1 **Using the Tea Bag Index to determine how two human pharmaceuticals affect**

2 **litter decomposition by aquatic microorganisms.**

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11 **Abstract**

12 This study demonstrates that independent additive effects of two human

13 pharmaceuticals, the antibiotic trimethoprim and the artificial estrogen 17a-

14 Ethinylestradiol (EE2), inhibit plant litter decomposition by aquatic microorganisms.

15 The constant release of pharmaceuticals, such as these, has the potential to affect

16 aquatic microbial metabolism and alter biogeochemical cycling of carbon and

17 nutrients. Here we advance the Tea Bag Index (TBI) for decomposition by using it in

18 a series of contaminant exposure experiments testing how interactions between

19 trimethoprim and EE2 affect aquatic microbial activity. The TBI is a citizen science

20 tool used to test microbial activity by measuring the differential degradation of green

21 and rooibos tea as proxies for respectively labile and recalcitrant litter

22 decomposition. Exposure to either trimethoprim or EE2 decreased decomposition of

23 green tea, suggesting additive effects upon microbial activity. Exposure to EE2 alone

24 decreased rooibos tea decomposition. Consequently, trimethoprim and EE2

25 stabilized labile organic matter against microbial degradation and restricted

26 decomposition. We propose that the method outlined could provide a powerful tool

27 for testing the impacts of multiple interacting pollutants upon microbial activity, at a

28 range of scales, across aquatic systems and over ecologically relevant time scales.

1. Introduction

Globally, human pharmaceutical use has increased by 3 % per annum since the year 2000, leading to constant discharge of these compounds into the aquatic environment from both point (Waste Water Treatment Works and Septic Tanks) and diffuse sources (e.g. agricultural run-off) (Gros et al, 2007; Rosi-Marshall & Royer, 2012; Van Boeckel et al, 2014;). Despite being present in the environment at low concentrations, pharmaceuticals have the potential to affect aquatic ecosystem processes because they are designed to be effective at micromolar or nanomolar concentrations (Rosi-Marshall & Royer, 2012; van Broeckel et al., 2014; Álvarez-Muñoz et al. 2015). The specific effects of pharmaceuticals in the environment are likely to be complex as a consequence of the myriad of potential interactions between these compounds, and their potential effects upon aquatic plants, animals and microorganisms (Hernando et al, 2006; Rosi-Marshall & Royer, 2012; Brodin et al, 2014). For example, the presence of antimicrobial compounds has the potential to affect the microbial processes that control aquatic carbon and nitrogen cycling (Rosi-Marshall & Royer, 2012; Brodin et al, 2014; Rosi et al., 2018), whilst other compounds such as artificial estrogens may actually stimulate microbial activity (Ribeiro et al., 2010; Pieper and Rotard, 2011; McClean and Hunter, 2020). As such, we need to understand how the interactions between pharmaceuticals influence microorganism-mediated biogeochemical processes, to safeguard ecosystem services.

Antibiotics and artificial estrogens represent some of the most widely detected pharmaceuticals in the environment (Rosi-Marshall & Royer, 2012; Álvarez-Muñoz et al, 2015; Archer et al, 2017; Gaston et al., 2019). Within aquatic systems, a wide range of human antibiotics such as tetracyclines, sulphonimides and trimethoprim are detectable in the water column, sediments and biota (Metcalfe et al., 2003; Ashton et al., 2004; Thomas and Hilton, 2004, Wiegel et al., 2004; Ebele et al., 2017; Kotke et al., 2019). Trimethoprim is of particular environmental concern, due to its widespread usage in human and veterinary healthcare, where it is mainly used to treat bladder and urinary tract infections, gastro-intestinal problems, ear infections and as prohylaxis against opportunistic infections (World Health Organisation, 2019). Approximately 50 – 60 % of any administered trimethoprim dose is excreted in a

61 subject's urine, combined with its widespread use this provides a definite route into

62 the environment via municipal wastewater and leaching of agricultural effluents. As

63 such, alongside its categorization as an essential medicine (World Health

64 Organisation, 2019), trimethoprim is recognized as a potential environmental hazard

65 (Straub, 2013; Tell et al., 2019).

