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1 **Organic composition of epilithic biofilms from agricultural and urban**
2 **watershed in south Brazil**

3
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30
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34 **Organic composition of epilithic biofilms from agricultural and urban watershed in**
35 **south Brazil**

36

37 **Abstract**

38 Active functional groups in biofilms determine the adsorption and desorption of contaminants
39 and nutrients. Epilithic biofilms were characterized in order to understand the association
40 between the chemistry alteration patterns and the surrounding anthropic activities of the
41 Guaporé River watershed. The instrumental analyses included pyrolysis coupled to gas
42 chromatography and mass spectroscopy, spectroscopy in the IR region with attenuated total
43 reflectance and two-dimensional nuclear magnetic resonance. Spectrometric techniques
44 demonstrated that epilithic biofilms are mainly composed of polysaccharides, nitrogen-
45 containing compounds, lipids, and aromatic and phenolic structures, which have functional
46 groups characteristic of alcohols, esters, ethers and amides. The polysaccharide levels reflect
47 well the environmental pressures. The chemical composition of epilithic biofilms can be an
48 effective tool for environmental assessment in watercourses, since the different anthropic
49 actions developed in watersheds, mainly agriculture and urban areas, can modify the organic
50 fraction of biofilms.

51

52 **Keywords:** environment; anthropic activities; epilithic biofilms; chemical composition;
53 functional groups; spectrometric techniques.

54

55 **Introduction**

56

57 Intensification of anthropic activities (urban or rural) continually exposes water
58 resources worldwide to different sources of pollution. Many contaminants (physical,
59 biological or chemical) reach water bodies through natural events, such as rainfall and surface
60 runoff, contributing to the degradation of water quality (Bhuyan et al. 2017). Microorganisms
61 present in rivers or lakes are among the first aquatic organisms to come into contact with such
62 contaminants (Sabater et al. 2007) and hence may present initial signs of modification at the
63 onset of environmental changes, such as changes in the structure of the population or
64 biogeochemical cycles (Sabater et al. 2007; Schneider and Lindstrom 2011). Recently,
65 attention has been focused on epilithic biofilms as bioaccumulators of organic compounds for
66 water quality studies (Aubertheau et al. 2017; Chonova et al. 2018; Fernandes et al. 2019;
67 Rheinheimer et al. 2020). These biofilms are communities of colonizing organisms formed on
68 submerged rocks (Aubertheau et al. 2017; Flemming et al. 2007), composed primarily of
69 microbial cells (bacteria, fungi, protozoa, and algae), extracellular polymeric substances (EPS)
70 produced by bacteria and algae, organic and inorganic materials, and detritus (Flemming et al.
71 2007).

72 The biofilm structure remains attached to the rock through the EPS matrix, which
73 includes polysaccharides, proteins, nucleic acids, phospholipids, and humic substances
74 (Flemming and Wingender 2010). EPS can represent 50-90% of the total organic carbon
75 (TOC) present in biofilms (Matin et al. 2011). This matrix is responsible for imparting
76 adhesive properties, surface fixation, and mechanical stability, allowing the organisms present
77 in the biofilm to develop in association (Wuertz et al. 2004). The heterogeneity of biofilms
78 and the metabolic processes of the various constituent microorganisms result in
79 physicochemical microzones with substrate gradients and metabolic products (Stewart and
80 Franklin 2008). In this sense, several biofilm components (living organisms, EPS, organic
81 compounds, and minerals) can interact with chemical compounds contained in water (Julien et
82 al. 2014), especially inorganic and organic contaminants.

83 The composition of the epilithic biofilm, especially the presence of distinct functional
84 groups present in EPS, can play an important role in the retention of, inorganic contaminants
85 (e.g.: heavy metals), organic contaminants (e.g.: pesticide residues and drugs) and nutrients
86 (Flemming et al. 2007). Thus, the characterization of the *in situ* epilithic biofilms
87 composition, especially if associated with the surrounding anthropic activities, could be an
88 important tool in determining its adsorption potential. The determination of the organic
89 molecular structure of EPS active functional groups can be carried out with standard
90 techniques used in organic geochemistry such as: (i) gas chromatography coupled to mass
91 spectroscopy (CG/MS), (ii) infrared spectroscopy (IR), (iii) nuclear magnetic resonance
92 (NMR) spectroscopy, (iv) liquid chromatography-organic carbon detection-organic nitrogen
93 detection (LC-OCD-OND) (Stewart et al. 2012) e (v) confocal laser scanning microscopy
94 (CLSM) (Schlafer and Meyer 2017).

95 Pyrolysis coupled to gas chromatography and mass spectroscopy (PY-CG/MS) can
96 establish the mean distributions of biopolymers present in biofilms (Julien et al. 2014).
97 Proteins, polysaccharides, and, to a lesser extent, nucleic acids are the major components
98 present in biofilms, indicating that their formation is associated with EPS production (Zhang
99 et al. 2015). Exposure of biofilms to different nutritional conditions – that is, to environments
100 with different anthropic pressures and soil uses – produces different macromolecular
101 compositions (Lin et al. 2014). With IR spectroscopy, the variability of the composition and
102 quantity of each component under the different conditions studied can be determined (Jiao et
103 al. 2010). The application of the Fourier Transform Infrared (FTIR) spectroscopic technique
104 to biofilms allows the detection of polysaccharides and nucleic acids (carbohydrates, DNA

105 and RNA in the region of 1300 to 900 cm^{-1}), as well as of proteins (1700 to 1500 cm^{-1}) and
106 of lipids (2930 to 2860 cm^{-1}) (Jiao et al. 2010). Two dimensional ^1H - ^{13}C NMR provides
107 details of the types and relative abundances of functional groups associated with the presence
108 of different biopolymers.

109 The study of the organic composition of epilithic biofilms can provide information
110 about the effects due to changes in anthropogenic pressure which took place before sampling,
111 i.e., epilithic biofilms are a more reliable matrix than active water sampling. Studies on the
112 composition and presence of functional groups of epilithic biofilms thus can contribute to
113 further knowledge about their interactions and ecological relationships with aquatic ecosys-
114 tems and the organisms that inhabit them. This knowledge should allow development of better
115 reliable biological indicators, based on the understanding of their interactions with the envi-
116 ronment and of the factors that influence the variability of a biofilm's ability to capture organ-
117 ic compounds (Bonnineau et al. 2020). The study of biofilms is of particular importance for
118 the Guaporé river watershed (Guaporé RW) where 90% of suspended sediments (part of
119 which can be retained in the epilithic biofilms) originate from crop areas (Tiecher et al. 2017);
120 the rate of water runoff is high (Didoné et al. 2015); and several small urban agglomerations
121 without treated domestic residues (Bastos et al. 2018) are located.

