**Short Communication**

# 17a-Estradiol limits the impact of ibuprofen upon community respiration by streambed biofilms in a sub-urban stream.

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**Co-first author note:** [1,2]These authors contributed equally to this work.

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**Disclaimer:** We declare no conflicts of interest relating to this study.

## Data Accessibility statement

All data related to this publication are available in the supplementary information and are also archived in the University of Ulster's PURE data archive and are available under a CC-BY creative commons licence. [add web link]

# ***Title:*** 17a-Estradiol limits the impact of ibuprofen upon community respiration by streambed biofilms in a sub-urban stream.

## Abstract

Pharmaceuticals compounds such as the non-steroidal anti-inflammatory drug ibuprofen and the artificial estrogen 17a-estradiol are contaminants of emerging concern in freshwater systems. Globally, the use of these compounds 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 17a-estradiol affect the growth and community metabolism 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 17a-estradiol on biofilm biomass (using ash free dry mass as a proxy) or gross primary production. Ibuprofen significantly reduced biofilm respiration. However, concomitant exposure to 17a-estradiol 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

17a-Estradiol; Biofilm; Contaminants of Emerging Concern; 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 [1, 2]. Since the year 2000, pharmaceutical use has grown by approximately 3% per year globally and this predicted to increase further as human populations grow [3]. Removal of Pharmaceuticals and personal care products (PPCPs) via waste-water treatment plants (WWTPs) is inefficient leading to constant release of compounds which are often specifically designed specifically to produce physiological effects within an organism, at ultra-low (nano-molar) concentrations [2, 4]; compounds such as non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. ibuprofen), antimicrobial compounds (e.g. triclosan, and trimethoprim) and endocrine inhibitors (e.g. Estradiol) into the aquatic environment [4-7]. 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 the functioning of aquatic ecosystems, alter carbon and nutrient cycling, and negatively affect water quality [8-12].

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 [13, 14]. Biofilms are composed of consortia of bacteria and unicellular eukaryotic algae bound within a complex matrix of extracellular polymeric substances (EPS) and play a key role in the functioning of fluvial ecosystems, controlling both the transport and degradation of organic matter within a stream [14]. Rosi-Marshall et al. [10] 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 [1, 10, 15]. 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 endocrine disruptors, such as 17a-estradiol, represent some of the most commonly detected compounds in aquatic systems [1, 4, 9]. NSAIDs are known to have antimicrobial properties, with ibuprofen exhibiting potential as a biofilm control agent [12, 16-18]. Conversely, oestrogens and other endocrine

disruptors may adsorb onto microbial biofilms facilitating their biological degradation [19-21]. As such, there is potential for antagonistic interactions between NSAIDs and Endocrine Disruptors to affect the growth and metabolism of streambed biofilms. Here we present the first data on how interactions between ibuprofen and 17a-estradiol affect the growth and community metabolism of streambed biofilms. We *conducted in situ* contaminant exposure experiments, following Costello et al. [22], to test how chronic exposure to ibuprofen, and 17a-estradiol, both individually and in combination, affected streambed biofilm growth, primary production and respiration.

## 2. Materials and Methods

All experiments were carried out between the 30$^{th}$ November 2018 and the 22$^{nd}$ 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 [23] 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 (Fig 1). 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. [22]. Briefly, forty 120 ml screw cap sample pots where filled with 2 % agar impregnated, of which 10 were impregnated a 0.5 mmol.l$^{-1}$ dose of ibuprofen, 10 with a 0.5 mmol.l$^{-1}$ dose of 17a-estradiol, 10 with a 0.5 mmol.l$^{-1}$ dose of both ibuprofen and 17a-estradiol, and 10 received no pharmaceutical treatment (control). Both ibuprofen and estradiol 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 in 70 % ethanol, with 1 ml aliquots 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 (dimensions) 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 and placed into one of the environmental chambers and allowed to acclimate over 24 hours. During the acclimation period each mesocosm was aerated using a Aquarline 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 xx l of streamwater and held at 7.7 $^{o}$C in one of the environmental chambers ([24, 25]. 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:

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

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.

All data analysis was conducted in the R statistical computing environment using the *base* and *ggplot2* packages [26, 27]. We tested for independent and combined effects of ibuprofen and estradiol 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. [28]. Microbial biomass data were $\log_{10}$ transformed to ensure the residuals of the ANOVA model conformed to a normal distribution.

## 3. Results

Using ash free dry mass as a proxy for microbial biomass we detected no significant effects of pharmaceutical exposure upon microbial biofilm growth (Fig 2 A; Table 1 a). We detected a significant interaction between ibuprofen and estradiol affecting microbial respiration (Fig 2 B; Table 1 b). Exposure to ibuprofen alone depressed microbial oxygen consumption by ~ 38 %, whilst exposure to estradiol alone resulted in a slight (non-significant) increase in oxygen consumption. In combination, estradiol counteracted the depressive effect of ibuprofen upon of microbial respiration. Gross Primary Production was negative in all treatments, with no significant effects of the pharmaceutical treatments detected (Fig 2 C; Table 1 c).

