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Abstract.

Fish can be exposed to a complex mixture of chemical contaminants, including pharmaceuticals, present in discharges of wastewater treatment works (WwTWs) effluents. There is little information on the effects of effluent exposure on fish metabolism, especially the small molecule signaling compounds which are the biological target of many pharmaceuticals. We applied a newly developed sensitive nanoflow-nanospray mass spectrometry non-targeted profiling technique to identify changes in the exposome and metabolome of roach (*Rutilus rutilus*) exposed to a final WwTWs effluent for 15 days. Effluent exposure resulted in widespread reduction (between 50% and 90%) in prostaglandin (PG) profiles in fish tissues and plasma with disruptions also in tryptophan/ serotonin, bile acid and lipid metabolism. Metabolite disruptions were not explained by altered expression of genes associated with the PG or tryptophan metabolism. Of the 31 pharmaceutical metabolites that were detected in the effluent exposome of fish, 6 were non-steroidal anti-inflammatory drugs but with plasma concentrations too low to disrupt PG biosynthesis. PGs, bile acids and tryptophan metabolites are important mediators regulating a diverse array of physiological systems in fish and the identity of wastewater contaminants disrupting their metabolism warrants further investigation on their exposure effects on fish health.

1. Introduction

Fish inhabiting lowland rivers are often exposed to reductions in water quality caused by discharges of effluents from wastewater treatment works (WwTWs). Treated wastewaters contain a wide range of biologically active man-made chemicals including pharmaceuticals, personal care products and pesticides, which maybe incompletely removed during influent treatment resulting in their temporally varying concentrations in the receiving waters.[1] Many studies have reported the presence of numerous pharmaceuticals from different therapeutic classes in final effluents and surface waters including non-steroidal anti-inflammatory drugs (NSAIDs), selective serotonin reuptake inhibitors (SSRIs), antipsychotics, statins and fibrates, beta blockers, and anticoagulants.[1-3] Pharmaceuticals are designed to interact with high affinity with specific biological targets (e.g. receptors, enzymes) in humans and livestock (for veterinary pharmaceuticals) and these targets can be evolutionary conserved and functional in fish and other vertebrates,[4] which may lead to unwanted pharmacological effects in non-target species. A challenge for the environmental community is to prioritize chemicals for risk assessment and monitoring in order to improve the quality of surface and ground waters.

The read-across hypothesis assumes that internal drug concentrations in blood plasma of wildlife which are similar to human therapeutic plasma concentrations (HtPC) may result in expected 'therapeutic' effects in non-target organisms.[5] For example, exposure to some anti-depressant SSRIs affect behavioural responses in fish at plasma concentrations similar to the HtPC.[6,7] However, because fish populations can be exposed to complex mixtures of biologically active chemicals,[8-10] identifying associations between exposure to their mixtures and health outcomes is extremely difficult.[11] To date, most analytical methods monitoring exposure of fish to effluents have used a targeted approach based on selection of key

59 contaminants and metabolite pathways and are thus limited in their assessments of the risk
60 of exposure to chemical mixtures.[e.g 8-.10] An untargeted analytical approach based on liquid
61 chromatography high resolution mass spectrometry (LC-MS) combined with chemometric
62 pattern recognition can identify chemical mixtures accumulating in fish (i.e., the exposome)
63 and simultaneously investigate for any associated disruption of metabolite profiles (the
64 metabolome). Using this approach, previous studies have demonstrated that exposure to
65 contaminants in wastewater effluents results in disruptions to lipid, steroid and bile acid
66 metabolism and uptake of a variety of household and personal care products in fish blood
67 plasma.[e.g 12-14] However, until now metabolite profiling methods have not been able to detect
68 trace levels of contaminants, endocrine or other signaling compounds present in tissues or
69 plasma extracts. Recently, we have demonstrated that the detection of less abundant
70 components in samples can be improved by using highly sensitive nanoflow/nanospray LC-
71 MS combined with appropriate sample preparation methods.[15-17] An additional advantage of
72 the method is that the chemical exposome, including pharmaceuticals, can also be profiled
73 alongside the metabolome in the same sample. Hence, an untargeted analytical approach,
74 based on nanoflow ultraperfomance liquid chromatography-nanoelectrospray ionization-
75 time-of-flight mass spectrometry (nUPLC-nESI-TOFMS) offers a very promising tool for
76 advancing ecotoxicological investigations in fish exposed to the complex mixtures of
77 contaminants present in WwTWs effluents.

