Short Communication

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

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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

52 **1. Introduction**

53 Human pharmaceuticals and personal care products (PPCPS) are contaminants of
54 emerging concern within the environment [1, 2]. Since the year 2000, pharmaceutical
55 use has grown by approximately 3% per year globally and this predicted to increase
56 further as human populations grow [3]. Removal of Pharmaceuticals and personal
57 care products (PPCPs) via waste-water treatment plants (WWTPs) is inefficient
58 leading to constant release of compounds which are often specifically designed
59 specifically to produce physiological effects within an organism, at ultra-low (nano-
60 molar) concentrations [2, 4]; compounds such as non-steroidal anti-inflammatory
61 drugs (NSAIDs) (e.g. ibuprofen), antimicrobial compounds (e.g. triclosan, and
62 trimethoprim) and endocrine inhibitors (e.g. Estradiol) into the aquatic environment
63 [4-7]. Eco-toxicological studies reveal that PPCPs at environmental concentrations
64 can have significant physiological effects on both aquatic fauna and microorganisms,
65 with the potential to disrupt the functioning of aquatic ecosystems, alter carbon and
66 nutrient cycling, and negatively affect water quality [8-12].

67 Headwater and lower order streams are the smallest tributaries of a river system,
68 which are typically closest to the rivers' sources. In these streams aquatic biofilms
69 attached to the streambed represent the dominant mode of microbial life [13, 14].
70 Biofilms are composed of consortia of bacteria and unicellular eukaryotic algae
71 bound within a complex matrix of extracellular polymeric substances (EPS) and play
72 a key role in the functioning of fluvial ecosystems, controlling both the transport and
73 degradation of organic matter within a stream [14]. Rosi-Marshall et al. [10] revealed
74 that aquatic PPCPs such as caffeine, cimetidine, ciprofloxacin, diphenhydramine,
75 metformin and ranitidine and negative effects upon biofilm growth, respiration, and
76 community composition. PPCPs, however, are diverse group of chemicals, which
77 may interact with each other in a multitude of different, and often-unexpected ways
78 [1, 10, 15]. Consequently, a mechanistic understanding of the interactions between
79 different PPCPs is needed if we are to fully understand their environmental impacts.

80 Within the broad spectrum of PPCPs the non-steroidal anti-inflammatories (NSAIDs),
81 such as ibuprofen, and endocrine disruptors, such as 17a-estradiol, represent some
82 of the most commonly detected compounds in aquatic systems [1, 4, 9]. NSAIDs are
83 known to have antimicrobial properties, with ibuprofen exhibiting potential as a
84 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 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 [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.

149 All data analysis was conducted in the R statistical computing environment using the
150 *base* and *ggplot2* packages [26, 27]. We tested for independent and combined
151 effects of ibuprofen and estradiol upon in microbial biomass (Ash Free Dry Weight),
152 Respiration and Net Ecosystem Production using two-way analysis of variance
153 (ANOVA). Post-hoc testing of significant interactions was conducted using Tukey's
154 test for Honest Significant Difference. All data were visually explored, to ensure they
155 conformed to the assumptions of normality and homoscedacity, following Zuur et al.
156 [28]. Microbial biomass data were $\log_{10}$ transformed to ensure the residuals of the
157 ANOVA model conformed to a normal distribution.

158 **3. Results**

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

168 **4. Discussion**

169 Our study demonstrates that interactions between an NSAID (ibuprofen) and an
170 endocrine disruptor (17a-estradiol) have a significant effect upon the metabolism of
171 streambed biofilms. Specifically, concomitant exposure to both 17a-estradiol and
172 estradiol reduces the depressive effect of ibuprofen upon biofilm respiration.
173 Ibuprofen is known to have antimicrobial properties and has been reported to inhibit
174 biofilm formation by both *Staphylococcus aureus* and *Escherichia coli* [16-18]. It is,
175 therefore, unsurprising that ibuprofen depressed microbial respiration within the
176 streambed biofilms. Estradiol has been observed to adsorb to microbial biofilms [19]
177 where it can then be used by the resident microorganisms as an organic matter
178 source [29, 30]. Consequently, biofilms have been proposed as a tool for the
179 removal of estradiol and other endocrine disruptors within wastewater treatment
180 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

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

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

3. 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.

