Sao Vicente, Santos Bay, in the coast of Southeastern Brazil. The area is highly urbanized and raw

sewage and WWTP effluents are frequently discharged in the bay. A total of 456 colonies of *E. coli* were analyzed and 56.6% demonstrated resistance to one or more antibiotics, like AMP, AMOX + clavulanic acid, STM, erythromycin (ERY) and others.

Another country that reported the occurrence of antibiotic resistance in surface waters was México. Mondragón et al. (2011) identified antibiotic-resistant *Enterococcus faecalis* in the Mololoa River in México. All sampling sites presented resistance to ciprofloxacin (CIPRO), 84.2% of the sampling sites presented resistance to KAN, 15.7% to vancomycin and 13.1% to gentamycin (GNT). In the agricultural area Culiacán, bacterial resistance to 16 antibiotics was tested. The bacteria presented higher resistance rates to AMP, neomycin and chloramphenicol and multi-resistance was also detected (Castaneda-Ruelas and Jiménez-Edeza, 2018).

Many authors reported antibiotic resistance in edible fishes, shrimps and aquaculture in general, which is a common economic activity in Brazil. Monteiro et al. (2016) determined the multiple antibiotic resistance (MAR) index from bacteria found in Nile tilapia collected from cage farms in Southern Brazil. The bacteria identified belonged to the genera *Pseudomonas*, *Burkholderia*, *Pasteurella*, *Streptococcus* and *Aeromonas*. The MAR index ranged from 0 to 0.86 and some of the bacteria were susceptible to all tested antibiotics. Most of the bacteria were resistant to SUF and TC. In Southeastern Brazil, the occurrence of antibiotic-resistant bacteria in the water of an experimental aquaculture farm in Minas Gerais was reported. The antibiotics with greater resistance rates were penicillin, azithromycin, AMP and GNT (Resende et al., 2012). In Northeastern Brazil, Vieira et al. (2010) tested the antimicrobial susceptibility of *E. coli* in shrimps and their culture environment in the state of Ceará. Pond water sediment and shrimp tissue samples were collected. The *E. coli* strains were mainly resistant to IMP, AMP and CPT, with 71.4%, 66.6% and 42.8% of strains resistance, respectively. Eight pond water samples and all shrimp tissues and sediment samples showed resistance to at least one antibiotic. In shrimp tissues, the presence of multidrug resistance was reported for all samples.

2.4 DISCUSSION

The review was able to gather a good amount of quality data regarding emerging contaminants, and the authors consider that the results are representative. According to the search, only 5 countries i.e., Brazil, Mexico, Argentina, Chile and Colombia, published articles fitting the search criteria. This does not mean that there are no publications about emerging contaminants in

these countries, only that they did not fit in the search criteria. Other countries published articles in this field, but most of them were exclusively about analytical methodology determination or bench-scale experiments, which were out of the scope of this research. According to the results, Brazil is the country that published the most, followed by Mexico, as seen in Figure 2-1. Despite the high amount of published articles in these two countries, the studies tend to focus on specific regions and areas, for example in the surroundings of Mexico City or in the southern and southeastern states of Brazil.

These are the most populated and developed areas of both countries, with the highest pressure on water resources. Also, it is where many universities and research institutes are located, which explains the higher number of studies performed in these areas. Latin America has a large territorial area and there are many differences in characteristics such as climate, economy, sanitation and consumption of emerging contaminants. This also means that the lack of information about certain substances or groups of substances does not mean that they are not present in the matrices. It is only an indication that more research should be performed in such areas, specially looking for substances that were frequently reported in Latin America before.

This represents both a challenge and an interesting opportunity in terms of environmental monitoring. This is one of the reasons why it is extremely important to create environmental monitoring programs, extend them to other countries in Latin America and decentralize research to other areas of México and Brazil. The main goals should be to get a better overview of the actual status of the most important water resources and later to focus on the most polluted and important areas.

To our knowledge, there are no official regulations, environmental laws or watch lists regarding emerging contaminants in environmental matrices in Latin America. Most regulations refer to drinking water and include nutrients, heavy metals and some pesticides (CONAMA, 2011), but pharmaceuticals, plasticizers, hormones, personal care products and other compounds are usually not regulated. However, the use of some compounds was banned or limited, like TCS in Argentina (ANMAT, 2016), Chile (ISP, 2017) and the Andean Community (Secretaría General de la Comunidad Andina, 2017). These countries followed previous regulations from Europe (EU, 2016) and the USA (FDA, 2017). Despite the efforts of other countries, Brazil and Mexico did not

ban TCS or limit its use. Also, in this review, TCS was one of the most frequently detected compounds, including drinking water sources.

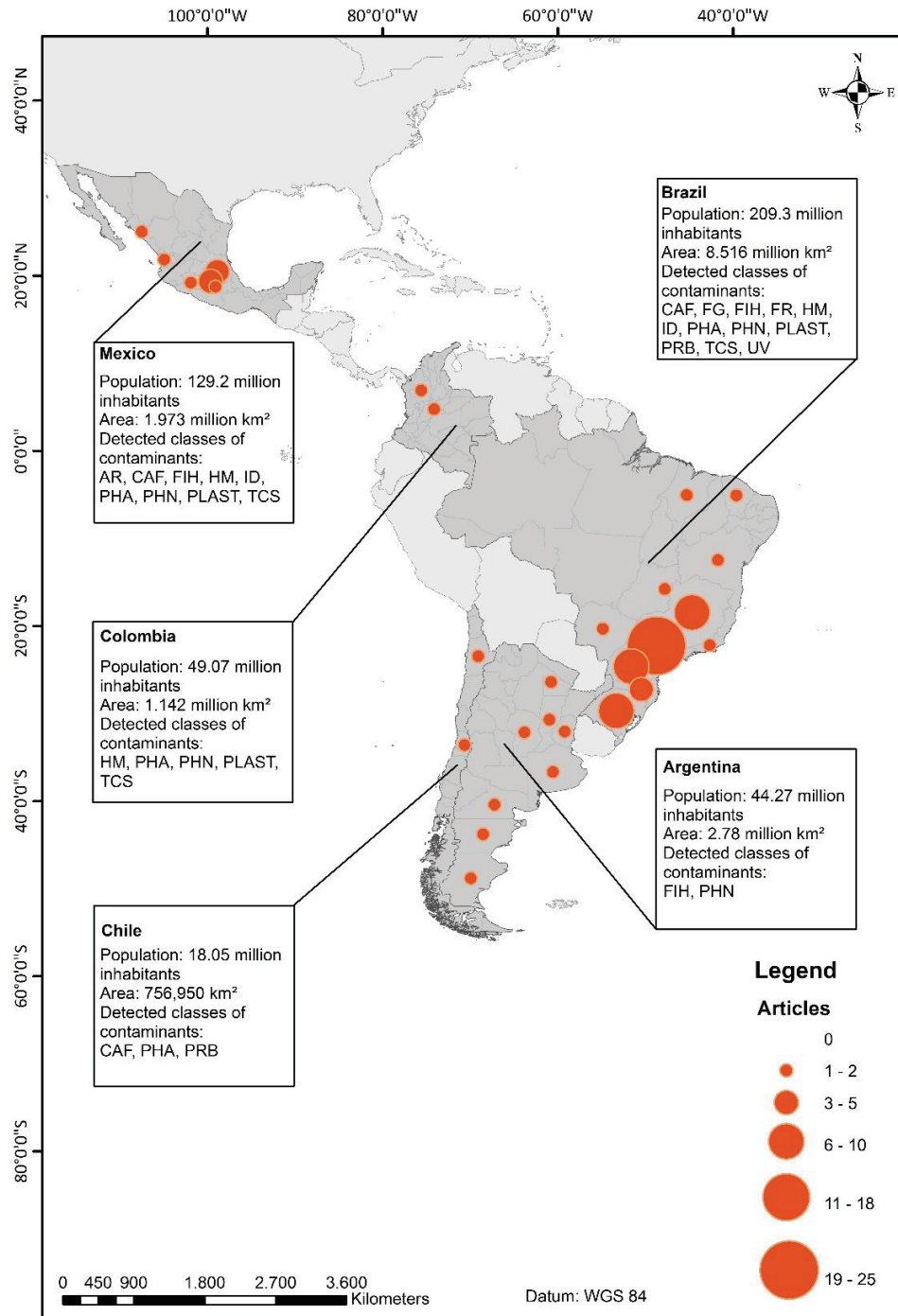


Figure 2-1: Distribution of published research regarding emerging contaminants in Latin America

Drinking water and its sources were the most frequently studied matrices in Latin America, according to this review. One of the most detected contaminants, besides TCS, was DEHP (Table S1). The US Environmental Protection Agency (EPA) developed the National Primary Drinking Water Regulation and established standard limits of contaminants in drinking water, which also included DEHP. The maximum allowed concentration of DEHP in drinking water is 6000 ng L^{-1} (EPA, 2009). In this study, DEHP concentrations of up to 2282 ng L^{-1} were detected in drinking water in Mexico (Félix-Cañedo et al., 2013) and up to $100970000 \text{ ng L}^{-1}$ in a reservoir in Colombia (Bedoya-Ríos et al., 2017), which is higher than the maximum concentration established by EPA for drinking water in public systems. EPA also proposed a Contaminant Candidate List (EPA, 2016) for contaminants that are still not subject to any proposed drinking water regulation, but may occur in public water systems and are a subject of concern. The following contaminants are included in the CCL4 list and were also frequently detected in drinking water in Latin America: E1, E2, EE2, E3 and NP. The CCL4 did not establish any maximum level or concentration (EPA, 2016), but the presence of these compounds in drinking water and its sources is concerning. These results highlight the importance of constant monitoring and protection of water sources like reservoirs, springs and others.

Surface water was also frequently investigated during the period under review. In order to compare and discuss the results, two official documents from the European Union were consulted. The European Directive 2008/105/EC established a list of environmental quality standards for priority substances as well as their maximum annual average values and maximum allowable concentrations in surface waters. The following contaminants are included in the list and were detected in environmental matrices in Latin America: Chlorpyrifos (30 ng L^{-1}), DDT total (25 ng L^{-1}), DEHP (1300 ng L^{-1}), 4-NP (300 ng L^{-1}), 4-OP (100 ng L^{-1}) and TBT (0.2 ng L^{-1}), where the concentration presented is the maximum annual average established by the Directive 2008/105/EC. Of those, 4-NP and 4-OP are also identified as hazardous substances and BP-A and PCB are in review for possible identification as priority substances. Chlorpyrifos, PCB, TBT and DDT were not detected in surface water, but in aquatic biota tissues, which indicate water contamination as well (S7). The reported concentrations of DEHP were lower than 1300 ng L^{-1} in surface water (S3), but were surprisingly higher in drinking water and its sources (S1): The contaminant 4-NP was detected in drinking water sources with concentrations of up to 1918 ng L^{-1} (Table S1), which are much higher than the allowed annual average of the European Directive. Although BP-A is still

not officially listed in the European Directive 2008/105/EC, it was one of the most detected contaminants in surface water (S2), with concentrations of up to 240000000 ng L⁻¹ in natural wetlands (Díaz-Torres et al., 2013) and 12610 ng L⁻¹ in rivers (Froehner et al., 2011a).

The watch list under the Water Framework Directive 2000/60/EC contains contaminants that should be monitored by the EU Member States to determine the risk they pose to the aquatic environment and to find out whether the EU should set quality standards for them. The first list was released in 2015 and updated in July 2018 and now includes E1, E2, EE2, ERY, AMOX, CIPRO, THIA, Clarithromycin, Azithromycin, Methiocarb, Imidacloprid, Thiacloprid, Clothianidin, Acetamiprid and Metaflumizone. The hormones E1, E2 and EE2 were frequently detected in drinking water (S1), WWTP (S2), surface water (S3) and even in groundwater (S4). Although only the antibiotic AMOX was detected in drinking waters (Monteiro et al., 2018), antibiotic resistance studies indicated the presence of AMOX, ERY, CIPRO and other antibiotics in the environment.

The occurrence of antibiotic-resistant bacteria and genes was extensively reported in Brazil, but there is a lack of information in the other countries. Since the presence of antibiotic-resistant bacteria and genes was reported in drinking water, surface water, wastewater, groundwater and even in edible species of fishes and shrimps, the matter attracts a lot of attention from the population, government and the health community. The antibiotics with higher bacterial resistance rates were TC, AMOX, AMP, IMP, GNT and Cephalotin, which are frequently employed to treat and prevent infections. The high percentage of bacteria resistant to these antibiotics shows that their release into the environment is constant and will affect the treatability of infections in Latin America in the future.

In previous review papers and long term studies, similar results were detected. For example, a ten years-study was performed in the Sao Paulo state, in Brazil (Montagner et al., 2019). 708 samples of wastewater, WWTP effluents, surface, groundwater and drinking waters were collected between 2006 and 2015. The compounds CAF, E1, E2, EE2, BP-A were frequently detected, as shown in the results of this review. In Brazil, Starling et al. (2019) evaluated the occurrence of emerging contaminants in the environmental compartments in Brazil. Their conclusions were that there is also a concentration of studies near capitals, mainly in the Southeast area, and that research in other areas should be performed. In addition, there is a lack of bioaccumulation studies of these

pollutants in Brazil. Also, Peña-Guzmán et al. (2019) wrote a review about the presence of emerging pollutants in the urban water cycle in Latin America. This review did not include other matrices, such as biota or soil, but they also found more papers in Brazil and Mexico than any other country in the Latin America. The authors also emphasized the importance of implementing regulations and directives regarding emerging contaminants.

2.4.1 Challenges and future perspectives

Although drinking water and surface water were widely investigated, some research gaps were identified. The low number of data regarding WWTPs and hospital effluents is concerning, since their effluents are considered the main source of emerging contaminants into aquatic environments. Also, these substances are not included in the usual monitoring program of WWTPs. In addition, this review showed that domestic effluent can contain equal or even higher concentrations of pharmaceuticals than hospitals effluent (S2). This reveals the importance of monitoring urban WWTP and establishing a priority list of contaminants. This gap may be explained by bureaucracy and other difficulties in accessing and monitoring WWTPs, another reason why is important to make the Latin American population and leaders aware of the emerging contaminants problem.

Other identified research gaps were the low number of studies in the soil, groundwater, coastal areas and within biota tissues. Studies regarding contamination of the benthic community or biofilms were not found in this research, even though these organisms play a very important role in the energy and food cycle. The possibility of bioaccumulation of a variety of substances is concerning, and it should be further investigated, mainly in Latin America, a continent with an extremely high biodiversity.

Since agriculture is one of the main economic activities in Latin America, monitoring the occurrence of emerging contaminants in soil is also very important. Also, groundwater is frequently used as a potable-water source in many areas in Latin America, and contamination of these sources could have a direct impact on human health, just like in coastal areas, where marine water is used for leisure activities like swimming, surfing and others. Such activities provide direct contact with water, which could be harmful to human health if the water is polluted by emerging contaminants. Another possible threat to human health is the use of wastewater for agricultural irrigation, for

example in Mexico. Further studies should be performed in these areas, assessing the contamination of soil, surface water, groundwater, springs, plants originated from wastewater irrigation and the possible hazards to human health.

The low number of studies regarding antibiotic resistance is also concerning, once the spread of antibiotic-resistant bacteria could be a big threat to human health in the next years. Most of the information about this matter was found in papers from Brazil and one paper from Argentina. However, more information should be gathered in the other countries regarding this issue.

According to the articles presented in this review, the detection frequency of the contaminants and the examination of regulations and watch lists from EPA and the European Union, a priority list with the most important and detected contaminants in Latin America was suggested. The chosen contaminants can be found in Table 2-2.

Table 2-2: Priority list: Compounds, chemical structure and properties

Compound	Molecular formula	Log Kow	Solubility Water (25°C)
4-n-nonylphenol (4-NP)	C ₁₅ H ₂₄ O	5.76	7 mg L ⁻¹
Bisphenol A (BP-A)	C ₁₅ H ₁₆ O ₂	3.32	120 mg L ⁻¹
Di-2-ethylhexyl phthalate (DEHP)	C ₂₄ H ₃₈ O ₄	7.60	0.27 mg L ⁻¹
Triclosan (TCS)	C ₁₂ H ₇ Cl ₃ O ₂	4.76	10 mg L ⁻¹
Estrone (E1)	C ₁₈ H ₂₂ O ₂	3.13	30 mg L ⁻¹
17β-estradiol (E2)	C ₁₈ H ₂₄ O ₂	4.01	3.6 mg L ⁻¹
17α-ethinylestradiol (EE2)	C ₂₀ H ₂₄ O ₂	3.67	11.13 mg L ⁻¹
Tetracycline (TC)	C ₂₂ H ₂₄ N ₂ O ₈	-1.37	231 mg L ⁻¹
Amoxiciline (AMOX)	C ₁₆ H ₁₉ N ₃ O ₅ S	0.87	3430 mg L ⁻¹
Ampicillin (AMP)	C ₁₆ H ₁₉ N ₃ O ₄ S	-1.13	10100 mg L ⁻¹
Imipenem (IMP)	C ₁₂ H ₁₇ N ₃ O ₄ S	-3.57	10000 mg L ⁻¹

Sources: www.pubchem.com and www.chemspider.com

We hope this suggested priority list could encourage governments to establish laws and directives with restrictions and guidelines to the Latin American countries, towards better water quality and higher environmental and human safety. However, the number of chemicals produced and consumed rises every day and new information about their consequences in the environment

are discovered. Therefore, priority lists must be updated periodically and should be always based on up-to-date information and data (occurrence, determination and toxicology) obtained in the country or target area.

2.5 CONCLUSION

Latin America has one of the greatest water availabilities per capita, however, the distribution is not equal and there are many issues regarding water quality, which could compromise the supply of potable water to the population. Several studies regarding emerging contaminants in Latin America were performed in the last two decades, but most of the monitoring studies are focused on specific areas in Brazil and Mexico. Therefore, it is necessary to monitor other countries and areas, as well as other matrices, like soil, biofilms, groundwater and coastal areas. It is also important to monitor WWTPs and encourage the improvement of their systems and treatment techniques in order to obtain higher removal efficiencies.

3 CHAPTER 2: DETERMINATION OF ANTIBIOTIC RESISTANCE GENES IN A WWTP-IMPACTED RIVER IN SURFACE WATER, SEDIMENT, AND BIOFILM: INFLUENCE OF SEASONALITY AND WATER QUALITY

This is a transcription of the following paper: Reichert, G., Hilgert, S., Alexander, J., Azevedo, J.C.R., Morck, T., Fuchs, S., Schwartz, T., 2021. Determination of antibiotic resistance genes in a WWTP-impacted river in surface water, sediment, and biofilm: Influence of seasonality and water quality, *Science of The Total Environment*, Volume 768. <https://doi.org/10.1016/j.scitotenv.2020.144526>.

3.1 INTRODUCTION

Antibiotics mark the beginning of modern medicine. Several diseases became treatable after the discovery of these substances and the use of antibiotics allowed to perform complex procedures, such as surgeries and chemotherapy (Friedman et al., 2016). Antibiotics were discovered from secretions of microorganisms, including fungi and bacteria themselves. The production of substances with such properties was a defense mechanism against other bacteria, and so is the ability to resist antibiotics (Friedman et al. 2016; Martínez, 2012). After several decades of extensive antibiotic use, resistant bacteria have become more and more frequent.

Bacteria can also develop resistance against multiple antibiotics and the number of multi-drug resistant bacteria resistant is increasing fast (Frieri et al., 2017). In former times, if treatment failed with one specific antibiotic, other antibiotics could be employed. Today, the health community is struggling to find efficient treatments for infections because of multiple antibiotic resistance, increasing the consumption of antibiotics (Macgowan and Macnaughton, 2017). This can cause negative consequences for the population, both on individual and public levels. Some consequences are: healthcare expenses (length of hospital stay, number, and higher complexity of procedures), need for intensive care and invasive devices, low efficiency of narrow-spectrum antibiotics, use of stronger medicines with possible negative consequences (such as nephrotoxicity), increase in mortality rates, and active population loss of productivity (Cassini et al., 2019; Friedman et al., 2016).

Since this is a matter of public safety and health, the consumption of antibiotics is monitored in European countries by the European Centre for Disease Prevention and Control (ECDC). Germany, for example, consumes around 11.9 DDD (defined daily dose) of antibacterials per 1000 inhabitants per day. With these numbers, Germany is the 7th lowest antibiotics consuming country in Europe. The main antibiotics consumed in the country are penicillin (31.5%), followed by other

β -lactams (20.9%), macrolides, lincosamides, and streptogramins (16.4%), tetracyclines (13.8%), quinolones (8.1%), and sulfonamides/trimethoprim (5.3%) (ECDC, 2019 - Report).

The ECDC also monitors the presence of antibiotic resistance in Europe. While the occurrence of antibiotic resistance in some organisms is not a public health problem, in others, such as in facultative pathogenic bacteria (FPB), it is highly concerning. The group of concern includes the following species: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Streptococcus pneumoniae* and enterococci (ECDC, 2018). The identification of antibiotic resistance in these microorganisms is very important, once they frequently cause human and animal infections and are easily detected in medical facilities. Some of these species are also adapted to live in other habitats, such as aquatic ecosystems.

Even if most of the concern about antibiotic resistance is towards pathogenic bacteria detected in medical facilities, patients, and infections, it is still necessary to pay attention to the growth of antibiotic resistance in other environments. Regarding this issue, surveillance of resistant bacteria in animals, food, and even in wastewater effluents is advancing fast, but monitoring efforts in the natural environment are still rudimentary and should be improved (Huijbers et al., 2019). The main reason to study antibiotic resistance in the natural environment, mainly in rivers and water bodies, is to better understand the threat to animal health, to detect possible reservoirs of resistance and to develop new techniques to prevent the spread and increase of antibiotic-resistant bacteria (Bengtsson-Palme et al., 2018; Von Wintersdorff et al., 2016). Also, there is a direct link from the natural environment (mainly aquatic ecosystems), and its resources, to human needs, such as the consumption of potable water, irrigation of crops and different kinds of plantations, and the direct consumption of fish and other organisms.

In urban rivers, the effluent of wastewater treatment plants (WWTP) and raw sewage are considered the main source of antibiotic resistance genes (ARG) (Auguet et al., 2017; Bengtsson-Palme et al., 2018; Hembach et al., 2019). Resistant bacteria and ARG were already detected in surface water, attached to suspended solids (Proia et al., 2018), in the sediment (Brown et al., 2019; Lopes et al., 2016) and natural biofilms (Guo et al., 2018; Proia et al., 2016; Subirats et al., 2017b). A biofilm is a complex mixture of microorganisms, including bacteria, that grow and attach to different surfaces, mainly when exposed to humidity and water. These microorganisms actively participate in the biological and biogeochemical fluxes, especially in aquatic ecosystems (Beaulieu et al., 2011; Mulholland et al., 2008; Raymond et al., 2013).

In rivers and streams, biofilms are attached to pebbles, tree branches, leaves, and even sediment. They are in constant contact with the nutrients, pollutants, and the microbiota present in the water, and have the ability to absorb substances and other microorganisms, incorporating them into their matrix (Battin et al., 2016; Bechtold et al., 2012). According to these features, biofilm seems to be a suitable medium to detect antibiotic-resistant bacteria and the spread of antibiotic resistance within a natural ecosystem (Balcázar et al., 2015). Most of the research in this field was performed with natural biofilms, collected from surfaces already incorporated in the environment, like rocks, cobbles, and others (Guo et al., 2018; Proia et al., 2016). However, biofilms grow fast on any kind of surface, which makes them good biological indicators. Also, they could be used as potential passive samplers for a wide range of substances.

Therefore, the goal of this research was to investigate different monitoring strategies to detect and quantify ARGs and FPB in aquatic compartments. In addition, multi-drug resistance was analyzed. Surface water (grab sampling), sediment (core sampling), and biofilm (passive sampling) were collected in a river impacted by human activities in southern Germany. Samples were collected upstream and downstream from a small WWTP (55 000 population equivalents, p.e.). Five sampling campaigns were performed from February to June 2019, to evaluate seasonality effects. The influence of physical and chemical parameters such as nutrients, water turbidity, dissolved oxygen, and other external parameters, such as UV radiation, were also evaluated.

3.2 EXPERIMENTAL DESIGN

3.2.1 Study area

The Kraichbach River is located in southern Germany, in the Baden-Württemberg state. In this area, the mean temperature reaches 0°C in January and 18°C in July. The river is approximately 60 kilometers long with a catchment area of 161 km². The lower part of the Kraichbach River is classified as a “heavily modified water body”, is strongly rectified with reduced morphological structures, and has a slow flow. The Kraichbach River is the effluent receptor of five WWTPs and since the sewer system is separated, there are contributions from combined sewer overflows in the area. The focus of this study is a WWTP in the lower Kraichbach River with a maximum treatment capacity of 55'000 p.e.. The WWTP has standard primary and secondary treatments, with nitrogen and phosphorus removal (83% and 96.6% of efficiency, respectively), but no advanced treatments,

such as ozonation, UV, microfiltration, etc. (Urban Wastewater Treatment Directive - <https://uwwtd.eu/Germany/uwwtps/treatment/>).

3.2.2 Sample collection

Five sampling campaigns were performed from February to June 2019. The samples were collected once a month upstream and downstream of the WWTP, to assess the influence of the effluent on the ARG and FPB. Water samples were collected with pre-washed and decontaminated plastic bottles. A core sampler was used to collect the first 5 cm of sediment from the river. Biofilm samples were collected from biofilm samplers made of a PVC box containing four glass sheets of 70 x 30 cm. The samplers were designed and built in our department for this purpose. The passive sampler was submerged for approximately a month while the biofilm grew on the glass sheets and was later scratched from the glass with a stainless steel spatula.

Samples of the sediment and biofilm samples were stored in previously washed and decontaminated plastic recipients and frozen at -20°C, to preserve the DNA content of the samples. The analyzed amounts of sediment and biofilm ranged from 0.10 to 0.41 g and 0.0048 to 0.078 g (dry weight), respectively. The water samples were filtered using a vacuum pump system and polycarbonate membranes, pore size of 0.2 µm (Whatman® Nuclepore™ Track-Etched Membranes, Sigma-Aldrich, Munich, Germany). The samples were filtrated until the membranes were clogged. The filtrated volume varied from 400 to 250 mL. The membranes containing the samples were frozen at -20°C in decontaminated plastic recipients until analysis. All the samples were filtered immediately after collection and arrival at the laboratory.

3.2.3 Physical and chemical parameters

In the water phase, pH, conductivity, temperature, and dissolved oxygen (DO) were measured in the field with a portable probe Multi 340i (WTW, Weilheim, Germany). Turbidity was measured with a portable turbidimeter (Hach, Loveland, United States). The concentration of suspended solids (SS) was measured by filtrating the samples with glass fiber filters (0.45 µm) and drying the filters at 110°C. In the water samples, orthophosphate, total phosphorus, ammoniacal nitrogen, nitrate, and total nitrogen were analyzed with ready-to-use cuvette tests and a DR2800 spectrophotometer (Hach Lange, Düsseldorf, Germany). In sediment, Kjeldahl nitrogen was analyzed according to the standard DIN-EN-25663 methodology, with a digestion unit (Büchi, Lawil, Switzerland). Total phosphorus was also analyzed, according to the standard methodology

DEV-D11-7. Both methodologies are from the *Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung*.