78 The aim of this study was to investigate the effects of exposure to a final treated
79 wastewater effluent and the resultant uptake of a complex chemical exposome on the tissue
80 and plasma metabolome of fish. Extracts of blood plasma and tissues from effluent-exposed
81 and control fish were profiled using our newly developed nUPLC-nESI-TOFMS method in order

to reach unprecedented levels of sensitivity and enable the detection of signaling compounds and contaminants present at very low concentrations. Our work revealed, for the first time, widespread disruption of signaling metabolites including prostaglandins (PGs), in tissues of effluent-exposed fish which could not be explained solely by the components of the pharmaceutical exposome known to target PG biosynthesis. As PGs are important signaling agents mediating a number of fish physiological processes, this study highlights the importance of understanding the mixture effects of contaminants present in wastewaters on fish health.

2. Materials and Methods

Sources of chemicals and standards are given in the Supporting Information.

2.1 Effluent characteristics

The WwTW chosen for this study received 95% influent from a domestic source (population equivalent of 117,574) and 5% input from industrial wastewaters. The influent was treated by fine screens, chemically assisted settlement, biological aerated flooded filter processes and UV disinfection. During the exposure period, the pH of the effluent was 7.3, concentrations of suspended solids 21 mg/l, biochemical oxygen demand 11 mg/l, chemical oxygen demand 67 mg/l and total ammonia 29 mg/L (South West Water data).

2.2 Fish exposure

A mixed-sex population of sexually mature roach [*Rutilus rutilus,* age 2+, length of 14.5 ± 1.3 cm and weight 45.4 ± 12.1 g (mean ± SEM)] was exposed to either a final treated WwTWs effluent or clean water (control). Roach were exposed in triplicate tanks (20 fish per 200 L

tank) containing either dechlorinated water or 100% effluent for 15 days. The flow rate was 10 L/min and the tanks were continually aerated with an average temperature of 12 ºC ± 1 and a light dark cycle of 15h:9h. During the first 7 days of exposure, mortality occurred amongst 8 out of 60 of effluent-exposed fish, while no mortalities occurred in the control group. At the end of the experimental period, fish were terminated using an overdose of phenoxyethanol according to UK Home Office regulations. Blood was collected from the caudal vein using heparinized needles and samples were centrifuged (5 min, 10,000 RCF) and the plasma supernatant collected. Gonads, kidney (posterior), gill and liver tissues were dissected out and immediately snap frozen in liquid nitrogen. All samples were stored at −70 °C until analysis.

Wastewater effluent samples (2 x 2.5 litres) were collected in solvent washed, acid rinsed amber glass bottles on days 0, 7, 15 of the exposure period. Samples were stored at 4 °C with the addition of acetic acid (1%) and methanol (5%) and were extracted within 12 h after collection.

2.3 Sample preparation for nUHPLC-nESI-TOFMS profiling

Plasma samples were analyzed from up to 5 fish per replicate tank and in total 30 females and 25 males were analyzed (males were present in lower proportion than females in the mixed sex population). Plasma samples (85-120 µL) were prepared as described in David et al.[15] Previous studies have shown that this method, using phospolipid removal followed by mixed-mode cation exchange plates, extracts non polar, cationic and anionic metabolites (e.g., amino acids, neurotransmitters, bile acids, lipids, eicosanoids and steroids) as well as contaminants such as pharmaceuticals from plasma samples. Briefly, methanol with 1%

125 formic acid (FA) was added to each sample to a final proportion of 80/20 methanol/plasma
126 (v/v). An internal standard (IS) mixture containing a mix of seven compounds (venlafaxine-d6,
127 propranolol-d7, carbamazepine-d10 and fluoxetine-d5 for the +ESI mode and prostaglandin
128 E2-d4, diclofenac-d4 and triclosan-C13 for the –ESI mode) was added to the plasma extracts.
129 The amount of IS added were adjusted to give a final concentration of 5 ng IS/120 µL of
130 plasma. Full details of plasma extraction using Phree and SPE plates are given in the
131 Supporting Information.

