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ÉCOLE DOCTORALE GAY LUSSAC

STUDY OF THE ENVIRONMENTAL CONTAMINATION OF HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

DOCTORAL THESIS IN JOINT SUPERVISION

Marília Camotti Bastos

Santa Maria, RS, Brasil 2017

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Présentée par :

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STUDY OF THE ENVIRONMENTAL CONTAMINATION BY HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

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STUDY OF THE ENVIRONMENTAL CONTAMINATION BY HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

Tese em cotutela apresentada ao Programa de Pós-Graduação em Ciência do Solo da Universidade Federal de Santa Maria e a École Doctorale Gay Lussac da Université de Poitiers, como requisito parcial para obtenção do título de **Doutora em Ciência do Solo** (UFSM) e **Chimie théorique, physique, analytique** (Univ. Poitiers).

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UNIVERSIDADE FEDERAL DE SANTA MARIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DO SOLO

UNIVERSITÉ DE POITIERS

ECOLE DOCTORALE GAY LUSSAC

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STUDY OF THE ENVIRONMENTAL CONTAMINATION OF HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

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Advisor at UFSM: Danilo Rheinheimer dos Santos
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Dedico essa tese aos meus amados pais,
Lucimar Aparecida Camotti Bastos e
Paulo Henrique Wiedmer Bastos.

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elaborated by Marília Camotti Bastos

as a partial requirement for the degree of Doctor in Soil Science and Chemistry, specialization in Theoretical, Physical and Analytical Chemistry

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RESUMO

Tese de doutorado em co-tutela
Programa de Pós Graduação em Ciência do Solo – UFSM - RS - Brasil
École Doctorale Science pour l'Environnement Gay Lussac – Université de Poitiers, France

STUDY OF THE ENVIRONMENTAL CONTAMINATION OF HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

Orientador no Brasil : Danilo Rheinheimer dos Santos
Orientadores na França : Jérôme Labanowski, Laurent Caner

O sul do Brasil é uma área agrícola, que tem experimentado forte crescimento de sua produção tanto animal quanto grãos. A intensificação das práticas agrícolas assim como o aumento da urbanização na região geram alta pressão antropica nos ambientes aquáticos e nos solos. Este trabalho tem o objetivo de melhor entender o impacto relacionado com o uso e a descarga de compostos farmacêuticos (humano ou veterinário) nos rios e solos através do exemplo da bacia hidrográfica do Rio Guaporé.

O estudo do solo mostrou contaminação das áreas agricolas por produtos farmacêuticos, onde a utilização de estrume está emergindo como uma importante fonte de contaminação e genes de resistência a antibióticos. As compostos detectadas variaram dependendo do tipo de uso do solo e do tipo de esterco usado como fertilizante. A erosão significativa sofrida pelos solos nesta região e sua lixiviação podem, então, promover o transporte destes compostos até ambientes aquáticos. O estudo dos ambientes aquáticos através de biofilmes epilíticos e amostradores passivos (POCIS) mostraram que áreas de maior produção agrícola são os mais contaminadas. Também foi destacada a elevada contaminação causada pelas áreas urbanas. Estes resultados devem estar relacionados com a falta de saneamento urbano na região. A natureza e extensão da contaminação são influenciados por variações sazonais. Além disso, a utilização de sucralose e carbamazepina como traçadores de atividade antrópica provou ser promissora quando avaliada sua presença em biofilmes e POCIS.

Palavras chave: Biofilme, POCIS, solo, antibioticos, agricultura, resistência, dejetos urbanos.

RÉSUMÉ

Thèse de Doctorat en Cotutelle
Pos Graduation en Science du Sol – Université Fédérale de Santa Maria - RS - Brésil
École Doctorale Science pour l'Environnement Gay Lussac – Université de Poitiers, France

STUDY OF THE ENVIRONMENTAL CONTAMINATION OF HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

Directeur thèse au Brésil : Danilo Rheinheimer dos Santos
Directeurs thèse en France : Jérôme Labanowski, Laurent Caner

Le sud du Brésil est une région agricole qui connait une forte croissance de sa production tant au niveau céréalier qu'animal. L'intensification des pratiques agricoles ainsi que l'urbanisation grandissante dans cette région génèrent également une forte pression anthropique pour les milieux aquatiques et les sols. Le présent travail vise à mieux comprendre l'impact lié à l'utilisation puis au rejet de composés pharmaceutiques (humain ou vétérinaire) sur les rivières et les sols à travers l'exemple du bassin versant du Rio Guaporé. L'étude des sols a montré une contamination par les composés pharmaceutiques dans les zones agricoles, où l'épandage de lisiers apparait comme une source importante de contamination et de gènes de résistance aux antibiotiques. Les molécules détectées varient selon le type d'exploitation des sols et les épandages effectués. L'érosion importante que subissent les sols de cette région et leur lessivage par les pluies peuvent favoriser ensuite le transport de ces contaminants vers les milieux aquatiques. L'étude des milieux aquatiques, à travers le biofilm épilithique et les POCIS, ont permis de montrer que les zones de plus forte production agricole sont les plus contaminées. Une forte contamination par les zones urbaines a été cependant aussi mise en évidence. Ces résultats sont à mettre en relation avec l'absence de réseaux d'assainissement urbains dans cette région. La nature et l'ampleur de la contamination sont influencés par les variations saisonnières. L'utilisation de la sucralose et de la carbamazépine comme traceurs de l'activité anthropique s'est avérée prometteuse de même que l'usage des biofilms et des POCIS comme échantillonneurs.

Mots clés: Biofilm, POCIS, sol, antibiotiques, agriculture, résistance, déchets urbains

ABSTRACT

Doctoral thesis in co-supervision
Graduate program in soil science – Federal University of Santa Maria, Brazil
Graduate school environmental science Gay Lussac – University of Poitiers, France

STUDY OF THE ENVIRONMENTAL CONTAMINATION OF HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

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Thesis advisor in France: Jérôme Labanowski, Laurent Caner

Southern Brazil is an agricultural region that is experiencing a strong growth in both cereal and animal production. The intensification of agricultural practices as well as the growing urbanization in this region also generates a strong anthropic pressure for the aquatic environments and the soils. This work aims to better understand the impact of use and release of pharmaceutical compounds (human or veterinary) on rivers and soils through the example of the Rio Guaporé watershed. Soil studies have shown contamination by pharmaceutical compounds in agricultural areas where spreading of manure appears to be an important source of contamination and antibiotic resistance genes. The compounds detected vary according to the type of land use and the spreading. The significant erosion of soils in this region and their leaching by rainfall can then promote the transport of these contaminants to aquatic environments. The study of aquatic environments, through epilithic biofilm and POCIS, showed that areas with the highest agricultural production are the most contaminated. However, high contamination by urban areas has also been highlighted. These results are related to the absence of urban sanitation networks in this region. The nature and extent of contamination are influenced by seasonal variations. The use of sucralose and carbamazepine as tracers of antropic activity proved promising when its presence was evaluated in biofilms and POCIS samplers.

Palavras chave: Biofilm, POCIS, sol, antibiotics, agriculture, resistance, urban waste

RÉSUMÉ LONG

STUDY OF THE ENVIRONMENTAL CONTAMINATION OF HUMAN AND VETERINARY MEDICINES IN THE SOUTH BRAZIL

1. INTRODUCTION ET CONTEXTE

La préservation de l'eau douce est un grand défi pour le maintien de la qualité des écosystèmes et l'approvisionnement de la population humaine. L'apparition de divers contaminants organiques a été signalés dans les eaux de surface dans le monde entier, provenant d'activités agricoles (ex. Produits chimiques, les médicaments vétérinaires et l'utilisation des pesticides) et urbain (ex:. Médicaments et produits de beauté). Souvent, ces produits sont trouvés en faibles concentrations ($\mu g\ L^{-1}$, $ng\ L^{-1}$) ce qui rend son identification parfois difficile. Dans la dernière décennie, les chercheurs ont travaillé à trouver un moyen pratique, efficace et peu coûteux d'identifier la pollution dans les cours d'eau, permettant de compléter les données fournies par les prélèvements ponctuels.

Des dispositifs d'échantillonnage passif ont été développés et sont en cours de test. Ces dispositifs ont un grand potentiel pour la détection en raison de la concentration très faibles des composés organiques présents dans l'eau. L'Echantillonneur Intégratif de Composés Organiques Polaire (POCIS) est un nouveau dispositifs largement utilisé capable de traiter les principaux problèmes associés à la surveillance de divers composés chimiques organiques polaires toxiques dans l'environnement, qui sont d'intérêt pour le monde entier. Cependant, l'utilisation de ces échantillonneurs ne reflète pas les interactions qui existent avec les organismes vivants des écosystèmes aquatiques.

Récemment, les biofilms ont été étudiés comme un nouveau réseau d'adsorbant naturel des produits pharmaceutiques. Ce sont des structures biologiques formées par les bactéries, les algues, les champignons et la microfaune en contact étroit, physique et chimique, noyées dans une matrice de polymères extracellulaires. Capable de capturer et d'accumuler des composés pharmaceutiques dans les systèmes aquatiques, les biofilms sont performants voire plus efficace que l'analyse spécifique de l'eau et sont moins chers que l'analyse d'un échantillonnage continu de l'eau en raison du grand volume d'eau nécessaire ou des échantillonneurs passifs commerciaux. En outre, le développement de la résistance génétique aux antibiotiques présents dans les biofilms peut être suivi, car les microorganismes constituant le biofilm sont en contact continu avec ces composés. Un des intérêts majeurs de l'étude de la contamination des biofilms est qu'ils sont parmi les premiers organismes à entrer en contact avec des contaminants dans les écosystèmes aquatiques et sont donc aussi les premiers organismes à être impactés par l'exposition à des contaminants. En outre, les biofilms font partie de la chaîne alimentaire en tant que producteurs primaires et par bioamplification trophique peuvent transférer à les effets chroniques ou aigus de la présence de ces composés bioconcentrés et avoir des impacts sur la chaîne alimentaire et conséquence direct de leur place dans la chaîne trophique.

Ainsi, l'utilisation intensive des médicaments vétérinaires dans la production animale peut contribuer à la dégradation des écosystèmes aquatiques. Les déchets animaux contaminés par des antibiotiques, qui peuvent être utilisés dans le cadre d'amendement de sols agricoles peuvent constituer une source importante de contamination des milieux aquatiques notamment des régions où la gestion des sols favorise l'érosion de ces sols, le ruissel des eaux plutôt que leur infiltration et donc un transfert important de matériaux vers les rivières. Dans le bassin de la rivière Guaporé, représentant du modèle agricole au sud du Brésil, ces phénomènes d'érosion des sols cultivés sont la source de 90% des sédiments en suspension, ainsi que le taux élevé de débit d'eau est de 31%.

1.1. Hypothèse

Les zones urbaines sans traitement des eaux usées et les zones rurales avec l'application des déchets animaux comme engrais sont sources de pollution et entraînent le développement de la résistance bactérienne aux antibiotiques dans les sols et les ressources en eau dans les bassins versants agricoles dans le sud du Brésil.

1.2. Objectifs

Le but de ce travail est de générer des informations sur la contamination de l'environnement par des composés pharmaceutiques à usage humain et vétérinaire dans les sols, l'eau et ses effets sur les organismes en contact avec ces molécules dans les bassins versants agricoles dans le sud du Brésil. Cette étude vise à contribuer à la connaissance sur la façon dont cette contamination se produit et les risques engendrés pour l'environnement et les êtres vivants.

2. MATERIELS ET METHODES

Le bassin de la rivière Guaporé est situé dans le nord-est de l'état du Rio Grande do Sul. L'agriculture est l'activité principale pour la plupart des résidents ruraux de cette région, les zones urbaines étant par ailleurs très limitées. L'occupation des sols agricoles est tournée principalement pour l'alimentation des animaux (vaches laitières, porcs et volailles) et la culture de céréales (principalement le soja, le maïs et le blé). La partie supérieure du bassin a des sols profonds et l'agriculture est principalement réalisée dans des systèmes sans labour. Dans les parties médianes et inférieures du bassin versant les terrains présentent un relief marqué, les sols sont peu profonds et l'activité principale est la culture du tabac par les exploitations familiales, de quelques hectares seulement. Les sols de ce bassin sont exposés à l'érosion et une perte de matériaux élevée.

Pour cette études de la contamination de l'environnement ont été échantillonnés: des sols, des biofilm, des eaux et des échantillonneurs intégratifs de type POCIS ont été placés. Les échantillons ont été prélevés dans quatre sous-bassins (sous-bassin Marau sous-bassin Capingui, sous-bassin Lajeado Carazinho, et sous-bassin Carazinho), le choix des sites d'échantillonnage définis en fonction de leur contamination.

Les composés sélectionnés pour les analyses ont été définies d'après une étude réalisée chez les agriculteurs dans le bassin versant du Guaporé, à savoir: le sucralose, la carbamazépine, le diclofénac, la sulfaméthazine, le sulfaméthoxazole, la sulfaquinoxaline, la norfloxacine, la ciprofloxacine, l'enrofloxacine, la lévofloxacine, l'érythromycine,

roxithromycine, tylosine et oxytétracycline. De plus, la résistance bactérienne aux antibiotiques a été analysés à travers l'étude de l'abondance des gènes *sul1* et *erm*.

2. 3. PRINCIPAUX RÉSULTATS

3.1. marqueurs anthropiques et l'utilisation de POCIS et biofilms

L'utilisation de marqueurs antropiques a permis de confirmer la conta humaine du bassin de la rivière Guaporé. Le bassin est anthropisé dans toute sa longueur, et l'absence de traitement des eaux usées dans les zones urbaines et les rejets des déchets ménagers sont effectués directement dans les rivières, ce qui constitue un facteur aggravant de la contamination des bassins étudiés. La proximité des zones urbaines a un fort impact sur la quantité des composés SCR et CBZ dans les systèmes aquatiques. De plus, des sites qui ont été considérés comme hors de portée de la contamination humaine, comme FLONA ont également montré la présence de composés marqueurs des activités humaines. Ainsi, un aperçu de la contamination du bassin Guaporé a montré que les zones les plus touchées sont situées dans la partie nord du bassin, en particulier en raison de la présence de la ville de Marau. Ainsi, il est possible de confirmer que les activités humaines sont en interaction avec les ressources en eau du bassin du Guaporé et que les biofilms et POCIS sont en mesure d'identifier les zones les plus impactées par les activités humaines. Les deux techniques sont complémentaires et ne peuvent être omises. Le temps d'exposition ne sont pas les mêmes pour les deux procédés qui signifie que les échantillons ne sont pas similaires. En raison de la spécificité de chaque composé, divers types d'interactions peuvent se produire entraînant plus ou moins d'adsorption de ces composés. En général, les biofilms peuvent être utilisés pour identifier les sites contaminés par la présence de SCR (marqueur d'activités humaines) et les POCIS sont capables de capturer CBZ avec une grande efficacité et à la différence des biofilms.

3.2. Les biofilms épilithiques comme indicateurs de contamination de l'environnement

Grâce à l'utilisation de biofilms était possible de vérifier que, en général, le bassin de la rivière Guaporé est polluée par des médicaments humains et vétérinaires. La concentration de ces composés dans le biofilm étaient très semblables à d'autres études menées dans le monde entier. Toutefois, le principal problème du bassin de la rivière Guaporé est la contribution générée par la ville de Marau. Les biofilms échantillonnés en aval de la ville présentent des concentrations de médicaments et des niveaux de résistance aux antibiotiques très supérieurs aux autres points d'échantillonnage, mettant l'accent sur la grande pollution potentielle d'une ville avec 37,145 habitants dépourvue de traitement des eaux usées.

Même si l'agriculture est une source de contamination comme le montre notamment le point en aval de la zone de production de tabac, la contribution de la ville Marau est beaucoup plus élevé. Ce résultat est en partie positif, puisque la pollution diffuse causée par l'agriculture est beaucoup plus compliquée à résoudre que l'installation d'un système d'égouts avec une station d'épuration des eaux usées dans une ville. En particulier, dans un bassin où l'agriculture est la principale forme d'utilisation des terres. Même si beaucoup de sensibilisation est faite auprès des agriculteurs locaux, la facilité d'offrir un bon service public pour les résidents est beaucoup plus facile à réaliser avec une bonne gestion des ressources municipales.

Ainsi, il a été vérifié que les biofilms épilithiques sont de robuste indicateurs on de la présence de polluants et le développement de la résistance bactérienne résultant de la pression anthropique locale. De plus, même si la relation entre les concentrations de médicament et la quantité du gène de résistance bactérienne n'a pas été obtenue, les deux sont individuellement fiables pour identifier les problèmes de pollution de l'environnement.

3.3. Epandage du fumier animal contaminé par des médicaments vétérinaires sur le terrain et leur contribution à la contamination de l'environnement

Au Brésil, les sols agricoles sont fertilisés annuellement avec des quantités très élevées de fumier (bovins et porcs) et de lit aviaire. Ce fumier largement épandu est la source des médicaments vétérinaires et des bactéries résistantes aux antibiotiques dans les sols agricoles. Dans ce travail de thèse, l'utilisation du lisier de porc était en lien avec de plus grandes concentrations de médicaments vétérinaires trouvés dans les sols agricoles, alors que le pâturage semble contribuer au développement de la résistance bactérienne. Les valeurs de résistance et les concentrations de médicaments obtenues dans cette étude sont similaires à celles obtenues dans d'autres études de sols agricoles ou forestiers dans d'autres régions du monde.

4. CONCLUSION

Cette thèse donne un aperçu de la contamination de l'environnement par les médicaments à usage humains et vétérinaires dans les sols et les eaux du bassin versant de la rivière Guaporé. Les résultats ont permis de réaliser une cartographie initiale de la contamination du bassin et ont permis d'identifier les zones les plus contaminées. Ainsi, il semble que les niveaux de contamination peuvent varier considérablement dans différentes conditions d'utilisation des terres, en particulier lorsqu'elles se rapportent à des zones agricoles et urbaines.

Des études dans les milieux aquatiques à proximité des zones agricoles ont montré que les niveaux de contamination par les antibiotiques sont plus faibles que dans les zones urbaines. Dans les zones agricoles, la contamination des biofilms est révélatrice des différences entre la haute technologie et faible utilisées par les agriculteurs. Un autre point fort de ce travail était la capacité de montrer comment la perte de sols contaminés par l'érosion dans les systèmes moins conservationnistes peut entraîner une contamination ultérieure des biofilms.

Des pratiques agricoles autres pourraient aider les agriculteurs, car le bassin de la rivière Guaporé beaucoup d'entre eux utilisent le système de plantation directe sans l'utilisation de terrasses pour ralentir l'eau. Un autre facteur important est l'utilisation des déchets animaux sans tenir compte des besoins du sol, ce qui entraîne souvent l'application d'un grand volume de fumier. Cette pratique favorise la dispersion de grandes quantités de médicaments et contribuent à l'érosion des sols.

Cette étude a montré aussi la possibilité d'utiliser biofilms comme indicateur de la pollution humaine, en particulier dans les zones qui ont des contributions importantes de la pollution urbaine, par exemple. Marau. Ces résultats constituent une aide à la sensibilisation de la pollution urbaine, car les Brésiliens ont une grande distance entre l'affichage de leur participation en tant que pollueur, en particulier lorsque la ressource est l'eau. Ces résultats sont un moyen de montrer que comme Marau, plusieurs villes brésiliennes ne possèdent pas encore réseau de traitement des eaux usées. Les risques ne sont pas seulement d'avoir des

ressources en eau potable de mauvaise qualité, mais aussi une incidence sur les organismes vivants dans le sol et l'eau en raison du développement de la résistance aux antibiotiques. Comme biofilms sont un maillon de base de la chaîne alimentaire, une fois contaminés, ils peuvent commencer le processus de bioaccumulation et peuvent contaminer les animaux supérieurs, voire les humains. De plus, plusieurs études menées avec l'intention de contrôler les problèmes de résistance bactérienne ont montré l'émergence de « super bactéries » présentant de multiples résistances aux antibiotiques.

Dans les sols, même si les concentrations des composés étudiés est relativement plus faible que les biofilms, les niveaux de résistance bactérienne mesurés sont élevés. Ainsi, l'utilisation intensive de fumier et de litière aviaire pour la correction de la fertilité du sol semble être un contexte favorable au développement et la diffusion de la résistance. En ce sens, des études supplémentaires sont nécessaires pour voir dans quelle mesure l'évolution des pratiques pourrait limiter ce risque en assurant la production agricole.

Il est prévu que les résultats de cette thèse servent d'avertissement de la nécessité de la recherche sur l'impact de l'homme sur l'environnement. Plus que cela, la nécessité d'étudier la pollution qui ne sont pas visibles à l'œil nu. Enfin, le problème existe et doit faire partie des discussions à venir sur la zone qui comprend le bassin du Rio Guaporé car elle est représentative d'autres régions du Brésil.

TABLE SUMMARY

FIGURE SUMMARY

SUMMARY

THESIS PRESENTATION

This thesis was developed in the Guapore River watershed, located in the state of Rio Grande do Sul, in Brazil. One of the future challenges in this region, as elsewhere in Brazil, is to avoid the emergence of sanitary risks induced by the pollution of waters and soils. The present work focused on pharmaceuticals residues that constitute emerging contaminants worldwide. Indeed, these contaminants are, to date, neglected in Brazil despite their wide utilization in agriculture and in human medicine. However, pharmaceuticals residues are toxic compounds for many animals species or can favor antibiotic resistance when they enter, persist and disseminates in the environment. Furthermore, these compounds could represent a serious risk especially in Brazil considering the local specificities : 1) many farms using large amounts of antibiotics or anti-inflammatory drugs for animal growth, 2) intensive crops production using animal wastes and manures - containing pharmaceutical residues, 3) urban or rural areas spreading directly wastewaters - without treatment - in the rivers. For this reason, a study should be done to evaluate the pharmaceutical pollution and to identify the main source. It is expected that the data obtained in this study could be an incentive for the financing of national research related to environmental problems caused by emerging contaminants that, to date, are neglected in Brazil.

Chapter 1 presents an overview of the knowledges on the contamination of pharmaceuticals, especially antibiotics, and the related risks of antibioresistance in Brazil. This chapter was written in Portuguese and published in the online book “Manejo e conservação do solo e da água em pequenas propriedades rurais no sul do Brasil: impacto das atividades agropecuárias na contaminação do solo e da água”.

Chapter 2 contains the material and methods sections of this work.

Chapter 3 aimed to use two anthropic markers (carbamazepine and sucralose) to identify the impact of urban area on river. For this purpose, epilitic biofilms and passive samplers (POCIS) were chosen to determine the contamination levels in sub-watersheds of the Guaporé River.

Chapter 4 uses epilitic biofilms to determine the impact areas of urban and agricultural pressures in the Guaporé River. The results of several sampling campaigns (two seasons) are used to discuss the quantities of pharmaceuticals and the bacterial resistance observed.

Given the large number of results presented in this chapter, it will not be presented as an article, due the need to better explain the data. Given the results obtained from drug

pollution and bacterial resistance in the agricultural areas, doubts arose as to how much the rural soils could be contaminated. Thus, **Chapter 5** was constructed aiming to know if pollution resulting from different types of animal production (dairy cow, swine and poultry) act on the supply of pharmaceuticals and development of resistance in soils. This study was possible because the Guaporé River Watershed has been monitored for many years by the team of Professor Danilo Rheinheimer dos Santos that monitors the erosion of agriculture soils to the rivers.

CHARPTER 1:CONTAMINAÇÃO DO SOLO E DA ÁGUA COM MEDICAMENTOS VETERINÁRIOS

RESUMO

Diante de um panaroma nacional e mundial de crescimento na produção animal, o mercado de produtos para a saúde animal nos últimos anos aumentou suas vendas. As atividades antrópicas atuam como base do aumento da presença desses contaminantes no meio ambiente que, em sua maioria, derivam de fontes pontuais e difusas atingindo diferentes matrizes ambientais. Os impactos relacionados ao uso desses compostos, frente as diferentes rotas de contaminação ambiental, transformações e destino final, são importantes para o conhecimento dos potenciais ambientais de contaminação. A persistência dos medicamentos veterinários em diferentes ambientes influencia especialmente os organismos vivos que se desenvolvem nessas zonas contaminadas e através da cadeia trófica atingem outros organismos vivos, como os seres humanos. Além disso, o desenvolvimento de estirpes bacterianas resistentes tem resultado em um elevado número de mortes no mundo representando risco a saúde humana e animal. Assim, através desse capitulo propomos uma síntese do estado atual do uso de medicamentos veterinários, suas rotas e transformações no ambiente, os riscos ambientais dessa prática e as formas de análise utilizadas para sua determinação no meio ambiente.

Palavras chave: contaminação, meio ambiente, resistência

ABSTRACT

In face with a national and worldwide panorama of increase in animal production, the market for animal health products in recent years had increase in sales. Anthropogenic activities act as a basis for increasing the presence of these contaminants in the environment, which, for the most part, derive from point and diffuse sources reaching different environmental matrices. The impacts related to the use of these compounds, considering the different routes of environmental contamination, transformations, and final destination, are important for the knowledge of the environmental potential of contamination. The persistence of veterinary medicinal products in different environments influences especially the living organisms that develop in these contaminated zones and through the trophic chain reach other living organisms, such as humans. In addition, the development of resistant bacterial strains has resulted in a high number of deaths worldwide posing a risk to human and animal health. Thus, through this chapter, we propose a summary of the current state of the use of veterinary drugs, their routes and changes in the environment, the environmental risks of this practice and the forms of analysis used for their quantification and qualification in the environment

Keywords: contamination, environment, resistance

Capítulo "*Contaminação do solo e da água com medicamentos veterinários*" do livro "*Manejo e conservação do solo e da água em pequenas propriedades rurais no sul do Brasil: impacto das atividades agropecuárias na contaminação do solo e da água*" disponivel em: http://www.fw.uri.br/NewArquivos/publicacoes/publicacoesarquivos//254.pdf.

1. INTRODUÇÃO

No século XIX a procura por princípios ativos presentes em plantas medicinais caracterizou o surgimento dos primeiros medicamentos. O pesquisador alemão Paul Erlich (1854 - 1915), ganhador do prêmio Nobel de Medicina e Fisiologia em 1908, estabeleceu um dos principais conceitos que permitiu o avanço da produção dos medicamentos ao afirmar que a ação de um medicamento somente é possível se houver sua ligação a um sítio específico (*"Corpora Non Agunt Nisi Fixate")*. No século XX, grandes descobertas casuais, como a penicilina e a sulfonamida, por Alexandre Fleming em 1928, vieram marcar a história da humanidade, especialmente pelo seu uso durante a segunda guerra mundial, salvando a vida de milhares de pessoas. Por consequência dessas descobertas, a indústria farmacêutica e suas corporações multinacionais estabeleceram relações importantes entre os institutos de pesquisa e as universidades em diversos países da Europa e Estados Unidos, dando início a descobertas e o desenvolvimento de novos produtos (Calixto e Siqueira Junior, 2008).

Hoje, estão disponíveis no mercado medicamentos com diferentes classes estruturais variando consideravelmente suas estruturas moleculares e propriedades físico-químicas (Thiele-Bruhn, 2003). Um exemplo são os produtos veterinários, administrados em todo o mundo para prevenção, diagnóstico, cura ou tratamento das doenças dos animais. No ano de 2014, a indústria mundial de saúde animal faturou 23,9 bilhões de dólares, com crescimento real de mais de 4,0% para o setor. As Américas e a Europa respondem por aproximadamente 80% do consumo mundial de medicamento veterinarios, participando os produtos farmacêuticos, biológicos e aditivos alimentares com 62%, 26% e 12% do volume total vendido, respectivamente (Sindicato nacional de produtos de saúde animal, 2017).

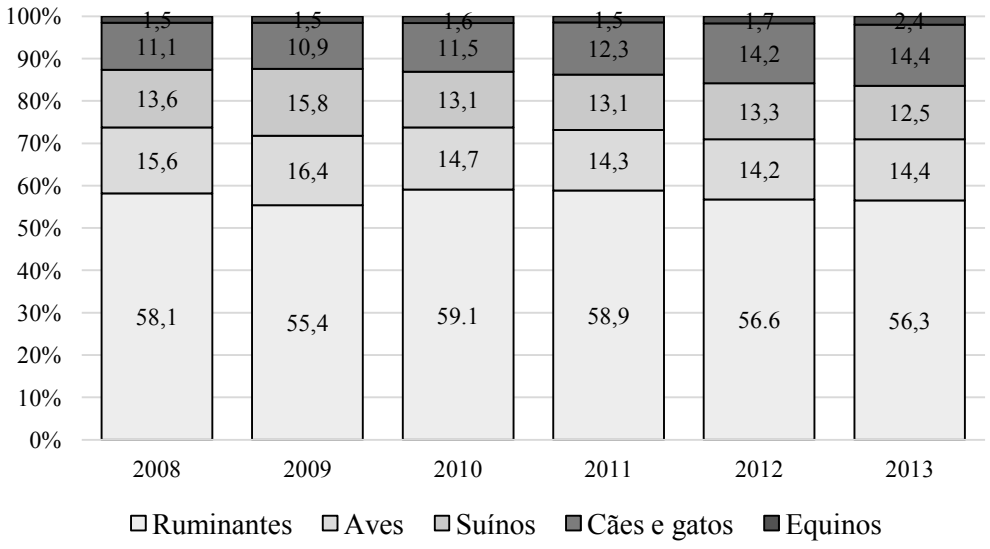
A fiscalização e a regularização desses produtos no Brasil são realizadas pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) através da lei n º 467, de 13 de fevereiro de 1969, em que foi estabelecida, no artigo 1º, a obrigatoriedade da fiscalização da indústria, do comércio e do emprego de produtos de uso veterinário, em todo o território nacional. Desses produtos de uso veterinário, o Brasil possui 6652 produtos autorizados para comercialização (Ministério da agricultura, pecuaria e abastecimento, 2013), destacando-se entre eles antibióticos e produtos de combate a ectoparasitas devido à grande expressividade do agronegócio no país (Regitano e Leal, 2010). Nos anos de 2010 e 2011, as criações de bovinos, suínos e aves contribuíram com mais de 90% da produção nacional de carnes (Tabela 1), onde a região sul consumiu mais de 80% dos medicamentos comercializados no país (Figura 1). Entre os anos de 2008 e 2013, o faturamento nacional da indústria brasileira

de produtos para a saúde animal aumentou 1,443 milhões de reais (Figura 2), sendo o grupo dos medicamentos biológicos e anti-parasitas responsáveis por mais de 50% das vendas nacionais (Figura 3) (Sindicato nacional de produtos de saúde animal, 2017).

Chapter 1 - Tabela 1- Média da população animal dos anos de 2010 e 2011 das regiões do Brasil

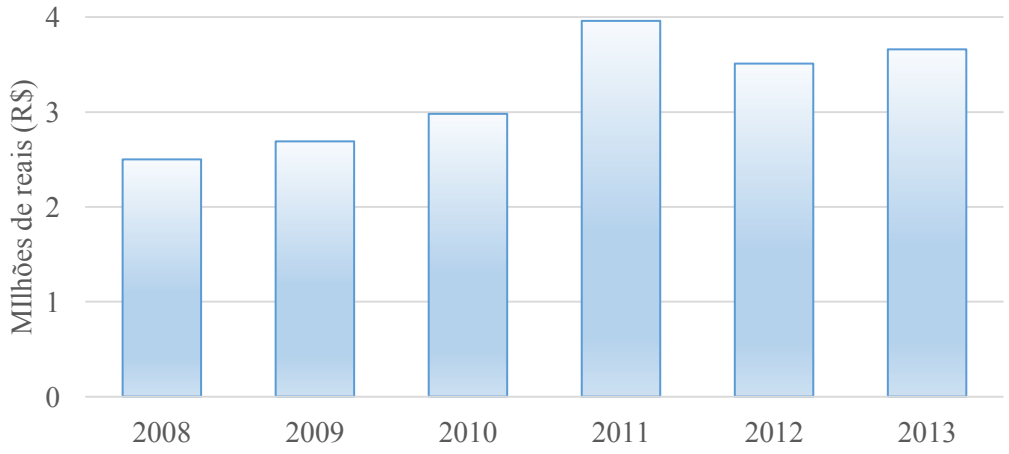
Criação	Norte	Nordeste	Sudeste	Sul	Centro Oeste	Brasil
	Número de animais (milhões)					
Bovinos	39,314	30,369	33,509	24,972	54,961	183,127
Suínos	1,669	6,801	6,770	18,459	4,591	38,292
Ovinos	0,586	9,857	0,781	4,886	1,268	17,380
Caprinos	0,164	8,458	0,233	0,343	0,113	9,312
Equinos	0,736	1,367	1,359	0,926	1,125	5,514
Matrizes aves de corte	0,189	3,030	13,303	27,174	5,855	49,556
Pintos de corte	93,281	521,057	1.434,856	3,489	689,449	6.227,853
Aves poedeiras	3,126	13,110	42,052	16,871	8,612	83.769

Fonte: Sindicato nacional de produtos de saúde animal (2017).



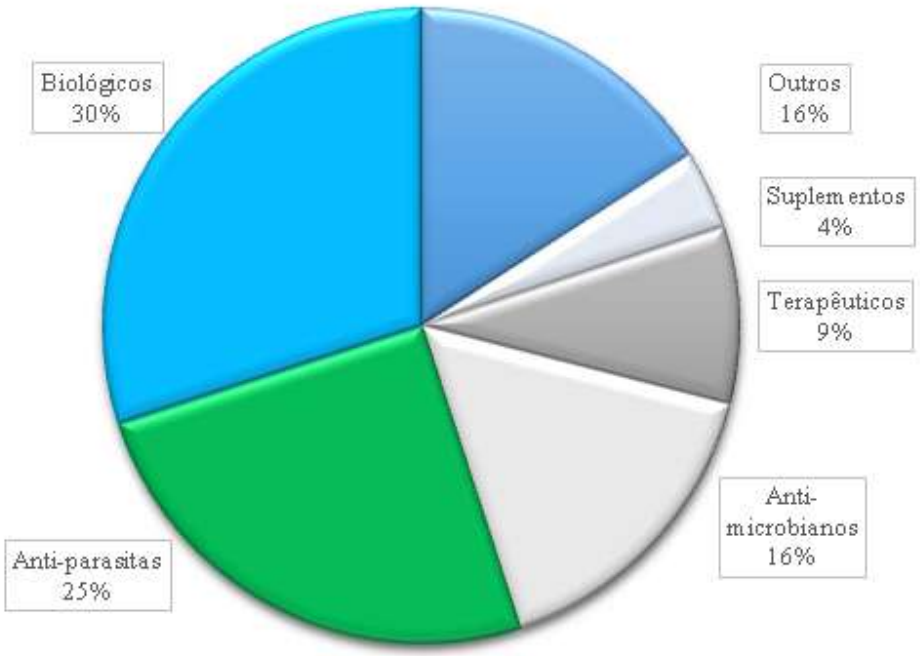
Fonte: Adaptado de Sindicato nacional de produtos de saúde animal (2017)

Chapter 1 - Figura 1- Participação nacional de espécies animais tratadas com produtos de uso veterinário no Brasil dos anos de 2008 a 2013 em porcentagem.



Fonte: Adaptado de Sindicato nacional de produtos de saúde animal (2017))

Chapter 1 - Figura 2- Faturamento nacional brasileiro da indústria de produtos para a saúde animal dos anos de 2008 a 2013 em milhões de reais.



Fonte: Adaptado de Sindicato nacional de produtos de saúde animal (2017))

Chapter 1 - Figura 3- Média da participação nacional das classes terapêuticas utilizadas no Brasil dos anos de 2008 a 2013 em porcentagem.

Sendo assim, diante da estimativa de crescimento nacional da produção animal para os próximos dez anos (abate de frangos aumento de 46,4%, de bovinos aumento de 22,5% e de suínos aumento de 20,6%) (Ministério da agricultura, pecuaria e abastecimento, 2013) far-se-á necessário seguir as exigências do mercado consumidor internacional, que se torna cada vez mais exigente em termos de segurança do alimento dos consumidores dos países compradores.

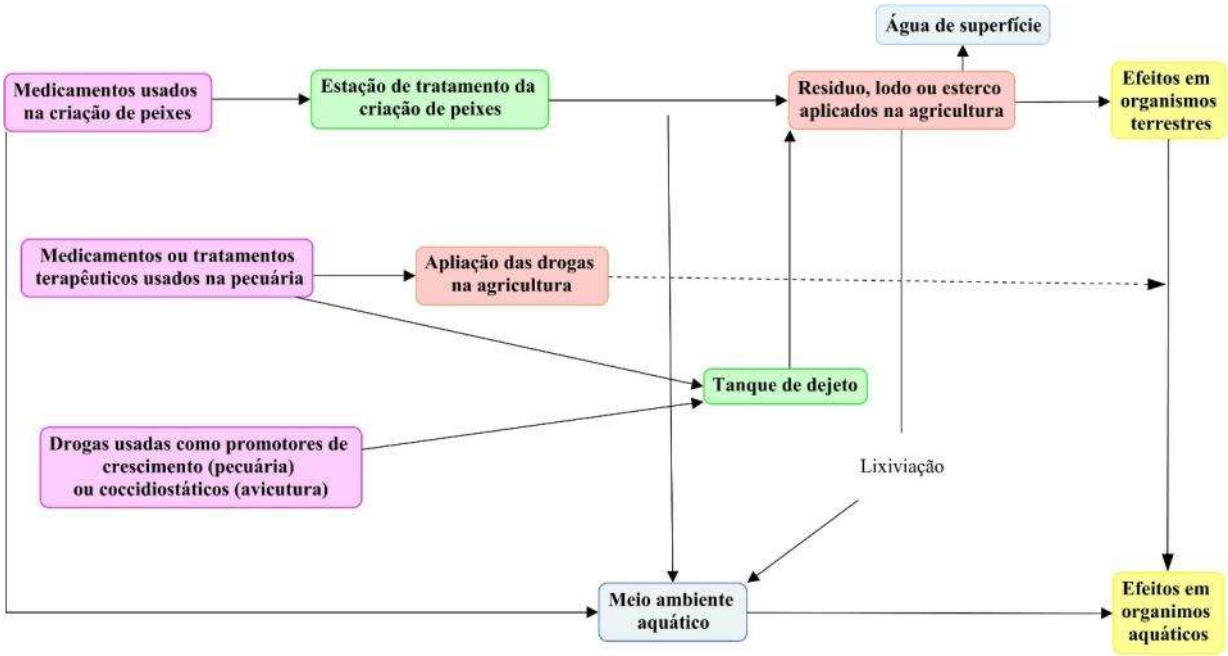
O presente capítulo visa contribuir para que grupos de pesquisas emergentes ou jovens pesquisadores possam ter uma síntese do estado atual do uso de medicamentos veterinários, suas rotas e transformações no ambiente além de riscos ambientais dessa prática. Além disso, busca promover o questionamento dos técnicos e produtores rurais sobre os possíveis riscos da presença de medicamentos veterinários no ambiente.

2. MEDICAMENTOS NO AMBIENTE

Os medicamentos veterinários ao serem administrados (via água, comida, injeção, pílulas) (Sarmah, Meyer e Boxall, 2006), mesmo quando em pequenas doses, terão parte do composto original ou seus metabólitos obrigatoriamente encontrados na urina ou fezes dos animais (Halling-Sørensen, 2001; Hirsch *et al.*, 1999). É bioquimicamente impossível metabolizar completamente esses compostos pelos animais. Sendo assim, as concentrações residuais de medicamentos nos excrementos animais é um componente a mais para a contaminação do solo e dos mananciais de água (Sarmah, Meyer e Boxall, 2006; Thiele-Bruhn, 2003). Considerando que o sistema de produção animal brasileiro predominante segue padrão veterinário baseado na intensificação do uso de medicamentos por causa do confinamento dos animais, indubitavelmente, vislumbra-se forte aumento nos níveis desses contaminantes no solo e nos mananciais aquáticos (Proia *et al.*, 2013). Monitorar o impacto provocado por essa atividade pecuária na qualidade do solo e da água, entendendo os destinos e as transformações desses medicamentos pode permitir a melhoria do seu uso, potencializando os sistemas de produção. A estabilidade dos compostos químicos com ação medicamentosa nos dejetos, ou nos locais de descarte, dependerá da sua estrutura química, de fatores microbiológicos e edafo-ambientais, como temperatura e umidade. As compostos dos princípios ativos ou de seus metabólitos podem sofrer transformações bioquímicas e físico-químicas, como hidrólise, oxidação, fotólise, adsorção, percolação, volatilização, entre outras (Aulton e Ortega, 2008; Velagaleti, 1997).

2.1. ROTAS DE CONTAMINAÇÃO

A contaminação dos mananciais aquáticos causada pelos medicamentos pode ocorrer quando: i) adicionados diretamente na água (aquicultura); ii) indiretamente através de: (a) eliminação pelas fezes e urina no solo em criações ao ar livre, (b) aplicação dos resíduos no solo como fertilizante que, na presença de erosão podem ser escoados superficialmente contaminando mananciais de águas superficiais, assim como por percolação alcançar o lençol freático e (c) descarte intencional ou acidental dos dejetos animais armazenados nos reservatórios de tratamento. Uma vez presentes no solo e na água, resultam em impactos negativos nos organismos terrestres e aquáticos (Figura 4) (Jørgensen e Halling-Sørensen, 2000).



Fonte: Jørgensen e Halling-Sørensen (2000).

Chapter 1 - Figura 4- Vias de exposição ambiental de medicamentos utilizados no tratamento veterinário

2.1.1. Vias de decomposição

Os medicamentos e seus metabólitos quando descartados no ambiente podem ser decompostos através de diferentes mecanismos: (i) biodegradação aeróbia, (ii) biodegradação anaeróbia e (iii) hidrólise e fotólise (Figura 5). Essas rotas podem ocorrer concomitantemente e a magnitude de cada uma delas é dependente do tipo de medicamento e das condições ambientais, inclusive da presença de microrganismos. A biodegradação é resultado do processo de biotransformação. Para que ocorra, o medicamento original deve ser convertido a metabólito dentro do corpo humano, no animal em que foi administrado ou no ambiente natural. Durante a biotransformação parcial o produto final é o medicamento inalterado e o seu metabólito. No entanto, durante a bio-transformação completa o medicamento pode ser convertido completamente ou formar compostos menores.

2.1.2. Biodegradação aeróbia

A biodegradação aeróbia ocorre na presença de oxigênio e pode conduzir a depleção completa da droga ou dos seus produtos biotransformados no ambiente, gerando água e dióxido de carbono. No entanto, pode haver biodegradação parcial e incompleta (Velagaleti, 1997), restando metabólitos dos medicamentos no ambiente. O tempo de biodegradação primária (quando a substância orgânica perde somente uma propriedade específica em decorrência da ação de um sistema biológico) de antibióticos advindos do escoamento de lavouras com dejetos animais pode variar entre os medicamentos e as condições ambientais. Por exemplo, para olaquindox a degradação ocorre entre 4 a 8 dias, para a tilosina de 9,5 a 45 dias, para a metronidazole 14 a 104 dias e para a oxitetraciclina de 42 a 46 dias (Ingerslev *et al.*, 2001).

2.1.3. Biodegradação anaeróbia

A biodegradação anaeróbia ocorre na ausência de oxigênio e resulta na bio-transformação parcial ou completa e na mineralização parcial. Nesse caso, os microrganismos usam outros aceptores finais de elétrons, em especial, nitrato e metais como manganês e ferro. Comumente ocorre a manutenção de parte do esqueleto carbonado do medicamento e o aparecimento de radicais livres. Alguns compostos podem estar sujeitos à perda de elétrons

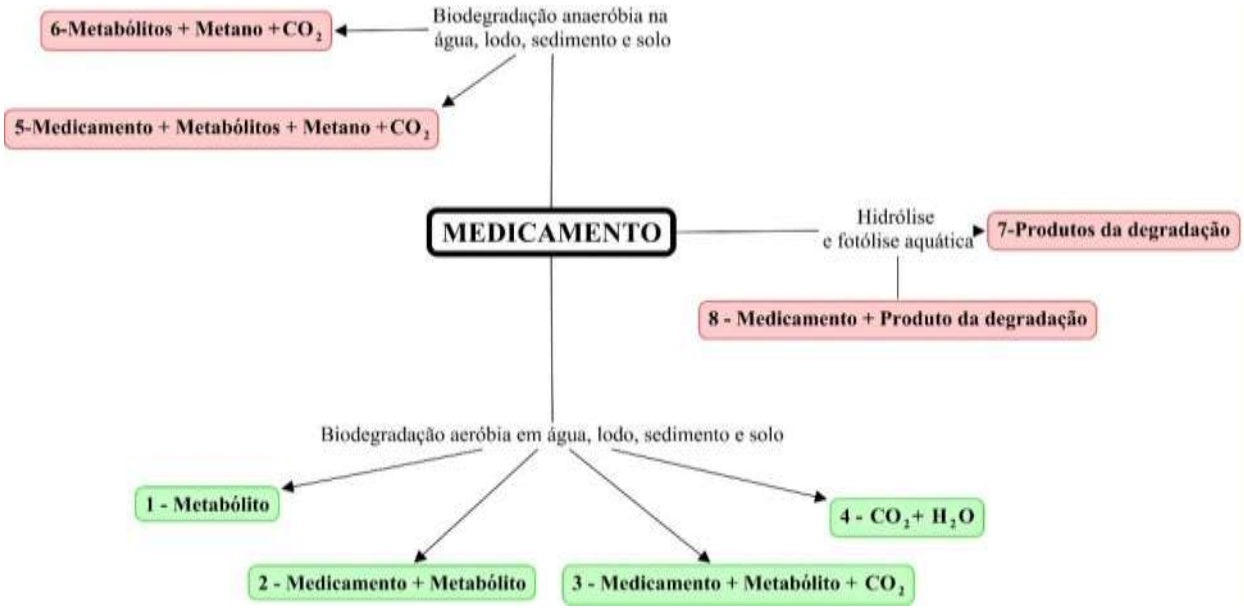
como: alcenos, aldeídos, heteroátomos adjacentes a anel benzênico (hidroquinonas), tióis e compostos de enxofre não totalmente oxidados, quelantes, EDTA, antioxidantes, sulfitos (sulfito e metabissulfito de sódio), ácido ascórbico e seus ésteres, tocoferóis, BHT (butilhidroxitolueno), BHA (butilhidroxianisol) e sulfoxilato.