122 Research on the chemical composition of biofilms mainly involves controlled
123 experimental systems (Lawrence et al. 2012; Fish et al. 2015). Some recent studies show
124 significant *in situ* results with regards to the composition of EPS (Sambalova et al. 2018;
125 Blanco et al. 2019). Studies which report the characterization of biofilms are scarce. Those
126 that do rarely use epilithic biofilms in their natural environment and/or do not extend the
127 research of their chemical constitution. Studies with emphasis on the characterization of
128 natural epilithic biofilms sampled in rural watersheds in Brazil have never been published.
129 The use of the chemical composition of epilithic biofilms is hypothesized to be as effective as
130 other methods for environmental assessment in water courses. The objective of the present
131 work is the characterization of epilithic biofilms to understand the alteration patterns
132 associated with surrounding anthropic activities.

133

134 **Materials and Methods**

136 **Study area**

137 The Guaporé RW, located in the state of Rio Grande do Sul, southern tip of Brazil
138 (Figure 1), has a Cfa climate according to the Köppen climatic classification (Kottek and
139 Rubel 2010). Its average annual rainfall ranges between 1,400 and 1,700 mm and its average
140 annual temperature is 17.9 °C. The watershed drains an area of 2490 km² and is 153 km long,
141 with topography predominately by little hilly relief (57%) and hilly relief (25%) in northern
142 part (Table 1) (Castro Lima et al. 2020). Agriculture and livestock are the main economic
143 activities, while urban areas occupy only 0.6% of the area (Tiecher et al. 2017).

144 Two sub-watersheds present in the northern part of the watershed, the Capingui River
145 sub-watershed and the Marau River sub-watershed, were selected for sampling biofilms due
146 to the varied sources of contamination (forest, agriculture, animal breeding, dam, urban – near
147 the river and riverbanks). The Capingui River sub-watershed has an area of 269 km², which
148 contains crop lands mostly used for soybeans (*Glycine max* (L.) Merrill) and corn (*Zea mays*,
149 L.) production in no-tillage systems, a preserved national forest and a dam. The Marau River
150 sub-watershed, which has an area of 257 km², had urban and rural activities. It is
151 characterized by the predominance of family agriculture and livestock production, based on
152 poultry, swine, and milk production. The soybean, corn and cereal production coexist with the
153 livestock production. The sub-watershed has a contribution of wastewater from urban areas of
154 the city of Marau (over 40 thousand inhabitants), in which there are agribusinesses, especially
155 large slaughterhouses for swine and poultry for export, and leather processing and mechanical
156 metal industry (Rheinheimer et al. 2020; Castro Lima et al. 2020).

157 The confluence of these two rivers (Capingui and Marau) gives rise to the Guaporé
158 River – an agronomically important river in the region, with a catchment area of 542 km² –
159 begins. This river is responsible for supplying the hydric needs of local agricultural activities
160 (soybean, corn, cereals, tobacco, vegetables and fruit), crops animal production (swine,
161 poultry and cattle), and, finally, for human consumption (Tiecher et al. 2017). The soil use is
162 composed of 66.8% crops and pastures; 22.4% forests; 5.2% exposed soil; 4.8% urban areas;
163 0.8% water bodies.

165 **Sampling sites description**

166 The descriptions of the nine sampled sites are present below and in Table 1.

167

168 The Capingui River sub-watershed sampled sites:

169 *C1* - the National Forest of Passo Fundo (FLONA), a Federal Conservation Unit with
170 1300 hectares. The site represents the headwaters of sub-watershed and is considered as a
171 control sample due to low anthropogenic action;

172 *C2* - the Capingui Dam site is located 600 m downstream from the Capingui River Dam
173 spillway. The riparian forest is limited to a strip between 15 and 60 m wide and is bordered by
174 grains production under no-tillage system. Currently, the dam is used for recreation and has
175 an increasing human activity specially in summer, resulting in the release of wastewater
176 directly into the dam reservoir;

177 *C3* - the Agriculture site located 17 km from the Capingui dam and represents the
178 contribution of a vast area of agricultural production. Crops occupy 69% of the area and are
179 managed in no-tillage system with soybean and corn in spring/summer and oats/wheat in
180 autumn/winter; and

181 *C4* - the Capingui sub-watershed Outlet site which the crops occupy 71% of the
182 neighboring area. The farming system is similar to that of the Agriculture site and, during the
183 sampling, there was a large concentration of residues from the rearing of confined animals
184 (mainly poultry and dairy cow).

185

186 The Marau River sub-watershed sampled sites:

187 *M1* and *M2* (Integrate Agriculture/Animal) sites – features predominantly integrated
188 agriculture-animal management systems. The applications of animal waste are frequent and
189 occur in greater volume around the breeding areas;

190 *M3* – the sample site is placed inside of Marau city and receives effluents from the city
191 of Marau (41,059 inhabitants) (Ramos and Abrantes 2017);

192 *M4* - Marau sub-watershed Outlet site at 14 km from the city of Marau, integrating
193 urban and rural pollutants; and

194 *G1* - The confluence of two sub-watersheds marks the northernmost point of the
195 Guaporé River.

Table 1 Catchment area, land use and slope class at the different sampling sites in the Guaporé River

Sub-watershed		----- Capingui River -----				----- Marau -----			Confluence	
Sample sites		C1	C2	C3	C4	M1	M2	M3	M4	G1
Main activities		Forest	Dam	Agriculture	Agriculture	Agriculture + animal	Agriculture + animal	Urban	Urban	
Catchment area (km ²)		1.3	123	201	267	13.4	165	227	256	542
Land use (%)	Forest	100	28	24	23	22	20	21	22	22
	Agriculture	0	64	69	71	72	74	72	72	70
Slope Class (%)	0 – 3%	5	10	9	8	6	7	6	6	7
	3.1 – 12%	49	60	59	58	56	60	57	56	57
	12.1 – 20%	35	22	23	23	25	23	24	24	24
	20.1 – 45%	12	8	9	10	13	9	12	13	11
	>45%	0	0	0	0	0	0	1	1	1

197 * Relief classification by EMBRAPA (2013): (0 – 3% flat relief; 3.1 – 12% little hilly relief; 12.1 – 20% hilly relief; 20.1 – 45% very hilly relief; >45% mountainous relief).

198 **Sampling, preparation, and storage of biofilm**

199

200 Epilithic biofilms were sampled in the autumn of 2016 (between May 26 and 28) and in
201 the spring of 2016 (between November 12 and 14). The sampling consisted of manual
202 brushing of the material adhered to submerged rock fragments with toothbrushes (2.54×1
203 cm^2) and 500 mL of deionized water (Aubertheau et al. 2017; Chonova et al. 2018). The rocks
204 were gently shaken in the river water prior to brushing to remove fine particles of suspended
205 sediment deposited on their surfaces. The number of brushed rocks and the total brushed area
206 (m^2) was recorded for each sample site. The brushed area for each rock was determined by
207 wrapping each rock in an aluminum foil with a defined grammage, and posterior laboratory
208 weighing of the foil and calculation of the area of the foil (Table 2).