132 Testes (0.28-0.47 g), kidney (0.09-0.17 g), gill (0.28-0.47 g) and liver (0.09-0.20 g),
133 tissues were extracted in glass tubes (5 mL) using three volumes of methanol and a Microson
134 XL2000 ultrasonic probe (Misonix Farmingdale) (15W x 30s).[18] Before extraction, the mix of
135 seven IS was added to each tissue extract to give a final concentration of 5 ng IS to 0.4 g of
136 testes, to 0.15 g of kidney or gill and to 0.35 g of liver. After extraction, samples were,
137 centrifuged (13,000 RCF for 20 min) and supernatants collected. The samples were diluted to
138 20% water (HPLC grade) and extracted using the Phree and SPE plates (see Supporting
139 Information).

140 2.4 Extraction of wastewater samples for nUHPLC-nESI-TOFMS analysis of NSAIDs.

141 Final wastewater effluent samples (1 L) were spiked with the IS mix, and filtered
142 through glass wool and filter paper Whatman No1 (Whatman, Maidstone, UK). Samples were
143 extracted through an Oasis HLB (20 mL, 1 g) cartridge, which was pre-conditioned with 10 mL
144 of methanol and 10 mL HPLC water. The cartridge was washed with 20 mL of distilled water
145 and dried under vacuum. Analytes were eluted with 10 mL of methanol and 10 mL of ethyl

146 acetate. Extracts were dried and reconstituted in 1 mL of methanol and 300 mL of the extract
147 evaporated and purified using the Strata-X-C SPE protocol as described for tissue samples.

148 2.5 nUHPLC-nESI-TOFMS

149 The methods used for the nUHPLC-nESI-TOFMS profiling of small molecules have been
150 established in our laboratory.[15, 17] The nUHPLC-nESI-TOFMS injections were performed on a
151 Waters nanoAcquity UPLC linked to a Waters Xevo G2 TOFMS, equipped with a nESI source
152 (Waters, Manchester, U.K) (see Supporting Information for analytical details).

153 2.6. Quality control

154 For the metabolomics analysis, two work-up samples (i.e. extraction without sample)
155 per analytical batch were prepared to monitor for background contaminants. Quality control
156 (QC) samples comprising a composite sample of the control extracts (diluted two fold) were
157 prepared in order to monitor for nUHPLC-nESI-TOFMS repeatability and sensitivity during
158 analysis of a sample run. Each run commenced with the injection of the blank work-up
159 samples followed by injection of 3 QC samples to equilibrate the source. The samples were
160 injected randomly with QC samples analysed after every 6 samples. In total, 151 samples
161 (excluding QCs) were injected in both +ESI and -ESI MS modes.

162 2.7 Chemometric analyses

163 Mass spectra were collected in full scan mode (50 to 1000 m/z) and spectral peaks
164 were deconvoluted and aligned using TransOmics algorithm (Waters, Manchester, UK). Peak
165 picking was performed creating an aggregate dataset from all sample files in which the MS
166 features were binned into retention time (Rt) x m/z values. Datasets were exported to SIMCA

167 software (Umetrics, Crewe, UK) for multivariate analysis (principal component analysis, PCA)
168 in order to examine class separating differences between control and exposed groups. Data
169 sets were log transformed and pareto scaled, and models were developed using the database
170 of *n* (from 10 to 15) different fish per class. PCAs were performed in order to observe
171 clustering between treatments, and models were evaluated through an examination of
172 explained variation (R2X) and predictive ability (Q2X) parameters. In order to determine
173 which contaminants and metabolites influence discrimination between two classes, the
174 datasets were modelled using orthogonal partial least squares to latent structures (OPLS)
175 method which were assessed by the explained variation (R2Y) and predictive ability (Q2Y). Rt
176 x m/z signals pertaining to discriminatory contaminants or metabolites were detected using
177 an 'S' plot of the OPLS model. [e.g. 13, 18, 19] Signals associated with the presence of chemical
178 contaminants arising from effluent exposure were identified as only present in effluent-
179 exposed fish and not detected in any replicates from control fish or from work-up blank
180 samples. Signals corresponding to metabolite markers were either decreased in control fish
181 or increased in effluent-exposed fish but also present in control fish but not in work-up blanks.
182 The signal intensity of suspected metabolite markers of effluent exposure were normalized
183 to the area of the closest IS. Analysis, using the Kruskal-Wallis test, of replicate fish from each
184 of the triplicate tanks from the two exposure treatments revealed no significant intertank
185 variability for any metabolite marker. Hence, metabolites from fish from replicate tanks were
186 analyzed as one treatment for each exposure by non-parametric Mann-Whitney U-test (SPSS,
187 IBM) with a correction for a false discovery rate of 5%.[20]

