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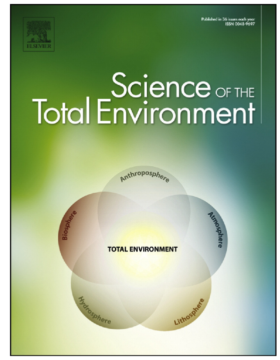

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Gracieli Fernandes[a]; Virginia Carolina Aparicio[b]; Marilia Camotti Bastos[c]; Eduardo De Gerónimo[d]; Jérôme Labanowski[e]; Osmar Damian Prestes[f]; Renato Zanella[g]; Danilo Rheinheimer dos Santos[h]

Affiliations

[a] Universidade Federal de Santa Maria, Centro de Ciências Rurais, Departamento de Solos, Avenida Roraima, n° 1000, Bairro Camobi, CEP 97105-900, Rio Grande do Sul, Brazil, gracieligfer@gmail.com
[b] Instituto Nacional de Tecnología Agropecuaria INTA EEA Balcarce, Ruta Nacional 226, Km 73,5, Balcarce CP 7620, Buenos Aires, Argentina, aparicio.virginia@inta.gob.ar
[c] Université de Lorraine, IUT Thionville/Yutz, France, mcamotti@hotmail.com
[d] Instituto Nacional de Tecnología Agropecuaria INTA EEA Balcarce, Ruta Nacional 226, Km 73,5, Balcarce CP 7620, Buenos Aires, Argentina, degeronimo.eduardo@inta.gob.ar
[e] Université de Poitiers, Institut de Chimie des Milieux et Matériaux de Poitiers, IC2MP, Poitiers, France, jerome.labanowski@univ-poitiers.fr
[f] Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Departamento de Química, Avenida Roraima, n° 1000, Bairro Camobi, CEP 97105-900, Rio Grande do Sul, Brazil, osmar.prestes@ufsm.br
[g] Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Departamento de Química, Avenida Roraima, n° 1000, Bairro Camobi, CEP 97105-900, Rio Grande do Sul, Brazil, renato.zanella@ufsm.br
[h] Universidade Federal de Santa Maria, Centro de Ciências Rurais, Departamento de Solos, Avenida Roraima, n° 1000, Bairro Camobi, CEP 97105-900, Rio Grande do Sul, Brazil, danilonesaf@gmail.com.

Corresponding author

GRACIELI FERNANDES
Full postal adress: Universidade Federal de Santa Maria, Avenida Roraima nº 1000, Cidade Universitária, Bairro Camobi. Centro de Ciências Rurais, Prédio 42, Departamento de Solos, Santa Maria – Rio Grande do Sul, Brazil. CEP: 97105-900.
E-mail adress: gracieligfer@gmail.com.

Indiscriminate use of glyphosate impregnates river epilithic biofilms in southern Brazil

ABSTRACT: Epilithic biofilms are communities of microorganisms composed mainly of microbial cells, extracellular polymeric substances from the metabolism of microorganisms, and inorganic materials. Biofilms are a useful tool to assess the impact of anthropic action on aquatic environments including the presence of pesticide residues such as glyphosate. The present work seeks to monitor the occurrence of glyphosate and AMPA residues in epilithic biofilms occurring in a watershed. For this, epilithic biofilm samples were collected in the Guaporé River watershed in the fall and spring seasons of 2016 at eight points. Physicochemical properties of the water and biofilms were determined. The determination of glyphosate and AMPA was performed using an ultra-high performance liquid chromatograph coupled to a tandem mass spectrometer. The concentrations of glyphosate and AMPA detected in epilithic biofilms vary with the season (from 90 to 305 μg kg^{-1} for glyphosate and from 50 to 240 μg kg^{-1} for AMPA, in fall and spring, respectively) and are strongly influenced by the amount of herbicide applications. Protected locations and those with poor access not demonstrate the presence of these contaminants. In the other seven points of the Guaporé River watershed, glyphosate was detected in concentrations ranging from 10 to 305 μg kg^{-1}, and concentrations of AMPA ranged from 50 to 670 μg kg^{-1}. An overview of the contamination in the Guaporé watershed shows that the most affected areas are located in the Marau sub-watershed, which are strongly influenced by the presence of the city of Marau. This confirms the indiscriminate use of glyphosate in the urban area (weed control, domestic gardens and horticulture) and constitutes a problem for human and animal health. The results showed that biofilms can accumulate glyphosate resulting from the contamination of water courses and are sensitive to the sources of pollution and pesticides present in rivers.

Keywords: environmental contamination, glyphosate, AMPA, epilithic biofilms, human health.

1. Introduction

The use of pesticides is part of the technology package of the predominant agricultural model in the large food, energy, and fiber production areas. Even more serious is the application of highly polluting compounds to crops by technicians and farmers at doses which are above the recommended levels. This practice is not allowed in Brazil, according to article 65 of Decree 4.074/2002, which regulates the Agrochemicals Law, but is carried out (Londres, 2011). The misuse of these compounds impacts the society in its proximity due to the presence of residues of these substances and their derivatives in food, water, and air.

The introduction, adoption, and popularization of transgenic varieties has resulted in the most successful agricultural technology in the history of agriculture (ISAAA, 2016). This agricultural package is very well developed with synchronism between companies, governments, banks, and farmers. The immediate result has been an increase in the consumption of pesticides, mainly in corn, canola, and cotton crops (Aparicio et al., 2013).

Worldwide, there are 185.1 million hectares of biotech crops in 26 countries, led by the United States and Brazil. The planted area of genetically modified soybeans is the most significant with a size larger than 91.4 million hectares, representing 50% of the global area for all biotech crops. Based on the global area for individual crops, in 2016 78% of the soybeans planted were the issue of biotechnology. In Latin America in the same year, ten countries planted approximately 80 million hectares of biotech crops. In Brazil, the final monitoring of the 2016/2017 harvest showed that the cultivation of genetically modified soybeans increased its total area of 29.4 million hectares in 2014/2015 to 32.7 million hectares in the current harvest, representing 93.5% of the area planted and maintaining leadership in the adoption of transgenic crops in the country (Céleres, 2016; ISAAA, 2016). In Brazilian agriculture, pesticide consumption increased by 147% ten years after the planting of transgenic crops was approved. In just 2014, more than 508 thousand tons of active substances were commercialized, with glyphosate being the most sold active ingredient, corresponding to 194,877 tons, 39% of the total marketed. Of this total, 28,683 tons of the active ingredient are sold only in Rio Grande do Sul state, corresponding to 14.72% of the total marketed (IBAMA, 2016).

Glyphosate (N- [phosphonomethyl] -glycine) is a broad-spectrum, systemic, non-selective, and post-emergence herbicide, and once in the topsoil, its horizontal mobility is related to the sediment transport and flow process (Yang et al., 2015). This herbicide is applied intensively in agricultural crops, mainly in transgenic corn and soybean crops. The low price of this total action herbicide facilitated the expansion of the no-tillage system (NTS), paving the way for the development of genetically modified plants resistant to glyphosate. The low-price supply of glyphosate, control of no-tillage systems, development of tolerant plants, and the concentration of production areas in a few world producers has led to an enormous expansion in the cultivated area and, consequently, indiscriminate use of this substance. The most fundamental principles of agronomy were replaced by the purchase of the technological package, without applying the principle of environmental precaution and human health protection (Londres, 2011; Aparicio et al., 2013; Lupi et al., 2015; Bain et al., 2017).

Applications with lower volumes of glyphosate in non-agricultural areas include weed control on railway lines and in parks, domestic gardens (Benbrook, 2016), and horticulture (Uren Webster et al., 2014). This means that urban areas can also contribute to the contamination of the aquatic environment with glyphosate residues. However, it is important to emphasize that the practice of chemical weeding is prohibited in urban environments where there is free circulation (squares, gardens, backyards) and where there is no means to ensure adequate isolation (ANVISA, 2016).