2.1.4. Fotólise e hidrólise

A fotólise é um mecanismo de alteração da molécula do medicamento e de seus metabólitos que não envolvem a presença de microrganismos e suas enzimas. A fotodegradação direta ocorre quando uma molécula adquire excitação através da luz natural transformando quimicamente a molécula em um ou mais produtos. A fotodegradação indireta ocorre quando a molécula recebe energia a partir de um sensibilizador que absorveu luz solar (Velagaleti, 1997). A luz UV afeta as ligações químicas fornecendo energia para a separação dos elétrons compartilhados entre os dois átomos dessa ligação. O resultado é a formação de radicais livres no processo de oxidação, lise da molécula formando dois radicais e quebra da molécula podendo causar isomerização. Alguns medicamentos são sujeitos à fotólise: vitaminas (A, B1, B12, D e E), ácido fólico, corantes, dipirona, ácido meclofenâmico, metotrexato, fenotiazinas, corticoides (hidrocortisona e metilprednisolona) (Sanches *et al.*, 2013). Na água, com aumento da turbidez e profundidade ou na presença de árvores, a incidência de luz é menor e as reações de fotodegradação ocorrem em menor quantidade ou podem não ocorrer (Kümmerer, 2009; Lunestad *et al.*, 1995). No solo, o efeito desse processo na concentração dos medicamentos não é significante, especialmente quando espalhados sobre o solo através da aplicação de lodo ou dejeto (Kümmerer, 2009).

A hidrólise é uma reação chave para compostos orgânicos em ambientes aquosos através da quebra da ligação química de uma molécula com a adição de uma molécula de água. Essa reação é mediada pelo deslocamento direto de um grupo químico, através da quebra da molécula de água em íons de hidrogênio (H^+) e hidroxila (OH^-), e a ligação dos compostos resultantes dessa quebra (Velagaleti, 1997). Compostos mais apolares geralmente sofrem menos hidrólise que compostos mais polares. Sendo assim, o tipo de ligação entre o medicamento e a molécula de água permitirá que a hidrólise ocorra em maior ou menor quantidade. Formas hidratadas, em que as compostos se encontram ligadas a estrutura cristalina do medicamento, somente entram no processo de degradação quando água que fica adsorvida a superfície é liberada. Ou seja, apenas as compostos de água que não fazem parte

da estrutura cristalina e que ficam adsorvidas à superfície do sólido, podem originar degradação do mesmo (Yoshioka e Stella, 2000). Alguns grupos funcionais favorecem a hidrólise: lactonas (ésteres cíclicos), lactamas (amidas cíclicas), ésteres e amidas.



Fonte: Adaptado de (Velagaleti, 1997)

Chapter 1 - Figura 5 -. Vias de degradação de medicamentos através da biodegradação aeróbia: (1) Biotransformação completa, biotransformação parcial (2), Mineralização parcial (3) e mineralização completa (4); Vias de degradação de medicamentos através da biodegradaçã**o** anaeróbia: bioransformação completa (5) e biotransformação parcial (6); Vias de degradação de medicamentos através da hidrolise e fotólise em meio aquoso: transformação completa pela hidrolise e transformação química completa mediada pela luz ou por fotodegradação indireta (7) e transformação parcial pela hidrólise e transformação química parcial mediada pela luz (8).

2.2. MEDICAMENTOS NOS AMBIENTES AQUÁTICOS

Nos ambientes aquáticos diversos mecanismos podem limitar a persistência dos medicamentos. No entanto, o aporte constante é o principal fator que define o caráter de pseudo-persistência ambiental. Os teores de medicamentos presentes nas águas podem variar com as estações do ano, caso sejam rapidamente degradados, como é observado para a cafeína e a sulfametoxazol (Conley *et al.*, 2008). Em contrapartida, há compostos mais estáveis, como a carbamazepina (Hua *et al.*, 2006), que são menos alterados e sua persistência na água é maior. Alterações no regime hídrico, devido a diferenças de sazonalidade, interferem

igualmente nas concentrações de medicamentos na água levando a maiores teores na época de menor precipitação devido a menor quantidade de água na superfície de rios (Vieno, Tuhkanen e Kronberg, 2005).

Nas zonas urbanas, mesmo que as águas servidas aos usuários passem por estações de tratamento, os medicamentos podem alcançar os cursos de água devido ao limitado potencial de descontaminação (Kümmerer, 2009). No Brasil, esse cenário é muito mais preocupante diante da carência de coleta e de tratamento de esgotos urbanos. Dados de mais de 10 anos atrás mostravam uma realidade brasileira preocupante. Somente 20,2% dos municípios tinham estações de tratamento; em 32% havia apenas o serviço de coleta sem tratamento e em 47,8% dos municípios o esgoto sequer era coletado e, portanto, lançado diretamente nos mananciais aquáticos (Bila e Dezotti, 2003). Felizmente, na última década, o governo federal tem disponibilizado grande volume de recursos financeiros para elaboração do projeto de coleta e tratamento de esgoto. Vários desses projetos estão sendo executados, como é o caso da cidade de Porto Alegre em que em 2014 o percentual de tratamento era de apenas 27% de seus dejetos. Após a inauguração da Estação de Tratamento de Esgotos Serraria, na Zona Sul, o tratamento superou a meta contratada (56%) com a gestão central da prefeitura e praticamente dobrou o volume de esgoto tratado em Porto Alegre na comparação com o final do ano anterior (2014). Atualmente, a capital do Rio Grande do Sul está tratando 66% do esgoto produzido.

Nas zonas rurais a situação é tão crítica quanto nas cidades. As regiões produtoras de animais, como a região sul do Brasil, responsável por 55, 48 e 14% da produção nacional de leite, carne de aves e suínos, respectivamente, (Sindicato nacional de produtos de saúde animal, 2017) tratam de forma inadequada seus dejetos. Grande parte das agroindústrias de processamento animal não possuei sistema de tratamento de seus resíduos e quando presentes são ineficazes para eliminar antibióticos e outras compostos medicamentosas (Kümmerer, 2009). Os dejetos aplicados sobre o solo como fertilizantes (Burton e Turner, 2003) são parcialmente transferidos aos sistemas aquáticos pelo escoamento superficial, em decorrência do regime hídrico da região e da falta de práticas conservacionistas adequadas. Ao mesmo tempo, o armazenamento dos efluentes animais em tanques ou lagoas, antes da aplicação em campos agrícolas (Jørgensen e Halling-Sørensen, 2000) podem resultar em problemas de vazamento devido à má vedação dos tanques de tratamento (Burton e Turner, 2003). Outra frequente realidade é o descarte clandestino realizado pelos produtores de animais que causa aporte direto dos contaminantes aos cursos d'água.

A regularização e a orientação sobre o tratamento e a disposição dos resíduos sólidos no Brasil são regulamentadas através de órgãos como a Agência Nacional de Vigilância Sanitária (ANVISA) e o Conselho Nacional do Meio Ambiente (CONAMA). As resoluções RDC nº 306, de 7 de dezembro de 2004 (ANVISA) (Ministério da Saúde e Agência Nacional de Vigilância Sanitária, 2004) e Resolução nº 358, de 29 de abril de 2005 (CONAMA) (Conselho Nacional do Meio Ambiente, 2005) enfatizam a responsabilidade dos geradores de resíduos em relação ao gerenciamento do início até sua disposição final (ex.: exigência de segregação da fonte de poluição, tratamento dos resíduos havendo necessidade, local de destinação final).

Diferentemente do curso atual do agronegócio brasileiro, alguns países desenvolvidos têm usado as preocupações ambientais para nortear as decisões governamentais. Especial atenção tem sido dada a manutenção da "qualidade" da água. Na Europa, por exemplo, a Comissão de Execução de 20 março 2015 (EU - 2015/495) listou as substâncias sujeitas a fiscalização no domínio da política da água nos termos da Diretiva 2008/105 / CE. Deverão ser analisados, para informar futuros exercícios de definição de prioridades referidas no artigo 16, parágrafo 2, da Diretiva 2000/60 / CE do Parlamento Europeu e do Conselho, as substâncias: 17-alfa-etinilestradiol (EE2), 17-beta-estradiol (E2), estrona (E1), diclofenaco, 2,6-ditert-butil-4-metilfenol, 4-metoxicianamato de 2-etilhexilo, antibióticos macrolídeos (Eritromicina, Claritromicina, azitromicina), metiocarbe, neonicotinoides (imidaclopride, tiaclopride, timetoxame, clotianidina, acetamipride), oxadiazão e trialato. Concomitantemente, alguns países decidiram investir na pesquisa relacionada com a contaminação dos sistemas hídricos, como é o caso do Ministério Federal Alemão de Educação e Pesquisa que iniciou um quadro de financiamento chamado Gestão de Riscos Emergentes de Compostos e Patógenos no Ciclo da Água (RiSKWa), o qual é formado por 12 projetos conjuntos de pesquisa.

2.2.1. Efeitos e riscos da interação com seres aquáticos

A disponibilização de compostos de medicamentos e seus metabólitos podem alterar o metabolismo dos seres vivos que habitam o meio aquático. Há concordância no meio científico que: modulações endócrinas em vertebrados podem ocorrer devido à presença de estrógeno (Crane, Watts e Boucard, 2006); o aumento no tempo de desenvolvimento embrionário e indução de vitelogenina pode aparecer em peixes machos (Carlsson *et al.*,

2006); acumulação de resíduos de oxitetraciclina na carapaça e nos músculos de animais como o camarão *Litopenaeus vannamei,* servindo de entrada para os medicamentos em animais de níveis tróficos superiores (Lavorante *et al.*, 2009); problemas de alterações estruturais, funcionais e até a morte de organismos constituintes dos biofilmes podem se desenvolver e que essas compostos podem ser transferidas dentro da cadeia trófica, como para as sulfonamidas, quinolonas e macrolídeos (Proia *et al.*, 2013).

Pesquisadores de diferentes países usam a dinâmica populacional dos macro invertebrados como medida da integridade biológica de rios e córregos. Como exemplo de método de verificação de qualidade ambiental através dos macroinvertebrados no mundo, a Suíça utiliza o RIVAUD (Rivers of Vaud index); a Austrália o AUSRIVAS (Australian River Assessment Scheme); o Reino Unido o BMWP scoring system (Biological Monitoring Working Party); os Estados Unidos o ICI (Invertebrate Community Index) e a França o IBGN (Indice Biologique Global Normalisé). Esses índices são baseados no exame global da macrofauna bêntica, analisada de acordo com um protocolo padrão de amostragem. Para tal, considera-se que as populações de um habitat são a expressão sintética de um conjunto de fatores ecológicos que condicionam o sistema. O índice é calculado tendo base uma tabela referente à fauna amostrada em relação aos grupos indicadores de sensibidade a diferentes perturbações e sua variedade (DIREN Haute-Normandie, 2013).

No caso do IBGN francês, após a coleta e a triagem dos animais, calculam-se:

Variedade taxonômica (∑t): número total de táxons independente do número de indivíduos e;

Grupo indicador (GI): grupo mais sensível a poluição (no mínimo 3 ou 10 indivíduos de acordo com o táxon) e possuindo o índice mais elevado possível.

Esses dados serão utilizados para o calculo o IBGN a partir de uma tabela de dupla entrada onde, a abcissa comporta as classes de variedade taxonômica (classificadas de 1 a 14) e na ordenada os grupos faunísticos indicadores, classificados por ordem de crescimento de sensibilidade de poluição (9 a 1 – Tabela 2). De acordo com a diversidade taxonômica do sítio e a presença e ou ausência de táxons indicadores, uma nota é atribuída a qualidade hidrobiológica que varia de 1 a 20 (Tabela 3).

Chapter 1 - Tabela 2 - Valor de IBGN de acordo com a natureza e variedade taxonomica da macrofauna (norma NFT 90-350 março 2004).

Classe da variedade		14	13	12	11	10	9	8	7	6	5	4	3	2	1
Taxons	GI\Σt	>50	49 45	44 41	40 37	36 33	32 29	28 25	24 21	20 17	16 13	12 10	9 7	6 4	3 1
Chloroperlidae (Plécoptères) Perlidae (Plécoptères) Perlodidae (Plécoptères) Taeniopterygidae (Plécoptères)	9	20	20	20	19	18	17	16	15	14	13	12	11	10	9
Capniidae (Plécoptères) Brachycentridae (Trichoptères) Odontoceridae (Trichoptères) Philopotamidae (Trichoptères)	8	20	20	19	18	17	16	15	14	13	12	11	10	9	8
Leuctridae (Plécoptères) Glossosomatidae (Trichoptères) Beraidae (Trichoptères) Goeridae (Trichoptères) Leptophlebiidae (Ephéméroptères)	7	20	19	18	17	16	15	14	13	12	11	10	9	8	7
Nemouridae (Plecoptères) Lepdostomatidae (Trichoptères) Sericostomatidae (Trichoptères) Ephemeridae (Ephéméroptères)	6	19	18	17	16	15	14	13	12	11	10	9	8	7	6
Hydroptilidae (Trichoptères) Heptageniidae (Ephéméroptères) Polymitarcidae (Ephéméroptères) Potamanthidae (Ephéméroptères)	5	18	17	16	15	14	13	12	11	10	9	8	7	6	5
Leptoceridae (Trichoptères) Polycentropodidae (Trichoptères) Psychomydae (Trichoptères) Rhyacophilidae (Trichoptères)	4	17	16	15	14	13	12	11	10	9	8	7	6	5	4
Limnephilidae* (Trichoptères) Hydropsychidae (Trichoptères) Ephemerellidae* (Ephéméroptères) Aphelocheiridae (Hétéroptères)	3	16	15	14	13	12	11	10	9	8	7	6	5	4	3
Baetidae* (Ephéméroptères) Caenidae* (Ephéméroptères) Elmidae* (Coléoptères) Gammaridae* (Crustacés) Mollusques	2	15	14	13	12	11	10	9	8	7	6	5	4	3	2
Chironomidae* (Diptères) Asellidae* (Crustacés) Achètes (Annélides)	1	14	13	12	11	10	9	8	7	6	5	4	3	2	1
* Taxons representados por ao menos 10 individuos - Os outros por pelo menos 3 individuos															

Fonte: (DIREN Haute-Normandie, 2013)

Chapter 1 - Tabela 3 - Valores de interpretação do resultado do IBGN.

IBGN	Interpretação
≥17	Excelente
16–13	Boa
12–9	Aceitável
8–5	Medíocre
≤4	Ruim

Fonte: (DIREN Haute-Normandie, 2013)

2.3. MEDICAMENTOS NOS SOLOS AGRÍCOLAS

As bactérias no solo são capazes de produzir diversos antibióticos (ex.: β-lactanos, estreptomicina e amino glicosídeos) (Kümmerer, 2009) através de um mecanismo químico natural de regulação chamado antibiose (Gottlieb, 1976). Todavia, solos agrícolas possuem como fonte de entrada majoritária de medicamentos a aplicação de dejetos contaminados. O aporte nos campos de produção pode alcançar teores de até quilogramas por hectare e um nível de concentração similar ao de pesticidas (Winckler e Grafe, 2001), apresentando risco ambiental e a saúde humana. As propriedades físico-químicas e as estruturas das compostos dos antibióticos têm um papel importante na adsorção das compostos (Wang e Wang, 2015). Ao mesmo tempo, as propriedades individuais de cada medicamento são fortemente influenciadas pela interação das compostos com as propriedades físico-químicas do solo como o pH, os compostos iônicos, a textura e a matéria orgânica (Thiele-Bruhn, 2003). De forma geral, as relações sólido/soluto são complexas e sua modelagem igualmente. (Rheinheimer *et al.*, 2007) comentaram sobre essas relações:

> A relação entre a interação solo-solução pode ser modelada considerando o equilíbrio químico ou hipótese cinética. Em condições de equilíbrio químico, a adsorção pode ser descrita por modelos fenomenológicos ou mecanicistas. Em modelos fenomenológicos os metais pesados contidos na solução do solo são matematicamente ajustados considerando as propriedades do solo. Na literatura, existem diferentes tipos de isotérmas, cada um delas com as limitações e vantagens. No entanto, para simulações de destino desses metais pesados, as isotérmas não-lineares são mais realistas do que as lineares. Os modelos mecanicistas precisam de hipóteses matemáticas simplificadas devido a complexidade do sistema. No entanto, eles exigem um grande número de parâmetros de solo e da solução do solo. O acoplamento de modelos mecanicistas com fluxos de água ao metal é ainda bastante recente. Em modelos cinéticos, a dessorção do metal pesado pode ser originado pela

> cinética de limitações físicas ou químicas. Isso significa que uma abordagem de dois locais (equilíbrio químico e locais cinéticos) é mais apropriado do que o de um único site para melhorar o desempenho da simulação e, em seguida, prever as propriedades de transferência e destino de metais pesados nos solos.

Algumas substâncias podem ser hidrofóbicas ou não polares, enquanto outras são completamente solúveis em água ou dissociadas em valores de pH tipicamente encontrados em solos (Thiele-Bruhn, 2003). A capacidade de adsorção entre a fase da água e a fase sólida, como os solos e sedimentos, é definida através do coeficiente de adsorção K_d ($K_d = C_s / C_w$, onde C_s representa a massa do contaminante na fase sólida do solo (mg kg^{-1}) e C_w massa do contaminante na fase líquida (mg l^{-1}) (Wang e Wang, 2015). No solo, o teor de carbono orgânico pode influenciar no processo de sorção e distribuição de compostos orgânicos. Para isso, o coeficiente de distribuição (K_d) nessa situação é expresso pelo coeficiente de partição do contaminante na fração orgânica do solo (K_{oc}, $K_{oc} = K_d/f_{oc}$ onde, K_{oc} = coeficiente de partição normalizado pelo carbono orgânico (l kg^{-1} substância orgânica); f_{oc} = fração de carbono orgânico (kg substância orgânica/kg solo seco) (Tolls, 2001). No entanto, alguns autores consideram que a descrição do modelo de K_{oc} apresentado por Tolls (2001), sobre a sorção apolar orgânica através de forças de van der Walls, é falha para descrever a sorção de medicamentos veterinários a solos e sedimentos. Os pesquisadores comentam que o coeficiente de sorção de carbono normalizado (K_{oc}) para fármacos é muitas ordens de magnitude maior do que o previsto por esses modelos típicos e que o K_{oc} não demostra correlação com compostos hidrofóbicos (Figueroa, Leonard e MacKay, 2004). Mesmo assim, esse modelo continua sendo utilizado para a representação do comportamento das compostos nos solos e sedimentos. Dessa forma, compostos altamente solúveis tendem apresentar valores de K_{oc} menores que 150 cm^3 g^{-1} podendo ser rapidamente biodegradados no solo e na água. Compostos com K_{oc} entre 150 e 500 são consideradas moderadamente móveis e acima de 2.000 de baixa mobilidade no solo (Barceló e Hennion, 1997). Considerando-se essa classificação, os medicamentos veterinários utilizados como exemplo teórico na tabela 4 com menor mobilidade são a ciprofloxacina e norfloxacina, seguidos da tilosina e a oxitetraciclina com baixa a nenhuma mobilidade e mobilidade moderada para o diclofenaco e sulfaquinoxalina (Beek, aus-der- *et al.*, 2016).

Chapter 1 - Tabela 4 - Massa molar, fórmula química, classe terapêutica, pKa, Koc e Log Kow dos medicamentos estudados.

Medicamento	Fórmula química	Classe terapêutica	Massa molar ($g\ mol^{-1}$)	pKa1/pKa 2[a]	Koc [a]	log Kow [a]
Enrofloxacina	$C_{19}H_{22}FN_3O_3$	Antibiótico do grupo das fluoroquilonas	359,39	(-)	(-)	(-)
Ciprofloxacina	$C_{17}H_{18}FN_3O_3$	Antibiótico do grupo das quinolonas	331,34	6,09/8,74	61000	0,28
Diclofenaco	$C_{14}H_{10}C_{l2}NaNO_2$	Anti-inflamatório não esteroide	296,14	4,15	245	4,51
Flavomicina	$C_{69}H_{107}N_4O_{35}P$	Antibiótico não inóforo	1583,57	(-)	(-)	(-)
Norfloxacina	$C_{16}H_{18}FN_3O_3$	Antibiótico anti-bactericida classe quinolonas	319,33	6,34/8,75	61000	0,46
Oxitetraciclina	$C_{22}H_{24}N_2O_9$	Antibiótico fungicida e bactericida	460,43	9,5	195-93317	-0,9
Sulfaquinoxalina	$C_{14}H_{12}N_4O_2S$	Anti-protozoário	300,36	5,1	200	1,68
Tilosina	$C_{46}H_{77}NO_{17}$	Antibiótico do grupo dos macrolídeos	916,10	7,73	553-7988	1,63

[a]:PubChem (2014)
(-) Dados não disponibilizados em PubChem, 2014

Obs. : Os valores de pKa são valores calculados através de simulações, e não são necessariamente valores encontrados experimentalmente.

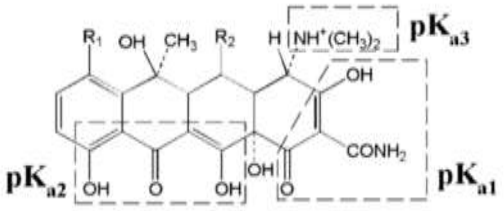
A associação de solutos com a matéria orgânica dissolvida pode ser comparada com a partição de equilíbrio, no qual a concentração associada a matéria orgânica dissolvida (C_{DOM}) é relacionada com a C_{aq} através $K_{d,DOM}$, a razão entre matéria orgânica dissolvida e o coeficiente de partição da água. Dessa relação pode-se concluir que medicamentos veterinários com um elevado valor de $K_{d,DOM}$ irão particionar significativamente para a matéria orgânica solúvel, possivelmente resultando no aumento da mobilidade dos medicamentos veterinários na sua presença (Tolls, 2001). A quantidade e a qualidade da matéria orgânica influenciará a adsorção dos medicamentos, pois ao se associarem com a matéria orgânica dissolvida do solo, os medicamentos podem ter sua mobilidade maior do que quando associados aos minerais do solo.

A solubilidade em água descreve o comportamento do medicamento em relação ao transporte e os possíveis destinos no ambiente. Os medicamentos podem ser definidos como muito solúveis (solúveis em proporções $g\ l^{-1}$) e muito insolúveis (solubilidade inferior a 0,5 a

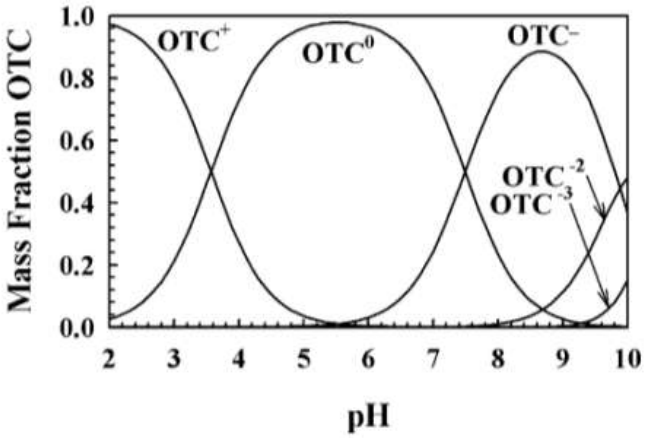
1 mg l^{-1}). Em complemento, usa-se o coeficiente de partição *n*-octanol–água (K_{ow}), que relaciona as propriedades hidrofílicas e lipofílicas, e demonstra a tendência à bioconcentração destes compostos. Geralmente substâncias com valor de log $K_{ow} > 3$ indicam acumulação (Barceló e Hennion, 1997). Os valores encontrados nos medicamentos listados na tabela 4 variam de 0,9 a 4,51; os mais hidrofílicos (log $K_{ow} < 1$) são a ciprofloxacina, a norfloxacina e a oxitetraciclina. Os compostos hidrofílicos tem maior solubilidade em água e uma menor tendência a se adsorver em sedimentos, no solo ou mesmo em organismos biológicos.

A constante de equilíbrio de ionização ácida (pKa) descreve a dissociação ácida dos medicamentos. Se o pH do meio for igual ao valor de pKa da espécie significa que 50% de suas compostos se encontram ionizadas e 50% apresentam-se não dissociadas. Contudo, se os valores de pH do meio forem superiores a pKa concentração da fração aniônica predomina na solução. Se os valores de pH forem inferiores, predomina a fração neutra (MILHOME, 2006). Nos ambientes naturais em que os valores de pH situam-se na faixa 5 – 8, grande parte das compostos de medicamentos tende a estar ionizada negativamente e podem migrar no perfil com maior facilidade (Barceló e Hennion, 1997). Exemplo de aplicabilidade do pKa é a tetraciclina (Figura 6): em soluções fortemente ácidas ela se encontra carregada positivamente e negativamente quando em condições alcalinas. Ainda, as espécies de tetraciclina catiônicas podem neutralizar sítios de carga negativa quando em condições ácidas e podem ser repelidos pelas superfícies de argila em pH ácido (Figueroa, Leonard e MacKay, 2004).

O uso das propriedades dos medicamentos e seus metabólitos auxiliam no entendimento da dinâmica deles no ambiente. Compostos antrópicas, como os medicamentos, presentes nos dejetos animais quando aplicados nos solos agrícolas, para alcançar os recursos hídricos superficiais, devem ser transportadas diretamente por escoamento e/ou erosão de dejeto ou dos solos contaminados (Koschorreck, Koch e Rönnefahrt, 2002). A percolação no perfil do solo de alguns medicamentos pode resultar em contaminação do lençol freático. Na Alemanha, por exemplo, a primeira constatação da presença de tetraciclina no sedimento retirado das valas de irrigação de área agrícola de alto uso de antibiótico veterinário oferece prova de que o transporte terrestre de antibióticos veterinários está ocorrendo (Bailey *et al.*, 2015). Assim, novos estudos vem sendo desenvolvidos em todo o mundo para servir de subsídio para a criação de leis referentes a entrada de medicamento através do dejeto animal aplicado nos campos de produção.



Antibiotic	R_1	R_2	pK_{a1}	pK_{a2}	pK_{a3}
OTC	H	OH	3.57	7.49	9.88
TET	H	H	3.3	7.7	9.7
CTC	Cl	H	3.6	7.52	9.88



Fonte: Figueroa, Leonard e MacKay (2004)

Chapter 1- Figura 6 - Química da tetraciclina e solução de especiação. Todos os antibióticos da classe das tetraciclinas têm sua estrutura de base comum com sítios de troca de próton relevantes no ambiente. Os grupos R1 e R2 são reportados para a oxitetraciclina (OTC), tetraciclina (TET) e a clorotetraciclina (CTC). A especiação demostrada acima é referente a oxitetraciclina, no entanto representa igualmente a TET e a CTC devido à proximidade dos valores de pKa de todos esses compostos.

3. MEDICAMENTOS NOS ALIMENTOS

A baixa solubilidade dos medicamentos na água no solo possibilita a sua bioacumulação pelas plantas e a transferência na cadeia trófica (Migliore, 1995), induzindo a contaminação indireta de organismos superiores da cadeia alimentar, inclusive, o homem. A análise de medicamentos veterinários e seus metabólitos em alimentos é justificada principalmente pela (o): i) utilização irregular dos medicamentos veterinários e a não observação de rótulos e período de carência dos compostos; ii) aparecimento de resistência, mutações, efeitos carcinogênicos e teratogênicos em organismos expostos aos medicamentos; iii) problemas endócrinos, alergias, toxicidade aguda e crônica (Sofos, 2005).

Os riscos à saúde humana devido à exposição de resíduos de medicamentos veterinários aumentam o interesse na certificação de alimentos orgânicos ou livres de resíduos (Prestes *et al.*, 2013). Para amenizar e controlar a presença de medicamento nos alimentos, o

governo federal através da Portaria n.º 51, de 6 de fevereiro de 1986 instituiu o Plano Nacional de Controle de Resíduos Biológicos em Produtos de Origem Animal - PNCRB, visando sistematizar o controle da contaminação de produtos de origem animal por resíduos de compostos de uso na agropecuária e/ou poluentes ambientais, onde podemos encontrar diversos programas, como por exemplo o Programa de Controle de Resíduos em Leite (PCRL) (Ministério da Agricultura, Pecuária e Abastecimento, 1986). Para atender essas necessidades os laboratórios do MAPA foram organizados visando atualizar e melhorar as políticas e atividades analíticas relacionadas à defesa sanitária vegetal e animal (Queiroz Mauricio, de e Lins, 2012). No ano de 1999, o PNCRB passou a se chamar "Plano Nacional de Controle de Resíduos em Produtos de Origem Animal – PNCR" através da Instrução Normativa nº 42, de 20 de dezembro de 1999 (Ministério da Agricultura e Abastecimento, 1999) com o propósito de atender nacional e internacionalmente as questões referentes a qualidade de alguns alimentos como carne, leite, mel e pescado. A partir desse momento, a ANVISA no ano de 2002 implantou o Programa de Análise de Resíduos de Medicamentos Veterinários em Alimentos de Origem Animal (PAMVet) com o objetivo de fortalecer os mecanismos de controle sanitário. Entre os alimentos selecionados, pode-se destacar o leite bovino, carnes (bovina, frango e suína), pescado, ovos e mel. Diante do cenário internacional, no ano de 2005, o MAPA decide atender as exigências da União Europeia relativa ao uso de medicamentos veterinários sugerindo que o Brasil aperfeiçoasse o programa de resíduos em mel, criasse um plano de resíduo em ovos, adicionando os resíduos máximos permitidos pela legislação europeia entre outras solicitações (Pacheco-Silva, Souza e Caldas, 2014). No mês de junho de 2009 foi iniciado o projeto "Uso racional de antibióticos e combate à resistência bacteriana". Esse projeto tem ganhado cada vez mais importância com suas ações voltadas ao uso de farmacêuticos recebendo destaque e deixando em evidência que, por mais difícil que seja de sanar um problema, com o uso da informação de forma adequada é possível amenizar a questão (Bisson, 2010). No ano de 2012, a lei n° 12.689/2012 altera o Decreto-Lei nº 467/1969, normatizando o uso e a produção de medicamentos genéricos veterinários e determina os critérios para registro, fabricação, distribuição/comercialização, prescrição e dispensação destes produtos (Ministério da Agricultura, Pecuária e Abastecimento, 2012).

Nesse sentido, a certificação foi implantada como uma forma de garantir a segurança da população a possíveis exposições acidentais e ao mau uso dos compostos químicos, além de permitir a comprovação de que o atual sistema de produção nacional contribui para a contaminação solo, água, sedimento e alimentos. Para a comprovação dessa realidade nacional, a regulamentação desses valores foi definida através do trabalho conjunto de

agências nacionais e do fórum Codex Alimentarius Brasil (Fórum Internacional de Normatização do Comércio de Alimentos) estabelecido pela Organização das Nações Unidas (ONU) por ato da Organização para a Agricultura e Alimentação (FAO) e Organização Mundial de Saúde (OMS) (Pacheco-Silva, Souza e Caldas, 2014). O Ministério da Saúde através da diretoria colegiada da Agência Nacional de Vigilância Sanitária aprovou o Regulamento Técnico Mercosul - Rdc N° 53, de 02 de Outubro de 2012- que dispõe de metodologias analíticas, quantidade diária admissível de ingestão e limites máximos de resíduos para medicamentos veterinários em alimentos de origem animal. Assim, assumindo a existência de resíduos de medicamentos veterinários em produtos de origem animal no Brasil, a seleção de bactérias resistentes aos medicamentos veterinários é um tema atual, discutido e estudado nacionalmente.

Os alimentos de origem animal como carnes, vísceras, leite, ovos e mel, dentre outros, são matrizes comumente analisadas em laboratórios de rotina, apresentando frequentemente resíduos de diversas classes de medicamentos veterinários. No ano de 2009, o Laboratório de Resíduos de Medicamentos Veterinários (LRM/PL, MG, Brasil) analisou 1519 amostras de tecido animal de dezesseis estados brasileiros. Dentre estes, 644 amostras (42%) foram positivas a presença de medicamentos, 240 amostras (16%) para resíduos de macrolídeos, 23 amostras (1.5%) para amino glicosídeos. Nenhuma amostra foi encontrada com resultados superiores para os seis maiores analitos encontrados pelo LRM (Tabela 5) (Nonaka *et al.*, 2012).

Chapter 1 - Tabela 5 - Resultado dos seis maiores analitos encontrados pelo Laboratório De Resíduos De Medicamentos Veterinários encontrados no ano de 2009 (LRM/PL, MG, Brasil) (Nonaka *et al.*, 2012).

Analítos	MRL[a]	Bovino	Cavalo	Porco	Aves	Máximo encontrado
	Valores ($\mu g\ kg^{-1}$)					
Lincomicina	1500	5	3	101	35	894,3
Tilcomisina	1000	4	4	37	14	235,5
Tilosina	100	1	0	28	4	<LOQ[c]
Clindamicina	n.e.[b]	2	1	9	3	<LOQ
Gentamicina	750	0	0	4	10	<LOQ
Eritromicina	200	1	1	7	1	<LOQ

[a.] Maximum Residue Level – CODEX e EEC 2377/90
[b.] Não estabelecido
[c] LOQ - Limite de quantificação

Outro alimento muito estudado no Brasil é o leite. De acordo com o relatório da Pesquisa de Orçamentos Familiares (IBGE/POF, 2008/2009)[1], o leite representa o segundo maior gasto com a alimentação na área urbana (11,0%) e o terceiro maior gasto na área rural (8,7%) [1]. Assim, Ferreira e colaboradores (2012) ao estabelecer um panorama da contaminação do leite com resíduos de medicamentos veterinários no Brasil verificaram que o número de ocorrências de resíduos de antimicrobianos como os β-lactâmicos e as tetraciclinas foram responsáveis pela maior incidência de amostras com resultados acima dos limites de detecção. Entre os artigos, dos nove estudos sobre anfenicóis, quatro apresentaram ocorrência de cloranfenicol e florfenicol e somente três referências apresentaram a ocorrência de amostras com teores acima do Limite Máximo de Resíduos (LMR). Para os analítos benzilpenicilina, da classe dos β-lactâmicos e o amino glicosídeo estreptomicina / diidroestreptomicina apenas sete das referências avaliadas apresentaram resultados insatisfatórios para antimicrobianos com relação à legislação brasileira. Os antiparasitários entre os 10 trabalhos foram encontrados em 5 com resultados acima do LMR.

[1]Dados disponíveis em www.ibge.gov.br/

4. RESISTÊNCIA A MEDICAMENTOS

A presença de mecanismos de resistência às compostos de medicamentos veterinários é comum nos microrganismos que vivem no solo, na água e nos sedimentos. No solo, por exemplo, metade das espécies de actinomicetos tem habilidade de sintetizar antibióticos (Topp, 1981), sendo a resistência responsável pela sua proteção a outros organismos suscetíveis ao ataque competitivo (Kemper, 2008). Alguns mecanismos de resistência podem ser intrínsecos às células dos microrganismos, como a alteração da redução da permeabilidade da membrana de lipopolissacarídeos, bomba de efluxo e alteração do sítio de ação. Outro mecanismo que pode ser desenvolvido pelas bactérias é o de degradação enzimática ou inativação do antibiótico. Existem três grandes estratégias para a degradação, a hidrólise e a transferência de um grupo ou processo redox (Džidić, Šušković e Kos, 2008).

Na natureza a troca de genes entre duas compostos de DNA é designada a formar novas combinações em um cromossomo, preservando sua integridade genética por meio da reparação de possíveis falhas no DNA bacteriano (Conley *et al.*, 2008) e servindo de fonte para a variação evolutiva da maioria dos procariotos (Tortora, Funke e Case, 2012). A

transferência de material genético pode ser vertical, quando os genes são passados de um microrganismo para seus descendentes, ou horizontal, quando os genes podem ser adquiridos de outros microrganismos da mesma geração (Bauman, 2008). Quando adquiridos horizontalmente efeitos deletérios podem ser ocasionados à célula bacteriana que os recebeu, resultando na eliminação da população ao qual está inserida. Em contrapartida, genes que conferem vantagem seletiva ao patógeno, em relação ao hospedeiro, podem potencialmente espalha-los rapidamente dentro da população bacteriana (Thomas e Nielsen, 2005). Após a mutação ser adquirida, sua transmissão é feita através de mecanismos normais de reprodução e a progênie integra aquela característica genética passando aos micróbios parentais. Tendo em vista a alta taxa de reprodução das bactérias, em um curto período quase toda a população passa a ser resistente a um novo antibiótico (Tortora, Funke e Case, 2012). Portanto, se os medicamentos forem utilizados indiscriminadamente poderão fornecer risco à população tanto pela presença nos alimentos como pela seleção de linhagens bacterianas resistentes a diferentes princípios ativos, dificultando o tratamento de infecções (Greeson *et al.*, 2013).

Hoje, o maior interesse em relação ao uso de antibióticos no tratamento humano e animal é o desenvolvimento de estirpes bacterianas resistentes que representem risco à saúde humana e animal (Kemper, 2008; Thiele-Bruhn, 2003). A ingestão de alimentos contaminados com medicamentos pode resultar em riscos para a saúde, tais como problemas de alergia, toxicidade e potencial de desenvolvimento de cepas bacterianas resistentes quando esses resíduos de antibióticos alcançarem os seres humanos através da cadeia alimentar (Fàbrega *et al.*, 2008). No Brasil, há vários estudos relativos a organismos causadores de infecção humana e animal, como, por exemplo, *Staphylococcus* spp., *Salmonella entérica* subs. *entérica* (S.) (Teixeira *et al.*, 1995), *Enterococus faecalism* (Campos *et al.*, 2013) e *Escherichia coli* (Baccaro *et al.*, 2002).

A maior preocupação é a resistência que esses organismos veem desenvolvendo aos antibióticos, causando risco de transmissão de genes de resistência antimicrobiana. Campos et al. (2013), por exemplo, isolaram e analisaram o perfil de resistência antimicrobiana de *Enterococcus* provenientes de carcaças de frango resfriadas e congeladas comercializadas no Distrito Federal. Todas as cepas que foram isoladas apresentaram resistência a, pelo menos um, antimicrobiano, sendo eles tetraciclina, eritromicina, ciprofloxacina e cloranfenicol. A principal preocupação abordada é o surgimento de sérios problemas para a saúde pública devido a capacidade de transmissão dos genes de resistência antimicrobiana para outros microrganismos presentes na microbiota intestinal de humanos e animais, podendo inviabilizar o uso destes medicamentos nos tratamentos clínicos (Campos *et al.*, 2013). (Silva,

Tejada e Timm, 2014) avaliaram a sensibilidade antimicrobiana de cepas de *Salmonella* spp. previamente isoladas de amostras de produtos oriundos de carne de frango, fezes de frango e fezes humanas. Dezessete cepas foram testadas e os autores observaram resistência à poliximina B e eritromicina em 94,1% das cepas e 58,8% apresentaram resistência à sulfonamida e penicilina G. Todas as cepas analisadas apresentaram multirresistência frente aos antibióticos. Assim, a ocorrência de cepas multirresistentes não é um fato raro (Silva, Tejada e Timm, 2014) e deve considerada uma ameaça global devido a capacidade do microrganismo de interromper a atuação de um determinado agente antimicrobiano sobre ele, resultando em tratamentos ineficazes, infecções persistentes e a possibilidade de transmitir essa característica a outros microrganismos (Organização Mundial de Saúde, 2012).

A OMS, após analisar a resistência de microrganismos em 114 países, constatou que todas as regiões do mundo estão passíveis de passar uma "era pós-antibiótico" (World Health Organization, 2014). O elevado número de mortes no mundo devido à resistência das bactérias (European Centre for Disease Prevention and Control, 2009; Klevens, 2007) tem suscitado discussões e publicações de trabalhos com o objetivo de aproximar os leitores ao tema, alertando sobre os riscos e discutindo medidas possíveis para redução dos problemas (Howard *et al.*, 2013; Tillotson, 2013; Wannmacher, 2004; Weckx, 2012; Young, Chopra e Ojoo, 2013). Conjuntamente, descobertas no mundo científico vem sendo realizadas, como é o caso teiobaxin, um novo antibiótico que ainda não foi testado em humanos porém é capaz de sanar infecções graves em ratos sem causar resistência (Ling *et al.*, 2015).

Diversos estudos referentes a resistência a medicamentos vêm sendo realizados nos últimos anos (tabela 6) e demostram que a falta de programas estratégicos para o controle de doenças resulta na administração de medicamentos em intervalos curtos de tempo, determinando a seleção de organismos (Nova *et al.*, 2014). Como alternativa a baixa resposta aos medicamentos uma das saídas de combate é a associação de medicamentos podem alcançar altos percentuais de eficácia e de redução das doenças (Buzzulini *et al.*, 2007).

Chapter 1 - Tabela 6 - Trabalhos brasileiros realizados com intuito de verificar resistência de organismos responsáveis por doenças animais em diferentes matrizes.

ANIMAL	MATRIZ(Referência)	MEDICAMENTO (Referência)	ESTADO(Referência)	REFERENCIA
Ovino	Fezes	Nitroxinil (1, 4) Ivermectina (1, 3, 4, 5, 6, 8, 10) Moxidectina (2, 4, 6, 8) Fosfato de levamisol (2) Benzimidazole (3) Levamisole (3, 4, 5) Closantel (3, 4, 5, 6) Abamectina (4) Sulfato de albendazole (4) Albendazole (4, 5, 6, 8, 9) Triclorfon (6) Tetraciclina (7) Sulfonamidas (7) Estreptomicina (7)	 PR (1,8) RS (2,3,5,7, 14, 20, 21,22 26) SC (4, 18, 25) MS (6) RJ (23) PE(9, 13) RN (10, 11) CE (12) SP (15,16,17,24)	(1) (Nova *et al.*, 2014) (2) (Cezar *et al.*, 2011) (3) (Echevarria *et al.*, 1996) (4) (Hammerschmidt *et al.*, 2012) (5) (Ramos *et al.*, 2002) (6) (Sczesny-Moraes *et al.*, 2010) (7) (Lopes, 2014) (8) (Filho, Pereira e Yamamura, 2009) (9) (Lima *et al.*, 2010) (10) (Pereira *et al.*, 2008) (11) (Coelho, 2009) (12) (Vieira e Cavalcante, 1999) (13) (Barros *et al.*, 2011) (14) (Silva, Tejada e Timm, 2014) (15) (Galdino *et al.*, 2013) (16) (Neves, 2014) (17) (Rangel *et al.*, 2005) (18) (Souza *et al.*, 2008) (19) (Melo *et al.*, 2009) (20) (Valbring, Souza e Silva, 2014) (21) (Colla *et al.*, 2014) (22) (Schmidt e Cardoso, 2003) (23) (Franco *et al.*, 2010) (24) (Baccaro *et al.*, 2002) (25) (Agnol *et al.*, 2014) (26) (Costa *et al.*, 2006)
Caprino	Fezes	Albendazole (9, 10, 11) Ivermectina (10, 11) Levamisole (12) Oxfendazole (12)		
Aves	*Swabs* (13, 15) Fezes e carne (14)	Clortetraciclina (13) Oxitetraciclina (13) Lincomicina (13) Emoxicilina (13)Enrofloxacina (13) Norfloxacina (13) Tiafenicol (13) Timetropim (13, 15) Sulfas (13) Poliximina B (14) Eritromicina (14) Sulfonamida (14, 15) Penicilina (14) Gentamicina (15), Ceftazidima (15) Amoxicilina (15)		
Bovinos	Fezes	Ivermectina (14, 17, 18, 19) Moxidectina (14, 19) Fosfato de levamisol (14, 18) Abamectina (14, 19) Doramectina (17, 19) Sulfóxido de albendozole (17)		
Suínos	Fezes (20, 23, 24, 26) Carcaças (21) Sistema de tratamento de dejeto (22) Carne (23) Tonsilas (24)	Apramicina (20) Florfenicol (20, 26) Trimetropina(20, 22, 26) Trimetropim (20, 26) Amplicina (21, 22, 24, 26) Estreptomicina (21, 22, 24) Sulfonamida (21, 22) Tetraciclina (21, 22, 25, 26) Ácido nalidíxico (22) Cloranfenicol (22, 26) Cefaclor (22)Tobramicina (22, 23) Gentamicina (22, 23, 24, 26) Amoxacilina (22) Neomicina (22) Amicacina(22, 26) Cetiofur (24) Kanamicina (24) Neomicina (24, 26) Oxitetraciclina (24)Eritromicina (25) Sulfamethoxazole (26) Colistina (26) Norfloxacina (26) Enrofloxacina (26)Cefalexina (26)		

5. AMOSTRAGEM, MÉTODOS DE EXTRAÇÃO E CONCENTRAÇÃO E TIPOS DE ANÁLISE DE MEDICAMENTOS VETERINÁRIOS NO AMBIENTE

5.1. AMOSTRAGEM

A escolha de um método de amostragem depende do tipo de amostra, das compostos alvo, do nível de contaminação e do objetivo final da análise. Existem diferentes tipos de amostras ambientais: **(*i*) líquidas**: água de superfície, subterrânea, salobra, salgada, potável, tratada, residual etc; **(*ii*) sólidas**: solo, sedimentos, materiais em suspensão, lama, esterco etc; e **(*iii*) organismos**: microrganismos, biofilmes, micro invertebrados, macro-inverterados, crustáceos, peixes etc.