188 The putative identities of contaminants and metabolites were determined from
189 screening the m/z of molecular ion using Metabosearch

(http://omics.georgetown.edu/metabosearch.html) which searched databases of the Human Metabolome Database (http://www.hmdb.ca/), PubChem (http://pubchem.ncbi.nlm.nih.gov/), METLIN (http://metlin.scripps.edu/) and LipidMaps (http://www.lipidmaps.org/). Chemical identity was confirmed from accurate mass, isotopic fit and fragmentation data obtained from high energy collisional-induced dissociation and from comparison in- house with standard compounds or with open source databases.

2.8 Targeted analysis of NSAIDs by nUHPLC-nESI-TOFMS

NSAIDs were quantified in the profiling datasets of tissues and plasma and also after nUHPLC-nESI-TOFMS analysis of wastewater extracts (see Supporting Information for details) Method detection and method quantification limits (MDLs, MQLs) are given in Tables S1 and S5.

2.9 Gene expression analysis

Quantitative real-time RT-PCR (RT-QPCR) was performed on male and female gill, liver and gonad tissue samples to quantify the expression profiles of transcripts encoding enzymes for which related metabolites were found to change in the prostaglandin and serotonin pathways. The targets were prostaglandin-endoperoxide synthase 1 (*ptgs1*), prostaglandin-endoperoxide synthase 2b (*ptgs2b*), prostaglandin E synthase (*ptges*) and cytosolic phospholipase A2 (*pla2g4*) in the prostaglandin pathway and monoamine oxidase (*mao*), dopa decarboxylase (aromatic amino acid decarboxylase, *ddc*), aldehyde oxidase 1 (*aox1*), tryptophan 2,3-dioxygenase a (*tdo2a*) and tryptophan 2,3-dioxygenase b (*tdo2b*) in the serotonin pathway. Details on RNA analyses, primer sequences, sizes of PCR products and PCR assay conditions are provided in Supporting Information and Table S2. Samples from 3

fish per tank were analysed and the Chauvenet's criterion[21] was applied to detect any outliers in the transcript expression data. Identified outliers were removed from the data set prior to statistical analyses. Data were analyzed using a t-test comparing transcript expression between all control and exposed fish and results of all tests were accepted as significant at $p < 0.05$ (IBM SPSS Statistics for Windows, Version 21.0.0).

3. Results and Discussion

3.1 nUHPLC-nESI-TOFMS profiling of roach plasma and tissues

Extracts of blood plasma from male and female roach exposed to either a treated WwTWs effluent or to clean water were profiled by nUPLC-nESI-TOFMS in both +ESI and -ESI modes. PCA modeling of the datasets revealed clear separation between control and effluent-exposed roach of both sexes on the first component of the scores plots (-ESI mode Figure 1, +ESI mode, Figure S1). In these plots, the QC samples clustered together indicating that the profiling methods were highly repeatable with little analytical variability. The datasets were further analyzed by OPLS modeling which revealed highly predictable models for control and exposed datasets from either male or female roach (R2Y of >0.99 and Q2Y>0.69). No separation according to sex was observed in effluent-exposed roach in either the PCA or OPLS models indicating that the response (in terms of plasma chemical profiles) to effluent exposure was similar in female and male fish. Therefore, female tissue samples (gills, liver and the posterior kidney which contains both renal cells and macrophages) were analyzed as they were available in greater numbers than males. Extracts of testes tissue from males were also profiled because it is known that PGs play important roles in sexual differentiation[22] and

234 the association between exposure to WwTWs effluents and feminization of male fish has
235 been well documented.[e.g.23] The PCA of the datasets of all tissue extracts revealed a clear
236 separation between samples from control and exposed fish (Figure S2).