After application, it is possible to find aminomethylphosphonic acid (AMPA) in the environment, which is one of the main metabolites from the microbial degradation of glyphosate (Dick and Quinn, 1995). Thus, the residues of glyphosate and AMPA can be found in all environmental compartments, according to studies of the soils, surface waters, suspended sediments, and bottom sediments in Argentina (Aparicio et al., 2013; Lupi et al., 2015; Giaccio et al., 2016; Primost et al., 2017); rivers, rainwater, and groundwater in the United States

(Battaglin et al., 2014); soil and groundwater in Europe (Poiger et al., 2017; Silva et al., 2017); and even fish in Brazil (Moura et al., 2017).

Recent studies are using epilithic biofilms as a matrix of adsorption of contaminants, allowing the detection of compounds not captured by active water sampling (Huerta et al., 2016; Aubertheau et al., 2017). Epilithic biofilms dominate microbial life in streams and rivers, conduct crucial ecosystem processes, and contribute substantially to global biogeochemical flows (Battin et al., 2016), since they are developed using mineral and organic elements from water or rocks (Julien et al., 2014).In most cases, they have been described as communities of microorganisms composed mainly of microbial cells (mainly bacteria but also fungi, protozoa, and algae), extracellular polymeric substances (SPE) from the metabolism of microorganisms, and inorganic materials (Flemming et al., 2007). The adsorbent biofilms transform organic substances and nutrients in the matrix as well as accumulate substances that, in the water flow, would be highly diluted, such as dissolved organic carbon or contaminants (Flemming and Wingender, 2010). It should be noted that in rivers, epilithic communities are the first organisms exposed to pesticides from the terrestrial environment. They can show the first signs of changes in the environment with changes in their structure and function (Sabater et al., 2007). In this way, biofilm is a useful tool to assess the impact of anthropic action in aquatic environments (Huerta et al., 2016; Aubertheau et al., 2017), including the presence of pesticide residues such as glyphosate and its AMPA metabolite.

The physicochemical characteristics of glyphosate are very different from other pesticides: it has high solubility in water, a low octanol / water partition coefficient (K_{ow}), and a high organic carbon partition coefficient (K_{oc}) (Mayer et al., 2006). The biofilm–water interaction, with an emphasis on the adsorption of glyphosate and AMPA, must be extremely complex due to the great variety of active functional groups, mainly in the SPE (Wei et al., 2011). In this sense, several components of the biofilm (bacteria, SPE, organic compounds, and minerals) can interact with chemical species (molecules) contained in the water (Julien et al., 2014). Thus, the present study seeks to monitor the occurrence of glyphosate and AMPA residues in epilithic biofilms occurring in eight points of the Guaporé watershed, located in the state of Rio Grande do Sul, Brazil, in the fall and in the spring of 2016, due to the use of the transgenic plants technological package – direct planting system.

2. Materials and Methods

2.1 Guaporé watershed and sampled sites

The Guaporé watershed (BHRG) is located in the state of Rio Grande do Sul, Brazil (Figure 1), and is representative of the heterogeneity of the set of environmental conditions found in southern Brazil in terms of the type, use, and management of the soil and relief. For this study, the upper third of the basin was selected, including the Capingui River sub-watershed and the Arroio Marau sub-watershed. The climatic classification of the region is Cfa according to the Köppen classification, with average annual rainfall ranging between 1,400 and 1,700 mm and an average annual temperature of 17.9 °C.

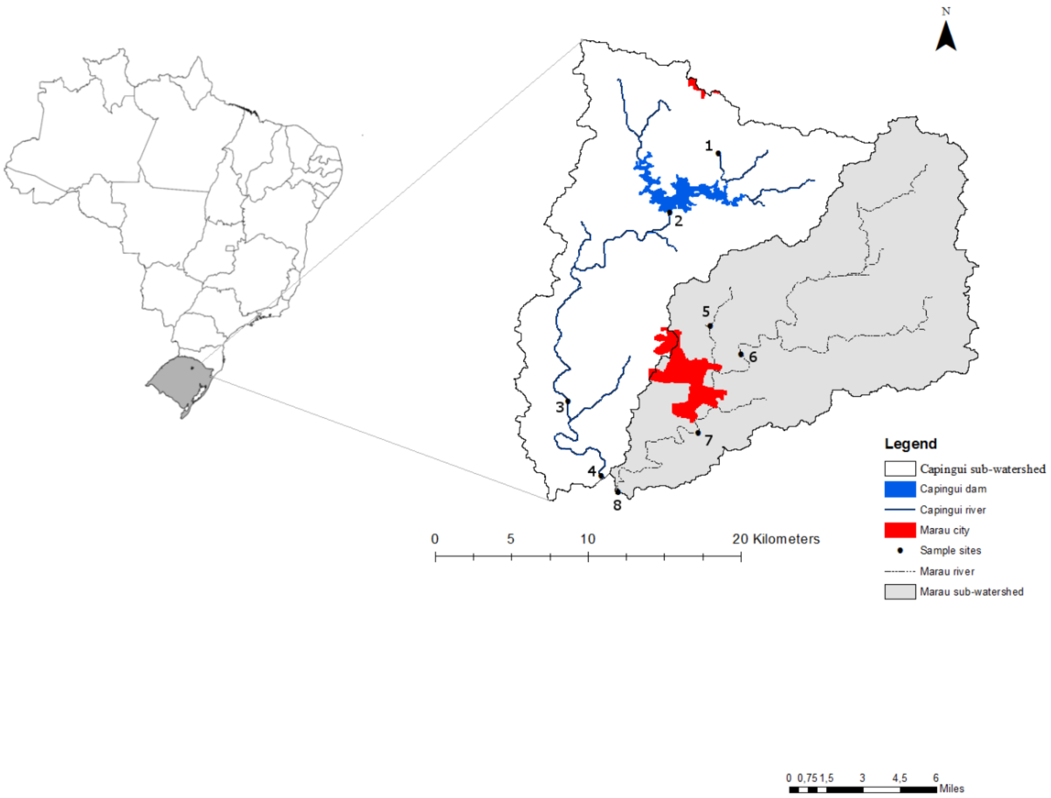

Figure 1. Location of the Guaporé River watershed in Brazil and sampling points in the Capingui River sub-watershed and the Arroio Marau sub-watershed.

The Capingui River sub-watershed has an area of 269 km^2, which is mainly used for soybean and maize crops in a direct planting system. In this basin, four sampling points were monitored, the first one located in the Passo Fundo National Forest (FLONA – point 1, Figure 1). FLONA is a Federal Conservation Unit with 1300 hectares, and therefore, this point was considered as a control sample due to the low anthropogenic action. The second sampling point is 600 m downstream from the Capingui dam (point 2, Figure 1). The ciliary forest is restricted between 15 and 60 m in width and is bordered by cropland managed in a no-tillage system (NTS). Currently, the reservoir is used for recreation and presents a growing increase in human occupation, resulting in the release of wastewater directly into the reservoir. The third point is located 17 km from the Capingui dam and represents the contribution of a large area of agricultural production (Agriculture, point 3, Figure 1). Crops occupy 69% of the area and are managed in an NTS with soybean and corn in the spring / summer and oats / wheat in the fall / winter. In this region, maize cultivation is more pronounced than in the other parts of the Capingui River in addition to the breeding of poultry and pigs in integrated systems and cattle breeding. The fourth point is at the outlet of the Capingui River (Capingui Outlet, point 4, Figure 1) and crops occupy 71% of the area. The culture system is similar to that of point 3 (Agriculture) and there is a high concentration of residues from the rearing of confined animals (mainly birds and dairy production).