Daily values for UVA and UVB radiation (J/m^2) were obtained from *Umweltbundesamt* and *Bundesamt für Strahlenschutz* (Oberschleißheim, Germany). Mean values for the sampling periods (while the biofilm was submerged) were calculated.

3.2.4 Extraction of DNA and quantification of ARG

DNA from sediment, biofilm, and filtration membranes was extracted using the FastDNATM Spin Kit for soil (MP Biomedicals, Illkirchen France) according to the kit protocol. After extraction, DNA concentration and purity were measured with Nanodrop ND-1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). For the samples with low concentrations, concentration was measured with QubitTM 3.0 (Thermo Fisher Scientific, Nidderau, Germany). When necessary, samples were diluted with Nuclease-Free water (Thermo Fisher Scientific) to reach $\sim 50 \text{ ng}/\mu\text{L}$ of DNA concentration.

The quantification of ARG was performed by qPCR (quantitative polymerase chain reaction) in a Cycler CFX96 TouchTM Deep Well Real-Time PCR Detection System (Bio-Rad, Munich, Germany). The samples were analyzed in technical duplicates. The mixture consisted of $2 \mu\text{L}$ of the sample (or template DNA), $10 \mu\text{L}$ of Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific), $1 \mu\text{L}$ of Primer FW ($10 \mu\text{M}$), $1 \mu\text{L}$ of Primer Rev ($10 \mu\text{M}$) and $6 \mu\text{L}$ of Nuclease-Free water (Thermo Fisher Scientific). For each ARG, a control (blank sample) was added to the plate.

The thermocycler conditions were: heating at 95°C for 10 min, 95°C for 15 s and cooling down to 60°C for 1 min for primer annealing and elongation. The melting curve, for control of specificity, consisted of 65°C for 5 s and heating until 95°C (increasing $0.5^\circ\text{C}/\text{s}$). The data analysis was performed using the Bio-Rad CFX Manager Software (version 3.1). Antibiotic resistance markers were analyzed, as well as taxonomical marker genes of FPB. The analyzed parameters are listed in Table 3-1 and Table 3-2.

The cell equivalency methodology was performed according to Hembach et al. (2017) and Hembach et al. (2019). To calculate the number of gene copies, reference strains carrying the genes and the genome sizes were used to calculate the number of cell copies. A base pair average molecular weight of 650 g/mol , and a converting factor of 10^9 ng/g were also used in the equation:

$$\text{Cell equivalents} = \frac{\text{amount of DNA [ng]} * \text{Avogadro's number}}{\text{average size of genome[bp]} * 10^9 * 650}$$

Avogadro's number = 6.022×10^{23} molecules/mol

The primers sequences, coefficient of determination, curves, limits of detection (LOD), and genome sizes can be found in Appendices I and J.

Table 3-1: Analyzed parameters in qPCR – genes and related facultative pathogenic bacteria

Gene marker	Bacteria
<i>ddl</i>	<i>Enterococcus faecalis</i>
<i>yccT</i>	<i>Escherichia coli</i>
<i>ecfX</i>	<i>Pseudomonas aeruginosa</i>
<i>gltA</i>	<i>Klebsiella pneumoniae</i>
<i>secE</i>	<i>Acinobacter baumannii</i>
23S rRNA	Enterococci
16S rRNA	Eubacteria

Table 3-2: Analyzed parameters in qPCR – genes and related antibiotic resistance

Target gene	Resistance
<i>bla</i> _{TEM}	β-Lactam – ampicillin
<i>ermB</i>	Erythromycin (macrolide)
<i>tetM</i>	Tetracyclin
<i>sulI</i>	Sulfamethoxazole
<i>CMY-2</i>	Cephalosporines
<i>bla</i> _{CTX-M}	Carbapeneme
<i>bla</i> _{CTX-M-32}	Carbapeneme
<i>bla</i> _{OXA-48}	Extended-spectrum β-lactams/Carbapenems
<i>mecA</i>	β-Lactams, Methicillin
<i>bla</i> _{NDM-1}	Highly potent extended-spectrum β-lactams/ Carbapenems
<i>bla</i> _{KPC-3}	Extended-spectrum β-lactams/ Carbapenems
<i>mcr-1</i>	Colistin/polymyxin

3.2.5 Antimicrobial susceptibility test

This test was performed to detect multi-drug resistant bacteria. Only sediment and biofilm samples were analyzed since the water samples did not have enough material to perform both of the analyses. The multi-drug resistance test was performed according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) disk diffusion test (EUCAST, 2017).

Small shares of the samples were collected with disposable pre-sterilized sticks and placed in falcon tubes in Muller Hinton-Bouillon broth (10.5 g in 500 mL of deionized water, placed in the autoclave at 121°C). The falcon tubes were placed in a shaker (37°C) and left overnight. The bacteria were inoculated in CHROMagar ESBL (Extended Spectrum Beta-Lactamase) plates (Mast Diagnostica, Reinfeld, Germany) and grew overnight at 37°C. Of the 20 samples, four samples were selected for analysis, and 10 isolates of each sample were picked (with sterile pipette tips) and cultivated in tubes with Luria-Bertany broth in a shaker overnight at 37°C. Afterward, the colony suspension was diluted with Luria-Bertany broth to achieve an absorbance of 0.1 at 625 nm in a U-5100 Spectrophotometer (Hitachi, Tokyo, Japan). The bacteria were inoculated in Muller Hinton agar plates with sterilized cotton swabs, antibiotic disks (Mastdiscs™ AST, Mast Group Ltd, Merseyside, United Kingdom) were applied to the agar plates and were left overnight at 37°C.

The following 16 antibiotics were tested: Cefotaxime (5 µg), Ceftazidime (10 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Imipenem (10 µg), Meropenem (10 µg), Piperacillin (30 µg), Piperacillin/Tazobactam (30/6 µg), Temocillin (30 µg), Amikacin (30 µg), Tigecycline (15 µg), Trimethoprim/Sulfomethoxazole (1.25/23.75 µg), Chloramphenicol (30 µg), Colistin sulfate (10 µg), Colistin sulfate (25 µg) and Fosfomycin/ Glucose-6-phosphate (200/50 µg). The inhibition zones diameters were measured and compared to the breakpoint tables, giving the susceptibility of the bacteria to each antibiotic (EUCAST). When no breakpoint was provided for determined species and there was no inhibition zone (diameter was zero) the isolate was considered resistant.

The bacteria were classified according to the Commission for Hospital Hygiene and Infection Prevention (*Kommission für Krankenhaushygiene und Infektionsprävention* - KRINKO). It considers 4 relevant antibiotic groups and some substances: (i) acylureidopenicillins with the substance piperacillin, (ii) 3rd /4th generational cephalosporins with the substance cefotaxime and/or ceftazidime, (iii) carbapenems with the main substance imipenem and/or with meropenem, and (iv) fluoroquinolones the substance ciprofloxacin. It classifies resistant bacteria in 3MRGN

(multidrug-resistant Gram-negative strains with resistance to 3 of the 4 antibiotic groups) and 4MRGN (multi-resistant Gram-negative strains resistant to all 4 antibiotic groups including pan resistance) (Robert-Koch-Institute, 2012).

3.2.6 Statistical Analysis

Correlation analysis was performed with Statistica 10.0 software (StatSoft. Inc., Tulsa, USA). Correlations between parameters were considered significant with a $p < 0.05$. The boxplots graphs were built with Python libraries Matplotlib and Pandas. The rectangle represents values from the first quartile (Q1) to the third quartile (Q3) of the data. The mean values are represented by the orange line inside the rectangle and the whiskers indicate the data range (minimum and maximum values).

3.3 RESULTS AND DISCUSSION

3.3.1 Physical and chemical parameters

According to the results, several physical and chemical parameters had similar values upstream and downstream of the WWTP (Appendix K). The concentrations of dissolved oxygen (DO) and ammoniacal nitrogen were the most affected by the effluent discharged in the river. The mean concentration for DO upstream of the WWTP was $9.28 \pm 1.13 \text{ mg L}^{-1}$, while in the downstream samples it was $8.86 \pm 1.64 \text{ mg L}^{-1}$. Ammoniacal nitrogen concentrations were lower upstream, with a mean concentration of $0.06 \pm 0.08 \text{ mg L}^{-1}$, and higher downstream, with a mean concentration of $0.24 \pm 0.27 \text{ mg L}^{-1}$. Total nitrogen and nitrate had similar values both upstream and downstream, and the values did not vary during the sampling campaigns. The decrease of DO and the increase in nitrogen concentrations in surface water is a common effect of the discharge of WWTP effluent in rivers (Atashgahi et al., 2015; Santos et al., 2016).

Besides the influence of the WWTP, many parameters showed great variation throughout the sampling period, most likely due to seasonal changes. Turbidity, for example, was around 25 NTU in February and March and decreased to 2 NTU in June. The concentration of SS was also high in winter, reaching 38 mg L^{-1} in February, and decreasing to 1.23 mg L^{-1} in June. This difference is related to the decreasing river flow and the increasing abundance of macrophytes in the river starting in April. The macrophytes lower flow velocity, facilitating the sedimentation of

solid particles, and also act as a filter for SS. The DO concentration also decreased in the warmer months, going from 9.60 mg L⁻¹ in February to 6.10 mg L⁻¹ in June, due to the higher temperatures. Nitrate concentrations also decrease from February (6.9 mg L⁻¹) to June (3.5 mg L⁻¹), while orthophosphate had a peak concentration in June (0.2 mg L⁻¹). The other nutrients, both in water and sediment samples, did not show important variations during the analyzed months.

3.3.2 Quantification of facultative pathogenic bacteria gene markers in water, sediment, and biofilm

The mean occurrence of the eubacterial 16S rRNA gene throughout the sampling period was $4.81 \times 10^5 \pm 4.08 \times 10^5$ cell equivalents/100 mL in water, $3.77 \times 10^7 \pm 5.60 \times 10^7$ cell equivalents/g of sediment, and $2.83.77 \times 10^8 \pm 3.50 \times 10^8$ cell equivalents/ g of biofilm samples. The boxplots for the FPB quantified in the samples are depicted in Figure 3-1, Figure 3-2, and Figure 3-3. The detailed results can be found in the Appendix L.

In water samples (Figure 3-1), the abundance and diversity of species increased after the WWTP. There was already a background concentration for most analyzed species in the surface water of the river, most likely because of previous wastewater emissions upstream of the investigation site. Except from *gltA*, the marker of the bacterium *K. pneumoniae*, that was not detected in any of the samples collected upstream of the WWTP, but it was detected in three sampling campaigns in downstream water samples, with high abundances. For the genes detected both upstream and downstream of the WWTP, the mean concentrations of all taxonomic marker genes were higher after the effluent discharge. In previous studies, the increase in the abundances of FPB in the environment was found due to the discharge of WWTPs, even with advanced treatments, like ozonation (Brown et al., 2019; Jäger et al., 2018).

In sediment (Figure 3-2), only a few FPB were detected in the samples. The mean values for the gene markers *23S rRNA* (*enterococci*) and *16S rRNA* were similar before and after the WWTP. Other gene markers were detected occasionally, mainly in February and March (Table S4). The gene markers for *E. coli* and *K. pneumoniae* were detected only after the discharge of the effluent, but it is not possible to see a strong influence of the WWTP in the FPB detected in sediment samples. Usually, WWTPs with higher treatment capacities have a stronger influence on the presence of FPB in environmental samples. For example, in similar research in Germany, the abundances of three gene markers in sediment samples were strongly affected by the effluent

discharge, however, the size of the WWTP was much bigger, approximately 875'000 p.e. (Brown et al., 2019).

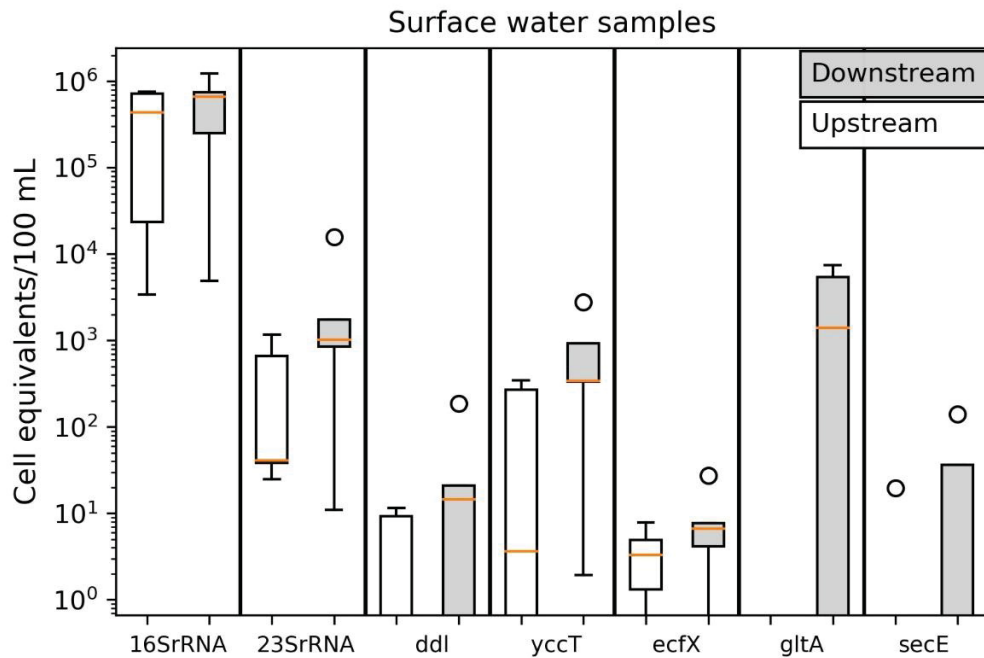


Figure 3-1: Absolute abundance of taxonomical gene markers in water samples from the Kraichbach River

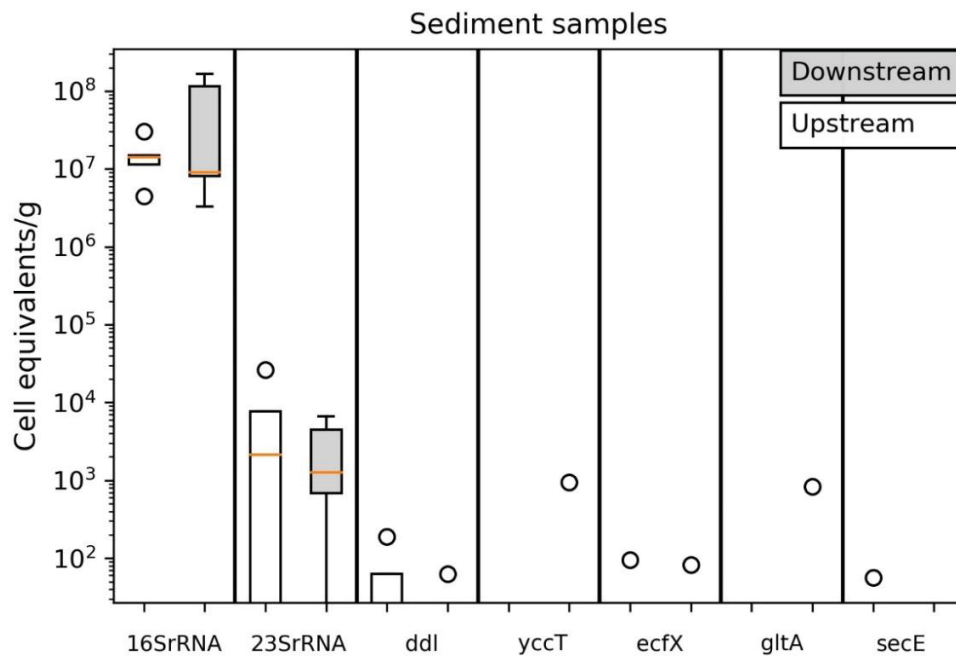


Figure 3-2: Absolute abundance of taxonomical gene markers in sediment samples from the Kraichbach River (values in dry weight)

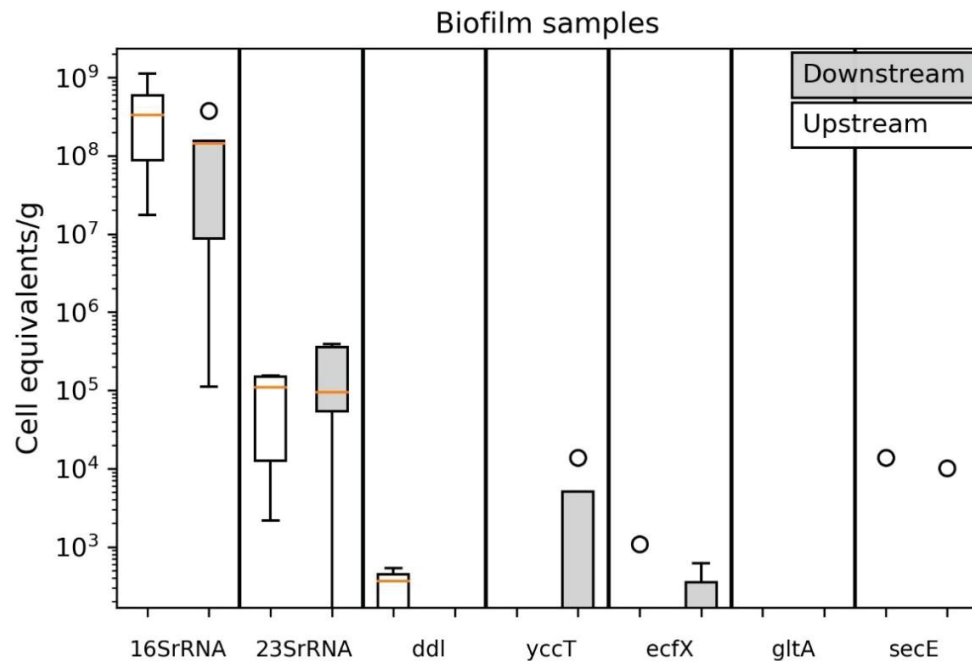


Figure 3-3: Absolute abundance of taxonomical gene markers in biofilm samples from the Kraichbach River (values in dry weight)

Similar to sediment, biofilms can also act as reservoirs and sources of important pathogenic species, and aquatic biofilms were already identified as important niches for pathogenic bacteria (Abraham, 2011). In biofilm samples (Figure 3-3) the detection frequency and abundances of FPB were higher than in sediment. Although the mean values of *23S rRNA* and *16S rRNA* were similar before and after the discharge of the WWTP, the occurrence of FPB was higher in samples collected downstream of the WWTP. The only FPB that was not detected above the limit of detection (LOD) in any biofilm sample was *K. pneumoniae* (*gltA*). *E. faecalis* (*ddl*) was detected in three samples upstream of the WWTP, and *E. coli* (*yccT*) was detected downstream of the WWTP only.

3.3.3 Quantification of ARG in water, sediment, and biofilm

The boxplots for the ARG quantified in the samples are depicted in Figure 3-4, Figure 3-5, and Figure 3-6. The detailed results can be found in the Appendix M.

Because of the intense use of some antibiotics, the resistance genes linked to them are usually detected more frequently (Hembach et al., 2019). This is the case of the *bla_{TEM}*, *ermB*, *tetM*, and *sulI* genes, encoding resistance to β -lactams, erythromycin, tetracycline, and sulfamethoxazole

(Hembach et al., 2019). These ARGs were detected in all the water, sediment, and biofilm samples analyzed (Figure 3-4, Figure 3-5, and Figure 3-6).

According to the results, the abundance of ARGs was higher in water samples collected downstream of the WWTP than the ones collected upstream of the WWTP (Figure 3-4). The highest concentrations detected in water samples were of *ermB* and *sulI*, with mean concentrations of 3.94×10^5 and 2.50×10^5 cell equivalents/100 mL in samples collected downstream of the effluent discharge. In previous work, *ermB* was very abundant in a German river, also reaching approximately 10^6 gene copies/100 mL in surface water samples (Brown et al., 2019). However, *ermB* is not always detected in other countries, but the gene *sulI* was frequently detected in China. In the Yangtze Estuary, *sulI* was detected with a concentration of up to 3.19×10^7 cell equivalents/100 mL (Guo et al., 2018) and in northeastern China, the concentrations of *sulI* reached 3×10^5 cell equivalents/100 mL (Lu et al., 2015).

The other ARGs were usually only detected in water samples collected downstream of the WWTP, while most of the concentrations detected upstream were below the LOD. Of these less frequent genes, *bla_{OXA-48}* was detected with higher concentrations, of up to 1.29×10^4 cell equivalents/ 100 mL in the Kraichbach River. In a recent study in Europe, *bla_{OXA-48}* was detected in surface waters with an approximate median of 5×10^4 cell equivalents/mL and was the 6th most abundant gene after genes like *sulI*, *tetM*, and *bla_{TEM}*, (Cacace et al., 2019). A very important ARG, *mcr-1*, was detected in two samples collected downstream of the effluent discharge. This gene was discovered recently in China and is linked to the resistance against a last resource antibiotic. It was already detected in WWTP effluents in Europe (Cacace et al., 2019; Hembach et al., 2017; Lekunberri et al., 2017) but is hardly detected in environmental samples (Yang et al., 2017).

In sediment samples, only the most frequent ARGs were detected above the LOD (Figure 3-5). The mean concentrations detected in samples collected upstream and downstream of the WWTP were very similar and the values did not have a great variation during the sampling period. Like in water samples, the highest concentrations were of *ermB* and *sulI*. The mean concentrations for *ermB* and *sulI* were 4.82×10^6 and 2.69×10^6 cell equivalents/g for both locations, while mean concentrations of *tetM*, and *bla_{TEM}*, were approximately 10^4 cell equivalents/g. In another river in Germany, impacted by a bigger WWTP, the concentrations of *tetM*, and *bla_{TEM}* were also similar, around 10^5 gene copies/g upstream and 10^6 gene copies/g downstream of the WWTP (Brown et al., 2019).

This pattern for *sul1* and other ARGs is comparable with recent studies. The authors believe urban rivers have background contamination and are already filled with ARGs related to commonly prescribed antibiotics. In this case, the discharge of effluents would not significantly influence the occurrence of ARGs (Cacace et al., 2019; Marti et al., 2013), mainly in sediment, which slowly responds to changes in the environment. Since the Kraichbach River has other effluent sources upstream of the studied section, as well as agricultural areas, these ARGs may be abundant in the whole basin, as the high detection frequencies and concentrations upstream of the WWTP suggest.

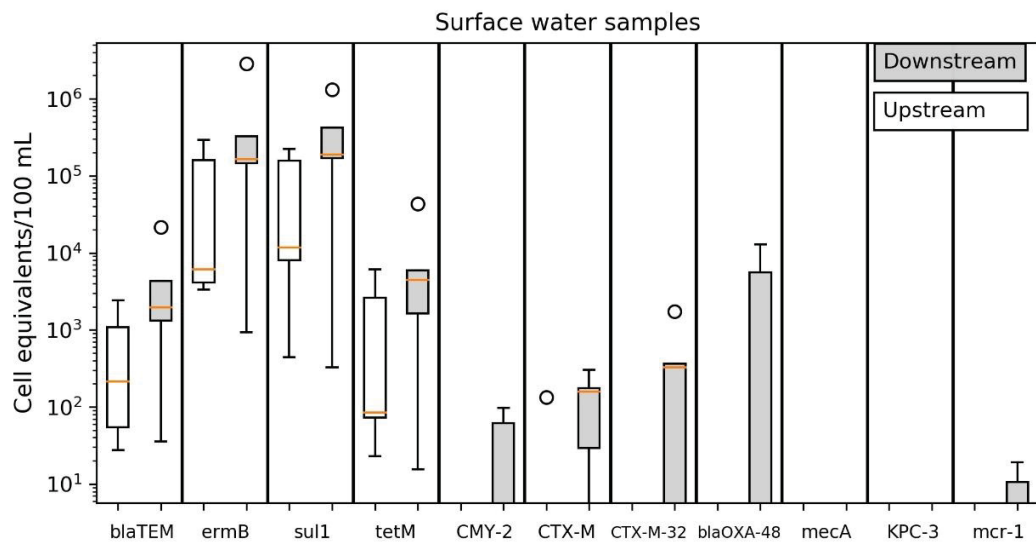


Figure 3-4: Absolute abundance of ARGs in water samples from the Kraichbach River

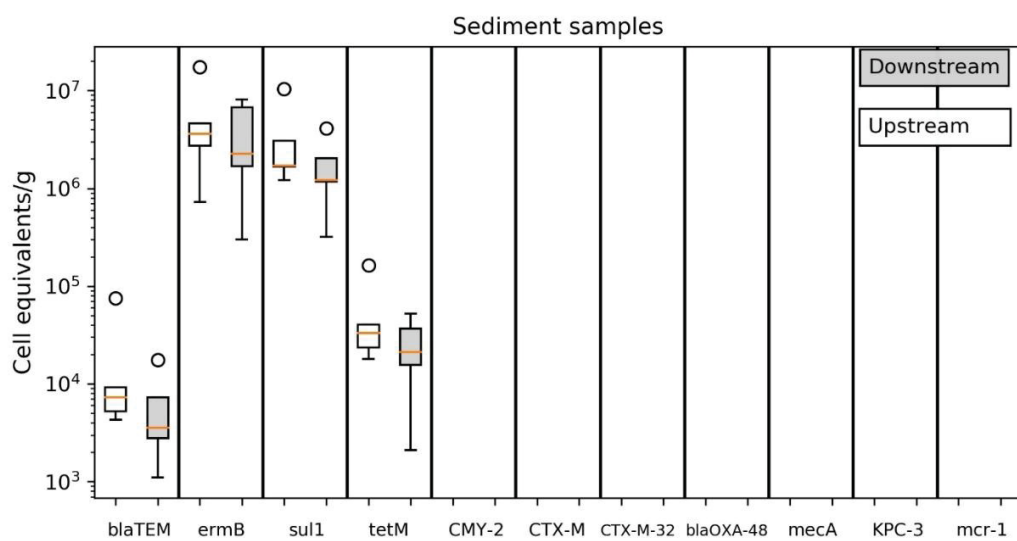


Figure 3-5: Absolute abundance of ARGs in sediment samples from the Kraichbach River

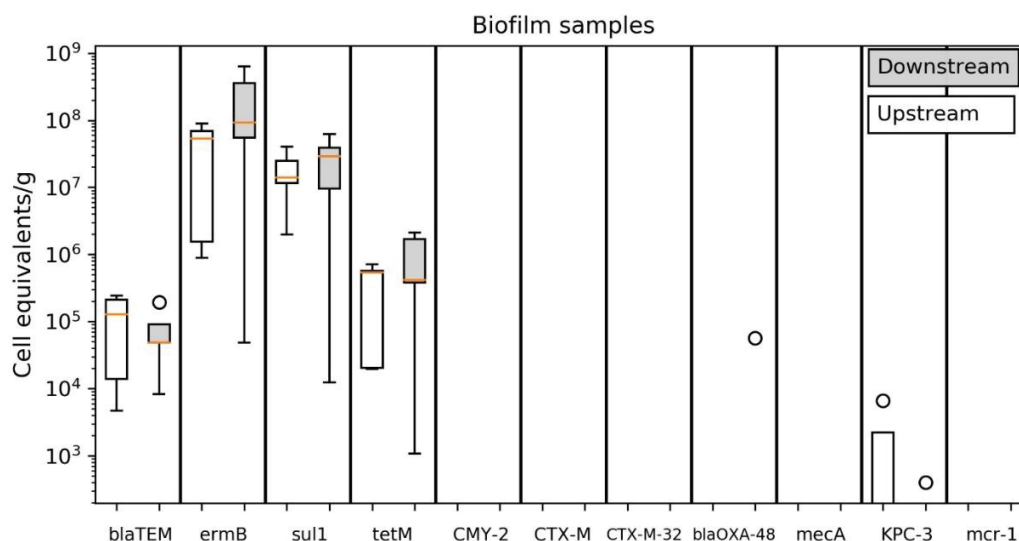


Figure 3-6: Absolute abundance of ARGs in biofilm samples from the Kraichbach River

The ARGs *ermB*, *sul1*, *tetM*, *blaTEM*, *blaOXA-48*, and *blaKPC-3* were detected in biofilm samples (Figure 3-6). Many of the other ARGs were detected in the samples, but the values were below the LOD. The abundances of the main four ARGs were higher in biofilm than in sediment samples, for at least one order of magnitude. Similar results were reported by Guo et al. (2018), that also found higher concentrations of *sul1* and other ARGs in biofilm than in sediment samples. The abundances of *blaOXA-48*, and *blaKPC-3* were lower, reaching 5.67×10^4 cell equivalents/g of *blaOXA-48* and 6.61×10^3 cell equivalents/g of *blaKPC-3*. These two genes were also analyzed in biofilm samples from two rivers in Spain, impacted by raw and treated sewage. The concentrations of *blaKPC-3* were approximately 10^5 gene copies/g, but *blaOXA-48* was not detected in any sample (Subirats et al., 2017a). Although the occurrence and abundance of ARGs may be different in biofilm samples from different locations, the results show that biofilms can be important disseminators of antibiotic resistance in urban rivers and they may play an important role in the spread of these genes in the environment because of their dispersion mechanisms and adaptability to other ecosystems.

