Short Communication

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

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Abstract

Pharmaceutical 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 use 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 EE2 affect the growth and respiration of streambed biofilms. We used contaminant exposure experiments to quantify how these compounds affected biofilm growth (biomass), respiration, net primary production (NPP) and gross primary production (GPP), both individually and in combination. We found no effects of either ibuprofen or EE2 on biofilm biomass (using ash free dry mass as a proxy) or gross primary production. Ibuprofen significantly reduced biofilm respiration and altered NPP. Concomitant exposure to EE2, however, counteracted the inhibitory effects of ibuprofen upon biofilm respiration. 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 is predicted to increase further as human populations grow (Van Broeckel et al. 2014). Removal of 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; Gallagher and Reisinger, 2020).

In headwater 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 had 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; Robson et al., 2020). 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 estrogens 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). There are currently no known therapeutic interactions between NSAIDs and artificial estrogens in animal systems. The fact that these compounds elicit different microbial responses, however, suggests 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. (2016), 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. (2016). Briefly, forty 120 ml screw cap sample pots where filled with 2 % agar, of which ten were spiked with a fixed 0.5 mmol.l^{-1} concentration of ibuprofen, ten with a fixed 0.5 mmol.l^{-1} concentration of of EE2, ten spiked with fixed 0.5 mmol.l^{-1} concentrations 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 transparent 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. Biofilm net primary productivity (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 productivity (GPP) by the biofilms was then calculated from NPP and R as:

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

In cases where NPP was more negative that R (indicating greater oxygen consumption under artificial illumination) the baseline for GPP defaulted to zero.

Microbial biomass within each Contaminant Exposure Experiment was quantified as Ash Free Dry Mass of the GF/F filters. These were dried for 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. (2016), 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 microbial biomass (Ash Free Dry Weight), Respiration and NPP and GPP 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

Using ash free dry mass as a proxy for microbial biomass we detected no significant effects ($p > 0.05$) of pharmaceutical exposure upon microbial biofilm growth (Fig 1 A; Table 1 a). We detected a significant interaction ($p < 0.001_{df = 1; F = 18.75}$) between

ibuprofen and EE2 affecting microbial respiration (Fig 1 B; Table 1 b). Exposure to ibuprofen alone inhibited 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 inhibitory effect of ibuprofen upon of microbial respiration, resulting in no significant change in respiration relative to the control. Biofilm NPP was negative in all treatments, with ibuprofen exposure resulting in a significant decrease in oxygen consumption ($p = 0.009_{df = 1; F = 7.483}$), reflecting the effect on biofilm respiration (Fig 1 C; Table 1 c). Across all treatments GPP was close to zero, with no significant effects ($p > 0.05$) of either ibuprofen or EE2 We did, however, detect a non-significant increase in oxygen production by biofilms exposed to both ibuprofen and GPP (Fig 1 D; Table 1 d).

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 inhibited 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). The presence of EE2 as an energy source may, therefore, counteract the inhibitory effects of ibuprofen (Combalbert et al., 2010), whilst sorption of EE2 to the biofilm matrix may protect the microbial cells by reducing the space available onto which ibuprofen molecules may bind (Writer et al. 2012; Zhang et al. 2014). These mechanisms, however, remain speculative and require further investigation within controlled laboratory experiments.

The negative NPP within the experiment suggests that our biofilms were heterotrophic, relying on organic matter from the surrounding environment to provide energy and nutrients for biofilm growth. The significant effects of ibuprofen upon

NPP, therefore, provide further evidence of this specific PPCP inhibits heterotrophic metabolism in streambed biofilms. Autotrophic activity was low, within our study, limiting our ability to infer how either ibuprofen or EE2 affects the algal component within our biofilms. Nevertheless, the non-significant increase in GPP within biofilms exposed to both pharmaceuticals further suggests that EE2 may mediates microbial responses ibuprofen exposure. This experiment was, however, conducted during the winter, when algal growth within streambed biofilms is typically low (e.g. Duncan and Blinn, 1989; Francoeur et al., 1999). To adequately test how interactions between ibuprofen and EE2 affect autrophic biofilms, requires repetition of the study during spring or summer, when longer day length is likely to promote higher algal growth at the streambed.