The Arroio Marau sub-watershed has an area of 257 km^2 and stands out for the integrated system of chicken and pig breeding and for the production of dairy cattle, but agriculture is also intensively practiced with an emphasis on the soybean and maize direct sowing system (Tiecher et al., 2017). This sub-basin receives effluents from some cities, the largest of which is Marau with a population estimated at 41,059 inhabitants (IBGE, 2017) which does not have any wastewater treatment plants, resulting in a huge increase in river pollution from the urban area (Sá and Gerhardt, 2016). In this basin, four sampling points were also monitored. The first one is located along the urban perimeter of the municipality of Marau (Marau Grain, point 5, Figure 1) and represents the water that comes mainly from the drainage of grain production areas (soy and maize) using a direct planting system. The second point, besides the production of grains, is also notable for the breeding of poultry, pigs, and dairy cattle (Marau Animal, point 6, Figure 1). The third point is just downstream from the city of Marau and receives the full burden of urban pollution (Marau City, point 7, Figure 1). Agricultural activities are similar to those of point 6. The fourth point includes the Marau sub-watershed (Marau Outlet, point 8, Figure 1), representing a zone with soybean and corn crops in NTS in spring / summer, areas of oats in winter, and dairy cattle and bird production in an integrated system.

2.2 Sampling, preparation, and storage of biofilm

The collection of epilithic biofilm samples in the Guaporé River fluvial network was carried out in the fall of 2016 between May 26 and 28 and in the spring of 2016 between November 12 and 14. Eight points distributed along the sub-watershed of the Capingui River (FLONA, Capingui Dam, Agriculture, and Capingui Outlet) and the Marau sub-watershed (Marau Grain, Marau Animal, Marau City, and Marau Outlet) were sampled.

Sampling consisted of brushing several rocks at each station that remain submerged with toothbrushes. The material adhered to the rock was rinsed with 500 mL of deionized water (Aubertheau et al., 2017). The aqueous solution containing the biofilm (800 mL in each sampling site and ± 4 g of dry biofilms) was stored in thermal boxes with ice (± 5°C) and transported to the laboratory. The samples were transferred to individual high-density polyethylene bottles, frozen at -80°C and lyophilized at -40°C. After lyophilization, the samples were processed with the aim of removing any possible impurities (leaves, branches, invertebrates) and homogenized in agate grade to obtain a representative sample.

2.3 Physicochemical properties of water and biofilms

Determination of the chemical composition of the epilithic biofilms was carried out to better interpret the results of the occurrence and concentration of glyphosate and AMPA. The pH, dissolved oxygen (DO), and water temperature were obtained at the collection sites using a Multiparameter Meter HI 9829. Water flow and water depth were determined using a Flow Probe water flow meter. The monthly precipitation and the averages of the maximum and minimum temperature in Marau during 2016 were obtained from the records of INMET - National Institute of Meteorology (INMET, 2018). In addition, the number of brushed rocks at each sampling point was counted, and the total area of these rocks (m^2) was determined by wrapping them with foil and then measuring the area of the aluminum foil used for this purpose. From these data, the biofilm density on the rocks was calculated.

The total organic carbon (TOC) and total nitrogen (TN) were quantified by dry combustion using a Flash EA 1112 model elemental analyzer (Thermo Finnigan, Milan, Italy), with 5 mg of dry and lyophilized biofilm sample. The percentage of organic material and mineral material was determined by calcining one gram of biofilm in a muffle furnace at 550°C for 2 hours. The determination of the mass of mineral material was defined by the difference between the weighing before and after the calcination. For the determination of the particle size distribution, biofilm samples were analyzed by laser granulometry after the oxidation of the organic matter with 5% H_2O_2 (Muggler et al., 1997), chemical dispersion with 1 mol L^{-1} NaCl (aiming to increase the repulsion between particles), and physical dispersion with ultrasound.

2.4 Extraction of glyphosate and AMPA

For the glyphosate and AMPA determination, 0.1 g of dry and lyophilized biofilm was used. After weighing, the biofilm samples were fortified with 50 μL of isotope-labeled glyphosate stock solution (10 mg L^{-1}) (1.2-^{13}C, ^{15}N) to determine the matrix effects and recovery. After 30 min, the samples were extracted with 10 mL of 0.5 mol L^{-1} potassium hydroxide solution (KOH) in an ultrasonic bath for 30 min and centrifuged at 3000 rpm for 10 min to separate the suspended material. Then, an aliquot of 5 mL was taken from the supernatant, treated with 50 mg of activated carbon to remove co-extractives, and kept at rest for 60 min followed by 10 min of centrifugation. A 3 mL aliquot was taken from the supernatant to be adjusted to pH = 11 with concentrated hydrochloric acid (HCl). Then, 1 mL of buffer solution (100 mM $Na_2B_4O_7.10H_2O$/100 mM K_3PO_4, pH= 9) was added to the sample before derivatization with 2 mL of 9-fluorenylmethylchloroformate (FMOC-Cl) reagent in acetonitrile (1 mg mL^{-1}). The samples were shaken vigorously and left overnight (between 12 and 15 h) at room temperature. After that, in order to eliminate the excess FMOC-Cl and remove any interference from the matrix, a liquid–liquid extraction with 5 mL of CH_2Cl_2 and centrifugation at 3000 rpm for 10 min was performed. Finally, the aqueous phase was filtered through a 0.22 µm nylon filter and 20 µL of the final extract was injected into the ultra-high performance liquid chromatograph coupled to a tandem mass spectrometer (UHPLC-ESI-MS/MS) system.

2.5 Instrumental analysis

A standard curve for glyphosate and AMPA in water (0.5, 1, 10, 20, and 50 µg L^{-1}) was prepared for each set of samples to compensate the FMOC-Cl degradation. An amount equivalent to that expected in the analyzed samples of isotope-labeled glyphosate was added at each point in the curve. Satisfactory linearity using weighed (1/X) least squares regression was assumed when the determination coefficient (r^2) was higher than 0.99, based on measurement of the analyte peak areas, and the residuals lower than 30%.

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was 3 µg kg^{-1} for glyphosate and AMPA. The limit of quantification (LOQ), defined as the smallest value of analyte that can be determined quantitatively, was 10 µg kg^{-1}.

The determination of glyphosate and AMPA concentrations was performed by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-ESI-MS/MS) analysis using an ACQUITY UPLC™ system coupled to a Quattro Premier™ XE tandem quadrupole mass spectrometer (Waters).

For the chromatographic separation, an Acquity UPLC BEH C18 column (1.7 µm, 50 x 2.1 mm) (Waters) fitted with an Acquity Van Guard BEH C18 pre-column (1.7 µm, 5 x 2.1 mm) (Waters) was used. The flow rate for the mobile phase was 0.4 mL min^{-1}. The mobile phase was a time-programmed gradient using organic-free water modified with 5 mM ammonium acetate (phase A) and methanol modified with 5 mM ammonium acetate (phase B). The percentage of organic modifier (B) was changed linearly as follows: 0 min, 0%; 0.2 min, 0%; 2.5 min, 70%; 3.5

min, 100%; 4.5 min, 100%; 5.0 min, 0%; and 6 min, 0%. The column was kept at 60°C and the sample manager was maintained at 8°C. The injection volume was 20 µL. The drying as well as nebulizing gas was nitrogen, obtained from a nitrogen generator. The cone gas and desolvation gas flows were optimized at 2 L h^{-1} and 600 L h^{-1}, respectively. For operation in MS/MS mode, the collision gas was 99.995% Argon with a pressure of 4.04×10^{-3} mbar in the T-Wave cell. The positive ionization mode was performed using a capillary voltage of 3.0 kV. The desolvation gas temperature was set to 400°C and the source temperature to 120°C. Dwell times of 0.10 $sscan^{-1}$ were chosen. The Masslynx NT v 4.1 (Waters) software was used to process the quantitative data obtained from the calibration standards and from the samples (Lupi et al., 2015).

