Using the Tea Bag Index to unravel how human pharmaceuticals affect aquatic microbial activity.

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Abstract

The constant release of complex mixture of pharmaceuticals, including antimicrobials and endocrine disruptors, has the potential to affect aquatic microbial metabolism and alter biogeochemical cycling of carbon and nutrients. Here we advance the Tea Bag Index (TBI) for decomposition by using it in a series of contaminant exposure experiments testing how interactions between two human pharmaceuticals, the broad spectrum antibiotic trimethoprim and the artificial estrogen 17a-Ethinylestradiol (EE2), affects microbial activity in an aquatic system. The TBI is a citizen science tool used to test microbial activity by measuring the differential degradation of green and rooibos tea as proxies for labile and recalcitrant organic matter decomposition. Exposure to trimethoprim and EE2 had significant independent negative effects upon decomposition of labile organic matter (green tea), suggesting additive effects upon microbial activity. Exposure to EE2 alone negatively affected the degradation of more recalcitrant organic matter (rooibos tea). Consequently, trimethoprim and EE2 stabilized labile organic matter against microbial degradation and restricted degradation rates. We propose that the method outlined could provide a powerful tool for testing the impacts of multiple interacting pollutants upon microbial activity, at a range of scales, across aquatic systems and over biogeochemically 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). 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), whilst other compounds such as artificial estrogens may stimulate microbial activity (Ribeiro et al., 2010; Pieper and Rotard, 2011; McClean and Hunter, 2020). As such, we need to understand how pharmaceuticals influence microorganism-mediated ecosystem processes.

Antibiotics and artificial estrogens represent some of the most widely detected pharmaceuticals in the environment (Álvarez-Muñoz et al, 2015; Archer et al, 2017; Rosi-Marshall & Royer, 2012). Within aquatic systems, a wide range of human antibiotics such as tetracyclines, sulphonimides and the broad spectrum antibiotic trimethoprim (Metcalfe et al., 2003; Ashton et al., 2004; Thomas and Hilton, 2004, Wiegel et al., 2004) are detectable. Their presence will, therefore, directly affect the structure of aquatic microbial communities (Wieser et al 2016; Yuan et al 2017), potentially altering the microbial processes which drive carbon and nitrogen cycling (Roose-Amsleg and Laverman, 2016). Whilst bacterial populations can rapidly evolve antibiotic resistance, this comes at a physiological cost. As a consequence antibiotic resistant bacteria tend to grow more slowly and use nutrients less efficiently than their non-resistant conspecifics (Anderson and Levin, 1999, Jansen et al 2013).

EE2 and other artificial estrogens, by contrast, can provide a novel carbon source for aquatic microorganisms, supporting increased microbial metabolism (Ribeiro et al., 2010; Pieper and Rotard, 2011), whilst their adsorption onto microbial biofilms may, also, provide protection against antimicrobial agents (Writer et al., 2012; Zhang et al., 2014). Artificial estrogens (such as EE2) may mediate the effects of antimicrobial agents upon aquatic microbial communities (McClean and Hunter, 2020). Consequently, interactions between antibiotics and artificial estrogens are of wider environmental relevance.

Contaminant exposure experiment provides a powerful tool for testing the sensitivity of aquatic microbial communities to pharmaceuticals and other pollutants (Tank et al, 2006; Costello et al, 2016). A well-refined method is provided by Costello et al (2016) to test how pharmaceuticals affect microbial biofilm growth and community structure and ecophsysiological responses of the biofilm, such as community respiration (Rosi-Marshall et al, 2013; McClean & Hunter, 2020). These methods, however, do not provide information on heterotrophic processes such as microbial degradation organic matter, which occurs over times-scales measured in weeks or months (Vannote et al, 1980; Raymond & Bauer, 2001). The Tea Bag Index (TBI) provides a powerful low-cost tool for investigating microbial activity in soils and aquatic systems, based upon the traditional use of leaf-litter bags in ecology (Keuskemp et al., 2013; Whigham et al., 2017; Mueller et al., 2018; Seelen et al., 2019. The key strength of TBI is its ability to provide a standardized method of quantifying microbial activity by comparing the relative degradation of a labile (green tea) and recalcitrant (rooibos tea) organic matter source. The use of TBI within contaminant exposure experiments could, therefore, allow the impacts of exposure to a pollutant to be quantified in terms of microbially-mediated organic matter degradation.