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. This, however, may reflect the development of microbial resistance to anthropogenic stressors such as pharmaceuticals in agricultural and urban catchments to (e.g. Drury et al., 2013; Cai et al., 2016; Qu et al, 2017; Roberto et al. 2018). Furthermore, siltation of fine particulate matter may affect the accuracy of ash free dry mass as a measure of biomass in urban and agricultural streams. This leads us to suggest that complimentary analysis of specific microbial biomarkers, such as polar lipid fatty acids (Middelburg et al 2000; Frostegård et al., 2010; Hunter et al., 2012, 2013) and extracellular polysaccharide quantification (Fish et al., 2017; Grzegorczyk et al., 2018) may provide further insight how these pharmaceutical may affect biofilm biomass and structure.

Within this short paper we demonstrate that interactions between NSAIDs and artificial estrogens could have important implications for aquatic ecosystem functioning during the winter, when lower water temperatures limit microbial activity within streambed biofilms (Ylla et al. 2012). Whilst the doses of ibuprofen and EE2 within our study appear high, they are broadly comparable with doses used in many other contaminant exposure experiments (Drury et al., 2013; Rosi-Marshall et al. 2013, Rosi et al., 2018; Gallagher and Reisinger 2020). Our experiment, thus, provides a reasonably realistic insight into of how interactions between these two PPCPs affect aquatic microbial activity.

Our study supports a growing body of evidence suggesting that PPCPs represent a major threat to ecosystem functioning in many streams and rivers (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). Interactions between PPCPs and their effects within the environment are potentially complex and mediated by changes in environmental context (Rosi-Marshall et al. 2013; Rosi et al., 2018 Gallagher and Reisinger, 2020). Future studies need to investigate how the interactions between different PPCPs affect aquatic microbial communities under different regimes of temperature, aquatic chemistry and ecological community structure. This demands the design of field-based contaminant exposure experiments that test the interactions between a range of PPCPs both within and between freshwater catchments. Here, we also highlight the need to identify what underlying biochemical mechanisms determine how interactions between different PPCPs affect aquatic microbial processes.

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Conflicts of Interest: The authors declare no conflicts of interest relating to this study.

Data Accessibility. All data related to this publication are available as a supplementary data file alongside this paper.

6. References

Adeel M, Song S, Wang Y, Francis D, Yang Y. (2017) Environmental impact of oestrogens on human, animal and plant life: A critical review. *Environment International* 99:107-119.

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

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, Besemer K, Bengtsson MM, Romani AM, Packmann AI. (2016) The ecology and biogeochemistry of stream biofilms. *Nature Reviews Microbiology* 14:251-263.

Besemer K, Peter H, Logue JB, Langenheder S, Lindstrom ES, Tranvik LJ, Battin TJ. (2012) Unraveling assembly of stream biofilm communities. *ISME Journal* 6:1459-1468.

Bott TL, Brock JT, Cushing CE, S. V. Gregory SV, King D, Petersen RC. (1978) A comparison of methods for measuring primary productivity and community respiration in streams. *Hydrobiologia* 60:3-12.

283 Cai W, Li Y, Wang P, Niu L, Zhang W, Wang C (2016). Revealing the relationship
284 between microbial community structure in natural biofilms and the pollution level in
285 urban rivers: a case study in the Qinhuai River basin, Yangtze River Delta. *Water*
286 *Science and Technology* 74:1163–1176. DOI: 10.2166/wst.2016.224.

287 Combalbert S, Hernandez-Raquet G. (2010) Occurrence, fate, and biodegradation of
288 estrogens in sewage and manure. *Applied Microbiology and Biotechnol*ogy 86:1671–
289 1692. DOI: 10.1007/s00253-010-2547-x.