5.1.1. Amostras líquidas

A amostragem líquida geralmente é de fácil obtenção, porém sua representatividade depende do modo que ela é realizada.

i) Amostragem pontual: A amostragem pontual é a mais fácil de ser efetuada, pois, geralmente, não necessita de material específico e tem baixo custo para a sua obtenção. Uma amostra pontual representa o estado de contaminação em um ponto e em um determinado momento. No entanto, não leva em consideração a evolução do meio (ex.: a flutuação da poluição em função da vazão do rio, a eficiência das estações de tratamento). Diversas amostragens pontuais podem ser realizadas em diferentes pontos e/ou a diferentes momentos com o motivo de integrar variações espaciais e/ou temporais.

ii) Amostragem média: A amostragem média consiste em coletar periodicamente (ex.: todas as horas durante 24h, ou todos os dias durante 15 dias) dentro de uma mesma zona um volume de água. Ela pode ser efetuada manualmente ou através da implantação de amostrador programado. Essa amostragem pode ser ponderada, ligada ou não a variação da vazão. A utilização da vazão pode ser aplicada quando ocorrem variações importantes, como por exemplo, nas estações de tratamento de esgoto ou nos rios. As amostras são misturadas (automaticamente ou manualmente) a fim de obter uma amostra única, representativa dos fluxos de poluentes em um período de tempo. Assim, o fluxo de poluentes pode ser muito variável num mesmo período, especialmente para os efluentes hospitalares (LINDBERG et al., 2004).

iii) Amostragem passiva: A amostragem passiva utiliza um material específico, dependendo da natureza do poluente (Greenwood, Mills e Vrana, 2007). Para acompanhar a poluição com medicamentos, dois tipos de amostradores podem ser usados: o Chemcatcher® (amostradore passivo equipado com uma membrana que limita a difusão) (Lissalde *et al.*, 2016; Vermeirssen *et al.*, 2009) e o POCIS (Amostrador Integrativo de Compostos Orgânicos Polares) (Brown, 2010; Togola e Budzinski, 2007). O POCIS é atualmente o mais utilizado para o estudo de medicamentos em rios. Ele é composto por uma fase absorvente recoberta por uma membrana de proteção. O dispositivo é depositado no local desejado e deixado durante alguns dias ou semanas, dependendo da natureza dos compostos, o nível de contaminação e as características do ambiente (temperatura, pressão). Um equilíbrio é gradualmente estabelecido entre a fase adsorvente e o meio até à retirada do dispositivo. Após a recuperação, a fase contida dentro do POCIS é recuperada e pesada antes de extrair os medicamentos (ver item 2.2 Métodos de extração e concentração). A amostragem passiva dá uma representação do fluxo de contaminantes veiculados através da água.

5.1.2. Amostras sólidas

Diversas estratégias podem ser estudadas para a amostragem de solo e de sedimentos. A heterogeneidade espacial e temporal é fator complicador na amostragem e geralmente passa a ser o ponto crítico nos estudos de monitoramento ambiental de compostos de medicamentos.

i) Solos e sedimentos: Podem ser escolhidas para trabalhar amostras de superfície (primeiros centímetros de espessura) ou em profundidade, utilizando a tradagem. A profundidade pode variar até vários metros, permitindo remontar a níveis mais antigos de contaminação. Assim que a zona contaminada é conhecida e constitui o objeto de estudo, uma amostragem pontual pode ser indicada. Na presença de poluição difusa ou desconhecida, um mapeamento permite o conhecimento da contaminação do local como um todo. Neste caso, várias amostras são coletadas a partir de várias áreas do sítio. As amostras podem ser constituídas de diversas amostragens pontuais ou de uma amostragem média na qual elas são misturadas.

ii) Sólidos em suspensão: Os sólidos em suspensão, que são encontrados nos rios, podem ser um estudo de caso interessante por seu papel como fase portadora (Guyot, Jouanneau e Wasson, 1999) através do transporte de potenciais poluentes (Foster e Charlesworth, 1996). Esses sólidos consistem em sedimentos superficiais remobilizados ou

partículas de solo arrastadas para rios através de escoamento superficial. No entanto, as concentrações de sólidos em suspensão podem sofrer grandes variações espaciais e temporais devido ao uso e manejo do solo, conformação da paisagem, condições climáticas e fase da chuva, entre outros. A amostagem manual desses sólidos pode ser considerada, mas é muito localizado e não integrativa para as condições ambientais e variações temporais. Assim, um coletor pode ser instalado visando a amostragem durante vários dias. Esses coletores podem ser constituídos por um tubo no qual é colocado um filtro de pano na extremidade ou, outro sistema que foi descrito por Phillips et al. (2000), em que o coletor possui dois pequenos furos, localizados nas extremidades do coletor, permitindo a entrada e saída de água. Estes furos permitem a entrada de água e a sedimentação dos sólidos em suspensão na parte inferior do coletor.

5.1.3. Amostras biológicas

Os organismos vivos captam e acumulam muitos contaminantes em seus tecidos ao longo de suas vidas. A análise química destes contaminantes é capaz de fornecer informações em relação a poluição recebida por esses organismos.

i) Biofilmes: Os biofilmes são constituídos por microrganismos (eucariotos, procariotos, protozoários e vírus) que secretam uma matriz protetora de consistência viscosa orgânica e mineral (Watnick e Kolter, 2000). Eles se desenvolvem na superfície de rochas e plantas, participando de forma significativa da cadeia trófica e dos ciclos bioquímicos do ambiente (Battin *et al.*, 2003). Eles estão em interação permanente com a água e seus poluentes, podendo acumulá-los de acordo com seu desenvolvimento (Drury, Stewart e Characklis, 1993). A amostragem de biofilmes pode ser feita diretamente nas rochas e plantas ou através da instalação de suportes de rochas mantidos constantemente em imersão. O biofilme natural ou desenvolvido é recuperado através da escovação das rochas e plantas, recuperando o líquido que é em seguida congelado e liofilizado antes de ser preparado para a análise (Laurent, 2013).

ii) Macro invertebrados/moluscos: A utilização de um Surber permite a coleta de macro invertebrados presentes no meio. O Suber é constituído por um quadro com uma malha coletora de malha variável, mas geralmente de 250 µm. Porém, o tamanho da malha pode variar dependendo do objetivo da pesquisa, tais como a importância ou não da coleta de indivíduos muito pequenos e imaturos, número de espécimes coletados entre outros (Silveira,

Queiroz e Boeira, 2004). Ele é posicionado contra a correnteza e a área de amostragem é fixada no leito do rio. A área de amostragem do Surber é de 900 cm^2 e a coleta deverá representar todo o rio. Assim, vários lugares da região devem ser escolhidos para amostrar diferentes substratos (ex.: sedimentos, rochas e plantas) e áreas de alta e de baixa velocidade do fluxo.

iii) Organismos superiores (peixes, mamíferos, etc.): Na maioria dos casos, é necessário capturar indivíduos em seu ambiente natural ou nos locais onde eles se alimentam. Dependendo das espécies a serem estudadas, são realizados somente amostragens de fluídos biológicos (sangue, urina) antes de colocá-los novamente em liberdade ou então o organismo é sacrificado para a realização de testes a partir da remoção de tecidos ou órgãos receptivos à presença de contaminantes.

5.2. MÉTODOS DE EXTRAÇÃO E CONCENTRAÇÃO

A fim de analisar os resíduos de produtos veterinários contidos nas matrizes sólidas (sedimentos e solos), biológicas (biofilmes e tecidos animais) ou líquidas (água) por meio de análise cromatográfica, o analíto tem que ser isolado da sua matriz. Da mesma forma, uma etapa de concentração é necessária devido à baixa concentração destes compostos em matrizes ambientais (da ordem de ng g^{-1}). Existe, portanto, a necessidade de um plano de preparação de amostras, levando em consideração a extração, a purificação e a concentração da substância a analisar.

5.2.1. Matrizes sólidas

No caso das matrizes sólidas (sedimento e solo) ou biológicas (biofilmes e tecidos animais), a extração sólido-líquido permite a dissolução do composto orgânico em um solvente. A técnica de Soxhlet compreende o aquecimento a refluxo de um solvente que, ao se condensar, atinge o corpo do extrator contendo o sólido a ser extraído e a sonicação no caso dos ultrassons em que o sólido é colocado num solvente sonicado. A sonificação é utilizada para desencadear cavitações ultrassônicas no meio, aumentando a temperatura e a pressão promovendo a solubilização de compostos solúveis no solvente. Este método é simples e barato, no entanto, tem desvantagens como a dimensão limitada da amostra e tempo de extração (várias horas). Atualmente, as técnicas de extração tendem para uma maior automatização e utilização de quantidade reduzida de solvente.

i) Extração líquida por pressão (pressure liquid extraction, PLE) : A PLE consiste em fazer percolar um solvente aquecido sob alta pressão através do sólido a ser extraído. A célula extratora é colocada dentro do extrator onde ela é preenchida com solvente durante um tempo de contato definido, para então ser esvaziado e o extrato ser recolhido num frasco. Vários ciclos de extração podem ser realizados, bem como misturas de solventes quando os compostos são compatíveis. Diversos fatores podem influenciar na extração e devem ser ajustados como: o volume e a natureza do solvente, a temperatura e pressão, o número de ciclos de extração, a duração destes ciclos e a velocidade de drenagem de célula. Este método reduz significativamente o tempo de extração (cerca de uma hora para uma amostra de sedimento) e é possível trabalhar com diferentes volumes de amostra (células podem conter um volume entre 1 e 100 mL). Além disso, é possível criar extrações sucessivas, utilizar diferentes solventes ao mesmo tempo através de um sistema automatizado. No entanto, este tipo de dispositivo não é adequado para compostos termolábeis. Os métodos de PLE aplicados a extração de medicamentos veterinários em amostras ambientais solidas está apresentado na tabela 7.

Chapter 1 - Tabela 7- Métodos de PLE aplicados a extração de medicamentos veterinários em amostras ambientais solidas.

Composto	Matriz	Solvente (v/v)	T (°C)	P (bar)	Ciclos	Tempo estático (min.)	Flushing (%)
Macrolídeos Ionóforos Tiamulina	30 g solo	1% amônia em MeOH	80	140	2	10	70
Sulfonamidas Penicilinas	5 g lama de bacia de infiltração	Acetona/MeOH 50:50	75	150	3	5	60
Sulfonamidas Tetraciclinas Macrolídeos	10 g solo	MeOH/solução tampão de ácido cítrico (pH 4.7) 50:50	Ambiente	150	-	3	-
Antimicrobianos (diversas classes terapêuticas)	10 g solo	MeOH/água 80:20	100	140	3	10	50

Fonte: (Díaz-Cruz e Barceló, 2007)

ii) Extração por fluído supercrítico (SFE): Esta técnica é semelhante ao PLE com a utilização de pressões mais elevadas, mas temperatura mais baixa e, portanto, adequado para os compostos termolábeis. Este método baseia-se nas propriedades dos fluídos supercríticos, entre o líquido e o gás correspondente. Os principais parâmetros em relação a extração são a natureza do fluído (geralmente é usado o dióxido de carbono), a pressão e a temperatura. Além do tempo reduzido de extração, este método utiliza pouco, ou nenhum, solvente orgânico. No entanto, a coleta dos extratos continua problemática.

iii) Extração por micro-ondas (MAE): Nessa técnica, o solvente e a amostra são aquecidos por micro-ondas. A economia de tempo é importante e é possível extrair várias amostras ao mesmo tempo com uma quantidade mínima de solvente. No entanto, é possível apenas a utilização de solventes polares (Sanchez-Prado *et al.*, 2015) e este método não é automatizado.

Um resumo comparativo entre as técnicas de extração é apresentado na tabela 8.

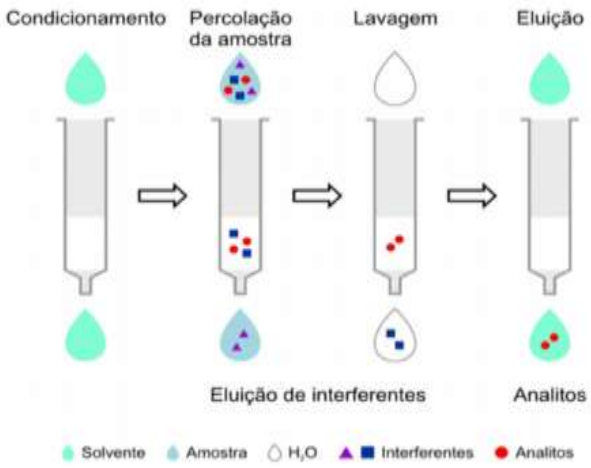
Chapter 1 - Tabela 8 - Comparação entre as técnicas de extração (Kinsella *et al.*, 2009).

Técnica de extração	MAE	PLE	SFE
Tempo de extração	3–30 min	5–30 min	10–60 min
Massa de amostra	1–10 g	1–30 g	1–5 g
Solvente usado	10–40 ml	10–100 ml	2-20 ml
Investimento	Moderada	Alta	Alta
Vantagens	•Extrações rápidas e múltiplas •Baixo volume de solvente •Altas temperaturas	•Extrações rápidas •Baixo volume de solvente •Temperaturas elevadas •Filtragem desnecessária • Sistema automatizado	•Extrações rápidas •Volume mínimo de solvente •Temperaturas elevadas •Baixa interferência da matriz •Filtragem e limpeza desnecessária • Sistema automatizado
Desvantagens	• Solvente Polar • Limpeza necessária • Tempo de espera necessário para resfriar seus recipientes	• Limpeza necessária	• Muitos parâmetros para aperfeiçoar especialmente o conjunto de analítos.

5.2.2. Extração de poluentes orgânicos em matrizes líquidas e purificação

i) Extração líquida: Esta técnica simples e antiga permite a transferência seletiva de compostos presentes numa solução para uma segunda solução imiscível com a primeira. Na prática, as duas soluções são colocadas em contato, agitadas e separadas. A natureza e o volume da solução de extração e o pH são parâmetros ajustáveis. Devido à baixa seletividade deste método, impurezas podem subsistir após a separação.

ii) Extração em fase sólida (solid phase extraction, SPE): O princípio deste método baseia-se na distribuição de compostos entre uma fase estacionária sólida (sorvente) e uma fase móvel líquida. Na prática, a amostra penetra através de uma fase sólida (pré-condicionada) contida num cartucho. Os compostos de interesse são retidos pelo adsorvente que é então lavado para remover impurezas. Finalmente, os compostos de interesse são eluídos em um pequeno volume de solvente orgânico. A respresentação dos quatro estágios para a recuperação dos compostos de medicamentos estão apresentados na figura 7.



Fonte: (Caldas *et al.*, 2011)

Chapter 1 - Figura 7 -. Representação dos quatro estágios para a recuperação dos compostos desejados.

A escolha dos solventes (em todas as etapas) é um fator importante, assim como a escolha do adsorvente. No caso da análise de resíduos de produtos veterinários, resinas poliméricas permitem absorver grandes famílias de compostos. Esse método é atualmente muito utilizado. Ele apresenta a vantagem de ser automatizado, de aceitar volumes de cargas importantes e permite igualmente uma boa purificação dos extratos dos tecidos extraídos das amostras solidas, liquidas ou biológicas.

iii) Secagem e restituição: Uma vez purificado, o extrato pode ser seco para remover a água residual. O solvente orgânico é então evaporado a uma temperatura moderada, vácuo ou fluxo de nitrogênio. Finalmente, o volume da amostra é ajustado permitindo o acesso ao fator de concentração dos processos de extração e purificação.

Desde que estabelecido o protocolo de preparação de uma amostra, é importante otimizar cada etapa. É importante notar que quando uma análise de multi-resíduos é realizada, a otimização se torna um compromisso. Além disso, é necessário conhecer a eficiência da extração de cada etapa de preparação. Um rendimento de 80% a 120% é geralmente considerado satisfatório (Rodier, Legube e Merlet, 2016). Existem inúmeros métodos de extração e purificação que podem ainda ser combinados. É importante que cada etapa seja aperfeiçoada a fim de facilitar a análise das amostras. A má preparação levará a uma análise ruim. Existem inúmeras publicações científicas tratando sobre a análise de resíduos farmacêuticos e /ou veterinários nas quais os métodos descritos neste capítulo são utilizados.

5.3. TIPOS DE ANÁLISE

Os espectrômetros de massa de alta resolução são utilizados para a análise de poluentes orgânicos. Diferentes tecnologias são possíveis e a escolha é geralmente feita considerando o objetivo de quantificação e/ou identificação almejada.

5.4. Análise quantitativa

A análise quantitativa permite determinar com segurança concentrações de compostos químicos. Esta análise é geralmente utilizada para medir compostos de interesse a partir de métodos de análise sensíveis que permitem a detecção de concentrações muito pequenas. Essa sensibilidade é obtida através de tecnologias seletivas (ex. triplo quadruplo, Orbitrap) onde, espectrômetros de massa dedicados à análise de um composto alvo utilizam os fragmentos da massa específica da molécula para conseguir detectá-la e identificá-la.

5.4.1. Screening

A análise conhecida como "*screening*" é cada vez mais utilizada nos estudos de contaminantes. Ela permite colocar em evidência compostos que não são pesquisados através da análise quantitativa. A técnica de screening chamada de "target screening" refere-se a busca específica de substâncias previamente conhecidas enquanto o screening chamado de "non – target screening" se refere a pesquisa de substâncias desconhecidas. Para realizar um screening, os métodos de análise utilizam os espectrômetros de massa de alta resolução permitindo a obtenção de grande precisão da massa (noção de "massa exata") e a associação de uma massa específica relacionada à fórmula bruta (ex.: Time of Flight, Orbitrap) (Munaretto *et al*., 2016) .

i) Screening direcionado (targeted screening): O screening direcionado é geralmente utilizado quando se buscam compostos selecionados dentro de uma lista específica como, por exemplo, as principais famílias de contaminantes referenciadas (ex.: hidrocarbonetos aromáticos policíclicos, pesticidas, bifenilas policloradas, dioxinas, furanos, substâncias farmacêutica e cosmética). Assim, essa análise permite que o pesquisador consiga uma indicação da presença ou ausência das compostos almejadas e a concentração estimada possibilita igualmente a obtenção de informações semi-quantitativas.

ii) Screening não-direcionado (non-targeted screening): O screening não-direcionado, comparado com o screening direcionado, permite a realização de um inventário maior de compostos e suas classes. Ele permite que inicialmente não seja realizada a escolha de compostos alvo, porém permite que sejam evidenciadas compostos desconhecidas (não identificadas) relacionadas com a poluição. No entanto, ele não permite detectar e identificar todas as substâncias presentes em uma amostra. Dessa forma, a análise por screening não direcionado depende das etapas de preparação das amostras (extração com auxílio de um solvente, concentração/enriquecimento da fase sólida) e das condições de medida (parâmetros do espectrômetro de massa, biblioteca de espectros de referência).

CONSIDERAÇÕES FINAIS

Esse capítulo foi escrito com o objetivo de instigar os leitores a temática de poluição ambiental por compostos emergentes. Essa temática tem ganhado espaço para discussões em

todo mundo pois existe uma grande preocupação em relação aos riscos que o homem pode oferecer através de suas interferências no meio ambiente.

Considerando os avanços no setor nacional e mundial de produção animal, espera-se que haja aumento na produção animal, na utilização de medicamentos e no volume de dejetos produzidos. Esses resíduos serão aplicados nos campos de produção e poderão atingir diferentes matrizes ambientais. A grande preocupação está relacionada com os potenciais de contaminação ambiental de medicamentos veterinários no meio ambiente pois, organismos vivos se desenvolvem em zonas contaminadas e através da cadeia trófica podem atingir os seres humanos. Dessa forma, é compromisso de todos conhecer os riscos de nossas práticas agrícolas para que possamos no futuro criar práticas agrícolas conservacionistas e preventivas.

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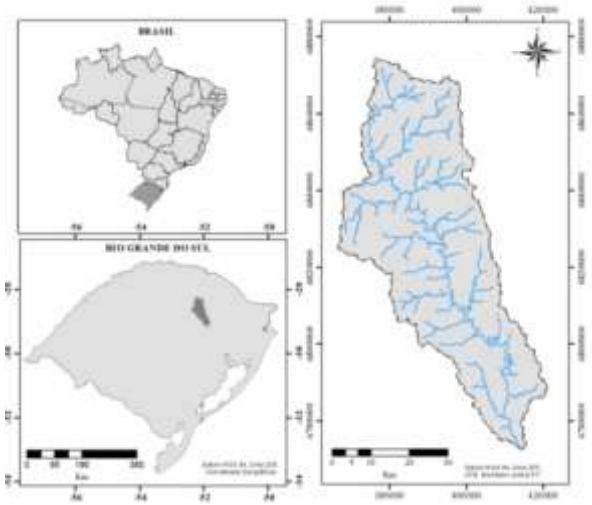
CHAPTER 2 - GENERAL MATERIAL AND METHODS

1. GUAPORÉ WATERSHED AND SAMPLED SITES

The watershed of the Guaporé River is located in the northeastern of the state of Rio Grande do Sul, Brazil (Figure 1).



Fonte: (Lima, 2017)

Chapter 2 - Figure 1 - Geographical position of the Guaporé River basin - Brazil.

This watershed drains an area of about 2,030 km^2. Water bodies represent approximately 0.57% of the watershed, and urbanization is not significant (~ 0.60% of total area). Agriculture is the main activity for most of the rural residents of this region, which comprises crop fields (32.5%), grasslands (9.7%) and forests (56.68%). The climate is subtropical super-humid mesothermal, with cool summers and too frequent severe frosts without dry seasons. The average annual rainfall varies between 1,400 mm yr^{-1} and 2,000 mm yr^{-1} and the annual mean temperature is 18.4 ºC. The topography is undulating to hilly. In this region, the waste water from the cities of Marau (37,145 inhabitants), Ilópolis (4,098 inhabitants), Arvorezinha (10,229 inhabitants), Itapuca (2,337 inhabitants), União da Serra (1,620 inhabitants), Nova Alvorada (3,177 inhabitants), Montauri (1,542 inhabitants), Vila Maria (4,221 inhabitants), Camargo (2,591 inhabitants) and Mato Castelhano (2,470 inhabitants) are directly released (without treatment) inside the rivers. The farmers mainly grow pasture for feeding their animals (dairy cattle, swine and poultry farming) and cultivate grain crops (mainly soybeans, corn and wheat). The upper portion of the watershed with undulated relief and deep soils is cultivated using no-tillage systems. In the middle and lower parts of the watershed with sloping relief and shallow soils, agricultural activities, especially

the cultivation of tobacco, are developed in small farms. The soils of this watershed are exposed to erosion and high sediment loss (Tiecher, 2015). The losses of soil material are greater for shallow soils and hilly areas. Still, in many areas, crop and soil management does not take into account the fragility of the soils, resulting in intense erosion areas (Figure 2).

Chapter 2 - Figure 2 - Landscapes of Guaporé watershead (Left represent north and right represent south watershed region).

1.1. CAPINGUI WATERSHED

The Capingui sub-watershed is located in the northern portion of the Guaporé's watershed. Nowadays, most of the area is used for growing soybeans (*Glicine max* (L.) Merrill) and corn (*Zea mays* L.) crops using no-tillage systems. In this sub-watershed, four points with different characteristics were monitored.

The northern point is the National Forest of Passo Fundo (FLONA – point 1, Figure 17) (Figure 3). This point is a Federal Conservation Unit of 1,300 hectares, created in the 1940s, including 450 hectares of native forest belonging to the biome of the Atlantic Forest. In this study, this site will be considered as the control sample due the low anthropic action. However, it is important to consider that the waters of other rivers (eg.: Tingatu, Cachoeirinha, Capingui and Rio Branco) have a direct influence on FLONA. The sample point is placed in the João de Barro Stream, classified as 1st order tributary river of the Capingui River. The catchment area of this point have 1.3 km^2 covered by Araucarias (*Araucaria angustifólia* Kuntze); Eucalyptus (*Eucalipytus* sp); Pinus (*Pinus elliotti*) and Mixed Ombrophilous Forest. However, there are unpaved roads that pass through the catchment area, establishing connectivity to the farming areas around Flona with the João de Barro Stream (Lima, 2017).

Chapter 2 - Figure 3 - Flona sampling site

The water of FLONA drain into the reservoir of Capingui Dam. Therefore, downstream of the Capingui Dam a sampling site was located (Capingui Dam – point 2 Figure 17) (Figure 4). Today, Capingui Dam is used only for recreation, with a rampant increase human occupation without a network waste collection, resulting in wastewater release directly on the dam. This point is located 600 m downstream of the Capingui River dam. Its catchment area is 123 km^2, however the dam limits water and sediment flow in

approximately 95% of the area. The presence of the riparian forest is restricted between 15 and 60 m wide, being rounded by the plantations managed in no-tillage system. Soya and corn crops (spring / summer) and oats and wheat (autumn / winter) occupy 64% of the area. In this region poultry and pig breeding is inexpressible, but despite the limited presence of cattle. The roads in this region channel the runoff directly into the river network (Lima, 2017).

Chapter 2 - Figure 4 – Outlet of Capingui Dam sampling site

Downstream of the Capingui dam, two sites were also monitored. The first site represents the contribution of a vast area of agricultural production (Agriculture – point 3, Figure 17) (Figure 5). It comprises the second point sampled in the Capingui River located 17 km from the Capingui River dam. The catchment area of this point is 201 km^2. Along this river the riparian forest has a length of 15 m on both sides, and no damping zone can be seen in the drainage lines. Crops occupy 69% of the area and are managed under no-tillage system with soybean and corn in spring / summer and oats and wheat in autumn / winter. Unlike the other points located in the Capingui River, corn cultivation near this point is more expressive. In this region of the Capingui River basin the cultivation of maize, poultry and pig farming in integrated systems, and cattle breeding are superior to the other points. There is a wide network of drainage systems in floodplain areas and erosive processes in furrows, channeling the waters and sediments to Capingui river and its tributaries (Lima, 2017).

Chapter 2 - Figure 5 - Agriculture sampling site

The second site is the outlet of sub-watershed of Capingui (Capingui outlet - point 4, Figure 17) (Figure 6). This site receives runoff water from the grain production region, however, with a large concentration of residues coming from breeding of confined animals, pig poultry and dairy production. It is located 26 km from the dam with 267 km^2 of catchment zone. Crops occupy 71% of the area and the riparian forest remains restricted to 15 m in length. The cultivation system is similar to that of point 3, but drainage systems are less noticeable. Animal husbandry is more focused on poultry and cattle breeding (Lima, 2017).

Chapter 2 - Figure 6 - Capingui outlet sampling site

1.2. MARAU WATERSHED

The Marau sub-watershed is characterized by the predominance of family agriculture and livestock production, with the contribution of wastewater from urban areas of the city of Marau (37,145 inhabitants). The economy of this sub-watershed is based in animal production

and dry grains coexist with livestock production on almost all properties. In the urban areas, there are agribusinesses, especially large slaughterhouses for pigs and poultry for export (Prefeitura Municipal de Marau, 2013). The impact of human activities, rural and urban, was monitored at four sites.

The first site, in addition to grain production, has a lot of pigs and dairy cows (Marau upstream Animal - point 5, Figure 17) (Figure 7). Located in Marau River, 3.5 km upstream of the confluence with Cestiado stream, this basin drains 165 km^2. In the lower third of this contribution area there are fragments of forest between the crop areas, differently than in the upper two thirds, which present a greater uniformity in the spatialization of the crops. The riparian forest is present on both sides, but in most cases it does not exceed 15 m wide. The main agricultural and livestock activities are poultry farming and cattle raising, no-tillage cultivation of soybeans and corn in spring / summer, oats and ryegrass in fall / winter. In this region, oat cultivation is predominantly for grazing, and areas with corn planting for grain production and silage are also highlighted. It should be noted that 3 km from this point there is the water intake system, operated by Corsan, to supply 31,145 inhabitants of the city of Marau. From this stream are pumped up to 300 L s^{-1} to the conventional type treatment plant (Lima, 2017).

Chapter 2 - Figure 7 – Marau upstream Animal sampling site

The second one represents the water coming predominantly from runoff of grain production areas in no-tillage (Marau upstream - Grain - point 6, Figure 17) (Figure 8). It is situated upstream of the urban perimeter of the municipality of Marau in Cestiado stream, a tributary of Marau River. This point has 13.4 km^2 of catchment area, the distance between the river and the fields is restricted to the presence of 15 meters of riparian forest on both banks.

Agricultural activity is developed in 72% of the area. Soya and maize production is carried out in spring / summer under no tillage system and in succession in the autumn / winter the planting of oats / ryegrass for dairy cattle pasture. The breeding systems are focused on the production of bovine milk and poultry farming in an integration system (Lima, 2017).

Chapter 2 - Figure 8 - Marau upstream – Grain sampling site

Two sites were monitored downstream of the city of Marau, which receive all the urban pollution load: the first site is immediately downstream of the town (Marau city – point 7, Figure 17) (Figure 9). Located in Marau River, 10 km from point 6, downstream of the urban area of Marau, totaling a drained area of 227 km^2. This point receives the launch of the industrial, pluvial and sewage depletion of the city that does not have a sewage treatment system. Marau does not have any sewage treatment system, which results in a huge increase of pollution in the river arising from the urban area. The path of the river into the city suffers various forms of environmental degradation. There is evidence of wastewater, municipal solid waste and industrial effluents, such as observed fish death along the river (Prefeitura Municipal de Marau, 2013).

Chapter 2 - Figure 9 - Marau city sampling point

The outlet of the sub-watershed of Marau (Marau Outlet – point 8, Figure 17) (Figure 10) aims to check the river resilience potential when the entry of urban pollutants decreases. It is located in Marau river, 0.9 km from the confluence with the Capingui River and 23 km from point 6, comprising the outlet of this river basin and draining 256 km^2. The margins of this point have areas of soy and maize grown under no-tillage system in the spring / summer, and winter is grown oats. Dairy farming and integrated poultry farming are among the main agricultural activities (Lima, 2017).

Chapter 2 - Figure 10 – Marau Outlet sampling sites.

A site was also monitored at the confluence of the Capingui and Marau sub-watersheds, (Guaporé River - point 9, Figure 17) (Figure 11). From this point, the river is called the Rio Guaporé. The site is located 1.2 km downstream of the confluence of the Capingui River and Marau River. This area integrates the anthropic pressures of the two sub-watersheds, totaling 542 km^2 of catchment area. At its margins crop and breeding systems are similar to point 4 and 7, and the riparian forest area has 15 meters wide (Lima, 2017).

Chapter 2 - Figure 11 – Confluence sampling site

1.3. LAJEADO CARAZINHO WATERSHED

The Lajeado Carazinho sub-watershed is a small region located in the central part of the Guaporé River watershed. It is a fragile region characterized by sloping relief, impoverished and predominantly used for tobacco cultivation (Didoné *et al.*, 2014). In this zone three sites were monitored.

The first site is located upstream of the tobacco-producing areas (Upstream Lajeado Carazinho – point 10, Figure 17) (Figure 12), located in Lajeado Carazinho River with a drainage area of 30 km^2. In the upper third of this catchment area the relief is less sloping and it is still possible to find areas cultivated by soybeans. However, corn and tobacco cultivation is predominant. Soil use is very heterogeneous, with expressive areas of riparian forest, tobacco cultivation and corn managed in conventional plantation. In winter the areas are fallowed with oats or under grazing. The presence of soy is more restricted. The total area cultivated corresponds to 40% (Lima, 2017).

Chapter 2 - Figure 12 - Upstream Lajeado Carazinho sampling site

The second site receives the water passing by the tobacco producing area (Tobacco – point 11, Figure 17) (Figure 13). Located in a tributary locate 0.2 km of the Lajeado Carazinho River, its catchment area is 3.2 km^2 in a V-shaped valley, which has 32% of its area occupied by tobacco and corn crops in spring / summer, and in winter it remains fallow with oats. The crops are located in the middle third of the slope between the riparian forest from 15 to 60 m and the upper third covered by forest (Lima, 2017).

Chapter 2 - Figure 13 - Tobacco sampling site

This point represents the downstream of tobacco contribution (Downstream of tobacco in Lajeado Carazinho – point 12, Figure 17) (Figure 14). Located at Lajeado Carazinho river, 2.7 km from Guaporé River and 3.2 km from point Upstream Lajeado Carazinho, it has a contribution area of 39 km^2. Around this point, the cultivation of corn and tobacco in conventional plantation, and the rearing of pigs and cattle predominates. In general, 40% of the area is occupied by crops and the banks of the river are covered by forests. However, there are clearings near the banks of the river where maize is grown. The smoke for the most part is located at the top of the slope (Lima, 2017).

Chapter 2 - Figure 14 - Downstream of tobacco in Lajeado Carazinho sampling site

Two sites were selected on the main river (Guaporé's River).

One site is placed upstream of this sub-watershed (Upstream Lajeado Carazinho sub-watershed – point 13, Figure 17), on the Guaporé River, 50 km from the point 9, with a draining area of 1,442 km^2. Along these 50 km the width of the riparian forest in the two margins remains in 15 m. In this region there are contributions from the tributaries that pass through the cities of Montauri, Camargo, Vila Maria, Nova Alvorada and Santo Antônio do Palma. Soya and corn cultivation is carried out in 67% of the no-tillage area and, in the areas closest to that point, maize cultivation is intensified in space and time. In autumn / winter the areas are cultivated with oats for pasture formation. Dairy farming is also intensified in this area. Next to the point it is perceived that the crops are fragmented between the forest (Lima, 2017).

The second point is placed downstream of the tobacco cultivation in Lajeado Carazinho River (Downstream Lajeado Carazinho sub-watershed – point 14, Figure 17).

Located on the Guaporé River 55 km from the confluence (point 9). This point receives the waters of Lajeado Carazinho River, extending its catchment area to 1,505 km^2. The width of the riparian forest between this point and the point Upstream Lajeado Carazinho is higher than 60 m. On the slope there are fragments of crops interspersed with forest areas. Cumulative area of cultivation represents 66% and the areas under cultivation of maize overlap the areas cultivated by tobacco and soy. The no-tillage system around this point is restricted to the top of the mountains (Lima, 2017).

1.4. CARAZINHO WATERSHED

The sub-watershed of the Carazinho River is located in the southern part of the Guaporé River watershed. This sub-watershed located near the Lajeado Carazinho sub-watershed was historically focused on food production. Characterized by a mountainous region, Carazinho sub-watershed is occupied by native forest and reforestation areas due to rural exodus. The sampled area was chosen to represent low human impact (pressure) and lower intensification of agriculture (crops and animal production) in the studied region. The Lajeado stream is a tributary of the Guaporé River that passes through the Carazinho subwatershed.

In the Lajeado stream, the Carazinho sub-watershed outlet (Carazinho Outlet – point 16, Figure 17) is located in the Lajeado River catchment, two km from the Guaporé River, draining an area of 144 km^2. In this basin 50% of the land is under cultivation. The presence of riparian forest and forest fragments is quite significant in the lower two thirds of the main river. However, agriculture is practiced more intensively in the headwaters, where maize, tobacco and soya crops border the streams. Also in this region it is possible to verify the breeding of poultry in integration systems (Lima, 2017).

The confluence of the Carazinho River with the Guaporé River (Figure 15) is located on the Guaporé River, 73 km from the Confluence (Central Carazinho – point 17, Figure 17), downstream of the confluence with the Carazinho watershed. The area around the point has similar characteristics to that surroundings Carazinho sub-watershed outlet, but receives the influences of the anthropic activities developed in the Carazinho River hydrographic basin presented in point Downstream of tobacco in Lajeado Carazinho (point 18, Figure 17), totaling 1850 km^2 (Lima, 2017).

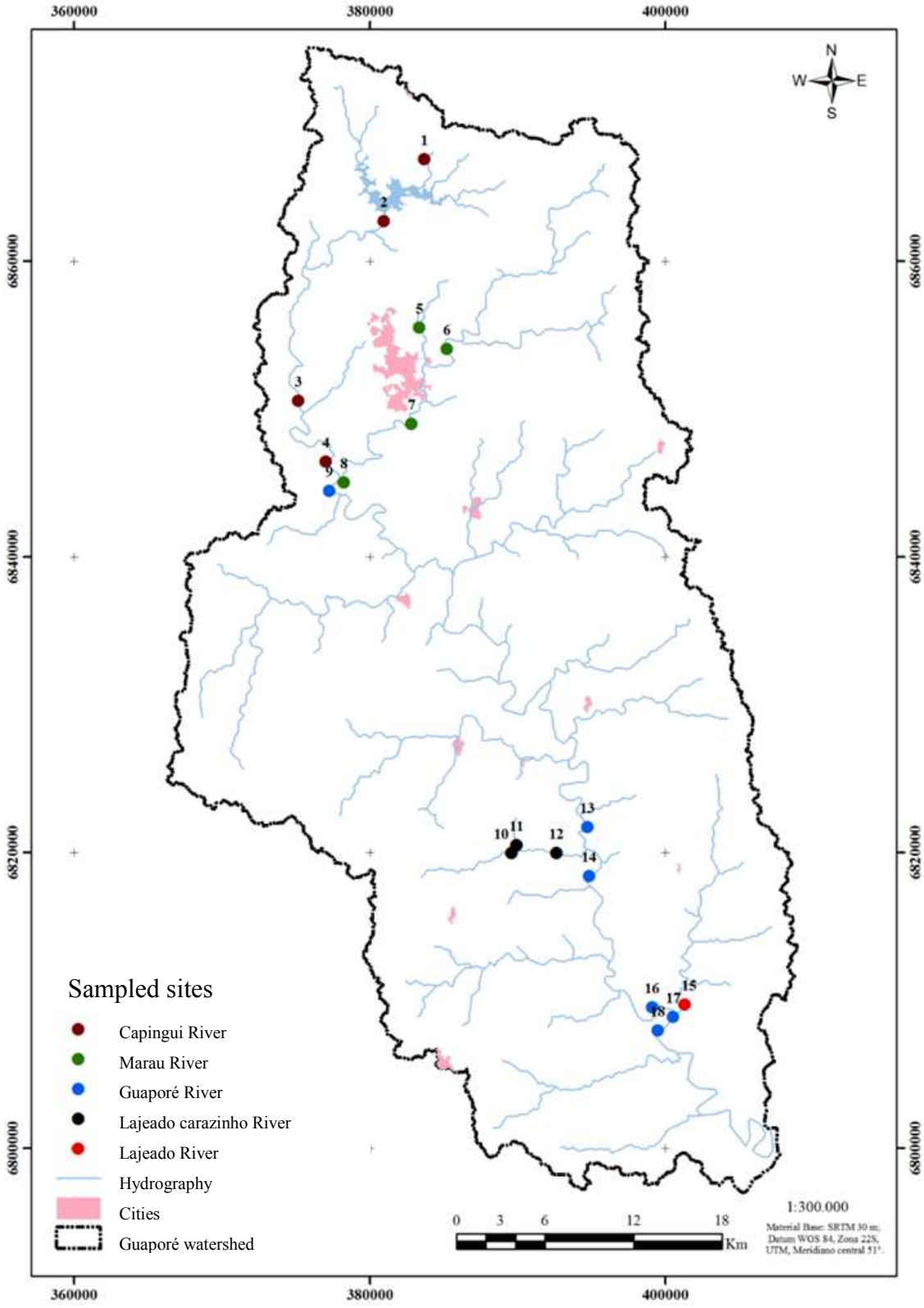
Chapter 2 - Figure 15 – Central Carazinho sampling site

In the Guaporé River, one point was placed upstream and another downstream of the Carazinho sub-watershed. The upstream (Figure 16) is located on the Rio Guaporé, 71 km

from Confluence of Marau and Capingui Rivers (upstream Carazinho sub-watershed – point 16, Figure 17). The surrounding area is quite steep and mostly covered by riparian forest with width often exceeding 100 m. The landuse, around the point, forms a true mosaic of forests, plantations of yerba mate, tobacco and corn in conventional plantation integrating the 1,697 km^2 of catchment area. In the vicinity of this point the animals' breeding is of less expressiveness. But dairy cattle still stand out over other creations (Lima, 2017).

Downstream of Carazinho watershed (Downstream Carazinho watershed – point 18, Figure 17) is located on the Guaporé River at 75 km from the Confluence (point 9, Figure 17). It is the most downstream monitoring point in the Guaporé River basin, comprising 1,853 km^2. The characteristics of the areas around this point resemble those of points Upstream Carazinho (point 16) and Central Carazinho (point 17) sampling sites.

Chapter 2 - Figure 16 - Downstream Carazinho watershed sampling site



Fonte: (Lima, 2017)

Chapter 2 - Figure 17 – Sampling points to POCIS and biofilms in Guaporé watershed, Rio Grande do Sul - Brazil

2. PHARMACEUTICALS AND SUCRALOSE

2.1. PHYSICAL AND CHEMICAL PROPERTIES

The acidic and/or basic pharmaceuticals functionalities is controlled by both solution pH and acidic dissociation constants (i.e. $K_a$values), whereas antimicrobial activities of antibiotics are associated with different functional groups of the molecular structure (Babić *et al.*, 2007; Thiele-Bruhn, 2003). Acid ionization equilibrium constant (pKa) describes the acid dissociation of pharmaceuticals and this constant enable to estimate the major species of pharmaceuticals present in the environment (Barceló and Hennion, 1997). The solubility in water describes the pharmaceutical's behavior in relation to transport and possible environmental destinations, and the *n*-octanol-water partition coefficient (K_{ow}), which lists the hydrophilic and lipophilic properties, and is related to the tendency of bioconcentration of these compounds. The sorption potential of hydrophobic contaminants according to their K_{ow} is considered with low sorption potential when log K_{ow} < 2.5, those with log K_{ow} between 2.5 and 4.0 have a medium sorption potential and those with log K_{ow} >4.0 have a high sorption potential (Rogers, 1996).

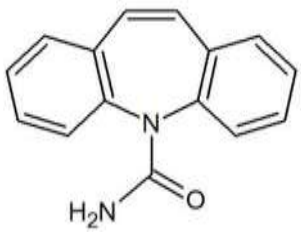
Chapter 2 - Table 1- Proprieties of the studied pharmaceuticals[1].

Family	**Compounds**	**pKa (pKa_1 / pKa_2)**	**log K_{ow}**	**K_{oc}**
	Sucralose	11.91	-1.00	10
	Carbamazepine	13.90	2.45	520
-	Diclofenac	4.15 (4/-2.1)	4.51	245
Sulphonamide	Sulfamethazine	7.59 (2.65/7.65)	0.14	98
	Sulfamethoxazole	(6.16/1.97)	0.89	72
	Sulfaquinoxaline	5.10	1.68	200
Quinolones	Norfloxacin	(6.34/8.75)	0.46	61,000
	Ciprofloxacin	6.09 (6.09/8.74)	0.28	61,000
	Enrofloxacin	(6.43/7.76)	2.31	16,500 to 770,000[2]
	Levofloxacin	(6.24/8.74)	2.10	44,143
Macrolides	Erythromycin	8.88	3.06	570
	Roxithromycin	(12.45/9.08)	1.70	364,760
	Tylosin	7.73	1.63	553 to 7,988
Tetracycline	Oxytetracycline	3.27	-0.90	195 to 93,317

Fontes: [1] https://pubchem.ncbi.nlm.nih.gov; [2] Tolls, 2001

2.1.1. Carbamazepine

Carbamazepine (Figure 18) is one of the well-studied and well-known drugs belonging to the class of antiepileptics and anticonvulsants, widely used in the treatment of epilepsy. In Brazil, this drug is included in the list of Pharmaceutical Assistance of the Unified Health System, and its use is approved by the National Agency of Sanitary Surveillance (ANVISA) for: (1) Epilepsy (Complex or simple partial seizures, with or without loss of consciousness) (2) Acute mania and maintenance treatment in bipolar affective disorders to prevent or attenuate recurrences, (3) Alcohol withdrawal syndrome (4) Idiopathic trigeminal neuralgia and trigeminal neuralgia (5) Diabetic neuropathy, (6) Diabetes insipidus centralis, polyuria, and polydipsia of neurohormonal origin (SAUDE, 2017). This is due to multiple sclerosis (typical or atypical) and idiopathic glossopharyngeal neuralgia.