2.6 Statistical analysis

The non-parametric Mann–Whitney U test was applied to evaluate the statistical difference between the sampling seasons. The similarity analysis of the sampled sites was performed using the clusters method. Statistical analyzes were performed using XLSTAT software.

3. Results and Discussion

3.1 Physicochemical characteristics of the biofilm sampling site

The water of the João de Barro stream – sampled within FLONA – had a water column with a depth of 9 cm in fall and 14 cm in spring, low temperatures (13.0 to 15.5°C), and a low flow rate (0.2 m s^{-1}) compared to the other sampling points (Table 1). The spring harvest was preceded by a large volume of rainfall (Figure 2), causing an increase in the water column and DO contents. The depth of the river affects the intensity of light that reaches the river bed and therefore the development of biofilms. An increase in light availability favors the growth of the autotrophic component of the biofilm, and this effect is more noticeable under higher nutrient conditions (Prieto et al., 2016).

The river sampled at the Capingui Dam sampling site, located just below the dam, had the deepest water column. The river flow rate at this sampling site was higher than at the FLONA river sampling site, but it remains constant in time as do the pH values. The faster the water flow, the greater the biofilm development, the more organisms begin to colonize it, and the larger the interactions with the aquatic environment (Hunt and Parry, 1998). In spring, the increase in the DO content was significant. At the Agriculture point, a greater depth of the water column was observed in fall since the distribution of precipitation varied from one season to another. At that point, the flow rate and the pH values were higher compared to the water collected at the other points of the stream. Higher pH values may be associated with plant proliferation in general or the presence of a more enclosed riparian forest over the river, as with the increase of photosynthesis there is the consumption of carbon dioxide, reduction of the carbonic acid in the water, and the consequent increase of pH (Von Sperling, 2005). In addition, residues from limestone applications in the soil can reach the water resources through erosive processes and increase the pH of the water. Subtle variations were found in the DO contents in fall, contrasting the water DO values obtained at this point (4.9 mg O_2 L^{-1}) with the water sampled within FLONA (4.1 mg O_2 L^{-1}). The same occurred with the values of DO in the water collected at the Capingui Outlet point (5.0 mg O_2 L^{-1}), probably due to the higher volume of rainfall causing a deeper water column without, however, changing the flow rate (Table 1).

In the Arroio Marau sub-watershed, the Marau Grain sampling site was characterized by a water column similar to FLONA, i.e., shallow, in addition to low values of the water flow rate and DO. In contrast, at the Marau Animal sample site, although located upstream of Marau City, the water table of the stream was high, and the water had higher levels of DO. The water collected at the Marau City and Marau Outlet points had physicochemical parameters, water depth and flow rate, similar to those observed at the other sampling points in this sub-watershed.

Table 1. Physicochemical parameters of Marau and Capingui sub-watershed water: water depth, water flow rate, pH, water temperature, and dissolved oxygen*

Sampling sites	Water depth (cm)		Water flow rate (m s^{-1})		pH		Water temperature (°C)		Dissolved oxygen (mg O_2 L^{-1})	
	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
1 - FLONA	9	14	0.2	0.2	7.1	6.9	13.0	15.5	4.1	4.8
2 - Capingui Dam	55	48	0.5	0.5	7.1	7.1	14.8	18.0	4.3	5.3
3 - Agriculture	48	29	0.7	0.9	7.2	7.3	14.6	17.3	4.9	5.0
4 - Capingui Outlet	41	62	0.3	0.3	7.3	7.2	14.5	18.3	5.0	4.9
5 - Marau Grain	15	18	0.4	0.3	7.1	7.0	14.0	15.5	4.3	4.5
6 - Marau Animal	49	44	0.3	0.4	7.2	7.1	13.0	18.2	4.7	4.9
7 - Marau City	36	31	0.5	0.3	7.2	6.8	13.8	16.3	4.8	4.7
8 - Marau Outlet	26	30	0.5	0.5	7.1	6.8	14.0	17.5	4.6	4.5

*No significant differences were observed (Mann-Whitney U test, $p>0.05$).

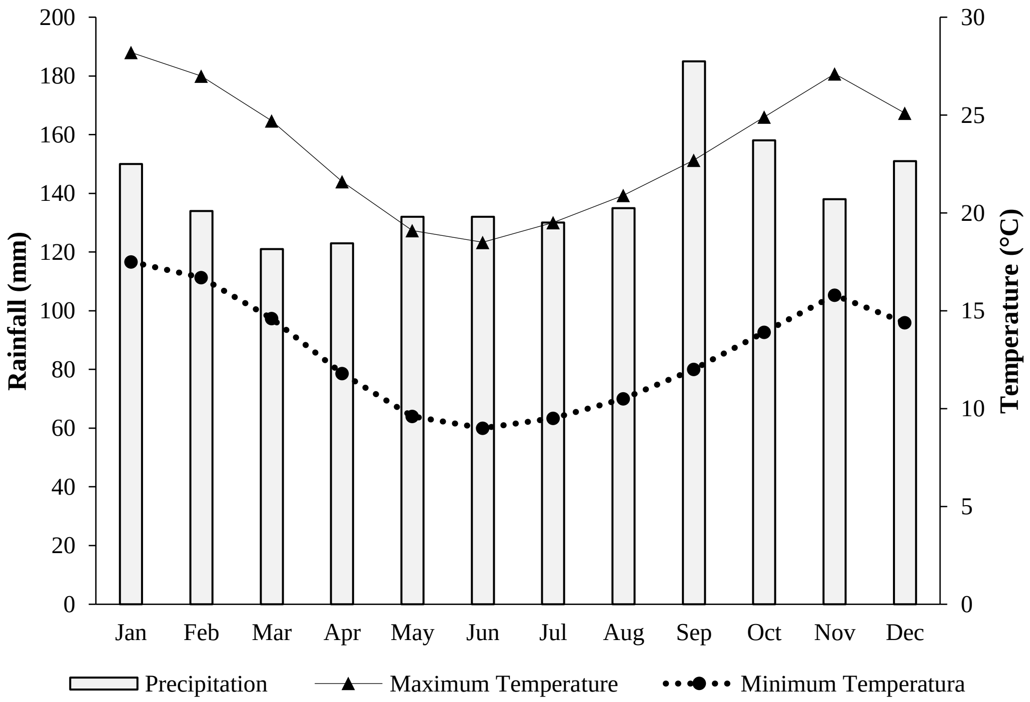

Figure 2. Total rainfall (mm) and averages of the maximum and minimum temperature in Marau during 2016 – Rio Grande do Sul (RS).

The temperature of the water affects the formation of epilithic biofilms as it influences the species of microorganisms present and the types of compounds produced by them, which will compose the SPE (Zeraik and Nitschke, 2012). The temperature of the water sampled ranged from 12.0 to 13.6°C in May and from 15.5 to 18.3°C in November. The water sampled inside FLONA in May and November was 1.6 and 2.8ºC lower than all other sample sites, respectively (Table 1). The variations in the temperature of the water follow the variations in the air temperature, whose maximum and minimum averages were 12 and 15ºC in May and 15 and 27ºC in November, respectively (Figure 2).

The DO is essential for aerobic organisms since, during the stabilization of organic matter, bacteria make use of oxygen in their respiratory processes which can lead to a reduction of its concentration in the environment (Von Sperling, 2005). Thus, DO levels may have seasonal variations with short periods, varying even with the water depth and flow. In unpolluted rivers, DO contents are between 8.0 and 12.0 mg O_2 L^{-1}. However, values higher than 4.0 mg O_2 L^{-1}, as observed in the water sampled in this study, are considered adequate for the development of aquatic microorganisms. In addition, the distribution of rainfall during the year may affect the physicochemical characteristics of the environment. The greater rainfall observed in the spring months implies a greater loss of water and sediments due to flow from the crops to the water bodies.