209

210 **Table 2** Number of brushed rocks and total brushed area at the sampling sites in the Guaporé River

Sample site	Number of rocks brushed		Total brushed area (m^2)	
	Autumn	Autumn	Autumn	Autumn
C1	28	33	2.10	2.55
C2	31	32	1.71	1.37
C3	49	45	2.49	4.77
C4	40	5	2.56	4.72
M1	40	96	3.44	6.52
M2	70	100	3.70	7.57
M3	15	33	1.66	3.87
M4	22	33	1.73	3.76
G1	23	25	1.59	2.94

211

212 The obtained “biofilm suspension” was then poured into glass containers, stored in ice-
213 cold boxes ($4\text{ }^\circ\text{C}$), and transported to the laboratory. Samples were then transferred to
214 individual high-density polyethylene bottles, frozen at $-80\text{ }^\circ\text{C}$ and lyophilized at $-40\text{ }^\circ\text{C}$.
215 After lyophilization, fragments leaves, twig, and invertebrates were manually removed from
216 the samples with a pair of tweezers. With the aid of an agate mortar, samples were ground to
217 obtain a homogenized material.

218

219 **Pyrolysis coupled to gas chromatography and mass spectroscopy (PY-GC/MS)**

220

221 The relative distribution of biopolymers in the biofilms was obtained by PY-GC/MS
222 analysis for only in the biofilm samples sampled in the autumn. The analyses were carried out
223 at the Université de Poitiers (France). For each sample, about 10 mg of lyophilized, ground

224 and dried material was introduced into a small cylindrical quartz tube whose ends were
225 plugged with quartz wool. The tube was placed inside the pyrolyzer Pyroprobe 2000 (CDS
226 Analytical, Inc., Oxford, USA) equipped with a platinum filament. The filament was heated
227 rapidly ($20\text{ }^{\circ}\text{C ms}^{-1}$ ramp) and held at a high temperature ($200\text{--}650\text{ }^{\circ}\text{C}$) for 10 s. Separation
228 and analysis of the products of the thermal decomposition were performed using a gas
229 chromatograph (Hewlett-Packard® HP G1800A operating at $\text{EI} = 70\text{ eV}$, with scanning from
230 $40\text{--}450\text{ a.m.u.}$ (atomic mass units) at 1 scan per s). The system is equipped with BP 20 (SGE
231 Analytical Science) fused silica capillary column ($30 \times 0.25\text{ mm}^2$ with $1.0\text{ }\mu\text{m}$ film thickness).
232 The temperature of the injector remained at $280\text{ }^{\circ}\text{C}$ and the temperature used for the detector
233 was $300\text{ }^{\circ}\text{C}$. The oven was programmed with an initial temperature of $50\text{ }^{\circ}\text{C}$ and the
234 temperature was raised to $250\text{ }^{\circ}\text{C}$ with a ramp of $4\text{ }^{\circ}\text{C min}^{-1}$, then held for 2 minutes. Helium
235 was used as carrier gas at a constant flow rate of 1.0 mL min^{-1} . Thermal decomposition
236 products (pyrolytic fragments) were identified after examining the NIST (National Institute of
237 Standards and Technology) mass spectral library. The fragments were grouped into seven
238 different families of biopolymers: N-containing compounds, polysaccharides, aromatic
239 compounds, phenolic compounds (polyhydroxy aromatic), fatty acids, lignin, and other
240 compounds (Bruchet et al. 1990), and the relative percentage of each family was calculated.

241

242 **Nuclear Magnetic Resonance**

243

244 The solubility of lyophilized epilithic biofilms samples was tested in several organic
245 solvents: acetone, chloroform, acetonitrile, deuterated water, methanol, and blends of these
246 solvents. We chose methanol (CH_3OH) for the extraction of organic compounds from the
247 lyophilized biofilms and deuterated chloroform (CDCl_3) for the NMR analysis. Three serial
248 extractions were made with 20 mL each of methanol on 2 g of lyophilized biofilm, stirring for
249 24 h and filtering through qualitative filter paper. The three extractions were combined and
250 evaporated to dryness in a rotary evaporator. The samples were dissolved in deuterated
251 chloroform (CDCl_3) with 1% tetramethylsilane (TMS) as an internal reference and transferred
252 to 5 mm NMR tubes. ^1H and $^{13}\text{C}\{^1\text{H}\}$ spectra were acquired at 600.13 MHz and 150.92 MHz,
253 respectively, at 298 K. For the $^{13}\text{C}\{^1\text{H}\}$ spectra, the proton couplings were decoupled with
254 power-gated decoupling using a waltz16 pulse sequence. The HSQC $^1\text{H}\text{--}^{13}\text{C}$ NMR spectra
255 were acquired at 298 K with the hsqcetgpsi2 pulse program ($2\text{D } ^1\text{H}/\text{X}$ correlation via double
256 inept transfer using sensitivity improvement, phase sensitive using echo/antiecho-TPPI

257 gradient selection with decoupling during acquisition using trim pulses in inept transfer with
258 gradients in back-inept echo-anti echo) using 16 dummy scans and 16 acquired scans and $^1J_{CH}$
259 = 145 Hz on a 600 MHz Bruker Avance III HD spectrometer located at the Nucleus for
260 Analysis and Organic Research of the Federal University of the Santa Maria (Brazil).

261

262 **Spectroscopy in the IR Region with Attenuated Total Reflectance**

263

264 Organic constituents and distribution of functional groups were realized with the aid of
265 an Agilent Cary 630 FTIR spectrophotometer from Agilent Technologies was used with a
266 horizontal ATR employing a diamond crystal and zinc selenide (ZnSe) optics and windows.
267 The spectra were obtained in the region of 4000–650 cm^{-1} , with a resolution of 4 cm^{-1} and 32
268 scans. Before the readings of each sample, the background IR spectrum was measured using
269 freshly cleaned crystal. Afterward, the direct analysis of the dry samples and lyophilized (in
270 powder form) at room temperature was performed, each sample was spread and compacted on
271 the diamond crystal surface of the ATR and the attenuated total reflectance spectrum was
272 obtained and converted to absorbance by the Kubelka-Munk method. The analyses were
273 carried out at the Laboratory of Inorganic Materials of the Chemistry Department of Federal
274 University of the Santa Maria (Brazil).

275

276 **Statistical procedure**

277

278 Principal components analysis (PCA) were performed with data obtained from the
279 infrared and pyrolysis analyses of the biofilms. The objective of these analyses was to relate
280 these results with the environmental characteristics of each sample site. Statistical analyses
281 were performed using XLSTAT software in Excel for Windows.