Chapter 2 - Figure 18 – Structural formula of Carbamazepine

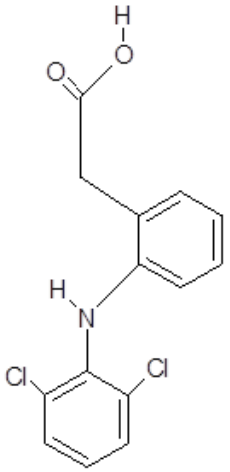
Due to pKa values (-3.8/15.98) carbamazepine could only be found as neutral form in environment. Studies have stated that carbamazepine may be a better chemical indicator of sewage contamination than caffeine due to its restriction to anthropogenic origins and its low degradability (Daneshvar *et al.*, 2012; Sousa *et al.*, 2014). Its half-life in the environment is considered to be long, averaging 50% of the dissipation time of 82 ± 11 days, under semi-field conditions, allowing its persistence and detection in aquatic ecosystems (Calisto and Esteves, 2009).

With an estimated K_{oc} value of 520 (determined from a log K_{ow} of 2.45) a moderate mobility of carbamazepine in soil is expected. In sewage treatment plants only 7% of this compounds can be removed (Tixier *et al.,* 2003). Biodegradation and transformation has a significant impact on surface water impacted by effluents and groundwater. Biodegradation depends on the presence of a community of organisms capable of transforming the contaminants through metabolic networks and the bioavailability of contaminants (Geissen *et al.*, 2015).

In water, its ratio of concentration in an organism (obtained with its log K_{ow}), suggests a low potential for bioconcentration in aquatic organisms. However, this drug can be transferred along the food chain and affect non-target organisms because of its high ability to bioaccumulate (Almeida *et al.,* 2014). Even if carbamazepine may be found at low concentrations in the aquatic environment (μg L^{-1} or ng L^{-1}), it may present risks to biota because they are continuously discarded, causing bioaccumulation and toxicity in different matrices (Beretta *et al.,* 2014), Such as bacteria, algae, macrophytes and invertebrates (Cleuvers, 2003; Ferrari *et al.*, 2003), inhibition of diatom growth (Claessens *et al.*, 2013), mortality, emergence and fertility of mosquitoes with semi-larvae (Heye *et al.*, 2016), oxidative stress in mollusks (Almeida *et al.*, 2014) and ecotoxicological problems in fish can be observed (Triebskorn *et al.*, 2007). Thus, it is important to be concerned about human health since people can consume drinking water and food contaminated by this compounds (Geissen *et al.*, 2015).

2.1.2. Anti-inflammatory – Diclofenac

Diclofenac (Figure 19) is an anti-inflammatory nonsteroidal from the group of aryl carboxylic acids. Its possible metabolites excreted are: 3'-hydroxy-, 4'-hydroxy-, 5-hydroxy-, 4 ', 5-dihydroxy- and 3'-hydroxy-4'-methoxy-diclofenac. Four of the metabolites have a short plasma half-life of 1 to 3 hours, while the metabolite 3'-hydroxy-4'-methoxy-diclofenac have longer plasma half-life. About 60% of the administered dose is excreted in the urine as the glucuronide conjugate of the intact compound and as metabolites, most of which are also converted to glucuronide conjugates. Less than 1% is excreted as unchanged drug. The remainder of the dose is eliminated through the bile as metabolites in feces (Novartis Pharmaceuticals Corporation, 2016).

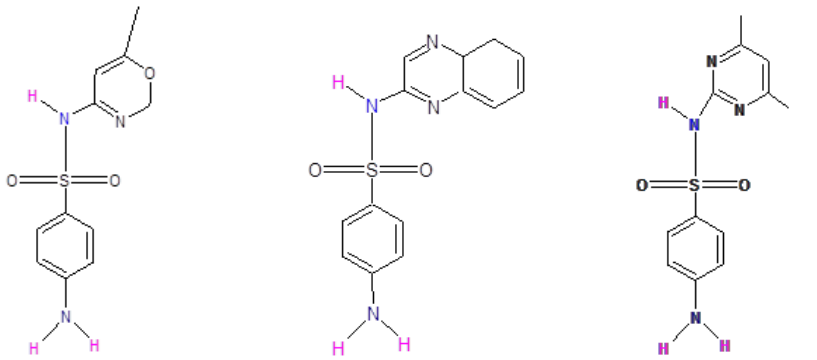


Chapter 2 - Figure 19 - Structural formula of Diclofenac.

Anti-inflammatories are sparingly soluble in water. Considering the pH of the studied soils range from 4.4 to 6.4, and the pka value of diclofenac (4.15), the compound will be majoritary in its anionic form, also reducing volatilization in moist soil surfaces. The K_{oc} estimated value of 245 L kg^{-1} suggests that diclofenac is expected to have moderate mobility in soil. However, with a log K_{ow} equal a 4.51, the hydrophobicity of these compounds is considering potentially high (Babić *et al.*, 2007).

2.1.3. Sulphonamides

Sulphonamides metabolization rates is high, considering that 80% of the administered dose is assimilated (Boxall *et al.*, 2003). Sulphonamides contains one basic amine group ($–NH_2$) and one acidic amide group (O=C–NH–) (Figure 20). The amine group is able to gain a proton and the amide group is able to release proton under specific pH conditions (Babić *et al.*, 2007). In soil, they have low affinity to the particles and there are therefore considered mobile in the soil profile as well described by the moderate hydrophobicity (log K_{ow} 0.89 – 1.68). This can be ratified by the observation that sulphonamide residues were detected in four groundwater samples (Hirsch *et al.*, 1999). At higher pH values (> pKa + 1), most of the compounds (> 90%) is found in anionic form. The pKa1 is the dissociation constant for equilibrium between the positively charged, protonated amino group of sulphonamide and its electrically neutral conjugate base. The pKa 2 refers to an equilibrium involving the loss of the sulphonamide proton to yield its negatively charged conjugate (Babić *et al.*, 2007).



A B C

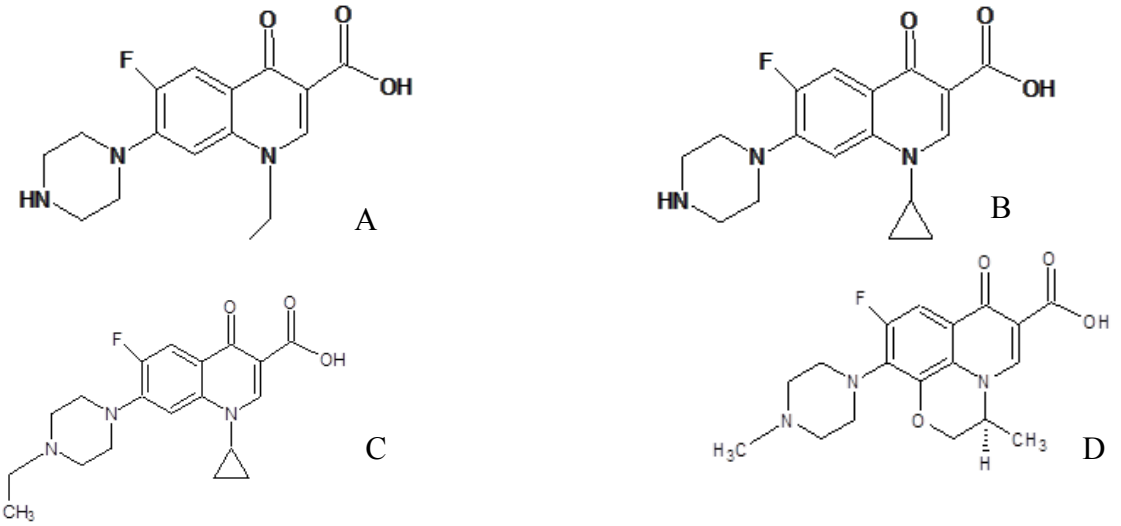
Chapter 2 - Figure 20 - Structural formula of (A) Sulfamethazine, (B) Sulfamethoxazole and (C) Sulfaquinoxaline.

If released in soil, sulfamethazine is expected to have very high to moderate mobility based upon K_{oc} values ranging from 49 to 208. Considering the pKa 2 of 7.65 (sulphonamide nitrogen), it is possible to have an indication that this compound will exist partially as an anion in the environment. The high reactivity of the aromatic amino group represented in this pharmaceuticals by anilines (aromatic amines) aid it to bind strongly to organic matter, reducing the mobility in soils. Based in K_{oc}, suspended solids and sediment will have less importance in its adsorbation.

The values for sulfamethoxazole pK_a1 of 1.6 and pKa2 of 5.7 and sulfaquinoxaline 5.1 indicate that it will partially exist in the anionic form in the environment. The estimated sulfamethoxazole K_{oc} value of 72 and sulfaquinoxaline K_{oc} value of 200, this compounds are expected to have high and moderate mobility in soil, respectively. However, aromatic amines may bind strongly to humus and organic matter in soils due the amino group, suggesting that mobility may be much lower in some soils.

2.1.4. Quinolones and Fluoroquinolones

After being administered, fluoroquinolones and quinolones (Figure 21) present an high rate of metabolism, between 20% and 80% of the applied dose (Boxall *et al.*, 2003). The animal dung used in the soil directly or after composting may cause the entering in the environment of these compounds.



Chapter 2 - Figure 21 - Structural formula of (A) Norfloxacin, (B) Ciprofloxacin, (C) Enrofloxacin and (D) Levofloxacin

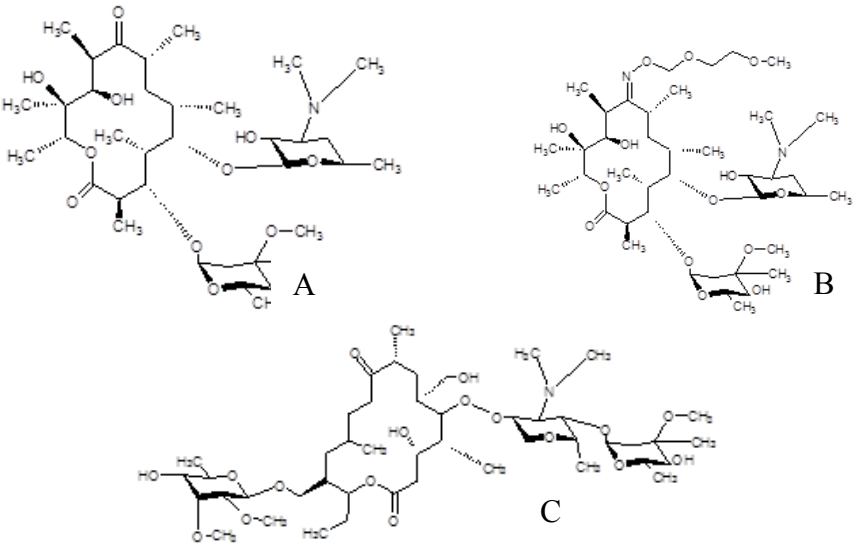
Ciprofloxacin and norfloxacin are first generation of fluoroquinolones antibiotic. First generation is an old class of synthetic antimicrobial agents having nalidixic acid in the composition (Suh and Lorber, 1995). The nalidixic acid caused the rapid development of bacterial resistance and therapeutic limitation of quinolones. Thus, studies were conducted with the intention of modifying its chemical structure, in anticipation of creating analogs of fluoroquinolones with low side effects, less reactive in order to ensure optimal efficiency in the diagnosis and prognosis of the individual. Recent studies showed that enrofloxacin, a third generation of fluoroquinolone with a very large spectrum of activity may form distinct metabolites (Morales-Gutiérrez *et al.*, 2014), however, ciprofloxacin is its main active metabolite and is associated with the antimicrobial activity of the drug (Mengozzi *et al.*, 1996).

All the compounds presented a large value of K_{oc} (16,500 to 770,000) indicating that the compound can be adsorb in suspended solids and sediment. Also, with rate of accumlation of 3, obtained from the log K_{ow}, the potential for bioconcentration of all compounds are considered low in aquatic organisms. All the quinolones have the pKa1 analogus, resulting in the same dissociation reaction of protonation with a pH below 6.

2.1.5. Macrolides

A macrolide contains a basic dimethylamine [–$N(CH_3)_2$] group, which is able to gain a proton (Figure 22), so, according to their chemical structure, macrolides have pKa value around 9 (Table 1). However, Tylosin is an exception once its pKa2 value is about 7.50, corresponding to the dimethylamine group, and the pKa1 value of tylosin is 3.31, corresponding to the tartarate moiety (Babić *et al.*, 2007).

Considering the pH of the studied soils ranging from 4.4 a 6.4, the tylosin, erythromycin and roxihromycin pKa suggests that all this compounds will be majoritary in their anionic form, also reducing volatilization in moist soil surfaces, permitting their adsorption. Hydrophobicity of these compounds (log K_{ow} around 1.7) suggests an affinity for organic matter as well as K_{oc} (between 553 and 7,988 for tylosin)



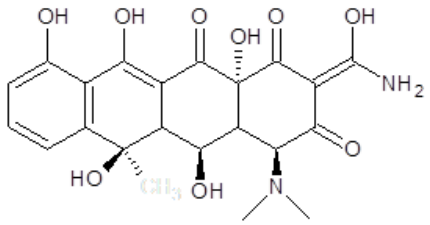
Chapter 2 - Figure 22 - Structural formula of (A) Erythromycin, (B) Roxithromycin and (C) Tylosin.

2.1.6. Tetracyclines

In contact with soil, tetracyclines are quite soluble and dissociate at pH values found in the environment or more polar, thereby presenting different potentials and sorption in soil mobility. Its sorption is influenced by the soil ionic strength, with the higher potential sorption observed in the presence of higher valence cations (Ca^{2+} instead of Na^{+}, for example) due to

complex formation between the tetracycline and multivalent cations. In soil, the volume of macropores will influence the transport of tetracyclines, even strongly sorbed compounds may optionally be carried into solution by the rapid preferential flow (Thiele-Bruhn, 2003). Abiotic degradation process, such as hydrolysis and photodegradation may occur (Halling-Sørensen, 2001; Sarmah, Meyer and Boxall, 2006), however photobleaching does not appear to be relevant (Thiele-Bruhn, 2003).

Tetracyclines have three pKa values (3, 7 and 9) representing different dissociation time (Figure 23). Ka1 is associated with the deprotonation of C 3 hydroxyl. The loss of protons from O 12 and dimethyl ammonium constitutes Ka2 and Ka3. As indicated by their acid-dissociation constants, tetracyclines contain localized charges across all pH values and only achieve an overall neutral state as zwitterions in the approximate range of pH 3–9 (Babić *et al.*, 2007).



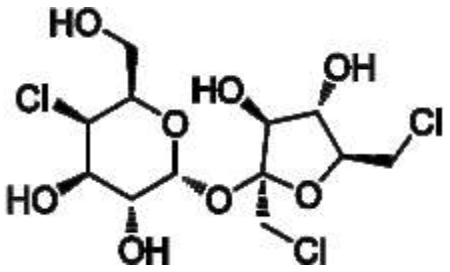
Chapter 2 - Figure 23 - Structural formula of Oxytetracycline.

Considering the pH of the studied soils ranging from 4.4 to 6.4 and the pKa of oxytetracycline 3.27, oxytetracycline will be always in its anionic form. The K_{oc} estimated value of 195 to 93,317 suggests that oxytetracycline is expected to have moderate to no mobility in soil. However, with a log K_{ow} equal to -0.9 the tendency of bio concentration of these compounds is considering low.

2.1.7. Sucralose

Sucralose (1,6-dichloro-1,6-dideoxy-BD-fructofura-nosyl-4-chloro-4-deoxy-α-D-galactopyranoside) is a sweetener with a sweetness power about 600 times higher than sucrose (Hough and Khan, 1989). In the body, sucralose is not hydrolyzed in the intestinal lumen and is hardly metabolized by humans (<8%), allowing it to be excreted almost metabolized in the urine (mean 14.5%) and feces (mean 78.3%) (Roberts *et al.*, 2000; Wood,

John and Hawkins, 2000). Recent publications have shown that artificial sweeteners (SEAs), such as SCR, have emerged as a new class of compounds applicable as anthropogenic markers due to their persistence in the environment (Scheurer, Brauch and Lange, 2009).



Chapter 2 - Figure 24 – Structural formula of Sucralose

The presence of this compound into natural waters occurs through the disposal of urban wastewater treatment effluents. Its destination, once in aquatic ecosystems, is still not well clarified and studies are being developed aiming to understand its occurrence and water distributions (Mead *et al.*, 2009). It is suggested that sucralose is not biologically inert, but its degradation occurs in variable rates that depends of bacteria and fungi presence (Labare and Alexander, 1993). In soil, with an estimated K_{oc} of 10, the sucralose are expected to have a very high mobility. If released into water, the sorption in suspended solids and sediment is not expected. The wastewater treatment plants is not able to eliminate this compound, is shown to be very persistent in surface waters.

3. SAMPLE COLLECTION

3.1. SOILS SAMPLES

Sampling of soils was performed with an auger at a depth of 0 to 20 cm (Malik *et al.*, 2008). In order to obtain representative composite, 30 sub-samples of soil were collected at each site and dried inside an air oven (40 °C and constant ventilation). After reaching constant weight, the material was sieved with a mesh of 630 μm and stored in glass bottles until the analysis. A final soil sample was composed by mixing the 30 sub-samples.

3.2. BIOFILMS SAMPLES

At each site, rocks that remained submerged in all seasons were sampled (between 50 to 100) (Figure 25). The biofilm was obtained through brushing rocks with a toothbrush. The

material adhered to the rock was rinsed with 1 L of deionized water into a glass jar (Aubertheau *et al.*, 2017).

Chapter 2 - Figure 25 - Biofilm sampling

The aqueous solution containing the biofilm was stored on ice coolers (± 4 °C) and transported immediately to the laboratory. In the laboratory, the samples were transferred to individual high density polyethylene jars and frozen at -80 °C for subsequent lyophilization (freeze LS3000 - Terroni). After being freeze-dried, samples were homogenized in an agate mortar to obtain a representative sample for posterior analysis (Figure 26)

Chapter 2 - Figure 26 – Biofilm sampling after lyophilization method.

3.3. POCIS SAMPLES

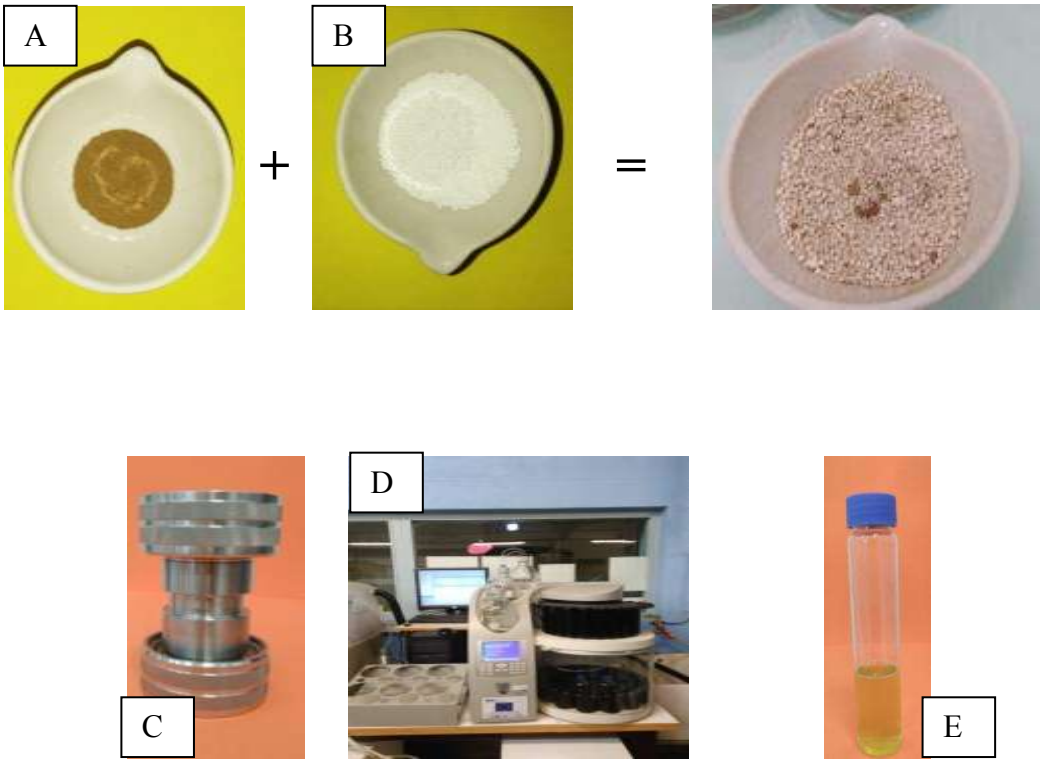
The POCIS sampler is a metallic structure formed by two steel rings, two membranes with 0.1 µm disk-shaped and 90 mm diameter (hydrophilic polyethersulfone - Supor®) and 200 mg of resin (Oasis® HLB sorbent, with hydrophilic / lipophilic characteristics) (Alvarez *et al.*, 2004) (Figure 27). The Oasis HLB sorbent was chosen because it sorbs a wider range of compounds and polar compounds better than C18 (Liu, Zhou and Wilding, 2004; Zhang and Zhou, 2007). The Oasis HLB sorbent was spread evenly between the membranes and fixed with the metallic structure. The POCIS were placed submerged in the river water for 15 days.

Chapter 2 - Figure 27 – POCIS sampling method

4. ANALYTICAL PROCEDURE

4.1. EXTRACTION OF PHARMACEUTICAL COMPOUNDS FROM SOLID MATRICES AND POCIS

Pharmaceuticals were extracted from biofilms and soils using Pressurised Liquid Extraction (PLE) technique. Briefly, sample was introduced into stainless steel cell and extracted with solvent at high temperature and under pressure (Figure 28).



Chapter 2 - Figure 28– Biofilm extraction and purification method. (A) Biofilm; (B) Diatomaceous earth; (C) stainless steel cell; (D) ASE system; (E) Biofilm extract

All parameters were validated in a previous study (Aubertheau *et al.*, 2017) for biofilm matrix, based on literature and experimental study. Method was adapted for soils samples. Extractions were carried using an ASE (Accelerated Solvent Extractor^{TM} 350, ThermoScientific, Waltham, USA). Parameters used are listed in table 2.

Chapter 2 - Table 2 - Parameters used for pharmaceuticals extractions of biofilms and soils matrices using pressurised liquid extraction

Equipment	ASE 350 (thermoscientific)
Temperature	80 °C
Pressure	100 bars
Static cycles number	4
Cycle time	5 min
Flush volume	60% loaded volume
Solvents	Methanol / Water
Volume ratio	1 / 2
Cells type	Cell inox 10 mL
Sample mass	500 mg dry biofilm or 1 g dry soil
Dispersive phase	Diatomaceous earth

Fonte: (Laurent, 2013)

Extracts from PLE were purified using a Solid Phase Extraction method. Oasis® HLB 6 cm^3cartdriges, containing 200 mg of sorbent phase (Waters, Milford, USA) choosed according to previous studies (Laurent, 2013). Purifications were carried out using an automatic extractor Autotrace™ 150, (ThermoScientific, Waltham, USA) (Figure 28). Before purification, extracts were diluted in water to obtain a methanol volumetric ratio below 5%.

The purification and extraction of compounds present in the POCIS resin was performed directly through the SPE. The parameters used in the process are the same as those used for solid samples. Solid phase extraction parameters are listed in table 3.

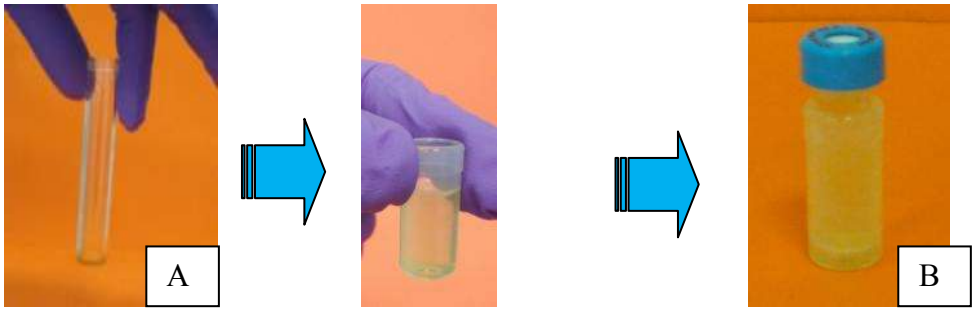
Chapter 2 - Table 3 - Parameters used for pharmaceuticals extractions and purification of biofilms soils matrices and POCIS using solid phase extraction

Apparatus	Autotrace 150 (thermoscientific
Cartridges	Oasis HLB 6 cc, 200 mg (Waters)
Loading flow	10 ml/min
Conditioning	5 ml Méthanol + 5 ml Eau ultrapure
Loaded volume	250 ml
Cartridge drying	5 minutes under 10 ml/min N2 flow
Elution	8 ml Methanol

Fonte: Laurent (2013)

4.2. EVAPORATION / RESTITUTION

Purified extracts were concentrated by evaporation under gentle N_2 flow. Residues were recovered in 500 µL Water/Methanol solution (90/10 v/v). For biofilms and soils analytical standards were also added during this step. The recovered liquid was then filtered one last time with the aid of medium filters Mini-Uni prep™ (PVDF Filter Media with polypropylene Housing, pore 0.45µm, Durapore®, Millipore, Billerica, USA) (Figure 29)



Chapter 2 - Figure 29 – Biofilm restitution (A) and Mini-Uni prep™ filter (B) for purification.

4.3. PHARMACEUTICALS QUANTIFICATION

All pharmaceuticals were detected using an electrospray ion source operating in positive modes. Pharmaceuticals were separated by high pressure liquid chromatography on an Acquity UPLC®BEH C_{18} column (2.1×100 mm, 1.7 µm; Waters) with methanol and water (both acidified with 0.3% formic acid) as the mobile phase. The liquid chromatography was coupled to a Q-Exactive Orbitrap™ mass spectrometer (Thermo Fisher Scientific) that combines high-performance quadrupole precursor selection with high-resolution/accurate-mass detection. Mass parameters for pharmaceutical quantification are given in table 4.

Chapter 2 - Table 4 – Chromatographic Separation Parameters

Chromatography equipment	Q-Exactive Orbitrap™		
column	Acquity UPLC®BEH C_{18}		
Mobile Phase	Methanol + 0.1% formic acid (A) Water UltraPure + 0.1% Formic Acid (B)		
Rate flow	0,45 mL/min		
Gradient	Time (min)	A(%)	B%
	Initial	10	90
	0.50	10	90
	1.50	60	40
	6.0	70	30
	9.0	100	0
	9.5	100	0
	10.5	10	90
	13.0	10	90
Duration of injection analysis	13 minutes		

The quantification was performed by the standard addition procedure with increasing concentrations of a standard mix of the 12 pharmaceuticals. Finally, the concentration of pharmaceuticals in the soil is expressed in milligrams of pharmaceutical per kilogram of dry soil and in nanograms per gram of dry biofilm.

Data acquisition and processing were treated using Xcalibur 2.2 software (Thermo Fisher Scientific Inc). Q Exactive 2.0 SP 2 (tune application) (Thermo Fisher Scientific) was used to control the mass spectrometer.

Chapter 2 - Table 5 – Compounds properties and analytical conditions of the studied pharmaceuticals.

Therapeutic class	Family	Compounds	Abbr	Chemical formula	Molar Mass	Parent ion (m/z)	Product ion (m/z)	Collision energy (V)
		Sucralose	SCR	$C_{12}H_{19}Cl_3O_8$	397,64	397.02183	-	40
		Carbamazepine	CBZ	C15H12N2O	236.27	237.10224	-	35
Anti-inflammatory	-	Diclofenac	DCF	$C_{14}H_{11}C_{l2}NO_2$	296.14	318.00591	261.1040	13
Antibiotic	Sulphonamide	Sulfamethazine	SMZ	$C_{12}H_{14}N_4O_2S$	278.33	279.08962	204.03990	35
		Sulfamethoxazole	SMX	$C_{10}H_{11}N_3O_3S$	253.28	254.05972	156.01137	30
		Sulfaquinoxaline	SQX	$C_{14}H_{12}N_4O_2S$	300.33	301.06751	156.01138 275.8543	25
	Quinolones	Norfloxacin	NOR	$C_{16}H_{18}FN_3O_3$	319.33	342.12244	312.92000	85
		Ciprofloxacin	CIP	$C_{17}H_{18}FN_3O_3$	331.34	332.14000	231.05600	60
		Enrofloxacin	ENR	$C_{19}H_{22}FN_3O_3$	359.39	360.17000	296.09900	65
		Levofloxacin	LVF	$C_{18}H_{20}N_3FO_4$	361.37	362.15000	318.16100	30
	Macrolides	Erythromycin	ERM	$C_{37}H_{67}NO_{13}$	733.93	734.46800	576.37000	14
		Roxithromycin	ROX	$C_{41}H_{76}N_2O_{15}$	837.05	837.52766	679.43600	13
		Tylosin	TIL	$C_{46}H_{77}NO_{17}$	916.10	916.52399	174.11276	21
	Tetracycline	Oxytetracycline	OXY	$C_{22}H_{24}N_2O_9$	460.43	483.13740	443.14426 426.12000	15

Chapter 2 - Table 6 - Chromatography conditions

Chromatographic Parameters	
Column	Acquity UPLCTM BEH C18 (100 × 2.1 mm d. i., 1.7 µm particle size)
Oven temperature	40 °C
Mobile Phase	Ultrapure water + 0.3% formic acid; Methanol + 0.3% formic acid
Flow rate	0.45 mL min^{-1}
Injection volume	2 µL
Elution	Ultrapure water + 0.3% formic acid; Methanol + 0.3% formic acid
Spectrometer Parameters	
Sheath gas flow rate	55 L h^{-1}
Auxiliare gas flow rate	20 L h^{-1}
Spray voltage	3.8 kV
Capillary temperature	320 °C
Heat temperature	400 °C

Each compound was added at the end of sample preparation in final concentrations ranging from 0 to 15 ng L^{-1} (4 additions). The obtained analytical curve were linear so that the concentration could be calculated. Each concentration level was injected three times to determine the average concentration in ng g^{-1} of dry biofilm (Aubertheau *et al.*, 2017).

4.4. POCIS EXTRACTION

POCIS extraction was performed by cartridge elution on the AutoTrace ™ 150 Solid-Phase Extraction (SPE) with methanol. The obtained extracts were evaporated under N_2 flow until they reached a volume of 100 µL for posterior restitution in a mixture of methanol / water (10/90; v/v). For the quantification, a standard curve method was used for CBZ and SCR at concentrations ranging from 5 to 20 µg L^{-1} of methanol and water mixture (10:90, v / v).

Quantification was performed with the same parameters as described above.

4.5. ANALYTICAL METHOD EFFICIENCY

In order to validate the analytical method (extraction and quantification) detection limits and extraction efficiency were determined. For both soil and biofilm matrices a “matrix sample” was selected. Due to the presence of pharmaceuticals in these samples, deuterated analogues were added as analytical standards to obtain samples without the presence of the compounds studied in their usual form.

4.5.1. Detection limit

Detection limits were calculated based on results obtained for "matrix samples" calibration (quantity and confidence range) and blanks. The calculation is based on a standard method (Neuilly, 1998). Corrected variance (Sc) is needed to apply this method. Briefly, detection limit (LD) could be expressed as:

$$LD = \frac{4\,Sc}{k}$$

With: k slope of calibration curve

and

$$Sc^2 = \frac{Q + Q_b\,b}{n - 2 + n_b - 1}\,(1 + \frac{1}{n} + \frac{\bar{x}}{W_{11}})$$

With $W_{11} = \sum(x_i - \bar{x})$; x_i standard concentration; $\bar{x}$standards mean; n number of standard; n_b number of blank; Q calibration residue; Q_b Residue to blanks

It was possible to obtain the most accurate detection limit with four calibrations. The standard curve method was used with concentrations ranging from 5 to 20 µg L^{-1} with three replicates of injections per point. Ten blanks were also analyzed to be use in the statistic calculus.

4.5.2. Rendment of extraction

Global recoveries were estimated by comparison of peak areas extracted from chromatogram acquired from spiked matrix samples in a hand and samples spiked with the same amount of deuterated standard (Table 7)

Chapter 2 - Table 7 - Global recoveries of soil and biofilm during the pharmaceutical extraction

Pharmaceutical	Deuterated analog	Global recovery (%)	
		Soil	Biofilm
Carbamazepine	D_{10}	184	43
Diclofenac	D_4	160	37
Sulfamethazine	$^{13}C_6$	89	28
Sulfamethoxazole	D_4	77	27

Global recovery from soil samples varies between 77 and 184 %. In literature, value between 80 and 120 % are considered as satisfying for quantification method. For sulfamethazine and sulfamethoxazole recovery values are acceptable when carbamazepine and diclofenac are overestimated in this study.

In biofilm case, recovery varies between 27 and 43 %. The relative homogeneity of the recovery yield permits a comparison of contamination level and a satisfying evaluation of the range of contamination level.

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CHARPTER 3: PRESENCE OF ANTHROPIC MARKERS IN WATER: A CASE STUDY OF THE GUAPORE RIVER WATERSHED – BRAZIL

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ABSTRACT

Southern Brazil is an agricultural region that is experiencing strong growth in both cereal and animal production. The intensification of agricultural practices, as well as the growing urbanization in this region, also generate strong anthropic pressures on the aquatic environment. In Brazil, the lack of sewage treatment facilities and the wide use of waste (liquid or solid) for the fertilization of soils are likely to favor the spread of pharmaceuticals in the surrounding environment. Considering the benefits of the determination of anthropic markers in the environment, the study aimed to monitor the presence of carbamazepine and sucralose in the Guaporé River, representative of a southern Brazilian rural watershed. Both carbamazepine and sucralose were measured in water by polar organic chemical integrative samplers (POCIS) and in the river biofilms to give an overview of the level of aquatic contamination. The use of sucralose and carbamazepine as tracers of anthropogenic activity proved promising, as was the use of biofilms and POCIS as samplers. Biofilm and POCIS are two complementary techniques. Biofilms are able to identify contaminated environment sites by the capture of SCR as a human marker and POCIS are capable to capture CBZ with high efficiency in polluted sites and with more ability than biofilms.

Keywords: Carbamazepine; Sucralose; Biofilm; POCIS; Pollution.

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1. INTRODUCTION

The increasing world population and growth of urban areas has led to the diversification of chemical usage, resulting in the dumping of various contaminants in water sources [1]. Among these, the residues of pharmaceuticals represent an emerging problem [2]. These compounds are widely used in human healthcare, agricultural and animal production [3], however they are also largely released through human and animal excretion. The United Nations estimate that every day two million tons of sewage (urban and rural) are dumped into the waters of the world. Therefore, the annual production quantity of residual water is approximately 1,500 km^3, representing six times the total amount in all the planet's rivers [4].

In Brazil, the National Sanitation Information System (SNIS) reported that, in 2014, only 49.8% of total Brazilian sewage was collected, amo,g them 70.9% are treated [5]. Thus, there is no doubt that widespread contamination of rivers, shallow reserves and probably also subsurface water has occurred due to the direct release of effluents [6]. Concern about water quality is a major issue for the scientific community, particularly when it comes to public supply [7] and the ability to affect one or more organisms of the aquatic food chain [8 - 10]. Therefore, the study of the dispersal and behaviour of pharmaceuticals in the environment remains of major importance. For this purpose, compounds with low degradation rates, such as carbamazepine (CBZ) and sucralose (SCR), are efficient and low cost persistent tracers of human activity [11 - 12] to evaluate the dissemination of pharmaceutical compounds in aquatic environments.

Carbamazepine is an antiepileptic with a long half-life, allowing its persistence and detection in aquatic ecosystems [13], and permitting its transfer along the food chain due to its high ability to bio accumulate [14]. Problems like mortalit and fertility of mosquitoes with semi-ground larvae [15], oxidative stress in molluscs [14], and ecotoxicological problems in fish [16] are also presented in the literature. In 2007, the consumption of CBZ in Brazil reached 30.4 tons [17], thanks to the free distribution in the Pharmaceutical Assistance list of the Unified Health System, and the approval of its use by the National Health Surveillance Agency (ANVISA) [18]. The presence of CBZ in sewage effluents, as a pharmaceutical indicator of contamination, has been widely studied especially because of its low degradability and its restriction to anthropogenic sources [19], [20]. Thus, even if CBZ is frequently found at low concentrations in the aquatic environment (e.g.: ng L^{-1} to µg L^{-1}), it can pose risks to the biota by being continuously discharged, causing bioaccumulation and toxicity [21]. Thus, besides the protection of ecological communities, human health should be

taken into account, as people who drink contaminated water, and eat food irrigated with it, can be adversely affected [22].

Recent publications have shown that artificial sweeteners, such as SCR, have emerged as a new class of applicable anthropogenic marker compounds due to their persistence in the environment [24 - 25]. Sucralose (SCR) is an artificial sweetener, with a sweetening power about 600 times higher that of saccharose [23], that has also emerged as an anthropogenic marker [24 - 25]. If ingested, SCR cannot be hydrolyzed in the intestinal lumen and is hardly metabolized by humans (<8%) and is thus excreted in urine (±14.5%) and feces (±78.3%) [26 - 28]. Labare & Alexander (1993) [29] suggested that SCR is not biologically inert, but that the degradation rate happens at a variable speed due to the presence of bacteria and permitting the persistence of this compound in the environment..

In Brazil, the lack of sewage treatment facilities and the wide use of waste (liquid or solid) for the fertilization of soils are likely to favor the spread of pharmaceuticals in the surrounding environment. In most cases, studies have reported a decrease in contamination as the distance from the source of discharge increases [30]. Considering the benefits of the determination of anthropic markers in the environment, the study aimed to monitor the presence of CBZ and SCR in a southern Brazilian rural watershed using river biofilms and POCIS. For that, the captors POCIS and biofilm were choosen in this study.

2. MATERIALS AND METHODS

2.1. GUAPORÉ WATERSHED AND SAMPLED SITES

The watershed of the Guaporé River is located in the northeastern of the state of Rio Grande do Sul, Brazil. The topography of the northern part of the watershed is undulating and the southern part is mountainous with steep slopes. Agriculture and livestock rearing are the main economic activities, while urban areas occupy only 0.60% of the area [31]. The presence of anthropic markers was monitored in four sub-watersheds of the Guaporé River watershed named Capingui, Marau, Lajeado Carazinho and Carazinho (Figure 1).

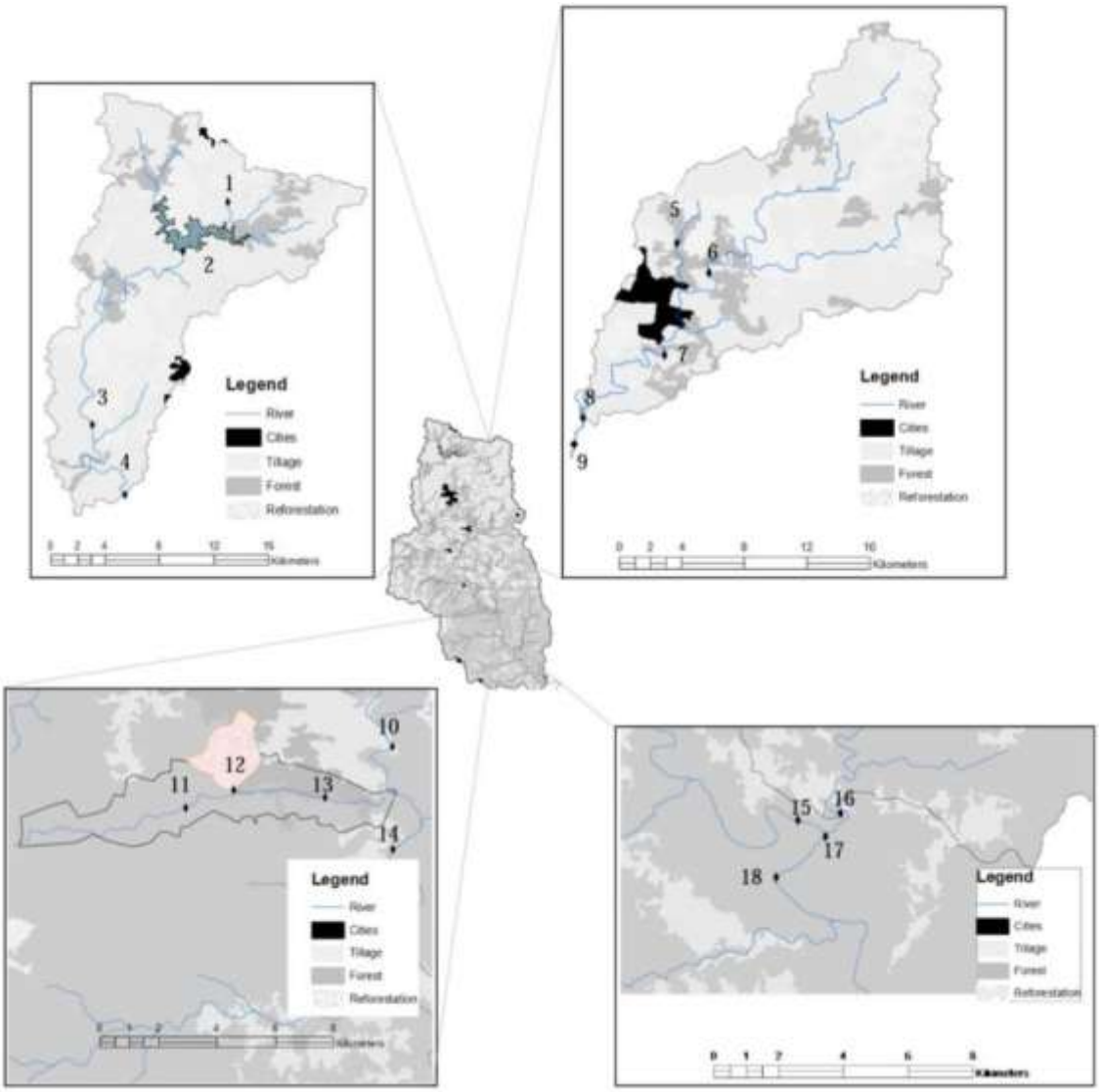
The Capingui sub-watershed is located in the northern portion of the Guapore's watershed. Nowadays, most of the area is used for growing soybeans (*Glicine max* (L.) Merrill) and corn (*Zea mays* L.) crops using no-tillage systems. In this sub-watershed, four points with different characteristics were monitored. The northern point is the National Forest of Passo Fundo (FLONA – point 1, Figure 1). This point is a federal conservation unit of

1300 hectares, created in the 1940s, including 450 hectares of native forest belonging to the biome of the Atlantic Forest. In this study, this site will be considered as control due the low anthropic action. However, it is important to consider that the waters of other rivers (eg.: Tingatu, Cachoeirinha, Capingui and Rio Branco) have a direct influence on FLONA. The waters of FLONA reach the reservoir of Capingui. Therefore, a sampling site was located downstream of the Capingui Dam (Capingui Dam – point 2 Figure 1). Today, Capingui Dam is used only for recreation, with a considerable growth in human occupation resulting in a wastewater release directly into the dam. Downstream of the Capingui dam, two sites were also monitored. The first site represents the contribution of a vast area of agricultural production. This site is used for grain production under the no-tillage system, especially soybean, corn and wheat (Agriculture –item 3, Figure 1). The second site located at the outlet of sub-watershed of Capingui (Capingui outlet point 4, Figure 1). This site receives runoff water from the grain production region, however, with a large concentration of residues coming from breeding of confined animals pig, poultry and dairy production.

The Marau sub-watershed is characterized by the predominance of family agriculture and livestock production, with the contribution of wastewater from urban areas of the city of Marau (36,000 inhabitants). The economy of this sub-watershed is based in animal production and dry grains coexists with livestock production on almost all properties. In the urban areas, there are agribusinesses, especially large slaughterhouses for pigs and poultry for export [32]. The impact of human activities, rural and urban, was monitored at four sites. The first one represents the water coming predominantly from runoff of grain production areas in no-tillage (Marau upstream - Grain - point 5, Figure 1). The second site, in addition to grain production, has a lot of pigs and dairy cows (Marau upstream Animal - point 6, Figure 1). Two sites were monitored downstream of the city of Marau, which receive all the urban pollution load: the first site is immediately downstream of the town (Marau city – point 7, Figure 1) and the second is at the outlet of the sub-watershed of Marau (Marau Outlet – point 8, Figure 1) and aims to check the river resilience potential when the entry of urban pollutants decreases. Marau does not have any sewage treatment system, which results in a huge increase of pollution in the river arising from the urban area. The quality of the river into the city suffers various forms of environmental degradation. There is evidence of wastewater, municipal solid waste and industrial effluents, such as observed fish death along the river [32]. A site was also monitored at the confluence of the Capingui and Marau sub-watersheds, (item 9, Figure 1). From this point, the river is called the Rio Guaporé.