3.3.4 Antimicrobial susceptibility test – Multi-drug resistance

Two sediment samples and two biofilm samples (one upstream and one downstream) were chosen to perform the antimicrobial susceptibility tests with cultivable isolates previously enriched from population communities. However, not all of the isolates presented bacterial growth in the last stage of the analysis (antibiotic application). The detailed results from the antimicrobial

susceptibility test, including inhibition zones and MRGN results, are displayed in the Appendix N, O and P.

The isolates chosen in the sediment sample collected upstream of the WWTP were classified as KEC (3 isolates), *E. coli* (3 isolates), and *Acinetobacter* (3 isolates, one did not present any bacterial growth). Many isolates were resistant to cephalosporins (either cefotaxime or ceftazidime, or both). However, some *E. coli* and *Acinetobacter* were resistant against colistin and fosfomycin. No isolate collected upstream of the WWTP was classified as 3MRGN or 4MRGN. Downstream of the WWTP, the isolates belonged to the species *P. aeruginosa* (5 isolates) and *Acinetobacter* (5 isolates, 3 did not present any bacterial growth). Two *P. aeruginosa* isolates were classified as 3MRGN, one was resistant to cephalosporins, meropenem and piperacillin and the other was resistant to cephalosporins, ciprofloxacin, and meropenem.

E. coli was the only specie in the biofilm sample collected upstream of the WWTP and only 3 isolates had bacterial growth. These 3 isolates were resistant to cefotaxime, ceftazidime, colistin, and fosfomycin. Downstream of the WWTP, *Acinetobacter* (3 isolates), and KEC (6 isolates) were analyzed. One of the KEC isolates in the biofilm sample collected downstream of the WWTP was classified as 3MRGN and was resistant to cefotaxime, ciprofloxacin, and piperacillin.

This was a qualitative test since not all of the samples and isolates were analyzed, but still, there was a clear impact of the WWTP on the dissemination of multi-resistant bacteria into the aquatic environment. 3 MRGN isolates were only detected downstream of the effluent discharge both in sediment and biofilm. The presence of multi-resistant bacteria is a potential health risk in case of human infection. Since biofilms can survive in almost every ecosystem and the microorganisms found in biofilms may be mobile, the ARGs could overpass the natural/anthropogenic frontiers. Therefore, the presence of antibiotic resistance and multi-drug resistant bacteria in stream biofilm can represent a threat to human and animal health.

3.3.5 Correlations of ARGs and FPB with physical and chemical parameters

The antimicrobial susceptibility test was not included in the statistical analysis because it was not quantitative and only four samples were analyzed. We performed the correlation analysis separately for the samples collected upstream and downstream of the WWTP since the results seemed to be different.

Many parameters in the studied river had a great variation along the year, such as turbidity, SS, water temperature, radiation, the presence and quantity of macrophytes, etc. During the winter, turbidity and SS reach their peaks in the Kraichbach River. We found that turbidity was strongly correlated ($r > 0.906$, $p < 0.034$) with several ARGs and FPB detected in water samples collected upstream of the effluent discharge (except for *CTX-m*, *16S rRNA* and *secE*). In these samples, it is possible to notice that the diversity and abundance of ARGs and FPB were higher at the beginning of the year (February, March), but there is a decreasing tendency until June. However, these correlations were not detected for the water samples collected downstream of the WWTP.

The concentration of SS also correlates with the ARGs *ermB*, *sul1*, *tetM*, *bla_{TEM}* ($r > 0.883$, $p < 0.047$) and the gene markers *23S rRNA* and *yccT* ($r > 0.880$, $p < 0.049$) detected in water samples upstream of the WWTP. Downstream of the WWTP, no correlation with SS in water samples was found, but significant correlations were detected for *ermB*, *16S rRNA* and *23S rRNA* collected from sediment ($r > 0.887$, $p < 0.045$). These positive correlations indicate turbidity and SS are possible causes of higher ARGs and FPB abundances during winter before the effluent discharge, but not after. A significant part of the riverine microbial community attaches to suspended solids, because of the organic matter fraction, a source of food for these organisms (Peduzzi and Luef, 2008). Also, higher turbidity and SS values prevent sunlight and radiation from penetrating the water column.

Significant negative correlations ($p < 0.05$) between UVA radiation, ARGs and FPB (*23S rRNA*, *ddl*, *yccT*, *ermB*, and *sul1*) in upstream water samples were also detected. These correlations also do not apply for samples collected downstream of the WWTP. UV radiation is known to decrease freshwater bacterial growth and production (Hörtl et al., 2011) and as the temperature (and UV radiation) gets higher closer to spring and summer, macrophytes grow on the bottom of the river, filtrating the water and decreasing the river flow. This can also help in the self-cleaning capacity of the river, which may act like a sedimentation basin, reducing the concentrations of suspended solids in water (Franklin et al., 2008).

Regarding nutrients, only ammoniacal nitrogen seems to influence the results. It correlated with some ARGs and FPBs in water samples (*23S rRNA*, *ddl*, *yccT*, *ermB*, and *sul1*, $r > 0.892$ $p > 0.041$) and biofilm samples (*bla_{TEM}* and *sul1*, $r > 0.934$ $p > 0.020$) collected upstream. Downstream of the WWTP, the influence was stronger and ammoniacal nitrogen had significant correlations with all the ARGs and FPB, except for *16S rRNA* and *bla_{CTX-M}*. Since ammoniacal nitrogen is an wastewater indicator in water resources (Ide et al., 2017), and the correlations were stronger after

the effluent discharge, the results suggest that the detection of ARGs and FPB are connected to effluent or sewage discharges into the river, mainly downstream of the WWTP. Higher bacterial and ARG diversity were also detected downstream of a WWTP in Spain (Marti et al., 2013), supporting the idea that the nutrients, bacteria and ARGs released by the WWTP have an important influence in the microbial community of streams and rivers (Brown et al., 2019; Cacace et al., 2019; Lapara et al., 2011).

Significant correlations happened more frequently in ARGs and FPB detected in water samples because the occurrence of the target genes was higher in this media. Also, the occurrence of ARGs and FPB in sediment was at the same level during the sampling campaigns, indicating the microbiota in this media is not influenced by external parameters. These results indicate that the occurrence of ARGs and FPB upstream and downstream of the WWTP, especially in surface water, is influenced by water quality, but in different ways. Parameters that changed according to seasons, like turbidity, SS, and UVA had stronger correlations with ARGs and FPB detected in samples collected upstream of the effluent discharge. In these samples, higher abundances of ARGs and FPB were detected during winter, when turbidity and SS values were also high, and the UVA radiation was low. This indicates that high turbidity and concentration of suspended solids, along with low sunlight and UVA radiation, can favor the presence and the spread of FPB and ARGs in surface water. Downstream of the WWTP, ammoniacal nitrogen, a parameter linked to water quality and the presence of domestic effluents, had higher correlations with the abundance of ARGs and FPB. This indicates that in this section of the river, seasonal parameters do not have a major impact in the microbiota, and that the input of effluent by the WWTP has a strong influence on the presence of ARGs and FPB.

3.4 CONCLUSION

Our results showed that the presence and abundance of ARGs and FPB in the Kraichbach River were influenced by water quality. The ARGs and FPB detected in water samples upstream of the WWTP were more susceptible to changes in the environment, and had higher correlations with parameters that showed seasonal variations, such as turbidity, suspended solids, and UVA radiation. Downstream of the WWTP, the concentration of ammoniacal nitrogen, and consequently the presence of effluent in the river, seemed to have a major role on the occurrence of ARGs and FPB. The genes detected in biofilm and sediment samples presented few significant correlations

with water quality or seasonal parameters, indicating water grab samples can be used to detect temporal changes, contamination points and sensitive areas.

The occurrence of ARGs in sediment was low and did not vary much during the sampling campaigns. One recommendation to improve and maximize the use of sediment as monitoring media for ARGs and FPBs is to collect only the upper layer of sediment (less than 1 cm), where bacteria usually are more abundant. Biofilm had higher abundances of the target genes when compared to sediment, indicating biofilm can act as a sink of ARGs and FPB in aquatic ecosystems. The occurrence of the target genes in sediment and biofilm samples were similar before and after the effluent discharge. However, multi-drug resistance was mainly detected in the isolates collected from the biofilm and sediment samples collected downstream of the WWTP, reinforcing the idea that WWTPs are hot spots for antibiotic resistance. Also, biofilm samples can be a more accurate representation of the presence of ARGs and FPB in the basin, since it is intrinsically connected to the microbiota in the river.

The biofilm sampler was a cheap and useful tool that allows collecting biofilm in rivers, streams, and even in artificial channels and waterways, where there are no cobbles or other submerged structures to collect biofilm from. It also acted as a passive sampler and helped to collect biofilm from pre-determined time frames and seasons along the year, integrating time, location, and water flow, giving a more complex response than water and sediment sampling. However, when planning biofilm monitoring campaigns in other areas, differences in temperature, sunlight, and nutrient availability should always be considered to determine the adequate minimum exposure time for biofilm to grow inside a river.

4 CHAPTER 3: BIOACCUMULATION OF ALKYLPHENOLS AND PLASTICIZERS IN BIOFILM: IMPACTS OF A SMALL WWTP IN SOUTHERN GERMANY

4.1 INTRODUCTION

Alkylphenols and plasticizers are compounds extensively consumed in the industry. Alkylphenols are organic phenolic compounds used in products such as detergents, dispersants, emulsifiers, paintings, personal care products, herbicides, and pesticides. They are also employed in the paper, leather, textile, and oil industries (Asimakopoulos et al., 2012; Cheng et al., 2017). Although alkylphenols comprise many compounds, 4-n-nonylphenol (4-NP) and 4-tert-octylphenol (4-OP) are the most used. Also, they are the major degradation products of other complex alkylphenols (Z. Wang et al., 2014) and were extensively used in Europe and in many other countries for decades.

Similarly, plasticizers were also widely commercialized in many countries. These substances promote flexibility in plastic materials and the most commonly used plasticizers are bisphenol A (BP-A) and di-2-ethylhexyl phthalate (DEHP). BP-A can be found in food cans, plastic bottles, water pipes, electronic equipment, automobiles, and even medical devices (Im and Löffler, 2016). DEHP can be found in medical devices, any soft and flexible plastic product, furniture, mattresses, floor tiles, and vinyl flooring (Dobrzyńska, 2016). Despite their frequent use in different products and industries, plasticizers and alkylphenols are considered endocrine disruptors (Bergé et al., 2014; Dévier et al., 2013).

In addition, plasticizers are not chemically bound to plastic, so they can easily be transferred to humans or the environment (J. Wang et al., 2014). Because of its properties, DEHP, 4-NP, and 4-OP are listed as priority pollutants by EPA (Environmental Public Agency – USA) and by EU (Directive 2013/39/EU). The use of DEHP and other phthalates was also limited in toys in the EU, USA, Japan, and Canada (EU/2005/84/EC, CPSIA 2008, HPA, 2010). BP-A was also banned from plastic infant feeding bottles in the EU due to concerns about its safety (Directive 2011/8/EC).

Several studies point that the main source of alkylphenols and plasticizers in the environment are domestic and industrial sewage. Despite the high concentrations detected in industrial sewage, the larger volume of domestic sewage in urban areas makes domestic effluent and wastewater treatment plants (WWTP) the main contributors to the contamination of urban rivers by alkylphenols and plasticizers (Bedoya-Ríos et al., 2017; Bergé et al., 2014; Huerta et al., 2016). These compounds were already detected in sediments and surface water in Argentina

(Babay et al., 2014), Colombia (Bedoya-Ríos et al., 2017), China (Li et al., 2017a; Luo et al., 2017), and Europe (Domínguez-Morueco et al., 2014; Salgueiro-González et al., 2015). However, there is a lack of studies in other matrices, such as in invertebrates, biota muscles and other tissues, and biofilms.

Biofilms are an assemblage of bacteria, algae, fungi, and other microorganisms (Pandey, 2017) that form a film covering submerged surfaces like rocks, leaves, and others. They are easily found in streams and rivers and have important roles in nutrients and organic matter availability (Beaulieu et al., 2011; Mulholland et al., 2008; Raymond et al., 2013). They are continuously exposed to the dynamic flow of the water, including other microorganisms and pollutants that are present in the area. For this reason, they are good biological indicators, since the exposure of biofilm to nutrients and pollutants may affect their structure and composition (Corcoll et al., 2015; Proia et al., 2013; Rosi-marshall et al., 2017; Sabater et al., 2007). Furthermore, they are able to retain and accumulate substances by concentrating compounds into the matrix or incorporating into microbial biomass, via adsorption, absorption, and diffusion (Battin et al., 2016; Flemming and Wingender, 2010; Fuchs et al., 1996).

The easy and fast growth of biofilm on any surface and the capacity to absorb substances give the possibility to use biofilm in biomonitoring. When aligned with other devices, such as samplers, the biofilm analysis could provide a better understanding of the ecosystem and integrate parameters such as the concentration of pollutants in the environment and the bioaccumulation during a certain period of time. Therefore, this paper aims to: (i) use the biofilm as a potential passive sampling media for pollutants in urban rivers and to assess the use of a biofilm sampler to collect biofilm from rivers, (ii) determine the concentrations of 4-n-nonylphenol (4-NP), 4-tert-octylphenol (4-OP), bisphenol A (BP-A), and di-2-ethylhexyl phthalate (DEHP) in biofilm, sediment, and water samples collected upstream and downstream of a small WWTP, and (iii) compare the concentrations found in the samples with the ones detected in the effluent of the WWTP and evaluate the capacity of each matrix to respond to the presence and inputs of pollutants.

4.2 EXPERIMENTAL DESIGN

4.2.1 Study area

The Kraichbach River is a tributary of the Rhine River, located in the Upper Rhine River basin in the Baden-Württemberg state, in southern Germany (Figure 4-1). In this area, the mean

temperature is 10°C and annual precipitation is between 700 and 900 mm/year. The river is approximately 60 kilometers long and has a basin of 388 km². The water has a low gradient and slow flow, because most of the course of the river was modified and rectified. The area is formed by loess sediments, which are aeolian sediments, formed by wind transportation and deposition in an environmental sink – a valley, for example. The loess has in its composition silt, clay, and sand, trapped by a layer of calcium carbonate. It is highly porous and susceptible to transport and erosion. The presence of calcium carbonate in surface water can increase the hardness, as well as the alkalinity and pH.

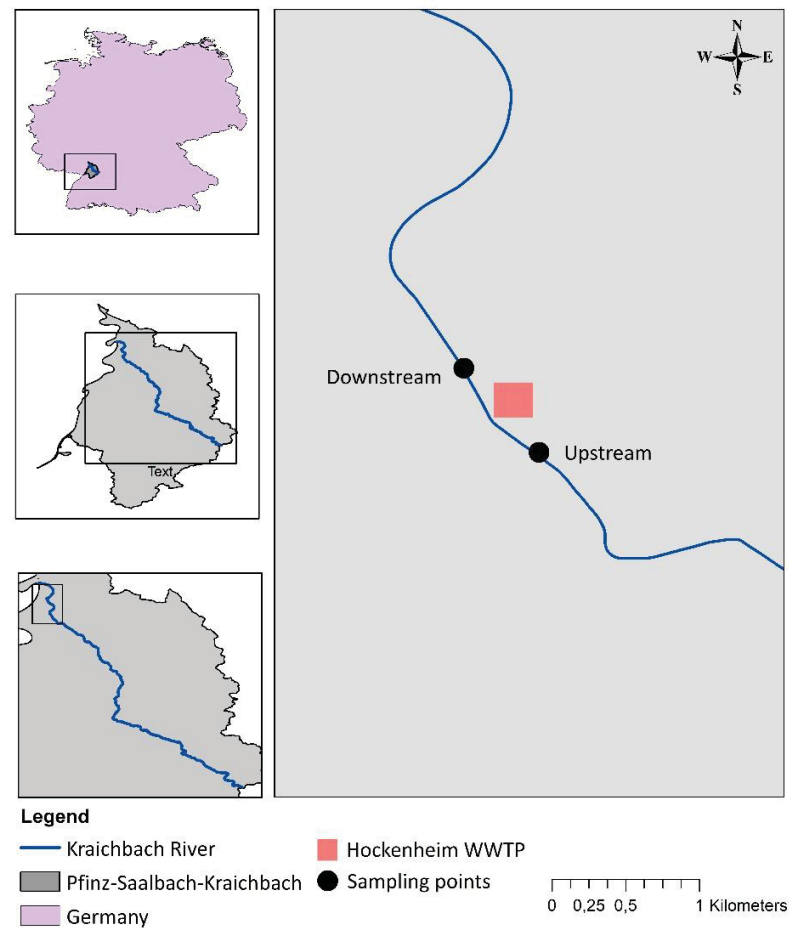


Figure 4-1 Study area – Kraichbach River and the Hockenheim WWTP

The Kraichbach River can be divided into two sections, the Upper Kraichbach and the Lower Kraichbach. From the source of the river until the beginning of the Oberrhein plain (lower areas close to the Rhein River) there is an altitude difference of 190 meters. In the other half of the

basin, inside the Oberrhein plain, the difference is only 19 vertical meters. Besides altitude, there are also differences in the land use of the Upper and Lower Kraichbach.

In the Upper Kraichbach, 49% of the area is agricultural land, 34% is forestry, 10% are classified as industrial and urban areas, and 7% is pasture. In the Lower Kraichbach, 35% of the area is agricultural land, 33% is forestry, 19% are urban and industrial areas, and 10% is pasture (LUBW). There are seven wastewater treatment plants (WWTP) in the Kraichbach River basin. The Kraichbach River is the effluent receptor of five of them, three in the Upper Kraichbach and two in the Lower Kraichbach. Also, there are several contributions from combined sewer overflows in the area (LUBW).

The intensive agricultural and urban land use, the wastewater treatment plants, and the combined sewer overflows lead to environmental and ecological problems in the area (LFU, 2004). For example, in the Upper Kraichbach, the estimated load of total nitrogen and total phosphorus is 525 tons/year and 38 tons/year, while in the Lower Kraichbach, the estimated loads are 442 tons/year and 15 tons/year, respectively. The main contributions of these nutrients are the WWTPs (LUBW, 2015).

This study focuses on the last WWTP located in the Lower Kraichbach. The river flow in this section is $1.22 \text{ m}^3\text{s}^{-1}$ and the velocity 0.44 m s^{-1} , approximately. The WWTP has a capacity of 55000 p.e. (population equivalent) and treats $2.917,883 \text{ m}^3/\text{year}$. The WWTP is part of a combined sewer system and receives both wastewater and rainwater runoff. It has primary and secondary treatments, with nitrogen and phosphorus removal. A previous study performed in the WWTP analyzed several contaminants in the effluent (Toshovsik et al, 2020). The samples were collected from November 2017 to November 2018, in dry and rainy weather, which were classified according to the WWTP inflow. During dry weather, the inflow of the WWTP was below $5000 \text{ m}^3 \text{ day}^{-1}$ and during rainy weather, the inflow was above $8500 \text{ m}^3 \text{ day}^{-1}$. The results of the WWTP monitoring are used for comparison with the results of this study.

4.2.2 Sampling collection

Six sampling campaigns were performed in the period of January/2019 to June/2019. The schedule with the dates and the name of the samples is presented in Table 4-1. The samples were collected upstream and downstream of the WWTP, to assess the influence of the effluent on the concentration of the pollutants. The names of the samples are formed by the kind of sample (W –

water, S-sediment, and B-biofilm), the sampling site (U – upstream, D – downstream), and the sampling date (day and month). For example, the water sample collected upstream of the WWTP in January is named W-U 2301.

Table 4-1 Schedule of sampling campaigns and collected samples

Date	Days*	Temperature**	Rainfall***	Water	Sediment	Biofilm
23/01/2019	40	3.6 °C	68.4 mm	W-U 23.01	S-U 23.01	B-U 23.01
				W-D 23.01	S-D 23.01	B-D 23.01
22/02/2019	30	3.2 °C	13.4 mm	W-U 22.02	S-U 22.02	B-U 22.02
				W-D 22.02	S-D 22.02	B-D 22.02
18/03/2019	25	8.5 °C	27.3 mm	W-U 18.03	S-U 18.03	B-U 18.03
				W-D 18.03	S-D 18.03	B-D 18.03
16/04/2019	29	8.2 °C	22.9 mm	W-U 16.04	S-U 16.04	B-U 16.04
				W-D 16.04	S-D 16.04	B-D 16.04
16/05/2019	30	11.4 °C	46.2 mm	W-U 16.05	S-U 16.05	B-U 16.05
				W-D 16.05	S-D 16.05	B-D 16.05
13/06/2019	28	16.25 °C	71.4 mm	W-U 13.06	S-U 13.06	B-U 13.06
				W-D 13.06	S-D 13.06	B-D 13.06

Days*: days between sampling campaigns, duration of the biofilm growth

Temperature**: mean air temperature for the growth period of the biofilm

Rainfall***: total accumulated rainfall for the growth period of the biofilm

Temperature and rainfall data were obtained from *Deutscher Wetterdienst*.

Sediment, water, and biofilm samples were collected once a month. A core sampler was used to collect the sediment samples. Only the first 5cm of the sediment core were analyzed, to assess the most recent accumulation of solid particles. The sediment samples were stored in glass jars until analysis. Water samples were collected in glass bottles (1.5 L for the alkylphenols and plasticizers).

Biofilm samples were collected from a biofilm sampler, developed for this purpose. The biofilm sampler consisted of a box that accommodated four flat glass plates (joined surface area of 1.68 m²). The glass plates were not treated or modified in any way (for example, to produce grooves on the glass surface, or to apply substances to promote the biofilm growth). They were only washed and decontaminated with organic solvents before the sampler was taken to the river. The box is closed but the front- and backsides remain open, so water can flow through, allowing the biofilm to attach and grow on both sides of the glass plates (Figure 4-2). The box was first submerged and

fixed to the riverbed with metal poles on the 14th of December of 2018. Previous experiments showed that in this area, during winter, the biofilm usually took 20-30 days to reach peak growth. Since the glass plates were completely clean, it would have taken a few more days until the microorganisms could attach to the surface and start to grow and multiply. Therefore, the first biofilm sample was collected on the 23rd of January of 2019, totalizing 40 days of maturation. After that, the samples were collected between 25-30 days of biofilm growth.

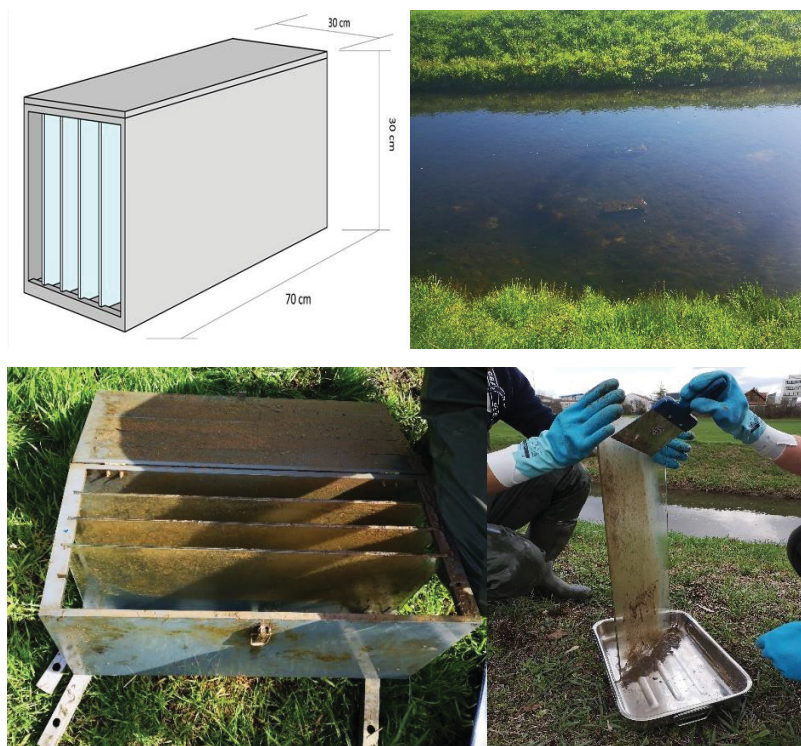


Figure 4-2 Sampling and collection of biofilm

In every sampling campaign, the box was removed from the river bed and the biofilm samples were collected by scraping the glass with a stainless steel spatula. The samples were stored in amber glass jars. Then, the box was fixed in the riverbed again, until the next sampling campaign. The jars and bottles where the water, sediment and biofilm samples were stored were previously washed and decontaminated with organic solvents, to avoid any contamination.