3.2 Physicochemical characteristics of epilithic biofilms

The TOC and TN values for the total mass of epilithic biofilms sampled vary more due to the large differences in the amount of inorganic material than due to the environmental conditions or anthropic pollution of each site. In biofilms collected in the fall and spring seasons, the TOC levels ranged from 7.92 to 22.09% and from 6.46 to 16.43% and TN contents ranged from 1.42 to 4.09% and from 0.87 to 3.05%, respectively (Table 2). These variations are negatively correlated with the percentage of inorganic material in the biofilms. The low percentage of organic material indicates that epilithic biofilms are mainly composed of minerals. When eliminating the presence of mineral material, the differences in organic C and organic N concentrations between biofilms sampled at different sites become very small. In the soil, for example, organic matter contains about 58% carbon, relative to the total mass (Nelson and Sommers, 1982), with the microbial biomass composed of organic material with contents lower than 50% (bacteria, fungi, and microfauna) contributing 2 to 5% of this C percentage (Gama-Rodrigues, 1999). The low percentages of organic carbon in the biofilm can be explained by the fact that the rest of the organic matter consists of other elements, mainly N, sulfur (S), phosphorus (P), and silicon (Si).

The epilithic biofilms collected at the Agriculture sample site had lower TOC and TN contents compared to the biofilms sampled at the other sites of the two sub-basins, most probably due to changes in the flow rate (Table 1). The biofilms sampled in fall, on average, had higher N contents and, consequently, lower C / N ratios compared to those sampled in the spring (Table 2).

The organic density of the epilithic biofilm directly influences the amount of C and N present *in situ*, expressed as mg of C per m^2 and mg of N per m^2, and the differences between the points and between the seasons are significant (Table 2). The watercourses within FLONA and downstream of the Capingui Dam provided a more stable and constant growth of the biofilms between both seasons since they were less influenced by the anthropic action and had little variation in the level and flow of the river when compared with the other sites. At these sites, the biofilms collected in the spring contained the highest amounts of organic compounds due to the presence of ciliary vegetation, low anthropogenic interference, and small variations in the flow level, which provides adequate conditions for the development of microorganism communities.

In contrast, the rocks found at the Agriculture, Capingui Outlet, Marau Grain, and Marau Animal sample sites had significantly less biofilm biomass (Table 2). These points represent anthropized environments with the development of intensive agriculture (mainly soy and corn) and animal husbandry. Although biofilms have a protective character with regard to microorganisms (Caixeta et al., 2012), the use and management of the soil weakens this protection, and environmental disturbances may affect the quantity and composition of the biofilms. The variations due to anthropic action, associated with the greater exposure of rocks to the sun, changes in the flow, and changes in the availability of nutrients, among others, are not favorable for the development of biofilms. Thus, once communities of mature biofilms are established, changes in the volume and extent of the biofilm are the result of the interaction between the biofilm and the environment (Tortora et al., 2017).

The entry of urban pollutants into the watercourses (Marau City and Marau Outlet) enriched the C and N contents of biofilms compared to agricultural areas. The Marau City point represents the urban part of the Marau sub-watershed and receives effluent from the city of Marau, which has no sewage treatment system, and even though the city's master plan requires the presence of fossils, the practice of clandestine sewage release is evident.

In fall, the FLONA and Capingui Dam points also had large amounts of C and N in the biofilms, greater than those in the spring, emphasizing the importance of the protection provided by the forest in the quality of the water courses. At the Agriculture point, the C and N values of the biofilms indicate that agricultural activities cause changes in biofilms, reducing the quantity of colonies adhered in terms of C and N, respectively, by 59.8% and 55.1% in comparison with the FLONA point. On the other hand, compared to spring, the amount of biofilm at the Agriculture point increased by 53.5% and 63.6% in terms of C and N, respectively. In Marau City there was a significant increase in the organic density and in the amount of C and N per square meter of the biofilms, exceeding the values found for the natural areas. These changes in the environment may have repercussions on the quantity and functioning of biofilms and even on ecosystem processes (Bier et al., 2015; Faure et al., 2015; Zhang et al., 2015; Battin et al., 2016).

The distribution of particle sizes in the epilithic biofilms showed little expressive variations between the monitoring sites and between the seasons of the year (Figure 3). The differences between the two seasons are mainly related to the water cycle of the region and to the greater or lesser aggregation between the soil particles, which causes erosion to be higher in periods with higher rainfall.

In epilithic biofilms, the largest fraction is mineral, and this fraction has a high clay particle content (<2 μm), varying from 12.2 to 27.6%. Clay particles come from basalt and other basic rocks occurring in the waterways where biofilms are established. In basalt, for example, the fraction <2 μm represents 10 to 13% of the rock granulometry (Korchagin, 2018). Even if these are high values for rocks and even if all rock material was "disaggregated," the clay fraction would still be lower than the contents found in the epilithic biofilms. On the other hand, the soils formed from this type of rock have extremely high levels of clay (40 to 70%), typical of the Red Latosols (Wiethölter, 2007) predominant in the Capingui and Marau sub-watersheds. These clay particles are transferred from the soil to the watercourses by water erosion, even if adequate soil conservation systems are used (Lupi et al., 2015). Roads and the watercourse beds are also sources of sediment that can be deposited and retained in biofilms (Tiecher et al., 2017). In this sense, factors such as the intensity and amount of rainfall, slope of the terrain, and speed and depth of the river water can influence the granulometry of the eroded material and, consequently, the biofilm granulometry (Kochem, 2014).

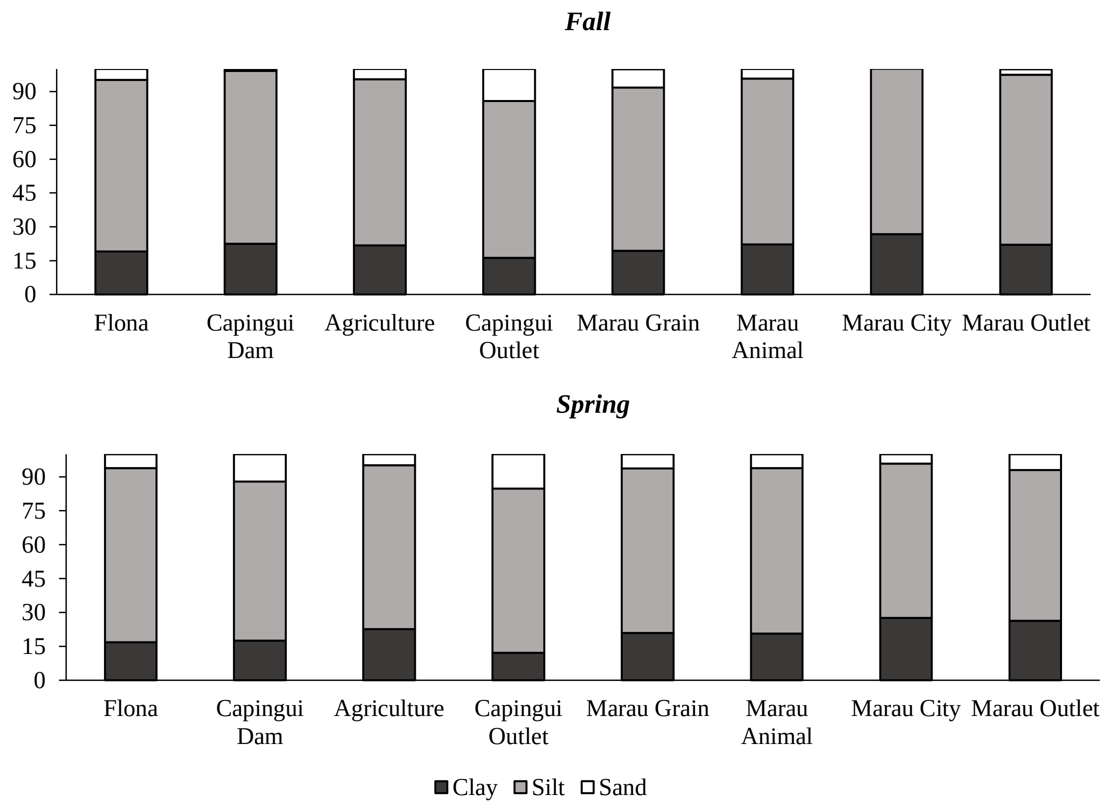

Figure 3. Particle size distribution in epilithic biofilms in the fall and spring seasons.

