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ABSTRACT

Present study evaluated the relationship between estrogenic hormones concentrations (17α-ethinylestradiol and 17β-estradiol) in surface waters in the Metropolitan Region of São Paulo (Brazil) and environmental variables. Four sampling stations were monitored ranging from a protected area to streams discharging human effluent in and around Billings Reservoir. Four sampling campaigns were carried out in each seasonal period: DRY and WET. Samples for hormone analysis (in ng L^{-1}) were concentrated (1000X) using solid phase extraction C_{18} cartridges and analysed by liquid chromatography coupled to quadrupole mass spectrometry detection, with 100 ng L^{-1} limit of quantification. Water temperature, pH, electrical conductivity (EC) and total dissolved solids were determined *in situ*; total phosphorus and *Sinapis alba* bioassays were performed subsequently. Reservoir active capacity (AC) and precipitation were also obtained. Estrogenic hormones concentrations were always below limit of quantification at pristine site; at the other sampling stations, 17β-estradiol concentrations varied from below limit of quantification to 1,720 ng L^{-1} and 17α-ethinylestradiol from below limit of quantification to 1,200 ng L^{-1}, with the highest concentrations found in the streams discharging into the reservoir. These streams showed higher Pearson's correlation between 17α-ethinylestradiol, total phosphorus, and electrical conductivity when compared with reservoir stations. Germination index and EC presented negative correlation (Pearson's r = -0.61), denoting a phytotoxicity increase with EC increment. AC influenced the dilution of pollutants and showed negative correlations with total phosphorus (Pearson's r = -0.56). These results highlight the relevance of including streams in water monitoring programs, since they are important pollutants loads into watersheds.

Keywords: *17 α-ethinylestradiol; 17β-estradiol; electrical conductivity; endocrine disruptors; streams monitoring; water pollution.*

1. Introduction

In the last decades, an emerging issue in the area of environmental management is the contamination of the water sources with micropollutants (or so-called pollutants of emerging concern), defined as organic and inorganic compounds that, even at low concentrations (in the order of μg L^{-1} and ng L^{-1}), present ecotoxicological risks. These contaminants comprehend a wide range of natural or synthetic chemical compounds, including pharmaceuticals, personal care products (PCPs), hormones, surfactants, flame retardants, pesticides and nanoparticles (Barceló and Petrovic 2008; Quadra et al. 2017). Some chemicals such as phthalates and polychlorinated biphenyls (PCBs) have industrial effluents as their main source of discharge; while pharmaceuticals and personal hygiene products commonly originate from domestic effluents (Ebele et al. 2017).

Among these micropollutants, the group known as endocrine disrupters (EDs) includes exogenous substances that have the capacity to alter organisms endocrine functions, thus causing adverse effects on human health (if ingested) and the aquatic ecosystem (Johns et al. 2011; Rani and Karthikeyan 2016). Although the concentration of these contaminants in the environment is typically low (ng range), adverse effects due to chronic exposure cannot be excluded (Adeel et al. 2017).

Estrogenic hormones are considered responsible for the majority of endocrine effects triggered by the disposal of effluents contaminated with these compounds, since they are very active biologically and are related to the etiology of various types of cancers (Aris et al. 2014; Adeel et al. 2017). Estrogens introduced into the environment may be natural, such as 17β-estradiol (E2), estriol, estrone, or synthetic, such as 17α-ethinylestradiol (EE2) and levonorgestrel, developed for use in hormone replacement therapies and contraceptive methods (Reis-Filho et al. 2006; Pereira et al. 2015). These contaminants may occur as parent compounds or in a partially metabolized form. In general 50-80% of the total parent

66 compounds are excreted in the urine and partly in the animal feces as a mixture of
67 metabolized conjugated compounds (Lienert et al. 2007). Women who do not use
68 contraceptives excrete between 10 and 100 μg of estrogenic hormones daily, whereas women
69 who are pregnant may excrete up to 30 mg of estrogens per day (Baronti et al. 2000).

70 Once released into water bodies, compound's physicochemical properties govern its
71 partition between the water, sediment and biomass matrices in an ecosystem. Compounds
72 with low water solubility and high organic phase partition coefficient (k_{ow}) are generally more
73 present in the adipose tissue of organisms, promoting bioaccumulation along the trophic
74 levels. For example, estrogenic hormones have log k_{ow} values ranging from 2.8 to 4.1,
75 denoting that they are lipophilic (affinity for lipids), poorly soluble in water and more likely
76 to be present in higher concentrations in the biomass, the organic matter that makes up the
77 sediment, or adhered to suspended solids present in the liquid phase of a waterbody (Ebele et
78 al. 2017). However, when excreted by mammals, estrogenic hormones are not in their
79 parental form, but conjugated (i.e. glucuronic acid or sulfate conjugates) which makes them
80 10 to 50 times more soluble in water (Birkett and Lester 2002; Ebele et al. 2017).

81 In the European Union (EU), these substances are currently on a Watch List in order to
82 gather monitoring data for the purpose of facilitating the determination of appropriate
83 measures to address the risk posed by those substances (Directive 2013/39/EU). The United
84 States Environmental Protection Agency (USEPA) included these substances in their Third
85 Unregulated Contaminant Monitoring Rule (USEPA 2012). In Brazil, there are still no
86 national regulations that define concentration limits for these compounds in environmental
87 matrices (Padhye and Tezel 2013).

88 Electrical conductivity (EC) is a normalized measure of the ability of water to conduct
89 an electric current and is directly related to the concentration of dissolved salts in water (Hem
90 2012). According Su et al. (2017), the monitoring of in-stream EC is a feasible alternative to

multi-sampling of hydrogeochemical parameters. EC has been widely investigated as a marker of pollution from wastewater discharges (Chalupová et al. 2012; Thompson et al. 2012). EC measurements are therefore useful as a screening tool of pollution levels, indicating loads of anthropogenic contribution. Bonvin et al. (2011) found a strong correlation between EC and concentrations of wastewater-derived micropollutants in Lake Geneva. Total dissolved solids (TDS) measures the combination between all inorganic and organic dissolved substances contained in the water and directly correlates with EC values.

Ecotoxicological bioassays for water quality assessment are an environmental monitoring tool that relate the concentration of xenobiotics to a response in the test organisms (Magalhães and Ferrão-Filho 2008; Silva et al. 2015). Belo (2011) recommends that ecotoxicological bioassays with seeds should be used in an integrated way with other chemical parameters for a better understanding. As an example, Dash (2012) verified the toxicity level of raw sewage samples from Bhubaneswar, India, using rice seeds. The germination index (GI) of rice seeds was 70% after 3 days incubation, which could be considered as moderately phytotoxic.

Seasonality may affect the water bodies' quality through greater dilution in rainy periods (Girard et al. 2016; Ling et al. 2017). However, Gomes et al. (2019) found inferior water quality in wet season, elucidating impact of catchment laden pollutant runoff. This was in contrast to the common local perception that rainy season would flush out pollutants.

This study is a part of the project Water Environmental Micropollutant Scientific Initiative (WEMSI), a collaborative partnership between Brazil and Scotland.The work objectives were: (I) evaluate the concentrations of selected estrogenic hormones in surface waters of Rio Grande, a Billings Reservoir Branch, , an important drinking water source for Metropolitan Area of São Paulo (MASP) and in streams which inflows this waterbody; (II) examine correlations between the concentrations of these hormones and environmental

variables, as well as with *Sinapis alba* bioassays; (III) evaluate the influence of seasonality and reservoir active capacity (AC) in pollutant dilution; (IV) evaluate the relevance of streams monitoring as source of pollutants to the reservoir.

2. Material and Methods

2.1 Study Area

Billings Reservoir (Figure 1), located in the Tietê River Basin (Cardoso-Silva et al. 2014), is the largest reservoir in MASP with a surface area of 127 million m² and a maximum depth of 19 m. One of the Billings Reservoir Branches: the Rio Grande, the focus of the present study, is used to supply water to 1.2 million inhabitants of the Greater São Paulo ABC Region (Cardoso-Silva et al. 2014). The waters of Rio Grande Branch are also valuable for amateur fishing; recreation (swimming and boating); landscape aesthetics and agriculture. Unfortunately, this branch also receives raw and treated sewage effluents from legal and illegal surrounding settlements. Preliminary EC monitoring along Rio Grande Branch allowed the selection of four sampling stations reported in Table 1.