4.2.3 Physical, chemical, and chromatographic analyses

All the analyses performed in water, sediment, and biofilm are cited in

Table 4-2. In the water phase, the parameters pH, conductivity, temperature, and dissolved oxygen were analyzed in the field with a portable probe Multi 340i (WTW, Weilheim, Germany). Turbidity was analyzed when the samples arrived at the laboratory, with a portable turbidimeter (Hach, Loveland, United States). Suspended solids (SS) and loss on ignition (LOI) were also analyzed in the water samples. For these analyses, the samples were filtrated with glass fiber filters (0.45 μm), previously decontaminated. After filtration, the filters were dried (110°C) for 24 hours and weighed to determine the concentration of SS in the samples. After the determination of SS, the filters were burned at 550°C in a laboratory furnace and weighted again to determine the loss on ignition of the suspended solids in water. The weight loss of the suspended solids after burning is the LOI (in percentage). This parameter is usually correlated with organic carbon content (Hoogsteen et al., 2015). LOI was also analyzed in the sediment and biofilm samples, following the same procedure as described for the water samples (drying the solid samples at 110°C, weighting, burning the samples at 550°C and weighting again to determine the weight percentage loss).

Table 4-2 Analyzed parameters on water, sediment and biofilm

Parameters	Methodology	Water	Sediment	Biofilm
pH	Portable probe	X		
Conductivity	Portable probe	X		
Turbidity	Turbidimeter	X		
Temperature	Portable probe	X		
Dissolved Oxygen	Portable prove	X		
Loss on Ignition (LOI)	Ignition	X	X	X
Nitrate	TNTplus Nitrate 835*	X		
Ammoniacal Nitrogen	TNTplus Ammonia 830*	X		
Kjedahl Nitrogen	DIN-EN-25663**		X	
Total Nitrogen	TNTplus Total Nitrogen 826*	X		
Orthophosphate	TNTplus Phosphorus 843*	X		
Total Phosphorus	TNTplus Phosphorus 843*/ DEV-D11-7**	X	X	
Alkylphenols	GC-MS/MS	X	X	X
Plasticizers	GC-MS/MS	X	X	X

* Hach Lange Kits: <https://www.hach.com/tntplus&tab=QBS>

** Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung

In the water samples, orthophosphate, total phosphorus, ammoniacal nitrogen, nitrate, and total nitrogen were analyzed with ready-to-use cuvette tests and a DR2800 spectrophotometer (Hach Lange, Düsseldorf, Germany). In sediment, Kjeldahl nitrogen was analyzed according to the

standard DIN-EN-25663 methodology, with a digestion unit (Büchi, Lawil, Switzerland). Total phosphorus was also analyzed, according to the standard methodology DEV-D11-7. Both methodologies are from the *Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung*.

A partner institute called *Technologiezentrum Wasser* (TZW) analyzed the alkylphenols and DEHP. The biofilm and sediment samples were freeze-dried prior to analysis. Two analytical methodologies were employed to detect the contaminants. BP-A, 4-NP, and 4-OP were analyzed by gas chromatography/ tandem mass spectrometry (GC/MS-MS) after solid-phase extraction and derivatization. The analytes were extracted to a nonpolar phase, eluted with acetone, and evaporated to dryness. The samples were derivatized with MSTFA, reconstituted with cyclohexane/MSTFA, and analyzed with a Trace GC Ultra system with a TSQ Quantum XLS ultra mass spectrometer. DEHP was analyzed by gas chromatography/ mass spectrometry (GC/MS) after liquid-liquid extraction with cyclohexane. The cyclohexane phase is concentrated and analyzed with a GC Autosystem XL a with TurboMass mass spectrometer (PerkinElmer, Massachusetts, United States).

4.2.4 Statistical analysis

Correlation analysis was performed with Statistica 10.0 software (StatSoft. Inc., Tulsa, USA).

4.3 RESULTS

4.3.1 Physical and chemical analyses

The complete table with the physical and chemical parameters for the two sampling points can be found in Appendix K. Many of the analyzed parameters had similar values upstream and downstream of the WWTP, which indicated a weak influence of the effluent discharge for these parameters. In water samples, conductivity, dissolved oxygen (DO), suspended solids (SS) and loss on ignition (LOI) showed more clearly the influence of the WWTP effluent. The conductivity values were slightly higher downstream of the WWTP, indicating a higher concentration of ions in the water. The increase in the conductivity could be caused by the high number and quantity of chemical compounds released with the effluent.

The decrease of DO concentration is a normal effect of the discharge of WWTP effluent in rivers (Atashgahi et al., 2015; Santos et al., 2016). In the Kraichbach River, the DO concentrations were lower downstream of the WWTP, however, it was not a significant difference. The mean concentration of DO in the sampling point upstream of the WWTP was 9.28 mg L^{-1} and after the discharge was 8.86 mg L^{-1} . The main difference in the DO levels were due to the water temperature. While in January the DO concentration was above 10 mg L^{-1} in both sampling sites, in June the concentrations were 7.39 mg L^{-1} and 6.10 mg L^{-1} upstream and downstream of the WWTP, respectively.

Similar results were obtained for the SS concentrations. The Kraichbach River has a very high SS concentration during the winter, reaching 71.25 mg L^{-1} of SS in January, but a very low concentration during spring, around 2 mg L^{-1} of SS. The values were lower downstream of the WWTP, mainly in winter. The results suggest that the effluent of the WWTP may cause the dilution and the decrease of this parameter during this period, causing a lower SS concentration downstream of the discharge.

The LOI analysis is an indirect method to determine the organic matter content of a solid sample, such as soil, sediment, or SS (Hoogsteen et al., 2015). For water samples, we measured LOI using the SS collected from the samples. The LOI values detected were similar upstream and downstream during all the sampling period, except for June, where LOI was 50% and 66.6% upstream and downstream, respectively. Also, there is an increase tendency of the organic matter content in the warmer months (May and June).

The nutrients values in water samples (Appendix K) had a similar pattern to the other physicochemical parameters. According to the detected values, the mean concentrations were very similar between upstream and downstream samples. This was expected, once the WWTP has nitrogen and phosphorus removal. The only exception was the ammonium concentration. While the concentrations upstream of the WWTP reached 0.23 mg L^{-1} of ammonium, the downstream concentration reached 0.77 mg L^{-1} . These values indicated that the WWTP may be releasing amounts of ammonium in the effluent. The reason may be the combined sewer overflows, which are collected by the WWTP, but may not be treated if the flow exceeds the WWTP capacity.

The physical and chemical parameters analyzed in sediment were LOI, total phosphorus (TP) and Kjeldahl Nitrogen (TKN), which is the sum of organic nitrogen and ammoniacal nitrogen (NH_3 and NH_4^+). The complete values can be visualized in Appendix K. The values of LOI, TP,

and TKN were slightly higher in the downstream samples than upstream of the WWTP, showing that the sediment has the capacity to absorb part of the organic matter and the nutrients released during the discharge of the effluent. The results of TKN presented the biggest difference in the values upstream and downstream. The mean values had a difference of almost 200 mg kg^{-1} of TKN. No seasonal trends were identified for the physical and chemical parameters in sediment.

Due to the low amount of sample collected, only LOI was analyzed in biofilm. The results can be seen in Appendix K. Downstream of the WWTP, the percentage of organic content was higher than in the samples collected upstream of the effluent discharge. The higher LOI values indicate that the effluent may influence in the organic content of the biofilm.

4.3.2 Concentrations of DEHP, BP-A, 4-NP, and 4-OP detected in the samples

From November 2017 to November 2018, the effluent of Hockenheim WWTP was analyzed to evaluate the presence of several pollutants. Among these pollutants were DEHP, 4-OP, 4-NP, and BP-A. In total, effluent samples were collected 24 times, both in dry weather (12 samples) and in rainy weather (12 samples). The differentiation is important because in this area in Germany the sewage collection system is combined and designed to collect rainwater runoff. Therefore, during heavy rain events, the volume of rainwater could dilute some pollutants, while increasing the concentrations of others. The mean concentrations detected in the effluent of Hockenheim WWTP are depicted in Figure 4-3.

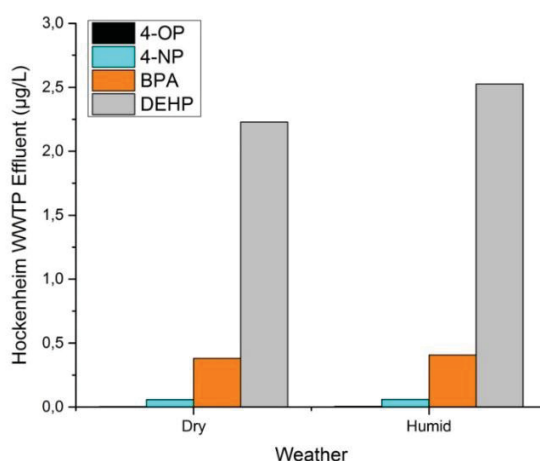


Figure 4-3: Mean concentrations for target compounds detected in the effluent of Hockenheim WWTP in rainy and humid weather. 4-OP: 4-tert-octylphenol, 4-NP: 4-n-nonylphenol, BP-A: bisphenol A, DEHP: di-2-ethylhexyl phthalate.

According to the results, the rain events did not change the concentration of the target compounds in the effluent of the WWTP. Also, the results showed a very interesting concentration pattern in the effluent. DEHP was detected with the highest concentrations, with mean concentrations around $2.5 \mu\text{g L}^{-1}$, while the other compounds were detected in lower concentrations. The mean concentrations of BP-A and 4-NP were lower than $0.5 \mu\text{g L}^{-1}$ and $0.1 \mu\text{g L}^{-1}$, respectively. DEHP, BP-A, and 4-NP were detected in all 24 samples, but 4-OP was detected above the quantification limit in only 5 out of 24 samples (3 in rainy weather and 2 in dry weather), always in very low concentrations, around $0.02\text{-}0.03 \mu\text{g L}^{-1}$.

The concentrations of BP-A and DEHP found in the effluent of the Hockenheim WWTP are very similar to the ones detected by Tran et al. (2015) in a small WWTP in France. The mean concentrations detected for BP-A and DEHP in the effluent were 0.4 and $2 \mu\text{g L}^{-1}$, respectively. The occurrence and concentrations of DEHP detected in WWTPs are reported to be high, despite the efficient removal of this compound by different types of treatment. The main removal pathways for DEHP are biodegradation and absorption, due to the high partition coefficient of this compound ($\log K_{ow}=7.60$) (Gao and Wen, 2016). In the USA, BP-A, 4-NP, and 4-OP were analyzed in several WWTPs and the mean concentrations detected were 0.46 , 1.4 , and $0.69 \mu\text{g L}^{-1}$ (Vidal-Dorsch et al., 2012). BP-A and 4-OP were detected in 100% of the samples, while 4-NP was detected in 94% of the samples, but in higher concentrations. The results suggest, both from the USA and from the Hockenheim WWTP, that 4-NP is released in higher quantities than 4-OP.

The data about these contaminants in the effluent of the Hockenheim WWTP is valuable information for monitoring in natural ecosystems. Emerging pollutants are usually not continuously monitored inside a WWTP, mainly because there are few laws about the release of these substances in the environment. Even when they are, frequently for research purposes, only a few samples are taken. Therefore, the opportunity to compare environmental concentrations with the WWTP emissions from a long monitoring period is unique.

In Figure 4-4 A, the detected concentrations in the water samples upstream and downstream of the WWTP can be visualized. The only compound detected in all water samples was BP-A. The mean concentrations detected upstream and downstream of the WWTP were 0.032 and $0.051 \mu\text{g L}^{-1}$. The concentrations were higher in the samples downstream of the WWTP, as expected. Also, the $\log K_{ow}$ of BP-A is 3.32 , the lowest $\log K_{ow}$ of the target compounds, meaning that is the least hydrophobic and more likely to be detected in water samples.

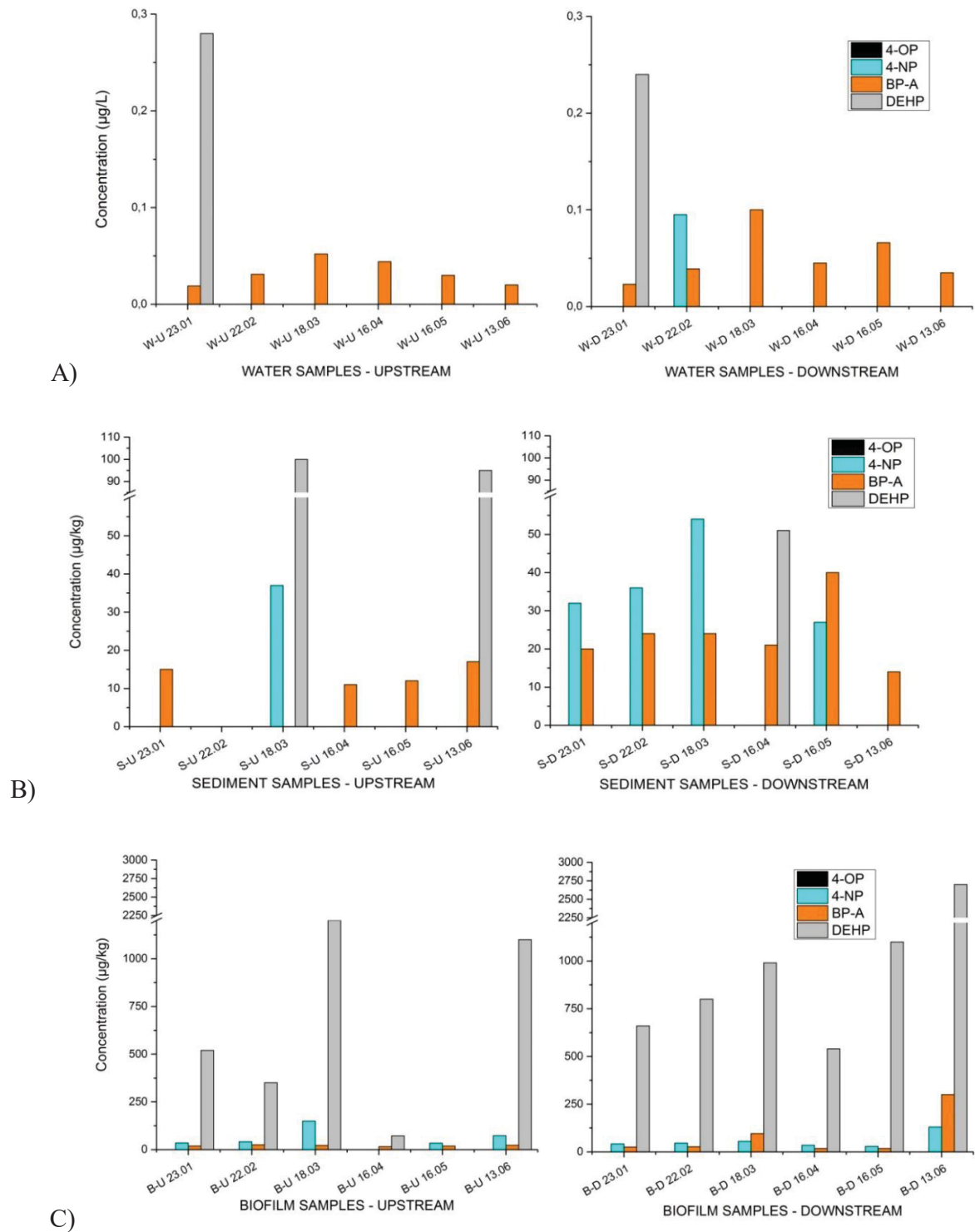


Figure 4-4: Concentration of target compounds in A) water, B) sediment and C) biofilm samples collected upstream and downstream of Hockenheim WWTP. 4-OP: 4-tert-octylphenol, 4-NP: 4-n-nonylphenol, BP-A: bisphenol A, DEHP: di-2-ethylhexyl phthalate. W: water, S: sediment, B: biofilm, U: upstream, D: downstream

DEHP was detected only in the samples collected in January 2019, at the beginning of the monitoring period. In this case, the concentration detected upstream and downstream was 0.28 and 0.24 $\mu\text{g L}^{-1}$, respectively. Since DEHP has a high log Kow of 7.6, it is not expected to find this compound in the surface water samples, but adsorbed to organic matter. The alkylphenol 4-NP was detected only in one sample, collected downstream of the WWTP in February, with a concentration of 0.095 $\mu\text{g L}^{-1}$, while 4-OP was not detected in any water sample. In a comparing with the results from the effluent of Hockenheim WWTP (4-OP was only detected in 5 out of 24 samples), it was expected that the concentrations would be very low or even that 4-OP would not be detected at all in water samples.

In France, the concentrations of BP-A and DEHP in a small river (less than 0.2 $\text{m}^3 \text{s}^{-1}$) impacted by the effluent of a small WWTP were analyzed. Upstream of the discharge the reported concentrations of BP-A and DEHP varied from 0.002-0.175 $\mu\text{g L}^{-1}$ and 0.16-0.90 $\mu\text{g L}^{-1}$, respectively. Downstream of the WWTP, they ranged from 0.11-0.79 $\mu\text{g L}^{-1}$ and 0.31-1.7 $\mu\text{g L}^{-1}$, respectively (Tran et al., 2015). The concentrations reported were higher than in the Kraichbach River, as well as the increase of concentration levels from the upstream to the downstream sites. This may happen because of the low flow of the studied river.

In China, 4-OP, 4-NP, and BP-A were analyzed in the tributaries of the Pearl River, which is located in a highly urbanized area, with more than 14 million inhabitants. The concentrations of 4-OP, 4-NP, and BP-A were up to 0.3, 1.2, and 1.4 $\mu\text{g L}^{-1}$, respectively. In summer, concentrations were usually higher (Gong et al., 2016). In this case, concentrations were also higher than the ones detected in the Kraichbach River, but the not proportional to the population difference.

The concentrations detected in the sediment samples are depicted in Figure 4-4 B. According to the results, the concentrations and frequency detection of BP-A and 4-NP were higher in the samples collected downstream of the WWTP than in samples collected upstream. In sediment, the concentrations of 4-NP were usually higher than of BP-A, a different result from the analyzed water samples. This is probably due to the higher log Kow of 4-NP, indicating a tendency of adsorbing to solid particles and organic matter. 4-OP was not detected in any sediment sample, as expected according to the lack of presence of this pollutant in the water samples and the low concentrations and detection frequency in the effluent of the WWTP. DEHP was only detected in two samples upstream and one sample downstream of the WWTP, but reached a concentration of 100 $\mu\text{g kg}^{-1}$, higher than the other pollutants. Also, the concentrations of DEHP were higher

upstream than downstream of the WWTP. This may indicate background DEHP pollution in the Kraichbach River. There are other WWTPs and several combined sewer overflows (CSO) upstream the sampling points that could cause this kind of influence. Another possibility is that since DEHP tends to attach to solid particles and organic matter, as indicated by its log Kow, after its release by the WWTP, it may take longer to settle than the distance to the downstream sampling point for sediment.

There are a few studies where both water and sediment samples were analyzed for these compounds. In a research performed in the Minho River, in Spain, 4-OP and 4-NP were detected in all the surface water samples collected (n=13), ranging from 0.018-0.088 and 0.033-1.03 $\mu\text{g L}^{-1}$. BP-A was detected in only three samples, but with concentrations of up to 4.8 $\mu\text{g L}^{-1}$. In sediment, 4-OP and 4-NP were also detected more frequently than BP-A. Their concentrations ranged from 9.3-74.5, 21-4460 $\mu\text{g kg}^{-1}$, and 4.3-130 $\mu\text{g kg}^{-1}$, respectively (Salgueiro-González et al., 2015).

In China, water and sediment samples from Taihu Lakes and two tributaries were analyzed for 4-NP, 4-OP, and BP-A. In water, the compounds were detected in all samples, with mean concentrations of 0.057, 0.104, 0.092 $\mu\text{g L}^{-1}$. In sediment, 4-OP was the most detected, followed by 4-NP with mean concentrations of 38.18, and 20.66 $\mu\text{g kg}^{-1}$. BP-A was rarely detected in sediment, but it reached a concentration of 99.20 $\mu\text{g kg}^{-1}$ (Liu et al., 2016). In the Jiulong River, also in China, DEHP concentrations ranged from 0.79 to 10.9 $\mu\text{g L}^{-1}$ in water samples, while in sediment they ranged from 7 to 1280 $\mu\text{g kg}^{-1}$ (Li et al., 2017b). In the Panlong River, in China, 4-NP, 4-OP, and BP-A were analyzed in water samples and the concentration ranged from 0.003-0.017, 0.0019-0.0046, and 0.012-0.079 $\mu\text{g L}^{-1}$, while in sediment they ranged from 0.3-18, ND-14, and 6.6-125 $\mu\text{g kg}^{-1}$, respectively (Wang et al., 2016). We can see in all of the previous cited studies and in the present study that the concentration levels of the compounds were higher in the sediment than in the water samples. This is explained by the physicochemical properties of these compounds, mainly the log Kow, which indicates they tend to be absorbed by solid particles, such as suspended particulate matter and organic matter. However, the concentrations found in sediment were usually lower in the Kraichbach River.

While the maximum concentration of DEHP was 100 $\mu\text{g kg}^{-1}$ of sediment, in biofilm there was a much higher accumulation (Figure 4-4 C). The DEHP concentrations reached 2700 $\mu\text{g kg}^{-1}$ and the BP-A concentrations reached 300 $\mu\text{g kg}^{-1}$, all downstream of the WWTP. According to the results, the concentrations of BP-A, 4-NP, and DEHP are usually higher downstream of the

WWTP. 4-OP was not detected, as expected by the low concentrations detected in the effluent and the lack of this contaminant in water and sediment.

There are very few studies that detected these compounds in biofilm. Huerta et al. (2016) analyzed BP-A in biofilm, among other substances, but it was not detected. However, the sorption of 4-NP, BPA, and DEHP in natural biofilms was previously studied (Wang et al., 2019; Writer et al., 2011). With bench experiments, these authors concluded that the sorption of these compounds is fast and the biofilm has the ability to uptake a great amount of these compounds. Also, the sorption capacity was correlated with the organic matter content of the biofilm, which can explain why the biofilms collected downstream of the WWTP had higher concentrations of DEHP, which has a high coefficient partition ($\log K_{ow} = 7.60$). These studies also indicated that the sorption capacity of the biofilm is greater than of sediment (Wang et al., 2019; Writer et al., 2011), which agrees with the results of the present study.

Also, analyzing the data obtained from the effluent of the WWTP, the biofilm samples were the ones that better showed the WWTP influence in the Kraichbach River. The concentration pattern detected in biofilm samples collected downstream of the WWTP was very similar to the concentration pattern detected in the effluent (Figure 4-3). DEHP was detected with a very low frequency in the other matrices, water, and sediment, even being the main pollutant released by the WWTP, but it was the most detected contaminant in biofilm. Downstream of the WWTP, BP-A was the second most detected contaminant, both similar patterns from the effluent.

Because of the sorption capacity of the biofilm, there are concerns about bioaccumulation. For example, in the Minho River in Spain, BP-A, 4-NP, and 4-OP were analyzed in fish samples, water and sediment samples. 4-NP was the most detected compound in the biota samples, despite not being the most detected compound in water and sediment, indicating a high possibility of bioaccumulation (Salgueiro-González et al., 2015). The results in the present study also show that the possibility of bioaccumulation of these contaminants is high since the biofilm has a key role in the food web and is a food source for several micro- and macroinvertebrates species.

4.3.3 Correlation analysis

The correlation analysis was performed between the concentrations of DEHP, BP-A, 4-NP, 4-OP found in the samples, physical and chemical parameters, nutrients and meteorological parameters. Meteorological parameters included air temperature, and precipitation. Besides the

total rainfall for each period of biofilm growth, we calculated the correlation for the accumulated rainfall for the last 2, 5, 10, 15 and 20 days, prior to the biofilm sampling. The goal was to evaluate if recent rainfall would influence in the accumulation of the studied pollutants in biofilm. We performed the correlation analysis separately for the upstream and downstream samples, to verify if there was any difference.

Upstream of the WWTP, DEHP and 4-NP were intrinsically correlated. Their concentrations detected in biofilm had a significant correlation ($r = 0.85$, $p = 0.029$). In addition, the quantity of 4-NP in biofilm correlated with the concentrations of 4-NP and DEHP found in sediment ($r > 0.85$, $p < 0.031$). The concentrations of DEHP in biofilm also correlated with the ones found in sediment ($r = 0.92$, $p = 0.007$). Although in many of the sediment samples these pollutants were not detected, they were present in the sediment when the biofilm had high concentrations as well. This may indicate that these pollutants are accumulating in these matrices, since no correlations were found in the water. Regarding nutrients, the only significant correlation in the samples collected upstream of the WWTP was total phosphorus (TP) detected in sediment, that had a positive correlation with DEHP, also detected in sediment ($r = 0.88$, $p = 0.019$). For meteorological parameters, only the concentrations of BP-A and DEHP detected in sediment had correlations with precipitation. While DEHP correlated with the precipitation that happened 5 days before the biofilm sampling ($r = 0.83$, $p = 0.039$), BP-A correlated with the total precipitation ($r = 0.83$, $p = 0.037$). These correlations may indicate that rainfall influences in the transport and accumulation of these pollutants in the studied area. No correlations with the temperature were found.

Downstream of the WWTP, 4-NP, BP-A and DEHP detected in biofilm samples presented high positive correlations ($r > 0.83$, $p < 0.04$) with the LOI analyzed in the SS present in the water samples. They also strongly correlated with the orthophosphate concentrations in water samples ($r > 0.94$, $p < 0.005$). Also, DEHP in biofilm had a significant negative correlation with DO ($r = -0.84$, $p = 0.034$). The pollutants concentrations found in biofilm also strongly correlated with each other ($r > 0.93$, $p < 0.006$). This may indicate an accumulation of these pollutants in the biofilm along time. In water samples we did not find any significant correlation, since BP-A was the only pollutant detected frequently above the LQ. In sediment, 4-NP correlated with TP and TKN detected in sediment ($r > 0.92$, $p < 0.008$). Regarding meteorological parameters, the precipitation that occurred 2 days prior to the biofilm collection correlated with the concentrations of 4-NP and

BP-A in biofilm ($r > 0.8$, $p < 0.04$). However, when analyzing the precipitation that happened 5 days prior to the sampling, the correlations were higher and included the DEHP ($r > 0.90$, $p < 0.013$). This indicates that rainfall have an important role in the transport of these pollutants into to Kraichbach river. This influence is even stronger after the discharge of the WWTP, probably because of the combined sewer overflows that are collected by the WWTP, when the effluent flow increases.