Table 2. Physicochemical properties of epilithic biofilms.

Sampling sites	Total organic carbon (TOC)	Total nitrogen (TN)	Mineral material	Organic material	Organic Carbon	Organic Nitrogen	C/N Ratio	Biofilm density	Organic density	Carbon per area *in situ*	Nitrogen per area *in situ*
	--------------------------		%		--------------------------			-------- $g\ m^{-2}$	--------	$mg\ C\ m^{-2}$	$mg\ N\ m^{-2}$
					Fall						
FLONA	16.54	2.64	62.14	37.86	43.7	7.0	6.3	2.05	0.78	339.1	54.1
Capingui Dam	12.60	1.96	69.76	30.24	41.7	6.4	6.5	2.50	0.76	315.0	48.8
Agriculture	7.92	1.42	78.03	21.97	36.0	6.4	5.6	1.72	0.38	136.2	24.2
Capingui Outlet	13.80	2.31	-*	-	-	-	-	1.28	-	-	-
Marau Grain	13.95	2.59	-	-	-	-	-	0.56	-	-	-
Marau Animal	10.23	1.87	-	-	-	-	-	0.68	-	-	-
Marau City	13.75	2.62	67.35	32.65	42.1	8.0	5.2	3.04	0.99	418.0	79.6
Marau Outlet	22.09	4.09	-	-	-	-	-	1.60	-	-	-
					Spring						
FLONA	7.64	1.11	79.85	20.15	37.7	5.5	6.9	2.76	0.56	209.8	30.4
Capingui Dam	10.26	1.52	73.65	26.35	38.7	5.7	6.8	2.65	0.70	270.3	39.7
Agriculture	7.19	1.03	81.23	18.77	38.4	5.3	7.2	0.88	0.17	63.4	8.8
Capingui Outlet	9.94	1.69	76.25	23.75	41.7	7.2	5.8	0.75	0.18	74.2	12.7
Marau Grain	7.73	1.28	81.42	18.58	41.4	7.0	5.9	0.63	0.12	48.5	8.2
Marau Animal	6.46	0.87	84.45	15.55	41.8	5.8	7.2	0.71	0.11	46.1	6.4
Marau City	11.18	2.00	72.66	27.34	41.0	7.3	5.6	0.93	0.25	104.2	18.6
Marau Outlet	16.43	3.05	63.19	36.81	44.6	8.1	5.5	0.68	0.25	111.5	20.4

*Amount of sample insufficient for analysis

Biofilms have a very large adsorption capacity due to the high clay content. The reactivity of clay-sized particles facilitates biofilm fixation on rocks and the adsorption of organic contaminants, such as glyphosate herbicide molecules and its main metabolite AMPA. Glyphosate adsorption in biofilms is maximized because sediments and all material reaching these communities are rich in 1:1 clays (kaolinite) and oxides whose functional groups interact with the functional groups of glyphosate and AMPA (the phosphate group, for example) (Toni et al., 2006; Aparicio et al., 2013).

After oxidation of the organic matter in the epilithic biofilms to determine the particle size distribution, there was a decrease in the TOC (Table 3). The oxidized TOC percentage varied among the samples of epilithic biofilms indicating that the physical protection of organic matter is related to organo-mineral interaction. The more intense the interaction, the greater the possibility of the formation of minerals (silt or sand) whose stability physically protects the organic material (Dick et al., 2009). The lower percentages of organic matter destruction observed at the Capingui Dam, Agriculture, and Capingui Outlet points in fall did not have an effect on the percentage of the clay fraction (Figure 3) since the differences between these three points in fall and spring (higher percentage of oxidation) are minimal. The difference in the clay content between the eight points in fall is also not significant, indicating that even in biofilms with a low percentage of oxidation it was possible to extract the clay from the organo-mineral complexes.

Table 3. Percent of TOC oxidized before and after burning of organic matter from epilithic biofilms

Sampling sites	% TOC Fall		% oxidized	% TOC Spring		% oxidized
	Before	After		Before	After	
FLONA	16.54	3.34	79.81	7.64	1.33	82.59
Capingui Dam	12.60	6.26	50.32	10.26	0.95	90.74
Agriculture	7.92	4.65	41.29	7.19	1.88	73.85

Capingui Outlet	13.80	7.58	45.07	9.94	1.07	89.24
Marau Grain	13.95	1.98	85.81	7.73	1.07	86.16
Marau Animal	10.23	6.53	63.83	6.46	1.69	73.84
Marau City	13.75	3.08	77.60	11.18	1.82	83.72
Marau Outlet	22.09	2.84	87.14	16.42	2.19	86.66

3.3 Glyphosate and AMPA in epilithic biofilms

The concentrations of glyphosate and AMPA in epilithic biofilms varied with the season, showing a higher accumulation in the spring. Sowing of corn and soybeans (> 95% of the cultivated area) begins in September (early spring), and the application of pesticides begins during this period or before, continuing until full bloom. In addition to the high agronomic rates and frequencies of application, farmers use the "agronomic precautionary principle" stimulated by the agrochemical industries, resulting in enormous amounts of pesticides and nutrients being applied to the fields. In the late summer and early fall, applications of glyphosate are dramatically reduced, and epilithic biofilms may respond differently to the aquatic ecosystem.

The concentrations of glyphosate and AMPA in the epilithic biofilms varied with the location of the sampling point within the basin, showing a greater accumulation downstream. Epilithic biofilms sampled at the FLONA river point are not contaminated by glyphosate and AMPA (Table 4). This sampling point, located in the Capingui River sub-watershed, is located in a natural, preserved area (Passo Fundo National Forest) with little current anthropogenic action. Soils at the site were occupied by agriculture until 1946 and by timber exploitation until 1968. However, in the 1970's, the site was transformed into a preservation area characterized by having part of the area occupied by native forest and part by the presence of exotic trees planted by man (Sá and Gerhardt, 2016). The result of this lower human interference is the maintenance of high water quality and the reduction of the volume of sediments available through erosion and mass movements, controlling the availability of some chemical elements and reducing their presence in the environment (Bacellar, 2005). Thus, in FLONA glyphosate is not applied, there is no drift and no water inlets from external flows from crops, and therefore, this herbicide was not detected in epilithic biofilms.

In the other seven points sampled in the Guaporé River watershed, glyphosate was detected in concentrations ranging from 10 to 305 μg kg^{-1}, and concentrations of AMPA ranged from 50 to 670 μg kg^{-1} (Table 4).

Table 4. Glyphosate and AMPA concentrations in epilithic biofilms*.

Sampling sites	Glyphosate (μg kg^{-1})		AMPA (μg kg^{-1})	
	Fall	Spring	Fall	Spring
FLONA	<LD	<LD	<LD	<LD
Capingui Dam	<LQ	110±0	50±10	240±30
Agriculture	<LQ	130±10	100±10	220±10
Capingui Outlet	30±0	10±2	210±20	110±0
Marau Grain	40±0	140±0	205±5	195±5
Marau Animal	170±0	140±0	340±0	150±0
Marau City	250±0	305±5	670±0	400±0
Marau Outlet	90±0	305±5	510±0	385±25

*No significant differences were observed (Mann-Whitney U test, p>0.05).