66 The presence of antibiotics and their residues, both in the water column and aquatic

67 sediments may, directly affect the structure (Wieser et al 2016; Yuan et al 2017) and

68 functioning of aquatic microbial communities. In inland waters, leaf litter and other

69 terrigenous phytodetritus represent an important energy and nutrient source within

70 the food-web (e.g. Vannote et al, 1980; Raymond & Bauer, 2001; Raymond et al.,

71 2016). Microbial decomposition is an essential process, which supports the transfer

72 of litter-derived energy and nutrients to organisms at higher trophic levels.

73 Consequently, antimicrobials are likely to directly affect the processes that heavily

74 depend on microbial communities, and alter ecosystem-scale carbon and nitrogen

75 cycling will be affected (Roose-Amsleg and Laverman, 2016; Robson et al., 2020).

76 Furthermore, whilst bacterial populations rapidly evolve antibiotic resistance, this

77 comes at a physiological cost. Antibiotic resistant bacteria tend to grow more slowly

78 and use nutrients less efficiently than their non-resistant conspecifics (Anderson and

79 Levin, 1999, Jansen et al 2013). As such, longer-term exposure to antimicrobial

80 contaminations is likely to have implications for the efficiency of microbially-mediated

81 processes in aquatic ecosystems (e.g. Rosi et al. 2018).

82 By contrast, artificial estrogens, such as EE2, may provide a novel carbon source for

83 aquatic microorganisms, supporting increased microbial metabolism (Ribeiro et al.,

84 2010; Pieper and Rotard, 2011), their adsorption onto microbial biofilms can provide

85 protection against antimicrobial agents (Writer et al., 2012; Zhang et al., 2014).This

86 was recently demonstrated by McClean and Hunter (2020), who showed that

87 exposure of streambed biofilms to EE2 counteracted the inhibitory effects of

88 ibuprofen upon microbial respiration. Consequently, the interactions between

89 antibiotics and artificial estrogens upon aquatic microbial processes are likely to

90 have wider environmental relevance.

91 Contaminant exposure substrata (CES) experiment provides a powerful tool for

92 testing the sensitivity of aquatic microbial communities to pharmaceuticals and other

93   pollutants (Tank et al, 2006; Costello et al, 2016). A well-refined method is provided

94   by Costello et al (2016) to test how pharmaceuticals affect microbial biofilm growth

95   and community structure and ecophsysiological responses of the biofilm, such as

96   community respiration (Rosi-Marshall et al, 2013; Rosi et al., 2018; Gallagher and

97   Reisinger, 2020; McClean & Hunter, 2020 Robson et al., 2020). These methods,

98   however, provide insufficient information on heterotrophic processes such as

99   microbial litter decomposition, which occurs over times-scales measured in weeks or

100 months (Vannote et al, 1980; Raymond & Bauer, 2001). The Tea Bag Index (TBI)

101 provides a powerful low-cost tool for investigating microbial activity in soils and

102 aquatic systems, based upon the traditional use of leaf-litter bags in ecology

103 (Keuskemp et al., 2013; Whigham et al., 2017; Mueller et al., 2018; Seelen et al.,

104 2019. The key strength of TBI is its ability to provide a standardized method of

105 quantifying microbial activity by comparing the relative degradation of green tea

106 (easy to decompose, or labile material) and rooibos tea (resistant to decomposition,

107 recalcitrant, lignified material) organic matter source. The use of TBI within

108 contaminant exposure substrata experiments will allow quantification of the impacts

109 of pollutant exposure on microbially-mediated litter decomposition.

110 Within this study we investigate how interactions between the antibiotic trimethoprim

111 and the artificial estrogen EE2 affect aquatic litter decomposition, using modified

112 contaminant exposure experiments. We integrate the TBI (Keuskemp et al., 2013)

113 into the existing CES methodology developed by Tank et al (2006) and Costello et al

114 (2016). This will provide a simple, low-cost tool for assessing how pharmaceuticals

115 (and other contaminants) may affect microbial litter decomposition. Using the TBI-

116 CES method we test how exposure of the microbial community to both trimethoprim

117 and EE2 affects the decomposition of both labile and recalcitrant litter in a mixed-use

118 catchment stream. We hypothesize that trimethoprim will inhibit decomposition while

119 EE2 may enhance it. We therefore expect that EE2 may mediate against any

120 inhibitory effects of trimethoprim upon the microbial activity that drives litter

121 decomposition.