282

283 **Results**

284

285 **PY-GC/MS analysis**

286

287 Epilithic biofilms were sampled in different environments throughout the Guaporé RW
288 and, as the amount of anthropic pressure differs in each sampling site, variations in the
289 organic constituents of the biofilms are expected, which the PY-CG/MS analysis should

290 reveal. The fragments identified in the pyrochromatograms were grouped into seven different
291 families of biopolymers. Their relative distributions varied significantly throughout the sub-
292 watersheds. Overall, the biofilms were composed of 22.1–56.8% of N-containing compounds
293 (C-N); 17.6–41.5% polysaccharides (PS); 2.3–27.2% of vegetables (lignin-containing
294 compounds - VEG); 0–8.4% aromatic compounds (CA); 3.5–21.0% phenolic compounds
295 (CF); 6.1–14.9% of fatty acids (lipids - GA), and 0–3.6% of other organic compounds (Figure
296 2).

297 In the pyrochromatograms of the epilithic biofilm samples collected in the Guaporé RW,
298 the main fragments identified were (Figure 3): (A) 3-methylbutanal (PS); (B) Acetonitrile (C-
299 N); (C) 1-hydroxy-2-propanone (PS); (D) acetic acid (PS); (E) hexanoic acid (GA); (F)
300 acetamide (C-N); (G) phytol (VEG); (H) 4-methyl phenol (CF); (I) 4-ethyl phenol (CF); (J) 3-
301 decen-2-one (others); (K) indole (C-N); (L) methyl indole (C-N); (M) tetradecanoic acid (GA);
302 (N) hexadecanoic acid (AG); (O) 3-methyl-butanenitrile (C-N); (P) pyrrole (C-N); (Q) methyl
303 pyrrole (C-N); (R) decandinitrile (C-N); (S) toluene (CA); (T) phenol (CF); (U) phytol (VEG).
304 The fragments observed confirm the microbial activity in biofilms due to the presence of N-
305 containing compounds (Julien et al. 2014), which include proteins, heterocycles, DNA, RNA,
306 among others (fragments B, F, K, L, O, P, Q and R, identified in Figure 3).

307 In the Capingui River sub-watershed, the fragments of 3-methylbutanonitrile (O) and
308 pyrrole (P) found in the biofilms sampled at the Capingui Dam (C2) site confirming the
309 highest percentage of the N-containing compounds found in the site (31.1%), in relation to the
310 biofilms sampled in National Forest of Passo Fundo, FLONA (22.1%); in Agriculture site, the
311 value increased to 56.8%, with the presence of fragments of methyl pyrrole (Q) and
312 decandinitrile (R).

313

314 **NMR analyses**

315

316 The structural characterization of epilithic biofilms was performed by ^1H and ^{13}C NMR
317 spectroscopy. Due to the complexity of the matrix of epilithic biofilms, it was difficult to find
318 a methodology capable of allowing for the visualization of the spectra and their respective
319 peaks of functional groups with minimum interference. The one-dimensional spectra did not
320 allow the unique identification of characteristic peaks of the functional groups. The two
321 dimensional ^1H - ^{13}C Heteronuclear Singular Quantum Correlation (HSQC) experiment was
322 chosen. The experiment correlates carbon atoms with their bound hydrogen atoms, as seen by

323 cross peaks between the ^1H and ^{13}C spectra (acquired independently) which separates
 324 functional groups and spreads the spectra in two dimensions. Proton signals of hydrogen
 325 atoms not bound directly to carbon atoms and ^{13}C signals of quaternary carbon atoms are not
 326 seen as cross peaks. In the ^{13}C NMR, the aliphatic hydrocarbons constituting the lipids
 327 typically have chemical shifts in the range of 0 to 40 ppm; carbon-bonded oxygen bonds (C-O,
 328 CH-O, $\text{CH}_2\text{-O}$, and $\text{CH}_3\text{-O}$) are in the range 45–60 ppm; carbohydrate alcohols ($-\text{CH}_2\text{OH}$), 60
 329 to 90 ppm; and the glycosidic carbon (anomeric carbon that is bound to the alcohol oxygen)
 330 of the polysaccharides, 95 and 106 ppm (Jiao et al. 2010; Fialho et al. 2010).

331 The 2D spectra of ^1H - ^{13}C HSQC showed some similarities in the relative abundance and
 332 composition of biopolymers present in biofilms (Table 3). The cross peak between 77 ppm
 333 (for ^{13}C) and 7.26 ppm (for ^1H) corresponds to the chemical shifts of the residual chloroform
 334 (CHCl_3) in the deuterated solvent (CDCl_3), and that between 0 ppm for both nuclei
 335 corresponds to the internal reference, tetramethylsilane (Figures 4, 5 and 6). In all spectra,
 336 cross peaks at the chemical shifts between 0 and 50 ppm (for ^{13}C in F1) and between 0 and 2
 337 ppm (for ^1H , in F2), which characterize the lipid structures, were observed. They can be
 338 identified by cross peaks of the HSQC spectra in the aliphatic region (0–40 ppm for ^{13}C and
 339 0–2 ppm for ^1H) indicating C-H bonds of $-\text{CH}_3$, $-\text{CH}_2$ and $-\text{CH}$ groups (Silverstein et al. 2006).

340
 341

Table 3 Characteristics of C and H displacements detected by NMR – HSQC

C chemical shift (ppm)	H chemical shift (ppm)	Biofilm constituent	Functional group
0–50	02	Lipids	Alkanes (C–H)
50–60	> 4	Lipids	Ester (C–O)
50–60	3,2–3,8	Polysaccharides	Ethers (C–O)
60–70	3,5–4	Polysaccharides	Alcohol (CH_2OH)
129	5,3	Lipids, polysaccharides, N-containing compounds, aromatic compounds	Alkenes (C=C)

342

343 The alkyl carbon atoms of the ester groups can be seen in the ^{13}C spectra of all the
 344 samples, around 60 ppm, at slightly different positions according to the effect of the
 345 substituent, but with greater intensity in the Agriculture (C3), Capingui Outlet (C4), Marau
 346 integrated agriculture-animal production (M1 and M2) and Marau Outlet (M4) sites. Ethers
 347 (C-O-C bond), functional groups that are part of the structure of polysaccharides, also have
 348 resonances between 50 and 60 ppm. There are structural differences between the samples,
 349 since the sites C1, C2, M3, and M4 do not have cross peaks related to the ether functional

350 group. By contrast, the epilithic biofilms sampled in C3, C4, M1, and M2 sites showed both
 351 ester and ether cross peaks. Esters can be distinguished in the ^1H part of the HSQC spectrum,
 352 since the hydrogen atoms of the α -carbon atom of the acid side of the ether generally exhibit
 353 chemical shifts less than 4.0 ppm (3.2 to 3.8 ppm), whereas the hydrogen atoms of the oxygen
 354 atom bonded to the carbon atom of the ether have shifts greater than 4.0 ppm due to the
 355 electronegativity of the oxygen atom (Silverstein et al. 2006).