LD = 3 μg kg^{-1} and LQ = 10 μg kg^{-1} for both glyphosate and AMPA.

In the Capingui River sub-watershed, the Capingui Dam point, located 600 m downstream from the Capingui Dam, has a catchment area of 123 km^2, and the barrage limits the flow of water and sediment from approximately 95% of the area before reaching the Capingui Dam point. Soya and corn crops in spring / summer and oats and wheat in fall / winter occupy 64% of the area and are grown under NTS. In addition, the roads present in this region channel the runoff directly into the river network, which explains the detection of glyphosate and AMPA in epilithic biofilms, especially in the spring. At the Agriculture point, concentrations were similar to those found at Capingui Dam (Table 4) and this sampling point has a catchment area corresponding to 201 km^2, and it is not possible to visualize damping zones in the drainage lines along the river. Crops occupy 69% of the area and are managed in NTS with soybean and corn in spring / summer and oats / wheat in fall / winter. In addition, poultry and pig breeding in integrated systems and cattle breeding are common. In the monitored area, it is possible to visualize a wide network of drainage systems in the floodplain areas and the presence of erosive processes in furrows which channel the water and sediments to the Capingui River and its tributaries. Thus, the higher levels of glyphosate and AMPA found in the spring for the Capingui Dam and Agriculture points confirm the greater amount of the herbicide applied by the farmers in that period. The Capingui Outlet point was the only point in the Capingui River sub-basin where glyphosate was detected in fall. The Capingui Outlet point integrates an area of 267 km^2, and the crops occupy 71% of the area. Despite the similarity of the cultivation system to that at the Agriculture point, in this area the drainage systems are less noticeable as is the presence of erosive processes.

In the Marau sub-watershed, the lowest concentrations of glyphosate and AMPA in epilithic biofilms were detected at the Marau Grain sample site (Table 4). Marau Grain is located at the height of the urban perimeter of the municipality of Marau. At that point, agricultural activities occupy 72% of the area, and the crops are managed under NTS with soybean and corn in spring / summer and oats / wheat in fall / winter. Livestock breeding systems are focused on the production of dairy cattle, pigs, and poultry in an integrated system. Despite the presence of agriculture and the use of herbicides, this point represents the smallest catchment area among the points sampled in the sub-watershed (13.4 km^2), which may explain the low levels of glyphosate and AMPA. At the Marau Animal point, the concentrations found for glyphosate and AMPA in fall were higher than the concentrations found in spring. This point is also situated upstream of the urban perimeter of the municipality of Marau in Arroio Marau but has a drainage area of 165 km^2. Poultry farming, swine farming, and cattle breeding activities are greatly expressed in the area, as well as the raising of soybean and corn (grain and silage production) in spring / summer and oats / ryegrass in fall / winter (no tillage). The values of glyphosate and AMPA found at the Marau Animal point pose a risk to the health of the population since the system of water abstraction operated by Corsan is located 3 km from this point and supplies more than 41,000 inhabitants of the city of Marau. As glyphosate and AMPA are accumulated in epilithic biofilms, they are also present in the water that is captured by Corsan. In addition, in Brazil, water quality control for the public supply follows a flawed regulation which excludes harmful contaminants such as the pesticide glyphosate from its list of toxic products (Oliveira, 2018).

On the other hand, the highest concentrations of glyphosate and AMPA were detected at the Marau City site, both in fall and spring. This point represents the urban part of the Marau sub-watershed and receives effluents from the city of Marau. This causes the city's organic waste to be dewatered in the rivers and accumulate in the biofilms of the submerged rocks, forming thicker layers of biofilm when compared with the other sampling sites. The contamination of biofilms by pollution from the city was discussed by Bastos et al. (2018), and the authors were able to confirm the effects of the presence of the city on the quality of the organisms present at the same sampling point (e.g., bacterial resistance and the presence of different drugs) through the use of biofilms. At Marau Outlet, the concentrations found for glyphosate and AMPA were also high, however they are lower than at the previous point upstream. This point, located 14 km from the city of Marau, drains 256 km^2 and integrates the pollution of both urban and rural areas, accumulating pressure due to the presence of the city of Marau and many other housing developments located downstream of the hydrographic basin (Bastos et al., 2018). At the margins of the watershed draining to this point, there are areas of soybean and maize grown under NTS in the spring / summer and areas of winter oats. In addition, we highlight the production of dairy cattle and the breeding of birds in an integrated system.

The contamination of epilithic biofilms with glyphosate and AMPA shows that the use of this herbicide in the agricultural areas, mainly for soybeans and corn which are predominant in the watershed, at high and repeated doses affects the quality of the aquatic ecosystem (Girotto et al., 2010; Fernandes et al., 2017). Agrochemicals applied in agriculture can reach surface waters through different transport processes, mainly through surface runoff due to heavy rains or irrigation, rapid leaching through preferential inflows such as cracks in the soil, or by direct point losses through the drift of (Tang et al., 2011). However, the indiscriminate use of glyphosate in the urban environment is also evident, even with the prohibition of the use of this pesticide in urban areas (ANVISA, 2016). Although municipalities do not carry out applications of glyphosate, it is observed that the population in general accomplishes this practice in the cities, which was also verified in the city of Marau. Biofilms sampled at sites crossing the city of Marau or that are downstream of the urban area are more contaminated than at sites in areas of intensive agriculture. This is because the great majority of the watersheds of Rio Grande do Sul are composed of multi-functional pluri-productive local arrangements, combining agricultural and non-agricultural activities for the generation of income, which can be called a “rurban” (rural–urban) area. The concept of rurban is an indication that disturbances to the environment, especially to the water quality, are a reflection of the socio-productive dynamics established in the territory (Veiga, 2002).

In Rio Grande do Sul, glyphosate consumption is 9 to 19 kg per hectare, with the Brazilian average being between 5 and 9 kg per hectare. The municipalities that are part of the area monitored in this study, including the municipality of Marau, present a percentage of pesticide use, with an emphasis on glyphosate, for the total establishment between 65.3 and 78.8%, on a scale of 0 to 100%. The high concentration of pesticide use in this region is mainly due to their application on soybean and corn crops (Bombardi, 2016). Comparing the two sub-basins of the Guaporé River, the general results indicate that the glyphosate and AMPA contamination is higher in the Marau sub-watershed (rurban), since it is characterized by the massive large-scale application of glyphosate and by the presence of a small strip of riparian forest around the rivers.

Thus, increased use of glyphosate-based herbicides in agricultural areas increases the possibility of disposal in and contamination of the adjacent aquatic ecosystems. Glyphosate was found in the epilithic biofilms of the Guaporé River watershed because it was applied to crops and then transferred from the crops to the aquatic ecosystem. Human action through the use and management of the soil and the environmental factors themselves, such as the intensity and amount of rainfall and slope of the terrain, may contribute to the greater deposition or incorporation of sediments containing pesticides in epilithic biofilms. Therefore, the amount of sediment incorporated into biofilms is related to the absence of mechanical barriers and the low soil cover index controlling nutrient losses along with sediment and water through runoff during periods of higher rainfall intensity. The glyphosate content can be increased by the addition of glyphosate to the soil surface. In addition, the glyphosate can also be desorbed, biodegraded, and accumulated (Aparicio et al., 2013) in epilithic biofilms (Shaw, Mibbayad, 2016). In addition, there is the greater proximity and lateral connectivity of tillage areas to the fluvial network (Didoné et al., 2014; Tiecher et al., 2017). This problem becomes important for the area of study due to the considerable volume of soil eroded annually, which can reach more than 300 Mg km^{-2} $year^{-1}$ of sediment from the crop and road areas (Tiecher et al., 2017).