2.2. Sampling

The climate in MASP is typically dry in winter and wet in summer (Lima and Rueda 2018). Thus, to evaluate the influence of seasonality in pollutant dilution, eight sampling campaigns were undertaken from June 2017 to February 2018. The dry period (April to August) was identified with "D" and in wet period (September to March) was identified with "W". The dry period sampling campaigns were conducted: DI on 13/6/17; DII on 19/6/17; DIII on 28/7/17 & DIV on 23/8/17. The wet period sampling campaigns were conducted: WI on 27/10/17; WII on 10/11/17; WIII on 7/12/17 & WIV on 22/2/17. Rainfall days were avoided.

141 Subsurface water samples were collected in triplicate (n = 3) using a Van Dorn bottle
142 and stored in 5-L capacity glass bottles previously cleaned with 1 mol L^{-1} HCl (10% v/v) and
143 rinsed with deionized water. Field blank containing free water poured into the container in the
144 field was also preserved and shipped to the laboratory. All samples were refrigerated during
145 transportation to the Laboratory of Environmental Analyses of Federal University of ABC for
146 estrogenic hormones analysis, total phosphorus and *Sinapis alba* bioassays.
147
148 ***2.3. Reagents***
149 Analytical standards (EE2 and E2) with purity levels greater than 98% obtained from
150 Sigma Aldrich were used for the chromatographic analyzes. Organic solvents of High
151 performance liquid chromatography (HPLC) grade with purity > 99.9% (JT Baker) were used
152 for extraction and elution and American Chemical Society reagent grades (Sigma Aldrich) for
153 total phosphorus analysis and phytotoxicity bioassays.
154
155 ***2.4. Estrogenic hormones analysis***
156 Analytical determination of estrogenic hormones was based in EPA Method 539
157 (USEPA 2010). The glassware used during the analysis were washed with Extran detergent in
158 tap water (8% v/v) and then rinsed with deionized water. The concentration of the estrogenic
159 hormones (E2 and EE2) in water samples was performed using solid phase extraction (SPE)
160 as described by Machado et al. (2014). Briefly the collected samples were filtered through a
161 cellulose acetate membrane (0.45 μm porosity) to remove particulate material and the pH
162 adjusted to 3.0. SPE cartridges (C_{18}, 500 mg, 6 mL, Strata Phenomenex) were conditioned
163 with solvents of increasing polarity (methanol and ultra-pure water) at a flow rate of 5 mL
164 min^{-1}.

After conditioning, 1-liter of filtered aqueous sample was passed through the cartridge to extract the target compounds. The cartridges were stored in a refrigerator prior to Liquid chromatography–mass spectrometry (LC-MS) analysis where the retained compounds were eluted with acetonitrile. The solvent was evaporated in an inert atmosphere at 40 °C and residual content suspended in 1 mL of methanol (achieving a 1000X concentration factor).

To assure and control data quality, a QA/QC protocol was planned and systematically implemented along the analytical processes. For SPE blanks were used to assess potential contamination as well as spikes of hormones standards with two-level concentrations used to obtain recovery and accuracy. All experiments were conducted in duplicates to determine analytical precision.

The concentrations of estrogenic hormones were determined using liquid chromatograph (Agilent 1200 Infinity, USA) coupled to a triple quadrupole type mass spectrometer (Agilent 6130, USA).

The simultaneous determination of EE2 and E2 by LC-MS was performed utilizing a C_{18} column (Zorbax Eclipse Plus, 100 mm x 4.6 mm x 3.5 μm, Agilent, USA) at 40 °C with a mobile phase flow of 0.3 mL min^{-1}. The mobile phases consisted of 0.02% NH_4OH in water and 0.02% NH_4OH in methanol under gradient elution (USEPA 2010). The injection volume was 20 μL and detection wavelength at λ = 281 nm. After the chromatographic separation, the mobile phase containing the analytes was subjected to the electrospray ionization stage in the mass spectrometer. The ionization source was operated in the negative mode at a voltage of 4000 V. Dry nitrogen gas was used as the carrier at a flow rate of 10 L min^{-1} and a temperature of 350 ^{o}C. Estrogenic hormones were identified and quantified using an external calibration method over a concentration range of 100 to 5000 μg L^{-1}. Mass spectra quantification and confirmation were acquired in multiple reaction monitoring (MRM) mode using the m/z transitions 295>145 for EE2 and 271 > 159 for E2.

190 The limits of detection (LOD) and limits of quantification (LOQ) were determined by
191 injecting progressively lower concentrations of the standard solutions of E2 and EE2 under
192 the chromatographic condition described above. LOD and LOQ were calculated directly from
193 the calibration plot, considering LOD and LOQ as 3 and 10 ρ/S, respectively, where ρ is the
194 standard deviation (SD) of intercept and S is the slope of the calibration curve. For
195 repeatability evaluation, 10 replicate of both compounds were analyzed at three concentration
196 levels (100, 500 and 1000 μg L^{-1}).

197 LC-MS linear calibration curves resulted in Pearson-r = 0.999 for E2 and 0.997 for
198 EE2. For both compounds, the LOD and LOQ were 30 μg L^{-1} and 100 μg L^{-1}, respectively.
199 Considering the concentration factor of 1000, the LC-MS method enabled quantification of
200 100 ng L^{-1} of each hormone in water samples. The LC-MS analysis of the 10 replicates at the
201 three selected concentrations showed accuracies between 90 and 97%, demonstrating good
202 repeatability of the chromatographic method.

203 The mean recovery for E2 using the Strata SPE cartridges was 89.3 ± 4.1% and for
204 EE2 it was 75.7 ± 4.5%. EPA method 539 (USEPA 2010) outlined the acceptance recovery
205 efficiencies for steroid hormones in water samples. The acceptance criteria are generous, for
206 example, for analysis of E2, the EPA acceptance criteria ranged from 55 to 108% with
207 Relative Standard Deviations (RSD) of 30% and from 55 to 110% with a RSD of 30% for
208 EE2. Results indicate little sample matrix effect on the extraction procedure and the non-
209 necessity of further sample clean-up steps which would be more laborious, expensive and
210 time consuming, besides being an extra source of sample contamination.

211

212 ***2.5. Environmental variables***

213 Daily precipitation and reservoir active capacity data were obtained from Sabesp
214 (2019). Water temperature, pH, EC and TDS were evaluated in the field using a pre-calibrated

multiparameter probe (Horiba U50, Japan). Total Phosphorus (TP) analysis was conducted at UFABC, with all glassware previously decontaminated with 10% (v/v) HCl and rinsed with deionized water. Analytical blanks and a calibration curve (0.01-1.0 mg L^{-1}). Water samples were digested in an autoclave using oxidation reagents and the concentrations determined spectrophotometrically (Hach DR 5000, USA) in the visible ultraviolet region (APHA 2012).

2.6. Sinapis alba bioassays

Sinapis alba bioassays were performed to assess the phytotoxicity of water samples and followed an adapted methodology based on Belo (2011), ISO 11269-1: 2012, Vieira (2016) and Kohatsu et al. (2018). The experiment consisted of placing a filter paper in a 90 mm diameter glass Petri dish and moistening with sample (3 mL) to be tested. Mustard seeds were placed centrally and uniformly (in-line) on the wetted filter paper in each Petri dish. Subsequently, the Petri dishes were capped and wrapped in plastic film to avoid evaporative loss of the sample. The Petri dishes were placed in an upright position with no exposure to light and at (21 ± 2) °C for three days. International Standard Organization (ISO) water (ISO 11269-1: 2012) was used as positive control to determine normal root lengths under optimum conditions and evaluate the seeds quality. The experiments were performed in triplicate.

After the three day period, germination was determined and root length measured with a digital calliper (Mtx, 150 mm, 0.01 mm resolution). Any potential toxic effect was assessed as relative percentage of seed germination (%RSG), relative percentage of root growth (%RRG) and the germination index (GI).