237 3.2 Metabolite markers of effluent exposure

238 From analysis of the 'S' loading plots of the OPLS models of the plasma datasets, it was
239 apparent that most of the chemicals driving the separation between control and effluent-
240 exposed fish were contaminants which were only detected in plasma of exposed fish (see
241 section 3.3 below). However, ten metabolite structures were identified as significant markers
242 of effluent exposure (Table 1). In agreement with a previous study,[14] exposure to WwTWs
243 effluent resulted in disrupted metabolism of plasma bile acids and lipids. After effluent
244 exposure, the plasma concentrations of two conjugated secondary bile acids were increased
245 by 5-12 fold (female and male fish) for taurodeoxycholic acid, and 39-56 fold for a
246 taurolithocholic acid-like structure. No changes in the levels of primary bile acids, such as
247 taurocholic acid and cyprinol sulfate, were detected. Little is known about the effect of
248 chemical exposures on disruption of bile acid profiles in fish or mammals, however secondary
249 bile acids are formed by the metabolism of gut microflora.[24,25] It is possible that the observed
250 increase in metabolism of secondary bile acids was a result of disruption of the community or
251 gene expression of resident gut bacteria as a result of exposure to chemical components in
252 the effluent. Increases in the lipid metabolite sphinganine (9- fold) and its potential precursor,
253 a palmitoyl serine type metabolite (177-227 fold), were also observed indicating disruption in
254 sphingolipid signaling.

255 In our study, the use of more sensitive nanoflow/nanospray LC-MS profiling methods
256 resulted in detection of new metabolites in effluent-exposed fish that were associated with

other signaling pathways. These included a 5-11 fold increase in the plasma concentration of tryptophan and a 2-fold decrease in its metabolite acetylserotonin, indicating effects on the serotonin pathway (Table 1). 'S' plot analysis of OPLS models of metabolite profiles in tissues revealed that concentrations of indolepyruvate, a metabolite of tryptophan, were increased by 13 and 32-fold in female gills and male testes, respectively (Table S3), indicating that tryptophan metabolism was also altered in these tissues as well as in blood plasma of effluent-exposed roach. In order to explore the mechanisms responsible for these metabolite changes, selected gene transcripts associated with tryptophan/serotonin metabolism were analyzed by qRT-PCR in gills, liver and gonads of male and female fish (Figure S3). Tryptophan can be metabolized by dioxygenases and a decrease in their expression would result in an increased substrate for metabolism to acetyl serotonin or indolepyruvate.[26] However, the dioxygenase (*tdo2a*) transcript was only significantly decreased in male and female liver from exposed fish and was increased in male gills. Significant increases in the dopa decarboxylase transcript levels (*ddc*) which metabolizes tryptophan to serotonin, and monamine oxidase expression (*mao*) which further converts serotonin to hydroxyindole acetic acid,[27] were observed only in female gills and for *ddc* also in female liver. Taken together, effluent exposure did not result in a widespread alteration in the transcription of key genes that could explain the changes involved in tryptophan metabolism in roach tissues.

Concentrations of a number of eicosanoid type metabolites were also altered in plasma samples from effluent-exposed male and female fish. These included a 10-15-fold reduction in prostaglandin F2α (PGF2α) levels, a 5-fold decrease in a tetranor PGE1-like structure, and a 13-16- fold increase in an oxygenated C20 metabolite (dicarboxy leukotriene B4-like) (Table 1). Examination of the 'S' plots from OPLS models of female gills, liver, kidney

and male testes revealed numerous signals, mainly corresponding to prostaglandin structures, which were prevalent (depressed) markers of effluent exposure (Table S3). PGE2 and PGF2α levels were reduced by 52-90% in all the tissues analyzed, together with a mean 93% reduction observed for PGF2α in plasma (Figure 2). Reductions in levels of a further 7 different prostaglandin metabolites were detected in various body tissues (Table S3) and further work is needed to determine their structural identity.

The widespread reduction of prostaglandin structures in tissues and in blood plasma indicates a general inhibition of the prostaglandin metabolism in effluent-exposed fish. Prostaglandins are formed from the action of prostaglandin endoperoxide synthases (PGTS), also termed cyclooxygenase enzymes, on the C20 polyunsaturated fatty acid, arachidonic acid, which in turn is released from membrane phospholipids by the action of cytosolic phospholipase A2 (PLA2). At least two PTGS genes (*pgts* 1 and 2) are present in fish and qRT-PCR analysis of gills, liver and gonads of male and female fish revealed that their expression was not altered as a result of effluent exposure (Figure S3). Expression of *pla2g4*, associated with substrate release by PLA2, was reduced in liver and gills of males but not in females or in gonads of either sex. Likewise, *pgtes*, which encodes PGE synthase and conversion of the PGH2 intermediate to PGE2, was reduced in liver and gills of males but not females, nor in gonads of either sex. These data indicate that the widespread reduction of PG levels in the various body tissues were not due to changes in expression of genes associated with their biosynthesis, but rather may arise from direct inhibition of enzyme activity instead.