4.3.4 Biofilm growth, hydrodynamic, and other influencing parameters

One of the concerns of collecting biofilm in natural streams is the possibility of biofilm detachment, which can happen due to active or passive dispersion. Active dispersion can happen when microorganisms reach their maturity peak and choose to leave the colony, either to live in the free form or to colonize other surfaces. This could be influenced by temperature, nutrient availability, oxygen and other factors. Passive dispersion is when the microorganisms are detached from the surface, a process called sloughing or erosion, due to external forces and physical factors, such as shear forces (Fang et al., 2017; Toyofuku et al., 2016).

Shear forces depend on the viscosity of the fluid and its velocity. When the fluid velocity is high, so are the forces imposed on a surface. When these forces are strong enough, they can cause the detachment of biofilm from the surface or from the inner layers of biofilm. This is why shear forces and hydrodynamics are so important for biofilm studies. These forces can influence not only biofilm detachment but also the initial attachment, growth, density, porosity, thickness, nutrient and oxygen dispersion inside the biofilm, etc. (Allen et al., 2018; Paul et al., 2012).

However, the study of the hydrodynamic effects on biofilms in natural ecosystems is challenging, since some parameters are difficult to measure and control, such as water flow and velocity, shear stress and nutrient availability. Because of this, many researchers focus their efforts in laboratory experiments. Paul et al., (2012) performed an experiment with reactors in low and high shear stresses, varying from 0.3 to 37 Pa. The researchers observed that detachment happened in all ranges of shear stress, but the remaining biofilm dry mass on the studied surface was lower when shear stress was higher. However, the detachment was limited to the superficial layers of biofilm. A process of compaction happened in the inner layers, increasing biofilm density and decreasing porosity and biofilm thickness. Nevertheless, even when high shear stress (above 9 Pa)

was applied, still a thin layer of biofilm could be detected on the studied substrates, and the biofilm was able to initially attach to the substrates with shear stress of up to 37 Pa.

Thomen et al. (2017) also studied biofilm initial attachment under different conditions in artificial channels. They discovered that when flow velocity is high, biofilm starts to grow near the edges of the channel, where shear stress is slightly lower. Then, the biofilm starts to grow from this initial settlement, advancing on the channel and eventually covering the entire surface.

Usually we can see the formation of biofilm even in very turbulent rivers, which shows, in agreement with the studies mentioned, that the structure of the biofilm can adapt to changes in flow velocity and shear stress, as well as in nutrient and oxygen availability. Also, the flow velocity of the studied section is low, approximately 0.44 m s^{-1} . During the warm months, this velocity can be even lower when macrophytes grow and the water becomes almost static.

Another parameter that usually influences biofilm growth is temperature. Usually, when the temperature is higher, inside an acceptable environmental range, biofilm grows faster and the biofilm density is higher (Toyofuku et al., 2016; Villanueva et al., 2011). From our observations, temperature was not a relevant factor for biofilm growth, since in the periods with low temperatures we were able to collect more biofilm (Figure 4-5). Nutrient availability was also not a contributing factor, once the concentrations remained stable throughout the sampling period.



Figure 4-5: Amount of biofilm collected from one of the glass plates in January (left) and May (right)

During the sampling campaigns, we noticed that the biofilm biomass was proportional to the concentration of suspended solids and turbidity, which could indicate a possible absorption and incorporation of these particles. We noticed that in January and February, during the winter, the turbidity and concentration of suspended solids were higher, and so was the amount of biofilm

collected, even with higher accumulation of rainfall. However, even when suspended solids were almost absent, the biofilm covered the glass and the amount collected was still enough to perform the analyses.

Biofilm growth and detaching can be influenced by many parameters that are difficult to control in the natural environment. The water flow and velocity were low in the Kraichbach River, as well as the intensity of rainfalls, which could have had low impact on biofilm erosion and sloughing. In fact, as mentioned before, rainfalls could have increased the concentrations of the pollutants in the biofilm. We believe that the loss of biomass due to sloughing or erosion can be considered negligible for this case. Nevertheless, in rivers with higher water flow and intense rain events, the biofilm growth and sampling could be impacted.

4.4 CONCLUSION

The results showed that the WWTP influenced the presence of nutrients, organic matter, and the studied contaminants. However, there is already background contamination, probably from other WWTPs and non-point sources upstream of the river. The concentrations detected in the biofilm were higher than in sediment and water, showing a higher sorption potential of the studied contaminants. The concentration patterns on biofilm were very similar to the ones detected in the effluent of the WWTP, with higher concentrations of DEHP, followed by BP-A and 4-NP. Indicating that biofilm may better represent the pollution status of the river. The results also demonstrate that rainfall can have an influence in the transport and contamination of the studied pollutants in the Kraichbach River. Therefore, we consider that the biofilm sampler was effective and the biofilm collected was a suitable and reliable matrix to monitor the substances analyzed in this research.

5 CHAPTER 4: BIOACCUMULATION OF EMERGING CONTAMINANTS IN BIOFILM COLLECTED FROM A SUB-TROPICAL URBAN RIVER IN BRAZIL

5.1 INTRODUCTION

Emerging contaminants are substances present in the environment that were recently detected or identified as pollutants. They comprise a group of compounds that belong to several classes: pharmaceuticals, personal care products, fragrances, UV filters, plasticizers, hormones, perfluorinated compounds, drugs of abuse, flame-retardants, antimicrobial agents, etc. (Agüera et al., 2013). They are frequently consumed on a daily basis by the population, and are employed in industry, livestock farming, veterinary, and medicine.

Their main source in the environment is effluents, easily reaching soil and water (Jiang et al., 2013; Yang et al., 2012). The concentrations detected in raw sewage, sludge, and the effluent of wastewater treatment plants (WWTP) are usually high (Martín et al., 2012), since most of the treatments do not efficiently remove these compounds from sewage (Basile et al., 2011; Martín et al., 2015; Verlicchi et al., 2012). Emerging compounds were already detected in several ecosystems in surface water, groundwater, sediment, soils, and even aquatic organisms (Azzouz and Ballesteros, 2012; Durán-Álvarez et al., 2015; Pinheiro et al., 2013; Sui et al., 2015; Xue and Kannan, 2016). Many of these substances are toxic or classified as endocrine disruptors and their presence in the environment can affect the ecological balance (Diniz et al., 2015; Fent, 2015; Stancová et al., 2015). Another concern is the bioaccumulation capacity of some contaminants (Xue and Kannan, 2016) and possible trophic magnification inside the food chain (Ruhí et al., 2016).

In aquatic ecosystems, the concern of bioaccumulation starts with biofilms, which are at the bottom of the food chain. Biofilms are an assemblage of microbial cells (Pandey, 2017), forming a complex community of bacteria, algae, fungi, and other microorganisms (Weber, 1973). They can be found attached to many underwater surfaces, such as pebbles, tree branches, leaves, and others. Therefore, they are continuously exposed to the dynamic flow of the water, associated nutrients, other microorganisms, and pollutants.

Biofilms are able to retain and accumulate substances, including nutrients (Bechtold et al., 2012) and pollutants, by concentrating compounds into the matrix or incorporating into microbial biomass, via adsorption, absorption, and diffusion (Battin et al., 2016; Flemming and Wingender,

2010). Therefore, biofilm is a suitable biological indicator and could be an appropriate matrix for monitoring and assessing contaminants (Katerina Kohušová et al., 2011). Besides, the biofilm grows at a fast rate, is easy to collect, and can be found in almost every environment (Fuchs et al., 1997; Huerta et al., 2016; Sabater et al., 2007). Their use, along with other analyses, may provide better results than traditional chemical and hydrological monitoring techniques (Lear and Lewis, 2009), especially for pollutants detected in low concentrations.

The detection of pollutants in biofilm was already performed in some countries, like Spain (Huerta et al., 2016), France (Dorigo et al., 2010), Czech Republic (Kateřina Kohušová et al., 2011), USA (Du et al., 2015; Writer et al., 2011) and Germany (Fuchs et al., 1997). However, most of the research was performed analyzing pesticides, heavy metals and polycyclic aromatic hydrocarbons (PAHs) and there is no research available in tropical and subtropical areas, where the conditions for the biofilm growth, like temperature and nutrient availability, are very different.

Therefore, the goals of this research are: (i) to develop a methodology to analyze pharmaceuticals, caffeine and UV filters in water and biofilm (ii) to detect emerging contaminants in biofilm collected from a subtropical urban river located in southern Brazil, (ii) compare the concentrations detected in water samples with biofilm collected with a sampler constructed for this purpose.

5.2 MATERIALS AND METHODS

5.2.1 Study Area

The Barigui River has 66 km and a 279 km² catchment area, mainly located inside urban areas, crossing the cities of Almirante Tamandaré, Curitiba, and Araucária, in southern Brazil. There is a Karst aquifer in the Upper Barigui River Basin, currently used as a potable water source for the population in the area. To protect the sources of the river and the aquifer, environmental protection areas were created. However, the presence of urban areas and industries down the river endanger the water quality.

The estimated population in the basin for the year 2020 is approximately 860 thousand inhabitants. Although most of the area is provided by sewage collection systems, which is a separate sewer system, many households are not connected to the sewage pipelines and only 64% of the domestic sewage is treated (SUDERSHA 2002; IAP 2009).

For the present study, two sampling points were chosen in the Upper Barigui River Basin (Figure 5-1). The first sampling point (BA1) is located after the Barigui water treatment plant (WTP), that supplies potable water for the Almirante Tamandaré population, and the other (BA2) is located after the São Jorge wastewater treatment plant (WWTP). The WWTP has an anaerobic reactor followed by a physico-chemical treatment to remove phosphorus (decantation/flotation) and treats 48.3 L s^{-1} of effluent, while the river has a flow of 347.1 L s^{-1} at the discharge point (ANA, 2017).

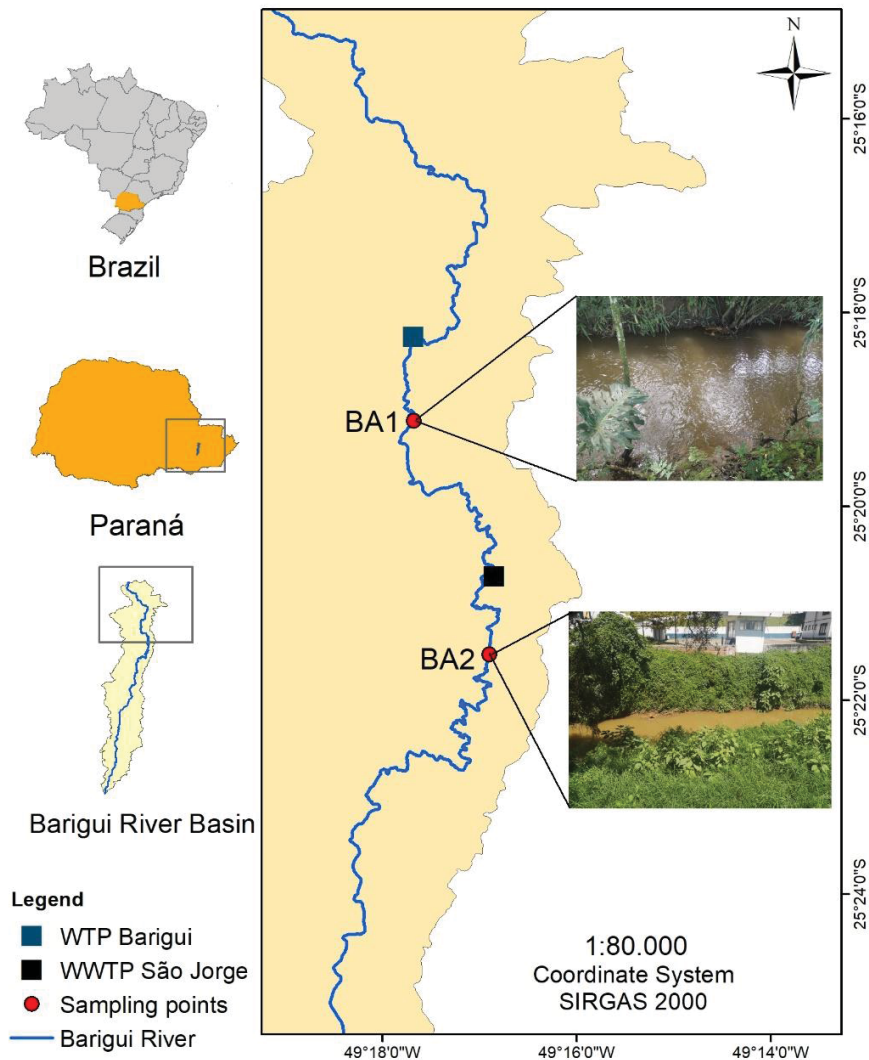


Figure 5-1: Map of the studied area and location of sampling points

5.2.2 Sampling collection

Two biofilm samplers were built (Figure 5-2), adapted from the model previously used in Chapter 2. Each sampler consisted of a wood box containing four glass sheets. The samplers were kept underwater for a month, so the biofilm could properly grow on the glass plates. The samplers were firstly placed inside the Barigui River on 22 February and the biofilm was collected on 29 March, as well as water and sediment samples.



Figure 5-2: Adapted biofilm sampler – project and

The biofilm was scrapped off the sampler with a previously decontaminated stainless-steel spatula and was kept in amber glass jars. Water samples were also collected from the sampling points and they were stored in previously decontaminated amber glass bottles. The samples were kept refrigerated until arriving at the laboratory. The water samples were analyzed immediately to avoid degradation. The biofilm samples were frozen and then freeze dried.

5.2.3 Chemical and physical parameters

All the chemical and physical parameters analyzed are displayed in Table 5-1. In the surface water samples, pH, conductivity, temperature, dissolved oxygen, and salinity were analyzed in the field with a portable multiparameter probe Hanna HI9828 (Hanna Instruments, Rhode Island, United States). Turbidity was analyzed with a portable turbidimeter Hanna HI98703 (Hanna Instruments, Rhode Island, United States). Ammoniacal nitrogen, nitrite, nitrate, and orthophosphate were measured in the samples using ultraviolet-visible (UV-Vis) absorption spectrophotometry, following methods of the “Standard methods for the examination of water and wastewater” (APHA, 2005).

Table 5-1 Analyzed parameters on water samples

Parameters	Methodology
pH	Multiparameter probe
Conductivity	Multiparameter probe
Temperature	Multiparameter probe
Dissolved Oxygen	Multiparameter probe
Turbidity	Turbidimeter
Nitrate	4500-NO ₃ ⁻ E (APHA, 2005)
Ammoniacal Nitrogen	4500-NH ₃ F (APHA, 2005)
Orthophosphate	4500-P E. (APHA, 2005)

Also, precipitation data from the biofilm growth period was monitored, due to concerns of biofilm detachment and sloughing, because of the increase of water flow and velocity. Data was obtained from Águas Paraná Institute/SIMEPAR (Paraná Environmental Technology and Monitoring System). The closest monitoring station was “Colombo Copel”. Data was downloaded from <http://www.simepar.br/aguasparana/relatorios/estacoes/horaria/pluviometricas.shtml>.

5.2.4 Chemicals and reagents

High purity standards of gemfibrozil ($\geq 99\%$), diclofenac ($\geq 99\%$), naproxen (≥ 98 -102%), ketoprofen ($\geq 99\%$), acetyl salicylic acid ($\geq 99\%$), caffeine ($\geq 99\%$), 4-methylbenzylidenecamphor ($\geq 98\%$), benzophenone-1 ($\geq 99\%$), benzophenone-3 ($\geq 98\%$), octocrylene ($\geq 98\%$), and octyl methoxycinnamate ($\geq 98\%$), were purchased from Sigma Aldrich (Steinheim, Germany).

The solvents employed in chromatographic analyses (acetone, acetonitrile, methanol, ethyl acetate, and hexane) were HPLC-grade, and the phosphoric acid (LiChropur™, 85%) was also purchased from Sigma Aldrich (Steinheim, Germany).

5.2.5 Extraction

5.2.5.1 Water samples

The methodology was adapted from Ide et al. (2017). Water samples (500 mL) were filtrated (cellulose acetate, 0.45 μm) and acidified with HCl 6.0 mol L⁻¹ until pH ± 3 . Solid-phase cartridges purchased from Chromabond C18 (500 mg, 6 mL, Macherei-Nagel, Düren, Germany) were conditioned with 6 mL of hexane, 6 mL of acetone, 6 mL of methanol, and 6 mL of acidified ultrapure water. The samples were extracted under vacuum in a flow rate of 6 to 8 mL min⁻¹. The

cartridges were dried and the content was eluted with 10 mL of acetonitrile. An aliquot of 2 mL was stored in vials and injected in the chromatograph.

5.2.5.2 *Biofilm samples*

The methodology was adapted from Huerta et al. (2016). The samples were freeze-dried, sieved (120 μm) and weighted (1g) in polypropylene tubes. Two extraction steps were performed with two aliquots of acetonitrile (5 mL). The solvents were added to the samples, shaken, ultrasonicated for 16 min, and centrifuged at 3600 rpm for 20 minutes. The supernatant was evaporated, reconstituted to 1mL of acetonitrile, and added 250mL of ultrapure water. The samples were acidified with HCl until ($\text{pH} \pm 3$). Then, solid-phase extraction and derivatization were performed according to the previous section.

5.2.6 Development and optimization of the chromatographic method

Solutions of 1 mg L⁻¹ of each analyte were prepared in acetonitrile and injected directly in the equipment in the scan mode, to identify the molecular ion. Then, the solutions were injected in the product ion mode and multiple reaction monitoring (MRM) mode, to identify and optimize the transitions and collision energies. Other parameters were tested and optimized, such as injection volume, gas temperature, gas flow, nebulizer pressure, column temperature, mobile phase flow, solvent gradient, mobile phase additives, etc.

Two chromatographic methods were developed in an Agilent 1260 Infinity liquid chromatograph with a quaternary pump (G1311B) coupled with a triple quadrupole mass spectrometry (Agilent Ultivo LC/TQ). A nitrogen generator model Genius 1024 LC-MS acquired from Peak Scientific (Scotland, United Kingdom) produced high purity nitrogen. An InfinityLab Poroshell 120 EC-C18 (2.1x100 mm, 2.7 μm) column was employed as stationary phase to separate the analytes.

5.2.7 Validation and quality assurance/quality control protocols

In order to prevent contamination in the analyses, all the glassware was washed with Extran 5%, ultrapure water, dried and carefully washed with a mixture of acetonitrile and isopropanol (1:1), sonicated and dried. Blank samples were included in every batch to evaluate possible contaminations.

Due to technical problems, only the water extraction methodology was properly validated. For validation purposes, samples were collected from a non-urban area in the Barigui River, near the source of the river. Linearity was assessed using at least 5 concentration levels in triplicate and it was determined using linear regression. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as $3.3SD/S$ and $10SD/S$, where SD is the standard deviation of the response and S is the slope of the calibration curve. Specificity was determined by comparing a calibration curve made in solvent and one made with a mixture of the extraction of the samples diluted 1:10, because we were not able to find a real matrix with the absence of the investigated compounds. The results were compared with the t Student test ($p < 0.05$, 95% of significance).

Intra- and inter-day precision were assessed injecting three concentration levels in the equipment. For intra-day 6 replicates were analyzed and for inter-day 3 replicates were analyzed in 3 different days and a relative standard deviation (RSD) $< 20\%$ was accepted. Recovery was assessed spiking the target analytes in samples with a concentration of $5 \mu\text{g L}^{-1}$ in triplicate. A RSD $< 20\%$ was accepted.

5.3 RESULTS AND DISCUSSION

5.3.1 Chemical and physical parameters

For a complete discussion, the physical and chemical parameters analyzed in the water samples (

Table 5-2) will be discussed according to the Brazilian CONAMA (Conselho Nacional do Meio Ambiente) Resolution nº 357/05. This resolution establishes environmental guidelines for the legal framing of rivers and proposes parameters for river classifications. The Barigui River is officially framed as a Class 2 River in the section where this study took place. Class 2 rivers can be used for human consumption after conventional treatment, leisure activities (swimming and diving), irrigation, and fishing. The classification is a measure for establishing a goal for the river water quality, after an evaluation of the current river status and an expectation for its future. The Barigui River classification is from 1992 (SUREHMA N°020/92).

The dissolved oxygen (DO) was measured as 7.77 mg L^{-1} in BA1 and 3.31 mg L^{-1} in BA2, downstream of the WWTP. According to the CONAMA (Conselho Nacional do Meio Ambiente) Resolution nº 357/05, Class 2 rivers must have at least 5 mg L^{-1} of DO, while Class 3 and 4 rivers must have at least 4 mg L^{-1} and 2 mg L^{-1} , respectively. Therefore, the BA1 river section has higher

DO concentration levels than its legal framing, while BA2 has lower DO levels than the established for Class 2 rivers. The oxygen saturation is also lower in BA2, reaching 40.6%, while in BA1 it was 97.7%. Lower DO and oxygen saturation levels can indicate pollution, mainly because of organic matter inputs in the river. Temperature, salinity, conductivity, and TDS were higher in BA2, also indicating higher pollution levels in this river section.

Turbidity is also a classifying parameter of the CONAMA Resolution n°357/05, and according to the text, Class 2 rivers must have a maximum of 100 NTU. Both BA1 and BA2 were below this limit.

Table 5-2: Physical and chemical parameters analyzed in the water samples

Parameter	BA 1	BA 2	Class 2 Rivers*
DO (mg L ⁻¹)	7.77	3.31	5.0
Saturation (%)	97.7	40.6	-
pH	7.18	7.5	6.0-9.0
Temperature (°C)	20.67	21.16	-
Salinity (g L ⁻¹)	0.14	0.20	0.5
Conductivity (mS cm ⁻¹)	0.286	0.416	-
Resistivity (MΩxcm)	0.0035	0.0024	-
TDS (g L ⁻¹)	144	209	-
ORP	110	89.3	-
Turbidity (NTU)	3.62	9.57	100
Ammoniacal-N (mg L ⁻¹)	0.57	4.43	3.7 (for pH ≤ 7.5)
Nitrite (mg L ⁻¹)	0	0.16	1.0
Nitrate (mg L ⁻¹)	0.51	1.91	10.0
Orthophosphate-P (mg L ⁻¹)	0.047	0.20	0.1 (Total P)

DO: dissolved oxygen, TDS: Total dissolved solids, ORP: oxidation reduction potential

*CONAMA Resolution n° 357/05

The presence of nutrients in a river is also indicative of the water quality and pollution levels, especially the presence of domestic sewage in urban areas (Ide et al., 2017). According to the analyses, the concentrations of ammoniacal nitrogen, nitrite, nitrate, and orthophosphate are much higher in BA2 than in BA1, indicating the WWTP can impact negatively the water quality in BA2. Of the analyzed nutrients, both BA1 and BA2 are fitting Class 2 parameters of nitrite and nitrate. However, only BA1 is in agreement with ammoniacal nitrogen and phosphorus levels. It is important to say that the CONAMA Resolution n°357/05 only addresses total phosphorus, while

our analyses are of orthophosphate-P, a fraction of total phosphorus found in surface waters. According to the resolution, a Class 2 river must have a maximum of 3.7 mg L⁻¹ of ammoniacal nitrogen ($\text{pH} \leq 7.5$) and 0.1 mg L⁻¹ of total phosphorus. The BA1 river section had lower concentrations of these parameters, but the BA2 river section presented higher concentrations.

Precipitation data was obtained from 22 February to 29 March, the same period the biofilm sampler was kept underwater. Daily precipitation can be visualized in Figure 5-3. During the period, a total precipitation of 144.8 mm was registered in the area. The highest daily precipitation was registered in 1st March (34 mm), followed by 28 February (22.6 mm). Also, there were events closer to the biofilm sampling, in 15 March (18.8 mm) and 27 March (11.8). The precipitation events did not affect the biofilm sampling and enough material was collected to perform the analysis.

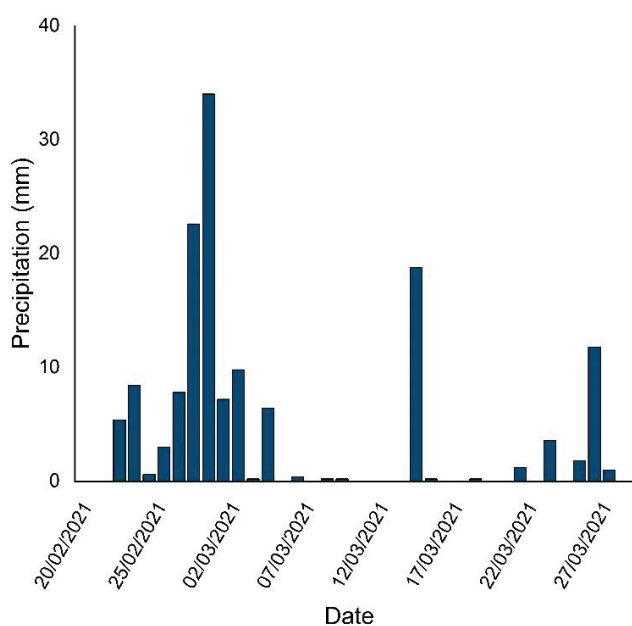


Figure 5-3: Daily precipitation in the studied area.

5.3.2 Optimization of the chromatographic methods

Two chromatographic methods were developed. Five pharmaceuticals (gemfibrozil, ibuprofen, diclofenac, naproxen, ketoprofen, and acetyl salicid acid) were analyzed in the first chromatographic method developed. In this method the temperature column was 50°C, the capillary voltage was 3500 V in negative mode, the gas temperature was 200°C, the gas flow was 9 L/min and the nebulizer was at 40 psi. The samples were injected (10 µL) into the system. The solvents

employed were A: water 0.04% formic acid and B: acetonitrile. The method had 10 minutes. Initially, the solvent proportion was 90% A and 10% B (1 min), then changed to 60% A and 40% B (2 min), changed to 30% A and 70% B, increasing to 10% A and 90% B in two minutes, and going back to initial conditions in one minute, maintaining this proportion for 4 min, until the end of the run.

The second chromatographic method analyzed caffeine and five UV filters (4-methylbenzylidenecamphor, benzophenone-1, benzophenone-3, octocrylene, and octyl methoxycinnamate). The method had the temperature column at 30°C, the capillary voltage was 4800 V in positive mode, the gas temperature was 300°C, the gas flow was 7 L/min and the nebulizer was at 15 psi. The samples were injected (10 µL) into the system. The solvents employed were A: water 0.04% formic acid and B: acetonitrile. The method had 13 minutes. Initially, the solvent proportion was 30% A and 70% B (2.5 min), then changed to 5% A and 95% B (4.5 min), returning to 30% A and 70% B until the end of the run.

The precursor and product ions, the collision and fragmentator energies employed in the detection and quantification of the compounds can be visualized in Table 5-3 (pharmaceuticals) and Table 5-4 (caffeine and UV filters).