## 4. Discussion

Our study demonstrates that interactions between an NSAID (ibuprofen) and an endocrine disruptor (17a-estradiol) have a significant effect upon the metabolism of streambed biofilms. Specifically, concomitant exposure to both 17a-estradiol and estradiol reduces 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* [16-18]. It is, therefore, unsurprising that ibuprofen depressed microbial respiration within the streambed biofilms. Estradiol has been observed to adsorb to microbial biofilms [19] where it can then be used by the resident microorganisms as an organic matter source [29, 30]. Consequently, biofilms have been proposed as a tool for the removal of estradiol and other endocrine disruptors within wastewater treatment facilities [31]. Furthermore, we propose that the sorption of estradiol to the biofilm

may protect the microbial cells, by reducing the space available within the EPS matrix onto which ibuprofen molecules may bind. This mechanism, however, remains speculative and would require investigation within controlled laboratory experiments.

Given ibuprofen's potential as a biofilm control agent [12, 16-18], 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 of estimating microbial biomass and so may not be able 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 [32], quantification of EPS polysaccharides [32, 33] or other biomarkers, such as polar lipid fatty acids [34-37] may have provided a more accurate proxy for biomass. Thus, we cannot reliably infer whether interactions between ibuprofen and estradiol 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 [10, 22], 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 endocrine disruptors have important implications for aquatic ecosystem functioning during the winter period, when lower water temperatures limit microbial activity within streambed biofilms [38]. Overall, this suggests that PPCPs represent a major threat to ecosystem functioning in many streams and rivers. The interactions between different PPCPs, however, are potentially complex. Whilst identification of the underlying biochemical mechanisms is beyond the scope of this study, our results highlight the need for detailed ecotoxicological analysis of the multiple interactions between PPCPs and how this effects microbial and faunal activity, in the environment.

## 5. References

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342 **Table**

343 Table 1. ANOVA summary tables of the effects of Ibuprofen and 17a-Estradiol upon
344 a) biomass (ash free dry weight), b) respiration and c) Gross Primary Production of
345 cultured streambed biofilms.
346

| a) Biomass (Ash Free Dry Weight) | | | | | |
|---|---|---|---|---|---|
| | Df | SS | MS | F | *p* |
| Ibuprofen | 1 | 0.001 | 0.00086 | 0.008 | 0.931 |
| Estradiol | 1 | 0.006 | 0.00586 | 0.051 | 0.822 |
| Ibuprofen : Estradiol | 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** |
| Estradiol | 1 | 5085 | 5085 | 32.26 | **<0.001** |
| Ibuprofen : Estradiol | 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 |
| Estradiol | 1 | 2612 | 2611.7 | 2.403 | 0.130 |
| Ibuprofen : Estradiol | 1 | 2318 | 2318.1 | 2.133 | 0.153 |
| Residuals | 36 | 39121 | 1086.7 | | |

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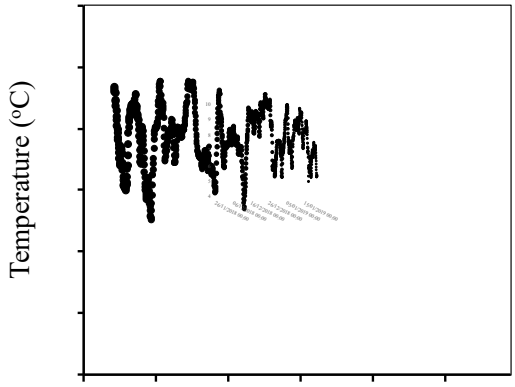
348 **Figures**

349 Figure 1. Change in water temperature over the biofilm growth period, measured at 1
350 hour intervals using a HOBO MX2204 Bluetooth temperature logger

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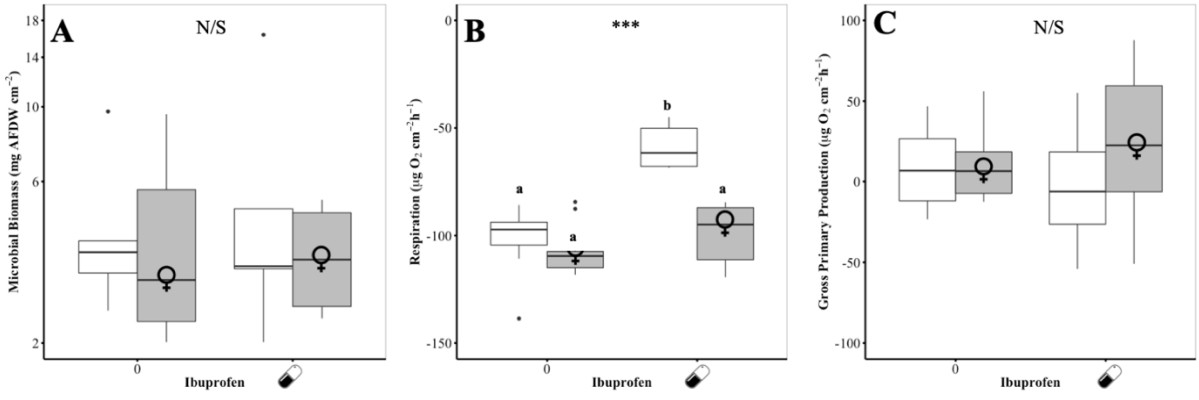
353 Figure 2. Effects of Ibuprofen (♂) and Estradiol (♀) upon the (A) biomass (ash free
354 dry weight), (B) respiration and (C) Gross Primary Production of cultured streambed
355 biofilms. Significance levels: ***p < 0.001; **p < 0.01; *p < 0.05; N/S p > 0.05.Where
356 significant interactions were identified, groups labelled with the same lowercase
357 letter are not significantly different (p > 0.05; Tukey's tests).

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