Short Communication

17α-ethynylestradiol (EE2) limits the impact of ibuprofen upon respiration by streambed biofilms in a sub-urban stream.

Peter McClean[1]; William Ross Hunter[1,2]*

[1]University of Ulster, School of Geography and Environmental Science, Coleraine, BT52 1SA, United Kingdom.

[2]Northern Ireland Agri-Food and Bioscience Institute, Fisheries and Aquatic Ecosystems Branch, Belfast, BT9 5PX, United Kingdom.

*Corresponding author. Email: Billy.Hunter@afbini.gov.uk

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Abstract

Pharmaceuticals compounds such as the non-steroidal anti-inflammatory drug ibuprofen and the artificial estrogen 17α-ethynylestradiol (EE2) are contaminants of emerging concern in freshwater systems. Globally, human pharmaceutical is growing by around ~3 % per year, yet we know little about how interactions between different pharmaceuticals may affect aquatic ecosystems. Here we test how interactions between ibuprofen and 17α-ethynylestradiol affect the growth and respiration of streambed biofilms. We used contaminant exposure experiments to quantify how these compounds affected biofilm growth (biomass), respiration and gross primary production, both individually and in combination. Within our study, we found no effects of either ibuprofen or 17α-ethynylestradiol on biofilm biomass (using ash free dry mass as a proxy) or gross primary production. Ibuprofen significantly reduced biofilm respiration. However, concomitant exposure to 17α-ethynylestradiol counteracted the depressive effects ibuprofen upon biofilm metabolism. Our study, thus, demonstrates that interactions between pharmaceuticals in the environment may have complex effects upon microbial contributions to aquatic ecosystem functioning.

Key Words

17α-ethynylestradiol; Biofilm; EE2; Ibuprofen; Microbial Metabolism; Pharmaceuticals and Personal Care products

1. Introduction

Human pharmaceuticals and personal care products (PPCPS) are contaminants of emerging concern within the environment (Rosi-Marshall and Royer 2012; Gaston et al. 2019). Since the year 2000, pharmaceutical use has grown by approximately 3% per year globally and this predicted to increase further as human populations grow (Van Broeckel et al. 2014). Removal of Pharmaceuticals and personal care products (PPCPs) via waste-water treatment plants (WWTPs) is inefficient leading to the constant release of low doses of compounds such as non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen), antimicrobial compounds (e.g. triclosan, and trimethoprim) and artificial estrogens (e.g. 17α-ethynylestradiol) into the aquatic environment (Gros et al. 2007; Álvarez-Muñoz et al. 2015; Archer et al. 2017). This is potentially problematic because these compounds are specifically designed specifically to produce physiological effects within an organism, at ultra-low (nano-molar) concentrations (Rosi-Marshall and Royer, 2012; Van Broeckel et al. 2014; Álvarez-Muñoz et al. 2015). Eco-toxicological studies reveal that PPCPs at environmental concentrations can have significant physiological effects on both aquatic fauna and microorganisms, with the potential to disrupt aquatic ecosystem functioning altering carbon and nutrient cycling, and negatively affect water quality (Jobling et al. 2003; Hernando et al. 2006; Rosi-Marshall et al. 2013; Drury et al. 2013; Źur et al. 2018).

Headwater and lower-order streams are the smallest tributaries of a river system, which are typically closest to the rivers' sources. In these streams aquatic biofilms attached to the streambed represent the dominant mode of microbial life Besemer et al. 2012; Battin et al. 2016). Biofilms, composed of consortia of bacteria and unicellular eukaryotic algae bound within a complex matrix of extracellular polymeric substances (EPS), play a key role in the functioning of fluvial ecosystems, controlling both the transport and degradation of organic matter within a stream (Battin et al. 2016). Rosi-Marshall et al. (2013) revealed that aquatic PPCPs such as caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin and ranitidine and negative effects upon biofilm growth, respiration, and community composition. PPCPs, however, are diverse group of chemicals, which may interact with each other in a multitude of different, and often-unexpected ways (Rosi-Marshall et al. 2013; Gerbersdorf et al. 2015; Gaston et al. 2019). Consequently, a mechanistic

understanding of the interactions between different PPCPs is needed if we are to fully understand their environmental impacts.