The functional role and the identities of the full complement of biologically active prostaglandin structures has not been not fully determined in fish systems. However, PGE2 regulates ion transport in fish gills and kidney[28], macrophage differentiation in the posterior

303 kidney, and is an important inflammatory and blood clotting mediator produced by leucocytes
304 and thrombocytes.[29] PGF2α is can regulate reproductive behavior, synchronous spawning,
305 and ovulation in females.[30] PGs are also important signaling agents during cell development
306 and differentiation.[31] This indicates that widespread reduction of PG levels may have
307 deleterious effects affecting numerous endpoints in fish including development,
308 reproduction, the immune response and osmoregulation.

309 The role of other metabolites identified in our study has yet to be fully understood in
310 fish physiology. However, there remains the possibility that the roles of serotonin, bile acid
311 and PG metabolites maybe linked, or act in concert, to affect health in fish. For instance, a
312 follow-up study of brain tissue sampled from fish used in this study revealed significant
313 increases of serotonin concentrations in selected brain regions of the effluent-exposed fish
314 (A David, pers comm). Serotonin regulates aggression[32] and PGF2α mediates courtship in
315 male fish,[33] which indicates that further investigations on the effects of wastewater exposures
316 on disruption of fish behaviour are warranted. Similarly, serotonin and PG metabolites are
317 both signalling agents in the hypothalamus-pituitary-gonad axis of fish and regulate gonadal
318 development.[22,34] They are also both important modulators of the immune and inflammatory
319 response of vertebrates, [29, 35] and both regulate epithelial cell function in gills. [28,36] In
320 mammals, some secondary bile acids, including taurodeoxycholic acid, can act as anti-
321 secretory agents in the colon,[37] whereas both serotonin and prostaglandin metabolites
322 promote epithelial mucosal secretion and gastrointestinal protection[38,39] raising the question
323 as to whether interactions occur between these different metabolites affecting
324 gastrointestinal function in fish.
325

326 3.3 The link between the exposome and metabolome of effluent-exposed fish

327 Interrogation of the 'S' loading plots of signals unique to effluent-exposed fish
328 revealed the accumulation of a complex mixture of contaminants in blood plasma and tissues.
329 These contained 31 pharmaceuticals from 11 different classes including NSAIDs (naproxen,
330 mefenamic acid, diclofenac, ibuprofen, felbinac, celecoxib) and SSRIs (paroxetine, citalopram,
331 sertraline, fluoxetine, norfluoxetine, norsertraline and norcitalopram) (Figure 3 and Table S4).
332 In addition, a wide range of endocrine disrupters, personal care products, pesticides,
333 antibacterial and human dietary products were also detected in the plasma and tissues of
334 effluent-exposed but not control fish (Table S4).

335 Due to matrix interferences, the concentrations of SSRIs in the different sample types
336 could not be determined in this study and their quantitative analysis required further method
337 development. However, the concentrations of NSAIDs, which are potent inhibitors of PGTS
338 enzymes in vertebrate systems, were determined in effluent and fish samples. During the
339 exposure period, NSAIDs levels in the effluent were all below 0.5 µg/l, except for naproxen
340 which was present at a mean ± SD of 3.7 ± 2.3 µg/l (Tables 2 and S5). Studies to date indicate
341 that these concentrations of NSAIDs were too low to affect PG or PGTS gene expression levels
342 in fish.[40-42] In addition, NSAID concentrations in plasma and tissues of effluent-exposed fish
343 were determined and compared with reported human therapeutic plasma concentrations,
344 HtPC[43] (Table 2). NSAIDs were detected in plasma and most of the tissues analyzed, but with
345 the exception of naproxen and ibuprofen, their mean concentrations were < 1ng/ml plasma
346 or 1 ng/g tissue. Mean concentrations of naproxen and ibuprofen in the plasma were higher
347 than in the effluent indicating bioconcentration of these contaminants in the fish. The effluent
348 concentrations of naproxen were above the MQL, which allowed the determination of a
349 plasma bioconcentration factor of 4, similar to that already reported for this compound.[44]

However, the plasma concentrations of all NSAIDs were between 650 and 14,0000-fold below the HtPC. As PG levels were significantly reduced in blood plasma and body tissues, this would indicate that either NSAIDs and their mixtures were more potent in fish compared with humans, or that other contaminants were present that inhibited PG biosynthesis in the fish. Previous reports suggest that many other compounds can inhibit PGTS enzyme activity, including pesticides and some of the endocrine disrupting chemicals detected in fish in this study (Table S4).[45,46] Further work is needed to identify other PGTS inhibitors amongst the diverse range of contaminants which bioconcentrate in fish exposed to WwTW effluents.