The Lajeado Carazinho sub-watershed is a small region located in the central part of the Guaporé River watershed. It is a fragile region characterized by sloping relief, impoverished and predominantly used for tobacco cultivation [33]. In this zone three sites were monitored. The first site is located upstream of the tobacco-producing areas (Upstream Lajeado Carazinho – point 11, Figure 1). The second site receives the water passing by the tobacco producing area (Tobacco – point 12, Figure 1) and represents the downstream of tobacco contribution (Downstream of tobacco in Lajeado Carazinho – point 13, Figure 1). Two sites were selected on the main river (Guaporé's River), one upstream of this sub-watershed (Upstream Lajeado Carazinho sub-watershed – point 10, Figure 1) and the other downstream (Downstream tobacco Lajeado Carazinho sub-watershed – point 14, Figure 1). These two sites were chosen in order to ascertain the effect of human activities in the Lajeado Carazinho sub-watershed on the main river (Rio Guaporé).

The sub-watershed of the Carazinho River is located in the southern part of the Guaporé River watershed. This sub-watershed, located near the Lajeado Carazinho sub-watershed, was historically focused on food production. Characterized by a mountainous region, Carazinho sub-watershed is occupied by native forest and reforestation areas due to rural exodus. The sampled area was chosen to represent low human impact (pressure) and lower intensification of agriculture (crops and animal production) in the studied region. The Lajeado stream is a tributary of the Guaporé River that passes inside the Carazinho sub-watershed. In the Lajeado stream, the Carazinho sub-watershed outlet was sampled (Carazinho Outlet – point 16, Figure 1), as well as the confluence of the Lajeado stream with the Guaporé River (Central Carazinho – item 17, Figure 1). In the Guaporé River, one point was placed upstream and another downstream of the Carazinho sub-watershed (upstream Carazinho sub-watershed – point 15 and downstream Carazinho watershed – 18, Figure 1, respectively) to evaluate the effects of this sub-watershed on the main river.



Capingui Sub-Watershed (A): (1) Flona, (2) Capingui Dam, (3) Agriculture e (4) Capingui outlet.

Marau Sub-Watershed (B): (5) Marau upstream - Grain (6) Marau upstream – Animal (7) Marau city (8) Marau Outlet and (9) Marau and Capingui confluence.

Lajeado Carazinho Sub-Watershed (C): (10) Upstream Lajeado Carazinho sub-watershed, (11) Upstream Lajeado Carazinho (12) Lajeado Carazinho (13) Downstream of Lajeado Carazinho (14) Downstream Lajeado Carazinho sub-watershed.

Carazinho Sub-Watershed (D): (15) upstream Carazinhosub-watershed (16) CarazinhoOutlet (17) Central Carazinho(18) downstream Carazinhowatershed

Chapter 3 - Figure 1 - Sites sampled in Guaporé sub-watershed, Brazil.

2.2. SAMPLING, PREPARATION AND STORAGE OF BIOFILM AND POCIS

Biofilm sampling was carried out in summer 2014 and winter 2015 in all monitored sites. One additional sampling was performed in fall 2014 for the sites of the Capingui and Marau sub-watershed. At each site, several rocks that remained submerged in all seasons were sampled. The biofilm was obtained through brushing rocks with a toothbrush. The material adhered to the rock was rinsed with deionized water into a glass jar [30]. The aqueous solution containing the biofilm was stored in ice coolers (± 4 °C) and transported immediately to the laboratory. The samples were then transferred into individual high density polyethylene jars and frozen at -80 °C for subsequent lyophilization (freeze LS3000 - Terroni). After being freeze-dried, samples were homogenized in an agate mortar to obtain a representative sample for posterior analysis of CBZ and SCR.

Complementary measurements were performed with POCIS to gives an indication of the amount of markers flowing in the river. The POCIS sampler is a metallic structure formed by two steel rings, two membranes with 0.1 μm of porosity and 90 mm diameter (hydrophilic polyethersulfone - Supor®) and 200 mg of resin (Oasis® HLB sorbent, with hydrophilic / lipophilic characteristics) [34]. This resin was chosen because it sorbs a wider range of compounds and polar compounds better than C18 [35 - 36]. The Oasis® HLB sorbent was spread evenly between the membranes and fixed with the metallic structure. The POCIS were placed submerged in the river water for 15 days in June for all 18 sites. After this period, they were cleaned and the resin recovered in the laboratory for analysis of the presence of CBZ and SCR. POCIS samplers were installed at the 18 sampling sites in June 2015. The structures were submerged during the sampling period, protected by an iron box with aluminum screen, in order to allow the water flow and act as a physical barrier against twigs and rocks. All samples were removed and placed in plastic bags inside cooler and transported to the laboratory.

2.3. EXTRACTION OF PHARMACEUTICALS FROM BIOFILM AND POCIS

Biofilm extraction procedure was performed using the method described by Aubertheau et al. (2016) [30], adapted from Jelić et al. (2009) [37]. Five hundred milligrams of biofilms were extracted by extraction liquid at high pressured (ASE™ 350, Thermo Fisher Scientific Inc, Waltham, USA) at 80 °C using methanol / water (1/2; v/v) as the extraction solvent. The extracts were purified by solid phase extraction (Autotrace™ 150, Thermo Scientific,

Waltham, USA) using Oasis® HLB cartridges (6cc, 200 mg of sorbent; Waters, Milford, USA) using methanol as eluent. The final extracts were evaporated under mild nitrogen steam until they reached a volume of 100 µL for posterior restitution to 500 µL with a mixture of methanol / water (10/90; v/v).

POCIS extraction was performed on the AutoTrace ™ 150 Solid-Phase Extraction (SPE) system with methanol. In the laboratory, the resins were firstly transferred to cartridges with the aid of ultrapure water, dried under gentle nitrogen flow with subsequent weighing and stored at -20 °C. The obtained extracts were evaporated under N_2 flow until they reached a volume of 100 µL for posterior restitution in a mixture of methanol and water (10/90; v/v).

2.4. QUANTIFICATION OF ANTHROPIC MARKERS IN BIOFILM AND POCIS

CBZ and SCR quantification was performed by liquid chromatography coupled to mass spectrometry (LC-MS) using a Q-Exactive Orbitrap (Thermo Fisher Scientific™, USA). Both were detected using an electrospray ion source operating in positive mode. Q-Exactive 2.0 SP 2 (Thermo Fisher Scientific™) (application tune) software was used to control the mass spectrometer and the acquisition and data processing were carried out using Xcalibur 2.2 software (Thermo Fisher Scientific, USA). The separation was done on a Acquity column UPLC® BEH C18 (2.1 × 100 mm, 1.7 µm, Waters, Milford, USA) with methanol and water (both acidified with formic acid 0.3%) as mobile phase. Table 1 presents the physical-chemical properties and parameters used in the analysis of CBZ and SCR.

Chapter 3 - Table 1 - Characteristics of the parameters of the studied compounds used in the LC-MS analysis.

	Therapeutic class	Molar Mass ($g\ mol^{-1}$)	n° CAS	Chemical formula	pKa	Ion adducted	Parent ion (m/z)	Collision energy (v)
CBZ	Antiepileptic and anticonvulsant	236.269	298-46-4	$C_{15}H_{12}N_2O$	15.96	H^+	237.10224	35
SCR	Sweetener	397.640	56038-13-2	$C_{12}H_{19}Cl_3O_8$	11.91	Na^+	419.00377	40

The quantification of anthropic markers in biofilms was performed with the aid of the standard addition method. Each compound was added at the end of sample preparation in final concentrations ranging from 0 to 15 µg L^{-1} (4 additions). The obtained analytical curve was

linear so that the concentration could be calculated. Each concentration level was injected three times to determine the average concentration in ng g^{-1} of dry biofilm [30].

For POCIS compounds quantification, four concentrations (5, 10, 15 and 20 µg L^{-1}) of external standard were used to performed a standard calibration curve and each injected 3 times, with 3 replicates. For biofilm the quantification, a standard curve method was used for CBZ and SCR at concentrations ranging from 5 to 20 µg L^{-1} of methanol and water mixture (10/90, v/v).

3. RESULTS AND DISCUSSION

3.1. KEYS OF INTERPRETATION FOR ANTHROPIC MARKERS IN THE BIOFILM AND POCIS SAMPLES

All CBZ and SCR concentrations values in biofilms are presented in Table 2. The results show that SCR (18/90 biofilms studied) was more frequently observed than CBZ (7/90 biofilms studied) in the samples. Thus, CBZ and SCR were found in only a few biofilm samples, suggesting the presence of more preserved than impacted area in the sampling sites.

Recent work dealing with the biofilms as captor have pointed out that the accumulation of pharmaceuticals depends on the affinity constant between the biofilm structure and the compound [30, 38, 39]. Thus, Huerta et al. (2016) [40] and Aubertheau et al. (2016) [30] have explained low accumulation of CBZ in their samples by the low affinity of CBZ for the biofilm despite the large distribution of this compound in the considered river. This finding indicates that the small presence of CBZ in the biofilms represents a significant presence of CBZ in the corresponding site. Furthermore, the "< LD" values should be interpreted as the possible absence of CBZ or the presence of this compound at low concentration or only temporarily in the river. Complementary results obtained with POCIS, collected at the period in the June 2015 sampling, confirm that high detections of CBZ in POCIS match the detection of CBZ in biofilms (Table 3). In the same way, low detections or absence of CBZ in POCIS match absence of CBZ in biofilms. Hence, the presence of CBZ in biofilms is not a sensitive indicator. CBZ is a aquatic contaminant [17], is what makes it a great anthropic marker.

In June 2015, SCR was not detected in all the POCIS deployed in the watershed. However, many occurrences of this compound were found in the biofilm, suggesting that the biofilm is sensitive to this compound (Table 3). Many studies have looked at the *in situ*

application of POCIS for organic micro pollutant monitoring in surface water [41]. Based on the results from field deployments and mass balance, it is now well accepted that POCIS is not suitable for all compounds. Some tests performed in aquatic environments confirmed the low recovery of SCR by POCIS. Metcalfe et al. (2014) [42] showed that SCR was not detected, despite the large potential of this device to capture it. Hence, the use of biofilms as captors represents an alternative to track the presence of SCR in the environment. Huerta et al. (2016) [40] comment that the water samples were collected at a single time while biofilms are grown for a longer period and thus were exposed to different concentrations of contaminants. Therefore correlation between water and biofilm concentration should only be considered as a possible tendency of these compounds to accumulate and used as a tool for the detection of compounds in areas where they are not found in water.

Chapter 3 - Table 2 - Concentration of sucralose and carbamazepine (µg g $^{-1}$) present in epilithic biofilms sampled in summer, fall and winter saisons in Guaporé watershed, Brazil.

Sampled sites	Carbamazepine			Sucralose		
	µg g^{-1}					
	Summer	Fall	Winter	Summer	Fall	Winter
Flona	<LD [a]	<LD	<LD	<LD	0.0179	0.0060
Dam capingui	<LD	<LD	<LD	0.0035	0.1393	<LD
Agriculture	<LD	<LD	<LD	<LD	<LD	<LD
Capingui outlet	0.0023	<LD	<LD	0.0020	<LD	<LD
Marau upstream - Grain	0.0036	<LD	<LD	0.0123	0.0055	0.0025
Marau upstream Animal	<2.5	<LD	<LD	<LD	<LD	0.0085
Marau city	<LD	<LD	0.0029	<LD	<LD	0.0055
Outlet of Marau	<LD	0.002	0.0027	0.0079	0.0038	0.0058
Confluence Marau and Capingui Watershed	<LD	<LD	0.0024	<LD	<LD	<LD
Upstream Lajeado Carazinho	<LD	-	<LD	0.0482	-	0.0031
Tobacco	<LD	-	<LD	<LD	-	<LD
Downstream of Lajeado Carazinho	<LD	-	<LD	<LD	-	<LD
Upstream Lajeado Carazinho watershed	<LD	-	<LD	<LD	-	<LD
Downstream Lajeado Carazinho watershed	<LD	-	<LD	<LD	-	<LD
Carazinho Outlet	<LD	-	<LD	<LD	-	0.0049
Central Carazinho	<LD	-	<LD	<LD	-	0.0022
Upstream Carazinho watershed	<LD	-	<LD	<LD	-	<LD
Downstream Carazinho watershed	<LD	-	<LD	<LD	-	0.0038

[a]LD=0.0002 µg g^{-1}

Chapter 3 - Table 3 - Concentration of sucralose and carbamazepine (µg g-1) in epilithic biofilms and POCIS sampled in winter in Guaporé watershed, Brazil.

Sampled Sites	Carbamazepine µg g^{-1}		Sucralose	
	Biofilm	POCIS	Biofilm	POCIS
Flona	<LD [a]	<LD	0.0060	<LD
Dam capingui	<LD	<LD	<LD	<LD
Agriculture	<LD	2.7	<LD	<LD
Capingui outlet	<LD	17.2	<LD	<LD
Marau upstream - Grain	<LD	39.4	0.0025	<LD
Marau upstream Animal	<LD	23.7	0.0085	<LD
Marau city	0.0029	285.6	0.0055	<LD
Outlet of Marau	0.0027	490.8	0.0058	<LD
Confluence Marau and Capingui Watershed	0.0024	746.8	<LD	<LD
Upstream Lajeado Carazinho	<LD	2.1	0.0031	<LD
Tobacco	<LD	<LD	<LD	<LD
Downstream of Lajeado Carazinho	<LD	<LD	<LD	<LD
Upstream Lajeado Carazinho watershed	<LD	12.5	<LD	<LD
Downstream Lajeado Carazinho watershed	<LD	29.1	<LD	<LD
Carazinho Outlet	<LD	<LD	0.0049	<LD
Central Carazinho	<LD	<LD	0.0022	<LD
Upstream Carazinho watershed	<LD	<LD	<LD	<LD
Downstream Carazinho watershed	<LD	<LD	0.0038	<LD

[a]LD=0.0002 µg g^{-1}

3.2. CONTAMINATION OF THE GUAPORÉ RIVER WATERSHED: CONCLUSIONS OF THE BIOFILM CAPTOR

Situated in the most northern of the Capingui sub-watershed, the FLONA was initially considered as the site with the lowest anthropic influence. This forest is a conservation unit protected since the 1940s. Thus, the FLONA is environmentally protected and one of the main objectives is the protection of the sources of water [43]. However, SCR was identified and quantified in biofilms collected in fall (0.0179 µg g^{-1}) and in winter (0.0060 µg g^{-1}). With the increase of human activities, Flona is now surrounded by several cities: Mato Castelhano that has a population of 2,470 inhabitants that do not have any Wastewater Treatment Plant and Passo Fundo with 196,739 inhabitants and only 25% of treated sewage [44]. The lack of Wastewater Treatment Plant and the disposal of human wastes from these cities seems contribute strongly to the contamination of this protected area. A recent study of Sá and Gerhardt (2016) [45] suggests that the fragmentation of the surface resulted in difficulties in establishing an efficient buffer zone, which reduced efficiency in protecting the forest from

anthropic pressures. Nevertheless, the buffer zone probably limits the contamination since the absence of CBZ in the biofilms (confirmed by the absence in the POCIS) suggests that the contamination reaching the sampling site is probably lower for certain compounds.

In the dam located downstream of the FLONA, SCR was also found in considerable quantities (0.1393 μg g^{-1}) in the biofilms in fall and in small amounts in summer (0.0035μg g^{-1}). Dams are known to concentrate various compounds resulting from drainage of disturbed areas [46]. This ability could explain the increased presence of SCR concentration in biofilms, especially during the fall period. It should be noted that the increase in SCR is not accompanied by an increase in CBZ concentration, both in biofilms and POCIS. This finding suggests the same sources of pressure and/or buffer mechanisms for the FLONA and the dam sites.

Further downstream in the watershed, the agricultural site Agriculture is characterized by the absence of detectable concentrations of CBZ or SCR in all seasons, suggesting the absence of anthropic pressure related to urban discharges. Furthermore, these results indicate that agricultural practices from local farmers (eg. manure application) do not impact the aquatic environment with anthropic markers. Nevertheless, POCIS deployed in the river show the presence of CBZ (2.7 μg g^{-1}) in June 2015, suggesting rather an intermittent contamination because of a rural way of life (ie. low population density). In South Korea, the occurrence of CBZ was not reported downstream a large agricultural area in Mankyung River watershed while soils received sewage treatment plant effluents with CBZ at concentrations up to 0.595 μg L^{-1} [47]. On the contrary, this compound had been found in water samples collected from other agricultural sites located downstream of a dam [48].

In the Capingui Outlet, CBZ and SCR were detected in the biofilms in summer (0.0023 and 0.0020 μg g^{-1}, respectively) and in the POCIS (17.2 μg g^{-1}). Although the river and this sampling site are not inside the Marau sub-watershed, it is possible to associate the contamination to the lack of wastewater management in this area close to Marau [32] which allows the accumulation of anthropic markers in the surrounding environment. Furthermore, increases in river flow that occur at the outlets of watersheds or sub-watersheds probably contribute to the dilution of the contamination.

The Marau sub-watershed is the most contaminated area studied in the present work. Indeed, it presents the greatest number of CBZ and SCR detection in biofilms and also in POCIS sampled. It is worth noting that the contamination fluctuates with the seasons. At points upstream of Marau (grains and animals), CBZ was only detected in the biofilms of summer (0.0036 μg g^{-1} and 0.0025 μg g^{-1}, respectively) showing a preferential or highest

contamination during this period. Indeed POCIS measurements performed in winter (June 2015) detect also the presence of CBZ, but at low amounts in the water transiting in these sites. SCR was quantified for the Marau (grains) biofilm samples in all the seasons (summer, fall and winter: 0.0123; 0.0055 and 0.0025 μg g^{-1}, respectively) while of the Marau (Animal) biofilm samples, only those obtained in winter contain this compound (0.0085 μg g^{-1}). This difference suggests that these two sites may be contaminated by different sources of pollution (ie. with more or less SCR in the discharge). Nevertheless, it is interesting to consider that these sites are characterized by intensive agricultural production with large quantities of manure discharged on soils. The agriculture is both rural and urban because of the vicinity of Marau city. Thus, the significant contamination observed in these places suggests the importance of this "near urban agriculture" system in the contamination of the aquatic environment.

The accumulation of thousands of people in cities remains, however, an important source of contamination. Indeed, the evaluation of the biofilms shows that Marau city releases significant amount of CBZ in the river. Thus, in winter, the biofilms present between 0.0029 to 0.0024 μg g^{-1} from the Marau city point to the confluence of the Marau/Capingui watershed. These observations were confirmed by the POCIS results showing the presence of very high amounts of CBZ (between 285.6 μg g^{-1} and 746.8 μg g^{-1}, respectively). High amounts of CBZ detected in POCIS were also found in Ukraine (Lopan River) and France (Jalle River) downstream of WWTP. The values reached approximately 6 μg g^{-1} [17], a value lower than those obtained downstream of the city of Marau. In summer and fall, CBZ was not observed in the biofilms of Marau city and only once in the biofilms of the Marau outlet. This trend was also observed with SCR that was found in winter in the biofilms of Marau and the Marau outlet (between 0.0055 and 0.0058 μg g^{-1}, respectively) but only in the biofilm samples of the Marau outlet site during the summer and fall periods. This highest contamination in the Marau outlet may be attributed to the accumulation of pressures consequent on the presence of Marau city and the many other housing developments located downstream in the watershed.

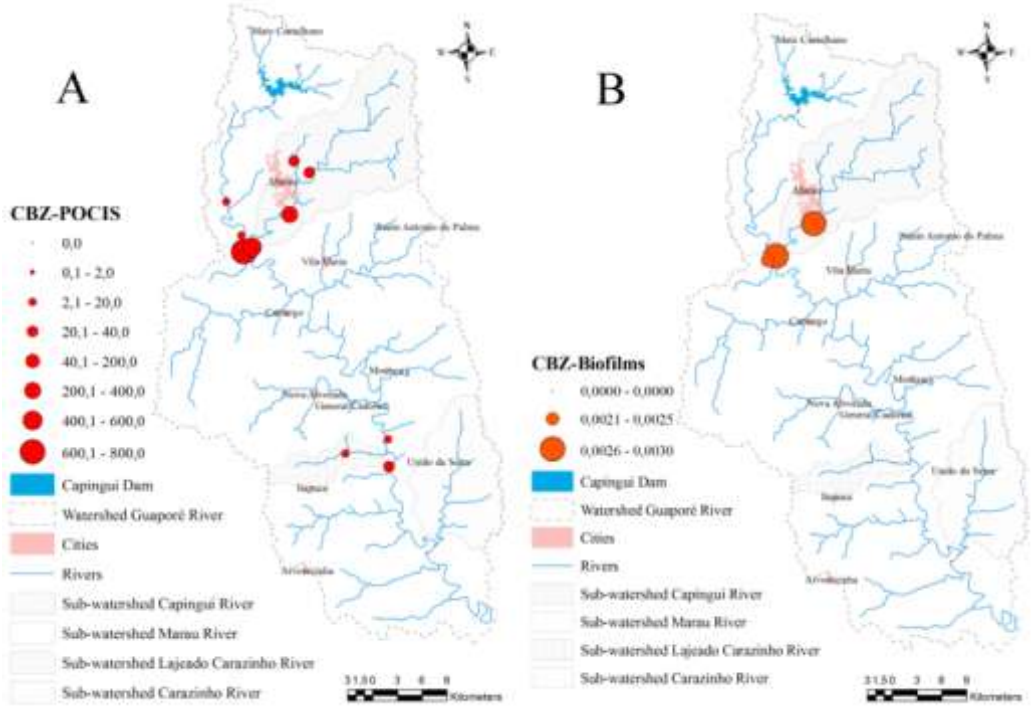
The confluence point of the two sub-watersheds Marau/Capingui was characterized by the presence of CBZ in biofilms (0.0024 μg g^{-1}) and in POCIS (746.8 μg g^{-1}) in winter only. This results suggest that the anthropic pressure is sufficiently high in winter to be detected by natural indicator, like biofilms (Figure 2). Although the human presence is not as high as in a city, the two main studies published with the use of biofilms in natural rivers, conclude that

the pharmaceutical compounds discharged from wastewater can be captured far from the discharge point [30, 40].

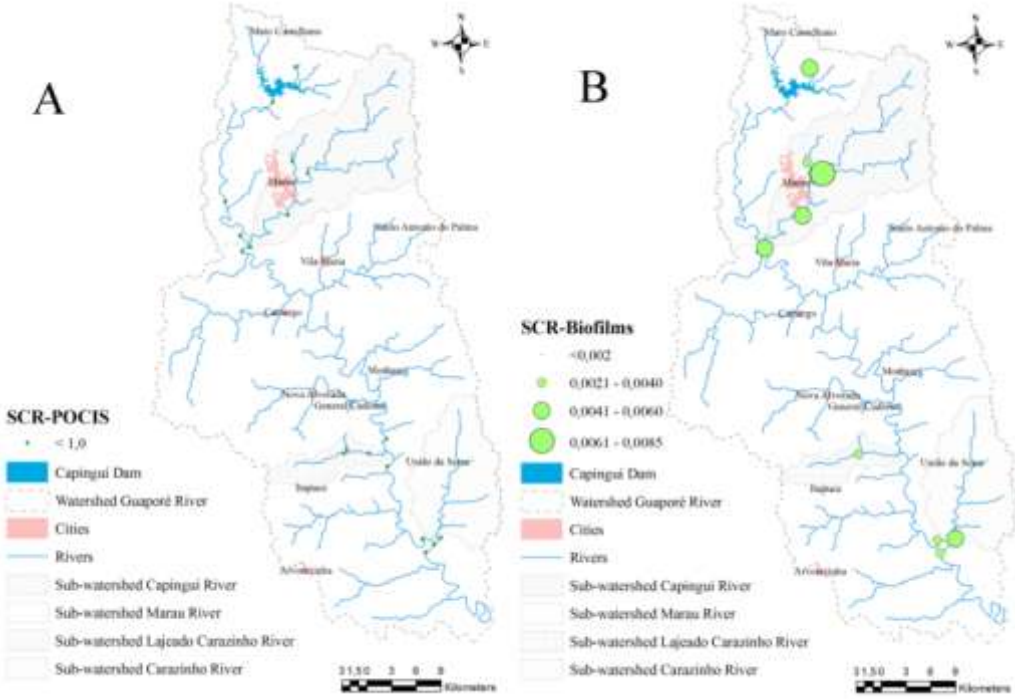
The concentrations of CBZ and SCR, present in the two southern portions of the Guaporé watershed (Lajeado Carazinho and Carazinho sub-watersheds), confirmed the role of urban area in the spread of anthropic compounds in water resources (Figures 2 and 3).

The sites sampled in the Carazinho sub-watershed were characterized by an absence of CBZ both in biofilms and POCIS, but also by the presence of SCR in different biofilms collected in winter. Thus, SCR was detected in the main river Guaporé upstream of the sub-watershed of Carazinho (0.0049 μg g^{-1}), downstream of the sub-watershed of Carazinho (0.0038 μg g^{-1}) and in the Carazinho outlet (0.0022 μg g^{-1}). The presence of SCR may be related to the proximity of the small cities of General Cadorna, Itapuca, and União da Serra. However, these values were not expected since these cities are at distance higher than 5 km from the sampled sites. Huerta et al. (2016) [40] verified the presence of pharmaceuticals in biofilms collected until 5 km downstream of a WWTP. It is worth noting that the Carazinho sub-watershed is predominantly a forested area with apparently low levels of human activity. Thus, the detection of anthropic makers in winter only suggests that the buffer ability of this area is sufficient in summer and fall but not in winter.

In the Lajeado Carazinho sub-watershed CBZ was not detected in all of the sites for all the seasons, but the POCIS installed in the Guaporé River upstream and downstream of the Lajeado Carazinho sub-watershed reported small amounts of CBZ (12.5 and 29.1 μg g^{-1}, respectively). This sub-watershed is characterized by small farms that discharge their housing effluents directly into small tributaries. As the results show, these effluents are particularly sensitive to pollution in certain seasons. The study of SCR in biofilms identified a pressure point in the upstream Lajeado Carazinho area in summer (0.0482 μg g^{-1}) and winter (0.0031 μg g^{-1}). These results and the presence of CBZ in the corresponding POCIS confirmed that this site is regularly contaminated by human discharges.



Chapter 3 - Figure 2 - Map of the Guaporé River sub-watershed with the concentrations of carbamazepine (µg g^{-1}) obtained in epilithic biofilms occurring in the Guaporé River watershed, Brazil.



Chapter 3 - Figure 3 - Map of the Guaporé River sub-watershed with the concentrations of sucralose (µg g^{-1}) obtained in epilithic biofilms occurring in the Guaporé River watershed, Brazil.

3.3. IMPLICATIONS FOR RIVER QUALITY MONITORING AT LOBAL SCALE.

This study shows that biofilm is an efficient natural bioindicator, which is easily available at distinct points of a watershed. This means that, the biofilm allows sampling campaigns because it is able to represent the pressures encountered in its close environment. Complementary to POCIS sampler, these both samplers allow access to sucralose and carbamazepine, known as anthropic markers.

These two compounds have the advantage of being spread in the environment and also discriminating zones more or less contaminated. Moreover they could be easily analyzed allowing sampling campaigns at different scales (e.g.: sub-watersheds, catchment basins and even countries). This study leaded in Rio Guaporé's watershed could be extended to larger scales and in other sites presenting different anthropic contexts more or less impacted by human activities.

CONCLUDING REMARKS

This study shows that anthropic markers may provide useful tools to investigate the contamination of watersheds and identify the pressure points. The Guaporé watershed is undergoing anthropic action, given that the lack of treatment of urban sewage is a national Brazilian problem. Sites that were considered as inaccessible to human contamination, such as FLONA, showed still the presence of human marker compounds. Moreover, the waste waters discharged into waterways constitute the major source of contamination provided by cities. Thereby, it is possible to confirm that human activities are interacting with the aquatic ressources of the Guaporé watershed. The proximity to urban areas has a strong influence on the amounts of CBZ and SCR in aquatic systems. An overview of the contamination of the Guaporé watershed shows that the most affected areas are located in the northern part, especially due to the presence of Marau city.

Biofilm and POCIS are two complementary techniques and neither can be omitted. The exposure time is not similar between the two sampling methods and each compound has a specificity that permits different kinds of interaction with each adsorbent. Biofilms can be used to identify environmentally contaminated sites by the capture of SCR as a human marker and POCIS are capable to capture CBZ with high efficiency in polluted sites and with more ability than biofilms.

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CHAPTER 4 - ANTIBIOTIC CONTAMINATION AND ARGSLEVELS IN THE GUAPORÉ RIVER (RIO GRANDE DO SUL) CATCHMENT BIOFILMS SAMPLES

1. INTRODUCTION

For more than ten years, Brazil is facing a high demographic growth leading to important economic changes in agriculture and urbanisation (Bastos *et al.*, 2017). The Brazilian government has made efforts to preserve the natural ressources but the public environmental policies are limited by the priority of the economic development. Brazilians think that environmental protection is poor, especially with regard to emerging sanitary problems observed worldwide. Thus, the presence of traces of human or veterinary compounds in natural waters is now a recurring problem. Indeed, these compounds are administered to humans or animals (via water, food, injection and pills - (Sarmah, Meyer and Boxall, 2006) but their metabolization is incomplete, resulting in the presence of the original compound and its metabolites in the urine and feces (Halling-Sørensen, 2001; Hirsch *et al.*, 1999). Consequently, significant amounts of pharmaceutical residues can reach waters and soils (Sarmah, Meyer and Boxall, 2006; Thiele-Bruhn, 2003) by the direct or treated discharges of feces and/or the erosion of contaminated soils.

The bioaccumulation of these compounds, due to their low solubility that occurs in plants and animals, are the main research targets specially because these compounds can be transferred into the trophic chain (Migliore, 1995). Nevertheless the main problem with the use of antibiotics is related to the development of resistant bacterial strains that represent a risk to human and animal health (Landers *et al.*, 2012). These problems are documented in studies related to ingestion of food contaminated with medicinal products and the health risks after its consumption, such as allergy, toxicity and the potential for development of resistant bacterial strains when those antibiotic residues reach the humans through the food chain (Fàbrega *et al.*, 2008). In Brazil, several studies related to organisms causing human and animal infection, such as *Staphylococcus spp., Salmonella enteric subsp., Enterococcus faecalism* (Campos *et al.*, 2013) and *Escherichia coli* (Baccaro *et al.*, 2002). Despite the large quantities of information about antibiotic pollution in water and food, the resistance in aquatic microorganisms has not yet been fully explored, needing more studies about the mecanisms of antibioresistance acquisition and dissemination (Balcázar, Subirats and Borrego, 2015).

Several international research programs have been lead to evaluate the concentrations of antibiotics or antibioresistant bacteria. Thus, many campaigns of water sampling have been

performed worldwide. Complementary passive or natural samplers have been also used to improve these evaluations. Thus, recent works have related the efficience of epileptic biofilms to monitor contaminated zones. Biofilms are capable of capturing compounds outcome of human pollution and results have been satisfactory for a large number of drugs, mainly at the outlets of sewage treatment plants (Aubertheau *et al.*, 2017; Huerta *et al.*, 2016). Furthermore the emergence of resistance genes can be also tracked in natural microbial communities present in biofilms (Andersson and Hughes, 2014; Aubertheau *et al.*, 2017).

These last years antibiotics consumption has increased in Brazil due to the use for the production of animals (pigs, catlles …) in addition to treatments delivered in human medicine. Consequently, monitoring the presence of both antibiotics and antibiotic resistance is essential to identify the most polluting human activities, arising from the cities and/or from the agriculture and then proposed strategies to limit the contamination. Thus, the objective of this work was to relate the quantities of pharmaceuticals sorbed in biofilms and the developpement of bacterial resistance with the main anthropic pressures occuring in Brazil. The study was performed in the Guaporé River watershed that is located in the northeastern region of the Rio Grande do Sul state. This region is submited to several sources of pharamaceutical contamination because the wastewaters from several cities are directly released (without treatment) inside the rivers and the farmers use animal manure and slurry for soil fertilization.

2. MATERIALS AND METHODS

2.1. GUAPORÉ WATERSHED AND SAMPLED SITES

The Guaporé River is one of the most important river in the south Brazil (Figure 1). Its catchment area drains an area of about 2,030 km^2 with around 0.57% of water bodies and 0.60% of urban areas. The upper part of the watershed presents an undulated relief and deep soils that are cultivated using no-tillage systems. In the middle and lower parts of the watershed with sloping relief and shallow soils, agricultural activities, especially the cultivation of tobacco, are developed in small farms. The soils of this watershed were exposed to erosion and high sediment loss (Tiecher, 2015). The losses of soil material are greater for shallow soils and hilly areas. Still, in many areas, crop and soil management does not take into account the fragility of the soils, resulting in intense erosion areas. The population is

mainly located in medium-sized cities: Marau (37,145 inhabitants), Ilópolis (4,098 inhabitants), Arvorezinha (10,229 inhabitants), Itapuca (2,337 inhabitants), União da Serra (1,620 inhabitants), Nova Alvorada (3,177 inhabitants), Montauri (1,542 inhabitants), Vila Maria (4,221 inhabitants), Camargo (2,591 inhabitants) and Mato Castelhano (2,470 inhabitants).

2.1.1. Capingui sub-watershed

The Capingui sub-watershed is located in the northern portion of the Guapore's watershed. Nowadays, most of the area is used for growing soybeans and corn crops in no-tillage system. In this sub-watershed, four points with different interests were monitored. The northern point is the National Forest of Passo Fundo (FLONA – point 1, Figure 1). This point is a Unit of Conservation Federal 1300 hectares, created in the 1940s, including 450 hectares of native forest belonging to the biome of the Atlantic Forest. In this study, this site will be considered as the control sample due the low anthropic action. However, it is important to consider that the waters of other rivers (eg.: Tingatu, Cachoeirinha, Capingui and Rio Branco) have a direct influence on FLONA. The water quality monitoring done by the Chico Mendes Institute (Instituto Chico Mendes de Conservação da Biodiversidade, 2011), classified the quality of FLONA waters as "good".

The water of FLONA drain into the reservoir of Capingui Dam. Thereupon, a sampling site was located downstream of the Capingui Dam (Capingui Dam – point 2, Figure 1). The Capingui Dam was the first large dam in southern Brazil, with a storage capacity of 40 million m^3 of water (Comitê Brasileiro de Barragens, 2011). Today, Capingui Dam is used only for recreation, with a rampant increase human occupation without a network waste collection, resulting in wastewater release directly on the dam.

Two sites were also monitored downstream of the Capingui dam. The first site represents the contribution of a vast area of agricultural production. This site is used for grain production under no-tillage system, especially soybean, corn and wheat (Agriculture –item 3, Figure 1). The second site is the outlet of sub-watershed of Capingui (Capingui outlet point 4, Figure 1). This site receives runoff water from the grain production region, however, with a large concentration of residues coming from breeding of confined animals, pig poultry and dairy production.

2.1.2. Marau sub-watershed

The Marau sub-watershed is characterized by the predominance of family agriculture and livestock production, with the contribution of wastewater from urban areas of the city of Marau (37,145 inhabitants). The economy of this sub-watershed is based on poultry, pork and milk production. The production of dry grains, especially maize in no-tillage system coexists with livestock production in almost all properties. In these urban areas, there are agribusinesses, especially with large slaughterhouses for pigs and poultry for export, leather processing and mechanical metal industry (Prefeitura Municipal de Marau, 2013).

The impact of rural and urban human activities was monitored at four sites. The first one represents the water coming predominantly from runoff of grain producing areas in no-tillage (Marau upstream - Grain - point 5, Figure 1). The second site, in addition to grain production, has loads of pigs and dairy cows (Marau upstream Animal - point 6, Figure 1). Two sites were monitored downstream of the city of Marau which receive all urban pollution load: the first site is immediately downstream of the town (Marau city – point 7, Figure 1) and the second is at the outlet of the sub-watershed of Marau (Marau Outlet – point 8, Figure 1) and aims to check the river resilience potential when entry urban pollutants decrease. Marau does not have any sewage treatment system that results in a huge increase of pollution in the river arising from the urban area. The path of the river into the city suffers various forms of environmental degradation. There are evidences of issue wastewater, municipal solid waste and industrial effluents, such as observed fish death along the river (Prefeitura Municipal de Marau, 2013). A site was also monitored at the confluence of Capingui and Marau sub-watersheds (item 9, Figure 1). From this point, the river is called the Rio Guaporé.

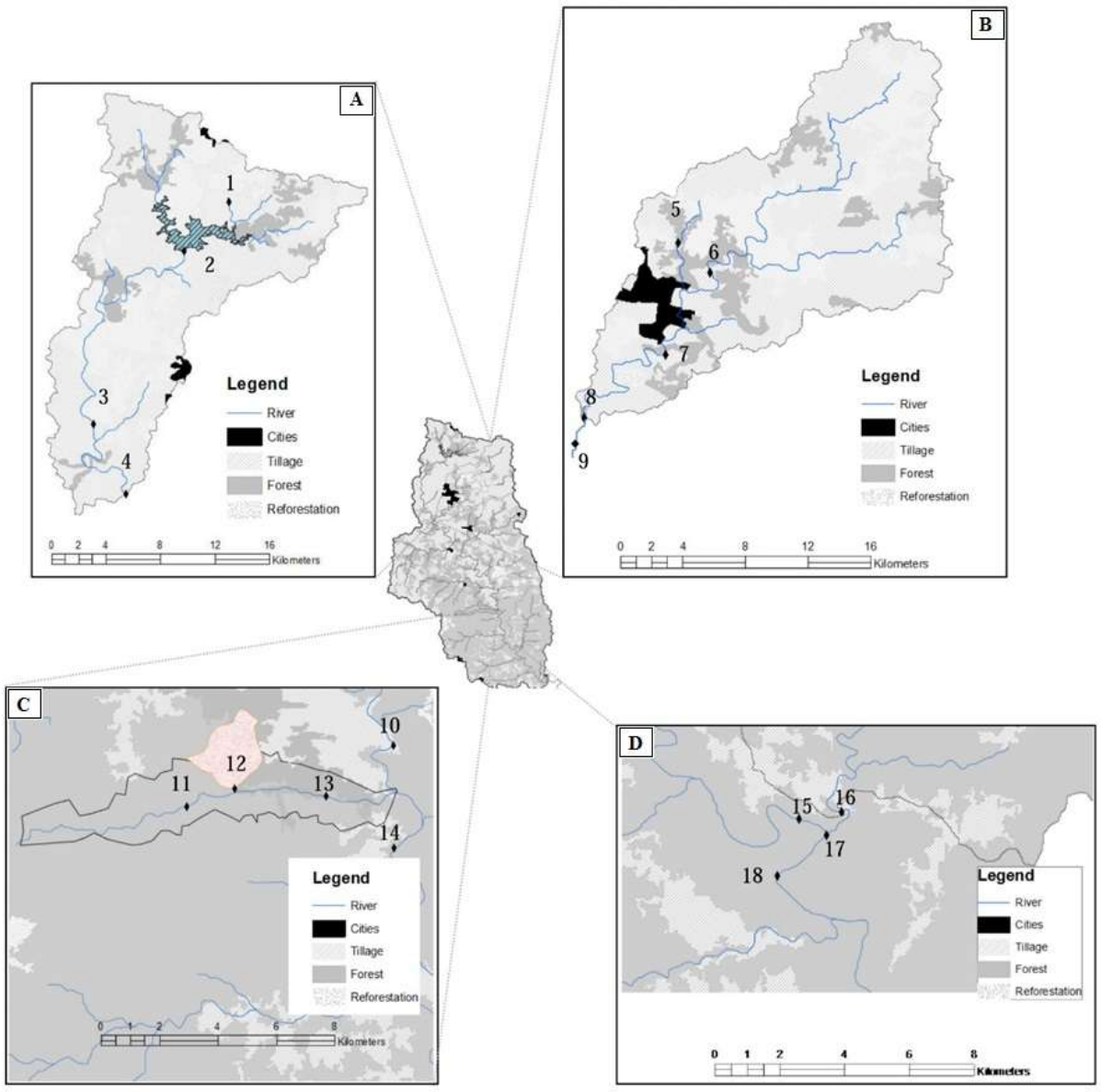
2.1.3. Lajeado Carazinho sub-watershed

Lajeado Carazinho sub-watershed is a small region located in the central part of the Guaporé River Basin. It is a fragile region characterized by sloping relief, impoverished and predominantly used for tobacco cultivation under system integration with tobacco international industries (Didoné *et al.*, 2014). Three sites were monitored in this zone. The first site is placed upstream of tobacco-producing areas contribution (Upstream in Lajeado Carazinho – point 11, Figure 1). The second site receives the water passing by tobacco producing area (Tobacco – point 12, Figure 1) and represents the downstream of the tobacco

contribution ((Downstream of tobacco in Lajeado Carazinho - Downstream in Lajeado Carazinho – point 13, Figure 1). Two sites were placed in the main river (Guaporé's River), one upstream of this sub-watershed (Upstream tobacco sub-watershed – point 10, Figure 1) and other downstream (Downstream tobacco sub-watershed – point 14, Figure 1). These two sites were chosen to know the participation of Lajeado Carazinho sub-watershed human activities in the main river (Rio Guaporé).

2.1.4. Carazinho sub-watershed

The Carazinho sub-watershed is located in the south part of the Rio Guaporé basin. This sub-watershed located near the Lajeado Carazinho sub-watershed was historically focused on the food production. Characterized by a mountainous region, União da Serra is occupied by native forest and reforestation areas due to the rural exodus. The sampled area was chosen to represent low human impact (pressure) and lower intensification of agriculture (crops and animal production) in the studied region. The stream Carazinho is a tributary of the Guaporé River that passes inside the Carazinho subwatershead. In Carazinho stream was sampled the Carazinho sub-watershed outlet (Carazinho Outlet – point 16, Figure 1) and the union of the stream Carazinho and Guaporé River (Central Carazinho – item 17, Figure 1). One point was placed upstream and another downstream of the Carazinho sub-watershed in the Guaporé River, (Upstream Carazinho sub-watershed – point 15 and downstream Carazinho sub-watershed – 18, Figure 1, respectively) to evaluate the effects of this sub-watershed in the main river.



Capingui Sub-Watershed (A): (1) Flona, (2) Capingui Dam, (3) Agriculture e (4) Capingui outlet.

Marau Sub-Watershed (B): (5) Marau upstream - Grain (6) Marau upstream – Animal (7) Marau city (8) Marau Outlet and (9) Marau and Capingui confluence.

Lajeado Carazinho Sub-Watershed (C): (10) Upstream Lajeado Carazinho sub-watershed, (11) Upstream Lajeado Carazinho (12) Lajeado Carazinho (13) Downstream of Lajeado Carazinho (14) Downstream Lajeado Carazinho sub-watershed.

Carazinho Sub-Watershed (D): (15) upstream Carazinhosub-watershed (16) CarazinhoOutlet (17) Central Carazinho(18) downstream Carazinhowatershed

Chapter 4 - Figure 1 - Sites sampled in Guaporé sub-watershed (Rio Grande do Sul – Brazil).

2.2. BIOFILMS SAMPLING

At each site, rocks that remained submerged in all seasons were sampled. The biofilm was obtained through brushing rocks with a toothbrush. The material adhered to the rock was rinsed with deionized water into a glass jar (Aubertheau *et al.*, 2017).

The aqueous solution containing the biofilm was stored in ice coolers (± 4 °C) and transported immediately to the laboratory. In the laboratory, the samples were transferred to individual high density polyethylene jars and frozen at -80 °C for subsequent lyophilization (freeze LS3000 - Terroni). After being freeze-dried, samples were homogenized in an agate mortar to obtain a representative sample for posterior analysis

2.3. PHARMACEUTICALS ANALYSES

Biofilm extraction procedure was performed using the method described by Aubertheau *et al.* (2017), adapted from Jelić, Petrović and Barceló (2009). Five hundred milligrams of biofilms were extracted by pressured solvent at high temperature (80 °C) using methanol / water (1/2; v/v) (ASE^{TM} 350, Thermo Fisher Scientific Inc, Waltham, USA). The extracts were purified by solid phase extraction (Autotrace^{TM} 150, Thermo Scientific, Waltham, USA) using Oasis® HLB cartridges (6ml, 200 mg of sorbent; Waters, Milford, USA) using methanol as eluent. The final extracts were evaporated under mild nitrogen steam until reaches a volume of 100 µL for posterior restitution to 500 µL with a mixture of methanol / water (10/90; v/v).

Twelve pharmaceuticals (eleven antibiotics and one non-steroidal anti-inflammatory drug) were identified and quantified in the biofilm samples (Table 1). The antibiotics were chosen after investigations with farmers and belong to four different families commonly used in the Guaporé region.