122

2. Materials and Methods

2.1. Site Description

Experiments were carried out between 22nd March and 13th June 2019 in the Ballysally Blagh (Latitude: 55°08'48.9"N Longitude: 6°40'08.0"W), a ground-water fed second-order stream draining a mixed agricultural (consisting of 21.9 % arable; 55.9 % grassland; 13.7 % heathland; 1.9 % woodland) and urban (7.3 %) catchment of 14.2 km^2. Mean volumetric water flow in the Ballysally Blagh is 0.21 (± 0.27) m^3 s^{-1}, measured at a V-shaped weir. Water temperatures at the study site (50 cm water depth) were recorded at 1-hour intervals throughout the experiment using a HOBO MX2204 Bluetooth temperature logger and ranged between 5.51 °C and 15.57 °C, with a mean temperature over the study period of 10.17 (± 2.08) °C.

2.2. Contaminant Exposure Substrata Experiments

Contaminant exposure substrata (CES) experiments were conducted using forty 120 ml screw cap sample pots, with a 35 mm hole drilled into the lid (following Costello et al, 2016). Each pot was filled with 100 ml of 2 % agar, with ten pots spiked with 688 μmol.l^{-1} Trimethoprim (Sigma-Aldich, product no: 92131), ten with 688 μmol.l^{-1} of EE2 (Sigma-Aldrich, Product No. E4876) and ten with 688 μmol.l^{-1} trimethoprim and 688 μmol.l^{-1} EE2. The remaining ten sample pots received no pharmaceutical treatment (control). Trimethoprim and EE2 stock solutions for were made up in 70 % ethanol, with 1 ml aliquots used to spike each contaminant exposure experiment, whilst the agar in the control treatments was spiked with 1 ml doses of 70 % ethanol. All pots were weighed to determine mass of agar within. One pre-weighed unused Lipton® Green Tea Sencha pyramidal tea bag with non-woven fabric (Product Number: EAN 87 22700 05552 5) and one pre-weighed unused Lipton® Rooibos tea bag with non-woven fabric (Product Number: EAN 87 22700 18843 8) were the placed into each pot screw cap secured in place. The contaminant exposure experiments were then secured to four L-shaped metal bars (Length = 1000 mm; Width = 35 mm; Height = 35 mm) and deployed onto the streambed at 30 – 40 cm depth (Figure 1).

CES experiments were left in place for 83 days. Upon recovery, the teabags were removed from the experiment and the pots were then weighed to determine the agar mass loss (following Costello et al, 2016). Teabags were dried to constant weight

5

over 72 hours at 60 °C, excess silt and detritus adhering to each teabag was gently removed, and their dry-weight determined (following Keuskamp et al, 2013). The tea was then removed from each teabag, re-weighed and combusted at 550 °C for four hours to determine ash free dry mass.

2.3. Data Treatment

We calculated percentage mass loss of green tea and rooibos tea in each contaminant exposure experiment as the difference between the dry weight of the teabag at the start and end of the incubation. We corrected for mass loss during the drying process based on the mean mass loss for five air-dry green teabags and five air-dry rooibos teabags, dried at 60 °C over 72 hours. Following Keuskamp et al. (2013) we then determined the organic matter stabilization factor (S_{TBI}) as:

[1] $$S_{TBI} = 1 - \frac{a_g}{0.842}$$

Where a_g is the decomposable fraction of the green tea (ash free dry weight) and 0.842 is the hydrolysable fraction of the green tea (from Keuskamp et al, 2013). Using S we then estimated the decomposable fraction of the rooibos tea (a_r) as:

[2] $$a_r = 0.552\,(1 - S_{TBI})$$

Where S_{TBI} is the stabilization factor and 0.552 is the hydrolysable fraction of the rooibos tea (from Keuskamp et al, 2013). The organic matter Initial decomposition rate of the labile material (k_{TBI}) can then be calculated as:

[3] $$k_{TBI} = \frac{\left(\ln\left(\frac{a_r}{W}\right)\right)}{t}$$

Where W is the mass loss of the Rooibos teabag in each experiment (in grams) and t is the duration of the experimental incubation (in days).