4. 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.

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

6. 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.

236 7. Archer E, Petrie B, Kasprzyk-Hordern B, Wolfaardt GM. 2017. The fate of
237 pharmaceuticals and personal care products (PPCPs), endocrine disrupting
238 contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental
239 waters. *Chemosphere* 174:437-446.

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

244 9. Hernando MD, Mezcua M, Fernández-Alba AR, Barceló D. 2006.
245 Environmental risk assessment of pharmaceutical residues in wastewater effluents,
246 surface waters and sediments. *Talanta* 69:334-342.

247 10. Rosi-Marshall EJ, Kincaid DWL, Bechtold H, Royer TV, Rojas M, Kelly JJ.
248 2013. Pharmaceuticals suppress algal growth and microbial respiration and alter
249 bacterial communities in stream biofilms. *Ecological Applications* 23:583-593.

250 11. Drury B, Scott J, Rosi-Marshall EJK, J.J. 2013. Triclosan Exposure Increases
251 Triclosan Resistance and Influences Taxonomic Composition of Benthic Bacterial
252 Communities. *Environmental Science and Technology* 47:8923-8930.

253 12. Żur J, Piński A, Marchlewicz A, Hupert-Kocurek K, Wojcieszyńska D, Guzik U.
254 2018. Organic micropollutants paracetamol and ibuprofen: toxicity, biodegradation,
255 and genetic background of their utilization by bacteria. *Environmental Science and*
256 *Pollution Research* 25:21498-21524.

257 13. Besemer K, Peter H, Logue JB, Langenheder S, Lindstrom ES, Tranvik LJ,
258 Battin TJ. 2012. Unraveling assembly of stream biofilm communities. *ISME Journal*
259 6:1459-1468.

260 14. Battin TJ, Besemer K, Bengtsson MM, Romani AM, Packmann AI. 2016. The
261 ecology and biogeochemistry of stream biofilms. *Nature Reviews Microbiology*
262 14:251-263.

263 15. Gerbersdorf SU, Cimatoribus C, Class H, Engesser K-, Helbich S, Hollert H,
264 Lange C, Kranert M, Metzger J, Nowak W, Seiler T-, Steger K, Steinmetz H,
265 Wieprecht S. 2015. Anthropogenic Trace Compounds (ATCs) in aquatic
266 habitats: Research needs on sources, fate, detection and toxicity to ensure timely
267 elimination strategies and risk management. *Environment International 79* 79:85-
268 105.

269 16. Reśliński A, Dábrowiecki S, Głowacka K. 2015. The impact of diclofenac and
270 ibuprofen on biofilm formation on the surface of polypropylene mesh. *Hernia* 19:179-
271 185.

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

276 18. Oliveira IM, Borges A, Borges F, Simões M. 2019. Repurposing ibuprofen to
277 control Staphylococcus aureus biofilms. *European Journal of Medicinal Chemistry*
278 166:197-205.

19. 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.

20. 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.

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

22. Costello DM, Rosi-Marshall EJ, Shaw LE, Grace M, Kelly JJ. 2015. A novel method to assess effects of chemical stressors on natural biofilm structure and function. *Freshwater Biology* 61:2129-2140.

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

24. 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.

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

300 26. R Development Core Team. 2009. R: A language and environment for
301 statistical computing.

302 27. Wickham H. 2016. *ggplot2: Elegant Graphics for Data Analysis.* Springer-
303 Verlag New York.

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

306 29. Stumpe B, Marschner B. 2009. Factors controlling the biodegradation of 17β-
307 estradiol, estrone and 17α-ethinylestradiol in different natural soils. *Chemosphere*
308 74:556-562.

309 30. Ribeiro AR, Carvalho MF, Afonso CMM, Tiritan ME, Castro PML. 2010.
310 Microbial degradation of 17β -estradiol and 17α -ethinylestradiol followed by a
311 validated HPLC-DAD method. *Journal of Environmental Science and Health, Part B*
312 45:265-273.