We tested how interactions between the broad-spectrum antibiotic Trimethoprim and artificial estrogen EE2 affect in-stream microbial activity, using a modified contaminant exposure experiments. These experiments integrate the TBI (Keuskemp et al., 2013) into the contaminant exposure experiment methodology developed by Tank et al (2006) and Costello et al (2016). Using this method we seek to test the hypothesis that concurrent exposure to EE2 will reduce the effects of

trimethoprim upon the microbial degradation pathways for both labile and recalcitrant organic matter in a mixed-use catchment stream.

2. Materials and Methods

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 ^oC and 15.57 ^oC, with a mean temperature over the study period of 10.17 (± 2.08) ^oC.

Contaminant exposure 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 % agar impregnated, with ten spiked with a 688 $\mu mol.l^{-1}$ dose of trimethoprim, ten with a 688 $\mu mol.l^{-1}$ dose of EE2 and ten with 688 $\mu mol.l^{-1}$ dose trimethoprim and a 688 $\mu mol.l^{-1}$ 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).

Contaminant exposure 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 over 72 hours at 60 ^oC, excess silt and detritus adhering to each

teabag was gently removed, and their dry-weight determined. The tea was then removed from each teabag, re-weighed and combusted at 550 ^{o}C for four hours to determine ash free dry mass (following Keuskamp et al, 2013).

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 ^{o}C over 72 hours. Following Keuskamp et al. (2013) we then determined the organic matter stabilization factor (*S*) as:

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

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] $$a_r = 0.552\ (1 - S)$$

Where S 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*) can then be calculated as:

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

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).

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 mass loss of the Green tea and Rooibos teabags, the stabilization factor (*S*) and Initial decomposition rate (*k*) of the labile organic material using two-way analysis of variance (ANOVA). Post-hoc testing of significant interactions were conducted using Tukey's test for Honest Significant Difference. All data were visually explored to ensure they conformed to the assumptions of normality and homoscedacity

(following Zuur et al, 2010), Initial decomposition rate (*k*) data were $\log_{10}$ transformed to ensure that the residuals from the ANOVA model conformed to a normal distribution. See supplementary table 1 for a summary of the ANOVA test results.

3. Results and Discussion

Over the course of the experiment, dissolution of the agar delivered estimated daily doses of approximately 275 nmol $.d^{-1}$ of trimethoprim and EE2 in both the single and combined pharmaceutical treatments. As such, the pharmaceutical doses which the microbial community with each teabag where exposed to was broadly comparable with the concentration of these compounds in inland waters (e.g. Álvarez-Muñoz et al, 2015; Archer et al, 2017; Tousova et al., 2017). Rooibos Tea mass loss decreased as a consequence of EE2 exposure, with no additional/significant effects of Trimethoprim (Figure 2 A, Table 1a). By contrast for green tea, we observed additive and inhibiting effects of both trimethoprim and EE2 (Figure 2 B, Table 1b). Based on these data we can demonstrate that EE2 and trimethoprim had significant independent effects upon both the organic matter stabilization factor (Figure 3 A, Table 1c) initial decomposition rate (Figure 3 B, Table 1 d).

Our results demonstrate that exposure to low doses of both an antibiotic (trimethoprim) and an endocrine disruptor (EE2) inhibits microbial degradation, with differential effects on different phases of the decomposition process. Our results, however, do not show that EE2 reduces the impact of trimethoprim upon aquatic organic matter degradation. Both trimethoprim and EE2 had significant additive positive effects upon the organic matter stabilization factor (s) and its initial decomposition rate (k). As such, our study indicates that pollution from both compounds 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 that pharmaceutical contaminants are likely to have important implications for 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).

Contaminant exposure experiments are typically used to determine the effects of a selected contaminant on biofilm forming bacteria and algae (Tank et al., 2006; Costello et al., 2016). By integrating the TBI (Keuskemp et al. 2013) into contaminant exposure experiments we have developed a low-cost tool to quantify how exposure to pollutants such as pharmaceuticals affects instream decomposition of organic matter. Whilst our study was restricted to one stream, the method could easily be replicated at multiple sites, within more complex experimental designs. We, therefore, foresee potential applications of this method to allow the effects of multiple pollutants upon organic matter degradation and residence times to be made across a range of spatial and temporal scales in both freshwater and marine systems. As our method is characterized by its low-cost, simplicity and use of readily available consumer products, it also provides a tool which can be used to achieve levels replications within experimental designs testing the interactions between a suite of multiple pollutants and their effects upon aquatic biogeochemical processes, Finally, we propose TBI Contaminant Exposure Experiments could provide a valuable pedagogical tool for research-led environmental science teaching across a range of education contexts, as well as in citizen science based initiatives to investigate the impacts of pollutants in aquatic systems.