2.4. Data Analysis

Data analysis and visualization was carried out in R using the *base* and *ggplot* packages (R Development Core Team, 2009; Wickham, 2016). We tested for independent and combined effects of trimethoprim and EE2 upon the percentage

182   mass loss of the Green tea and Rooibos teabags, the stabilization factor (S_{TBI}) and
183   Initial decomposition rate (k_{TBI}) of the labile organic material using two-way analysis
184   of variance (ANOVA). Post-hoc testing of significant interactions were conducted
185   using Tukey's test for Honest Significant Difference. All data were visually explored
186   to ensure they conformed to the assumptions of normality and homoscedacity
187   (following Zuur et al, 2010), Initial decomposition rate (k_{TBI}) data were $\log_{10}$
188   transformed to ensure that the residuals from the ANOVA model conformed to a
189   normal distribution. See supplementary table 1 for a summary of the ANOVA test
190   results.

191   **3. Results and Discussion**

192   Over the course of the experiment, dissolution of the agar was assumed to deliver
193   constant daily doses of approximately 275 nmol .d^{-1} of trimethoprim and/or EE2 in
194   both the single and combined pharmaceutical treatments. As such, each teabag
195   would have been exposed to a pharmaceutical dose that was broadly comparable
196   with the concentration of these compounds in inland waters (e.g. Álvarez-Muñoz et
197   al, 2015; Archer et al, 2017; Tousova et al., 2017). Rooibos Tea mass loss
198   decreased upon EE2 exposure, with no additional/significant effects of trimethoprim
199   (Figure 2 A, Table 1a). By contrast for green tea, we observed additive and inhibiting
200   effects of both trimethoprim and EE2 (Figure 2 B, Table 1b). Based on these data we
201   can demonstrate that EE2 and trimethoprim had significant independent effects upon
202   both the TBI stabilization factor (Figure 3 A, Table 1c) and initial decomposition rate
203   (Figure 3 B, Table 1 d).

204   Trimethoprim decreased mass loss of green tea but not of rooibos, while EE2
205   decreased both green tea and rooibos mass loss. Together, these pharmaceuticals
206   had additive negative effects on green tea mass loss but not on rooibos. No
207   significant interaction was observed for either green tea or rooibos. Further, EE2 and
208   trimethoprim had significant independent effects upon both the litter stabilization
209   factor (Figure 3 A, Table 1c) and initial decomposition rate (Figure 3 B, Table 1 d)

210   Our results demonstrate that exposure to low doses of both an antibiotic
211   (trimethoprim) and an endocrine disruptor (EE2) inhibit microbial litter decomposition.
212   Specifically, trimethoprim appears to affect decomposition of lignified material less

compared to more easy to degrade material, whilst EE2 had a broader effect across litter types. In counter to our hypothesis, EE2 did not reduce the negative impact of trimethoprim upon aquatic litter decomposition. Both trimethoprim and EE2 had significant additive positive effects upon the organic matter stabilization factor (S_{TBI}) and its initial decomposition rate (k_{TBI}). Our study, thus, indicates that pollution from both pharmaceuticals may increase the residence time for organic matter in aquatic systems. Inland waters (rivers, lakes and streams) typically receive large inputs of terrestrial organic matter, which is then partially metabolized, temporarily buried within the sediment or transported to the ocean (Battin et al, 2009). Our study adds further evidence to that pharmaceutical contaminants directly affect the rates and pathways for aquatic carbon and nutrient cycling (e.g. Jobling et al. 2003; Hernando et al. 2006; Gros et al. 2007; Rosi-Marshall and Royer 2012; Rosi-Marshall et al. 2013; Álvarez-Muñoz et al. 2015; Ruhí et al. 2016; Archer et al. 2017; Rosi et al. 2018; Gallagher and Reisinger 2020; McClean and Hunter, 2020).

Given the widespread use of trimethoprim as an antibiotic medication and its high solubility in water [ranging from 500 mg l^{-1} at pH 8 to 15,500 mg l^{-1} at pH 5.5 (Dahlan et al. 1987)], its effect upon microbial activity within the teabag experiment was expected. As a highly soluble contaminant trimethoprim is likely to leach rapidly from the agar within CES experiments, and thus affect microbial activity during the early phases of the experiment. Thus the effects of trimethoprim upon labile litter decomposition are likely to reflect the relatively high solubility of this contaminant. By contrast, EE2 exhibits relatively low solubility in water, ranging between 2.92 and 4.8 mg l-1 (Lai et al., 2000; Ying et al. 2002; Guo and Hu, 2014). This property of EE2, combined with its tendency to adsorb onto organic substances (Lai et al., 2000; Ying et al., 2002; Writer et al., 2012; Zhang et al., 2014), provides a mechanism through which the EE2 released by the agar would affect the degradation of more recalcitrant and lignified litter. The additive effects of trimethoprim and EE2 upon litter decomposition within our experiment were likely to be driven by differences in the solubility of each compound and their potential to adsorb onto the tea. This highlights how differences in the physico-chemical properties of pharmaceutical mediate their potential impacts in aquatic biogeochemical processes.