Table 5-3: Pharmaceuticals analyzed by HPLC-MS/MS – retention time, precursor and product ions, collision, and fragmentator energies

Compounds	Retention time (min)	Molecular Ion [M+H] ⁺	Transitions (m/z)	Collision Energy (V)	Fragmentator (V)
GFZ	6.47	249.1	121 106.3	20 20	100
AAS	3.67	136.9	93 -	20	80
KET	5.58	253	209 197	5 8	50
DIC	6.07	294	250 213.6	15 15	80
NAP	5.61	229	185.1 -	5	50

GFZ: gemfibrozil, ASA: acetyl salicylic acid, KET: ketoprofen, DIC: diclofenac, NAP: naproxen

The most abundant transition was used to quantify the target analyte and the second was used as a qualifier. For some compounds, the second transition was not stable and repetitive and only the first transition was used to quantify and identify the analyte. The transitions found are

similar from the ones reported in the literature (Gago-Ferrero et al., 2013; Jedziniak et al., 2012; Magi et al., 2012).

Table 5-4: Caffeine and UV filters analyzed by HPLC-MS/MS – retention time, precursor and product ions, collision, and fragmentator energies

Compounds	Retention time (min)	Molecular Ion [M+H] ⁺	Transitions (m/z)	Collision energy (V)	Fragmentator (V)
CAF	0.68	195	138 -	17	80
BP-1	1.37	183	105 77	18 18	80
BP-3	1.58	229	151 105	18 18	135
OC	5.89	362	250 232	17 14	80
OMC	5.90	291	179 161	5 18	70
4MBC	0.91	255	119 105	25 30	100

CAF: caffeine, BP-1: benzophenone-1, BP-3: benzophenone-3, OC: octocrylene, OMC: octyl methoxycinnamate, 4MBC: 4-methylbenzylidenecamphor

5.3.3 Method validation

Linearity was assessed with concentrations ranging from 1-50 $\mu\text{g L}^{-1}$ (pharmaceuticals) and 0.5-10 $\mu\text{g L}^{-1}$ (UV filters and CAF). The slope, intercept and R^2 values are displayed in Table 5-5, as well as LOD and LOQ values. All calibration curves had a R^2 above 0.99. LOD and LOQ values were between 1-2 and 4-8 $\mu\text{g L}^{-1}$ for pharmaceuticals and 0.2-0.5 and 0.6-2 $\mu\text{g L}^{-1}$ for CAF and the UV filters. According to the t student test, the method was specific for most of the contaminants, except for CAF ($p=0.11$) and DIC ($p=0.25$).

Intra- and inter-day precisions were between 1.89-19.06% and 2.69-24.55%, respectively (Table 5-6). NAP was the only contaminant with DPR >20%, at a concentration level of 1 $\mu\text{g L}^{-1}$, however its LOD was 2 $\mu\text{g L}^{-1}$. The recovery efficiencies varied from 12.66-132.66%. The lowest recoveries were from CAF (12.66%) and DIC (51.33%) and the highest were from OC (132.66%) and BP-1 (111.33%). The recoveries DPR varied from 5.12-24.11%. CAF was the only with a DPR > 20%, but all the others were reproducible, including DIC.

Table 5-5: Linearity parameters, LOD and LOQ of the developed chromatographic methods

Compounds	Calibration range ($\mu\text{g L}^{-1}$)	Slope (n=3)	Intercept (n=3)	R ²	LOD ($\mu\text{g L}^{-1}$)	LOQ ($\mu\text{g L}^{-1}$)
GFZ	1-50	160.23	57.69	0.9994	1	3
AAS	1-50	314.64	53.67	0.9984	2	6
KET	1-50	28.06	20.74	0.9976	2	6
DIC	1-50	119.44	86.70	0.9991	1	3
NAP	1-50	8.91	6.45	0.9986	2	6
CAF	0.2-10	1593.45	56.56	0.9996	0.2	0.6
BP-1	0.2-10	1154.21	29.26	0.9992	0.3	0.9
BP-3	0.2-10	1269.09	35.36	0.9994	0.2	0.6
OC	0.2-10	616.90	188.67	0.9931	0.5	1.5
OMC	0.2-10	1767.23	428.11	0.9943	0.5	1.5
4MBC	0.2-10	1075.46	36.36	0.9994	0.2	0.6

Table 5-6: Data for intra- and inter-day precisions and recovery of the analyzed contaminants

Concentration ($\mu\text{g L}^{-1}$)	Intra-day RSD (%)			Inter-day RSD (%)			Recovery (RSD) (%)
	1	10	50	1	10	50	5
GFZ	5.57	4.75	6.33	5.34	5.13	3.02	78 (5.12)
AAS	19.06	7.81	2.82	7.38	6.57	5.29	104 (20.35)
KET	12.47	7.51	3.75	8.14	9.79	5.35	89.3 (5.17)
DIC	15.53	3.41	1.89	17.43	5.82	2.69	51.33 (11.9)
NAP	16.88	4.97	5.58	24.55	8.68	6.58	87.33 (7.36)

Concentration ($\mu\text{g L}^{-1}$)	Intra-day RSD (%)			Inter-day RSD (%)			Recovery (RSD) (%)
	0.5	1	10	0.5	1	10	5
CAF	3.88	5.31	11.96	15.88	16.00	18.00	12.66 (24.11)
BP-1	1.35	3.10	7.38	19.08	17.57	18.16	111.33 (9.21)
BP-3	0.97	1.13	7.25	16.13	15.60	16.67	96.66 (15.25)
OC	4.01	18.80	7.78	14.27	14.09	14.15	132.66 (15.47)
OMC	2.00	3.18	8.54	17.85	17.42	13.80	86 (8.38)
4MBC	4.11	6.05	9.98	18.98	16.19	18.61	94.66 (15.85)

Chromatographs from the contaminants standards diluted in solvent and spiked real samples can be seen in Appendices Q and R.

5.3.4 Emerging contaminants detected in water and biofilm samples from the Barigui River

The concentrations of pharmaceuticals, caffeine and UV filters detected in the water samples collected in 29 March are described in Table 5-7. The concentrations found in water samples collected in the other two sampling campaigns can be found in Appendix S. However,

they will not be discussed in this chapter. Because of the higher LOD and LOQ of the pharmaceuticals method, all the pharmaceuticals analyzed in BA1 were below LOD. In BA2, KET, DIC, and NAP were below LOQ, while GFZ and AAS were below LOD. In a previous study in the Barigui River, GFZ was detected in concentrations varying from 0.02-0.06 $\mu\text{g L}^{-1}$ (Goulart et al., 2021). In a study performed in Spain, pharmaceuticals were analyzed in the Ebro River basin. DIC had concentrations varying from nd-0.07 $\mu\text{g L}^{-1}$, KET nd-0.13 $\mu\text{g L}^{-1}$, NAP nd-0.11 $\mu\text{g L}^{-1}$ (Silva et al., 2011).

Caffeine and UV filters were detected above the LOQ in almost all the samples, except for OC in BA1 and OMC in BA2 (Table 5-7). For most of the compounds, such as BP-1, BP-3, OC, and 4MBC, the concentrations detected in BA2 were higher than the ones found in BA1. However, with the exception of BP-1 and 4MBC, the concentrations were very similar. Also, CAF and OMC had higher concentrations in BA1. CAF is frequently used as a chemical tracer for recent contamination by raw sewage in surface water, because of its fast degradation (Mizukawa et al., 2019). In fact, CAF was the pollutant detected with the highest concentration, of 0.49 $\mu\text{g L}^{-1}$, even with the lowest recovery of all the compounds. The highest concentration of UV filters detected was of BP-1 with 0.12 $\mu\text{g L}^{-1}$.

Table 5-7: Concentrations of pharmaceuticals, caffeine, and UV filters detected in water and biofilm samples

Contaminants	Water (ppm- $\mu\text{g L}^{-1}$)		Biofilm (ppm- $\mu\text{g kg}^{-1}$ dw)	
	BA 1	BA 2	BA1	BA2
GFZ	<LOD	<LOD	nd	nd
AAS	<LOD	<LOD	<LOD	<LOD
KET	<LOD	<LOQ	nd	<LOD
DIC	<LOD	<LOQ	<LOD	34.66
NAP	<LOD	<LOQ	<LOD	<LOD
CAF	0.49	0.22	6.9	95.04
BP-1	0.10	0.12	8.81	8.15
BP-3	0.07	0.08	3.1	15.0
OC	<LOQ	0.04	105.8	586.4
OMC	0.07	<LOQ	53.4	33.4
4MBC	0.02	0.06	<LOD	9.6

nd: not detected, LOD: limit of detection, LOQ: limit of quantification
dw: dry weight

Previous studies analyzed UV filters in rivers from different countries, including Brazil. da Silva et al., (2015) analyzed BP-3, OC, and OMC in surface water samples collected from rivers that are source for water treatment plants (WTP). Usually OC and OMC were not detected, or were detected below LOQ, but OMC was detected in a few samples with concentrations of 0.05-0.06 $\mu\text{g L}^{-1}$, while BP-3 was detected in concentrations of 0.02 – 0.04 $\mu\text{g L}^{-1}$. In Switzerland, 4MBC, BP-3, OMC, and OC were analyzed in water samples collected from lakes. The concentrations varied from <0.002-0.03 $\mu\text{g L}^{-1}$ for 4MBC, <0.002-0.02 $\mu\text{g L}^{-1}$ for BP-3, <0.002-0.007 $\mu\text{g L}^{-1}$ for OMC, and <0.002-0.005 $\mu\text{g L}^{-1}$ for OC (Balmer et al., 2005). In Korea, OMC, BP-1, BP-3, and 4-MBC were analyzed in surface water samples. The highest concentration detected was 0.25 $\mu\text{g L}^{-1}$ of OMC, while BP-3 reached 0.15 $\mu\text{g L}^{-1}$, 4MBC 0.05 $\mu\text{g L}^{-1}$, and BP-1 0.04 $\mu\text{g L}^{-1}$ (Ekpeghere et al., 2016).

In biofilm samples, the only pharmaceutical detected above LOQ was DIC, with a concentration of 34.66 $\mu\text{g kg}^{-1}$. In the only other study that analyzed pharmaceuticals in river biofilms, Huerta et al. (2016) reported that DIC and GFZ were detected above LOQ in biofilm, with concentrations varying from 23.5-103 $\mu\text{g kg}^{-1}$ and 4-10.3 $\mu\text{g kg}^{-1}$, respectively. In our study, GFZ was not detected in the biofilm samples, but it was also not detected in the water samples. CAF and UV filters were detected in almost all biofilm samples, except for 4MBC in BA1. Most of the compounds had a higher accumulation downstream of the WWTP, except for OMC and BP-1. The highest concentrations detected were of OC that reached 586.4 $\mu\text{g kg}^{-1}$ in BA2 and 105.8 $\mu\text{g kg}^{-1}$ in BA1, followed by CAF with 95.04 $\mu\text{g kg}^{-1}$, also in BA2. We did not find data from other studies to compare the accumulation of UV filters in biofilm. However, Mizukawa et al. (2017) analyzed UV filters in sediment samples collected from nearby rivers. The concentrations found ranged from <LOQ -322.6 $\mu\text{g kg}^{-1}$ for OC, <LOQ -67.2 $\mu\text{g kg}^{-1}$ for BP-3, nd-61.8 <LOQ -322.6 $\mu\text{g kg}^{-1}$ for BP-1, nd- 49.3 $\mu\text{g kg}^{-1}$ for 4MBC, and nd- 166.8 $\mu\text{g kg}^{-1}$ for OMC.

According to the results, it is possible to see bioaccumulation in biofilm from all the quantified pollutants. Biofilm accumulated both hydrophobic (high log Kow) and hydrophilic (low log Kow) compounds, like UV filters, with log Kow in the range of 3-6 and CAF, with log Kow of -0.07. Also, pharmaceuticals like DIC can be amphiphilic, e.g. can act both as hydrophobic and hydrophilic compounds, because they contain hydrophilic and hydrophobic groups (Khan et al., 2013). This diverse bioaccumulation capacity happens mainly because of the extracellular polymeric substances (EPS) produced by the microorganisms. The EPS can act as a filter, trapping

cations, anions, apolar compounds and particles from the water phase (Flemming and Wingender, 2010).

In addition to bioaccumulation, compounds easily digested or partitioned by microorganisms can undergo biodegradation processes into the biofilm, which would make them harder to detect. This interesting biofilm capacity is already used in wastewater treatment processes, such as in moving bed bio-reactors (MBBR) and other biological reactors (El-taliawy et al., 2018). However, most of the toxic contaminants have complex molecules, and would hardly be degraded by the microorganisms present in river biofilms. In addition, there is the possibility of contamination of other species that feed from the biofilm, and even biomagnification of some pollutants along the food chain (Huerta et al., 2016).

According to the literature review, there are few studies regarding the accumulation of pollutants in natural biofilm. More studies should be performed with different classes of pollutants, especially emerging contaminants.

5.4 CONCLUSION

The proposed methodology was efficient to analyze pharmaceuticals, UV filters and CAF in water and biofilm samples. Also, biofilm was able to grow in the sampler and the collected amount was enough to analyze emerging contaminants. Intense rain events that happened during biofilm growth did not seem to strongly influence in the detachment or sloughing of biofilm. Results show that biofilm is a suitable biological indicator and can act as a natural passive sampler of UV filters, CAF, and pharmaceuticals, mainly DIC. In addition, biofilm seems to be an important environment compartment to accumulate emerging contaminants and an important key to better understand the partition behavior of many pollutants.

6 GENERAL CONCLUSION AND MAIN FINDINGS

The presence of pollutants, pathogenic organisms and antibiotic resistance in the environment is a worldwide problem that can cause negative effects and endanger animal and human health and safety. The constant release of these substances in the environment by anthropic activities is increasing and little effort is made to reverse or change this situation. This is a complex problem due to the variety of chemicals constantly released in the environment and their known and unknown consequences for the biota and even humans.

This thesis, with its different chapters, addressed different problems in the context of emerging contaminants and antibiotic resistance in the environment. The first chapter, named “Emerging contaminants and antibiotic resistance in the different environmental matrices of Latin America” gathered quality data about the occurrence of emerging contaminants in Latin America. The goal was to identify the most commonly detected contaminants and their patterns in the studied countries. What we found was that very few countries in Latin America published papers in peer-reviewed journals that fit our criteria (environmental monitoring, real matrices, etc.). In addition, the publications are related to very specific areas in these countries; especially the most urbanized and populated areas of Brazil and Mexico. Most of the papers analyzed drinking and surface water, mainly because of the possible direct risk to humans. However, we were able to propose a priority list for monitoring emerging contaminants in Latin America. The list includes plasticizers, hormones, antimicrobials, and antibiotics.

The other three chapters of this thesis are the result of an investigation about the use of biofilm in environmental monitoring. The detection of emerging contaminants in different media, besides water, sediment, and soil, is very important for understanding biogeochemical processes and the partition behavior of these substances in the environment. Biofilms, for example, can be found in almost every ecosystem and are easily collected from rocks and other natural substrates underwater, but also from man-made structures. Biofilms are made of living organisms and are profoundly influenced by water quality parameters, e.g. nutrients, organic matter content, temperature, turbidity, concentrations of suspended solids, etc. Therefore, the biofilm analysis can couple physical, chemical, and biological analyses, and may better reflect the real situation of an aquatic environment.

For this purpose, we analyzed the accumulation of different kinds of pollutants, facultative pathogenic bacteria and antibiotic resistance genes in the biofilm itself. We also evaluated the use of artificial samplers of biofilm and how they can help and influence the biofilm collection. Experiments took place in two different ecosystems, with different climate, hydrological, social and environmental features. We also wanted to evaluate if different climate and hydrological features would affect biofilm growth and collection. The results show that biofilm sampling was successful in both areas, despite low temperatures and intense rain events that could harm biofilm growth or cause biofilm detachment from the samplers. The samplers are useful especially in artificial channels and waterways, where there are no cobbles or other submerged structures to collect biofilm from. Furthermore, samplers allow to control the biofilm growth period and consequently the bioaccumulation period. However, the first sampler model we used accumulated a lot of sediment inside and around the glass plates, reducing the biofilm growth area. The second model, an adaptation from the first one where the glass plates are placed higher from the sampler bottom, solved the problem. It is also very important to fix the sampler to the bottom of the river, so the sampler does not move or is dragged down the river.

The results of the chromatographic analyses indicated that the biofilm has a very strong capacity of absorbing and retaining several pollutants, both hydrophobic and hydrophilic. In addition, biofilm proved to be a source for antibiotic resistance and facultative pathogenic bacteria in the environment. Biofilm was able to better reflect the inputs of plasticizers, alkylphenols, UV filters, and caffeine in urban rivers. The high concentrations found in this media is worrisome, since biofilm is an important matrix for the environment and the food web. This could cause the contamination of other species, such as microinvertebrates and fishes. Also, the possibility of biomagnification exists, which could, ultimately, endanger human health.

Our conclusion is that biofilm is an important tool for environmental monitoring, and it should be used together with more traditional practices, such as water and sediment analysis. The use of biofilm could be beneficial mainly when monitoring areas where the contamination is not so evident, and the concentrations are usually below the quantification limits in water and sediment samples. The results could bring a new level of understanding to the environment contamination issue, and help to bring awareness to this problem, as well as more efforts and technical solutions to the problem of emerging contaminants and antibiotic resistance in the environment.

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APPENDIX

Appendix A: Emerging contaminants in drinking water and potable-water resources

Drinking Water and Potable-water Sources						
Source	Location	Matrix	Compounds	Analytical method	LD - LQ (ng L ⁻¹)	Concentration range 3 most detected (ng L ⁻¹)
(Félix-Cañedo et al., 2013)	México México City	Dams Mixed tanks	Pharmaceuticals	GC-MS	0.50 -	DEHP (75-2282)
			Hormones		0.25 -	SA (29-309)
			Surfactants		0.25 -	NAP (52-186)
			Phthalates			
			Biocides			
(Bedoya-Ríos et al., 2017)	Colombia Bogotá	Reservoir	Pharmaceuticals	GC-MS	-	DEHP (nd-100970000)
			Phthalates		-	DPP (nd-13840)
			Plasticizers		-	BP-A (nd-64370)
			Hormones			
(Martínez and Peñuela, 2013)	Colombia Antioquia	Reservoir Tributaries	Triclosan	GC-MS/MS	70 - 230	TCS (nd-490)
			4-NP		42 - 140	4-NP (nd)
(Caldas et al., 2018)	Brazil Rio Grande do Sul	WTP	Pharmaceuticals	LC-ESI-MS/MS	8 -	MeP (15-840)
			Personal care products		4 -	NIM (70-730)
			Pesticides		40 -	GBC (50-120)
(Marta-Sánchez et al., 2018)	Brazil Rio Grande do Sul	WTP Bottled mineral water	Parabens	LC-MS/MS	12 - 80	MeP (nd-242)
(De Souza et al., 2018)	Brazil Rio Grande do Sul	River Tap water	Pharmaceuticals Hormones Plasticizers	UHPLC/MS-MS	12 - 2000	Nd
(Monteiro et al., 2018)	Brazil Rio de Janeiro	River Tap Water	Pharmaceuticals	HPLC-M/MS	4 - 13	CEF (nd-575.5)
					6 - 20	AMOX (nd-287.5)
					9 - 27	SUF (nd-105)
(Campestrini and Jardim, 2017)	Brazil Sao Paulo	Reservoir River Tap Water	Cocaine Benzoyllecgonine	LC/MS-MS	2 - 6	COC (nd-62)
					2 - 5	BLGN (nd-1019)
(Torres et al., 2017)	Brazil Sao Paulo	River Tap water	Antibiotics	LC-ESI-MS/MS	0.1 - 80	Nd
(da Silva et al., 2015)	Brazil Sao Paulo	River WTP	UV filters	GC-MS/MS	2 - 10	BP-3 (nd-105)
					2 - 50	EHMC (nd-101)

(de Sousa et al., 2014)	Brazil Sao Paulo	River	Pharmaceuticals Caffeine Triclosan Hormones	UPLC-MS	1.7 - 1.1 - 1.3 -	CAF (nd-213.6) CBZ (nd-14.4) DIC (nd-29.2)
(Melo and Brito, 2013)	Brazil Maranhao	River Tap Water	17 α -ethinylestradiol Bisphenol A	HPLC Fluorescence	650 - 2170 460 - 1530	EE2 (nd – 3610) BP-A (nd)
(Silveira et al., 2013)	Brazil Rio Grande do Sul	WTP	Pharmaceuticals PCPs	LC-ESI- MS/MS	20 - 100 200 - 1000 10 - 50	HAL (nd-100) MeP (<LQ-134000) NIM (nd-50)
(Maldaner and Jardim, 2012)	Brazil Sao Paulo	WTP	Paracetamol Herbicide	HPLC- MS/MS	- 6	ATZ (9,3 -81)
(Souza et al., 2012)	Brazil Sao Paulo	WTP Bottled mineral water	Plasticizers	GC-MS/MS	7 - 23 10 - 34 29 - 44	DEP (<LQ-143) DBP (80-454) BP-A (<LQ)
(Moreira et al., 2011)	Brazil Minas Gerais	River	Hormones Plasticizers Phtalates	ESI-LC-IT- TOF/MS	- 3.9 - 4.3 - 2.5	NP (25.9-1435.3) DEP (5-410) BP-A (8.6-168.3)
(Lopes et al., 2010)	Brazil Sao Paulo	River	17 β -estradiol 17 α -ethinylestradiol	LC-UV	3.7 - 7.5 300 - 600	E2 (nd – 30.6) EE2 (nd – 600)
(Verbinnen et al., 2010)	Brazil Maranhao	Reservoir WTP Tap Water	Hormones	HPLC-DAD	1250 - 62.5 -	E1/E2/EE2 E3 (nd)
(Sodré et al., 2010a)	Brazil Sao Paulo	Tap Water	Hormones Plasticizers Caffeine	GC-MS	40 - 130 50 - 160 30 - 100	STIG (<LQ-340) CHOE (<LQ-270) CAF (<LQ-220)
(Moreira et al., 2009)	Brazil Minas Gerais	River	4-Nonylphenol 17 β -estradiol 17 α -ethinylestradiol	LC-MS/MS	6.5 - 1.4 - 0.9 -	4-NP (40-1918) E2 (nd-36.8) EE2

Appendix B: Emerging contaminants in raw sewage, hospital effluent and WWTP effluent

Raw sewage						
Source	Location	Matrix	Compounds	Analytical method	LD – LQ (ng L⁻¹)	Concentration range 3 most detected (µg L⁻¹)
(Lesser et al., 2018)	Mexico Hidalgo Mezquital Valley	Raw sewage	Pharmaceuticals Antibiotics PCPs Hormones	GC-MS	5.9 - 25 - 14.8 -	MET (1.31-107) CAF (0.115-42.8) PARA (nd-67.2)
(Rivera-Jaimes et al., 2018b)	Mexico Morelos	Raw sewage	Pharmaceuticals	LC-MS/MS	14 - 46 67 - 262 5.5 - 18	NAP (0.82-4.21) PARA (2.33-14.9) DIC (0.56-2.47)
(Estrada-Arriaga et al., 2013b)	Mexico Mexico City	Raw sewage	Hormones	GC-MS/MS	-	E1 (0.004-0.093) E2 (0.004-0.093) EE2 (0.004-0.093)
(Gibson et al., 2010)	Mexico Hidalgo Tulla Valley	Raw sewage	Pharmaceuticals Plasticizers PCPs	GC-MS	-	4-NP (1.98-39.32) NAP (7.26-13.58) IBU (0.74-1.40)
(Gibson et al., 2007)	Mexico Hidalgo Tulla Valley	Raw sewage	Pharmaceuticals Plasticizers PCPs Phtalates Hormones	GC-MS	0.005- 0.025- 0.005-	NAP (15.22-16.65) 4-NP (11-22.4) SA (0.62-29.06)
(Bedoya-Ríos et al., 2017)	Colombia Bogotá	Wetland	Pharmaceuticals Plasticizers Hormones Phtalates	GC-MS	-	DEHP (nd-309.6) DPP (nd-309.6) BP-A (nd-22.73)
Hospital effluent						
(Porto et al., 2019)	Brazil São Paulo	Hospital Effluent	Antiparasitics	UHPLC- MS/MS	- 10	ABZ: (- 3.81) RBZ: (- 3.89) TBZ: (- 0.33)

(Chiarello et al., 2016)	Brazil Rio Grande do Sul	Hospital Effluent	Pharmaceuticals	LC-MS	900 - 2800 600 - 1600 130 - 400	PARA (7.5) MET (2.0) ENA (1.0)
(Minetto et al., 2012)	Brazil Rio Grande do Sul	Hospital Effluent	Diclofenac	HPLC-DAD	20 - 50	DIC (0.83-3.59)
WWTP effluent						
(Rivera-Jaimes et al., 2018a)	Mexico Morelos	Raw sewage	Pharmaceuticals	LC-MS/MS	1.2 - 4.2 13 - 44 1.5 - 4.9	NAP (0.049-0.392) SUF (0.44-1.21) DIC (0.46-21.8)
(Robledo Zacarías et al., 2017)	Mexico Michoacán	WWTP	Pharmaceuticals	ESI-MS-TOF	-	TC (2.7-11.94) Levotiroxina (7.3-37.60) Fexofenadina (1.20-33.60)
(Estrada-Arriaga et al., 2016)	Mexico Guanajuato State of Mexico	WWTP UV Disinfection	Pharmaceuticals Hormones Illegal drugs PCPs	HPLC-MS	- 11.2 - 0.47 - 4.44	MET (0.057-3.77) SUF (0.005-2.33) ERY (<LQ-6.48)
(Rozas et al., 2016)	Chile Antofagasta	WWTP UV/H ₂ O ₂	Pharmaceuticals Triclosan	LC-MS/MS	-	CAF (0.06-0.85) DIC (0.3-0.4) ATZ (0.07-0.15)
(Pessoa et al., 2014)	Brazil Ceará	WWTP	Hormones	GC-MS	35.3-117.9 11.2-37.58 28.9-62.9	E1 (nd-2.08) E2 (nd-0.39) EE2 (nd-0.17)
(Froehner et al., 2011c) A	Brazil Paraná	WWTP Aerobic Anaerobic	Hormones Caffeine Bisphenol A	HPLC-UV HPLC-DAD	-	E2 (0.49-0.76) EE2 (nd-0.47) BP-A (nd-0.020)