To calculate the %RSG for each plate, equation 1 was used, where $\bar{\bar{S}}g_s$ is the arithmetic mean of the number of germinated seeds in the sample; $\bar{\bar{S}}g_c$ is the arithmetic mean of the number of germinated seeds in the control.

$$\%RSG = \frac{\bar{\bar{S}}g_s}{\bar{\bar{S}}g_c} * 100\% \qquad \text{Equation 1}$$

The relative percentage of root growth (%RRG) for each plate was estimated by equation 2, where $\bar{\bar{L}}r_s$ is the arithmetic mean of the root length in the sample; and $\bar{\bar{L}}r_c$ is the arithmetic mean of the root length in the control.

$$\%RRG = \frac{\bar{\bar{L}}r_s}{\bar{\bar{L}}r_c} * 100\% \qquad \text{Equation 2}$$

After determining %RSG and %RRG, the GI was determined by equation 3:

$$GI = \frac{\%RSG * \%RRG}{100} \qquad \text{Equation 3}$$

The phytotoxicity of the samples was rated according to Belo (2011): GI > 100% - material enhances germination and root growth of seeds; 80 > GI < 100% - no phytotoxic, mature compound; 60 > GI < 80% - moderately phytotoxic; 30 > GI < 60% - phytotoxic; GI < 30% - strongly phytotoxic.

2.7. Statistical analysis

Analyses of Pearson product moment correlation coefficients were used to determine significant associations between limnological parameters (EC, pH, TDS and TP), hormones concentrations and phytotoxicity results. Strong correlation for Pearson coefficient value lies between ± 0.50 and ±1, for moderate degree between ± 0.30 and ± 0.49, and low degree below ± 0.29 (Statstutor 2020). For analysis of variance (ANOVA), a p value <0.05 was considered for a significant statistical difference between the evaluated groups. The Tukey's test results were shown by letters above boxplot representations. Levels that are not significantly different from each other are represented with the same letter (e.g.: a, b or c). Pearson correlation analyses, ANOVA statistics and Tukey's tests were done using Microcal Origin 8.1 software.

Principal Components Analysis (PCA) was done to facilitate interpretation of multivariate results and was performed using PAST 3.20 software (Hammer 2001). Limnological data were treated by PCA to identify possible temporal or spatial patterns

265 among the sampling stations. Factor loadings >0.7 are typically regarded as excellent and
266 <0.3 very poor. In this study, all principal factors extracted from the variables were retained
267 with eigenvalues >1.0 (Yongming et al. 2006).
268
269 **3. Results**
270 ***3.1. Estrogenic hormones concentration***
271 E2 and EE2 levels during the eight sampling campaigns are shown in Table 2.
272 Concentrations of both compounds at station S1 (Reference) were below LOQ (100 ng L^{-1}) in
273 all the sampling campaigns. Station S3 (Ribeirão Pires stream) measurable E2 was found in
274 all campaigns (110 – 1,700 ng L^{-1}) and EE2 in six (210 – 1,200 ng L^{-1}). The S4 sampling
275 station, Tubarão stream, yielded E2 in five campaigns (300 – 1,720 ng L^{-1}) and EE2 in seven
276 (120 – 650 ng L^{-1}).
277
278 ***3.2. Environmental variables monitoring***
279 Precipitation data (mm) within the studied area, together with the Rio Grande active
280 capacity (AC, %) and sampling campaign dates are presented in Figure 2. Total rainfall
281 during the study period was 1157 mm, with 39% in dry (April – August) and 61% in wet
282 (September-March) periods, respectively. The AC (%) showed a different pattern, with a
283 greater capacity (>70%) at sampling DI, DII and WIV. Whereas sampling campaigns WI, WII
284 and DIII occurred when the reservoir was operating at around 65% capacity. This indicates
285 environmental dilution during the dry sampling campaigns could be typically greater than that
286 in the wet sampling campaigns.
287 Figure 3 shows environmental variables boxplots and ANOVA results with Tukey's
288 test. Water temperature was higher in the wet than in the dry season, but no statistical
289 difference was observed seasonally or spatially. Considering seasonal variation no statistical

290 difference was observed within EC, TDS and TP, which might have been influenced by AC
291 variability. However, for these parameters, ANOVA results showed spatial significant
292 differences among stations located in the reservoir (S1 and S2) and in the streams (S3 and
293 S4), evidencing the contribution of the streams to pollution loads in the reservoir with
294 subsequent dilution effect.

295 The mean GI value for samples from the dry period was 83.16 ± 0.32% and the wet
296 season 82.74 ± 0.37% (both no phytotoxic). S3 was the only station where the GI value in
297 both periods was classified as moderately phytotoxic (GI between 60 and 80%). Overall, no
298 seasonal difference among GI values was observed.

299

300 ***3.3. PCA of Reservoir stations***

301 Figure 4 shows PCA results obtained using the general indicators of anthropogenic
302 pollution to reservoir stations (S1 and S2). The first two components explained 97.7% of the
303 total variance. The PC1 represents 78.4% of the data variability and the most influential
304 variables are EC, TDS, pH and GI. PC2 explained 19.2% of the variance, where EC, TDS,
305 and TP are the variables with more influence. Temperature (T) is the most important factor in
306 the PC3, which explains 1.8%. This PCA analysis shows clearly that TDS and EC are highly
307 correlated.

308 The score graph do not shows the separation of sites S1 and S2 into different groups,
309 which may not mean that there is no distinction, and could simply mean that largest sources
310 of variation are similar in both groups. Water samples from sampling campaigns D1-S1, D1-
311 S2, and WII-S2 showed the highest values of the EC, TDS and TP suggesting no existence of
312 spatial and seasonal trends in the pollutant concentrations in the reservoir. The loading
313 graphic permits to observe that EC and TDS showed high positive correlation among
314 themselves and strong negative with TP.

315 Figure 5 (a-c) shows the Pearson's correlation coefficients for AC (r_{S1} = -0.59 and r_{S2} =
316 -0.89), TP (r = -0.56) and GI (r = -0.61) with respect to EC values obtained to reservoir
317 stations (S1 and S2).
318
319 ***3.4. PCA of Streams stations***
320 Figure 6 shows PCA with estrogenic hormones concentration, GI and limnological
321 parameters for the streams stations (S3 and S4). The first two components explained 91.9 %
322 of the total variance. The PC1 represents 63.4 % of the data variability and the most
323 influential variables are E2 and EE2 concentrations and PC2 explained 28.7 % of the
324 variance, where TP most influenced the results.
325 The score graph do not shows spatial distinction between streams S3 and S4 which
326 might indicate that largest sources of variation are similar in both groups. Neither seasonal
327 group distinction was observed within data. The loading graphic permits to observe that E2
328 levels most influenced PCA with no significant correlation to common indicators of
329 anthropogenic pollution (e.g.: EC and TP).
330
331 **4. Discussion**
332 ***4.1. Presence of hormones in Rio Grande waters and adjacent streams***
333 The development of more selective and sensitive analytical techniques has allowed the
334 identification and quantification of these active estrogenic compounds in low concentrations
335 in aquatic environments from different parts of the world (Zuccato et al. 2005; Nakada et al.
336 2006; Kim et al. 2007; Benotti et al. 2009; Kummerer 2009; Sui et al. 2010; Gracia-Lor et al.
337 2012; Tran et al. 2018).
338 At present study, estrogenic hormones levels were not detected at station S1, reflecting
339 the preservation status of this area, which is less impacted by anthropogenic sources. Sousa et

al. (2014) evaluated different emerging pollutants in river water from metropolitan region of São Paulo and Campinas city and did not find detectable E2 and EE2. Even in very clear water, hormones have been reported in the literature, for example, Scala-Benuzzi et al. (2018) found 8.2 ng L^{-1} of EE2 in pristine river waters in Argentina. Caldwell et al. (2012) noted that reported concentrations in the United States and Europe reach about 0.2 or 0.3 ng L^{-1} in low flow conditions, and caution against studies that report higher concentrations as unrepresentative.