3.4 Implications of the work and future studies.

This study was conducted with undiluted effluent, which reflects the exposures of aquatic animals, including fish, in some countries where there is little or no dilution of the discharges into the receiving waters.[47] However, there was a mortality of 13% during effluent exposure indicating that fish were subjected to high physiological stress. Further studies are required to determine whether similar metabolite disruptions are observed with diluted effluents that typify ambient waters.

Through the application of newly developed sensitive non-targeted chemical profiling techniques, a novel finding emerging is the widespread reduction in PG metabolism in the roach exposed to a final treated WwTWs effluent. This finding could not have been predicted from an analysis of pharmaceuticals in the effluent which are designed to target PG metabolism, and there is a need to identify the key causative contaminants disrupting PG biosynthesis in WwTW effluents. Moreover, we highlight the need for further understanding on the identity and function of the numerous PG metabolites that were detected in fish tissues. Furthermore, PGs, serotonin and bile acid metabolites are components of key

signaling pathways which mediate gastrointestinal function, ion transport, immune and reproductive processes in vertebrates, and disruption in their levels may potentially impact on a range of health endpoints in contaminated fish.

Our study further emphasizes the importance of understanding mixture effects of contaminants (including their metabolites) on the health of wildlife. Improvements in discovery-based chemical profiling technologies are likely to increase coverage of the exposome and metabolome, and advance our understanding of the link between exposure to complex mixtures of contaminants and their impact on multiple metabolite pathways. However, it will require a step change in knowledge to understand the link between disruption of multiple interacting pathways and their consequent effects on wildlife or human health. Without this knowledge, environmental policymakers may have to consider whether regulation of chemical contaminants should be proactive and informed by changes in molecular profiles with the *potential* for physiological disruption, rather than a reliance on demonstrated health endpoints which may take many decades to investigate, particularly in respect to exposure of chemical mixtures.

Supporting Information

Supporting information comprises further analytical details, PCA score plots of metabolomics datasets and tables of identification of markers of effluent exposure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Table 1. The effect of exposure to wastewater effluent on blood plasma metabolite profiles of roach.

Retention time	Observed molecular ion	Molecular ion species	Δ ppm	Formula of ion	Fragment ions	Kegg no	Identity	Fold change	p value	Fold change	P value
Metabolites decreased in plasma								**Female**		**Male**	
6.19	219.1136	M+H	0.9	C12H15N2O2	160.0766	C00978	acetylserotonin [a]	1.6	0.014	2.7	$2.8x10^{-4}$
13.61	353.2326	M-H	-0.6	C20H33O5	309.2058	C00639	PGF2α [a]	10.6	$3.7x10^{-5}$	14.7	$2.6x10^{-4}$
14.20	297.1702	M-H	0	C16H25O5	nd	-	tetranor PGE1 like	5.2	$4.0x10^{-6}$	4.8	$8.5x10^{-5}$
Metabolites increased in plasma											
15.80	383.2068	M-H	-0.5	C20H31O7	nd	-	dicarboxy LTB4 like	16.6	6.1×10^{-6}	13.3	8.1×10^{-5}
14.74	498.2889	M-H	0	C26H44NO6S	nd	C05122	taurodeoxycholic acid [a]	5.1	$1.3x10^{-4}$	12.1	$1.2x10^{-4}$
19.29	482.2939	M-H	-0.2	C26H44NO5S	nd	C02592	taurolithocholic acid like	39.3	$2.0x10^{-6}$	56.0	$2.6x10^{-5}$
6.01	205.0979	M+H	0.1	C11H13N2O2	188.0713, 146.0606	C00078	tryptophan [a]	5.0	$4x10^{-3}$	10.7	$4.0x10^{-4}$
4.27	204.0662	M+H	0.5	C11H10NO3	158.0605, 130.0658	C00331	Indolepyruvate[a]	22.9	2.1x10-5	77.6	$5.6x10^{-5}$
15.59	302.3060	M+H	0.3	C18H40NO2	284.2952	C00836	sphinganine	9.1	6.4×10^{-5}	9.4	5.2×10^{-5}
25.19	352.2828	M+Na	0	C19H39NO3Na	312.2898		palmitoyl serine like	227	$4.5x10^{-5}$	177	$3.0x10^{-4}$