Appendix C: Emerging contaminants in surface water

Surface water						
Source	Location	Matrix	Compounds	Analytical method	LD - LQ (ng L ⁻¹)	Concentration range 3 most detected (ng L ⁻¹)
(Hernández-Quiroz et al., 2019)	Mexico Mexico City Xochimilco Wetland	Interstitial water from Agricultural Wetlands <i>Chinampas</i>	Hormone Personal care product Pharmaceutical Fragrance	GC-MS	20 - 260 -	IBU (0.5-13.9) MUK (nd-82.3)
(Lesser et al., 2018)	Mexico Hidalgo Mezquital Valley	Springs	Pharmaceuticals Antibiotics PCPs Hormones	GC-MS	0.4 - 25 - 80 -	DEET (12.7-5280) CAF (nd-171) Theophylline (nd-84.6)
(Rivera-Jaimes et al., 2018a)	Mexico Morelos	Raw sewage	Pharmaceuticals	LC-MS/MS	0.9 - 3.1 2.8 - 28 1.2 - 4.1	NAP (0.732-4.88) PARA (0.35-4.46) DIC (0.25-1.39)
(Díaz and Peña-Alvarez, 2017)	Mexico Hidalgo	River Sediment	Pharmaceuticals Bisphenol A Triclosan Hormones	GC-MS	0.11-0.3 0.04-0.1 0.1-0.3	NAP (99.2-251.2) IBU (56.1-101.3) TCS (27.3-32.5)
(Díaz-Torres et al., 2013)	Mexico Mexico City Xochimilco Wetland	Agricultural Wetlands <i>Chinampas</i>	Hormones Plasticizers	GC	0.001 - 0.01 - 0.01 -	E1 (1000000-22000000) BP-A (nd-240000000) TCS (nd-110000000)
(Gibson et al., 2007)	Mexico Hidalgo Tulla Valley	Springs	Pharmaceuticals Plasticizers PCPs Phtalates Hormones	GC-MS	0.01 - 0.005- 0.005-	DEHP (6.6-25) SA (7.8-9.6) NAP (0.8-8)
(Peteffi et al., 2019)	Brazil Rio Grande do Sul	River	Caffeine Plasticizer	HPLC-DAD GC-MS	1.2 -	CAF (0.041-28.43) BP-A (nd-0.51)
(Mizukawa et al. 2018)	Brazil Paraná	River	PCPs	GC-MS	6.9 - 22.9 7.3 - 24.5 4.0 - 13.4	TCS (120-10300) MeP (nd-3590) PrP (nd-2059)

(Torres et al., 2017)	Brazil Sao Paulo	River	Antibiotics	LC-ESI-MS/MS	0.8 - 8	NOR (8-26)
(Annuniação et al., 2017)	Brazil Brasília	Lake sediment	Polybrominated diphenyl ethers	GC Electron capture detection	0.69-2.31 ng g ⁻¹ 0.62-2.05 ng g ⁻¹	2,3',4,4'- Tetrabromodiphenyl ether (BDE-66) (8.1 ng g ⁻¹) 2,2',4,4'- Tetrabromodiphenyl ether (BDE-47) (2.51 ng g ⁻¹)
(Caldas et al., 2016)	Brazil Rio Grande do Sul	River	Pharmaceuticals PCPs Pesticides	LC-MS/MS	- 125 - 250 - 250	Bentazone (nd-6700) Avobenzone (nd-5930) BP-A (nd-4420)
(De Sousa et al., 2015)	Brazil Sao Paulo	River sediment	Pharmaceuticals Hormones Triclosan	UHPLC-MS/MS	0.01-0.05 ng g ⁻¹ 0.01-0.04 ng g ⁻¹ 0.02-0.04 ng g ⁻¹	TCS (3.53-176 ng g ⁻¹) CAF (nd-41 ng g ⁻¹) PROP (nd-28.5 ng g ⁻¹)
(Campanha et al., 2015)	Brazil Sao Paulo	River	Pharmaceuticals Hormones	UPLC-MS/MS	 0.04-5	CAF (<LQ-129585) ATEN (<LQ-8199) PARA (<LQ-30421)
(Galinaro et al., 2015)	Brazil Sao Paulo	River	Parabens	LC-DAD	500-1300 800-1900 800-2400	PrP (nd-55600) EtP (nd-33000) MeP (nd-29800)
(da Rocha et al., 2015)	Brazil Mato Grosso do Sul	River	Pharmaceuticals Inseticide	LC-DAD	360 - 370 -	CBZ nd THIA (1230-1580)
(de Sousa et al., 2014)	Brazil Sao Paulo	River	Pharmaceuticals Caffeine Triclosan Hormones	UPLC-MS	1.72 - 1.12 - 3.38 -	CAF (nd-19329.7) CBZ (nd-659.5) ATEN (nd-412.9)
(Froehner et al., 2011a) B	Brazil Paraná	River	Caffeine Bisphenol A Musk Xylene	HPLC-DAD GC-FID	- - 0.05 -	CAF (174-123450) BP-A (nd-12610) XYL (40-560)

(Froehner et al., 2011b) C	Brazil Santa Catarina	Mangrove sediment	Hormones	HPLC-UV	-	EE2 (ND-133.64 ng g ⁻¹) E1 (nd-50.75 ng g ⁻¹) E2 (nd-40.96 ng g ⁻¹)
(Sodré et al., 2010b)	Brazil Sao Paulo	River	Hormones Surfactants	LC-MS/MS	0.4 - 1.2 0.2 - 0.6 0.1 - 0.3	BP-A (2.5-84) E3 (nd-182) E1 (nd-39)
(Suazo et al., 2017)	Chile Santiago	River Lake	Pharmaceuticals E2 PCPs	GC-MS/MS	1600/2900	All compounds <LQ
(Rozas et al., 2016)	Chile Antofagasta	River	Pharmaceuticals Triclosan	LC-MS/MS	-	TCS (25-54) CAF (8-34) ATZ (20-38)
(Bedoya-Ríos et al., 2017)	Colombia Bogotá	River	Pharmaceuticals Plasticizers Hormones Phtalates	GC-MS	-	BP-A (nd-76820) CBZ (nd-36920) TRI (nd-3580)
(Babay et al., 2014)	Argentina Buenos Ayres	River	Surfactants	HPLC-FL	1300- 800 - 800 -	NP (300-7000) NP1EO (100-8600) NP2EO (100-5200)

Appendix D: Emerging contaminants in groundwater

Groundwater						
Source	Location	Matrix	Compounds	Analytical method	LD - LQ (ng L⁻¹)	Concentration range 3 most detected (ng L⁻¹)
(Lesser et al., 2018)	Mexico Hidalgo Mezquital Valley	Groundwater	Pharmaceuticals	GC-MS	0.4 -	DEET (nd-756)
			Antibiotics		33.1 -	TCS (nd-698)
			PCPs Hormones		1.8 -	CBZ (nd-99.7)
(Félix-Cañedo et al., 2013)	México México City	Groundwater	Pharmaceuticals	GC-MS	0.25 -	SA (nd-464)
			Hormones		0.1 -	TCS (nd-345)
			Surfactants		0.5 -	DEHP (nd-232)
			Phthalates Biocides PCPs			
(Melo and Brito, 2013)	Brazil Maranhao	Groundwater	17 α -ethinylestradiol bisphenol A	HPLC/MS-MS	650-2170	EE2 (<LQ)
					460-1530	BP-A (nd)
(da Silva et al., 2015)	Brazil Sao Paulo	Groundwater	UV filters	GC-MS/MS	2 - 50	EHMC (nd-137)
					2 - 10	BP-3 (<LQ)
					2 - 100	ES (<LQ)

Appendix E: Emerging contaminants in coastal water

Coastal water						
Source	Location	Matrix	Compounds	Analytical method	LD - LQ (ng L⁻¹)	Concentration range 3 most detected (ng L⁻¹ or ng g⁻¹)
(Salgado et al., 2019b)	Brazil Sao Paulo	Sediment	Pharmaceuticals Personal care products	GC-MS/MS	-	PROP (5410 – 66470) MTL (<LD-31340) FEN (<LD-21830)
(Pereira et al., 2016)	Brazil Sao Paulo	Sea water	Pharmaceuticals Illegal drugs	LC-MS/MS	10 - 35	IBU (326.1 – 2094.4)
					0.1 - 8.5	CAF (84.4-648.9)
					3 - 12	COC (12.6-400.5)
(Lisboa et al., 2013)	Brazil Bahia	Sea water	Surfactants Hormones	LC Fluorescence	4.5 - 15	4-OP (19.3-134)
					3.3 - 16	BP-A (<LQ-76.8)
					2.8 - 9	E3 (<LQ-37.9)
(De Waisbaum et al., 2010)	Argentina Argentinian coast	Sea water	Tribultyn	GC-MS	54.4 ng g ⁻¹	TBT sand (nd-5200 ng g ⁻¹)
		Sediment			56 ng L ⁻¹	TBT sea water (160-6700 ng L ⁻¹)

Appendix F: Emerging contaminants in soil

Soil						
Source	Location	Matrix	Compounds	Analytical method	LD - LQ (ng g⁻¹)	Concentration range 3 most detected (ng g⁻¹)
(Gibson et al., 2010)	Mexico Hidalgo Tulla Valley	Soil	Pharmaceuticals	GC-MS	-	4-NP
		Raw	Plasticizers			(nd-299)
		wastewater	PCPs			TCS
		irrigation	Phtalates			(0.4-35.3)
			Hormones			BP-A (1.6-30.2)
(Durán-Alvarez et al., 2009)	Mexico Hidalgo Tulla Valley	Soil	Pharmaceuticals	GC-MS	25 -	DEHP (820-2079)
		Raw	Phtalates		25 -	DBP (244-552)
		wastewater	Hormones		25 -	BuBeP (131-346)
		irrigation	PCPs			
			Surfactants			

Appendix G: Biological concentrations of emerging contaminants

Biological concentrations						
Source	Location	Specie	Compounds	Analytical method	LD - LQ (ng g ⁻¹)	Concentration range 3 most detected (ng g ⁻¹)
(Guidi et al., 2018)	Brazil Minas Gerais	<i>Oreochromis niloticus</i> <i>Oncorhynchus mykiss</i>	Antibiotics	LC-MS/MS	- 12.5	ENRO (<LQ)
(Monteiro et al., 2016)	Brazil Sao Paulo Mato Grosso do Sul	<i>Oreochromis niloticus</i>	Antibiotics	LC-MS/MS	-	OXTC (nd-1298.7) FFC (nd-524.7) TC (32.4)
(Rossato et al., 2016)	Brazil Santa Catarina	<i>Stramonita haemastoma</i>	Organotins	GC-MS	- 3.5 - 2.0 - 1.0	Monobultyn (<LQ-335.29) Dibultyn (<LQ-98.48) TBT (<LQ-75.08)
(Munaretto et al., 2013)	Brazil Rio Grande do Sul	<i>Oreochromis niloticus</i> <i>Pangasius hypophthalmus</i>	Endocrine disruptors	GC-MS/MS	0.3 - 1.0 0.3 - 1.0 0.3 - 1.0	BP-A (20.7-14.5) Chlorpyrifos (nd-34.7) Bifenthrin (nd-2.1)
(Lailson-Brito et al., 2011)	Brazil Sao Paulo Paraná	<i>Franciscana dolphins</i>	Organochlorine compounds	GC-ECD	-	PCB (909-5849) DDT (264-5811) HCB (10-61)
(Stoker et al., 2011)	Argentina Chaco, Santa Fé, Entre Ríos	<i>Caiman latirostris</i>	Organochlorine compounds	GC-MS	-	PCB (<LQ-136.6) Pesticides (28.9-189)

Appendix H: Presence of Antibiotic Resistance in Latin America

Antibiotic Resistance						
Source	Location	Matrix	Species	Antibiotics	Resistance	Multi-resistance?
Castaneda-Ruelas and Jiménez-Edeza, 2018	Mexico Sinaloa	River	<i>Salmonella</i> sp.	Apramycin	X	Yes
				Amikacin		
				Streptomycin	X	
				Gentamycin	X	
				Neomycin	X	
				Ampicillin	X	
				Amoxicilin + clavulanic acid	X	
				Cefoperazone	X	
				Ceftazidime	X	
				Chloramfenicol	X	
				Ceftazidime	X	
				Furazolidone		
				Colistin sulphate	X	
				Nalidixic acid	X	
				Trimethoprim	X	
				Sulfonamide	X	
				Tetracycline	X	
Mondragón et al. 2018	Mexico Nayarit	River	<i>Enterococcus faecalis</i>	Ampicillin	X	No
				Vancomycin	X	
				Gentamycin	X	
				Kanamycin	X	
				Ciprofloxacin	X	
(Brisola et al., 2019b)	Brazil Santa Catarina	Swine feces	<i>Escherichia coli</i>	Amoxicilin + clavulanic acid	X	Yes
		Water		Ceftiour	X	
		Soil		Enrofloxacin	X	
				Gentamicin	X	
				Trimethoprim + Sulfamethoxazole	X	
				Colistin	X	
Bortoloti et al. 2018	Brazil Minas Gerais	Potable water sources	<i>Escherichia coli</i>	Amoxicilin	X	Yes
				Gentamycin	X	
				Aztreonam	X	
				Cefalexin	X	
				Ciprofloxacin	X	
				Chloramfenicol	X	
				Penicilin	X	
				Vancomycin	X	
Nascimento et al. 2017	Brazil Sao Paulo	Reservoir Lake	<i>Escherichia coli</i>	Amoxicilin + clavulanic acid	X	Yes
			<i>Klebsiella pneumoniae</i>	Cefotaxime	X	
			<i>Pseudomonas aeruginosa</i>	Ceftriaxone	X	
			<i>Pseudomonas putida</i>	Ceftazidime	X	
			<i>Aeromonas hydrophila</i>	Cefepime	X	
			<i>Aeromonas hydrophila</i>	Cefoxitin	X	
			<i>Providencia rettgeri</i>	Amikacin	X	
			<i>Providencia rettgeri</i>	Gentamycin	X	

			<i>Enterobacter kobei</i>	Trimethoprim	X	
			<i>Stenotrophomonas maltophilia</i>	Ciprofloxacin	X	
				Ertapenem	X	
			<i>Citrobacter freundii</i>	Imipenem	X	
				Tigecycline	X	
Paiva et al. 2017	Brazil Minas Gerais	WWTP	<i>Escherichia coli</i>	Nalidixic acid	X	Yes
			<i>Proteus mirabilis</i>	Ciprofloxacin	X	
			<i>Providencia. rettgeri</i>	Ofloxacin	X	
			<i>Morganella morganii</i>	Norfloxacin	X	
			<i>Citrobacter freundii</i>	Levofloxacin	X	
			<i>Peusodmonas guandongensis</i>	Kanamycin	X	
				Gentamicin	X	
			<i>Comomonas sp.</i>	Amikacin	X	
			<i>Acidovorax sp.</i>	Streptomycin	X	
			<i>Alicaligenes faecalis</i>			
			<i>Shewanella xiamenensis</i>			
			<i>Enterococcus gallinarum</i>			
Oliveira et al. 2017	Brazil Minas Gerais	WWTP	<i>Pseudomonas aeruginosa</i>	Piperacillin+ tazobactam		Yes
				Gentamycin	X	
				Amikacin	X	
				Ciprofloxacin		
				Norfloxacin		
				Imipenem	X	
				Meropenem	X	
				Ceftazidime	X	
				Cephepime	X	
Lopes et al. 2016	Brazil Paraná	WWTP River sediment	<i>Escherichia coli</i>	Norfloxacin		Yes
				Ciprofoloxacin		
				Cephalotin	X	
				Gentamicin		
				Streptomycin	X	
				Imipenem		
				Cefaclor		
				Ampicillin		
				Cefoxitin		
				Tetracycline	X	
				Amoxicilin	X	
				Chloramphenicol		
Monteiro et al. 2016	Brazil Sao Paulo	Fish	<i>Pseudomonas</i>	Oxytetracycline		
			<i>Burkholderia</i>	Tetracycline	X	
			<i>Pasteurella</i>	Chlortetracycline	X	
			<i>Streptococcus</i>	Ciprofloxacin	X	
			<i>Aeromonas</i>	Enrofloxacin	X	
				Sarafloxacin		
				Norfloxacin	X	
				Sulfathiazole	X	
				Sulfadimethoxine		
				Florfenicol	X	

				Sulfamethazine Chloramphenicol		
Andrade et al. 2015	Brazil Sao Paulo	Sea water Sand	<i>Escherichia coli</i>	Amoxicilin + clavulanic acid Rifampicin Streptomycin Ampicillin Erytromycin Tetracycline Gentamicin Vancomycin Penicilin Amoxicilin	X X X X X X X X	Yes
da Rocha et al. 2015	Brazil Mato Grosso do Sul	River	<i>Escherichia coli</i> <i>Pseudomonas</i> <i>aeruginosa</i> <i>Pseudomonas putida</i>	Amikacin Ampicillin Ampicillin+ sulbactam Cefepime Cefoxitin Ceftazidime Ceftriaxone Cefuroxime Cefuroxime axetil Ciprofloxacin Colistin Etapenem Gentamicin Imipenem Meropenem Piperacillin+ tazobactam Tigecycline	X X X X X X X X X X X X X X X X X	Yes
Giowanella et al. 2015	Brazil Paraná	River	<i>Escherichia coli</i>	Nalidixic acid Ampicillin Amikacin Amoxicilin + clavulanic acid Cefalotin Ceftazidime Ciprofloxacin Chloramphenicol Streptomycin Gentamicin Sulfamethoxazole Tetracycline	X X X X X X X X X X	Yes
Heck et al. 2015	Brazil	Composting plant sludge	<i>Pseudomonas</i> <i>aeruginosa</i> <i>Escherichia coli</i> <i>Staphylococcus</i> <i>aureus</i>	Ampicillin Amoxicilin + clavulanic acid Cephalotin Cefoxitin Ceftriaxone Chloramphenicol	X X X X X X X	Yes

				Streptomycin	X	
				Gentamicin	X	
				Imipenem	X	
				Nitrofurantoin	X	
				Sulfamethoxazole	X	
				Tetracycline	X	
Martins et al. 2014	Brazil Sao Paulo	River Lake	<i>Pseudomonas aeruginosa</i>	Amikacin		No
				Amoxicilin		
				Aztreonam		
				Cefepime		
				Ceftazidime		
				Ceftriaxone		
				Cephalotin		
				Ciprofloxacin		
				Chloramphenicol		
				Gentamicin		
				Meropenem		
				Streptomycin		
				Tetracycline	X	
				Imipenem		
				Piperacilin- tazobactam		
				Ticarcillin- clavulanic acid		
				Polymycin		
				Rifampicin		
				Tobramycin		
				Trimethoprim		
Palhares et al. 2014	Brazil Santa Catarina	River Feces Soil	<i>Salmonella</i> sp.	Nalidixic acid	X	Yes
				Amoxicilin + clavulanic acid	X	
				Amikacin	X	
				Ampicillin	X	
				Kanamycin	X	
				Cephalotin	X	
				Ceftazidime	X	
				Ceftiofur	X	
				Ciprofloxacin	X	
				Chloramphenicol	X	
				Colistin	X	
				Doxycycline	X	
				Enrofloxacin	X	
				Streptomycin	X	
				Florfenicol	X	
				Gentamicin	X	
				Neomycin	X	
				Norfloxacin	X	
				Sulfametazol + trimethoprim	X	
				Tetracycline	X	
				Trimethoprim	X	

Rebello and Regua-mangia, 2014	Brazil Rio de Janeiro	River Agricultural and hospital effluents	<i>Escherichia coli</i>	Amikacin Ampicillin Cephalotin Cefepime Cefoxitin Ciprofloxacin Gentamicin Nitrofurantoin Norfloxacin Sulphazotrim Trimethoprim	X X X X X X X X X X X	Yes
Zanetti et al. 2013	Brazil Sao Paulo Santa Catarina	River Lake	<i>Pseudomonas aeruginosa</i>	Amikacin Amoxicilin Aztreonam Cefepime Ceftazidime Ceftriaxone Cephalotin Ciprofloxacin Chloramphenicol Gentamicin Meropenen Streptomycin Tetracycline Imipenen Piperacillin-tazobactam Polymyxin B Riphampicin Ticarcillin-clavunate acid Tobramycin Trimetropin	 X X X X X X X	Yes
Resende et al. 2012	Brazil Minas Gerais	Shrimp farm	<i>Enterococcus faecalis</i> <i>Staphylococcus epidermidis</i> <i>Enterococcus avium</i> <i>Micrococcus luteus</i>	Pinicilim Oxacilin Vancomycin Gentamycin Azithromycin Tetracycline Levofloxacin Trimetoprim Chloramfenicol Ampicillin Piperacillin-tazobactam Ceftriaxone Cefepime Meropenen Levofloxacin Amikacin	X X X X X X X X X X X X X X X X X	Yes
Da Silva et al. 2011	Brazil Paraná	River	<i>Escherichia coli</i>	Sulphazotrin Amoxicilin Imipenem	X X X	Yes

				Amikacin		
				Cefalotin	X	
				Gentamycin		
				Norfloxacin		
				Chloramphenicol		
				Ampicillin	X	
				Nitrofurantoin		
				Ciprofloxacin		
				Tetracycline	X	
				Ceftazidim		
Fuentefria et al. 2011	Brazil Rio Grande do Sul	Hospital Raw Sewage River	<i>Pseudomonas aeruginosa</i>	Amikacin	X	Yes
				Gentamicin	X	
				Ciprofloxacin	X	
				Ceftazidim	X	
				Piperacillin-tazobactam	X	
				Ticarcillin-clavulanic acid	X	
				Imipenem	X	
				Meropenem	X	
				Cephepim	X	
				Aztreonam	X	
				Polymyxin B	X	
Gambero et al. 2018	Argentina Córdoba	River Groundwater	<i>Escherichia coli</i>	Ampicillin	X	Yes
				Tetracycline	X	
				Cephalotine	X	
				Ciprofloxacin		
				Amoxicilin + clavulanic acid	X	
				Chloramfenicol		

Appendix I – Antibiotic resistance genes – primer sequences, calibration curves, r^2 , references

Target	Primer sequence	Calibration curve	amplicon	efficiency	R ²	LOD	Literature
<i>ermB</i>	Fwd: TGAATCGAGACTTGAGTGTGCAA Rev: GGATTCTACAAAGCGTACCTT	$F(x) = -3.328x + 35.901$	71 bp	100 %	1.000	16	(Alexander et al., 2015)
<i>tetM</i>	Fwd: GGTTCCTCTTGGATACTTAAATCAATC Rev: CCAACCATAAATCCTGTTCRC	$F(x) = -3.424x + 38.747$	88 bp	95.9 %	0.998	4	(Peak et al., 2007)
<i>bla_{TEM}</i>	Fwd: TTCCTGTTTTTGTCTACCCAG Rev: CTCAAGGATCTTACCGCTGTTG	$F(x) = -3.303x + 38.559$	112 bp	100.8 %	0.999	80	(Rocha et al., 2020)
<i>sul1</i>	Fwd: CGCACCGGAACATCGCTGCAC Rev: TGAAGTTCGGCCGCAAGGCTCG	$F(x) = -3.387x + 39.802$	161 bp	97.6 %	0.999	80	(Rocha et al., 2020)
<i>bla_{CTX-M15}</i>	Fwd: CGCTTTGCGATGTGCAG Rev: ACCGCGATATCGTTGGT	$F(x) = -3.504x + 34.255$	551 bp	92.9 %	1.000	93	(Paterson et al., 2003; Rocha et al., 2020)
<i>bla_{CTX-M32}</i>	Fwd: CGTCACGCTGTTGTTAGGAA Rev: CGCTCATCAGCACGATAAAG	$F(x) = -3.517x + 37.800$	155 bp	92.5 %	1.000	235	(Rocha et al., 2020)
<i>bla_{OXA-48}</i>	Fwd: TGTTTTTGGTGGCATCGAT Rev: GTAAMRATGCTTGGTTCG	$F(x) = -3.540x + 36.913$	177 bp	91.6 %	0.998	92	(Monteiro et al., 2012)
<i>bla_{CMY-2}</i>	Fwd: CGTTAATCGCACCATCACC Rev: CGTCCTTACTAACCGATCCTAGC	$F(x) = -3.591x + 34.026$	172 bp	89.9 %	0.998	71	(Kurpiel and Hanson, 2012)
<i>bla_{KPC-3}</i>	Fwd: CAGCTCATTC AAGGGCTTTC Rev: GGCGGCGTTATCACTGTATT	$F(x) = -3.457x + 38.837$	196 bp	94.6	0.999		(Szczepanowski et al., 2009)
<i>vanA</i>	Fwd: TCTGCAATAGAGATAGCCGC Rev: GGAGTAGCTATCCCAGCAT	$F(x) = -3.541x + 33.078$	376 bp	91.6 %	1.000	43	(Klein et al., 1998)
<i>mecA</i>	Fwd: CGCAACGTTCAATTAAATTTGTTAA Rev: TGGTCTTTCTGCATTCCTGGA	$F(x) = -3.327x + 34.887$	91 bp	99.8 %	1.000	11	(Volkmann et al., 2004)
<i>mcr-1</i>	Fwd: GGGCCTGCGTATTTAAGCG Rev: CATAGGCATTGCTGTGCGTC	$F(x) = -3.386x + 35.349$	183 bp	97.4 %	0.999	8	(Hembach et al., 2017)
<i>bla_{NDM-1}</i>	Fwd: TTGGCCTTGCTGTCTTG Rev: ACACAGTGACAAATACACCG	$F(x) = -3.293x + 35.877$	82 bp	101.2 %	0.999	66	(Monteiro et al., 2012)

Source: Hembach et al., 2019

Appendix J – Facultative pathogenic bacteria – primer sequences, calibration curves, r^2 , references

Target	Primer sequence	Calibration curve	amplicon	efficiency	R ²	LOD	Literature
enterococci (23S rRNA)	Fwd: AGAAATTCCAAACGAACTTG Rev: CAGTGCTCTACCTCCATCAT	$F(x) = -3.585x + 35.283$	93 bp	90.1 %	1.000	65	(Frahm and Obst, 2003)
<i>E. faecalis</i> (ddl)	Fwd: CACCTGAAGAAACAGGC Rev: ATGGCTACTTCAATTTCACG	$F(x) = -3.472x + 35.447$	475 bp	94.1	0.999		(Depardieu et al., 2004)
<i>P. aeruginosa</i> (ecfX)	Fwd: AGCGTTTCGTCCTGCACAAAGT Rev: TCCACCATGCTCAGGGAGAT	$F(x) = -3.282x + 35.276$	81 bp	101.7 %	0.999	3	(Clifford et al., 2012)
<i>K. pneumoniae</i> (gltA)	Fwd: ACGGCCCGAATATGACGAATTC Rev: AGAGTGATCTGCTCATGAA	$F(x) = -3.387x + 38.844$	68 bp	97.4 %	0.998	18	(Clifford et al., 2012)
<i>A. baumannii</i> (secE)	Fwd: GTTGTGGCTTTAGGTTTATTATACG Rev: AAGTTACTCGACGCAATTCG	$F(x) = -3.380x + 35.679$	94 bp	97.6 %	1.000	31	(Clifford et al., 2012)
<i>E. coli</i> (yecT)	Fwd: GCATCGTGACCACCTTGA Rev: CAGCGTGGTGGCAAAA	$F(x) = -3.361x + 35.797$	59 bp	98.4 %	0.994	4	(Clifford et al., 2012)
Eubacteria (16 rDNA)	Fwd: ACTCCTACGGGAGGCAGCAG Rev: ATTACCGCGGCTGCTGG	$F(x) = -3.406x + 36.360$	200 bp	96.6%	0.997		(Fierer et al., 2005)