356 In the chemical shift region of alcohol groups (65–90 ppm) of the carbohydrate groups
 357 (of which the polysaccharides are part) significant peaks for all biofilms were observed. In all
 358 biofilm samples was visualized the ^1H - ^{13}C cross peaks around 60-70 ppm and 3.5-4 ppm,
 359 respectively, indicative of oxygen-connected $-\text{CH}_2$ groups, that is, $-\text{CH}_2\text{OH}$ (Figures 4, 5 and
 360 6). Polysaccharide glycosidic carbon atoms were not seen in any of the samples, which may
 361 be due to a longer T1 relaxation time. In the biofilms sampled at Capingui Dam (C2),
 362 Capingui Outlet (C4), Marau City (M3), and Confluence (G1), C=C cross peaks for alkene
 363 groups at shifts of 129 ppm for ^{13}C and 5.3 ppm for ^1H were also observed.

364

365 IR analysis

366

367 The results of spectroscopy in the IR region obtained for the epilithic biofilms of the
 368 Guaporé RW reveal several characteristic IR absorption bands (Figure 7). Twelve main
 369 spectral characteristics were observed (Table 4), identified in Figure 7-A, which were grouped
 370 according to the main classes of biopolymers. The extracted spectra indicate the presence of
 371 polysaccharides, $1300\text{--}900\text{ cm}^{-1}$, as well as N-containing compounds, from $1700\text{--}1500\text{ cm}^{-1}$
 372 (Silverstein et al. 2006; Rossel and Behrens 2010).

373

374

Table 4 Characteristics of absorption bands detected by the IR technique

Spectral Characteristic	Wavenumbers (cm^{-1})	Wavenumbers from literature (cm^{-1})	Biofilm constituent	Functional group
1	3694	3670-3580 ^a	Phenolic compounds	O–H
2	3620	3670-3580 ^a	Phenolic compounds	O–H
3	3276	3278 ^b	Water	O–H
4	2925	2930-2840 ^a	Lipids	CH_2
5	1637	1640-1550 ^{a, b, c}	N-compounds	C=O
6	1541	1640-1550 ^{a, b, c}	N-compounds	N–H
7	1030	1300-1000 ^{a, b}	Polysaccharides	C–O
8	1005	1300-1000 ^{a, b}	Polysaccharides	C–O
9	910	910 ^a	Polysaccharides	C–H
10	795	900-650 ^{a, c}	Aromatic compounds	C–H

11	780	900-650 ^{a, c}	Aromatic compounds	C-H
12	746	900-650 ^{a, c}	Aromatic compounds	C-H

^aBarbosa (2007); ^bViscarra Rossel and Behrens (2010); ^cSilverstein et al. (2015).

375
376

377 Polysaccharides are identified by absorption bands between 1030 and 1005 cm⁻¹, which
378 correspond to C-O-C elongation vibrations of ethers, a group of organic fragments which
379 forms part of the polysaccharide structure (Figure 7). Acyclic and cyclic ethers, and related
380 compounds such as acetals and ketals, exhibit typical IR absorption patterns in the region of
381 1300-900 cm⁻¹ (Barbosa 2007). The absorption band at 1410 cm⁻¹ corresponds to hydroxyl
382 groups (OH) attached to the -CH₂ group of polysaccharides (Rossel and Behrens 2010). The
383 significant decrease in the intensity of these bands corresponds to the degradation of
384 carbohydrates (Fialho et al. 2010).

385 The 1637 and 1541 cm⁻¹ bands are attributed to the primary amides: amide I in proteins
386 and amide II in proteins, respectively (Silverstein et al. 2006; Rossel and Behrens, 2010).
387 Primary amides have a carbonyl group (C=O) attached to an NH₂ group and may also have
388 alkyl or aryl groups attached to the N atom. In all amides, the IR spectra have an absorption
389 band corresponding to the C=O stretching vibration, which is known as the amide band I
390 (band 5, of Figure 7). In the amide spectra with one or two nitrogen-bonded hydrogen atoms,
391 stretching and angular deformation bands of N-H bonds (amide band II, represented by band 6
392 of Figure 7) are also observed. The stretching bands of the C-N bond are less important, from
393 the point of view of identification of this class of compounds (Rossel and Behrens, 2010).

394 The spectra reveal bands typically associated with the aliphatic hydrocarbon portion of
395 fatty acid and lipid molecules (symmetric and asymmetric stretches of CH₃ and CH₂ in the
396 region of 2980–2800 cm⁻¹) at low absorbance intensities. In general, the positions of the
397 absorption bands relative to these stretches varied little, with methyl groups exhibiting the
398 asymmetric stretch absorptions around 2975-2950 cm⁻¹, which could easily be distinguished
399 from the asymmetric stretch of CH₂ that appeared around of 2930-2920 cm⁻¹ (Rossel and
400 Behrens 2010). The decrease in the intensity of the bands in this region, as is observed for
401 band 4 at the Capingui Dam (C2) and Agriculture (C3) sites in autumn, indicates the loss of
402 aliphatic structures (Fialho et al. 2010).

403 Absorption bands 10, 11, and 12 refer to C=CH bonds of aromatic compounds since the
404 angular deformations outside the plane of C=CH and the angular deformation vibrations
405 outside the ring plane result in absorption bands in the region of approximately 900–650 cm⁻¹.

406 Absorption bands 1 and 2 are referred to OH stretching vibrations of phenol groups
407 (Silverstein et al. 2006; Barbosa 2007).

408 Because extracellular polymeric substances are usually mixtures of various
409 biomolecules, some absorption bands may overlap, and even IR characteristic absorption
410 bands may interfere. In this way, some absorption bands could be assigned to different types
411 of compounds. The broad absorption band between 3000 and 3500 cm^{-1} , for example, was
412 attributed to stretching vibrations of both O-H of phenolic compounds and N-H. Absorption
413 bands observed below 1000 cm^{-1} (795, 780, 746 cm^{-1}) may belong to phosphoric groups of
414 DNA and RNA or to C-H bonds of aromatic compounds. There is also a possible overlap of
415 the amide band I at 1650 cm^{-1} with the absorption bands due to the axial deformation of C=C
416 of alkenes and the stretching of C=C bonds of aromatic compounds (Silverstein et al. 2006;
417 Barbosa 2007; Sun et al. 2009; Rossel and Behrens 2010; D'Abzac et al. 2010).

