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

Peter McClean¹ · William Ross Hunter¹,²

Received: 24 March 2020 / Accepted: 10 July 2020 / Published online: 17 July 2020
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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.

Keywords 17α-ethynylestradiol · Biofilm · EE2 · Ibuprofen · Microbial metabolism · Pharmaceuticals and personal care products

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 5% per year globally and this is predicted to increase further as human populations grow (Van Boeckel 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 Boeckel 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; Hernandez et al. 2006; Rosi-Marshall et al. 2013; Drury et al. 2013; Zür 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 (Rešliškis et al. 2015; Shah et al. 2018; Żur 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 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 biofilm colonization. Contaminant exposure experiments conducted following Costello et al. (2016). Briefly, forty 120-ml screw cap sample pots were 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 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. One-milliliter 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® GF/F filters were placed onto of the solid agar and secured using the screw cap, to provide a substratum for streamed biofilm colonization. Contaminant exposure experiments were then secured to 4-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.

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 groundwater-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 km². The mean volumetric rate for water flow in the Ballysally Blagh is 0.21 (± 0.27) m³ 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-h intervals throughout the experiment using a HOBO MX2204 Bluetooth temperature logger. Temperatures ranged between 9.35 and 5.16 °C, with a mean temperature of 7.72 (± 0.85) °C recorded over the study period.

Contaminant exposure experiments were conducted following Costello et al. (2016). Briefly, forty 120-ml screw cap sample pots were 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 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. One-milliliter 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® GF/F filters were placed onto of the solid agar and secured using the screw cap, to provide a substratum for streamed biofilm colonization. Contaminant exposure experiments were then secured to 4-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 W) aquarium heater was also attached to the Inkbird temperature controller of each unit to help stabilise 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 °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 h. During the acclimation period, each mesocosm was aerated using an 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 l of sterile-filtered streamwater and held at 7.7 °C in one of the environmental chambers (Bott et al. 1978; Fellows et al. 2006). Biofilm respiration (R) was quantified by measuring the change in oxygen concentrations over a 1-h period.
Environ Sci Pollut Res (2020) 27:37149–37154

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. 1A; Table 1a). We detected a significant interaction \((p < 0.001; \text{df} = 1; F = 18.75)\) between ibuprofen and EE2 affecting microbial respiration (Fig. 1B; Table 1b). Exposure to ibuprofen alone inhibited microbial oxygen consumption by \(\approx 38\%\), whilst exposure to EE2 alone resulted in a slight (non-significant) increase in oxygen consumption of \(\approx 5\%.\) In combination, EE2 counteracted the inhibitory effect of ibuprofen upon 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 \((p = 0.009; \text{df} = 1; F = 7.483)\), reflecting the effect on biofilm respiration (Fig. 1C; Table 1c). 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. 1D; Table 1d).

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 (Rešlífskí et al. 2015; Shah et al. 2018; Oliveira 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 and Marschner 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 and Hernandez-Raquet 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 that 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 mediate microbial response 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 autotrophic 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 (Rešiński et al. 2015; Shah et al. 2018; Żur 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. 2011; Hunter et al. 2012, 2013) and extracellular polysaccharide quantification (Fish et al. 2017; Grzegorczyk et al. 2018), may provide further insight into how these pharmaceuticals 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 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; Hernandez et al. 2006; Gros et al. 2007; Rosi-Marshall and Royer 2012; Rosi-Marshall et al. 2013; Álvarez-Muñoz et al. 2015; Rühi 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.

Acknowledgments This work was completed by PMcC during his final year undergraduate research project, supervised by WRH. It was funded through start-up funds provided to WRH by the University of Ulster’s School of Geography and Environmental Science. We acknowledge fieldwork assistance by Ashley Williamson, and technical support in the lab from Peter Devlin and Hugo McGrogan.

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

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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