Within the broad spectrum of PPCPs the non-steroidal anti-inflammatories (NSAIDs), such as ibuprofen, and artificial estrogens, such as 17α-ethynylestradiol, represent some of the most commonly detected compounds in aquatic systems (Álvarez-Muñoz et al. 2015; Gaston et al. 2019). NSAIDs are known to have antimicrobial properties, with ibuprofen exhibiting potential as a biofilm control agent (Reśliński et al. 2015; Shah et al. 2018; Źur et al. 2018; Oliveira et al. 2019). Conversely, artificial oestrogens and other endocrine disruptors may adsorb onto microbial biofilms facilitating their biological degradation (Writer et al. 2012; Zhang et al. 2014; Adeel et al. 2017). Whilst there are no known therapeutic interactions between NSAIDs and artificial estrogens in animal systems, the fact that these compounds elicit different microbial responses suggests that there may be potential for interactions between NSAIDs and artificial estrogens to affect the growth and metabolism of aquatic microorganisms. Here we present the first data on how interactions between ibuprofen and 17α-ethynylestradiol (hereafter, EE2) affect the growth and respiration of streambed biofilms. We *conducted in situ* contaminant exposure experiments, following Costello et al. (2015), to test how chronic exposure to ibuprofen, and EE2, both individually and in combination, affected streambed biofilm growth, primary production and respiration.

2. Materials and Methods

All experiments were carried out between the 30th November 2018 and the 22nd January 2019 in the Ballysally Blagh (Latitude: 55°08'45.1"N Longitude: 6°40'18.0"W), a ground-water fed second-order stream. The Ballysally Blagh is a tributary of the lower River Bann (Northern Ireland), 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. The mean volumetric rate for water flow in the Ballysally Blagh is 0.21 (± 0.27) m^3 s^{-1}, measured at a V-shaped weir (National River Flow Archive. 2019) and the stream is defined as eutrophic, with dissolved nitrate concentrations ranging between 1.37 and 14.15 ml.l^{-1} and soluble reactive phosphorus concentrations between 0.033 and 0.4 mg.l^{-1}. Water temperature at the study site was recorded at 1-hour intervals throughout the experiment using a HOBO

MX2204 Bluetooth temperature logger. Temperatures ranged between 9.35 ^{o}C and 5.16 ^{o}C, with a mean temperature of 7.72 (± 0.85) ^{o}C recorded over the study period.

Contaminant exposure experiments were conducted following Costello et al. (2015). Briefly, forty 120 ml screw cap sample pots where filled with 2 % agar impregnated, of which ten were spiked a 0.5 mmol.l^{-1} dose of ibuprofen, ten spiked with a 0.5 mmol.l^{-1} dose of EE2, ten spiked with a 0.5 mmol.l^{-1} dose of both ibuprofen and EE2, and ten received no pharmaceutical treatment (control). Both ibuprofen and EE2 have relatively low solubility in water (21 mg.l^{-1} and 3.6 mg.l^{-1} respectively). As such, stock solutions for each pharmaceutical treatment were made up by dissolving 159 mg of ibuprofen (Sigma-Aldrich, Product No. I4883), 105 mg of EE2 (Sigma-Aldrich, Product No. E4876) or both in 11 ml of 70 % ethanol. 1 ml aliquots of the stock solution were then used to dose each contaminant exposure experiment and the control treatments receiving a 1 ml aliquot of 70 % ethanol. Pre-combusted Whatman® 45 mm GF/F filters were placed onto of the solid agar and secured using the screw cap, to provide a substratum for streambed biofilm colonization. Contaminant exposure experiments were then secured to four L-shaped metal bars (l = 1000 mm; w = 50 mm; d = 50 mm) and deployed at 10 cm depth, in an area of turbulent flow (riffle) within the stream.

Environmental chambers were assembled from two Curry's Essentials® C61CF13 chest freezers, with the power source re-routed through Inkbird ITC-308 Digital Temperature Controller used to override the freezers internal thermostat. A single Tetra HT50 (50 Watt) aquarium heater was also attached to the Inkbird temperature controller of each unit to help stablise the internal temperature. Two NICREW planted aquarium LED strip lights were attached to the lid, providing a source of photosynthetically active radiation (– 106.0 µmol m^{-2} s^{-1}, measured using an Apogee Instruments Photosynthetically Active Radiation Meter). Environmental chambers were filled with 20 l of streamwater and the internal temperatures set to 7.7 ^{o}C. The contaminant exposure experiments were left *in situ* for 54 days, after which they were recovered from the stream, directly placed into one of the environmental chambers and allowed to acclimate over 24 hours. During the acclimation period each mesocosm was aerated using a Aqualine Hailea Aco-9630.