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Author Information

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

Conflict of Interest

The authors declare that we have no conflicts of interest.

Data Availability

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

Animal Research (Ethics)

No animals were used in this research.

Consent to Participate (Ethics)

No human subjects were used in this research.

Consent to Publish (Ethics)

This is not applicable to this study.

Plant Reproducibility

This is not applicable to this study.

Clinical Trials Registration

This is not applicable to this study.

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Table 1. Two-way analysis of variance testing for significant effects of trimethoprim and EE2 upon the percentage mass loss of the Green tea and Rooibos teabags, the organic matter stabilization factor (*S*) and initial decomposition rate (*k*).

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*) of the labile material					
	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) of the labile material					
	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				

Figures

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

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

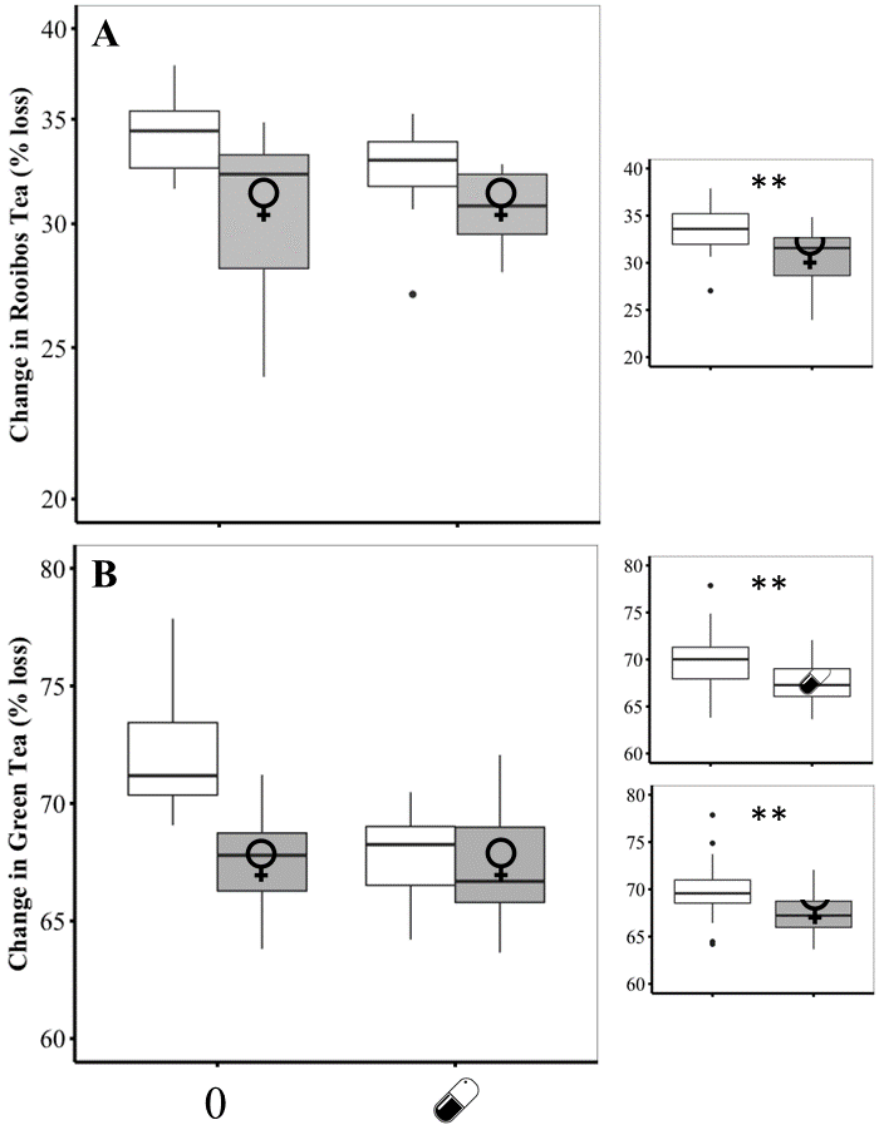
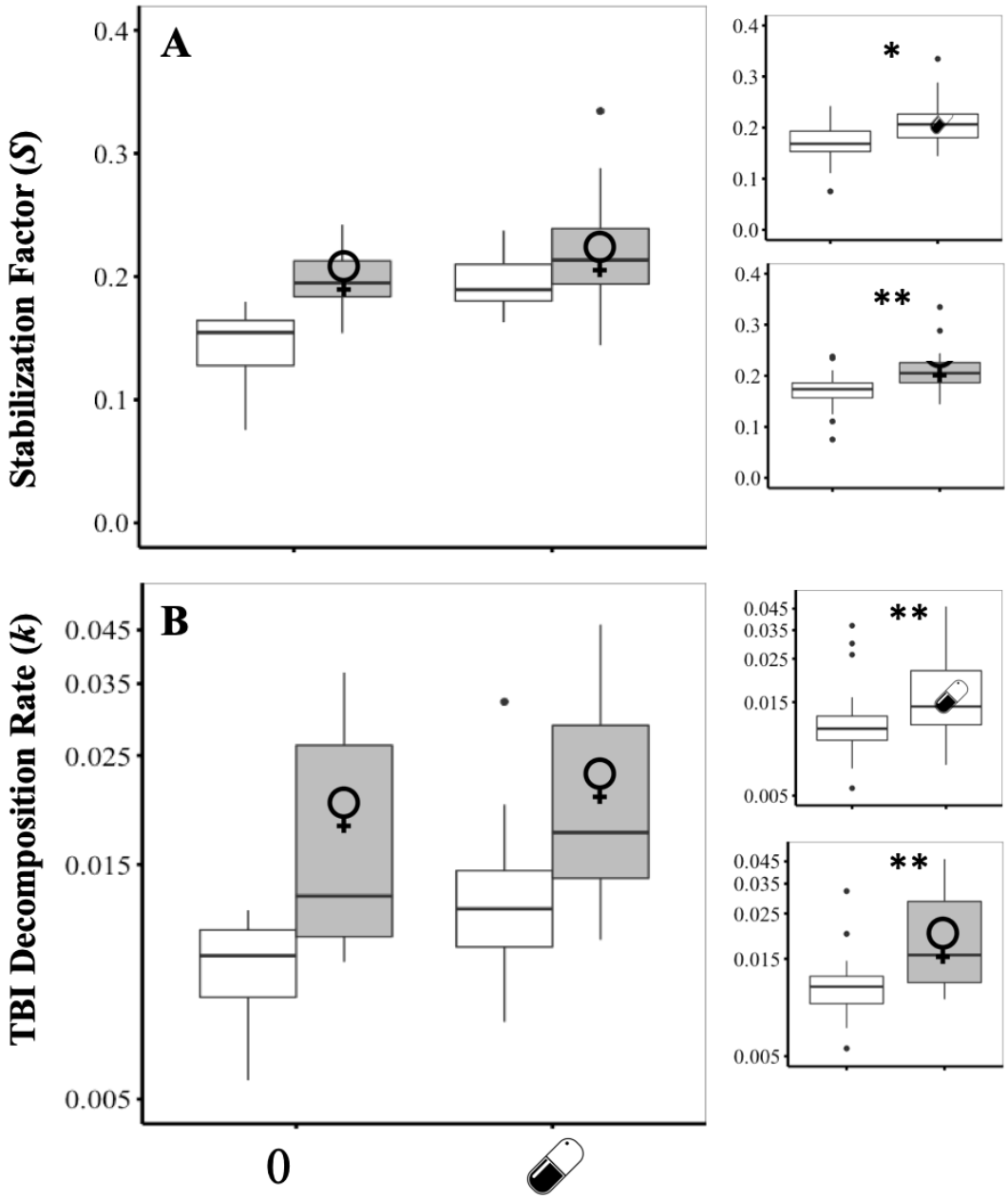
Figure 3. Effects of Trimethoprim (💊) and EE2 (♀) on the A) labile organic matter stabilization factor and B) organic matter degradation rate, calculated following Keuskamp et al (2013). Inserts show pooled data where significant independent effects of either Trimethoprim or EE2 where detected. Significance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