290 Costello DM, Rosi-Marshall EJ, Shaw LE, Grace M, Kelly JJ. (2016) A novel method
291 to assess effects of chemical stressors on natural biofilm structure and function.
292 *Freshwater Biology* 61:2129-2140. DOI: 10.111/fwb.12541

293 Drury B, Scott J, Rosi-Marshall EJK, J.J. (2013) Triclosan Exposure Increases
294 Triclosan Resistance and Influences Taxonomic Composition of Benthic Bacterial
295 Communities. *Environmental Science and Technology* 47:8923-8930.

296 Duncan SW, Blinn DW. (1989) Importance of physical variables on the seasonal
297 dynamics of epilithic algae in a highly shaded canyon stream. Journal of Phycology
298 25:455-461. doi:10.1111/j.1529-8817.1989.tb00250.x

299 Fellows CS, Clapcott JE, Udy JW, Bunn SE, Harch BD, Smith MJ, Davies PM.
300 (2006) Benthic Metabolism as an Indicator of Stream Ecosystem Health.
301 *Hydrobiologia* 572:71-87.

302 Fish K, Osborn AM, Boxall JB. (2017) Biofilm structures (EPS and bacterial
303 communities) in drinking water distribution systems are conditioned by hydraulics
304 and influence discolouration. *Science of The Total Environment* 593-594:571-580.

305 Francoeur SN, Biggs BJF, Smith RA, Lowe RL. (1999) Nutrient Limitation of Algal
306 Biomass Accrual in Streams: Seasonal Patterns and a Comparison of Methods.
307 Journal of the North American Benthological Society 18 (2):242-260

308 Frostegård A, Tunlid A, Bååth E. (2011) Use and misuse of PLFA measurements in
309 soils. *Soil Biology and Biochemistry.* 43:1621-1625.

310 Gallagher MT, Reisinger AJ. (2020) Effects of ciprofloxacin on metabolic activity and
311 algal biomass of urban stream biofilms. Science of the Total Environment
312 706:135728. DOI: 10.1016/j.scitotenv.2019.135728.

313 Gaston L, Lapworth DJ, Stuart M, Arnscheidt J. (2019) Prioritization Approaches for
314 Substances of Emerging Concern in Groundwater: A Critical Review. *Environmental*
315 *Science & Technology* 53:6107-6122.

316 Gerbersdorf SU, Cimatoribus C, Class H, Engesser K-, Helbich S, Hollert H, Lange
317 C, Kranert M, Metzger J, Nowak W, Seiler T-, Steger K, Steinmetz H, Wieprecht S.
318 (2015) Anthropogenic Trace Compounds (ATCs) in aquatic habitats: Research
319 needs on sources, fate, detection and toxicity to ensure timely elimination strategies
320 and risk management. *Environment International* 79:85-105.

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

324 Grzegorczyk M, Pogorzelski SJ, Pospiech A, Boniewicz-Szmyt K. (2018) Monitoring
325 of Marine Biofilm Formation Dynamics at Submerged Solid Surfaces With
326 Multitechnique Sensors. *Frontiers in Marine Science* 5:363.

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.

Hunter WR, Jamieson A, Huvenne VAI, Witte U. (2013) Sediment community responses to marine vs. terrigenous organic matter in a submarine canyon. *Biogeosciences.* 10:67-80.

Hunter WR, Veuger B, Witte U. (2012) Macrofauna regulate heterotrophic bacterial carbon and nitrogen incorporation in low-oxygen sediments. *ISME Journal* 6:2140-2151.

Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Baunbeck T, Turner AP, Tyler CR. (2003) Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquatic Toxicology* 65:205-220.

Middelburg JJ, Barranguet C, Boschker HTS, Herman PMJ, Moens T, Heip CHR. (2000) The fate of intertidal microphytobenthos carbon: An in situ ^{13}C-labeling study. *Limnology and Oceanography.* 45:1224-1234.

National River Flow Archive. (2019) Station 203050: Ballysally Blagh at University of Ulster. https://nrfa.ceh.ac.uk/data/station/spatial/203050.