The quantification of pharmaceuticals in biofilms was performed with a Q-Exactive Orbitrap™ mass spectrometer (Thermo Scientific, Waltham, USA) of the standard addition method. Each compound was added at the end of sample preparation in final concentrations ranging from 0 to 15 ng L^{-1} (4 additions). The obtained analytical curve was linear so that the concentration could be calculated. Each concentration level was injected three times to determine the average concentration in ng g^{-1} of dry biofilm (Aubertheau *et al.*, 2017).

Chapter 4 - Table 1 - Properties of the studied pharmaceuticals.

Therapeutic class	Family	Pharmaceuticals	Abbr	Chemical formula	Molar Mass	Parent ion (m/z)	Product ion (m/z)	Collision energy (V)
Anti-inflammatory	-	Diclofenac	DCF	$C_{14}H_{11}Cl_2NO_2$	296.14	318.00591	261.1040	13
Antibiotic	Sulphonamide	Sulfamethazine	SMZ	$C_{12}H_{14}N_4O_2S$	278.33	279.08962	204.03990	35
		Sulfamethoxazole	SMX	$C_{10}H_{11}N_3O_3S$	253.28	254.05972	156.01137	30
		Sulfaquinoxaline	SQX	$C_{14}H_{12}N_4O_2S$	300.33	301.06751	156.01138 275.8543	25
	Quinolones	Norfloxacin	NOR	$C_{16}H_{18}FN_3O_3$	319.33	342.12244	312.92000	85
		Ciprofloxacin	CIP	$C_{17}H_{18}FN_3O_3$	331.34	332.14000	231.05600	60
		Enrofloxacin	ENR	$C_{19}H_{22}FN_3O_3$	359.39	360.17000	296.09900	65
		Levofloxacin	LVF	$C_{18}H_{20}N_3FO_4$	361.37	362.15000	318.16100	30
	Macrolides	Erythromycin	ERM	$C_{37}H_{67}NO_{13}$	733.93	734.46800	576.37000	14
		Roxithromycin	ROX	$C_{41}H_{76}N_2O_{15}$	837.05	837.52766	679.43600	13
		Tylosin	TIL	$C_{46}H_{77}NO_{17}$	916.10	916.52399	174.11276	21
	Tetracycline	Oxytetracycline	OXY	$C_{22}H_{24}N_2O_9$	460.43	483.13740	443.14426 426.12000	15

2.4. QUANTIFICATION OF RESISTANCE GENES

DNA was extracted from biofilms samples using a Fast DNA® SPIN kit for feces that efficiently isolates PCR-ready genomic DNA from the samples. The extraction method was accomplished according to the manufacturer's instructions. Extraction was performed with a FastPrep®135 Instrument (MP Biomedicals, California, USA). Extracted DNA was quantified with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, USA) and stored at -80 °C until analysis. Three antibiotic resistance genes, largely described and observed in the environment (Zhang *et al*., 2009), were quantified from the DNA extracts: *sul1* - (sulphonamide resistance), *qnrA* - (quinolone resistance) and *erm* (erythromycin resistance). Genes were detected by using quantitative PCR and realized by INRA Transfert Environnement (Narbonne - France).

The 16S rRNA encoding gene was quantified by SYBR green assay using the universal primers 338F and 518R (MightCycler ® FastStart DNA Master plus SYBR Green I, Roche Life Science). The calculation of genes copies per bacteria was performed from the amount of DNA per mass of biofilm and the number of copies of DNA per resistant gene in a gram of biofilm. The final amount of bacteria was defined using estimated value from literature, where 4.1 gene copies represent a unit of bacteria (Klappenbach *et al.*, 2001; Stalder, 2012).

3. RESULTS AND DISCUSSION

3.1. PRESENCE OF PHARMACEUTICALS IN DIFFERENT AREA OF THE GUAPORÉ WATERSHED

3.1.1. Capingui sub-watershed

The pharmaceuticals concentrations found in the Capingui watershed biofilms are presented in Table 2. The biofilm developed in the submerged rocks within the João de Barro stream, located in Flona, had a high concentration of diclofenac (188.2 ng g^{-1}) in summer. Biofilms sampled in other seasons contained lowesr concentrations (fall: 8.2 ng g^{-1}) or were free of this drug. The presence of diclofenac in water occurring within a natural area, with low anthropogenic action, was also observed by Kasprzyk-Hordern, Kondakal and Baker (2010). These authors revealed the presence of diclofenac (1.0 ng L^{-1}) and sulfaquinoxaline (2.0 ng L^{-}

^{1}) in water samples collected in the Llantrisant Forest and attributed these values to the anthropic actions around the sampled area, such as agriculture and the presence of urban areas. The biofilms sampled in Flona watercourse contained also many traces of antibiotics: sulfaquinoxaline (4.6 ng g^{-1}) in summer; (2.3 ng g^{-1}), enrofloxacin (3.1 ng g^{-1}) and sulfamethoxazole (4.3 ng g^{-1}) in autumn and oxytetracycline (2.3 ng g^{-1}), norfloxacin (3.0 ng g^{-1}) and levofloxacin (12.8 ng g^{-1}) in biofilms sampled in winter.

The site is considered as preserved (Passo Fundo National Forest) with a history of land use turned to agriculture, until the year 1946, and wood exploitation, until the year 1968. Since the 70's, it has been transformed into a preservation area, resulting in a national forest with predominantly man-planted trees (Sá and Gerhardt, 2016). Therefore, biofilms collected within Flona site do not receive constant contributions of pharmaceuticals. However, the waters drained from surrounding agricultural sites supply the biofilms with compounds used by farmers in their rural activities. The contamination of this protected zone is the result of the low efficiency damping of the Flona zone. In the past, the Passo fundo forest was a wooden park. When the park was created, the National Pine Institute (INP) bought agricultural lands abble to produce wood. However, not all local farmers sold their properties to the institute resulting in a fragmented and discontinuous park surface. When the park became the national preservation area the fragmentation of the suface resulted in difficulties in establishing an efficient buffer zone, which reduced efficiency in protecting the forest from human activities pressure (Sá and Gerhardt, 2016). Special emphasis should be given to the rudimentary roads that cross the Flona area, that are preferred ways of transferring contaminated water and sediment from adjacent crops according to Tiecher (2015). The contribution of unpaved roads, in small and medium magnitude pluviometric events, can reach approximately 70% of the global sediment production (Tiecher, 2015).

The second site sampled downstream of Flona represents the outlet of the Capingui dam. The dam was created to feed the Capingui Hydroelectric Plant and part of its water supply assured by the water sources located in FLONA (Prefeitura Municipal de Marau, 2013). On the banks of the reservoir there are also several country houses belonging mostly to Marau, Mato Castelhano and Passo Fundo residents (Sá and Gerhardt, 2016). The biofilms collected downstream of the dam in summer had a great diversity of compounds (seven), which reflect high anthropic pressures around this dam, especially in this season. In this evaluation, all drug families had at least one of their compounds detected in the biofilms (Anti-inflammatory: diclofenac; Tetracycline: oxytetracycline; Macrolides: erythromycin and roxithromycin; Fluoroquinolones: norfloxacin and levofloxacin; Sulphonamides:

sulfamethazine). The contributions of agricultural activities can not be ruled out as the surface drainage is drained to the dam by several streams. The damming (ie. stagnation) of water in the dam can concentrate the chemical compounds coming from areas used for agricultural activities (Victoretti *et al.*, 1969) and large presence of people around the dam during the summer period. However, biofilms collected in fall and winter remained contaminated with diclofenac and norfloxacin compounds, even at extremely high concentrations during the winter period (181.4 and 186.9 ng g^{-1}, respectively). The levofloxacin compound was detected in the biofilms collected in the autumn and sulfamethazine and sulfamethoxazole in the biofilms sampled in winter.

The Capingui sub-watershed is covered by intensive agricultural activities. The dominant production are corn and soybeans in spring and summer, and forage species in winter. Farmers apply regurlarly manure and slurry for soil fertililization. There are also several farms for the breeding of poultry, pork, and dairy cattle in confinement systems at family-scale. All these activites can lead to the discharge of pharmaceuticals (especially antibiotics that are used to prevent diseases or to promote growth). All the twelve pharmaceuticals were present in the biofilms sampled in summer. Diclofenac was found at high concentrations (196.7 ng g^{-1}) in biofilms collected in summer, and the values were much lower in biofilms sampled in winter and autumn (26.1 and 3.3 ng g^{-1}, respectively). Oxytetracycline (2.7 ng g^{-1}) and all sulphonamides (sulfamethoxazole - 8.8 ng g^{-1}, sulfamethazine - 9.5 ng g^{-1} and sulfaquinoxaline - 7.6 ng g^{-1}) were found only in summer. The sowing of corn and other commercial crops takes place from begin in September. The application of animal waste begins before sowing and carry on until fully blooming. The doses applied are much higher than those recommended for nutrient supply (Sociedade Brasileira de Ciência do Solo, 2016), due to a need to dispose all the animal waste (Capoane *et al.*, 2015; Gatiboni *et al.*, 2014). Even though crops are conducted under no-tillage system, which drastically reduces soil losses by surface runoff (Didoné *et al.*, 2014), the transfers of water and extremely fine particles are still high specially because the soils had barely no mechanical barriers to contain the surface runoff. Thereby, soil with animal anf humam wastes can be transferred quickly into rivers taking nutriments (Girotto *et al.*, 2010; Rheinheimer *et al.*, 2007) and pharmaceuticals. During the final period of development of summer crops, the application of animal waste becomes impossible. Consequently, biofilms appear to respond positively to the lower flow of drugs to the aquatic system, since only two compounds were present when the biofilm was sampled in autumn and winter, among them diclofenac, which has a widespread occurrence in the sub-watershed Capingui.

The Capingui outlet biofilms sampled in the summer period still contained six compounds: 50% of the total found at the agricultural downstream site. The compounds found were diclofenac, oxytetracycline, erythromycin, roxithromycin, sulfaquinoxali and enrofloxacin (Table 2). The lower intensity of land use (in particular, the lower number of farmed animals in the area between the agricultural and the outlet sampled site) could have resulted in less compounds to be transported to the river. Another explanation is the metabolization of these compounds, once that in this study only the parent drug. In the two other periods of the year the number of compounds and their concentrations in the biofilms were very similar to those found downstream of the agricultural site, except for the high concentration of levofloxacin (105.50 ng g^{-1}) in the winter period. The lowest compound concentration can be explained by the increase of the river flow in the rainy season, leading to dilution.

Chapter 4 - Table 2 - Pharmaceuticals concentration found in biofilms sampled in summer, fall and winter in the sub-watershed of Capingui in Guaporé's watershed (Rio Grande do Sul – Brazil).

Sampled site	Flona		Capingui Dam		Agriculture		Capingui Outlet	
	Summer (ng g^{-1})							
Diclofenac	188.3	(± 16)	20.0	(± 50)	196.8	(± 31)	98.7	(± 248)
Oxytetracycline	<LD		47.2	(± 97)	2.7	(± 57)	2.1	(± 29)
Erythromycin	<LD		4.6	(± 4)	5.1	(± 6)	2.3	(±16)
Roxithromycin	<LD		3.5	(± 7)	6.2	(± 12)	2.5	(± 17)
Tylosin	<LD		<LD		7.6	(± 15)	<LD	
Enrofloxacin	<LD		<LD		24.2	(± 26)	5.9	(± 26)
Norfloxacin	<LD		3.1	(± 31)	28.7	(±11)	<LD	
Ciprofloxacin	<LD		<LD		32.7	(±44)	<LD	
Levofloxacin	<LD		4.8	(± 34)	9.9	(± 21)	<LD	
Sulfamethoxazole	<LD		<LD		8.8	(± 15)	<LD	
Sulfamethazine	<LD		44.2	(± 16)	9.5	(± 4)	<LD	
Sulfaquinoxaline	4.6	(± 4)	<LD		7.6	(± 15)	4.6	(± 17)
Σ concentrations	192.9		127.5		339.9		116.0	
Number of compounds	2		7		12		6	
	Fall (ng g^{-1})							
Diclofenac	8.3	(± 7)	13.7	(±38)	3.28	(± 29)	22.23	(± 7)
Oxytetracycline	<LD		<LD		<LD		<LD	
Erythromycin	<LD		<LD		<LD		<LD	
Roxithromycin	<LD		<LD		<LD		<LD	
Tylosin	<LD		<LD		<LD		<LD	
Enrofloxacin	<LD		<LD		<LD		<LD	
Norfloxacin	3.0	(± 8)	2.5	± 31	<LD		<LD	
Ciprofloxacin	<LD		<LD		<LD		<LD	
Levofloxacin	12.8	(± 10)	7.2	± 6	8.88	± 6	5.20	± 3
Sulfamethoxazole	<LD		<LD		<LD		<LD	
Sulfamethazine	<LD		<LD		<LD		<LD	
Sulfaquinoxaline	<LD		<LD		<LD		<LD	
Σ concentrations	24.1		23.4		12.16		27.43	
Number of compounds	3		3		2		2	
	Winter (ng g^{-1})							
Diclofenac	<LD		181.44	± 3	26.10	± 68	<LD	
Oxytetracycline	2.33	(± 7)	<LD		<LD		<LD	
Erythromycin	<LD		<LD		<LD		<LD	
Roxithromycin	<LD		<LD		<LD		<LD	
Tylosin	<LD		<LD		<LD		<LD	
Enrofloxacin	3.11	(± 6)	<LD		4.99	± 15	8.24	± 30
Norfloxacin	<LD		8.34	± 8	<LD		<LD	
Ciprofloxacin	<LD		<LD		<LD		<LD	
Levofloxacin	<LD		<LD		<LD		105.50	± 247
Sulfamethoxazole	4.34	(± 9)	67.15	± 5	<LD		<LD	
Sulfamethazine	<LD		186.98	± 6	<LD		<LD	
Sulfaquinoxaline	<LD		<LD		<LD		<LD	
Σ concentrations	9.78		443.91		34.09		113.74	
Number of compounds	3		4		2		2	

<LD : below the detection limit

3.1.2. Marau sub-watershed

The concentrations of the pharmaceuticals identified in the biofilms sampled in the Marau sub-watershed are presented in Table 3. This sub-watershed is characterized by large agricultural production in the upper part and by the presence of the Marau city with 37,145 inhabitants and no sewage collection and treatment network. In the rural area upstream of the city of Marau the biofilms were strongly contaminated with pharmaceuticals due to the integration of grain production with animal husbandry. Ten compounds were detected at the point surrounded by grain production and nine compounds at the point with predominance of animal husbandry. Even if farmers who grow and raise animals are not the same that those who apply the manure to the crops, it is extremely common to the familiar agricultors to apply these residues in the soils as fertilizers. There is a trade of waste between animal breeders and grain producers. In these two sites biofilms, were contaminated with diclofenac in the three seasons of the year, with the highest values obtained in summer (1148.2 ng g^{-1} and 295.2 ng g^{-1} for grains and animals biofilms sites, respectively). The high concentrations of diclofenac are related to the large production of dairy cattle in the region as the drug is frequently used as anti-inflammatory, antipyretic, non-steroidal analgesic and non-narcotic analgesics to treat pain, fever with or without inflammation. Diclofenac is administered to cattle with acute mastitis problems, postpartum paresis, acute infections and others disorders. The presence of veterinary antibiotics in water bodies adjacent to agricultural fields with application of animal waste was also showed in Germany by Bailey *et al.* (2015). The authors highlight for the first time that the use of manure contaminated with veterinary antibiotics for fertilization resulted in the contamination of rivers from land transport by surface runoff and / or soil erosion.

In the urban part of this sub-watershed, pollution from the city in summer resulted in large contamination of biofilms. Thus, there were nine compounds of drugs identified at that station (diclofenac, tylosin, enrofloxacin, norfloxacin, ciprofloxacin, levofloxacin, sulfamethoxazole, sulfamethazine, sulfaquinoxalin) (Table 3) and in the autumn (diclofenac, erythromycin, roxithromycin, enrofloxacin, ciprofloxacin, levofloxacin, sulfamethoxazole, sulfamethazine and sulfaquinoxalin) (Table 3). Several works, studying the presence of drugs in the waters of Brazilian rivers and streams, have also pointed out the presence of high concentrations of diclofenac at sampling points near urban areas (Almeida and Weber, 2009; Américo *et al*., 2013) which reached very high concentrations (12,000 µg L^{-1}) at sampling points near clandestine sewage discharge sites (Stelato *et al.*, 2016). In the present work, our results showed that a seasonal variation of the contamination occur in the river, inside the

biofilms. The highest concentrations of diclofenac in the biofilms sampled during the autumn season may be related to the no-dilution in dry seasons (Luque-Espinar *et al.*, 2015; Pereira *et al.*, 2015). In autumn, unlike the agricultural zones, the amount of compounds present in the biofilms remained as high as those sampled in summer (nine compounds). However, there was disappearance of tylosin and norfloxacin and the identification of two new macrolide compounds (erythromycin - 4.7 ng g^{-1} and roxithromycin - 4.0 ng g^{-1}). The variation of these amounts may probably result from the patients consumption, since their uses depends of the doctor prescription and the price variation of the drug in the market (Nicolini *et al.*, 2008). For example, disappearance of tylosin was concomitant with the appearance of other compounds of the same family (erythromycin - 4.7 ng g^{-1} and roxithromycin - 4.0 ng g^{-1}). Similarly, it could be noted that the disappearance of norfloxacin and the increase in the concentration of other compounds of the same family by more than 100% (enrofloxacin 2.5 to 5.5 ng g^{-1}, ciprofloxacin 7.8 to 44.6 ng g^{-1} and levofloxacin 30.5 to 214,0 ng g^{-1} in summer and fall respectively). In the biofilm sampled in winter, the number of compounds found was lower than in the other seasons. In addition, their concentrations were lower than those measured in biofilms of the other seasons (erythromycin - 3.15 ng g^{-1}, tylosin - 4.70 ng g^{-1}, levofloxacin - 26.69 ng g^{-1}, sulfamethazine - 3.76 ng g^{-1}; sulfaquinoxalin - 4.20 ng g^{-1}). Even though antibiotic use is more frequent in winter, due to the increase of diseases affecting the respiratory tract (Weber *et al.*, 2010), high precipitation affects river dynamics (dilution) and biofilms. In addition to the dilution of the main compound (Luque-Espinar *et al.*, 2015), the increase in river flow changes the potential for bioaccumulation, the structure and metabolism of natural biofilm communities (Corcoll *et al.*, 2015).

In the outlet of the Marau sub-watershed (located at 14 km from the city of Marau), the biofilms sampled in the summer (diclofenac, tylosin, norfloxacin, levofloxacin sulfamethazine) and fall (diclofenac, erythromycin, roxithromycin, tylosin, enrofloxacin, norfloxacin, ciprofloxacin, levofloxacin, sulfamethazine, sulfaquinoxalin) were contaminated by five and ten compounds, respectively. On the other hand, biofilms sampled in winter had only three compounds (enrofloxacin, levofloxacin and sulfamethazine) (Table 3). This "outlet" site probably integrates the pollution of both the upstream urban and rural areas. Thus, the concentrations are similar for most of the compounds when comparing the results of this site and those obtained upstream of Marau. It is worth noting that this observation is different from that found by Huerta *et al.* (2016), studying the impact of a treatment plant on the concentration of epileptic biofilms in the Segre river (Spain). The authors found an

opposite relation between the concentration of drugs and the distance from the source of contamination after 5 km.

3.1.3. Confluence Capingui/Marau

Biofilms sampled less than one kilometer dowstream of the confluence between the Capingui and Marau rivers had pharmaceuticals concentrations lower than the concentrations obtained in the outlets of the two sub-watersheds. In only a few kilometers distance between the entrance of huge urban pollution and the monitoring site there was a sharp decrease in the diversity and concentration of drug compounds impregnated in the biofilms (Table 3). It is clear that the rural pollution is linked to the dates of application of the waste in the crops and the concomitant transfer to the aquatic sources during the pluviometric events. On the other hand, urban pollution is much more constant and more diverse, since there is a strong population density whose residues are quickly transferred to the waterways due to the lack of a collection and treatment system. In addition, when considering areas with urban presence, large disparities may occur in effluent treatment efficiency, as approximately 50% of antibiotics are retained in sewage treatment plants (Miège *et al.*, 2009). These data highlight the importance of the treatment of urban effluents in the contamination of the rivers and the great problem of the city of Marau that discards its urban waste directly in the local rivers.

Furthermore, the results obtained at the confluence demonstrate that the Guaporé watershed system is capable of resilience for pharmaceutical compounds. This resilience can be mainly explained by the dilution caused by the addition of the sub-watershed waters.

Chapter 4 - Table 3 - Pharmaceuticals concentration found in biofilms sampled summer, fall and winter in the sub-watershed of Marau in Guaporé's watershed (Rio Grande do Sul – Brazil).

Sampled site	Marau upstream Grain		Marau upstream Animal		Marau City		Marau Outlet		Confluence	
	Summer (ng g^{-1})									
Diclofenac	1148.2	(± 458)	295.2	(± 111)	10.2	(± 2)	10.1	(± 3)	18.3	(± 8)
Oxytetracycline	5.5	(± 35)	4.6	(± 21)	<LD		<LD		<LD	
Erythromycin	3.0	(± 48)	<LD		<LD		<LD		<LD	
Roxithromycin	2.5	(± 45)	<LD		<LD		<LD		<LD	
Tylosin	2.6	(± 47)	3.4	(± 20)	16.8	(±58)	3.6	(± 10)	<LD	
Enrofloxacin	11.9	(± 62)	<LD		2.9	(±34)	<LD		5.9	(±33)
Norfloxacin	40.4	(± 104)	3.2	(± 30)	31.5	(± 11)	8.3	(± 10)	13.0	(± 25)
Ciprofloxacin	<LD		3.8	(± 37)	7.8	(±21)	<LD		<LD	
Levofloxacin	<LD		166.7	(± 743)	30.5	(± 44)	38.1	(±68)	24.2	(± 36)
Sulfamethoxazole	7.3	(± 45)	59.2	(± 74)	41.5	(± 94)	<LD		7.2	(± 5)
Sulfamethazine	6.0	(± 44)	4.6	(± 25)	2.2	(± 16)	2.9	(± 7)	<LD	
Sulfaquinoxaline	4.1	(± 41)	2.4	(± 2)	4.3	(± 2)	<LD		<LD	
Σ concentrations	1231.4		543.21		147.7		63.0		68.7	
Number of compounds	10		9		9		5		5	
	Fall (ng g^{-1})									
Diclofenac	63.8	(± 14)	4.0	(± 7)	642.4	(± 25)	41.8	(± 67)	8.8	(± 3)
Oxytetracycline	<LD		<LD		<LD		<LD		<LD	
Erythromycin	<LD		<LD		4.7	(± 3)	4.7	(± 15)	<LD	
Roxithromycin	<LD		<LD		4.0	(± 3)	6.7	(± 21)	<LD	
Tylosin	<LD		<LD		<LD		5.4	(± 18)	<LD	
Enrofloxacin	<LD		<LD		5.5	(± 4)	6.2	(± 23)	<LD	
Norfloxacin	<LD		3.8	(± 6)	<LD		33.1	(± 113)	<LD	
Ciprofloxacin	<LD		2.6	(± 13)	44.6	(± 25)	3.4	(± 32)	<LD	
Levofloxacin	<LD		5.8	(± 20)	214.0	(± 12)	34.2	(± 82)	4.2	(± 11)
Sulfamethoxazole	<LD		<LD		12.9	(± 9)	<LD		<LD	
Sulfamethazine	<LD		<LD		4.8	(± 4)	2.8	(± 17)	<LD	
Sulfaquinoxaline	<LD		<LD		5.1	(± 3)	6.0	(± 20)	<LD	
Σ concentrations	63.8		16.2		937.8		144.2		13.0	
Number of compounds	1		4		9		10		2	
	Winter (ng g^{-1})									
Diclofenac	17.49	(± 30)	163.92	(± 897)	<LD		<LD		<LD	
Oxytetracycline	<LD		<LD		<LD		<LD		<LD	
Erythromycin	<LD		<LD		3.15	(± 4)	<LD		<LD	
Roxithromycin	<LD		<LD		<LD		<LD		<LD	
Tylosin	<LD		<LD		4.70	(± 4)	4.73	(± 4)	<LD	
Enrofloxacin	<LD		4.22	(± 20)	<LD		<LD		7.40	(± 24)
Norfloxacin	22.00	(± 67)	6.11	(± 33)	<LD		<LD		<LD	
Ciprofloxacin	<LD		<LD		<LD		<LD		<LD	
Levofloxacin	<LD		3.84	(± 32)	26.69	(± 67)	31.72	(± 65)	42.74	(±103)
Sulfamethoxazole	<LD		<LD		<LD		<LD		<LD	
Sulfamethazine	2.94	(± 27)	<LD		3.76	(±6)	2.52	(± 6)	<LD	
Sulfaquinoxaline	<LD		<LD		4.20	(± 5)	<LD		<LD	
Σ concentrations	42.43		178.09		42.5		38.97		50.14	
Number of compounds	3		4		5		3		2	

<LD : below the detection limit

3.1.4. Lajeado Carazinho sub-watershed

The concentrations of the pharmaceuticals quantified in the biofilms collected in the watercourses of the Lajeado Carazinho sub-watershed are shown in Table 4. This region is characterized by a strong tobacco production (Carazinho sub-watershed), intersperse by pig, poultry and dairy cattle farmers, all of them integrated with multinational companies. The results show that biofilms sampled in a small stream that drains water from several tobacco fields, tributaries of Carazinho River, presented in summer 200.0 ng g^{-1} of all pharmaceuticals found and in winter 11.0 ng g^{-1}. The biofilms contamination in summer was marked by the great participation of levofloxacin (109.2 ng g^{-1}), which in was smaller winter (4.4 ng g^{-1}). In addition, biofilms was contaminated with norfloxacin (23.6 ng g^{-1}), diclofenac (9.3 ng g^{-1}), sulfamethoxazole (4.3 ng g^{-1}), sulfamethazine (2.5 ng g^{-1}) and sulfaquinoxaline (3.2 ng g^{-1}). These pharmaceuticals come from the tobacco crops that were fertilized with animal waste and suffered surface runoff. Tobacco is frequently conducted in large line spacing making impossible to fully cover the soil even when the plant is entirely developed. The intense and frequent precipitations, that occur in this period, cause very high sediment and water transfers from the tobacco plantations to the watercourses (Pellegrini *et al.*, 2008). Also, the high potential for pollution due to intensive tobacco production in steep areas can result in transport of contaminated material by surface runoff. If the river that receives this contaminated material is small, with a little flow, that increase the contact time between pharmaceuticals and biofilms (Comte, Guibaud and Baudu, 2006; Solís *et al.*, 2012). Indeed, the soil losses due to water erosion are enormous especially in spring and summer period (Didoné *et al.*, 2014; Tiecher, 2015), if associated with very high doses of animal waste application, certainly carry the residues of medicines to the drainage network. Upstream of Rio Carazinho, biofilms contained diclofenac (15.4 and 66.0 ng g^{-1}), enrofloxacin (11,6 and 9,8 ng g^{-1}), levofloxacin (25. 2 and 7.7 ng g^{-1}) and sulfamethoxazole (11.6 and 2.1 ng g^{-1}), in winter and summer, respectively (Table 4). Tylosin (2.1 ng g^{-1}) was only detected in biofilms collected in winter. These results indicate that the contamination of this area is also due to the surrounding cities and not only the Tobacco culture. Nevertheless, this family farming mode (integrated or not with multinational companies) to produce pigs, poultry, milk and tobacco contributes to the contamination of water with pharmaceuticals, in addition to other chemical elements and coliforms already been reported in several studies (Capoane, Tiecher and Santos, 2016; Mallmann *et al.*, 2014; Tiecher *et al.*, 2017; Zafar *et al.*, 2016).

In the Carazinho river outlet, the sum of pharmaceuticals present in the biofilms sampled was lower in the biofilms monitored upstream (33.0 and 17.0 ng g^{-1} in summer and winter, respectively). In winter, with the exception of sulfaquinoxaline, all the pharmaceuticals that were detected in the biofilms sampled immediately downstream of the tobacco fields were also found in the sub-watershed outlet (diclofenac - 5.3 ng g^{-1}; enrofloxacin - 9.1 ng g^{-1} and sulfamethoxazole - 2.8 ng g^{-1}). The outlet receives waters from other streams that drain areas without tobacco culture leading to a dilution of the anthropic pollution.

3.1.5. Confluence Carazinho river/Guaporé River

In the Guaporé River, a few meters upstream of the water discharge from the Carazinho river, the values obtained in the biofilms sampled in summer (62.0 ng g^{-1}) were higher than those sampled in winter (17.0 ng g^{-1}) (Table 4). In summer, the biofilms contained oxytetracycline (2.2 ng g^{-1}), enrofloxacin (8.6 ng g^{-1}), norfloxacin (5.9 ng g^{-1}), ciprofloxacin (7.4 ng g^{-1}), levofloxacin (24.0 ng g^{-1}) and sulfamethoxazole (4.3 ng g^{-1}). The biofilms collected in winter had only the presence of levofloxacin (16.9 ng g^{-1}). The biofilms sampled in the Guaporé River, immediately downstream of Carazinho river confluence, were less contaminated than those sampled upstream (33.0 ng g^{-1} in summer and 42.0 ng g^{-1} in winter). The pharmaceuticals quantified in summer were oxytetracycline (2.7 ng g^{-1}), enrofloxacin (11.4 ng g^{-1}), norfloxacin (6.0 ng g^{-1}), levofloxacin (7.8 ng g^{-1}) and sulfamethoxazole (4.8 ng g^{-1}) and in winter diclofenac (31.6 ng g^{-1}), oxytetracycline (4.5 ng g^{-1}) and sulfaquinoxaline (6.1 ng g^{-1}). All these findings suggest that the animal manure used in tobacco and grain crops, especially maize, have a local impact on the contamination of the biofilms. In addition, due to the low flow late of Carazinho River compared to the Guaporé River, even if the environmental contamination occurs, the impact on the biofilms of the Guaporé watershed main river is imperceptible.

Chapter 4 - Table 4 - Pharmaceuticals concentration found in biofilms sampled Summer and Winter in the sub-watershed of Carazinho river in Guaporé River watershed (Rio Grande do Sul – Brazil).

Sampled River	Lajeado Carazinho tributary		----Lajeado Carazinho River----				----------Guaporé River----------			
Sampled Site	Tobacco production		Upstream of tobacco production		Downstream of tobacco production		Upstream Carazinho Watershed		Downstream Carazinho Watershed	
	Summer (ng g^{-1})									
Diclofenac	9.0	(±3)	15.4	(±1)	4.9	(±18)	9.7	(±3)	<LD	
Oxytetracycline	35.1	(±5)	<LD		<LD		2.2	(±5)	2.7	(±4)
Erythromycin	<LD		<LD		<LD		<LD		<LD	
Roxithromycin	<LD		<LD		<LD		<LD		<LD	
Tylosin	<LD		<LD		<LD		<LD		<LD	
Enrofloxacin	<LD		11.6	(±7)	6.3	(±27)	8.6	(±8)	11.4	(±34)
Norfloxacin	26.6	(±5)	<LD		5.2	(±18)	5.9	(±16)	6.0	(±3)
Ciprofloxacin	<LD		<LD		<LD		7.4	(±18)	<LD	
Levofloxacin	109.2	(±26)	25.2	(±8)	8.8	(±6)	24.4	(±30)	7.8	(±7)
Sulfamethoxazole	14.3	(±18)	11.7	(±23)	2.3	(±16)	4.3	(±3)	4.8	(±4)
Sulfamethazine	2.5	(±3)	2.1	(±3)	<LD		<LD		<LD	
Sulfaquinoxaline	3.2	(±1)	<LD		5.1	(±18)	<LD		<LD	
Σ concentrations	200.1		66.0		35.5		61.9		32.6	
Number of compounds	7		4		6		7		5	
	Winter (ng g^{-1})									
Diclofenac	3.8	(±2)	65.9	(±5)	5.4	(±3)	<LD		31.6	
Oxytetracycline	<LD		<LD		<LD		<LD		4.5	(±6)
Erythromycin	<LD		<LD		<LD		<LD		<LD	
Roxithromycin	<LD		<LD		<LD		<LD		<LD	
Tylosin	<LD		2.06	(±2)	<LD		<LD		<LD	
Enrofloxacin	3.3	(±2)	9.8	(±6)	9.1	(±3)	<LD		<LD	
Norfloxacin	<LD		<LD		<LD		<LD		<LD	
Ciprofloxacin	<LD		<LD		<LD		<LD		<LD	
Levofloxacin	4.4	(±12)	7.7	(±7)	<LD		16.7	(±38)	<LD	
Sulfamethoxazole	<LD		2.1	(±5)	2.8	(±3)	<LD		<LD	
Sulfamethazine	<LD		3.0	(±5)	<LD		<LD		<LD	
Sulfaquinoxaline	<LD		<LD		2.0	(±2)	<LD		6.1	(±6)
Σ concentrations	11.5		90.6		19.2		16.7		42.2	
Number of compounds	3		6		4		1		3	

3.1.6. Sub-watershed of Lajeado

In the outlet of this sub-watershed the sums of contaminant concentrations present in the biofilms sampled in summer and winter were low and similar (Table 5). The compounds identified in summer were diclofenac, sulfamethoxazole and levofloxacin and only levofloxacin in winter. This low contamination may be explained by the soil use in this region. Indeed, Lajeado sub-watershed presents a vast area of natural forest (Atlantic Forest) inserted in a mountainous topography. There is no urban areas next to the point, only a few family farmers are in charge of the production of maize, beans and other subsistence crops. Consequently, this part of the Guaporé River watershed remains less anthropized than the other parts.

3.1.7. Confluence Lajeado Carazinho sub-watershed and Guaporé River

Biofilms on the Guaporé River prior to the dowstream of Lajeado Carazinho sub-watershed were already contaminated in summer and winter with diclofenac (8.1 ng g^{-1} and 39.5 ng g^{-1}, respectively) and levofloxacin (20.4 ng g^{-1} and 15.4 ng g^{-1}, respectively) (Table 5). Oxytetracycline (2.1 ng g^{-1}), norfloxacin (15.1 ng g^{-1}), ciprofloxacin (3.1 ng g^{-1}) and levofloxacin (15.4 ng g^{-1}) were present only in biofilms sampled in winter. The release of the waters of the Lajeado Carazinho sub-watershed on the Guaporé River was imperceptible in the dynamics of biofilms, since they contained only levofloxacin (20.6 and 5.5 ng g^{-1} in summer and winter, respectively) and diclofenac only in summer (6.3 ng g^{-1}).

Chapter 4 - Table 5 - Drugs concentration found in biofilms sampled Summer and Winter in the sub-watershed of União da Serra in Guaporé River watershed (Rio Grande do Sul – Brazil).

Sampled River	Lajeado River		----------Guaporé River----------			
Sampled Site	Outlet		Upstream Carazinho watershed		Downstream Carazinho watershed	
	Summer (ng g^{-1})					
Diclofenac	9.5	(±4)	8.1	(±22)	6.3	(±14)
Oxytetracycline	<LD		<LD		<LD	
Erythromycin	<LD		<LD		<LD	
Roxithromycin	<LD		<LD		<LD	
Tylosin	<LD		<LD		<LD	
Enrofloxacin	<LD		<LD		<LD	
Norfloxacin	<LD		<LD		<LD	
Ciprofloxacin	<LD		<LD		<LD	
Levofloxacin	14.0	(±26)	20.4	(±38)	20.6	(±78)
Sulfamethoxazole	2.4	(±24)	<LD		<LD	
Sulfamethazine	<LD		<LD		<LD	
Sulfaquinoxaline	<LD		<LD		<LD	
Σ concentrations	25.9		28.49		26.9	
Number of compounds	3		2		2	
	Winter (ng g^{-1})					
Diclofenac	<LD		39.5	(±20)	<LD	
Oxytetracycline	<LD		2.1	(±18)	<LD	
Erythromycin	<LD		<LD		<LD	
Roxithromycin	<LD		<LD		<LD	
Tylosin	<LD		<LD		<LD	
Enrofloxacin	<LD		<LD		<LD	
Norfloxacin	<LD		15.1	(±32)	<LD	
Ciprofloxacin	<LD		3.1	(±31)	<LD	
Levofloxacin	27.0	(±48)	15.4	(±41)	5.5	(±6)
Sulfamethoxazole	<LD		<LD		<LD	
Sulfamethazine	<LD		<LD		<LD	
Sulfaquinoxaline	<LD		<LD		<LD	
Σ concentrations	27.0		75.2		5.5	
Number of compounds	1		5		1	

3.2. EVALUATION OF THE PRESENCE OF *SUL1, ERM* AND *QNRA* ANTIBIORESISTANCE GENES

3.2.1. Capingui sub-watershed

The biofilms sampled in the Capingui sub-watershed presented only the resistance genes *sul1* and *erm* (Table 6). Gene copy values per bacterium ranged from 5.9 10^{-5} and 1.1 10^{-3} for *sul1* resistance gene and from 0 to 1.4 10^{-4} for the *erm* resistance gene. The *qnrA* gene, which confers resistance to quinolones, was not detected in the biofilms sampled in Capingui sub-watershed. This gene is generally mediated by different plasmids and can be not easily detected. Thus, Yang *et al.* (2014) studied the bacterial resistance to quinolones in *Escherichia coli* and *Klebsiella pneumoniae* and did not found the presence of the *qnrA* resistance gene. However, the authors were able to verify resistance to quinolones by the presence of *qnrB* genes in 50% of the isolates of *K. pneumoniae* and the *qnrS* genes in 2.5% of *E. coli* and 9.1% in *K. pneumoniae*. Therefore, even if the *qnrA* resistance gene has not been detected in the biofilm samples, the possibility of resistance to quinolones can not be ruled out.

At the Flona site, the *sul1* resistance gene was found in the biofilms sampled in summer and winter (8.8 10^{-5} and 4.6 10^{-4} gene copy per bacterium, respectively). Sulphonamides are synthetic antibiotics (Guimarães *et al.*, 2010) and their presence occurs only in environments with the presence of human activities. Therefore, the bacteria living in the biofilm must have been contaminated with residues from livestock activities in the vicinity of Flona. Indeed, the contamination came from the surrounding agricultures areas of the watercourses. The low efficiency of damping of the Flona zone cannot preserve the forest from the impacts of agricultural activities that are in the vicinity as already well documented by Sá and Gerhardt (2016).

The biofilms sampled in the Capingui dam, despite the large concentrations of sulphonamides found in summer and mainly in winter, showed no difference in the amount of resistance (log scale) between the seasons (10^{-4} gene *sul1* copy per bacterium). In addition, the biofilms of the Capingui dam were not those that presented the highest resistance to *sul1* within the sub-watershed. Resistance to *erm* in biofilms sampled in summer and winter had similar gene copy number per bacterium (10^{-5} gene copy per bacterium). This result shows that, even if the concentration of the macrolides in summer was superior to winter, the dynamics of resistance development is associated to several interactions between the

environment and the bacteria that colonize the biofilm (e.g.: temperature, flow rate, nutriments, etc) .

Downstream of the Capingui dam, the biofilms had a strong influence of the interaction of crop and animal production on the resistance genes in summer and in winter for *sul1* (10^{-5} and 10^{-4} copy of gene per bacterium, respectively) and *erm* (10^{-5} and 10^{-4} gene copies per bacterium, respectively) being most pronounced in biofilms collected in winter.

The biofilms sampled in the outlet of the Capingui sub-watershed had the resistance genes *sul1* and *erm* withthe highest value of resistance gene copy per bacterium within all the sampled sites in the Capingui sub-watershed in summer (10^{-3} copy of gene per bacterium). The sampling site is located downstream of a zone with high agricultural pressure, where the activities carried out during summer have a lot of expressiveness, resulting in the sulphonamides supply to the river, allowing the development of resistance. Even though the amount of sulphonamides found in the biofilms was lower than the other sampling sites, the presence of bacterial resistance reinforces that the outlet of the Guaporé River sub-watershed is contaminated and receives significant amounts of sulphonamides. Other sulphonamide compounds or metabolites of the studied compounds may be responsible for the presence of resistance genes. Furthermore, the dynamics of adsorption and desorption with the biofilms probably influence the presence of these compounds (Comte, Guibaud and Baudu, 2006; Solís *et al.*, 2012).

Chapter 4 - Table 6 - *Sul1*, *erm* and *qnrA* resistance genes per bacterium sampled in epilitic biofilms from the Capingui river sub-watershed, Guaporé River watershed - RS, Brazil.

Sampling site	*sul1*		*erm*		*qnrA*	
	Summer	Winter	Summer	Winter	Summer	Winter
	Genes of Resistance per bacteriium					
FLONA	$8.8\ 10^{-5}$	$4.6\ 10^{-4}$	n.d.	n.d.	n.d.	n.d.
Capingui Dam	$2.7\ 10^{-4}$	$2.6\ 10^{-4}$	$6.1\ 10^{-5}$	$2.3\ 10^{-5}$	n.d.	n.d.
Agriculture	$5.9\ 10^{-5}$	$5.3\ 10^{-4}$	$1.8\ 10^{-5}$	$1.4\ 10^{-4}$	n.d.	n.d.
Capingui Outlet	$1.1\ 10^{-3}$	$6.1\ 10^{-4}$	$6.1\ 10^{-5}$	$2.6\ 10^{-5}$	n.d.	n.d.

3.2.2. Marau sub-watershed

Biofilms sampled in the Marau sub-watershed present the same resistance genes (*sul1* and *erm*; Table - 7) as the biofilms sampled in Capingui sub-watershed. Gene copy values per bacterium ranged from $4.7\ 10^{-4}$ to $7.2\ 10^{-3}$ for the *sul1* resistance gene and from $2.3\ 10^{-5}$ to 9.3

10^{-4} for the *erm* resistance gene, as predominance of the *sul1* gene in order of one to two log. It is worth noting that the level of antibiotic resistance was the highest in the urban area (Marau), with gene copy values per bacterium of 7.2 10^{-3} for the *sul1* genes and 9.3 10^{-4} for the *erm* genes. This tendancy was observed for the two season (summer and winter). Urban areas release generally a great diversity and important concentration of drugs. Consequently, the resistance to antibiotics is generally high close to the discharge points (Baquero, Martínez and Cantón, 2008; Czekalski *et al.*, 2015). Thus, the study performed by Pruden *et al.* (2013) has shown that the wastewater treatment plants and their effluents (urban or hospital sewages) constitute preferential environments for antibiotic resistance. Indeed, wastewater treatment plants receive large amount of resistant bacteria excreted from the human digestive tract. However, significant amount of bacteria are not eliminated by the treatment processes and are then continuously released with the treated water. Thus, WWTPs favor the dissemination of antibiotic resistance. In Marau sub-watershed, like elsewhere in Brazil, a large number of sewage waters are not collected and directly dumped in the river. Nevertheless, few kilometers downstream from the city of Marau, biofilms were less contaminated with pharmaceuticals and with fewer resistance genes in bacteria. The values return to those observed upstream urban environment biofilms (7.10^{-4} copies of bacterial genes for *sul1* and 1.5 10^{-4} copies of genes per bacterium for *erm*). Consequently the resistance seems to be mainly brought by the city.

3.1.1. Confluence Marau/Capingui

Biofilms sampled downstream of the confluence of the two rivers draining the Marau and Capingui sub-watershed had a higher *sul1* resistance gene than those of the two outlet biofilms (1.5 10^{-3} gene copies per bacterium in summer and 4.2 10^{-3} gene copies per bacterium in winter). The increase in resistance followed the increase in sulphonamide concentrations. In contrast, macrolide resistance was lower than those found in the two downstream rivers biofilms sampled (7.1 10^{-5} gene copies of *erm* per bacterium in summer and 9.1 10^{-5} gene copies of *erm* per bacterium in winter). Similar to the other monitored sites, the *qnrA* resistance gene was not identified even though the biofilms were impregnated with high concentrations of fluoroquinolones.

Chapter 4 - Table 7 - *Sul1*, *erm* and *qnrA* genes sampled in epilitic biofilms from the Capingui river sub-watershed, River watershed - RS, Brazil.

Sampling site	*sul1*		*erm*		*qnrA*	
	Summer	Winter	Summer	Winter	Summer	Winter
	Genes of Resistance per bacteriume					
Marau US Grain	$2.3\ 10^{-3}$	$4.7\ 10^{-4}$	$1.5\ 10^{-4}$	$2.3\ 10^{-5}$	n.d.	n.d.
Marau US Animal	$4.6\ 10^{-4}$	$9.8\ 10^{-4}$	$5.6\ 10^{-5}$	$6.5\ 10^{-5}$	n.d.	n.d.
Marau City	$5.4\ 10^{-3}$	$7.2\ 10^{-3}$	$2.6\ 10^{-4}$	$9.3\ 10^{-4}$	n.d.	n.d.
Marau Outlet	$9.0\ 10^{-4}$	$7.0\ 10^{-4}$	$1.3\ 10^{-4}$	$1.5\ 10^{-4}$	n.d.	n.d.
Confluence	$1.5\ 10^{-3}$	$4.2\ 10^{-3}$	$7.1\ 10^{-5}$	$9.1\ 10^{-5}$	n.d.	n.d.