244 CES experiments are typically used to determine the effects of a selected
245 contaminant on biofilm forming bacteria and algae (Tank et al., 2006; Costello et al.,
246 2016). Integrating the TBI (Keuskemp et al. 2013) into CES experiments provides a
247 useful low-cost tool to quantify how exposure to pollutants such as pharmaceuticals
248 affects instream decomposition of organic matter. The inclusion of both a labile
249 (green tea) and more recalcitrant (rooibos tea) organic matter source within the
250 experiment allows inferences to made upon the effects of a contaminant upon the
251 phenology of the litter decomposition process. Refinement of the method is,
252 however, necessary and should include investigation of agar dissolution and teabag
253 degradation rates, and how these may vary under differing climatic and hydrological
254 regimes (following Costello et al., 2016).

255 Whilst our study was restricted to one stream, the method could easily be replicated
256 at multiple sites, within more complex experimental designs. We believe this method
257 will allow the effects of multiple pollutants upon organic matter degradation and
258 residence times to be made across a range of spatial and temporal scales in both
259 freshwater and marine systems. As our method is characterized by its low-cost,
260 simplicity and use of readily available consumer products, it could potentially be used
261 to achieve high levels replications within experimental designs testing the
262 interactions between a suite of multiple pollutants and their effects upon aquatic
263 biogeochemical processes. Furthermore, we propose that the TBI-CES method
264 could provide a valuable pedagogical tool for research-led environmental science
265 teaching and in citizen science based initiatives to investigate the impacts of
266 pollutants in aquatic systems.

Acknowledgements

Author Information

272 Experiments were designed by WRH following discussions with JS, and were
273 conducted by WRH and AW. All authors contributed to the writing of the manuscript.

274 **Conflict of Interest**

275 The authors declare that we have no conflicts of interest pertaining to this study.

276 **Data Availability**

277 All data are freely available Creative Commons (CC-BY) license through the Zenodo
278 data repository (DOI: 10.5281/zenodo.3528009).

279 **Animal Research (Ethics)**

280 No animals were used in this research.

281 **Consent to Participate (Ethics)**

282 No human subjects were used in this research.

283 **Consent to Publish (Ethics)**

284 This is not applicable to this study.

285 **Plant Reproducibility**

286 This is not applicable to this study.

287 **Clinical Trials Registration**

288 This is not applicable to this study.

289

4. References

Álvarez-Muñoz D, Rodríguez-Mozaz S, Maulvault AL, Tediosi A, Fernández-Tejedor M, Van den Heuvel F, *et al.* (2015). Occurrence of pharmaceuticals and endocrine disrupting compounds in macroalgaes, bivalves, and fish from coastal areas in Europe. Environmental Research 143:56-64.

Anderson DI, Levin BR (1999). The biological cost of antibiotic resistance. Current Opinion in Microbiology. 2:489-493.

Archer E, Petrie B, Kasprzyk-Hordern B, Wolfaardt GM (2017). The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters. Chemosphere 174:437-446.

Battin TJ, Luyssaert S, Kaplan LA, Aufdenkampe AK, Richter A, Tranvik LJ (2009). The boundless carbon cycle. Nature Geoscience 2:598.

Brodin T, Piovano S, Fick J, Klaminder J, Heynen M, Jonsson M (2014). Ecological effects of pharmaceuticals in aquatic systems: impacts through behavioural alterations. Philosophical Transactions of the Royal Society of London B: Biological Sciences 369:20130580.

Costello DM, Rosi-Marshall EJ, Shaw LE, Grace M, Kelly JJ (2016). A novel method to assess effects of chemical stressors on natural biofilm structure and function. Freshwater Biology 61:2129-2140.