The herbicide glyphosate and its metabolite AMPA remained accumulated in the epilithic biofilms, confirming that the biofilm is able to incorporate compounds present in natural waters. The concentrations detected are the result of the entire retention period at the rock surface (Aubertheau et al., 2017). The presence of glyphosate and its metabolite AMPA in epilithic biofilms can occur through different mechanisms: adsorption, electrostatic adsorption form complexes in water with metal ions of calcium and magnesium, which may increase its mobility in the soil profile (Toni et al., 2006), etc.

Even with the accumulation of glyphosate and AMPA, the biofilm matrix may still protect the microorganisms present against the negative effects of herbicides (Shaw and Mibbayad, 2016). However, the study by Bastos (2017) showed that in the same sub-basin, the presence of veterinary drugs in biofilms was able to promote bacterial resistance to antibiotics present in the water. That is, the protection of organisms is not fully assured and needs further study.

Through hierarchical agglomerative analysis it was possible to form classes of comparison between the biofilm sampling sites based on their contamination by glyphosate and AMPA. As for the comparison between the groups formed, it is possible to separate the collection sites into three classes of contamination (Figure 4a and 4b). The results obtained from this analysis show that the contamination is associated with seasonality and the source of the pollution (agricultural or urban).

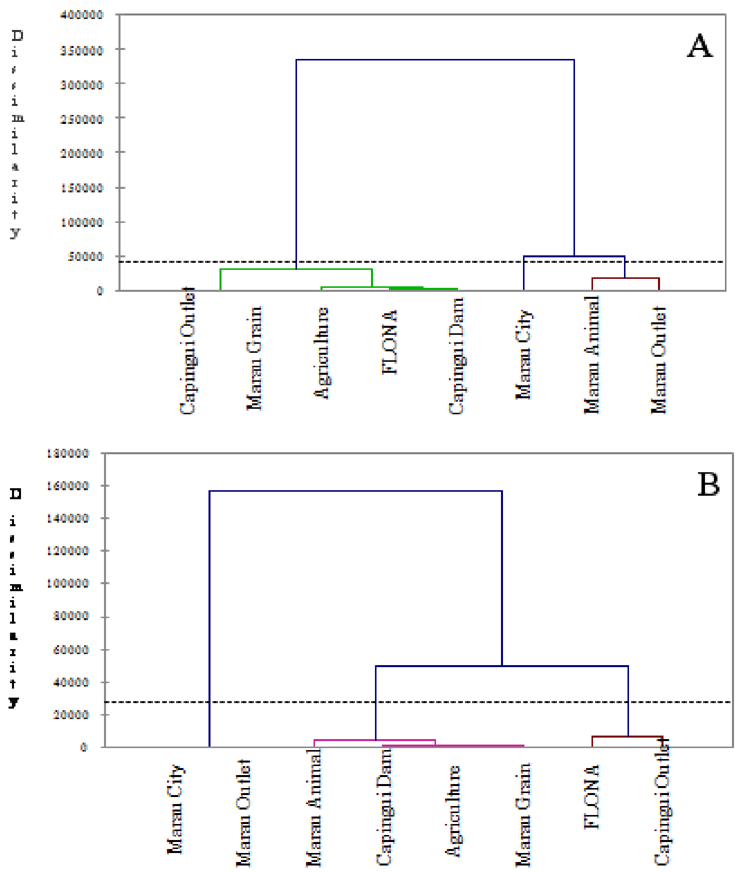

Figure 4. Dendrogram of the hierarchical agglomerative cluster analysis for the glyphosate and AMPA factors in the sub-watersheds of Capingui and Arroio Marau in the (a) fall and (b) spring seasons.

The present study shows that natural epilithic biofilms are efficient in accumulating the herbicide glyphosate and its metabolite AMPA. As verified by Bastos et al. (2018), epilithic biofilms are able to reflect the different anthropic pressures near rivers. The accumulation potential of biofilm was also observed by European teams who studied the presence of pharmaceutical products in biofilms developed in rivers receiving wastewater that has gone through all of the treatment processes before being discharged into the rivers (Huerta et al., 2016; Aubertheau et al., 2017).

Biofilms sampled in two distinct seasons of the year were able to accumulate glyphosate and AMPA and showed the great potential of this matrix as a source of information on anthropic actions that occurred prior to sampling, that is, as a more reliable source than water for environmental studies due to its lower mobility in space. Obtaining supplementary data from biofilms for the evaluation of environmental contamination can be easily performed. Sampling can be carried out at the same time as water sampling campaigns at different scales, e.g., sub-watersheds, catchments, and even countries; this study that was carried out in a southern Brazilian watershed could easily be extended to larger scales for the study of other sites with different anthropogenic contexts more or less impacted by human activities.

Conclusions

- The Guaporé river watershed is representative of most of the rural watersheds in the southern region of Brazil since the main agricultural activities are focused on soybean and corn crops and on pig, cattle, and poultry farming. Along with the development of these activities, there are cities and urban settlements which contribute significantly to the contamination of water resources.
- Anthropogenic activities, including those practiced in urban areas, are interacting with the water resources of the Guaporé River watershed and contaminating epilithic biofilms, communities that represent the base of the food chain in aquatic ecosystems.
- Epilithic biofilms are able to bioaccumulate the herbicide glyphosate and its metabolite AMPA and can be used to distinguish different anthropogenic pressures in the river watersheds.
- The glyphosate herbicide, adsorbed on soil particles, can move and reach surface water sources where it can also be biodegraded and accumulated in epilithic biofilms. This information did not exist until now and constitutes an important contribution to the understanding of the environmental distribution of glyphosate and AMPA.
- The concentrations of glyphosate and AMPA detected in epilithic biofilms vary with the season and are strongly influenced by the amount of herbicide applications as well as the interference of environmental factors. Protected locations and those with poor access such as FLONA did not demonstrate the presence of these contaminants.
- An overview of the contamination in the Guaporé watershed shows that the most affected areas are those located in the Marau sub-watershed that are strongly influenced by the presence

of the city of Marau. This confirms the indiscriminate use of glyphosate in the urban area and constitutes a problem for human and animal health, since the river water of the sub-basin is used for consumption and glyphosate is probably carcinogenic.

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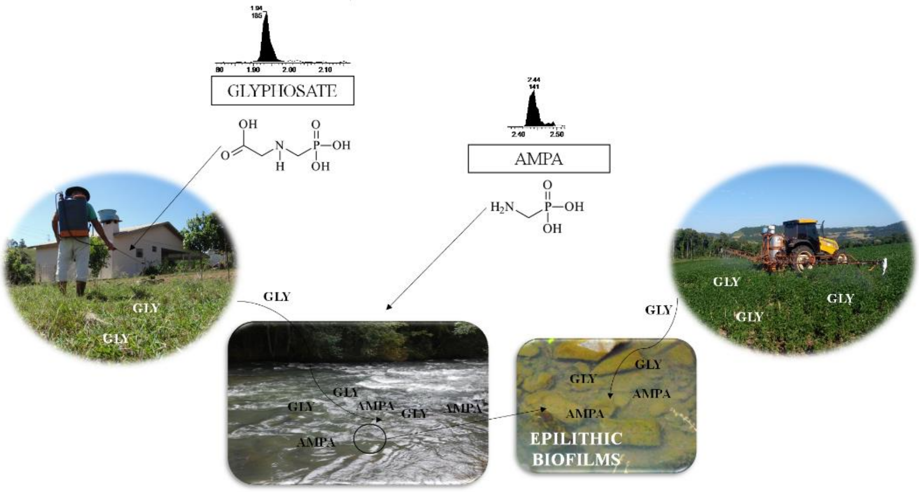

Graphical abstract

Highlights

- Epilithic biofilms are accumulators of glyphosate and AMPA.
- Epilithic biofilms are able to differentiate anthropic pressures.
- Glyphosate and AMPA concentrations vary with seasons.
- The indiscriminate use of glyphosate constitutes a problem for human health.