Pieper C, Rotard W. (2011) Investigation on the removal of natural and synthetic estrogens using biofilms in continuous flow biofilm reactors and batch experiments analysed by gas chromatography/mass spectrometry. *Water Research* 45:1105-1114.

Oliveira IM, Borges A, Borges F, Simões M. (2019) Repurposing ibuprofen to control Staphylococcus aureus biofilms. *European Journal of Medicinal Chemistry* 166:197-205.

Qu X, Ren Z, Zhang H, *et al.* (2017). Influences of anthropogenic land use on microbial community structure and functional potentials of stream benthic biofilms. *Scientific Reports* 7:15117. https://doi.org/10.1038/s41598-017-15624-x

R Development Core Team. (2009) R: A language and environment for statistical computing.

Reśliński A, Dábrowiecki S, Głowacka K. (2015) The impact of diclofenac and ibuprofen on biofilm formation on the surface of polypropylene mesh. *Hernia* 19:179-185.

Ribeiro AR, Carvalho MF, Afonso CMM, Tiritan ME, Castro PML. (2010) Microbial degradation of 17β -estradiol and 17α -ethinylestradiol followed by a validated HPLC-DAD method. *Journal of Environmental Science and Health, Part B* 45:265-273.

Roberto AA, Van Gray JB, Leff L. (2018) Sediment bacteria in an urban stream: Spatiotemporal patterns in community composition. Water Research 134:359-369.

Robson, SV, Rosi EJ, Richmond EK, Grace MR (2020). Environmental concentrations of pharmaceuticals alter metabolism, denitrification, and diatom assemblages in artificial streams. Freshwater Science. DOI: 10.1086/708893.

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

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

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

Ruhí A, Acuña A, Barceló D, Huerta B, Mor J-, Rodríguez-Mozaz S, Sabater S. (2016) Bioaccumulation and trophic magnification of pharmaceuticals and endocrine disruptors in a Mediterranean river food web. *Science of the Total Environment* 540:250-259.

Shah PAL, Marshall-Batty KR, Smolen JA, Tagaev JA, Chen Q, Rodesney CA, Le HH, Gordon VD, Greenberg DE, Cannon CL. (2018) Antimicrobial Activity of Ibuprofen against Cystic Fibrosis-Associated Gram-Negative Pathogens. *Antimicrobial Agents and Chemotherapy* 62:e01574-17.

Stumpe B, Marschner B. (2009) Factors controlling the biodegradation of 17β-estradiol, estrone and 17α-ethinylestradiol in different natural soils. *Chemosphere* 74:556-562.

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

Wickham H. (2016) *ggplot2: Elegant Graphics for Data Analysis.* Springer-Verlag New York.

Writer JH, Ryan JN, Keefe SH, Barb LB. (2012) Fate of 4-Nonylphenol and 17β-Estradiol in the Redwood River of Minnesota. *Environmental Science & Technology* 46:860-868.

Ylla I, Romaní AM, Sebater S. (2012) Labile and Recalcitrant Organic Matter Utilization by River Biofilm Under Increasing Water Temperature. *Microbial Ecology* 64:593-604.

Zhang X, Li Y, Liu B, Wang J, Feng C. (2014) The effects of estrone and 17β-estradiol on microbial activity and bacterial diversity in an agricultural soil: sulfamethoxazole as a co-pollutant. *Ecotoxicology and Environmental Safety* 107:313-320.

Żur J, Piński A, Marchlewicz A, Hupert-Kocurek K, Wojcieszyńska D, Guzik U. (2018) Organic micropollutants paracetamol and ibuprofen: toxicity, biodegradation, and genetic background of their utilization by bacteria. *Environmental Science and Pollution Research* 25:21498-21524.

Zuur AF, Ieno EN, Elphick CS. (2010) A protocol for data exploration to avoid common statistical problems. *Methods Ecology and Evolution.* 1:3-14.