418

419 **Discussion**

420

421 Relative distribution of the biopolymers can provide useful information about the origin
422 of the fragments (Julien et al. 2014). Likewise, the high percentage of polysaccharides
423 confirm the production of EPS substances by epilithic biofilms (Fragments A, C and D,
424 Figure 3). Polysaccharides are among the major constituents of extracellular polymeric
425 substances, including the biofilm development stage (Jiao et al. 2010) and are responsible for
426 determining the thickness and "consistency" of the layer of biofilm that develops on the rocks.
427 According to Jiao et al. (2010), thicker and more advanced biofilms present a more diversified
428 microbial community, influencing the production of polysaccharides. In general, bacteria
429 respond to environmental variations or stressing conditions by producing either more or less
430 EPS through their cellular membranes (Jiao et al. 2010; Colica et al. 2014). The composition
431 of EPS also changes to protect the bacterial cells and to favor their acclimatization to their
432 living environment. A high percentage of polysaccharides indicates more production of EPS
433 substances by epilithic biofilms which, in turn, suggests a higher stability and greater
434 protection provided by the environment.

435 The National Forest of Passo Fundo (FLONA) represents a "quasi-natural environment"
436 because the anthropic action is extremely low due to the establishment of mixed forest almost
437 a century ago. There is no nitrogen input in the system except the natural fixation of N_2 . The
438 "natural" epilithic biofilms are 22.1% of biopolymers N-containing and 41.5% of

439 polysaccharides (Table 5). The epilithic biofilms monitored in anthropized areas are richer in
 440 nitrogen compounds and contain lower levels of polysaccharides than the FLONA samples.
 441 The differences in the N-containing/polysaccharide ratios of biofilms are most likely related
 442 to direct nitrogen transfers from cultivated soils to watercourses. The agricultural model used
 443 in the south of Brazil is based on family agriculture with the integration of grain production
 444 (soybean and corn) and animal husbandry (swine, poultry and bovine milk) to industry. In the
 445 in anthropized areas, there are three sources of nitrogen to plants: biological fixation of N₂ by
 446 soybean, animal waste and industrialized fertilizers. In the Guaporé River watershed,
 447 representative of South-Brazilian agriculture and also the Mercosur region, there is an excess
 448 of nitrogen (Kaiser et al. 2015) and other nutrients (Zafar et al. 2017). Cultivated soils are
 449 responsible for more than 90% of the sediments and nutrients that are transferred to
 450 watercourses (Tiecher et al. 2017). The biofilm contents are more related with the anthropic
 451 activities along riversides, not having a strong direct relationship with the landscape (Table 5).
 452 Except for the FLONA, sampling site with preserved riparian forest, the other sites monitored
 453 have similar reliefs and widths of riparian forest and, therefore, are no obstacles to the transfer
 454 of pollutants from the crops.

455

456 **Table 5** Land use (agriculture and animal production), relief and riparian forest affecting the epilithic
 457 biofilms composition
 458

Site		Agriculture	Animal production*	Relief (3-12%)	Riparian Forest width	Biofilms composition (%)	
		%		%	m	N-containing	Polysaccharides
Capingui sub-watershed							
C1	Forest	0	–	49	> 800	22.1	41.5
C2	Dam	64	+	60	15-60	31.1	36.0
C3	Agriculture	69	+++	59	< 15	56.8	24.1
C4	Outlet	71	++	58	< 15	38.1	20.6
Marau sub-watershed							
M1	Integrated agriculture-animal	72	++++	56	< 15	40.0	17.6
M2	Integrated agriculture-animal	74	+++++	60	< 15	49.9	21.4
M3	Urban	72	†	57	< 15	38.7	26.5
M4	Outlet	72	+++	56	< 15	30.3	25.2
G1	Guaporé River	70	+++	57	< 15	22.8	32.5

459 * Swine manure equivalent (kg ha⁻¹ day⁻¹) = (number bovine × 5 + number swine + number of poultry × 0.005) ×
 460 6 kg manure day⁻¹ ha⁻¹). + < 10 and +++++ > 50. †Domestic sewage of 41.320 inhabitants.

461

462 The N-containing compounds contents varied from 31.1 to 56.8% and from 30.3 to 49.9%
463 in Capingui and Marau sub-watersheds, respectively. In the outlet of the two sub-watersheds,
464 the epilithic biofilms are less rich in nitrogen compounds, expressing a dilution effect since
465 the water flow increases exponentially. This emphasizes that natural resilience occurs in
466 watercourses and the tendency is that these return to their original physicochemical
467 characteristics. In contrast, polysaccharide contents decline linearly from FLONA
468 downstream of the Capingui sub-watershed, reaching only 50% of the content of "natural"
469 biofilms. Even at the head of the Marau River sub-watershed, the polysaccharide levels are
470 extremely low and are consistent with intense animal production and high cultivated area. In
471 addition, farmers remove the terraces from the no-till system and the width of the riparian
472 forest is insufficient to prevent sediments and animal waste from reaching the small
473 waterways.

474 In the Marau sub-watershed, the high percentages of phenolic compounds in the
475 biofilms sampled at Marau City (M3) and Marau Outlet (M4) can be explained by the
476 presence of the T fragment (phenol, Figure 3). For aromatic compounds, the M3 site,
477 characterized by the presence of the city of Marau, presented the second-highest concentration
478 of these compounds (5.4%). The integrated agriculture-animal production site (M1) presented
479 the highest concentrations (8.4%) of these compounds. Also, in both sites (M1 and M2), high
480 animal waste production could be responsible for the higher intensity of tetradecanoic acid
481 fragment, highlighting a higher percentage of lipids in these sites (14.30% and 14.89%,
482 respectively).

483 The principal component analysis (PCA) related to the pyrolysis results of the epilithic
484 biofilms sampled in the autumn and their interactions with the soil use and width of riparian
485 forest is represented at Figure 8. The first two components represent 67.12% of the
486 explanation capacity of the total variance of the set of samples. Through the PCA, it is
487 possible to verify the emergence of relationship among anthropic pressures and biofilms
488 composition. The ligninine derived material (VEG) and PS variables with less anthropized
489 areas (C1, C2 and G1) and C-N, CF and CA associated with the more anthropized ones.
490 These relations are supported by data, particularly showed by first PC, who explains 39,22%
491 of total variance. The distribution of vectors and sites highlights that soils with agricultural
492 activities increase the presence of nitrogen compounds in biofilms, whilst polysaccharides are
493 related to the forest and less intensive soil uses.