After the acclimation period, biofilm respiration and gross primary production were determined by changes in oxygen consumption by enclosing each contaminant

exposure experiment into a sealed Perspex push core (height = 30 cm, internal diameter = 7 cm) chambers containing 1 litre of sterile-filtered streamwater and held at 7.7 ^{o}C in one of the environmental chambers (Bott et al. 1978; Fellows et al. 2006). Biofilm respiration (R) were quantified by measuring the change in oxygen concentrations over a one-hour period (oxygen consumption in darkness (PAR ~ 0.0 µmol m^{-2} s^{-1}) using a Hach Sension 6 dissolved oxygen meter. Net primary production (NPP) was then quantified by measuring the change in oxygen concentration over a one 1-hour period, under artificial illumination (PAR ~ 106.0 µmol m^{-2} s^{-1}). Biofilm Gross Primary Production (GPP) was calculated from NPP and R as:

$$GPP = NPP - R \quad [1]$$

Microbial biomass within each Contaminant Exposure Experiment was quantified as Ash Free Dry Mass of the GF/F filters. These were dried four 48 hours at 65 ^{o}C and then subsequently combusted at 550 ^{o}C for 2 hours. We estimated the daily dose of the pharmaceuticals delivered within each treatment following Costello et al. (2015), assuming that ibuprofen and EE2 doses were proportional to the agar mass lost.

All data are available in the supplementary information. Data analyses were conducted in the R statistical computing environment using the *base* and *ggplot2* packages (R Development Core Team. 2009; Wickham, 2016). We tested for independent and combined effects of ibuprofen and EE2 upon in microbial biomass (Ash Free Dry Weight), Respiration and Net Ecosystem Production using two-way analysis of variance (ANOVA). Post-hoc testing of significant interactions was 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). Microbial biomass data were $\log_{10}$ transformed to ensure the residuals of the ANOVA model conformed to a normal distribution.

3. Results

Based on agar mass loss throughout the experiment, we estimated that the biofilms growing within our contaminant exposure experiments were exposed to a daily ibuprofen dose ~ 164 (± 14) nmol d^{-1}; and a daily estrogen dose ~ 115 (± 20) nmol.d^{-}

[1]. In the combined treatment the daily dose of ibuprofen ~ 155 (± 13), nmol d^{-1}, whilst the estrogen dose ~118 (± 10) nmol d^{-1}.

Using ash free dry mass as a proxy for microbial biomass we detected no significant effects of pharmaceutical exposure upon microbial biofilm growth (Fig 1 A; Table 1 a). We detected a significant interaction between ibuprofen and EE2 affecting microbial respiration (Fig 1 B; Table 1 b).

Exposure to ibuprofen alone depressed microbial oxygen consumption by ~ 38 %, whilst exposure to EE2 alone resulted in a slight (non-significant) increase in oxygen consumption of ~ 5 %. In combination, EE2 counteracted the depressive effect of ibuprofen upon of microbial respiration, resulting in no significant change in respiration relative to the control .

Gross Primary Production was negative in all treatments, with no significant effects of either ibuprofen or EE2 detected (Fig 1 C; Table 1 c).

4. Discussion

Our study demonstrates that interactions between the NSAID ibuprofen and the artificial estrogen EE2 have a significant effect upon the streambed biofilm respiration. Specifically, concomitant exposure to both ibuprofen and EE2 reduced the depressive effect of ibuprofen upon biofilm respiration. Ibuprofen is known to have antimicrobial properties and has been reported to inhibit biofilm formation by both *Staphylococcus aureus* and *Escherichia coli* (Reśliński et al. 2015; Shah et al. 2018; Oliviera et al. 2019). It is, therefore, unsurprising that ibuprofen depressed microbial respiration within the streambed biofilms. EE2 has been observed to adsorb to microbial biofilms (Writer et al. 2012) where it can then be used by the resident microorganisms as an organic matter source Stumpe et al. 2009; Ribeiro et al. 2010). Consequently, biofilms have been proposed as a tool for the removal of artificial estrogens and other endocrine disruptors within wastewater treatment facilities (Pieper and Rotard, 2011). Sorption of estrogens to the biofilm may protect the microbial cells, by reducing the space available within the EPS matrix onto which ibuprofen molecules may bind (Writer et al. 2012; Zhang et al. 2014). This mechanism, however, remains speculative and would require investigation within controlled laboratory experiments.