Table

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

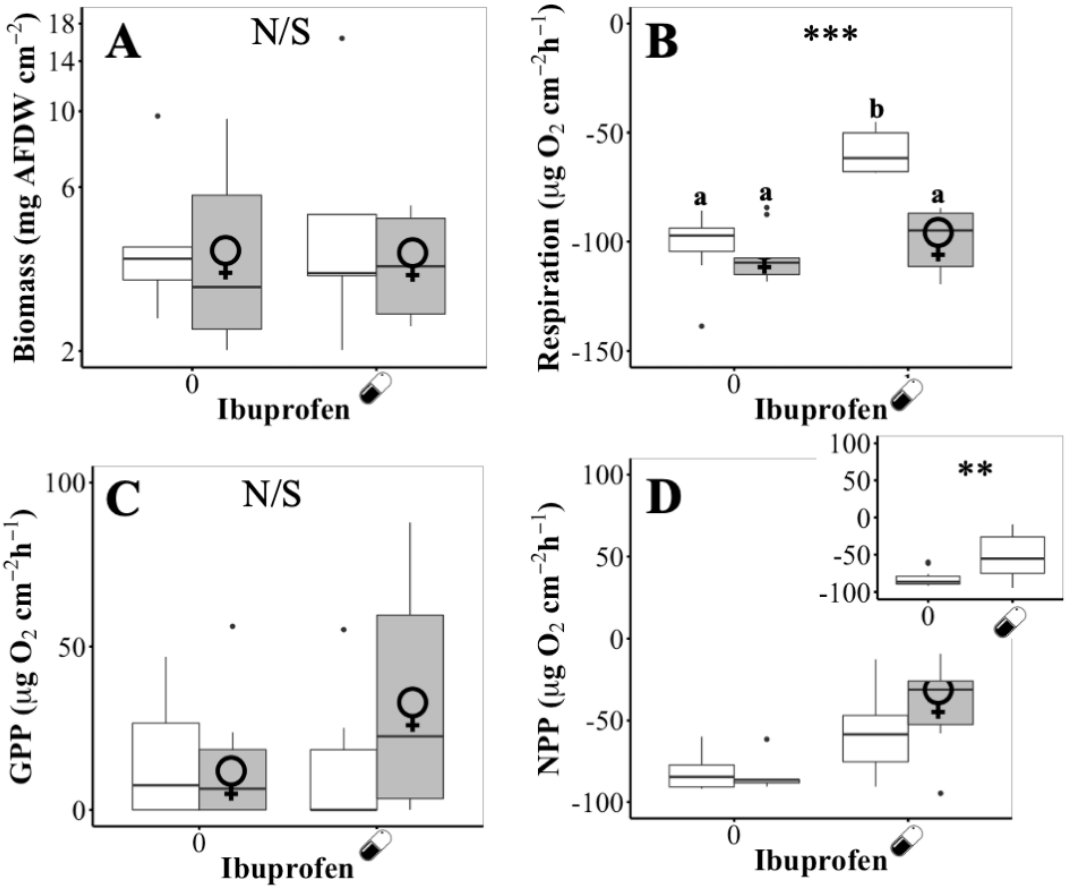
	Df	SS	MS	F	*p*
Ibuprofen	1	7546	7545.8	7.483	**0.009**
EE2	1	408	408.1	0.405	0.528
Ibuprofen : EE2	1	38	38.2	0.038	0.847
Residuals	36	36303	1008.4		