494 The high values of phenolic compounds observed in C3 (9,8%), C4 (12,4%), M1
495 (11,5%), M3 (12,0%) and M4 (21,0%) sites appear to be related to urban and in animal
496 activities (Figure 8). Their origin in the waters and, consequently, accumulation in biofilms
497 could be attributed to industrial waste, hydrolysis and photochemical oxidation of
498 organophosphorus agrochemicals and microbial degradation of herbicides (Archela et al.
499 2003). The monitored site is located immediately downstream of the city of Marau and
500 received the full burden of urban pollution. Aromatic compounds may also have originated
501 from the urban activities occurring in the watershed and can reach water bodies through
502 domestic and industrial effluents, atmospheric origins (through the precipitation of particles
503 and gases from the burning of fossil fuels and wood), and synthesis by organisms. Some
504 organisms such as bacteria, algae, and fungi can produce aromatic compounds naturally
505 through biogenic processes (Catter et al. 2007; Rocha et al. 2007).

506 Organic compounds that are known to arise from plants are referred to as vegetable
507 chains, which are the result of chlorophyll degradation. The G1 site presented the highest
508 vegetable content, with the presence of the U (phytol) fragment. The presence of a large
509 amounts of microorganisms inside the biofilm matrix leads the hypothesis that the
510 environments less impacted by environmental pressure favor the activity of microorganisms
511 which degrade vegetal pigments constituted by lignin. In this site, due to the great depth of the
512 river at the site, sampling was carried out at the river bank which increases the presence of
513 vegetal fragments (leaves, vegetal residues) and their uptake into the biofilms. The highest
514 concentrations of lipids were found in the confluence (G1), Capingui Dam (C2) and FLONA
515 (C1) biofilms. The microorganisms accumulate a maximum amount of oil to ensure that the
516 growth medium is limiting in the amount of available nitrogen (Ratledge 1991).

517 Epilithic biofilm structure was similar between the sites, consisting of a mixture of
518 polysaccharides, N-containing compounds, lipids, plant chains, aromatics, and phenolic
519 compounds. This is because the biofilm matrix is naturally (excluding human interference)
520 composed of biomolecules, mainly polysaccharides, proteins, and lipids (Lawrence et al.
521 2012). It is possible to affirm that the different activities near the sample points will influence
522 the composition of the biofilms developed under the rocks at these sites such as high
523 concentrate nitrate by leaching and runoff after nitrogen fertilizer applications. The presence
524 of nitrate anions may affect the growth of epilithic biofilms, limiting or significantly altering
525 the biomass response to nutrient addition. This process is dependent on the constituents and
526 microorganisms present in the biofilm (e.g., fungi or bacteria) and will influence the tolerance

527 of epilithic communities (Tank and Dodds 2003). Enrichment of the aquatic environment with
528 nitrogen sources has important implications for nitrogen cycling (Ribot et al. 2015), whereas
529 tolerance of epilithic biofilms suggests an epilithic community more metabolically diverse
530 and with the potential to carry out an active role in the biogeochemical nitrogen cycling (Vila-
531 Costa et al. 2014).

532 Biofilms from sites with greater development of agriculture will have their composition
533 more marked by higher percentages of N-containing compounds and lower percentages of
534 polysaccharides (Figure 8) because polysaccharide production can be related to the stability
535 and greater protection provided by forest environments or with less agricultural development.
536 Sampling sites with high input of clandestine urban waste have less marked characteristics
537 compared to agricultural areas, with the percentage of N-containing compounds decreasing
538 along the course of the river, as well as the percentage of fatty acids. Sites with less impact
539 will also have a more marked record, mainly by the decrease in the percentage of N-
540 containing compounds at these sites and the considerable increase in the percentages of
541 polysaccharides and vegetable compounds. Considering FLONA as a site of less anthropic
542 interference or "witness", one can verify a tendency of the biofilm to recover its initial
543 characteristics of composition, that is, potential resilience of the epilithic biofilms confronted
544 with the adversities presented in the water course in which they develop.

545 The presence of lipids shown in the NMR spectra indicates the predominance of long-
546 chain hydrocarbons such as tetradecanoic and hexadecanoic acids found in the PY-CG/MS
547 analysis fragments. The most obvious difference between samples is the difference in their
548 relative ethers and esters levels, both were found at points C3, C4, M1 and M2. For chemical
549 shifts in the region of the alcohol groups of the carbohydrate functions, differences in spectral
550 characteristics could be explained by significant differences in the distribution of sugar
551 monomers within polysaccharides (Jiao et al. 2010). Such hydrocarbons may be present in the
552 polysaccharide fragments, in the N-containing and benzene-linked compounds of the aromatic
553 compounds. The differences in polysaccharide peaks between samples are indicative of a
554 difference in carbohydrate composition or the local chemical environment (Reichhardt et al.
555 2015). As in this work, there was no distinction in the EPS binding to the bacterial cell wall,
556 the large contributions of polysaccharides are consistent with the thick hyphal cell walls of
557 biofilms. NMR techniques also indicated that biofilms exhibit various polysaccharides,
558 proteins, fatty chains, and aromatic structures probably responsible for their binding
559 properties with surfaces (rocks, sediment particles) or other compounds.

560 The IR spectra of biofilm samples are all similar, both in shape and in absorbance
561 intensity, indicating that the technique was non-discriminant in the composition of epilithic
562 biofilms in qualitative terms (Figure 7). IR analysis shows that polysaccharides absorption
563 bands were similar, both in autumn and spring and between sampled sites. Proteins and lipids
564 absorption bands were larger in the autumn and for virtually all samples.

565 The ACP was performed from the absorbances and wave numbers obtained in the IR
566 spectra, between the points sampled in autumn and spring (Figure 9). The first two main
567 components represent 82.58% of the capacity to explain the total variance of the data set. The
568 epilithic biofilms sampled in autumn at C1 (Flona), C2 (Dam) and G1 (Confluence) sites are
569 very similar and have high percentage of polysaccharides and ligninine derived material and
570 low percentages of compounds containing N. The C3 and M2 sites are located near each other
571 which explains the higher concentrations of compounds containing N. The biofilms collected
572 at M1, M3 and M4 sites are in the same quadrant and presented the highest concentrations of
573 phenolic compounds. The epilithic biofilms sampled in spring formed three distinct groups: (a)
574 M4, M1 and C2 sites; (b) M3, M2 and G sites; (c) C1 and C3 sites.

575 According to D'Abzac et al. (2010), interference between the absorption bands does not
576 allow quantification of the amounts of biochemical compounds present in biofilms, but the IR
577 spectra revealed an overview of the functional groups present. The analysis of PY-GC/MS, on
578 the other hand, had a significant variation in the quantity of each of the individual components
579 of the biofilms.

580 PY-GC/MS can be used to confirm lipids by the presence of tetradecanoic and
581 hexadecanoic acids in the pyrochromatogram. Lipids can be classified, according to their
582 chemical structure, into hydrolyzable (with ester groups) and non-hydrolyzable (without ester
583 groups). This finding was also reported by many authors (Branda et al. 2005; Jiao et al. 2010;
584 Lin et al. 2014; Zhang et al. 2015), but none of them investigated “*in situ*” epilithic biofilms
585 directly collected from rocks, especially in a watershed exposed to both to intense agricultural
586 and urban pressures like observed in south Brazil.