At present work, station S2, closer to precarious urbanization with inadequate wastewater disposal and a drinking water abstraction point, yielded measurable E2 levels in three campaigns (860 – 900 ng L^{-1}) and EE2 in two campaigns (210 and 630 ng L^{-1}). This is possibly due to input of raw or inefficiently treated wastewater into the ecosystem. Ghiselli (2006) reported E2 at 1,800 to 6,000 ng L^{-1} and EE2 at 1,000 to 3,500 ng L^{-1} in surface water in Campinas, Brazil. Other Brazilian studies have indicated levels in the order of 500 ng L^{-1} of estrogenic hormones in the Atibaia River, which supplies 92% of Campinas city (Ghiselli and Jardim 2007; Sodré et al. 2010; Maldaner and Jardim 2012). Sodré et al. (2010) identified E2 and EE2 levels, which reached 2,510 and 310 ng L^{-1}, respectively, at places located along the Atibaia River watershed.

Levels of estrogen hormones in effluents might be higher even after wastewater treatment by conventional technologies. Pessoa et al. (2014) found estrogen levels in the effluent of a Brazilian WWTP between <LOD to 776 ng L^{-1} and 3,180 ng L^{-1} for raw wastewater and 397 and 176 ng L^{-1} for activated sludge treated effluent (for E2 and EE2, respectively). These values are of similar magnitude to the concentrations detected in the present study at locations S3 and S4.

Ashfaq et al. (2018) quantified these hormones in influent wastewater in China and reported EE2 concentrations from <LOD to 4.04 ng L^{-1}, but much higher levels of E2 of 46 to

365 150 ng L^{-1}. In Europe, concentrations reported in a review paper by Schröder et al. (2016)
366 remain below 97 ng L^{-1} (E2) and 106 ng L^{-1} (EE2) in WWTP influent. Vymazal et al. (2015)
367 in Czech Republic didn't detected EE2 in influent wastewater but found levels of E2 from
368 <LOD to 199 ng L^{-1}. Adeel et al. (2017), in a global review paper, comment on the
369 particularly high levels of some estrogenic hormones in Brazil. Thus, in the Brazilian context
370 of inadequate sewage treatment, high concentrations should not be dismissed out of hand as
371 and may indeed be cause for concern.

372 All mammals excrete estrogenic hormones, which end up in the environment through
373 direct excretion or through animal waste disposal. Multiple biological effects resulting from
374 the exposure to E2 and EE2 have been described in the literature. In humans, the binding
375 affinity of EE2 to the estrogen receptor is higher than for E2, and has been shown to be up to
376 five times higher in some fish species (Aris et al. 2014). This higher affinity indicates that EE2
377 can be a more potent estrogenic compound in terms of endocrine disrupting effect compared
378 to the naturally produced E2 (Tomšíková et al. 2012). For example, when early life stages of
379 the zebrafish (*Danio rerio*) were exposed to 54 ng L^{-1} of E2, sex ratio was significantly
380 altered (Holbech et al. 2006). EE2 determined in water courses is cause for concern as its
381 estrogenic potency is twenty-five times higher in *in vitro* tests using Zebra fish compared to
382 E2 (van der Belt et al. 2004; Andrew et al. 2010).

383 E2 and EC had a weak correlation (Pearson's r = 0.21) (Figure 7c). This can be
384 explained by E2 sources and biodegradation rates in the environment. Ma and Yates (2018)
385 evaluated E2 degradation in batch assays using river and sediment samples from Santa Ana
386 River (USA). The study reported higher E2 degradation rates in the presence of
387 microorganisms, especially in assays with sediment samples where microbiological density
388 was higher: E2 degradation in water took 10 days and in sediment about 2 days. E2 is
389 typically excreted in conjugated forms such as sulfate and glucuronide which the

390 microorganisms de-conjugate back to parental estradiol. These results supports the low
391 correlation between E2 and EC in the present study, due to their excretion in the conjugated
392 form, which is not detected by the LC-MS method and also by the faster degradation rates of
393 this compound in polluted water containing microorganisms from wastewater, such as
394 *Escherichia coli*. However, as expected, EE2 and EC had a strong correlation (Pearson's r =
395 0.71). EE2 is more resistant to biodegradation than E2 and remains stable even after
396 wastewater treatment by activated sludge process (Panter et al. 1999; Aris et al. 2014). This
397 support the higher correlation between EE2 and EC in the present study that involved
398 sampling stations with raw wastewater disposal (S4) and treated effluent from WWTP (S3),
399 reinforcing the persistence and detectability of EE2.

400 At present study, given that half of the sampling sites were locations where human
401 pollutants would be well diluted (reservoir), the overall average E2 (650 ng L^{-1}) and EE2 (720
402 ng L^{-1}) is of environmental concern. These are 10x and 3000x the reported EC_{50} values to
403 Zebra fish for E2 and EE2 respectively (Figure 8) and are likely to occur in association with
404 other emerging pollutants (van den Belt et al. 2004). This clearly demonstrates the need to
405 eliminate discharge of raw sewage into Brazilian watercourses and to improve existing
406 wastewater treatment procedures and plants, as well include streams in environmental
407 monitoring to avoid pollution of waterbodies used to water supply.

408

409 ***4.2. Spatial distribution of pollutants***

410 During this study, EC and TDS values were one order of magnitude higher in the two
411 streams compared to sampling stations in the reservoir, indicating the influence of
412 anthropogenic sources on the streams. In fact, EC is pointed as a domestic sewage marker
413 (Sousa et al. 2014), since each person consumes an average of 6 g of chloride per day (WHO
414 2003), which increases chloride ion concentrations in effluents. In opposite, undisturbed

415 catchments are characterized by very low in-stream ionic concentrations and by EC < 10 μS
416 cm^{-1} (Markewitz et al. 2006). Yet, EC can be used too assessing water discharge, since these
417 parameters have an inverse relationship due to the fact that new water contributing to runoff
418 has shorter residence time than old water and hence lower ionic content (Weijs et al. 2013;
419 Cano-Paoli 2019).

420 Unexpected, S3 and S4 showed similar pollution levels, despite their difference in
421 discharge content. S3 is a water body which receives huge quantity of raw sewage from
422 Ribeirão Pires municipality. S4 receives effluent from a secondary level WWTP, rich in P.
423 This can be explained by the dilution effect in S3, which has a higher flow capacity, which
424 reduces pollution effects (Gomes et al. 2014, 2019). However, hydrological data of the
425 studied streams are not available, and this is a reality in tropical areas, where hydrological
426 data use to be very scarce (Hartemink et al. 2008, Rodrigues et al. 2018).

427 For all the monitoring stations, the concentration of TP exceeded the Brazilian
428 Standards limit value (Brasil 2005), even at the reference station (S1), reinforcing the
429 anthropogenic contribution by sewage in the reservoir and necessity of to improve sanitation
430 in the Rio Grande basin, aiming the eutrophication control (Mariani et al. 2006; Moschini-
431 Carlos et al. 2010; Wengrat and Bicudo 2011).

432

433 ***4.3. Seasonal variation of pollutants concentration***

434 Rangel-Peraza et al. (2009) showed that water quality parameters have seasonal
435 responses in large Tropical reservoirs, especially for temperature, electrical conductivity,
436 biochemical oxygen demand and total coliforms. In general, wet season may cause soil
437 erosion and bring eroded material to the water body. The dry season may cause river water
438 lost through evaporation, increasing concentrations of suspended materials and dissolved
439 elements (Nguyen et al. 2020).

Rio Grande basin presents two distinct rainfall seasons: wet and dry. Wet season is characterized by higher temperatures. AC presented a strong negative correlation with EC, especially for S2 station thus the lower volume of the reservoir concentrated chemical species enhancing electrical conductivity. For the estrogenic hormones, the weak correlations might be related to their lipophilicity (log $k_{ow, E2}$ = 4.0 and log $k_{ow, EE2}$ = 3.7), partitioning mainly into particle and sediment phases rather than being present in water column, thus not presenting a direct correlation with the dissolved ions responsible for EC. Regarding GI, a negative strong correlation with EC indicates that higher levels of pollution can increase water toxicity, reflecting in lower GI values. As expected, both in dry and wet seasons, GI was less affected at control station S1.