Dahlan, R., Mcdonald, C. and Sunderland, V.B. (1987), Solubilities and intrinsic dissolution rates of sulphamethoxazole and trimethoprim. Journal of Pharmacy and Pharmacology, 39: 246-251. https://doi.org/10.1111/j.2042-7158.1987.tb06261.x

Gallagher MT, Reisinger AJ (2020) Effects of ciprofloxacin on metabolic activity and algal biomass of urban stream biofilms. Sci Total Environ 706:135728. https://doi.org/10.1016/j.scitotenv.2019.135728

Gaston L, Lapworth DJ, Stuart M, Arnscheidt J (2019) Prioritization approaches for substances of emerging concern in groundwater: a critical review. Environ Sci Technol 53:6107–6122

Gros M, Petrović M, Barcelo D (2007). Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river basin (northeast Spain). Environmental Toxicology and Chemistry 26:1553-1562.

Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D (2006). Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. Talanta 69:334-342.

325 Jansen G, Barbosa C, Schulenburg H (2013). Experimental evolution as an efficient
326 tool to dissect adaptive paths to antibiotic resistance. Drug Resistance Updates 16:
327 96-107

328 Keuskamp JA, Dingemans BJJ, Lehtinen T, Sarneel JM, Hefting MM (2013). Tea
329 Bag Index: a novel approach to collect uniform decomposition data across
330 ecosystems. Methods in Ecology and Evolution 4:1070-1075.

331 Lai KM, Johnson KL, Scrimshaw MD, Lester JN (2000). Binding of waterborne
332 steroid estrogens to solid phases in river and estuarine systems Environ. Sci.
333 Technol., 34: 3890-3894

334 McClean P, Hunter WR (2019). 17α-ethynylestradiol (EE2) limits the impact of
335 ibuprofen upon respiration by streambed biofilms in a sub-urban stream.
336 Enviromental Science and Pollution Research. 27: 37149–37154.

337 Mueller P, Schile-Beers, LM, Mozdzer TJ, Chmura GL, Dinter T, Kuzyakov Y, de
338 Groot AV, Esselink P, Smit C, D'Alpaos A, Ibáñez C, Lazarus M, Neumeier U,
339 Johnson BJ, Baldwin AH, Yarwood SA, Montemayor DI, Yang Z, Wu J, Jensen K,
340 Nolte S (2018). Global-change effects on early-stage decomposition processes in
341 tidal wetlands: implications from a global survey using standardized litter.
342 Biogeosciences, 15, 3189–3202, 2018

343 Raymond PA, Bauer JE (2001). Riverine export of aged terrestrial organic matter to
344 the North Atlantic Ocean. Nature 409:497-500.

345 Raymond PA, Saiers JE, Sobczak WV. "Hydrological and biogeochemical controls
346 on watershed dissolved organic matter transport: pulse-shunt concept." Ecology 97:
347 15-16.

348 Robson SV, Rosi EJ, Richmond EK, Grace MR (202 (2016) Environmental
349 concentrations of pharmaceuticals alter metabolism, denitrification, and diatom
350 assemblages in artificial streams. Freshwat Sci 39:256–267.
351 https://doi.org/10.1086/708893

352 Roose-Amsaleg C, Laverman AM (2016). Do antibiotics have environmental side-
353 effects? Impact of synthetic antibiotics on biogeochemical processes. Environmental
354 Science and Pollution Research. 23:4000–4012.

355 Rosi EJ, Bechtold HA, Snow D, Rojas M, Reisinger AJ, Kelly JJ (2018) Urban stream
356 microbial communities show resistance to pharmaceutical exposure. Ecosphere
357 9:e02041

358 Rosi-Marshall EJ, Kincaid DWL, Bechtold H, Royer TV, Rojas M, Kelly JJ (2013).
359 Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial
360 communities in stream biofilms. Ecological Applications 23:583-593.

361 Rosi-Marshall EJ, Royer TV (2012). Pharmaceutical compounds and ecosystem
362 function: an emerging research challenge for aquatic ecologists. Ecosystems.
363 15:867-880.

Seelen LMS, Flaim G, Keuskamp J, Teurlincx S, Arias Font R, Tolunay D*, et al.*
(2019). An affordable and reliable assessment of aquatic decomposition: Tailoring
the Tea Bag Index to surface waters. Water Research 151:31-43,
doi:https://doi.org/10.1016/j.watres.2018.11.081.

Straub JO (2013): An environmental risk assessment for human-use Trimethoprim in
European surface waters. Antibiotics, 2, 115–162

Tank JL, Bernot MJ, Rosi-Marshall EJ (2006). Nitrogen limitation and uptake. In:
Methods in Stream Ecology. Academic Press: San Diego, CA., pp 213-238.