Source: Hembach et al., 2019

Appendix K: Physical and chemical parameters, UV radiation and macrophytes during the sampling campaigns

<i>Water samples</i>	<i>Sampling dates</i>	23/01	22/02	18/03	16/04	16/05	13/06
Turbidity (NTU)	Upstream	42.50	29.5	23.0	2.55	2.60	2.22
	Downstream	56.20	25.5	19.4	2.56	2.70	1.88
pH	Upstream	8.33	7.97	8.04	8.17	8.13	8.45
	Downstream	8.40	7.97	7.95	8.17	7.99	8.13
Conductivity (µS/cm)	Upstream	1236	1342	627	1212	1256	824
	Downstream	1262	1340	1053	1226	1275	795
Temperature (°C)	Upstream	2.5	10.6	8.10	15.0	14.8	13.5
	Downstream	2.5	9.40	8.80	14.5	15.0	13.7
Dissolved oxygen (mg/L)	Upstream	10.55	9.40	10.1	9.50	8.65	7.39
	Downstream	10.80	9.60	9.84	8.71	8.12	6.10
Suspended solids (mg/L)	Upstream	71.25	38.5	19.7	15.5	2.33	1.23
	Downstream	62.33	24.7	23.5	13.0	2.83	1.38
Total phosphorus (mg/L)	Upstream	0.27	0.21	0.15	0.12	0.11	0.25
	Downstream	0.35	0.19	0.21	0.11	0.12	0.23
Orthophosphate (mg/L)	Upstream	0.095	0.09	0.06	0.09	0.10	0.21
	Downstream	0.089	0.10	0.11	0.07	0.08	0.19
Total nitrogen (mg/L)	Upstream	8.67	8.91	6.71	6.15	7.20	7.20
	Downstream	11.50	7.67	6.29	6.04	6.85	6.85
Ammonium (mg/L)	Upstream	0.22	0.06	0.06	0.00	0.01	0.02
	Downstream	0.21	0.06	0.77	0.00	0.16	0.23
Nitrate (mg/L)	Upstream	-	6.91	5.42	5.88	5.40	3.47
	Downstream	-	6.95	4.11	5.90	5.32	3.55
LOI (%)	Upstream	18.60	18.83	16.46	19.35	35.71	50
	Downstream	18.18	19.19	14.89	19.23	35.29	66.67
<i>Sediment samples</i>							
Total phosphorus (mg/kg)	Upstream	941	698	1326	1038	928	1296
	Downstream	1084	1035	1279	833	1162	871
Total Kjeldahl nitrogen (mg/kg)	Upstream	3513	1273	4340	4052	3343	3119
	Downstream	4238	3901	4833	1589	4079	2032
LOI (%)	Upstream	5.96	3.59	6.78	6.34	6.50	6.42
	Downstream	7.94	6.85	6.60	3.32	7.72	5.64
<i>Biofilm samples</i>							
LOI (%)	Upstream	-	16.93	11.97	15.53	28.32	21.22
	Downstream	-	17.71	21.98	23.32	33.05	21.36
<i>Climate</i>							
UVA (J/m ²)	-	1.33E+05	2.48E+05	3.76E+05	7.44E+05	9.63E+05	1.12E+06
UVB (J/m ²)	-	8.27E+02	2.32E+03	4.34E+03	1.08E+04	1.58E+04	2.18E+04
Mean temperature (°C)	-	3.6	3.2	8.5	8.2	11.4	16.25
Total rainfall (mm)	-	68.4	13.4	27.3	22.9	46.2	71.4

<i>Others</i>						
Presence of macrophytes	-	No	No	No	Yes	Yes

Appendix L: Gene markers of eubacterial DNA and facultative pathogenic bacteria detected in sampling campaigns (cell equivalents/100 mL or cell equivalents/g)

<i>Water samples</i>	Location	22/02	18/03	16/04	16/05	13/06
16S rRNA	Upstream	7.57E+05	7.12E+05	3.41E+03	4.34E+05	2.33E+04
	Downstream	6.62E+05	1.22E+06	4.91E+03	2.49E+05	7.48E+05
23S rRNA	Upstream	1.17E+03	6.57E+02	2.46E+01	3.83E+01	4.08E+01
	Downstream	1.75E+03	1.58E+04	1.10E+01	8.50E+02	1.02E+03
ddl	Upstream	1.15E+01	9.33E+00	<LOD	<LOD	<LOD
	Downstream	2.11E+01	1.87E+02	<LOD	<LOD	1.45E+01
yccT	Upstream	3.43E+02	2.70E+02	3.66E+00	<LOD	<LOD
	Downstream	3.37E+02	2.80E+03	1.92E+00	3.34E+02	9.28E+02
ecfX	Upstream	7.90E+00	4.90E+00	<LOD	3.31E+00	1.32E+00
	Downstream	7.68E+00	2.72E+01	<LOD	4.13E+00	6.63E+00
gltA	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	7.39E+03	<LOD	1.41E+03	5.42E+03
secE	Upstream	<LOD	1.97E+01	<LOD	<LOD	<LOD
	Downstream	<LOD	1.41E+02	<LOD	<LOD	3.63E+01
<i>Sediment samples</i>						
16S rRNA	Upstream	1.14E+07	3.05E+07	4.45E+06	1.41E+07	1.49E+07
	Downstream	1.16E+08	1.65E+08	8.97E+06	8.08E+06	3.29E+06
23S rRNA	Upstream	7.71E+03	2.63E+04	2.11E+03	<LOD	<LOD
	Downstream	4.51E+03	6.71E+03	1.26E+03	<LOD	6.88E+02
ddl	Upstream	6.24E+01	1.88E+02	<LOD	<LOD	<LOD
	Downstream	<LOD	6.32E+01	<LOD	<LOD	<LOD
yccT	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	9.50E+02	<LOD	<LOD	<LOD
ecfX	Upstream	<LOD	9.47E+01	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	8.25E+01	<LOD
gltA	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	8.37E+02	<LOD
secE	Upstream	5.65E+01	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
<i>Biofilm samples</i>						
16S rRNA	Upstream	1.12E+09	8.76E+07	1.74E+07	5.96E+08	3.33E+08
	Downstream	1.54E+08	1.12E+05	3.76E+08	1.42E+08	8.69E+06
23S rRNA	Upstream	1.11E+05	1.48E+05	2.15E+03	1.26E+04	1.54E+05
	Downstream	3.57E+05	<LOD	9.50E+04	5.43E+04	3.90E+05
ddl	Upstream	5.35E+02	3.67E+02	<LOD	4.42E+02	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
yccT	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD

	Downstream	<LOD	<LOD	5.08E+03	<LOD	1.37E+04
ecfX	Upstream	1.09E+03	<LOD	<LOD	<LOD	<LOD
	Downstream	6.21E+02	<LOD	<LOD	<LOD	3.53E+02
gltA	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
secE	Upstream	<LOD	1.39E+04	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	1.02E+04

ND – non-detected; LOD – limit of detection

Appendix M: Antibiotic resistance genes detected in sampling campaigns (cell equivalents/100 mL or cell equivalents/g)

<i>Water samples</i>	Location	22/02	18/03	16/04	16/05	13/06
blaTEM	Upstream	2.41E+03	1.08E+03	2.74E+01	5.49E+01	2.15E+02
	Downstream	1.95E+03	2.17E+04	3.57E+01	1.32E+03	4.33E+03
ermB	Upstream	2.93E+05	1.59E+05	4.14E+03	3.32E+03	6.07E+03
	Downstream	3.27E+05	2.84E+06	9.33E+02	1.45E+05	1.64E+05
sul1	Upstream	2.22E+05	1.56E+05	4.43E+02	1.17E+04	7.97E+03
	Downstream	1.88E+05	1.32E+06	3.27E+02	1.70E+05	4.21E+05
tetM	Upstream	6.12E+03	2.62E+03	2.30E+01	8.48E+01	7.23E+01
	Downstream	5.94E+03	4.31E+04	1.55E+01	1.64E+03	4.42E+03
CMY2	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	9.78E+01	<LOD	<LOD	6.17E+01
CTX-M	Upstream	1.33E+02	<LOD	<LOD	<LOD	<LOD
	Downstream	1.57E+02	3.02E+02	<LOD	1.75E+02	2.92E+01
CTX-M-32	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	1.73E+03	<LOD	3.65E+02	3.28E+02
blaOXA-48	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	1.29E+04	<LOD	<LOD	5.57E+03
mecA	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
blaKPC-3	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
mcr1	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	1.90E+01	<LOD	<LOD	1.06E+01
<i>Sediment samples</i>						
blaTEM	Upstream	7.31E+03	7.49E+04	4.28E+03	5.26E+03	9.16E+03
	Downstream	7.26E+03	1.75E+04	2.78E+03	3.56E+03	1.10E+03
ermB	Upstream	4.64E+06	1.74E+07	2.73E+06	7.27E+05	3.60E+06
	Downstream	6.73E+06	8.10E+06	2.26E+06	1.69E+06	2.99E+05
sul1	Upstream	1.70E+06	1.04E+07	1.21E+06	1.66E+06	3.07E+06
	Downstream	2.03E+06	4.12E+06	1.18E+06	1.21E+06	3.20E+05
tetM	Upstream	4.06E+04	1.62E+05	2.35E+04	1.79E+04	3.32E+04
	Downstream	3.69E+04	5.22E+04	1.55E+04	2.12E+04	2.10E+03
CMY2	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
CTX-M	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD

	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
CTX-M-32	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
blaOXA-48	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
mecA	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
blaKPC-3	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
mcr1	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
<i>Biofilm samples</i>						
blaTEM	Upstream	2.11E+05	2.42E+05	4.68E+03	1.40E+04	1.29E+05
	Downstream	1.95E+05	8.26E+03	4.85E+04	4.82E+04	9.04E+04
ermB	Upstream	5.35E+07	6.94E+07	1.56E+06	8.96E+05	8.88E+07
	Downstream	3.55E+08	4.81E+04	9.23E+07	5.53E+07	6.31E+08
sul1	Upstream	2.47E+07	4.02E+07	1.99E+06	1.16E+07	1.40E+07
	Downstream	6.18E+07	1.24E+04	2.88E+07	3.87E+07	9.59E+06
tetM	Upstream	5.70E+05	7.10E+05	1.97E+04	2.05E+04	5.37E+05
	Downstream	2.12E+06	1.07E+03	4.17E+05	3.84E+05	1.69E+06
CMY2	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
CTX-M	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
CTX-M-32	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
blaOXA-48	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	5.67E+04
mecA	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD
blaKPC-3	Upstream	6.62E+03	<LOD	<LOD	<LOD	2.23E+03
	Downstream	<LOD	4.01E+02	<LOD	<LOD	<LOD
mcr1	Upstream	<LOD	<LOD	<LOD	<LOD	<LOD
	Downstream	<LOD	<LOD	<LOD	<LOD	<LOD

Appendix N: Susceptibility test and MRGN results: Inhibition zones (mm) caused by different antibiotics observed for isolates collected from sediment and biofilm samples collected upstream and downstream of the WWTP. Green circles = susceptible, standard dose regimen; yellow circles = susceptible, increased exposure; red circles = resistant. When no value was displayed, there was no bacterial growth in the Petri dish. Gray cells indicate that no breakpoints were available at EUCAST table for that species. In this case, the isolate was considered to be resistant when the antibiotic did not cause growth inhibition (diameter of the inhibition zone = 0 mm).

Samples	Isolate	Cephalosporin		Fluoroquinolone	Carbapenem		Penicillin	MRGN
		Cefotaxime	Ceftazidime	Ciprofloxacin	Imipenem	Meropenem	Piperacillin	
		5 µg	10 µg	5 µg	10 µg	10 µg	30 µg	
Sediment - Upstream	KEC	17	21	29	27	29	24	0
	KEC	0	0	32	26	26	10	2
	KEC	18	18	32	30	30	20	1
	E. coli	19	21	31	26	24	19	0
	E. coli	22	22	25	31	27	22	0
	E. coli	0	0	33	35	33	26	1
	Acinetobacter	0	0	28	37	36	27	1
	Acinetobacter	-	-	-	-	-	-	-
	Acinetobacter	0	14	30	30	15	16	1
Sediment - Downstream	P. aeruginosa	0	15	28	29	16	15	3
	P. aeruginosa	0	13	28	26	15	18	2
	P. aeruginosa	0	15	25	25	15	18	3
	P. aeruginosa	0	15	29	29	15	20	2
	P. aeruginosa	0	15	28	27	14	18	2
	Acinetobacter	0	0	22	26	12	16	2
	Acinetobacter	-	-	-	-	-	-	-
	Acinetobacter	-	-	-	-	-	-	-
	Acinetobacter	0	0	25	27	9	12	2
	Acinetobacter	-	-	-	-	-	-	-
Biofilm - Upstream	E. coli	0	0	29	36	34	26	1
	E. coli	6	0	30	36	28	26	1
	E. coli	0	0	29	36	30	26	1
	E. coli	-	-	-	-	-	-	-
	E. coli	-	-	-	-	-	-	-
	E. coli	-	-	-	-	-	-	-
	E. coli	-	-	-	-	-	-	-
	E. coli	-	-	-	-	-	-	-
	E. coli	-	-	-	-	-	-	-
	E. coli	-	-	-	-	-	-	-
Biofilm - Downstream	Acinetobacter	0	8	25	30	22	16	1
	Acinetobacter	0	9	24	30	23	12	1
	Acinetobacter	6	8	24	30	24	14	0
	KEC	0	20	36	26	29	0	2
	KEC	0	10	25	32	25	16	2
	KEC	0	19	34	26	28	0	2
	KEC	0	20	32	26	27	0	2
	KEC	0	21	15	28	31	0	3
	KEC	0	18	32	26	26	0	2
	KEC	0	18	32	26	26	0	2

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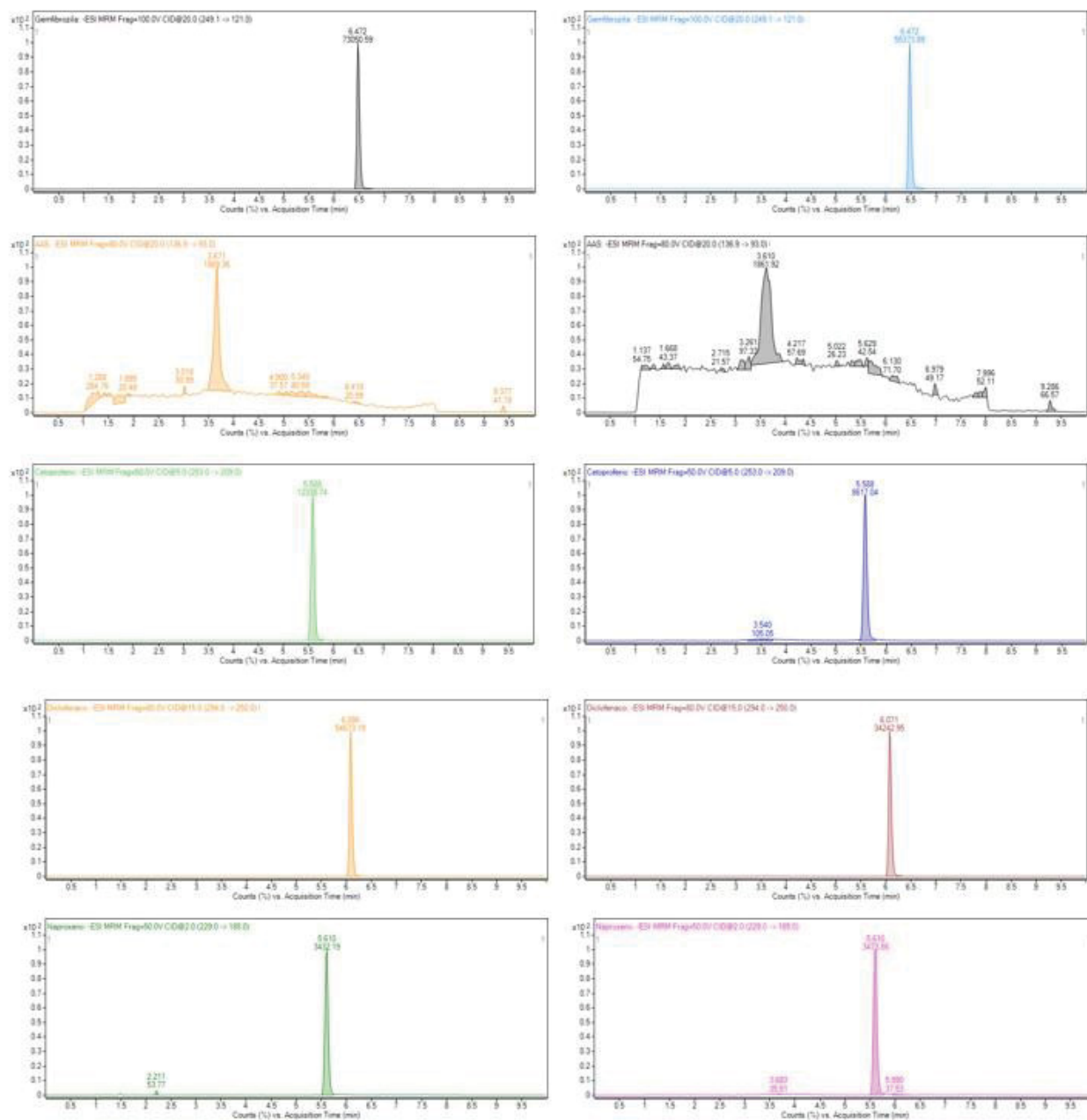
Appendix O: Susceptibility test results: Inhibition zones (mm) caused by different antibiotics observed for isolates collected from sediment and biofilm samples collected upstream and downstream of the WWTP. Green circles = susceptible, standard dose regimen; yellow circles = susceptible, increased exposure; red circles = resistant. When no value was displayed, there was no bacterial growth in the Petri dish. Gray cells indicate that no breakpoints were available at EUCAST table for that specie. In this case, the isolate was considered to be resistant when the antibiotic did not cause growth inhibition (diameter of the inhibition zone = 0 mm).

Samples	Isolate	Fluoroquinolone	Penicillin		Aminoglycoside	Tetracyclin
		Levofloxacin	Piperacillin/ Tazobactam	Temocillin	Amikacin	Tigecycline
		5 µg	30/6 µg	30 µg	30 µg	15 µg
Sediment - Upstream	KEC	29	24	27	21	22
	KEC	30	21	19	21	18
	KEC	26	26	23	20	24
	E. coli	31	24	19	19	21
	E. coli	27	27	22	19	23
	E. coli	32	30	9	23	27
	Acinetobacter	35	33	10	27	32
	Acinetobacter	-	-	-	-	-
Sediment - Downstream	Acinetobacter	25	0	0	21	16
	P. aeruginosa	23	20	0	18	9
	P. aeruginosa	23	21	0	20	14
	P. aeruginosa	21	21	0	19	15
	P. aeruginosa	23	22	0	19	14
	P. aeruginosa	23	24	0	20	14
	Acinetobacter	20	21	0	8	23
	Acinetobacter	-	-	-	-	-
	Acinetobacter	-	-	-	-	-
	Acinetobacter	18	17	0	0	23
Biofilm - Upstream	Acinetobacter	-	-	-	-	-
	E. coli	28	32	8	26	26
	E. coli	28	31	8	24	27
	E. coli	24	31	8	26	25
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
Biofilm - Downstream	Acinetobacter	26	24	0	22	18
	Acinetobacter	25	24	0	24	19
	Acinetobacter	24	22	0	24	18
	KEC	32	21	28	21	24
	KEC	28	22	0	23	19
	KEC	28	26	25	19	20
	KEC	26	24	26	19	22
	KEC	30	24	26	18	20
	KEC	30	24	26	17	15
	KEC	30	24	26	17	15

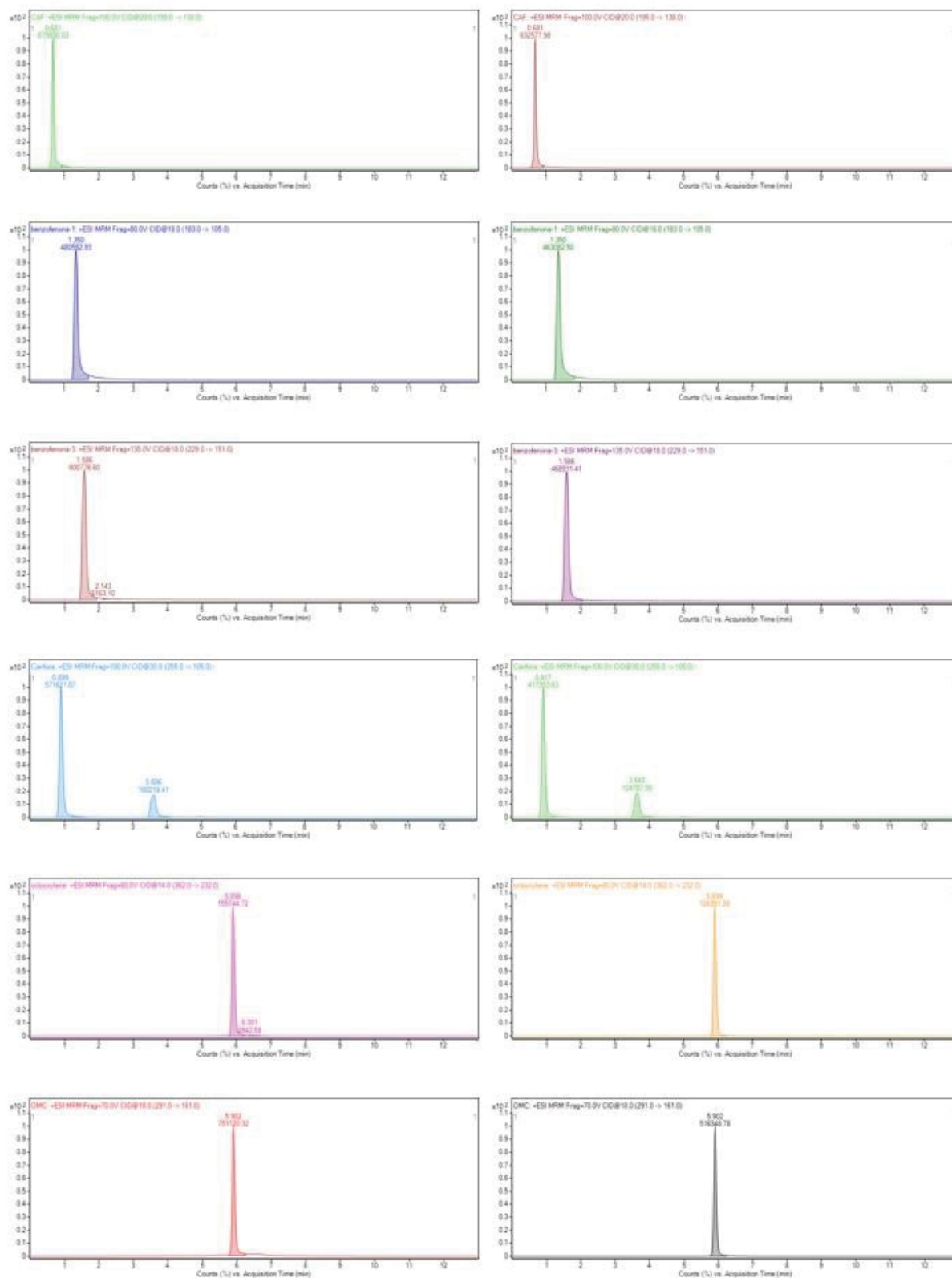
Appendix P: Susceptibility test results: Inhibition zones (mm) caused by different antibiotics observed for isolates collected from sediment and biofilm samples collected upstream and downstream of the WWTP. Green circles = susceptible, standard dose regimen; yellow circles = susceptible, increased exposure; red circles = resistant. When no value was displayed, there was no bacterial growth in the Petri dish. Gray cells indicate that no breakpoints were available at EUCAST table for that specie. In this case, the isolate was considered to be resistant when the antibiotic did not cause growth inhibition (diameter of the inhibition zone = 0 mm).

Samples	Isolate		Sulfonamide	Polymyxine		Epoxide-Antibiotic
		Chloramphenicol	Trimethoprim/ Sulfomethoxazol	Colistin	Colistin	Fosfomycin
		30 µg	1.25/23.75 µg	10 µg	25 µg	200 µg
Sediment - Upstream	KEC	21	31	15	18	17
	KEC	24	30	14	17	17
	KEC	22	34	15	17	25
	E. coli	20	30	16	19	10
	E. coli	28	38	16	18	25
	E. coli	35	27	0	0	14
	Acinetobacter	36	30	0	0	15
	Acinetobacter	-	-	-	-	-
	Acinetobacter	11	10	14	18	0
Sediment - Downstream	P. aeruginosa	0	10	15	17	0
	P. aeruginosa	0	9	14	16	8
	P. aeruginosa	0	0	14	16	9
	P. aeruginosa	0	11	14	18	9
	P. aeruginosa	0	9	14	16	9
	Acinetobacter	-	26	-	-	-
	Acinetobacter	-	-	-	-	-
	Acinetobacter	-	-	-	-	-
	Acinetobacter	-	23	-	-	-
Biofilm - Upstream	E. coli	34	26	0	0	13
	E. coli	33	25	0	0	16
	E. coli	35	28	0	0	15
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
	E. coli	-	-	-	-	-
Biofilm - Downstream	Acinetobacter	7	27	13	16	9
	Acinetobacter	6	27	13	17	6
	Acinetobacter	6	27	14	16	8
	KEC	27	34	16	19	18
	KEC	7	26	15	18	8
	KEC	28	33	16	17	19
	KEC	25	31	16	19	19
	KEC	27	30	14	16	18
	KEC	26	32	14	18	16

Appendix Q: Solvent (first column) and real water matrix (second column) chromatograms of the analyzed contaminants at the same concentration level - Pharmaceuticals



Appendix R: Solvent (first column) and real water matrix (second column) chromatograms of the analyzed contaminants at the same concentration level – UV filters and caffeine



Appendix S: Concentrations of emerging contaminants in water samples ($\mu\text{g L}^{-1}$)

Contaminants	C1 – 22 February		C3 – 26 April	
	BA 1	BA 2	BA1	BA2
GFZ	<LOD	<LOD	<LOD	<LOD
AAS	<LOD	<LOD	<LOQ	<LOQ
KET	<LOQ	0.17	<LOD	<LOD
DIC	<LOD	<LOD	<LOD	<LOQ
NAP	<LOD	<LOQ	<LOD	<LOD
CAF	0.17	0.32	0.10	0.18
BP-1	0.05	0.07	0.02	0.04
BP-3	0.03	0.04	0.02	0.02
OC	<LOD	<LOQ	<LOQ	<LOQ
OMC	<LOD	<LOD	<LOQ	<LOQ
4MBC	<LOQ	0.072	<LOQ	0.016