Our findings indicated higher mean values of EC, TDS, TP and low GI (more phytotoxic) to streams (S3 and S4) during the dry season, but without a statistical difference (Figure 3). This can be explained by low rain volume to dilute pollutants in the dry season in the streams. These findings are in according to Coulibaly et al. (2012) who found higher metal (Hg, Cd, Pb, and Zn) concentrations in the dry season than in the rainy season in sediments from Biétri Bay in Ebrié Lagoon, Ivory Coast. These authors attributed this to "reduced water volume during the dry season". Strong correlation between TP and EC and a medium one between GI and EC were observed, as expected, since streams receive domestic wastewater and treated effluent from WWTP loads and are more affected by precipitation dilution. It is noteworthy that only two abundant rainfalls (greater than 20 mm) occurred in the 2 days prior to sampling (DIV and WI). Rodrigues et al. (2018) found higher discharge values in the rainy season. To these authors, pH and EC were higher probably because sewers can discharge via combined sewer overflows an input of water flow to the main stream channel during intense rainstorms which was not observed in the study.

464 The pH had a statistical difference between stations and seasonality (Figure 3). This
465 could be due to eutrophication process. Algal blooms in Rio Grande Branch mainly occur in
466 summer, between the months of December and March (wet period), due to increased run-off,
467 but this also depends on reservoir active capacity, which might dilute these effects (Leal et al.
468 2018). These blooms tend to consume CO_2 from water during photosynthesis and thus
469 contribute to pH increase (Tailing 2010; Brasil et al. 2016). Bicudo et al. (2007) obtained a
470 strong positive correlation between CO_2 and water transparency, as well as strong negative
471 correlations of these variables with chlorophyll-a and pH, when evaluating data from a
472 hypereutrophic lentic aquatic ecosystem in Brazil before and after removal of aquatic
473 macrophytes and subsequent dominance of cyanobacteria. Before removal of the
474 macrophytes, the pH was always neutral to slightly acid and free CO_2 was around 30 mg L^{-1}.
475 After removal of the macrophytes and with the subsequent dominance of the cyanobacteria,
476 the pH was always above 7, reaching almost 11 and free CO_2 decreased dramatically.
477 Algal blooms frequently occur around station S2 which is close to the drinking water
478 abstraction location, therefore the Sanitation Company of the state of São Paulo applies
479 copper sulfate and hydrogen peroxide for phytoplankton and cyanobacteria control in this area
480 (Sabesp 2011). Cyanobacteria lead to socioeconomic problems once toxins released degrades
481 organoleptic water characteristics such as odor and taste, enhancing the cost of water
482 treatment (Leal et al. 2018). Chronic ingestion to levels of $CuSO_4$ may cause respiratory
483 inflammation, hepatotoxicity, and loss of fertility in humans (Huh and Ahn 2017). In
484 developed countries the use of $CuSO_4$ as an algaecide was abolished (Codd et al. 2005).
485 However, in Brazil, it is still one of the most used cyanobacteria control methods due to its
486 low cost and effectiveness (Leal et al. 2018). Additionally, pH might increase due to residual
487 copper hydrolysis (Souza and Wasserman 2015).

GI values for reservoir stations S1 and S2 were lower (i.e. more toxic) in the wet season than in the dry period. In the wet season, it was expected that the dilution factor would decrease the toxicity, given that the rainfall volume was generally higher, despite this AC was typically higher in dry period. Thus, the dilution factor was not the dominant factor in GI of the samples. The opposite was observed with samples taken from the streams S3 and S4 where, as expected, the dry season samples were more toxic.

4.4. Streams monitoring relevance

Stream monitoring supports water resource management in recognize segments severely impaired requiring remediation; identify priority pollutants that might achieve watersheds; prioritize watersheds for restoration; assess effectiveness of storm water management retrofits to capture, slow down, and filter water run-off; track progress in meeting water quality goals and legally mandated water quality standards; and assess and track the health of the watershed ecological functions (US Forest Service 1995; Ziemer 1998).

Freshwater environments offer many ecosystem services for the society such as water, food, water purification, genetic resources, regulation of water flow, recreation and aesthetic aspects (Harrison et al. 2010; Schäfer et al. 2012). In this context, streams have hydrological functions in the environment once they receive direct discharge of wastewater. These pollutant loads are diluted by stream flowrates and are biochemically transformed by the microorganisms present in these ecosystems (Thompson et al. 2018). However, environmental degradation have been threatening these ecosystems functions, especially by the provision of pollutants that change natural characteristics of the environment, as well as presenting some toxicity level to the biota (MEA 2005). For example the release of treated sewage (Pereda et al., 2020) and nanoparticles (Du et al. 2019) may affect ecosystem functions. Pereda et al. (2020) carried out an experiment in a stream, evaluating the influence

513 of effluent discharge from a WWTP on the biota and stream's function. They concluded that
514 structure and functioning of the ecosystem was affected even with well treated and highly
515 diluted discharges. Du et al. (2019) concluded that after a certain dosage of nanoparticles
516 (from 10 to 1000 mg L^{-1} of ZnO), there was a reduction in fungi diversity, which affects the
517 decomposition and the environment ecological functions.

518 These functions highlight the streams importance as attenuators to ingress of
519 pollutants into reservoirs. The present study found hormones levels in the same order of
520 magnitude of raw wastewater, which can cause hazards to biota. This reinforces the
521 importance of the present study of considering streams in water quality monitoring programs
522 aiming reservoirs management and protection especially those directly receiving pollution
523 from its drainage basin.

524

525 **5. Conclusions**

526 This leads us to following main conclusions:

527 • E2 concentrations varied from <LOQ to 1,720 ng L^{-1} and EE2 from <LOQ to 1,200 ng
528 L^{-1}, with the highest levels being present in the two streams, contaminated with raw
529 and treated domestic wastewater, that feed into Billings reservoir. For both of these
530 compounds, this exceeds PNEC and NOEC levels by at least an order of magnitude,
531 indicating a very high ecotoxicological risk in the investigated area and giving rise to
532 possible concern for concentrations in drinking water.

533 • Stream monitoring stations showed higher correlations between TP, GI and EE2 with
534 EC, which indicates wastewater discharge into the streams. Thus, EC is a good marker
535 for these variables in streams and reinforces their relevance for inclusion in water
536 quality monitoring programs. Seasonality influences precipitation and active capacity
537 of the reservoir. These variables influenced the pollutant dilution in present study.

538 This reinforces the necessity of including hydraulic and hydrological data in
539 environmental monitoring. Unfortunately, this kind of data is very scarce in tropical
540 regions yet.

541 • The findings in this paper emphasis the importance of including streams in water
542 quality monitoring programs; not only because of ecotoxicological impacts in these
543 water bodies themselves, but also because they are important as pathways for
544 pollutants to reservoirs, which are used for fishing, bathing and drinking water
545 abstraction.

546

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553

554 **Conflict of interest**

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557

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861 **Table 1.** Sampling stations coordinates and description (IBGE 2010, Trata Brasil 2018).

Sampling Station	Coordinates	Description
S1	-23,7150 S -46,4294 W	A pristine area of the Rio Grande Branch considered as reference at present study. There is no direct disposal of effluents and it is used for bathing and amateur fishing by nearby resident. Average depth is 10 m. Presence of native vegetation and aquatic macrophytes in some periods.
S2	-23,7511 S -46,4694 W	Water abstraction point for drinking water supply located in São Bernardo do Campo municipality. Densely occupied area with precarious urbanization. Application of copper-based algaecides to avoid algal bloom.
S3	-23,7714 S -46,4750 W	Ribeirão Pires stream that receives raw sewage from the municipality of Ribeirão Pires with a population of around 122.000 inhabitants of which only approximately 14% is connected to a sewer. This stream has about 10 m width and 3 m depth and enters the Rio Grande Branch from the North-East. The sampling spot was on a bridge that crosses the stream next to a residential neighborhood that dumps sewage directly into the water body. Presence of solid waste at the borders.
S4	-23,7692 S -46,5322 W	Tubarão stream that receives treated effluent from Parque Andreense Wastewater Treatment Plant (WWTP). This WWTP uses activated sludge technology and treats around 80% of the wastewater from the Parque Andreense neighborhood in Santo André-SP, amounting to a population of around 5,000 inhabitants. WWTP do not include tertiary treatment step, thus disposing high amounts of Phosphorus into the water body. The sampling spot was in a water column of about 20-cm depth close to a community with 20 residences that directly dispose wastewater on the stream.