Tell J, Caldwell DJ, Häner A, Hellstern J, Hoeger B, Journel R, Mastrocco F, Ryan J,
Snape J, Straub JO, Vestel J (2019): Science-based targets for antibiotics in
receiving waters from pharmaceutical manufacturing operations. Integr Environ Ass
Manag. 15(3):312–319. https://doi.org/10.1002/ieam.4141

Tousova Z, Oswald P, Slobodnika J, Blaha L, Muzc M, Huc M, Brack W, Krauss M,
Di Paolo C, Tarcai Z, Seilerd T-B, Hollert H, et al. (2017). European demonstration
program on the effect-based and chemical identification and monitoring of organic
pollutants in European surface waters. Science of the Total Environment. 601–602:
1849-1868.

Van Boeckel TP, Gandra S, Ashok A, Caudron Q, Grenfell BT, Levin SA*, et al.*
(2014). Global antibiotic consumption 2000 to 2010: an analysis of national
pharmaceutical sales data. Lancet Infectious Diseases 14:742-750.

Vannote RL, Minshall GW, Cummins KW, Sedell JR, Cushing CE (1980). The River
Continuum Concept. Canadian Journal of Fisheries and Aquatic Sciences 37:130-
137.

Waiser MJ, Swerhone GD, Roy J, Tumber V, Lawrence JR (2016). Effects of
erythromycin, trimethoprim and clindamycin on attached microbial communities from
an effluent dominated prairie stream. Ecotoxicology and Environmental Safety 132:
31-39. doi: 10.1016/j.ecoenv.2016.05.026.

Whigham DF, Walker CM, Maurer J, King, RS, Hauser W, Baird S, Keuskamp JA,
Neale PJ (2017). Watershed influences on the structure and function of riparian
wetlands associated with headwater streams - Kenai Peninsula, Alaska. Science of
the Total Environment. 599–600: 124-134

World Health Organization (2019). World Health Organization model list of essential
medicines: 21st list 2019. Geneva: World Health Organization. hdl:10665/325771.
WHO/MVP/EMP/IAU/2019.06.

Ying GG, Kookana RS, Ru YJ (2002). Occurrence and fate of hormone steroids in
the environment. Environ. Int., 28: 545-551

Yuan K, Xiao S, Jiang X, Yang L, Chen B, Luan T, Lin L, Tam NFY (2017). Effects of
endocrine disrupting chemicals (EDCs) on bacterial communities in mangrove
sediments. Marine Pollution Bulletin 122: 122-128

403

404 **Tables**

405 Table 1. Two-way analysis of variance testing for significant effects of trimethoprim
406 and EE2 upon the percentage mass loss of the Green tea and Rooibos teabags, the
407 organic matter stabilization factor (S_{TBI}) and initial decomposition rate (k_{TBI}).

a) Percentage mass loss of Green tea					
	Df	SSq	MSq	F	p
Trimethoprim	1	105.89	105.889	10.486	0.003 **
EE2	1	108.43	108.428	10.738	0.002 **
Trimethoprim:EE2	1	16.26	16.259	1.610	0.213
Residuals	36	363.52	10.098		
Adjusted R^2	0.3371				
b) Percentage mass loss of Rooibos tea					
	Df	SSq	MSq	F	p
Trimethoprim	1	8.430	8.430	1.342	0.254
EE2	1	70.510	70.510	11.225	0.002 **
Trimethoprim:EE2	1	8.399	8.399	1.337	0.255
Residuals	36	226.119	6.281		
Adjusted R^2	0.219				
c) stabilization factor (S_{TBI})					
	Df	SSq	MSq	F	p
Trimethoprim	1	0.015	0.015	10.486	0.003 **
EE2	1	0.015	0.015	10.738	0.002 **
Trimethoprim:EE2	1	0.002	0.002	1.610	0.213
Residuals	36	0.051	0.001		
Adjusted R^2	0.337				
d) Initial decomposition rate (k_{TBI})					
	Df	SSq	MSq	F	p
Trimethoprim	1	1.073	1.073	5.389	0.026 *
EE2	1	2.594	2.594	13.025	<0.001 ***
Trimethoprim:EE2	1	0.112	0.112	0.564	0.458
Residuals	35	6.972	0.199		
Adjusted R^2	0.29				

408

409 **Figures**

410 Figure 1. Images of (A) the TBI contaminant exposure experiments, (B) how they are
411 secured prior to the L-shaped metal bars and (C) their deployment at the streambed
412 (right panel)