3.1.2. Lajeado Carazinho sub-watershed

In the biofilms monitored immediately downstream of the drainage of the water from the area intensively cultivated with tobacco and with the use of high doses of animal wastes, the bacterial resistance genes *sul1* and *erm* were found. The amount of *sul1* genes per bacterium was higher in the biofilms sampled in winter (10^{-3} gene copies per bacterium) compared to summer biofilms (10^{-4} gene copies per bacterium)_. The *erm* resistance genes found in the biofilms sampled in winter and summer seasons had similar values (10^{-4} copies of genes per bacterium for both) (Table 8), even if the presence of the macrolides had not been recorded.

The discharge of waters from the small stream that drains the crop fields highly fertilized with animal wastes in the Carazinho River that contained a high pollutant load did not affect the diversity and concentration of drug compounds nor the amount of bacterial resistance genes in the biofilms. In addition, biofilms sampled upstream had a higher concentration of sulphonamides and *sul1* resistance genes in summer ($9.2\ 10^{-4}$ copies of genes per bacterium), compared to biofilms sampled in winter ($1.4\ 10^{-4}$ copies of genes per bacterium) (Table 8). The values of genes *erm* founded in biofilms was lower in winter ($3.4\ 10^{-6}$ gene copies per bacterium) compared to summer ($5.8\text{-}10^{-5}$ gene copies per bacterium). Also, the biofilms sampled downstream of the stream entrance had lower concentrations of sulphonamides than biofilms collected upstream from the stream entrance. Additionally, the bacterial resistance found for *sul1* and *erm* had lower values than those obtained in the biofilms sampled at the tobacco site, except for *sul1* in summer that had a similar value (10^{-4} gene copies per bacterium).

3.1.3. Confluence Carazinho River and Guaporé River

Similarly, the entrance of water from the Carazinho River into the Guaporé River did not affect the degree of contamination of the biofilms monitored. Resistance values upstream and downstream of the Carazinho River in the Guaporé River for *sul1* (10^{-4} gene copies per bacterium) and *erm* genes (10^{-3} gene copies per bacterium) were similar for the biofilms sampled in summer and winter. The resistance *qnrA* is not preferentially coming from the Carazinho sub-watershed, since the resistance genes were found in the biofilms collected upstream of the Carazinho River sub-watershed in summer ($1.0\ 10^{-6}$) and in winter ($1.8\ 10^{-6}$). In addition, the participation of the site with tobacco production, that has the most frequent application of manure, was not responsible for this contribution, once *qnrA* genes were not found in the biofilms sampled in this site and downstream of it into the Carazinho river (Table 8).

Finally, the outlet of Lajeado River, which has very low contamination of biofilms by drugs, did not alter the degree of contamination of the biofilms of the Guaporé River in the southern part of the hydrographic basin. The biofilms sampled in summer and winter contained only the resistance genes *sul1* ($7.2\ 10^{-4}$ and $4.2\ 10^{-4}$ copies of resistant genes per bacterium, respectively) and *erm* ($5.8\ 10^{-5}$ and $1.1\ 10^{-4}$ copies of resistant genes per bacterium, respectively) (Table 8).

The quality of the biofilms sampled throughout the watershed served as a tool to prove man's participation in environmental pollution. The most contaminated and resistant bacteria were found in sample sites were expected to be highly contaminated, while the less contaminated in the forest sites or in the sites that the contribution of pharmaceuticals are occurring in a no punctual form.

Chapter 4 - Table 8 - *Sul1*, *erm* and *qnrA* genes of bacteria sampled in epilitic biofilms from the Carazinho river sub-watershed, Guaporé River watershed - RS, Brazil.

Sampling site	*sul1*		*erm*		*qnrA*	
	Summer	Winter	Summer	Winter	Summer	Winter
	Genes of Resistance per bacterium					
Tobacco	$5.2\ 10^{-4}$	$2.1\ 10^{-3}$	$4.3\ 10^{-4}$	$1.7\ 10^{-4}$	n.d.	n.d.
Upstream Carazinho	$9.2\ 10^{-4}$	$1.4\ 10^{-4}$	$5.8\ 10^{-5}$	$3.4\ 10^{-6}$	n.d.	$1.0\ 10^{-6}$
Downstream Carazinho	$3.8\ 10^{-4}$	$8.3\ 10^{-5}$	$2.5\ 10^{-5}$	$5.8\ 10^{-6}$	n.d.	n.d.
Upstream Carazinho SW	$1.1\ 10^{-4}$	$2.7\ 10^{-3}$	$4.2\ 10^{-5}$	$4.2\ 10^{-4}$	$1.0\ 10^{-6}$	$1.8\ 10^{-6}$
Downstream Carazinho SW	$4.4\ 10^{-4}$	$2.1\ 10^{-3}$	$3.3\ 10^{-5}$	$1.5\ 10^{-4}$	n.d.	$1.5\ 10^{-6}$
Downstream Lajeado River SW	$7.2\ 10^{-4}$	$4.2\ 10^{-4}$	$5.8\ 10^{-5}$	$1.1\ 10^{-4}$	n.d.	n.d.

3.2. DISCUSSION

3.2.1. Are there areas more contaminated than others and where are they?

Using a clustering analysis approach (based on the Ward method of dissimilarity with euclidean distance - (Linden, 2009)), it was possible to group sampling sites with similar contamination patterns. Thus, five different classes of contamination were statistically identified (Hierarchical Aglomerative Cluster Analysis) from the number of pharmaceuticals compounds found and their concentrations in the biofilms (Figure 2):

- The first group corresponds to the urban area of Marau, where waster waters were released with poor or without treatment.

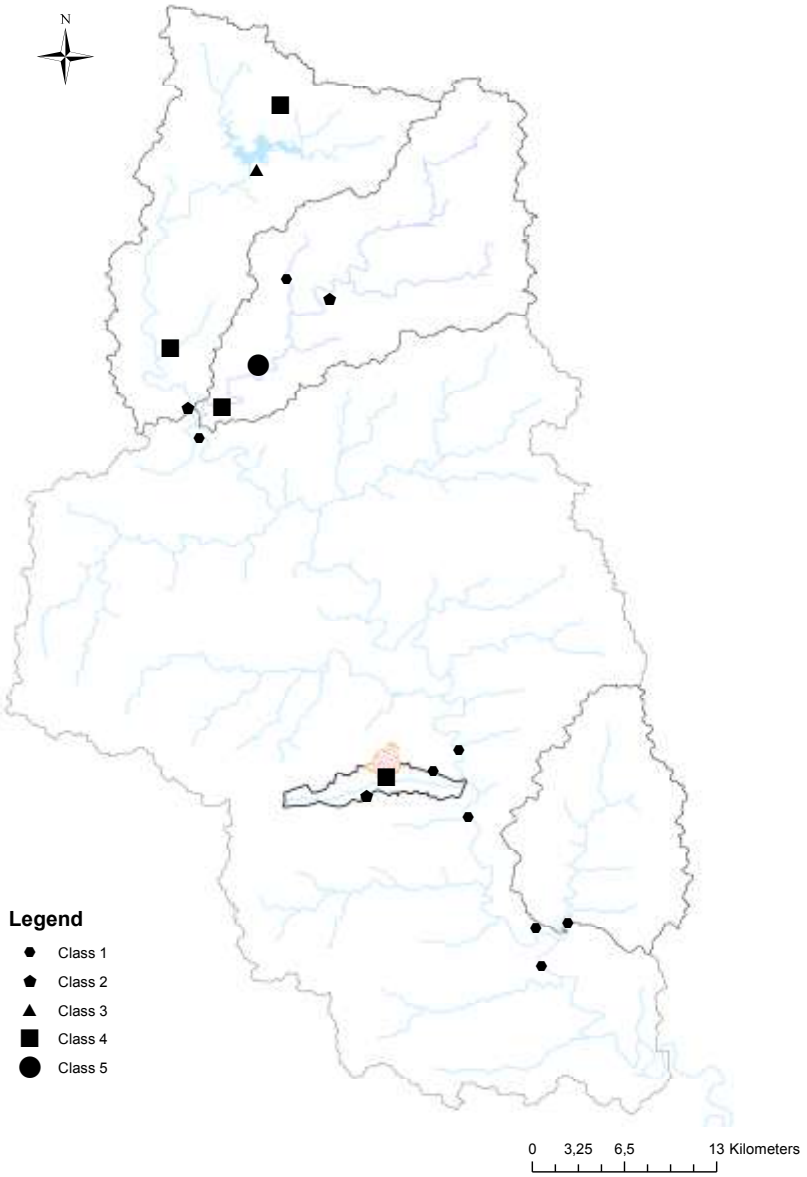
- The second group corresponds to the Flona, the Tobacco, the agriculture site in Capingui sub-watershed and the Marau Outlet. These sites are mainly characterized by direct or indirect exposition to animal waste application. The presence of the Marau outlet in this group suggests that this part of the Marau sub-watershed has lost the influence of the urban area but has gained the contribution of agricultural area.

- The third group corresponds to the Capingui dam showing that the contamination of this site is specific and different from the rest of the Capingui sites.

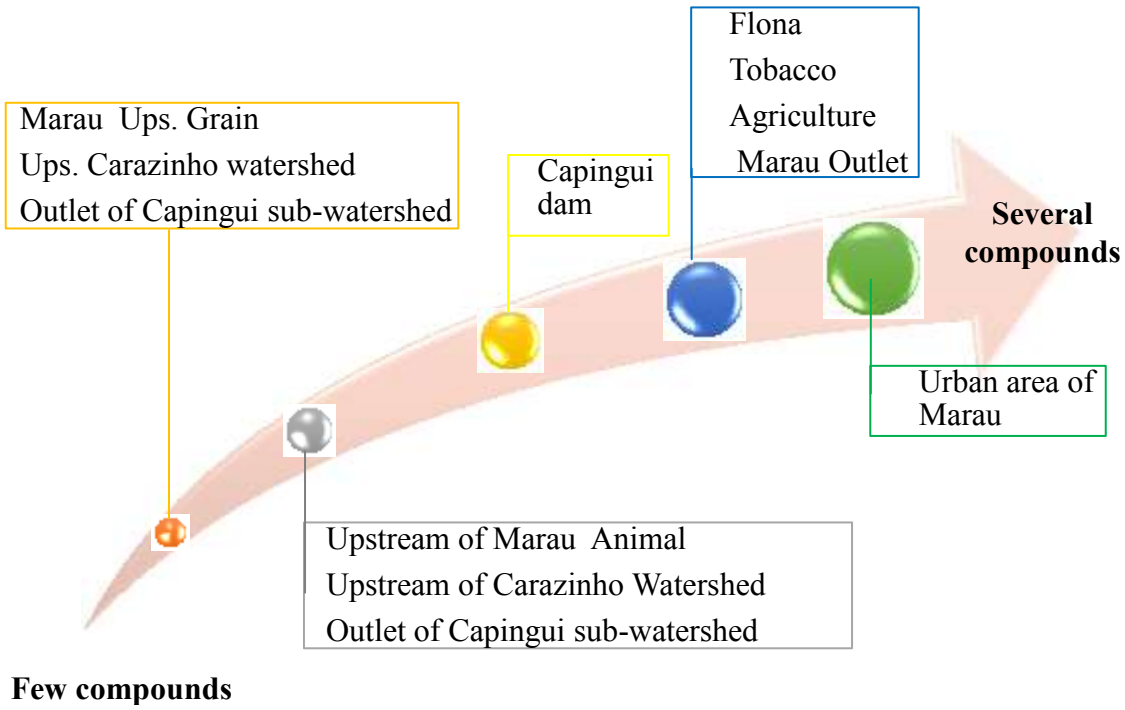
- The fourth group corresponds to the animal production area with the presence of many farms: upstream of Marau (Marau US Animal), upstream of Carazinho River in Guaporé River and the outlet of Capingui sub-watershed.

- The fifth group includes all the other sites sampled in the Guaporé River (confluence of the Capingui and Marau sub-watershed rivers, upstream and downstream of the Lajeado

sub-watershed, upstream and downstream of the Carazinho river sub-watershed), the site upstream of Marau (Marau US Grain), the site downstream of the tobacco stream in Carazinho River and the outlet of the Lajeado sub-watershed. All these sites are characterized by a low contamination of their biofilms, making their differentiation difficult depending on the sources of contamination. It is worth noting that these groups are classified according to the level of contamination. Thus the urban area of Marau correspond to the most polluted site (Figure 3).



Chapter 4 - Figure 2- Hierarchical Agglomerative Cluster classes obtained to pollution in epilithic biofilms sampled in Guaporé River watershed – RS, Brazil



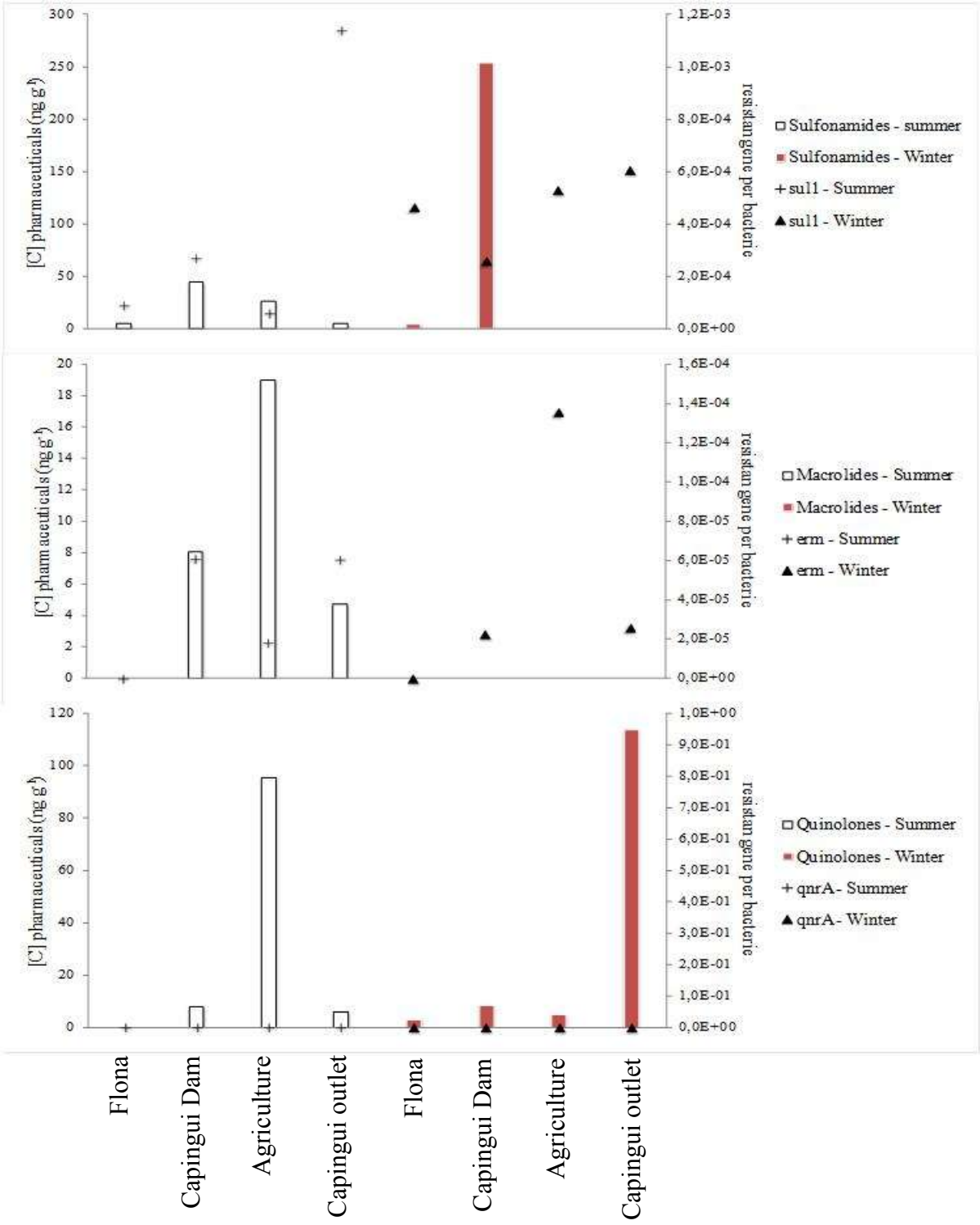
Chapter 4 - Figure 3 - Representation of the pollution levels of biofilm sampled in Guporé watershed.

The clusters obtained show also that biofilms are able to separate punctual sources (e.g.. urban environment, water reservoir for leisure use and application of manure on the banks of the watercourse) from those diffuse and seasonal sources (e.g. Guaporé River). This finding confirms that biofilms are good bioindicators, sensitive to variations in the diversity of compounds and the concentrations of pharmaceuticals released into the river.

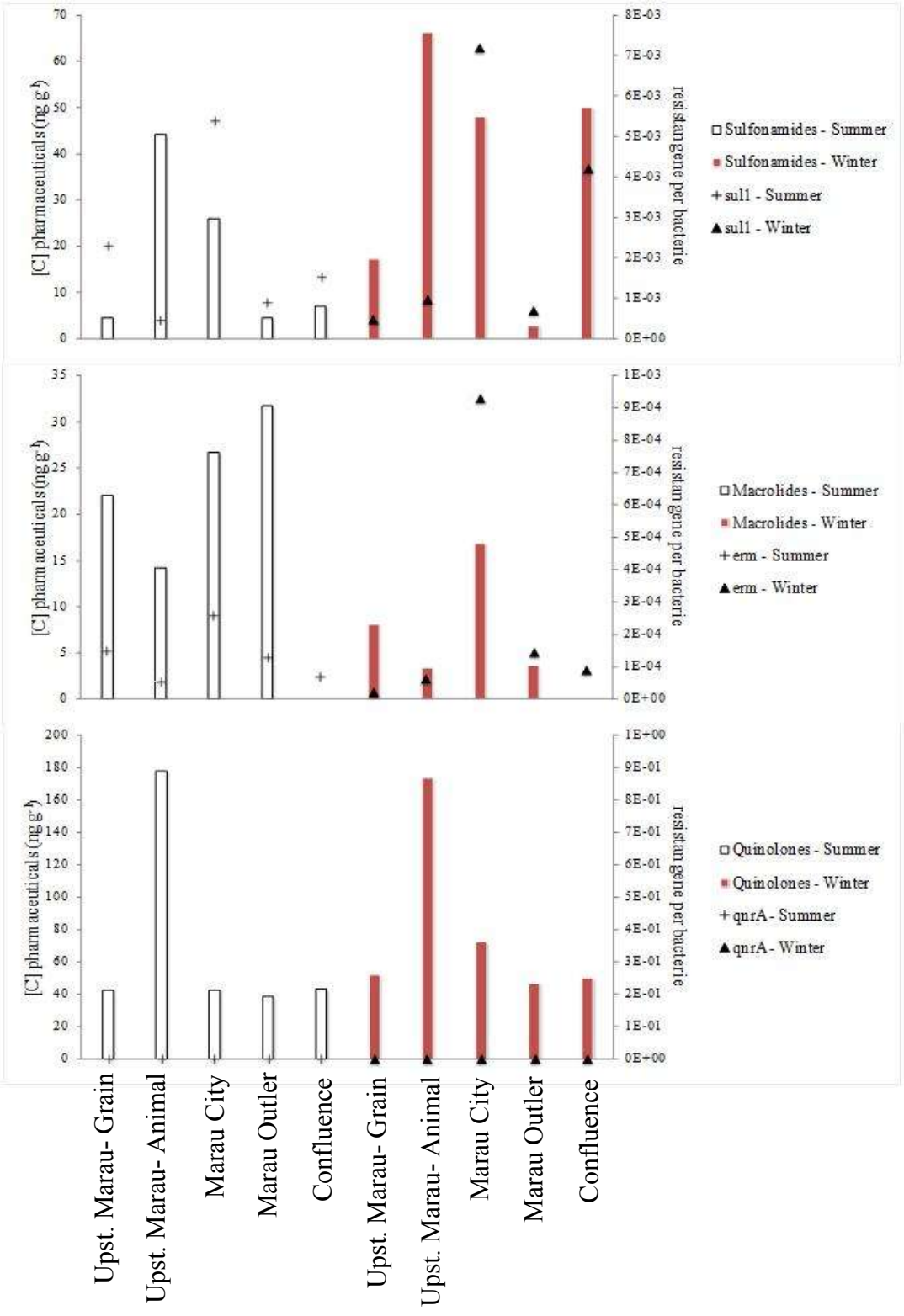
3.1.1. Does the presence of antibiotic favor the spread of antibiotic resistance in the Guaporé River?

As shown in the figures 4, 5 and 6 the concentration of antibiotic resistance genes was poorly correlated with the total amount of the corresponding antibiotic family. It is not yet very clear whether the presence of antibiotics in the aquatic environment results in developing resistance in the bacteria found in rivers (Diwan *et al.*, 2010). While very high levels of antibiotic contamination are likely to select for resistant bacteria directly, the role of sub-inhibitory concentrations of antibiotics in environmental antibiotic resistance dissemination remains unclear (Berglund, 2015). Thus, the increase in antibiotic resistance genes downstream wastewater treatment plants or cities may rather be due to accumulation

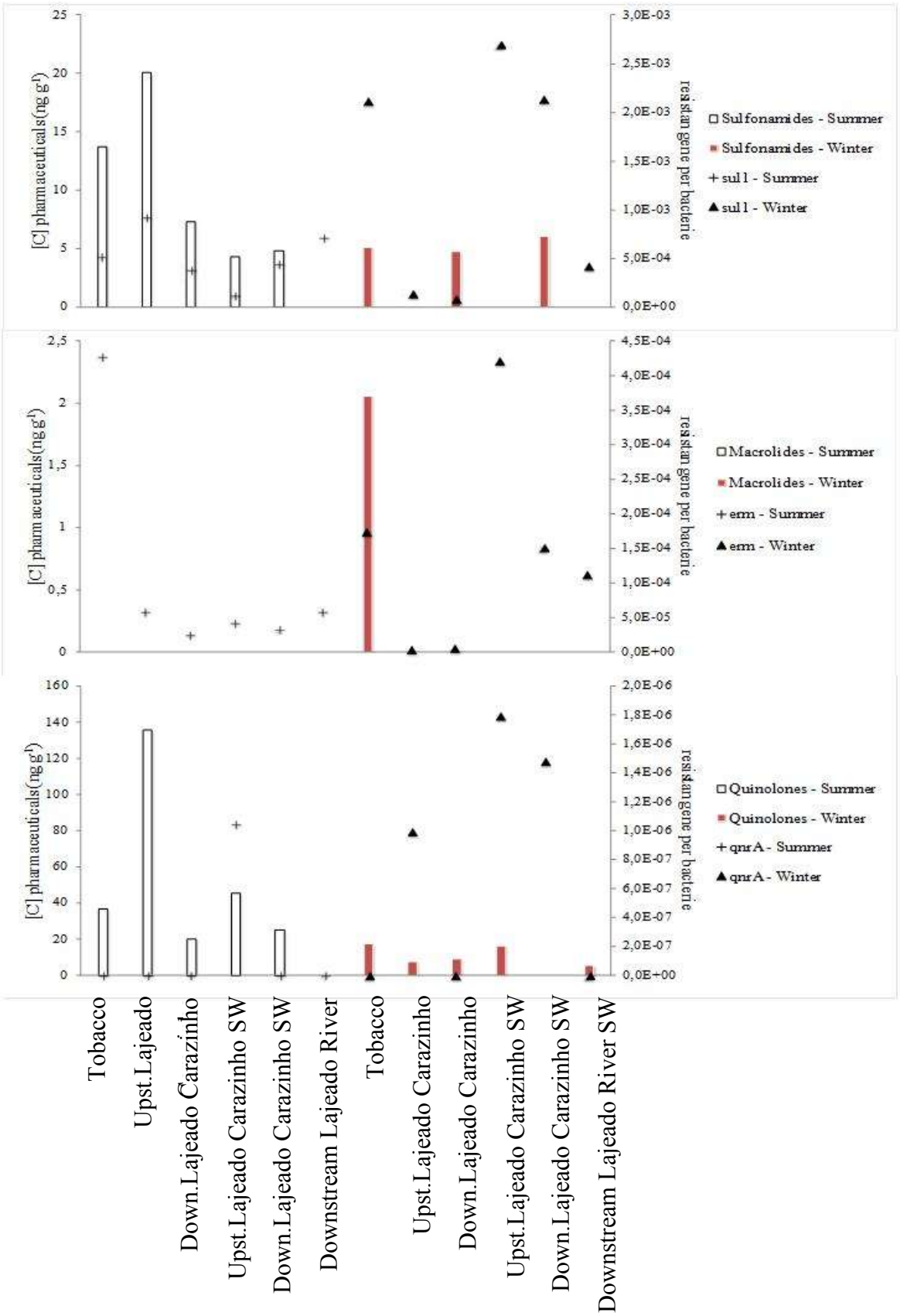
from anthropogenic sources rather than proliferation or selection of bacteria (Guerin *et al.*, 2009). Some authors, such as Bruchmann, Kirchen and Schwartz (2013), attributed the spread of antibiotic resistance genes to the difference in energy efficiency between groups of bacteria. Multiresistants consume less basic energy and have less transcription activity compared to normal bacteria, allowing better adjustment and adaptability in environments contaminated with drug residues.



Chapter 4 - Figure 4 – Concentration of pharmaceuticals and bacterial resistance genes obtained in epilithic biofilms sampled in Capingui sub-watershed (Guaporé River watershed) in winter and summer.



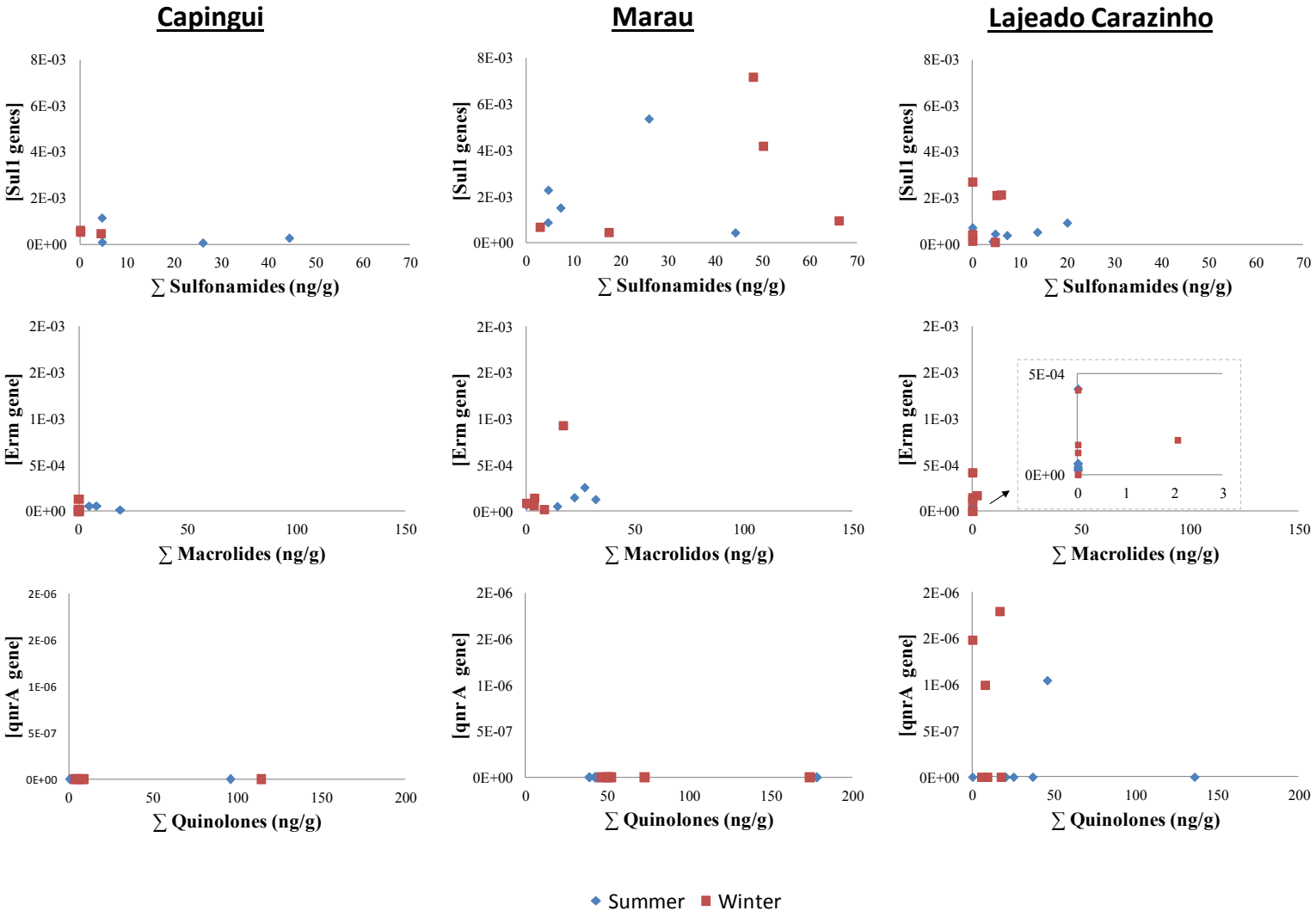
Chapter 4 - Figure 5 – Concentration of pharmaceuticals and bacterial resistance obtained in epilithic biofilms sampled in Marau sub-watershed (Guaporé River watershed) in winter and summer.



Chapter 4 - Figure 6 – Concentration of pharmaceuticals and bacterial resistance obtained in epilithic biofilms sampled in Capingui sub-watershed (Guaporé River watershed) in winter and summer.

In the present work, the absence of correlation may be also related to the limited number of antibiotics studied for each family, since not all the compounds of a family were analyzed. Nevertheless this finding may be also explained by the interaction between the biofilm matrix (Hall-Stoodley, Costerton and Stoodley, 2004) and the antibiotics. Thus, antibiotic compounds may be desorbed from the biofilm or degraded by bacteria, resulting in the decrease of the antibiotic concentration whereas antibiotic genes rest in the DNA of bacteria. Several works report also that heavy metals brought in animal farming and aquaculture might promote the spread of antibiotic resistance via co-selection of resistant bacteria (Seiler and Berendonk, 2012). Indeed chromosomal *erm AB* operon in *Staphylococcus aureus* conferred both ampicillin and chromate resistance to host cells inhabiting polluted environments (Zhang *et al.*, 2016). This phenomena is particularly observed in soil and water bodies impacted by agriculture and aquaculture.

In the Capingui samples, the concentration in resistance genes (*sul1, erm* or *qnrA*) do not vary greatly between samples independently of the sampling season and even if the concentration of antibiotics (especially sulphonamides and macrolides) are higher in summer than in winter. In the Marau sub-watershed, the relations between each resistance gene and its corresponding antibiotic are very different from each others. Thus, few samples (two in winter and one in summer) associated high sulphonamide concentrations and high *sul1* concentrations but some samples present also high concentrations without having high resistance. All the samples present similar *erm* or *qnrA* resistance gene concentrations (excepted one winter sample) althought concentrations of macrolides or quinolone present large variation range (Figure 7). It is worth noting that the high contamination in macrolides observed in summer samples is also not associated to an increase of *qnrA* genes. In the Lajeado Carazinho River sub-watershed, the highest resistance concentrations were observed for the winter samples althought antibiotic concentrations are low at this period. Also in this sub-watershed, the summer samples appeared more contaminated than the winter samples without resulting in a high resistance level.



Chapter 4 - Figure 7 – Relationship between the sum of antibiotics and resistance genes relative to the antibiotic group studied

4. CONCLUSION

Generally, the hydrographic basin of the Guaporé River is polluted with human and veterinary drugs. The values found in the epilitic biofilms of the Guaporé River hydrographic basin are very close to several works performed in the world.

However, the data obtained demonstrate that the main current problem of the Guaporé River watershed is the contribution generated by the city of Marau. The biofilms sampled at the sites located downstream of the city showed the great potential that a city with 37,145 inhabitants has to pollute and to be responsible for the development of bacterial resistance.

Even though agricultural points have shown pollution potential, such as the Tobacco point, the contribution of the city of Marau is very higher.

On the one hand this result is partly positive. Controlling problems caused by agriculture are much more complicated to solve than the installation of a sewage network with a wastewater treatment plant in a city. Especially in a basin where agriculture is the main form of land use. Even if a great deal of awareness-raising was done with local farmers, the ease of providing a public service to the villagers is much easier to achieve with good management of urban resources.

It is concluded from this chapter that epileptic biofilms were able to provide reliable data on the amounts of pollutants and bacterial development. In addition, even if the relationship between pharmaceuticals concentrations and the amount of bacterial resistance genes was not found, both were individually able to identify points with higher pollution.

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CHARPTER 5: ANTIBIOTICS AND MICROBIAL RESISTANCE IN BRAZILIAN SOILS UNDER MANURE APPLICATION

Marilia Camotti Bastos, Danilo Rheinheimer dos Santos; Élodie Aubertheau, José Augusto Monteiro de Castro Lima, Thibaut le Guet, Laurent Caner, Leslie Mondamert, Jérôme Labanowski.

Animal waste are commonly used as a source of nutrients in agriculture. This practice may cause the contamination of soils by pharmaceuticals residues used in animal production and the enhancement of soil microorganisms resistance to these compounds. Twelve pharmaceuticals (eleven antibiotics and one non-steroidal anti-inflammatory drug) were quantified in soil samples from fifteen sites regularly fertilized by animal manure (poultry litter, swine or dairy cow manure) and forest in south Brazil. Bacterial resistance gènes sul1, qnrA and erm were also quantified to verify the impacts of these compounds on the soil bacterial communities. The impact of anthropic actions was confirmed by the presence of antibiotics residues (up to 38.3 mg kg-1) and modifications in the Bacterial resistance of the community (up to 8.97 10-3 copies of gene per bacteria). Manure applications were responsible for introducing antibiotic compounds and develop microbial resistance into agricultural soils. The presence or absence of veterinary pharmaceutical concentrations was consistent with the resistance to sulfonamides, quinolones and macrolides in the agricultural or forest soils.

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1. INTRODUCTION

Modern agriculture uses a large variety of antibiotics for preventing and treating animals (Aitken *et al*., 2016). Moreover sub therapeutic levels of antibiotics are also used in feed (3–220 g of active molecule Mg^{-1} feed) to promote animal growth or to decrease stress-related diseases (Kumar *et al*., 2005). However, pharmaceuticals are partially metabolized, resulting in excretion of approximately 30 to 90% through urine and feces (Rang *et al*., 2007). Consequently, the deposition of animal manure on soils favors the entry of veterinary pharmaceuticals in the environment. Quantities and forms (active molecules or metabolites) will depend on the methods of animal husbandry, on the treatment received by the animal (e.g.: in food, or as an injection or bolus) (Boxall *et al*., 2003) and on the animal's metabolism (VICH, 2011). Thereby, it is estimated that 70% to 80% of veterinary pharmaceuticals administered are frequently released into the environment by farms (Halling-Sørensen, 2001).

Valorization of animal manure is also a common world practice in agriculture to supply the nutrient requirements of crops and to valorize waste. Animal biosolids – urine and feces - provide an important source of nutrients, improving the fertility of soil and maintaining and increasing soil organic matter levels (Davis *et al*., 2003, Kumar *et al*., 2005; Shappell *et al*., 2016). In Brazil, manure is considered as nutrient resource for agriculture, but represents challenges. Indeed, data reported from the Agricultural and Livestock Census shows that the number of swine in Brazil (in 2006) exceeded 31.1 million head, with more than half of them (16.7 million) concentrated in the Southern region (Paraná, Santa Catarina, Rio Grande do Sul) (Mathias, 2014). The number of cattle is far greater (nearly 200 million), with only 4 million head confined, including a little over 600 thousand head in the Southern Region.

Brazil produced 13.14 million megaton of chicken meat in 2015, with 62.83% of this production concentrated in the southern region *(ABPA, 2016).*

This livestock represents a potential manure production of 28.3 million Mg $year^{-1}$ and 1.1 billion Mg $year^{-1}$ of swine and cattle manure respectively (nb. considering that swine produce 2.3 to 2.5 kg of dry waste per day and that cattle produce 10 to 15 kg per day (Salomon & Lora, 2005) in southern Brazil. Manure application rates are based on the nutrient status of the soil and crop needs defined by the Committee on Soil

Chemistry and Fertility (CQFS-RS/SC, 2016) or limited by soil phosphorus status (Gatiboni et al., 2014).

In both case, high application rates of manure are recommended and will contribute to pollution owing to the presence of potentially toxic chemicals (e.g.: heavy or transition metals and organic chemicals such as pharmaceuticals) (Davis *et al*., 2003, Kumar *et al*., 2005). The chemical properties of veterinary pharmaceuticals (solubility in water, acidity constant and adsorption coefficient) also determine their dynamic with soil constituents (pH, ionic compounds, organic matter and type and content of clay minerals (Thiele-Bruhn, 2003)), playing an important role in the retention or the release of pharmaceuticals. Despite potential sorption to soil constituents, large amount of pharmaceuticals can reach both surface water through runoff or erosion (Koschorreck *et al*., 2002) and groundwater by infiltration (Avisar *et al*., 2009).

New studies have recently been conducted around the world to provide support for the creation of laws relating to contamination caused by the application of animal waste with veterinary pharmaceuticals. Indeed, one of the main threats of manure application to soils is associated with the development of resistant organisms. More specifically, the emergence of antimicrobial resistance among pathogenic bacteria to humans and animals could be a problem for the treatment of some life-threatening infections (Igbinosa & Odjadjare, 2015). Antibiotic resistance is defined as the ability of certain bacteria to evade the action of the antibiotic or to eliminate it from the cell. The resistance can be transmitted by descent or develop in living organisms. The development of resistance is usually caused by mutations or horizontal gene transfer (DNA exchanges) (Laurent, 2013). Many studies have been developed to determine the risk of migration of resistant bacterias or resistant genes from soils, waters, and air to humans and animals (Halling-Sørensen, 2001; Hirsch *et al*., 1999, Nwosu, 2001). Thus, the extended application of manure, known to be a reservoir of plasmids, on soils could favor the development and/or the spread of resistance (Bihn *et al*., 2008). Another problem is that horizontal gene transfer can efficiently be enhanced by factors like wind or water, resulting in the transport of aerosol or particles from soils to urban environments. Resistant bacteria can also be endophyte or attached to the surface of crops, permitting human exposure through direct food consumption (Dolliver *et al*., 2007; Heuer *et al*., 2011). The presence of bacteria in food could result in problems for

the treatment of infections after the development of organisms with resistance (Greenson *et al*., 2013).

Several health organizations survey the presence of antibiotics and antibiotic resistance in various aspects of life. However, environmental aspects remain negligible. The amounts of antibiotics in soil microhabitats may be largely underestimated, especially in an emerging country like Brazil, and data on the *in situ* bioavailability are still missing (Heuer *et al*., 2011). Agriculture to the economy, need to be studied becasue the use of manures is an integral part of almost all national food production, which entails the risk of dissemination of antibiotic resistance in the environment. This work was created to demonstrate a real problem caused by soils contaminated with animal residues in southern Brazil. As a pioneering work, the aim was to discover the environmental pollution caused by Brazilian agriculture. For this reason, all the samples were taken from agricultural land amended with animal waste in real conditions. In view of this, the aim of this study was to investigate Brazilian soils with animal waste application regarding the entries of antibiotics and development of bacterial resistance.

2. MATERIALS AND METHODS

2.1. The Guaporé catchment

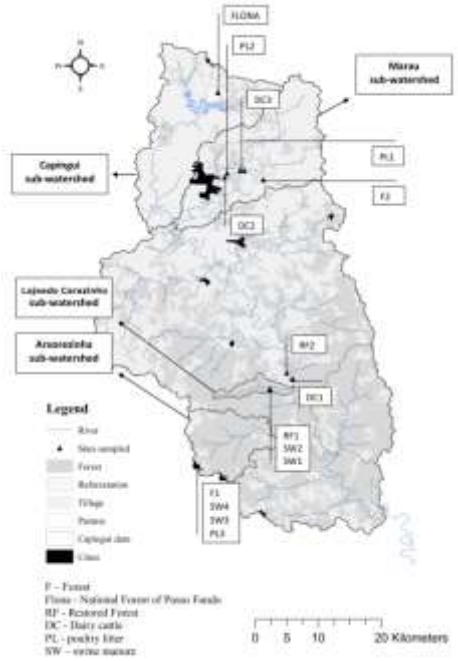
The soil samples were collected in the watershed of Guaporé situated in the northeastern region of the Rio Grande do Sul state (Brazil) that drains an area of about 2,030 km^2. The climate is subtropical super-humid mesothermal, with cool summers and too frequent severe frosts without dry seasons. The average annual rainfall varies between 1,400 to 2,000 mm yr^{-1} and the annual mean temperature is 18.4 ºC. The topography is undulating to hilly. Water bodies represent approximately 0.57% of the watershed, and urbanization is not significant (~ 0.60% of total area). Agriculture is the main activity for most of the rural residents of this region, which comprises crop fields (32.5%), grasslands (9.7%) and forests (56.7%).

The farmers mainly grow pasture for feeding their animals (dairy cattle, swine and poultry farming) and cultivate grain crops (mainly soybeans, corn and wheat). The upper portion of the watershed with undulated relief and deep soils soybeans and corn

are intensively cultivated using no-tillage systems. In the middle and lower parts of the watershed with sloping relief and shallow soils, agricultural activities, especially the cultivation of tobacco, are developed in small farms. The soils of this watershed were exposed to erosion and high sediment loss (Tiecher, 2015). The losses of soil material are greater for shallow soils and hilly areas. Still, in many areas, crop and soil management does not consider account the fragility of the soils, resulting in intense erosion areas.

2.2. Sites sampled

Fifteen sites were sampled in four sub-watershed areas of the Guaporé catchment: Lajeado-Carazinho, Marau, Capingui and Arvorezinha (Figure 1).



Chapter 5 - Figure 1 - Sites sampled in four sub-watershed areas of the Guaporé watershed

They are regularly fertilized (except forest soils) by animal manure or by the presence of animals grazing, but the nature and/or the time of application of manure differ between sites (Table 1 and 2).

All sites receiving swine manure are in the Lajeado-Carazinho and Arvorezinha sub-watersheds and have a conventional tillage cropping system. They can produce two to three grain crops per year which may or may not be alternated with the grazing of

animals. The first swine amendment site (SW1), used for tobacco production with conventional tillage, was amended with swine manure applications for 8 years without animal grazing. The second site (SW2), also used for tobacco production, received manure applications for 11 years, with the farmers growing annual pasture during the winter season. The SW3 and SW4 sites are used for corn production (grain and silage) in the summer and for forage production in the winter, with conventional tillage. Sites SW3 and SW4 have been treated with manure for 12 and 20 years respectively, with biennial application for SW3.

The two sites receiving poultry litter (PL1 and PL2) are located at the Marau sub-watershed, and are cultivated with conventional tillage; PL1 site has been treated with poultry litter for 5 years and PL2, for 6 years, both used for grazed annual pasture. PL3 site is located at Arvorezinha sub-watershed, receiving poultry litter for 8 years.

Three sites have been used for dairy cattle. The DC1, DC2, and DC3 sites were fertilized for more than 5, 15, and 40 years, respectively. All of them were then used for grazed annual pasture.

Several forest soils were also sampled to provide the reference “pharmaceutical background” of non-fertilized soil. Forests correspond to the Atlantic Forest, which is world-renowned for its rich biodiversity, ranking among the world's hotspots (Conservation International-Brazil, 2000). Forest F1 site is located in the southeastern part of the catchment in the Arvorezinha sub-watershed, while the Forest F2 site is located at Northwestern part of the catchment, in the Capingui sub-watershed. The Flona (Federal Conservation Unit) site is located at Capingui sub-watershed. The Flona site was created in the 1940s, with a surface of 1,300 hectares, including 450 hectares of native Atlantic Forest.

The Restored Forest (RF) sites are those which have actions to reinstate ecological processes, functioning and biodiversity levels. In this study, these zones are usually located between two crops that were used for agriculture in the past. However, these areas are now recomposed and have the forest covering soil at an advanced stage. The RF1 and RF2 sites are located at Lajeado-Carazinho sub-watershed and are located at central part of the catchment.

Chapter 5 - Table 1 - Land use and geographical position of soil samples.