862 **Table 2.** Estrogenic hormones concentrations and standard deviation (ng L^{-1}) in surface
863 waters of Metropolitan Area of São Paulo, MASP, Brazil (n = 3).

Sampling stations	DRY PERIOD			WET PERIOD		
	Sampling campaigns	E2 (ng L^{-1})	EE2 (ng L^{-1})	Sampling campaigns	E2 (ng L^{-1})	EE2 (ng L^{-1})
S1	DI	<LOQ	<LOQ	WI	<LOQ	<LOQ
S2		860 ± 120	630 ± 80		<LOQ	<LOQ
S3		1,700 ± 300	1,200 ± 140		180 ± 50	300 ± 90
S4		<LOQ	200 ± 80		<LOQ	250 ± 100
S1	DII	<LOQ	<LOQ	WII	<LOQ	<LOQ
S2		<LOQ	<LOQ		890 ± 240	<LOQ
S3		160 ± 30	460 ± 100		250 ± 80	<LOQ
S4		710 ± 160	340 ± 80		630 ± 130	320 ± 120
S1	DIII	<LOQ	<LOQ	WIII	<LOQ	<LOQ
S2		<LOQ	<LOQ		900 ± 110	210 ± 90
S3		120 ± 30	<LOQ		140 ± 50	210 ± 80
S4		300 ± 80	120 ± 30		1,720 ± 560	250 ± 100
S1	DIV	<LOQ	<LOQ	WIV	<LOQ	<LOQ
S2		<LOQ	<LOQ		<LOQ	<LOQ
S3		360 ± 90	450 ± 30		450 ± 130	240 ± 90
S4		<LOQ	650 ± 120		870 ± 150	<LOQ
S1	Number of occurrences	0	0	Number of occurrences	0	0
S2		1	1		2	1
S3		4	3		4	3
S4		2	4		3	3

864

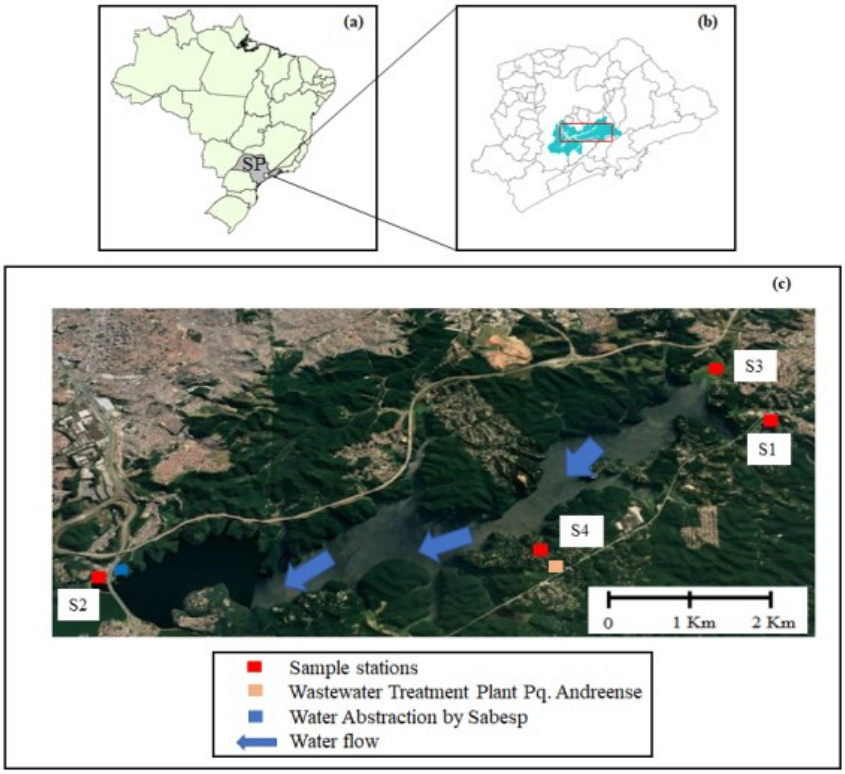

Figure 1. Study area location: (a) São Paulo State in Brazil; (b) Metropolitan Area of São Paulo and Billings Reservoir; (c) Sampling stations on the Rio Grande Branch.

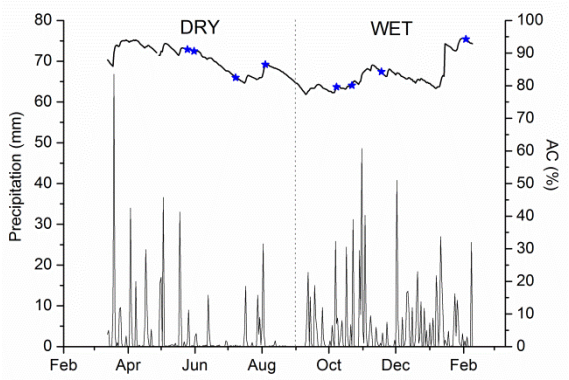

Figure 2. Precipitation (mm) at the studied area and Rio Grande Active Capacity (AC, %) in dry and wet seasons. Stars on the AC plot indicate sampling dates.

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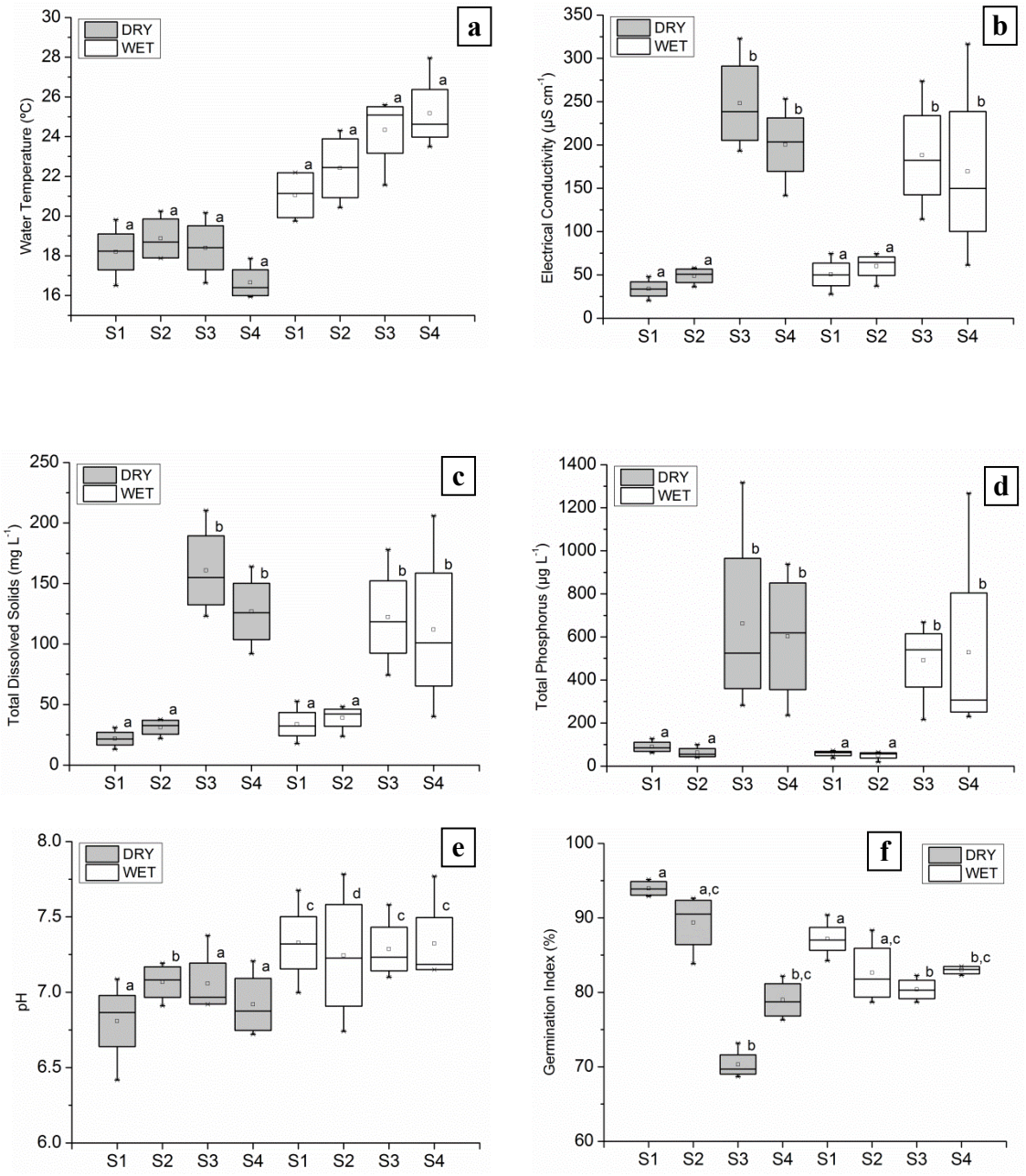

870 **Figure 3.** Boxplot of (a) Water temperature (ºC); (b) EC (µS cm^{-1}), (c) TDS (mg L^{-1}); (d) TP

871 (µg L^{-1}); (e) pH; (f) GI (%) in dry and wet periods, over the four sampling campaigns.