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415 Figure 2. Effects of TMP (✐) and EE2 (♀) on the degradation (% mass loss) of A)
416 Rooibos and B) Green tea, as labile and recalcitrant organic matter sources. Inserts
417 show pooled data where significant independent effects of either Trimethoprim or
418 EE2 where detected. Significance levels: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.
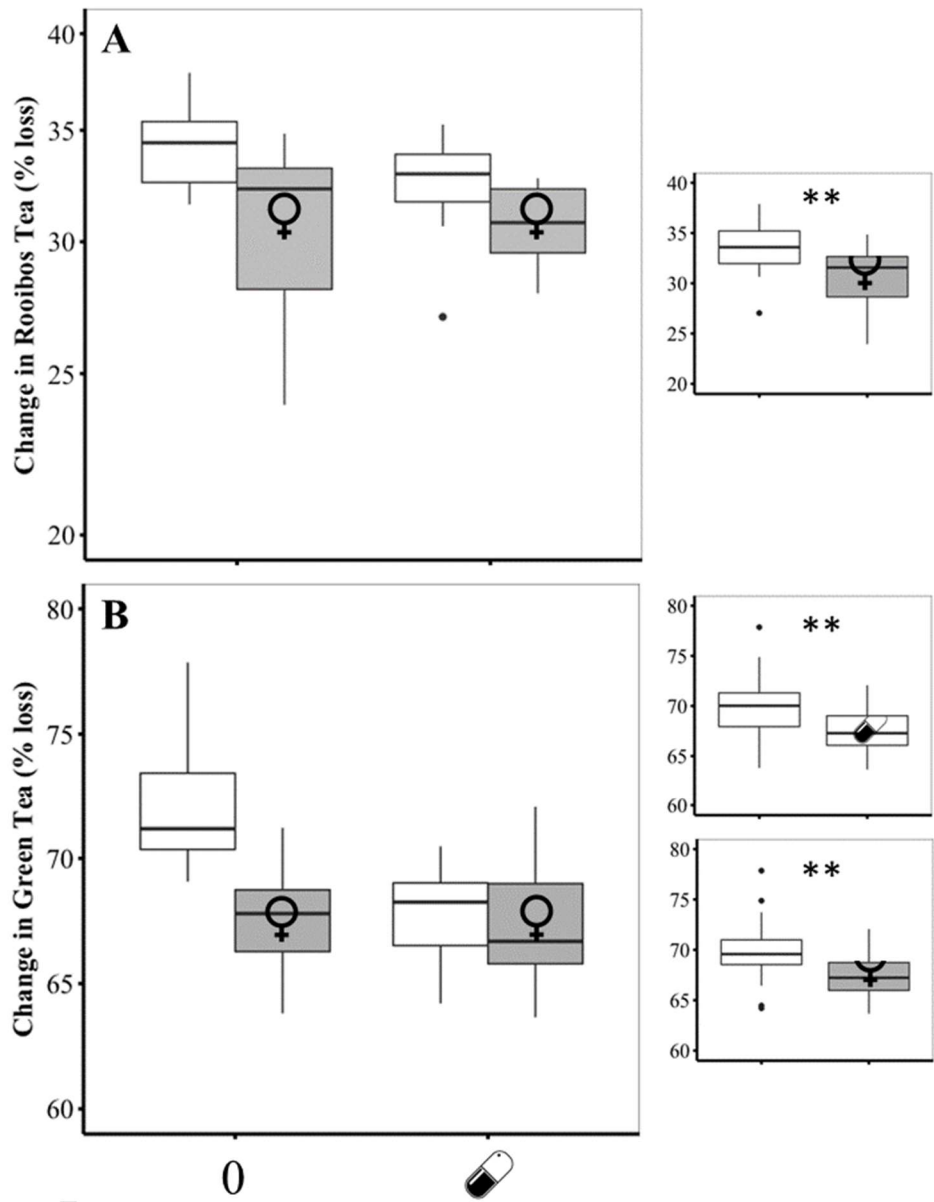

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Figure 3. Effects of TMP (🖊) and EE2 (♀) on the A) TBI stabilization factor and B)
initial decomposition rate, calculated following Keuskamp et al (2013). Inserts show
pooled data where significant independent effects of either Trimethoprim or EE2
where detected. Significance levels: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.