587 Due to their complexity, epilithic biofilms are particularly challenging to analyze
588 chemically, mainly because they represent environmental samples. However, naturally grown
589 epilithic biofilms can be efficiently utilized in reducing some water pollution issues. Epilithic
590 communities are sensitive enough to discriminate the different periods in which nutrients are
591 applied to the environment, mainly through agriculture (land use, seasons and type of crop)
592 (Rheinheimer et al. 2020) and the presence of urban areas (Fernandes et al. 2019), causing, for

593 example, eutrophication of water bodies near livestock farms. As biofilms show great
594 adaptability to its environment, they can undergo changes due to the capture of the
595 contaminants present in water bodies (Morin et al. 2010; Fechner et al. 2012; Lambert et al.
596 2012). Environmental heterogeneity can be considered an essential factor for the development
597 of spatial resilience in biofilms. In sites with eutrophication, epilithic biofilms can be used as
598 bioindicators of pollution and for water quality assessment. These communities can respond
599 positively to nutrient inputs to some extent and, subsequently, with further enrichment, can
600 lead to changes in the biomass, structure, and functions of the biofilm, all of which can be
601 measured and used in actions to combat and reduce pollution. The efficient use of biofilms at
602 these sites must consider the connections between sustainable farming practices, the nutrient
603 delivery, the biological response, and recovery trajectories in different types of water bodies.
604

605 **Limitations of the research and future research directions**

606

607 The use of epilithic biofilms as a matrix for monitoring the health of water resources is
608 promising, however, the elucidation of the processes of accumulation, transformation and
609 resilience are still insipient in the international literature. The knowledge of the variations in
610 the organic composition of biofilms already is useful as a bioindicator and may aid in
611 understanding their functionality in relation to specific types of pollutants. Brazil has
612 transformed its agriculture into large extensions of genetically modified crops to tolerate
613 pesticides (37 million ha of soybean and 14 million ha of corn in 2020). As a result, there is
614 an enormous diversity and quantity of pesticides being transferred to aquatic systems. The
615 detailing of the composition of epilithic biofilms is practically mandatory to the understanding
616 of the dynamics of pesticides in the aquatic environment, such as the accumulation of
617 glyphosate and its metabolite - AMPA (Fernandes et al. 2019). Similarly, Brazil is the largest
618 producer of meat in a confinement system (swine and poultry, specifically) with intensive use
619 of veterinary drugs. The waste is discarded in the soils and indirectly reaches the surface
620 aquatic reservoirs, carrying large quantities of drug residues (Bastos et al. 2018).

621 The applicability of these analyses *in situ* is a useful tool for the monitoring of sites
622 exposed with contaminants as, pesticides, drugs, and other organic compounds and the
623 subsequent adsorption of these residues to biofilms. Even with the use of sophisticated
624 techniques and equipment, it is not easy to elucidate in detail the organic complexity of both
625 living biofilm organisms and their exudates. There is a need to integrate studies of the organic

626 composition of epilithic biofilms with changes in the microbial community induced by
627 anthropic action (Carles and Artigas, 2020) and their resilience capacity, and even with the
628 appearance of microbial resistance to antibiotics, as demonstrated by Bastos (2017). Another
629 applicability of this analysis protocol is the potential use of biofilms in bioremediation and
630 biotransformation of organic pollutants, as they can tolerate toxic effects of chemicals due to
631 their natural properties and tenacity (Edwards and Kjellerup 2013). Therefore, it is important
632 to carry out the characterization of the entire biofilm by combining the two systems,
633 organisms, and extracellular material.

634

635 **Conclusions**

636

637 The PY-GC/MS, IR, and NMR techniques indicated that biofilms exhibit various
638 polysaccharides, proteins, fatty chains, and aromatic structures probably responsible for their
639 binding properties with surfaces (rocks, sediment particles) or other compounds. The use of
640 the chemical composition of epilithic biofilms has proved effective as auxiliary tool for
641 environmental assessment in water courses. Biofilms from more developed agricultural sites
642 will have their composition more marked by higher percentages of N-containing compounds
643 from nitrogen fertilizers applied to the soil and lower percentages of polysaccharides.
644 Biofilms from sites with lower soil use and management intensity, on the other hand, will
645 have higher percentages of lignin-containing compounds and polysaccharides in their
646 composition. Thus, the development of epilithic communities may be influenced by changes
647 that are occurring in the environment and at certain periods of the year, including changes due
648 to greater or lesser intensity of land use or greater or lesser discharge of urban effluents. The
649 study showed the applicability of the methods in in situ sampled epilithic biofilms and
650 confirmed the expected constituents based on controlled systems. Furthermore, observations
651 reinforce the complex biochemical nature of epilithic biofilm communities. Although
652 environmental biofilm systems have been studied for several decades, there are still research
653 gaps to be filled due to the typically heterogeneous composition of this matrix, from a spatial
654 point of view.

655

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821 **Figures**

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824 **Figure 1** Location of the Guaporé river watershed in Brazil and epilithic biofilm sampling
825 sites

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827 **Figure 2** Relative distribution of biopolymers in epilithic biofilms collected in autumn in the
828 Guaporé RW

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830 **Figure 3** Pyrochromatograms of biofilms samples from Guaporé RW

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832 **Figure 4** Carbon and hydrogen HSQC 2D spectra for biofilms of the Capingui River sub-
833 watershed

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835 **Figure 5** Carbon and hydrogen HSQC 2D spectra for biofilms of the Marau River sub-
836 watershed

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838 **Figure 6** Carbon and hydrogen HSQC 2D spectrum for biofilms of the main course of
839 Guaporé watershed

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841 **Figure 7** IR spectra for epilithic biofilms collected in autumn (A) and spring (B)

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843 **Figure 8** Analysis of main components of pyrolysis data from biofilm sampled in autumn at
844 the Guaporé River watershed. Site sampled: C1 to C4 sites in Capingui River watershed; M1 to M4 sites in
845 Marau River watershed; G1 site in confluence (Guaporé River). Organic groups: C-N = N-containing
846 compounds, PS = polysaccharides, VEG = lignin-containing compounds, CA = aromatic compounds, CF =
847 phenolic compounds, GA = fatty acids – lipids, Others = other organic compounds.

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849 **Figure 9** Analysis of main components of infrared spectra data from biofilm sampled in
850 autumn (blue rhombus) and spring (red rhombus) at the Guaporé River watershed. Site sampled:
851 C1 to C4 sites in Capingui River watershed; M1 to M4 sites in Marau River watershed; G site in confluence
852 (Guaporé River).

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