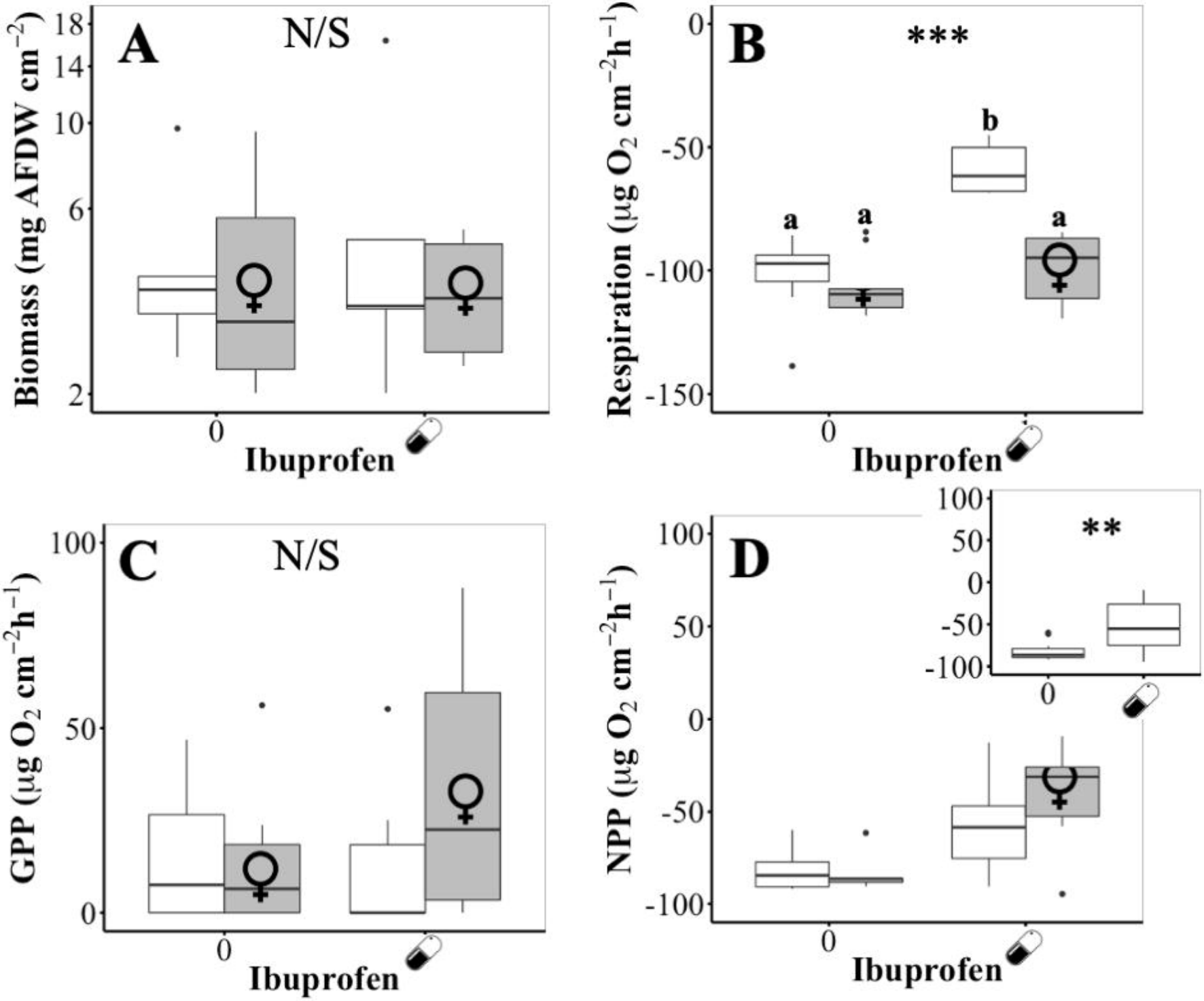
d) Gross Primary Production

	Df	SS	MS	F	*p*
Ibuprofen	1	931.5	931.46	1.737	0.196
EE2	1	1201.0	1200.96	2.240	0.143
Ibuprofen : EE2	1	1607.4	1607.40	2.998	0.092
Residuals	36 b	19300.8	536.13		

Figures

Figure 1. Effects of Ibuprofen (💊) and 17α-ethynylestradiol (♀) upon the (A) biomass (ash free dry weight), (B) respiration and (C) Net Primary Production and (D)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
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
GPP (μg O$_2$ cm^{-2}h^{-1})
100
50
0
0
Ibuprofen
D
**
NPP (μg O$_2$ cm^{-2}h^{-1})
100
50
0
-50
-100
100
50
0
-50
-100
0
0
Ibuprofen