Given ibuprofen's potential as a biofilm control agent (Reśliński et al. 2015; Shah et al. 2018; Źur et al. 2018; Oliveira et al. 2019), we were surprised to observe that it had no effect upon biofilm biomass within our experiments. Ash free dry mass is, however, a coarse method for estimating microbial biomass and so not suitable to detect small changes in the biofilm. This is likely to be of particular concern in urban and agricultural streams, where siltation may introduce a significant bias into weight-based estimates of biomass. Visual methods, such as microscopic cell counts (Grzegorczyk et al. 2018), quantification of EPS polysaccharides (Fish et al. 2017; Grzegorczyk et al. 2018) or other biomarkers, such as polar lipid fatty acids (Middelburg et al 2000; Frostegård et al., 2010; Hunter et al., 2012, 2013) would provide a more accurate proxy for biomass. Thus, we cannot reliably infer whether interactions between ibuprofen and EE2 may have altered biofilm biomass within this study.

The negative values for GPP within the present study suggest that the biofilms were net heterotrophic, relying on the supply of organic matter from the surrounding environment to provide energy and nutrients for biofilm growth. This may reflect the choice of agar as the carrier medium for the pharmaceuticals within the contaminant exposure experiments. The agar releases a constant supply of dissolved organic matter through the glass fibre filters (Rosi-Marshall et al. 2013; Costello et al. 2015), which may generate favorable microhabitat heterotrophic microorganisms. As such we were unable to determine whether chronic pharmaceutical exposure had any effects upon photosynthetic pathways within our biofilms.

Within this short paper we present preliminary results which demonstrate that interactions between NSAIDs and artificial estrogens could have important implications for aquatic ecosystem functioning during the winter period, when lower water temperatures limit microbial activity within streambed biofilms (Ylla et al. 2012). Whilst the doses of ibuprofen and estrogen within the CES experiments appear high, the daily dose delivered to the growing biofilm was comparable with the concentrations detected in many aquatic systems (Jobling et al. 2003; Hernando et al. 2006; Gros et al. 2007; Rosi-Marshall and Royer, 2012; Álvarez-Muñoz et al. 2015; Ruhí et al. 2016; Archer et al 2017), and lower than the doses used in previous contaminant exposure experiments (Rosi-Marshall et al. 2013). Our

234 experiment, thus, provides a realistic insight into of how interactions between these
235 two PPCPs affect aquatic microbial activity.

236 Overall, our study supports a growing body of evidence suggesting that PPCPs
237 represent a major threat to ecosystem functioning in many streams and rivers
238 (Jobling et al. 2003; Hernando et al. 2006; Gros et al. 2007; Rosi-Marshall and
239 Royer, 2012; Rosi-Marshall et al. 2013; Álvarez-Muñoz et al. 2015; Ruhí et al. 2016;
240 Archer et al 2017). The study, is, however, limited to a single urban stream, which
241 prevents wider interpretation of our results. Interactions between PPCPs and their
242 effects within the environment are potentially complex and mediated by changes in
243 environmental context (Rosi-Marshall et al. 2013). Future studies need to investigate
244 how the interactions between different PPCPs affect aquatic microbial communities
245 under different regimes of temperature, aquatic chemistry and ecological community
246 structure. This demands the design of field-based contaminant exposure
247 experiments to test the interactions between a range of PPCPs both within and
248 between freshwater catchments. Furthermore, this study highlights a clear need to
249 identify the underlying biochemical mechanisms which explain how interactions
250 between different PPCPs affect aquatic microbial processes.