313 31. Pieper C, Rotard W. 2011. Investigation on the removal of natural and
314 synthetic estrogens using biofilms in continuous flow biofilm reactors and batch
315 experiments analysed by gas chromatography/mass spectrometry. *Water Research*
316 45:1105-1114.

317 32. Grzegorczyk M, Pogorzelski SJ, Pospiech A, Boniewicz-Szmyt K. 2018.
318 Monitoring of Marine Biofilm Formation Dynamics at Submerged Solid Surfaces With
319 Multitechnique Sensors. *Frontiers in Marine Science* 5:363.

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

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

326 35. Frostegård A, Tunlid A, Bååth E. 2011. Use and misuse of PLFA
327 measurements in soils. *Soil Biology and Biochemistry.* 43:1621-1625.

328 36. Hunter WR, Veuger B, Witte U. 2012. Macrofauna regulate heterotrophic
329 bacterial carbon and nitrogen incorporation in low-oxygen sediments. *ISME Journal*
330 6:2140-2151.

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

334 38. Ylla I, Romaní AM, Sebater S. 2012. Labile and Recalcitrant Organic Matter
335 Utilization by River Biofilm Under Increasing Water Temperature. *Microbial Ecology*
336 64:593-604.

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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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Figures

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

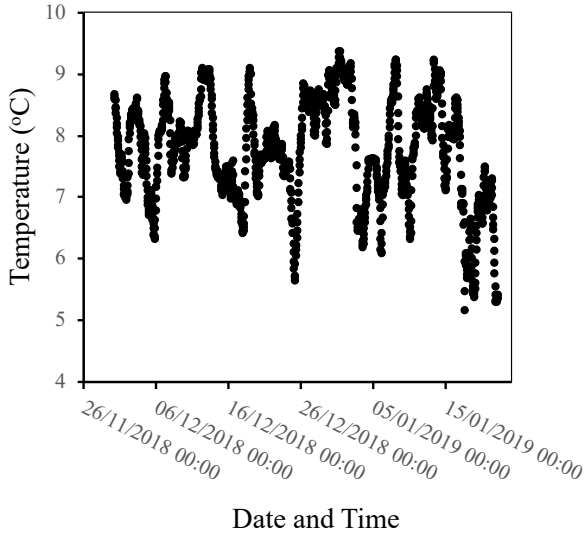
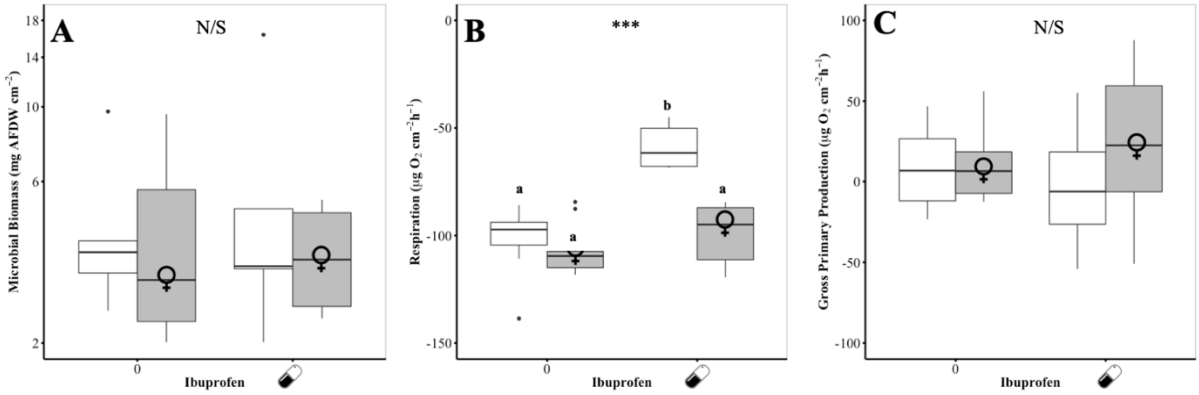
Figure 2. Effects of Ibuprofen (💊) and Estradiol (♀) upon the (A) biomass (ash free dry weight), (B) respiration and (C) Gross Primary Production of cultured streambed biofilms. Significance levels: ***p < 0.001; **p < 0.01; *p < 0.05; N/S p > 0.05.Where significant interactions were identified, groups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