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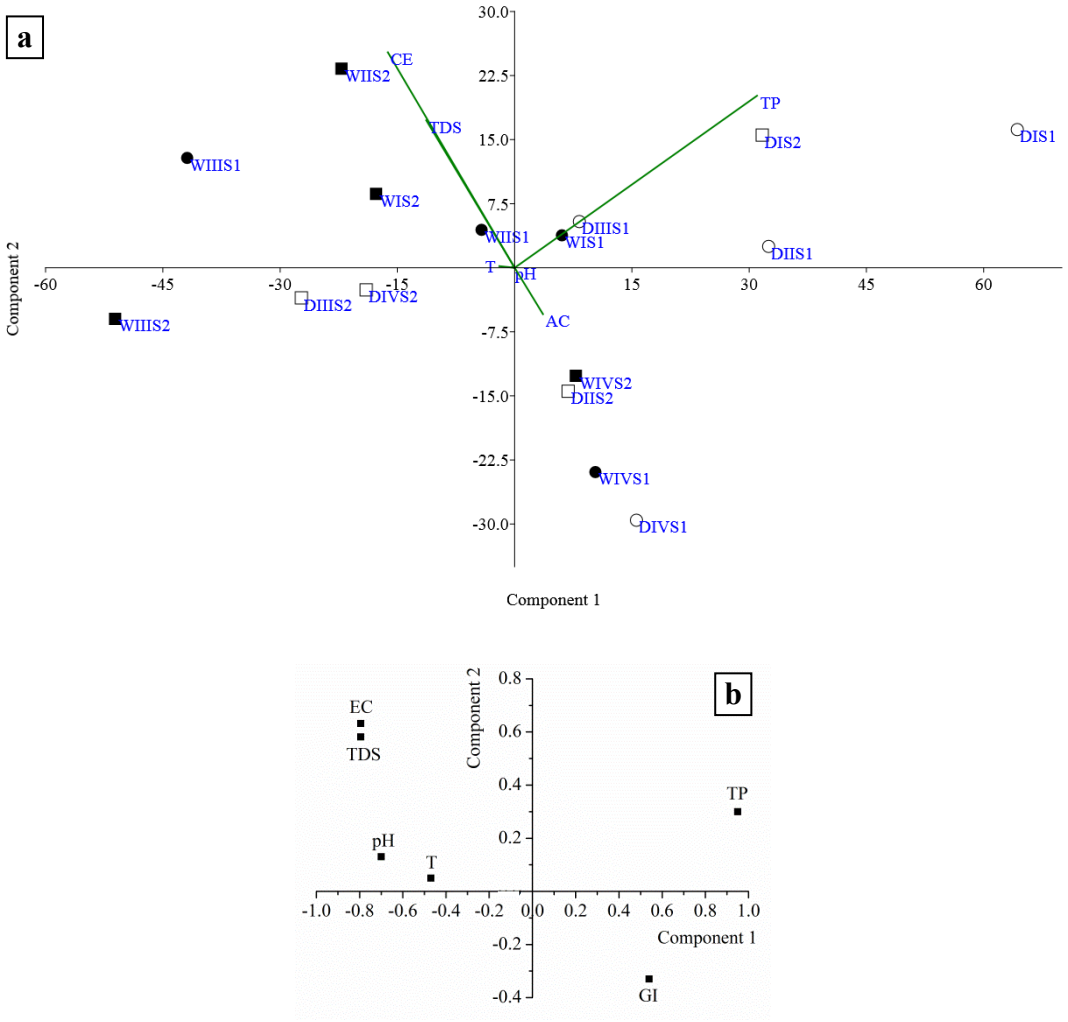

873 **Figure 4.** PCA scores (a) and loadings (b) for samples and variables studied at reservoir
874 stations (S1 and S2).

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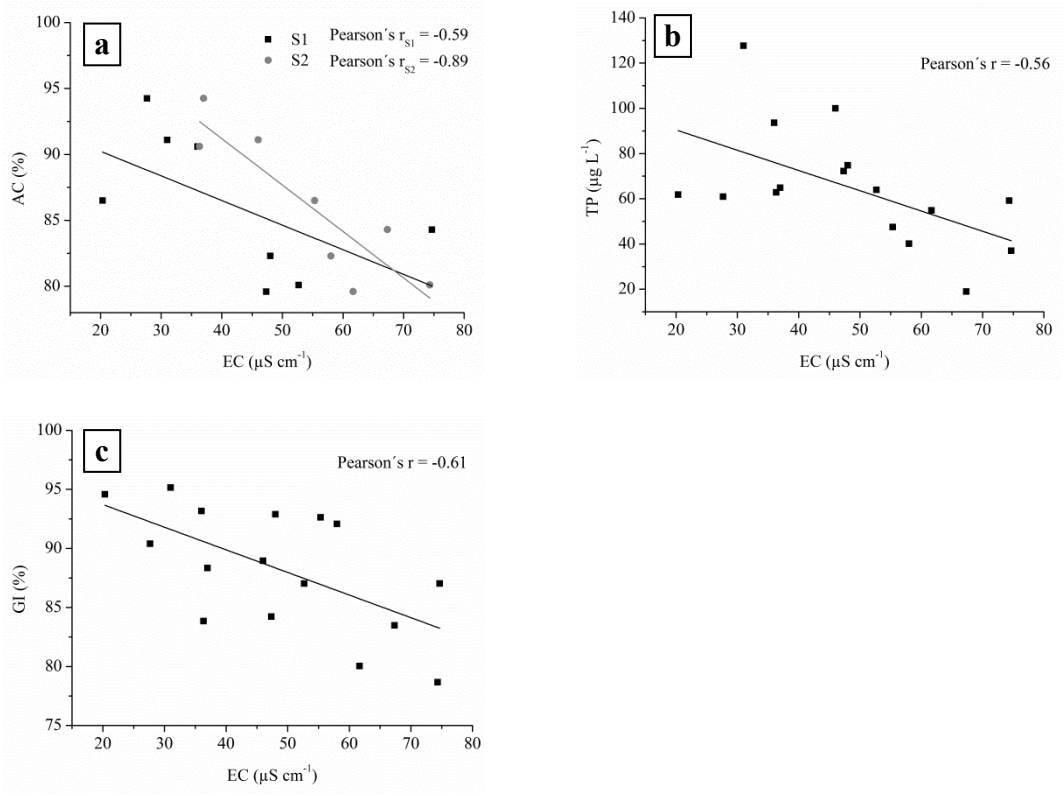

876 **Figure 5.** Pearson's correlations of EC (µs cm^{-1}) with: a) AC (%); b) TP (µg L^{-1}) and c) GI

877 (%) in reservoir stations (S1 and S2).