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257 **Conflicts of Interest:** The authors declare no conflicts of interest relating to this
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259 **Data Accessibility.** All data related to this publication are available as a
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261

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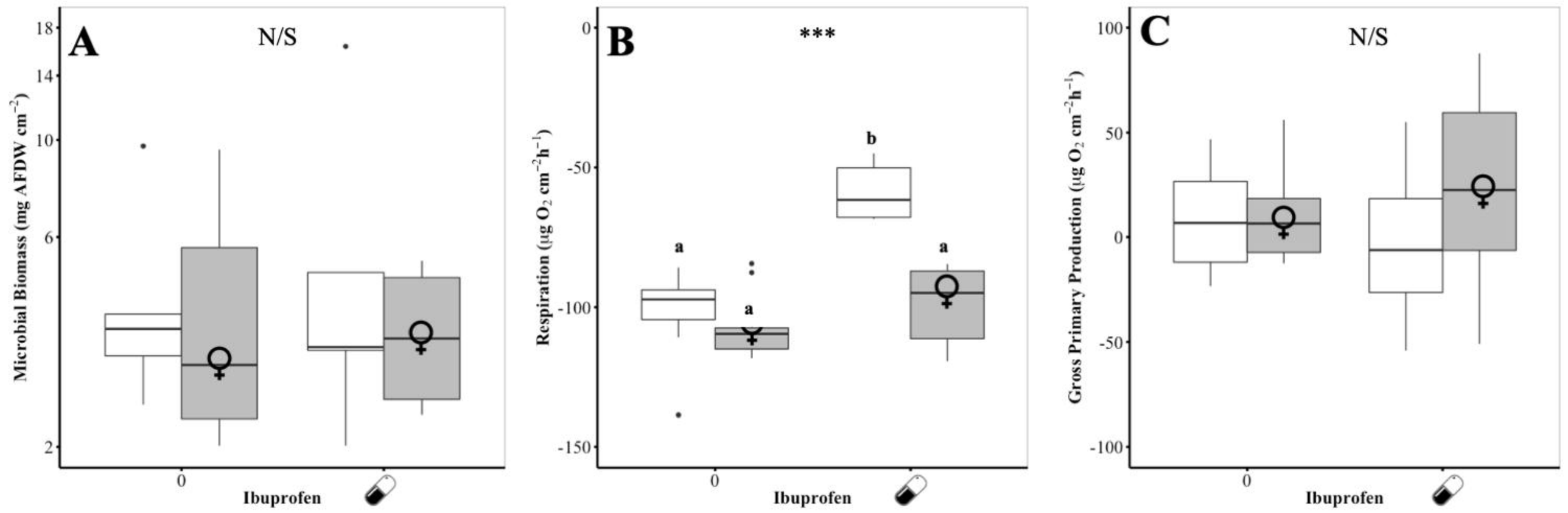
Table

Table 1. ANOVA summary tables of the effects of Ibuprofen and 17α-ethynylestradiol [EE2] upon a) biomass (ash free dry weight), b) respiration and c) Gross Primary Production of cultured streambed biofilms.

a) Biomass (Ash Free Dry Weight)					
	Df	SS	MS	F	*p*
Ibuprofen	1	0.001	0.00086	0.008	0.931
EE2	1	0.006	0.00586	0.051	0.822
Ibuprofen : EE2	1	0.151	0.15142	1.331	0.256
Residuals	36	4.097	0.11379		
b) Respiration					
	Df	SS	MS	F	*p*
Ibuprofen	1	6482	6482	41.13	**<0.001**
EE2	1	5085	5085	32.26	**<0.001**
Ibuprofen : EE2	1	2952	2952	18.73	**<0.001**
Residuals	36	5674	158		
c) Gross Primary Production					
	Df	SS	MS	F	*p*
Ibuprofen	1	40	40.4	0.037	0.848
EE2	1	2612	2611.7	2.403	0.130
Ibuprofen : EE2	1	2318	2318.1	2.133	0.153
Residuals	36	39121	1086.7		

Figures

Figure 1. Effects of Ibuprofen (♂) and 17α-ethynylestradiol (♀) upon the (A) biomass (ash free dry weight), (B) respiration and (C) Gross Primary Production of cultured streambed biofilms. Significance levels: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; N/S $p > 0.05$. Where significant interactions were identified, groups labelled with the same lowercase letter are not significantly different ($p > 0.05$; Tukey's tests).


A
N/S
Microbial Biomass (mg AFDW cm^{-2})
18
14
10
6
2
0
Ibuprofen
B
***
Respiration (µg O$_2$ cm^{-2}h^{-1})
0
-50
-100
-150
a
a
b
a
0
Ibuprofen
C
N/S
Gross Primary Production (µg O$_2$ cm^{-2}h^{-1})
100
50
0
-50
-100
0
Ibuprofen