Manure Application	Abbr	Exposure time to manure (years)	Land use	Time of manure application	GPS coordinates		Sub-watershed
Without Application	F1		Forest	-	28°51'21.50"S	52°13'29.44"W	Arvorezinha
	F2		Forest	-	28°26'40.91"S	52° 7'13.27"W	Marau
	FLONA		National Forest	-	28°19'12.14"S	52°11'4.61"W	Capingui
	RF1		Restored Forest	-	28°44'42.04"S	52° 6'40.10"W	Lajeado-Carazinho
	RF2		Restored Forest	-	28°43'13.16"S	52° 5'11.89"W	Lajeado-Carazinho
Poultry Litter	PL1	5	Conventional Yield	≥30 days	28°25'51.95"S	52° 8'50.58"W	Marau
	PL2	6	Annual Pasture	-	28°26'29.26"S	52°10'30.55"W	Marau
	PL3	8	Tobacco	≥30 days	28°51'37.90"S	52°13'35.29"W	Arvorezinha
Swine	SW1	8	Conventional Yield	≥30 days	28°44'35.51"S	52° 6'37.69"W	Lajeado-Carazinho
	SW2	11	Annual Pasture	-	28°44'40.21"S	52° 6'41.13"W	Lajeado-Carazinho
	SW3	12	Conventional Yield	≥30 days	28°51'37.90"S	52°13'35.29"W	Arvorezinha
	SW4	> 20	Conventional Yield	≥30 days	28°51'12.29"S	52°12'46.37"W	Arvorezinha
Dairy Cow	DC1	15	Annual Pasture	-	28°43'42.51"S	52° 4'42.63"W	Lajeado-Carazinho
	DC2	> 15	Annual Pasture	-	28°25'52.45"S	52° 9'11.33"W	Marau
	DC3	40	Annual Pasture	-	28°26'7.99"S	28°26'7.99"W	Marau

Chapter 5 - Table 2 - Proprieties of the studied soils.

Soils	Sand	Silt	Clay	S.S.A	pH	C	N	K	Ca	Mg	Na	P	Ecx. Al
	%			cm^2 mL	water	---------- g kg^{-1} ----------						mg kg^{-1}	$cmol_c$ dm^{-3}
F1	21.5	57.(	20.8	23450	4.4	3.92	0.40	154	0.74	1.82	0.22	4.42	4.66
F2	19.4	58.:	22.4	23925	4.6	3.55	0.29	88	1.18	1.70	0.27	17.80	3.41
FLONA	41.6	47.9	10.5	15976	5.0	4.60	0.18	132	0.97	2.04	0.70	2.54	2.15
RF1	43.3	46.7	9.9	16186	6.4	8.03	0.84	198	6.41	3.42	0.23	9.27	0.16
RF2	29.9	51.9	18.2	20912	5.7	9.98	0.98	336	4.64	3.24	0.83	9.63	0.27
PL1	26.8	55.:	17.8	22005	6.2	2.43	0.26	132	2.74	4.25	0.75	32.45	0.09
PL2	15.6	51.:	33.1	33297	5.3	1.93	0.19	378	0.87	1.67	0.52	33.01	0.36
PL3	21.1	60.:	18.8	20311	4.8	1.45	0.16	308	1.37	2.73	0.66	61.07	3.59
SW1	29.2	50.3	20.0	23840	5.4	2.63	0.28	154	3.13	5.18	0.44	18.45	0.18
SW2	28.5	53.9	17.6	21215	6.1	2.44	0.25	420	2.74	3.29	0.29	30.20	0.00
SW3	20.3	61.9	17.7	19722	4.7	1.95	0.17	176	0.82	2.02	0.69	28.51	2.33
SW4	36.1	47.:	16.4	22091	6.0	1.40	0.15	154	1.42	2.42	0.43	67.83	0.00
DC1	34.4	48.(	17.0	21561	5.4	1.66	0.18	336	1.25	2.08	0.59	13.38	0.45
DC2	19.9	61.	19.1	21083	5.8	2.34	0.28	504	1.71	2.91	0.67	13.30	0.18
DC3	31.1	51.4	17.4	22101	6.3	2.13	0.24	798	1.80	2.39	0.70	57.24	0.09

2.3. Pharmaceuticals analysis

Sampling of soils was performed with an auger at a depth of 0 to 20 cm (Malik *et al*., 2008). In order to obtain representative composite, 30 sub-samples of soil were collected at each site, transported immediately to the laboratory, transferred into individual high density polyethylene jars and frozen at -80 °C for subsequent lyophilization (freeze LS3000 - Terroni). After lyophilization, the material was sieved with a mesh of 630 µm and stored in glass bottles until the analysis. A final soil sample was composed by mixing the 30 sub-samples.

Twelve pharmaceuticals (eleven antibiotics and one non-steroidal anti-inflammatory drug) were quantified in the soil samples (Table 3). The antibiotics belong to different families (sulfonamide, quinolone, macrolide and tetracycline) commonly given to animals by the farmers of the Guaporé catchment.

Chapter 5 - Table 3 - Proprieties of the studied pharmaceuticals.

Therapeutic class	Family	Pharmaceuticals	Abbr	Chemical formula	Molar Mass	Provider	Purity (%)	Parent ion (m/z)	Product ion (m/z)	Collision energy (V)
Anti-inflammatory	-	Diclofenac	DCF	$C_{14}H_{11}Cl_2NO_2$	296.14	Sigma Aldrich	N.I. (1)	318.00591	261.1040	13
Antibiotic	Sulfonamide	Sulfamethazine	SMZ	$C_{12}H_{14}N_4O_2S$	278.33	Sigma Aldrich	≥ 99.7	279.08962	204.03990	35
		Sulfamethoxazole	SMX	$C_{10}H_{11}N_3O_3S$	253.28	Sigma Aldrich	P.S.Standard (2)	254.05972	156.01137	30
		Sulfaquinoxaline	SQX	$C_{14}H_{12}N_4O_2S$	300.33	Sigma Aldrich	≥ 96.0	301.06751	156.01138 275.8543	25
	Quinolones	Norfloxacin	NOR	$C_{16}H_{18}FN_3O_3$	319.33	Sigma Aldrich	≥ 99.8	342.12244	312.92000	85
		Ciprofloxacin	CIP	$C_{17}H_{18}FN_3O_3$	331.34	Sigma Aldrich	P.S.Standard	332.14000	231.05600	60
		Enrofloxacin	ENR	$C_{19}H_{22}FN_3O_3$	359.39	Sigma Aldrich	≥ 99.0	360.17000	296.09900	65
		Levofloxacin	LVF	$C_{18}H_{20}N_3FO_4$	361.37	Sigma Aldrich	≥ 99.0	362.15000	318.16100	30
	Macrolides	Erythromycin	ERM	$C_{37}H_{67}NO_{13}$	733.93	Sigma Aldrich	P.S.Standard	734.46800	576.37000	14
		Roxithromycin	ROX	$C_{41}H_{76}N_2O_{15}$	837.05	Sigma Aldrich	≥ 90.0	837.52766	679.43600	13
		Tylosin	TIL	$C_{46}H_{77}NO_{17}$	916.10	Sigma Aldrich	≥ 89.7	916.52399	174.11276	21
	Tetracycline	Oxytetracycline	OXY	$C_{22}H_{24}N_2O_9$	460.43	Sigma Aldrich	≥ 97.0	483.13740	443.14426 426.12000	15

(1) Not identified

(2) Pharmaceutical Secondary Standard.

One gram of soil was extracted in a pressurized liquid extractor (ASE ™ 350, Thermo Fisher Scientific) at 80 °C using methanol/water (1/2; v/v) as the extraction solvent. The extracts were then purified by solid phase extraction (Autotrace™150, Thermo Scientific) using Oasis® HLB cartridges (6cc, 200 mg sorbent; Waters) and eluted with methanol. The final extracts were evaporated under a gentle steam of nitrogen and then restituted in a methanol/water mixture (10/90; v/v) (Jelić *et al*., 2009). Pharmaceuticals were separated by high pressure liquid chromatography on an Acquity UPLC®BEH C_{18} column (2.1×100 mm, 1.7 µm; Waters) with methanol and water (both acidified with 0.3% formic acid) as the mobile phase. The liquid chromatograph was coupled to a Q-Exactive Orbitrap™ mass spectrometer (Thermo Fisher Scientific) that combines high-performance quadrupole precursor selection with high-resolution/accurate-mass detection. All pharmaceuticals were detected using an electrospray ion source operating in positive modes. Data acquisition and processing were treated using Xcalibur 2.2 software (Thermo Fisher Scientific ™). Q Exactive 2.0 SP 2 (tune application) (Thermo Fisher Scientific™) was used to control the mass spectrometer. The quantification was performed by the standard addition procedure with increasing concentrations of a standard mix of the 12 pharmaceuticals (Sigma Aldrich) (Diclofenac –DCF; Sulfamethazine – SMZ; Sulfaquinoxaline - SQX; Norfloxacin – NOR; Ciprofloxacin – CIP; Enrofloxacin – ENR; Levofloxacin – LVF; Erythromycin – ERM; Roxithromycin – ROX; Tylosin – TIL; Oxytetracycline – OXY). Finally, the concentration of pharmaceuticals in the soil is expressed in milligrams of pharmaceutical per kilogram of dry soil.

2.4. Quantification of resistance genes

DNA was extracted from soil samples using a Fast DNA® SPIN kit for feces that efficiently isolates PCR-ready genomic DNA from the samples. The extraction method was accomplished according to the manufacturer's instructions. Extraction was performed with a FastPrep®135 Instrument (MP Biomedicals, California, USA). Extracted DNA was quantified with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, USA) and stored at -80°C until analysis. Three antibiotic resistance genes, largely described and observed in the environment (Zhang *et al*., 2009), were quantified from the soil DNA extracts: *sul1* - (sulphonamide resistance), *qnrA* - (quinolone

resistance) and *erm* (erythromycin resistance). Genes were detected by using quantitative PCR and realized by I**NRA Transfert Environnement** (Narbonne - France).

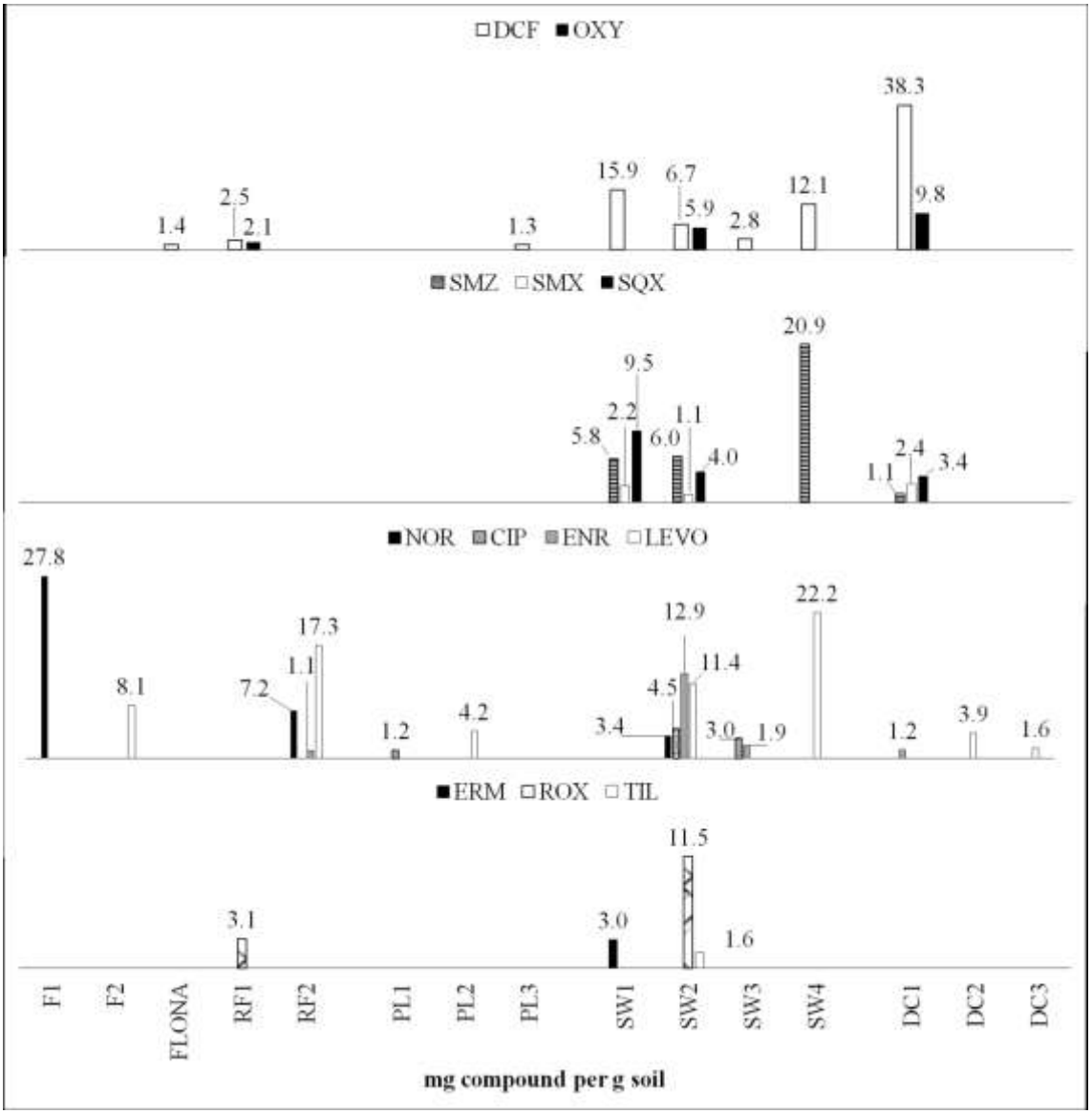
The 16S rRNA encoding gene was quantified by SYBR green assay using the universal primers 338F and 518R (MightCycler ® FastStart DNA Master plus SYBR Green I, Roche Life Science). Then, the 16S rRNA encoding gene quantities were divided by 4.1 (Hardwick *et al*., 2008, Klappenbach *et al*., 2001; Stadler et al., 2012).

3. RESULTS AND DISCUSSION

3.1. Incidence of manure type

The concentration of pharmaceuticals in each soil is shown in figure 2. The natural and regenerated forest sites, used as reference soil, indicate that soils which have not been amended for more than 50 years can also contain antibiotics. Sulfonamides were the only substances among those studied that are not found in these sites. The national forest (FLONA) was less contaminated and presents only DCF (1.4 mg kg^{-1}). The forest F1 present significant concentrations of LEVO (8.1 mg kg^{-1}) and NOR (27.8 mg kg^{-1}). Additionally, in RF1 site OXY (2.1 mg kg^{-1}), DCF (2.5 mg kg^{-1}) and ROX (3.1 mg kg^{-1}) were found. The soil under RF2 forest was contaminated by quinolones ENR (1.1 mg kg^{-1}), NOR (7.2 mg kg^{-1}) and LVF (17.3 mg kg^{-1}). The two regenerated monitored forests are small fragmented areas surrounded by fields in a hilly landscape. The high erosion rate and run off events can induce transfers of synthetic antibiotics (SMT, SMX, SMQ, ENR, NOR, CIP, and LEVO) from cultivated soils to forests. In addition, soil contamination can occur due to atmospheric transport during the application of animal waste (Dungan, 2014).

It is also well-known that soils are a natural reservoir of antibiotic-producing microorganisms which provides a natural antibiotic residue in soil (Madigan *et al*., 2009; Popowska *et al*., 2012). Approximately 50% of Actinomycetes organisms isolated from soil are capable of synthesizing antibiotics (Topp, 1981). Consequently, the presence of OXY, ERM, ROX, and TIL might also be partly explained by natural production.



Chapter 5 - Figure 2 - Concentration of pharmaceuticals in each soil

The soils receiving swine manure present the highest diversity of antibiotics. The soils of the SW1, SW2, and SW3 sites contain several sulfonamides (minimum-SMX: 1.1 mg kg^{-1}; maximum-SMZ: 20.9 mg kg^{-1}) or macrolides (minimum-TIL: 1.6 mg kg^{-1}; maximum-ROX: 11.5 mg kg^{-1}). The values found for TIL and ROX in this study were up to 100 times higher than those found in German (Hamscher *et al*., 2005) and Chinese (Hou *et al*., 2015) soils amended with swine manure. High concentrations of quinolones were found in the SW2, SW3, and SW4 soils (minimum-ENR: 1.9 mg kg^{-1}; maximum-LVF: 22.2 mg kg^{-1}). The values were higher than those found in the literature (Zhou *et al*., 2013). OXY, frequently used for the treatment of diseases of the respiratory and

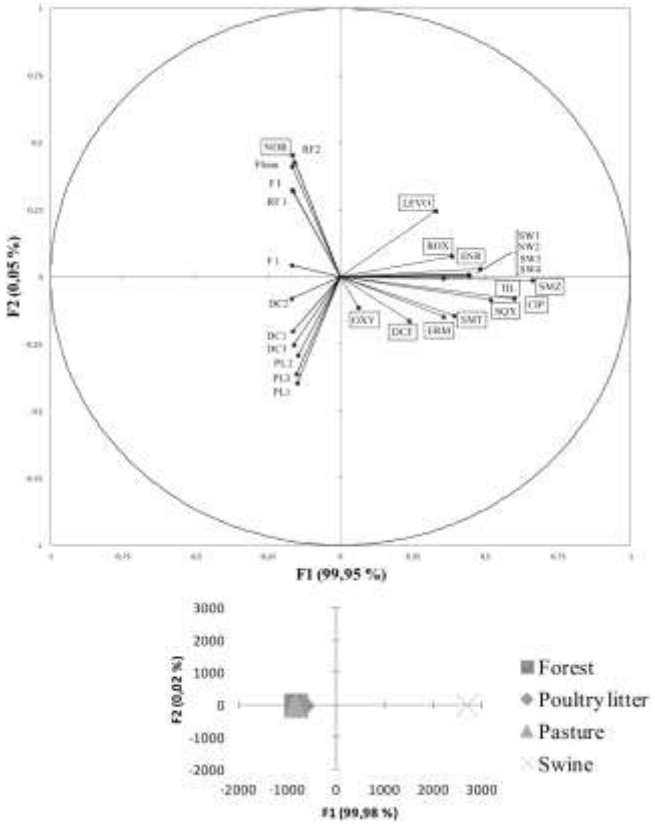
digestive tracts of pigs, especially diarrhea in piglets (Sato *et al*., 2015), was found only in the soil at site SW2 (5.9 mg kg^{-1}) at a value 20 times higher than those found in the USA (0.3 mg kg^{-1}) (Boxall *et al*., 2003). DFC was also systematically found (minimum: 2.8 mg kg^{-1}; maximum: 15.9 mg kg^{-1}) in the soils amended with swine manure. The presence of this compound is consistent with its wide utilization as an anti-inflammatory and painkiller in pigs and cattle.

The soils receiving poultry litter present globally lower pharmaceutical concentrations than the swine and dairy cow manure treated soil. However, poultry litter is generally suspected to have a high release potential of veterinary medicines (Wei *et al*., 2016). The poultry production system implemented in Brazil allows creating several batches of chickens without renewing the aviary. The characteristics of this manure (high nutrient concentration and low water content) reduce the rate and frequency of application compared to other manures. Besides, not all animal residue is applied near the poultry production farm, poultry litter can be sold and exported out of the area of production. The results highlight the presence of quinolones in the soils receiving poultry litter. Thus, LVF (4.2 mg kg^{-1}) was found in the PL2 soil and CIP (1.23 mg kg^{-1}) were found in the PL1 soil. The values found for CIP were lower than the values found in livestock manure and amended soils of Northern China (53.4 mg per kg soil) (Hou *et al*., 2015) and in French soils (30 mg kg^{-1}) (Pourcher *et al*., 2014). Different sulfonamides and macrolides were also found in each site. The PL3 soil contains DCF (1.3 mg kg^{-1}). For the other pharmaceuticals (DCF, SQX, ERM, TIL, SMZ, LVF, and NOR), all were below the values found in other studies (Hou *et al*., 2015; Wei *et al*., 2016; Zhou *et al*., 2013).

The soils amended with dairy cows manure also present low concentrations of pharmaceuticals. Only at site DC1 site the soil was contaminated with SMZ (1.1 mg kg^{-1}), ENR (1.2 mg kg^{-1}), SMX (2.4 mg kg^{-1}), OXY (9.7 mg kg^{-1}) and DCF (38.3 mg kg^{-1}). OXY and DCF are the most concentrated pharmaceuticals found in these soils. Only one sulfonamide was observed in soil - SQX - in the DC1 site (3.4 mg kg^{-1}). LVF was detected in the DC2 (3.9 mg kg^{-1}) and DC3 (1.55 mg kg^{-1}) sites. These results are consistent with literature, since bovine manure is known to contain high levels of sulfonamides. Li *et al*. (2013) studying the residues and ecological risks of veterinary antibiotics (VAs) also found OXY (5.10 mg kg^{-1}), SMZ (0.46 mg kg^{-1}), NOR (0.85 mg

kg^{-1}), CIP (0.53 mg kg^{-1}), ENR (1.18 mg kg^{-1}) and TIL (0.25 mg kg^{-1}) in dairy cow samples from concentrated animal feeding operations in north-eastern China.

Discriminant analyses were used to obtain a broader view of the relation between the different kinds of manure application and the presence of pharmaceuticals in soils. For that, soil samples were differentiated by the different kinds of management (forest, dairy cow, swine and poultry litter) and the concentration of the twelve pharmaceuticals. The high coefficient of correlation of the first function (99.95%) indicates a high degree of association between the first discriminant function. The spatial representation of DA displays all the analyzed soil separated from the soil with swine manure application (Figure 3). Except for NOR, all veterinary pharmaceuticals have a strong relationship between their concentrations in soil and the application of swine manure. This finding highlights the fact that swine manure is more polluting than other manure applied to soils. In China, a study of the occurrence of veterinary pharmaceuticals in soils amended with the manures of different animal species showed that the classes, amount and frequency of veterinary pharmaceuticals were considerable in soils applied with poultry and swine manure (Wei et al., 2016).



Chapter 5 - Figure 3 - Discriminant analysis.

In the water catchment, particles containing pharmaceuticals may be transported to rivers during soil erosion. The erosion of agricultural soils is estimated at an average of 394.6 Mg km^{-2} $year^{-1}$ from several measurements performed between the years 2003 and 2012 (Tiecher, 2015). Regardless of the potential export of these compounds, it is worth remembering that there is a very high risk of displacement of these chemicals in water systems and consequently a risk of contamination of aquatic trophic base, like biofilms and others (Laurent, 2013). Bailey (2016) investigating veterinary antibiotics from cow excrement to the German fields (using sorption isotherms and elimination constants), found in sediment rivers collected from the top centimeters of the bed or floodplain material studied the presence of SMX (30 a 37µg kg^{-1}), SMZ (>20µg kg^{-1}) and tetracyclines (<750µg kg^{-1}), which are also subject of the present manuscript. As an example, these pharmaceuticals can cause structural and functional problems, sometimes even death, in food chain base animals, for example, biofilms (Proia *et al*., 2013), and can also contaminate animals that will be consumed by humans, such as shrimp (Lavorante *et al*., 2009).

3.2. Resistance genes in soil

The evaluation of the bacterial resistance (expressed in copies of genes per bacteria in each soil) in the different soils is shown in Table 4. The measurement of resistance genes in forest soils show that the gene *sul1* is not found in these samples, except for the F1 soil that presents a very low value (10^{-8} copies of genes per bacteria). Resistance integrons carrying the *sul1* gene are generally abundant in natural environments affected by human activity (Wellington *et al*., 2013). Thus, based on the study of 21 Swiss lake sediments, the value of abundance of *sul1* gene below 1.5 10^{-2} could be considering as a typical baseline (Czekalski *et al*., 2015). In the present case, the absence of sulfonamide contamination in the forest soils (natural or reforested) may explain the absence of resistance marks. It is worth noting that the natural forest soils (F1, F2 and FLONA) present lower values of *erm* (10^{-7} copies of genes per bacteria) and *qnrA* genes *(*10^{-4} copies of genes per bacteria) than the restored forests (RF1 and RF2) (10^{-6} copies of genes per bacteria and 10^{-3} copies of genes per bacteria, respectively). Restored forests are sites that were previously used for agricultural activity, but that are no longer cultivated by farmers due to the steep gradients. During their past use, these

soils have been amended with animal manures. Although they do not receive direct amendments now, the application of manure in the fields all around them may expose them by run-off or aerosol deposition. The Lajeado-Carazinho sub-watershed is typical of this landscape where the highest relief areas are cultivated for crop production, and thus have received several applications of animal wastes. A study comparing the development of resistance in organisms living in untreated soils and manured soils showed that, after mixing these soils with manure containing pharmaceuticals, plasmids conferring resistance to multiple antibiotics were more pronounced from bacterial communities that did not receive manure in the previous years (Heuer & Smalla, 2007). The authors explained that soils that were periodically manured had antibiotic resistance genes more established in the bacterial community.

Chapter 5 - Table 4 - Quantification of the classes sul1, qnrA and erm resistance integrons in soils sampled in Guapore's watershed.

	sul1	qnrA	erm
	copies of genes per bacteria		
F1	$6.20\ 10^{-8}$	5.3710^{-4}	$2.21\ 10^{-7}$
F2	n.d.	$4.24\ 10^{-4}$	$8.86\ 10^{-7}$
Flona	n.d.	$2.79\ 10^{-4}$	$4.45\ 10^{-7}$
RF1	n.d.	$8.97\ 10^{-3}$	$5.74\ 10^{-6}$
RF2	n.d.	$2.53\ 10^{-3}$	$1.71\ 10^{-6}$
PL3	$1.48\ 10^{-7}$	$2.10\ 10^{-4}$	$3.10\ 10^{-7}$
PL2	$8.64\ 10^{-7}$	$1.08\ 10^{-4}$	n.d.
PL1	2.4110^{-7}	$1.70\ 10^{-4}$	$1.20\ 10^{-7}$
DC1	9.6010^{-8}	$3.89\ 10^{-5}$	$8.14\ 10^{-8}$
DC2	2.6610^{-6}	$9.09\ 10^{-4}$	$6.07\ 10^{-7}$
DC2	$1.87\ 10^{-5}$	$1.20\ 10^{-3}$	$1.54\ 10^{-6}$
SW2	$7.77\ 10^{-7}$	$1.22\ 10^{-4}$	$7.28\ 10^{-8}$
SW1	$1.77\ 10^{-6}$	$2.75\ 10^{-4}$	$1.12\ 10^{-7}$
SW3	$1.37\ 10^{-7}$	$1.18\ 10^{-5}$	$6.05\ 10^{-7}$
SW4	$1.54\ 10^{-6}$	$3.49\ 10^{-4}$	$3.98\ 10^{-7}$

The amount of resistance genes in all the soils receiving poultry litter residue is near identical (*sul1:* 10^{-7}; *qnrA*: 10^{-4}; *erm:* 10^{-7} copies of genes per bacteria). These values are among the lowest values observed. The *erm* gene was not found in sample PL2 located in Capingui sub-watershed. The use of poultry litter as enrichment seems to

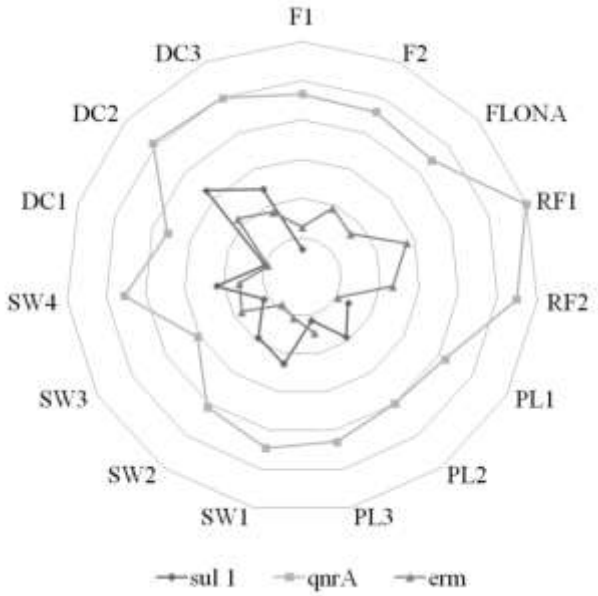
have a lower impact, compared with slurry from pigs and cattle, on antibiotic resistance dissemination. This finding may be related to the composition or the mode of application and the ratio of decomposition of the manure (Wei *et al*., 2016). Furthermore, the concentrations of veterinary pharmaceuticals were lower in soils amended with poultry litter residues comparing to swine manure soils. Hence these low levels of antibiotics may have favored lower development of resistance in bacteria.

Soils subjected to dairy cow grazing over long time periods (pastures with more than 15 years and 40 years) present higher concentrations of *sul1* genes (10^{-5} and 10^{-6} copies of genes per bacteria, respectively) than those soils which have been subjected to grazing for shorter periods (10^{-8} copies of genes per bacteria). Some differences were also observed between DC soils according to their location. Thus, pastures that are in the sub-watershed of Marau had higher *qnrA* and *erm* gene values (DC2: 10^{-3} and 10^{-6} copies of genes per bacteria, respectively; DC3: 10^{-4} and 10^{-7} copies of genes per bacteria) compared with the pasture in the Lajeado-Carazinho sub-watershed (DC1: 10^{-5} and 10^{-8} copies of genes per bacteria, respectively).The soils sampled with the presence of animals grazing for 15 years and for over 15 years have a similar production system. In these soils, animals graze in smaller areas using all the land area for grazing. However, the soil sampled that receive animals grazing for 40 years are placed in a larger area and the producer does not put the full load of animals on their land. Thus, grazing is carried out throughout the area and not in concentrated form as with the other producers. The dispersion of the manure over a larger area may be one of the reasons of the low resistance levels in these samples. However, these repeated inputs over almost half a century maintain this residual of resistance in soil bacteria.

In soil SW2 the application of manure is undertaken every year and the area is used for grazing, while for SW3 the application is undertaken every two years and the area is used for corn and soybeans. Such differences in soil management may have an impact on the level of antibiotic bacteria resistance. Indeed the biological cost of maintaining the integrity of organisms is generally postulated as the main driving force for the reduction of frequency of resistant bacteria in environments with lower intake of antibiotics (Levin, 2002). The presence of resistant genes was found in almost all the samples. Two of the soils (SW1 and SW2) receiving swine manure are characterized by low values of *sul1* gene (both, 10^{-7} copies of genes per bacteria). These two soils also

present similar values of *qnrA* (10^{-5} copies of genes per bacteria - SW2) and *erm* (10^{-8} copies of genes per bacteria – SW1) genes than the SW3 soil.

However, the results shown here demonstrate that a low intake of veterinary pharmaceuticals does not necessarily lead to the reduction of the resistant gene pool (Figure 4). Possible explanations may be related to the composition of manure of each producer or to the difference of land use: grazing or agricultural planting.



Chapter 5 - Figure 4 - Relative spacial variation of antibioresistant genes

3. CONCLUSIONS

Manure application is responsible for introducing antibiotic compounds into soil as demonstrated by the differences in resistance levels in agricultural and forested soils. Manure from antibiotic-fed animals exacerbates the spread of resistance, as demonstrated by the high levels found in manure-amended soils. Furthermore, agricultural practices seem to have an important role in the development of resistance. In general, the presence of cattle in agricultural areas also appears to influence the resistance values developed in soils. The resistance to sulfonamides, quinolones and erythromycins found in the agricultural or forest soils is consistent with presence or absence of veterinary pharmaceutical is consistent and with the history of land-use (manure application, crop rotations and reforestation). However, as not all pharmaceuticals and metabolites responsible for the development of resistant genes

were studied, it was not possible to correlate the concentrations of molecules and the bacterial resistance in the soil. In Brazil, agricultural soils are amended by several million to billion Mg of manure (swine and cattle) per year. This wide spread of manure is also an important source of antibiotics and antibiotic resistant bacteria for soils and neighboring waters.

The amount of antibiotics or resistant bacteria that are transported by km^2 through agricultural erosion could be a significant contribution to watershed contamination. Furthermore, soil runoff probably facilitates the exposure of soil bacteria to manure containing veterinary pharmaceuticals.

The consequence of antibiotic release in natural environments is a major issue worldwide for the future to prevent the expansion of resistant bacteria. However, even though soils with application of swine waste some drugs were detected in high concentrations, the situation in Brazil is not more alarming than elsewhere in the world, since the values of antibiotics and antibiotic resistant bacteria are smaller than those observed in amended agricultural soils in Europe or Asia for poultry litter and dairy cow.

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ANNEXE – EPILITHIC BIOFILMS AND WATER SAMPLES AS SUPPLEMENTARY SOURCES OF INFORMATION FOR AQUATIC CONTAMINATION STUDY

1. INTRODUCTION

Besides the pharmaceuticals, antibiotics are considerate an important medicinal group, use frequently in treatment of human and veterinary animal infections(Hirsch *et al.*, 1999). Over the past few years, studies about the presence of antibiotics in different compartments of the aquatic environment (e.g. water, sediments and biota) increased the awareness about the risks and effects that this compounds have on aquatic organisms (Ebele, Abou-Elwafa Abdallah e Harrad, 2017). This awareness was only possible due to the many improvements in analytical detection methods (Bartelt-Hunt *et al.*, 2009).

In the last decade researchers try to find a practical, efficient and inexpensive way to identify pollution in water courses. One of the most used methods is the collection of water from rivers with bottles. This method is a very widespread analysis and its main objective is the detection of compounds at the time of collection.

Recently, a new natural matrix has been studied as adsorbent of the pharmaceuticals. The epilithic biofilms are able to capture and accumulate pharmaceutical compounds in aquatic systems more efficiently that the water analysis (Aubertheau *et al.*, 2016; Huerta *et al.*, 2016). This annex reports few data acquired about the analysis of the contamination founded in biofilms and water samples sampled in the Guaporé river watershed.

2. MATERIALS AND METHODES

Sampling site are the same as described in Chapter 4.

Samples were collected in glass bottles with a capacity of one liter, amber, with a mouth of 2.54 cm in diameter. The flasks were previously washed with neutral detergent and 10% v / v nitric acid for twelve hours, rinsed with ultrapure water and methanol and then oven dried at 105 ° C. The maximum height of the water column sampled was 70 cm from the bed, and the collection procedure was performed from the bottom up at a constant speed and in the same place 4 to 5 times until the volume of the flask was complete. The samples were refrigerated in iceboxes and transported to the Laboratory of Analysis of Pesticide Residues (LARP) of the Department of Chemistry of the Federal University of Santa Maria.

The extraction of the active principles of pharmaceuticals present in the sampled water was performed by solid phase extraction (SPE) in Strada X cartridges containing 200 mg of adsorbent (Polymeric sorbent similar to Oasis) , using minifold systems (table 3).

Annexe - Table 1 - Parameters of extraction and purification by solid phase extraction.

Procedure	**Material Description**
Cartridges	Strada X, 200 mg.
Cartridge Conditioning	3 mL MeOH; 3 mL H_2O ultrapure; 3 mL H_2O ultrapure/pH 3,0 with H_3PO_4 (1/1,v/v)
Sample percolation	100 mL of filtered and acidified water sample with pH 3,0 H_3PO_4 (1/1,v/v)
Washing	3 mL H_2O ultrapure
Elution	2 mL of MeOH/ACN (1/1, v/v) solution, with 1% acetic acid.

Detection and quantification were performed in an ultra-high performance liquid chromatograph coupled to a tandem mass spectrometry. The device used for the separation of the compounds was the Acquity UPLC® (Waters, USA), with triple Xevo TQ triplequadrupole detector. The mass spectrometer operated in the Selected Reaction Monitoring (SRM) mode and the ionization was performed by positive-mode electrospray. The chromatographic parameters and the mass spectrometer are detailed in table 4.

Annexe - Table 2 - Parameters of chromatographic separation and mass spectrometer.

Chromatographic Parameters	
Column	Acquity UPLC™ BEH C18 (50 × 2,1 mm d. i., 1,7 µm particle size);
Oven temperature	45 °C
Mobile Phase	Ultrapure water + 0,1% formic acid + 5 mM Ammonium formate (A); Methanol + 0,1% formic acid + 5 mM Ammonium formate (B) ;
Flow rate	0,225 mL min^{-1}
Injection volume	10µL
Elution	2 mL of MeOH/ACN solution (1:1, v/v), com 1% acetic acid.
Spectrometer Parameters	
Capillary voltage	2,5 kV;
Font temperature	150 °C
Desolvation temperature	500 °C;
Desolvation gas flow (N2)	600 L h^{-1};
Cone gas flow (N_2)	80 L h^{-1}
Collision gas flow (Ar)	0,15 ml min^{-1}

The pharmaceuticals analysed were Ciprofloxacin (CIP), Norfloxacin (NOR), Oxytetracycline (OXY), Sulfamethazine (SMZ), Sulfamethoxazole (SMX), Sulfaquinoxaline (SQX) and Tylosin (TYL)

3. RESULTS AND DISCUSSION

Except ciprofloxacine and tylosin, all studied compounds were largely more frequently detected (47 to 65 %) in biofilms than in water (0 to 29 %) (Table 4).

Annexe - Table 3 - Frequency of detection of the pharmaceuticals obtained in water and biofilm samples sampled in the Guaporé River watershed – RS, Brazil.

Pharmaceuticals	% Detection frequency in water (n=17)	
	Biofilm	Water
Ciprofloxacin	24	94
Enrofloxacin	53	0
Norfloxacin	65	6
Oxytetracycline	47	12
Sulfamethazine	47	29
Sulfamethoxazole	65	6
Sulfaquinoxaline	47	0
Tylosin	2	6

Most part of the results had a great number of pharmaceuticals detected in biofilms (67%, n = 12) when compared with the water results (16%, n = 3). Huerta *et al.* (2016) studying discrete water and biofilms allowed to grow for a longer period in the River Segre (Spain) detected several compounds in the biofilm that were not correspondingly found in water. The authors argued that biofilm was able to uptake compounds present in water at very low concentrations resulting in its bioaccumulation in biofilms.

Only 16% (n = 3) samples had the numbers of pharmaceuticals founded major in water than those in biofilm samples. The same quantity of samples (16%, n = 3) had the same number of pharmaceuticals founded in water and biofilm samples. Thus, the matrix biofilm had a greater potential for detection of environmental pollution if compared with the water samples (Table 5). Still, a number of compounds detected in water were not detected in the biofilm, such as, citalopram, pravastatin, sulfamethoxazole, furosemide, carbamazepine, and bezafibrate, found in water at low concentrations (below 50 ng L^{-1}), even in the sampling sites close to the WWTP. Other compounds found at higher concentration in water, such as hydrochlorothiazide (max. conc. 361 ng L^{-1}) and ibuprofen (max. conc. 193 ng L^{-1}), were not detected either in biofilm.

Only four of the eight investigated compounds could be detected in both water and biofilm samples, CIP (Agriculture, Marau Up. Grain, Marau City, Up Carazinho Watershed), OXY (Lajeado-Carazinho), SMZ (Marau Up. Grain, Marau city) and SMX (Down. Lajeado Carazinho) (Table 5). As in the present study, Huerta *et al.* (2016) founded some compounds only in biofilms or water. They had also said that the correlation between water and biofilm concentration should not be considered as possible to almost all

Annexe - Table 4 – Pharmaceuticals concentrations in water and biofilms sampled in Guaporé sub-watershed.

Sampled site		CIP	ENR	NOR	OXY	SMZ	SMX	SQX	TYL	Σ	Number of compounds
Flona	Biofilm[1]	<LD	<LD	<LD	<LD	<LD	<LD	4.61	<LD	4.61	1
	Water[2]	0.07	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.07	1
Capingui Dam	Biofilm	<LD	<LD	3.10	47.18	44.23	<LD	<LD	<LD	94.5	3
	Water	0.17	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.17	1
Agriculture	Biofilm	32.71	24.21	28.74	2.67	9.50	8.82	7.62	7.62	114.2	5
	Water	0.13	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.13	1
Capingui Outlet	Biofilm	<LD	5.90	<LD	2.11	<LD	<LD	4.56	<LD	12.5	3
	Water	0.12	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.12	1
Marau	Biofilm	<LD	11.85	40.35	5.49	5.99	7.33	4.13	2.58	75.1	7
Up. Grain	Water	0.12	<LD	<LD	<LD	<LQ	<LD	<LD	<LD	0.12	2
Marau	Biofilm	3.82	<LD	3.22	4.63	4.60	59.15	2.42	3.41	77.8	7
Up. Animal	Water	0.12	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.12	1
Marau City	Biofilm	7.79	2.92	31.47	<LD	2.21	41.47	4.32	16.83	90.1	7
	Water	0.18	<LD	<LD	<LD	<LQ	<LD	<LD	<LD	0.18	2
Marau Outlet	Biofilm	<LD	<LD	8.30	<LD	2.87	<LD	<LD	3.64	11.1	3
	Water	0.14	<LD	<LD	<LQ	<LQ	<LD	<LD	<LD	0.14	3
Confluence	Biofilm	<LD	5.92	13.02	<LD	<LD	7.24	<LD	<LD	26.1	3
	Water	0.14	<LD	<LD	<LQ	<LQ	<LD	<LD	<LD	0.14	3
Tobacco	Biofilm	<LD	<LD	26.63	35.14	2.54	14.28	3.24	<LD	81.8	5
	Water	0.13	<LD	<LD	<LQ	<LD	<LD	<LD	<LD	0.13	2
Up.Lajeado	Biofilm	<LD	11.60	<LD	<LD	2.09	11.65	<LD	<LD	25.3	3
Carazinho	Water	0.20	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.20	1
Down.Lajeado	Biofilm	<LD	6.31	5.19	<LD	<LD	2.26	5.07	<LD	18.8	4
Carazinho	Water	0.12	<LD	<LD	<LD	<LD	<LQ	<LD	<LD	0.12	2
Up.Carazinho	Biofilm	7.35	8.62	5.85	2.15	<LD	4.32	<LD	<LD	28.2	5
Watershed	Water	0.15	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.15	1
Down.Carazinho	Biofilm	<LD	11.40	5.96	2.68	<LD	4.82	<LD	<LD	24.8	4
Watershed	Water	0.14	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.14	1
Carazinho	Biofilm	<LD	<LD	<LD	<LD	<LD	2.41	<LD	<LD	2.41	1
Outlet	Water	0.20	<LD	<LD	<LD	<LQ	<LD	<LD	<LD	0.20	2
Up.Carazinho	Biofilm	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.00	0
Watershed	Water	0.28	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.28	1
Down.Carazinho	Biofilm	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	0.00	0
watershed	Water	0.25	<LD	<LD	<LD	<LQ	<LD	<LD	<LD	0.25	2

(1) ng g^{-1}

(2) ng L^{-1}

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CONCLUSION

This thesis gives an overview of the environmental contamination by human and veterinary pharmaceuticals in soil and water of the Rio Guaporé River watershed. The results obtained made it possible to trace an initial mapping of the contamination of the watershed and aid to identify the most contaminated areas.

Thus, it appears that under different land use and pressure conditions, levels of contamination can vary significantly between areas under agricultural pressure and areas under urban pressure.

The study performed in aquatic environments close to agricultural areas shows that levels of antibiotic contamination are lower than in urban areas. Nevertheless, indicators of agricultural pressure have be highlighted in the biofilms sampled in sites of high agricultural technology. These sites present producers which use pharmaceuticals more frequently and destine their waste to the agricultural fields. In view of the high erosion of the Guaporé River watershed, it was possible to show that the contamination of biofilms results, inter alia, from the losses of the soil fertilized with animal waste. Environmental practices could help these agricultures. Many of them use the “no till” system but without use the construction of the terraces to reduce the water velocity. They also apply the manure without regarding the needs of the soil, normally applying a big volume of manure in once application. This practice favors the supply of a lot of medicaments to the river by run-off and/or soil erosion. Elsewhere in the world, some countries use a calendar that not allow the use of manure in the raining season. However, the control applications could be more difficult to be applied in Brazil, once that the agricultures hardly respect the laws related to the direction and the condition of the soil so that the waste is applied in a legal way.

The present work showed also that the use of epilithic biofilms is also a great tool to track the pollution caused by the human presence, especially the environmental problems caused by cities, eg. Marau.

The study of conventional anthropic markers was the beginning of this awareness, since it is very difficult for Brazilian people to believe that the pollution of a river is linked to their daily actions. The sampling campaigns showed that the anthropic pressure of the city resulted in the adsorption of several compounds in the biofilms due to the no-treatment of the Marau sewage and resulting in the development of bacterial resistance in the organisms present in these places.

These results serve as a way of showing that, like Marau, several Brazilian cities do not yet have a sewage treatment network and the risks are not only the pollution of drinking bad quality water, but also which is the strategies that the millions of organisms are creating to adapted themselves to keep living. Biofilms are part of the base of the food chain, once contaminated they can initiate the process of bioaccumulation and could contaminate the animals that the humans eat, so one day these problems can arrive to our “food dishes”. In addition, several works have been carried out with the intention of controlling the problems with super-resistant battles, so one day this can really be a problem to everybody.

In soils, even if measurements in terrestrial environments showed that agricultural soils have relatively low levels of antibiotics and other compounds, resistance levels could be founded in high levels. Thus, the intensive supply of soil amendment to support soil fertility seems to constitute a favorable situation for the development of resistance. In this sense, studies are still needed to see to what extent an evolution of practices could limit this risk while guaranteeing the same production.

It is hoped that the results of this thesis will come as a warning of the need for research on the impact of man on the environment. More than that, the need to study pollution that is not seen, or do not want to be seen, once everyone knows the problem. Finally, the problem exists and should be part of future discussions on the Guaporé region and on regions that resemble the problems encountered in this sub-watershed.