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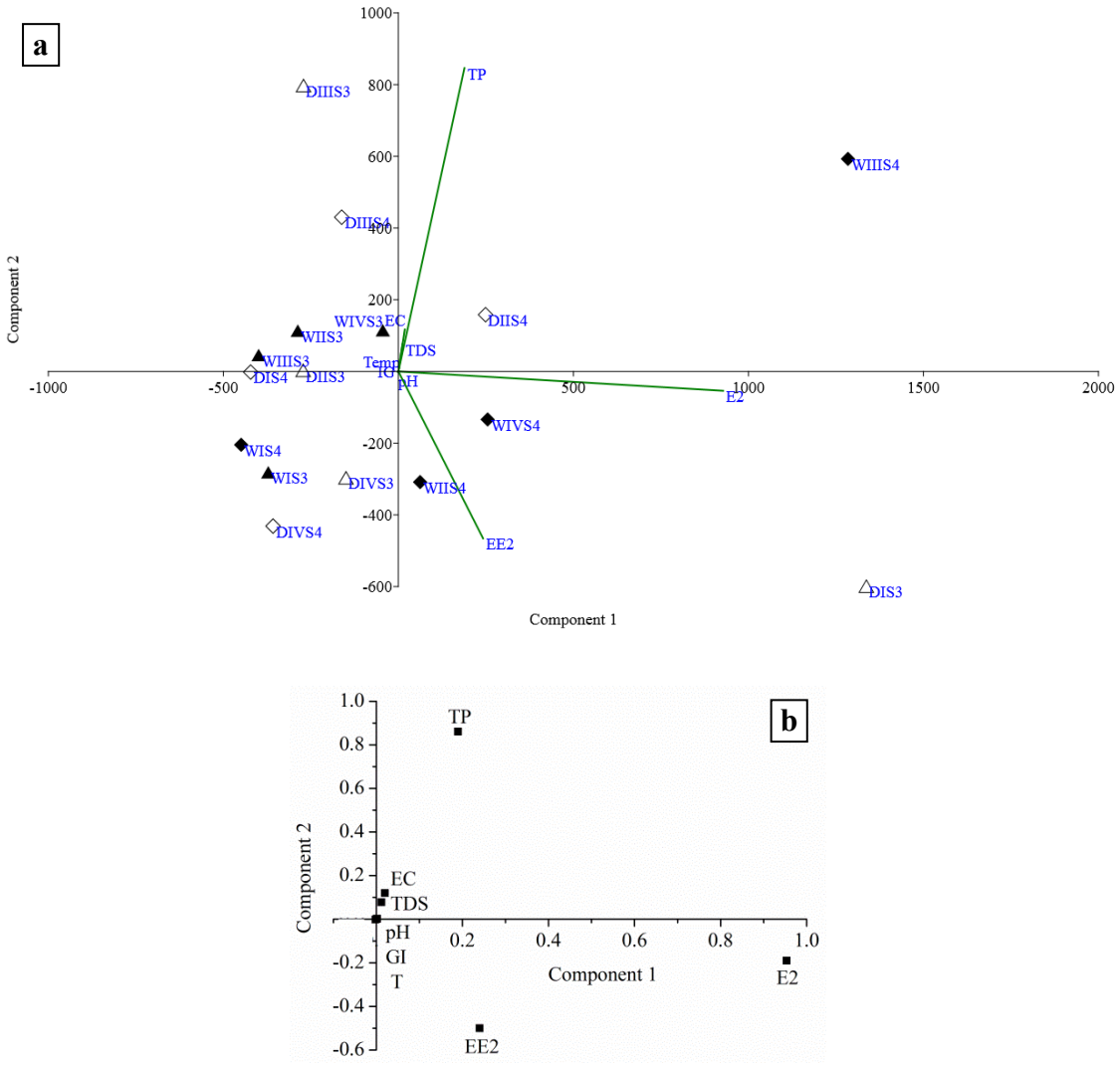
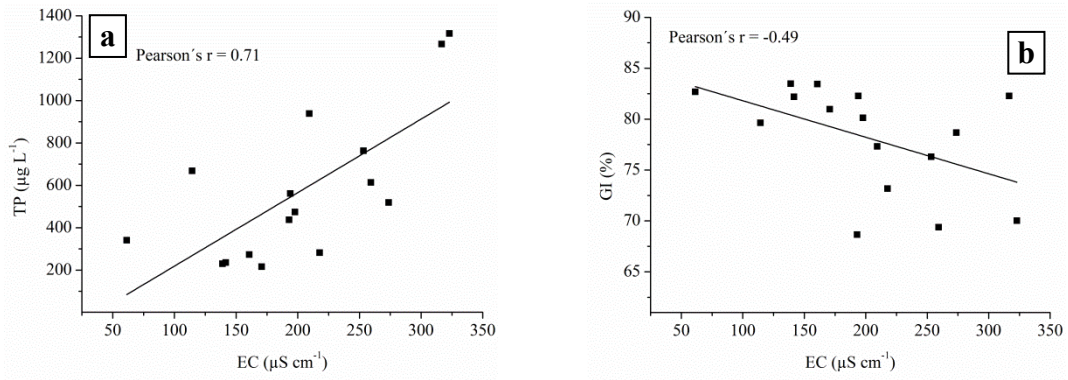
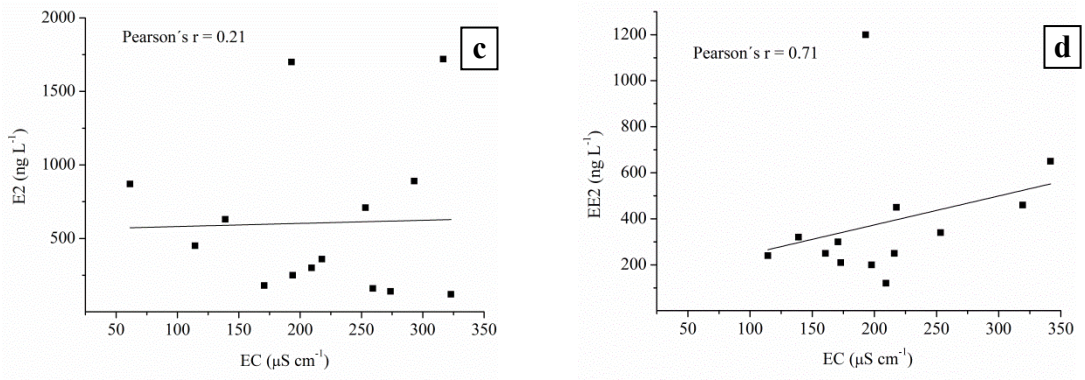
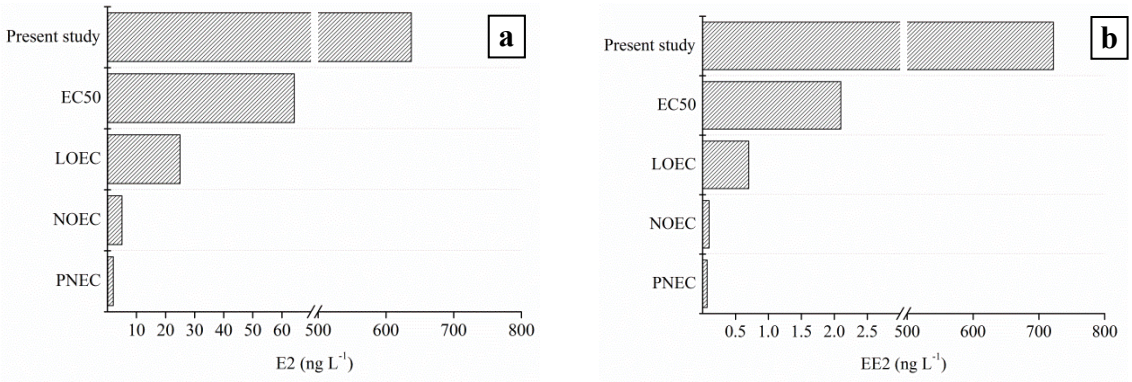


879 **Figure 6.** PCA for stream stations (S3 and S4): (a) Biplot scores (b) Loadings.





880 **Figure 7.** Pearson's correlations of Electrical conductivity with environmental data to stream
881 stations (S3 and S4): (a) TP (µg L^{-1}); (b) GI (%); (c) E2 (ng L^{-1}); (d) EE2 (ng L^{-1}).
882



883 **Figure 8.** E2 (a) and EE2 (b) levels and risk assessment for PNEC (Caldwell et al. 2012);

884 NOEC (Aris et al. 2014); LOEC (Aris et al. 2014) and EC_{50} (van den Belt et al. 2004).