[a] metabolite identity confirmed by a pure standard. Other metabolite structures determined from comparison of CID fragments with MS databases. Δ ppm difference between observed and calculated molecular ion. nd= not detected. Metabolites disrupted by effluent exposure were determined from P values calculated using Mann Whitney test and after correction for a false discovery rate of 5%. n=10 and n=13 for control and exposed males; n=13 and n=16 for control and exposed females.

Table 2. Concentrations of nonsteroidal anti-inflammatory drugs in effluent, blood plasma and tissues of effluent-exposed roach and human therapeutic plasma concentrations (HtPC).

		Effluent[a] ng/ml (n=6)	plasma ♀ ng/ml (n=16)	plasma ♂ ng/ml (n=13)	gills ng/g (n=10)	kidney ng/g (n=12)	liver ng/g (n=12)	testes ng/g (n=12)	HtPC [b] ng/ml
Naproxen	Range	2.0 - 7.7	5.2 - 27	6.7 - 22	3.3 - 6.9	3.5 - 16	12 - 351	<0.11[d] - 1.9	20000 - 50000
	Mean±SD	3.7 ± 2.3	13 ± 6.2	14 ± 4.6	5.1 ± 1.2	8.2 ± 3.8	75 ± 91	0.93 ± 0.48	
Felbinac	Range	<0.25[c]	0.36 - 1.1	<0.19[d]- 0.84	0.19-0.23	0.58 - 0.97	3.5 - 8.7	0.056 - 0.11	400 - 1000
	Mean±SD	<0.25[c]	0.65 ± 0.15	0.57 ± 0.27	0.21 ± 0.01	0.77 ± 0.1	5.7 ± 1.6	0.091 ± 0.013	
Diclofenac	Range	0.22 - 0.38	<0.30[d] - 1.9	<0.1 - 0.82	0.35 -1.0	<0.24[d]- 0.35	<0.21[d] - 0.31	<0.03[c] - 0.32	500 - 3000
	Mean±SD	0.35 ± 0.10	0.43 ± 0.45	0.39 ± 0.27	0.58 ± 0.21	0.13 ± 0.10	0.21 ± 0.09	0.09 ± 0.09	
Ibuprofen	Range	<0.01[c]	<7.5[d] - 13	<7.5[d] - 14	<2.0 [c] - <6.0[d]	<2.0 - <6.0[d]	<1.7[c] - <5.1[d]	<0.75[c] -< 2.3[d]	15000 - 30000
	Mean±SD	<0.01[c]	5.2 ± 3.7	5.8 ± 4.3	<2.0 [c]	<2.0 [c]	<1.7[c]	<0.75[c]	
Celecoxib	Range	0.013 – 0.018	0.30 - 0.32	0.29 - 0.31	0.23 - 0.25	0.35 - 0.37	0.36 - 0.41	<0.019[c] - 0.094	360 - 800
	Mean±SD	0.015 ± 0.002	0.31 ± 0.01	0.30 ± 0.01	0.24 ± 0.01	0.36 ± 0.003	0.38 ± 0.01	0.091 ± 0.001	
Mefenamic	Range	0.08 – 0.15	<0.94[d] - 1.2	<0.94[d] - 1.3	<0.25 [c] - 0.75[d]	0.94 - 1.0	1.0 - 1.7	<0.094[c] - <0.28[d]	2000 - 10000
acid	Mean±SD	0.10 ± 0.03	0.70 ± 0.40	0.79 ± 0.42	<0.25[c]	0.98 ± 0.03	1.2 ± 0.18	<0.09[c]	

[a]Effluent samples measured at 0, 7, and 15 days of exposure. Plasma and tissue samples analysed at the end of the 15 day exposure period. [b]HtPC from Shultz et al.[43] [c] = MDL value, [d] = MQL value. SD= standard deviation. For the mean calculations, all concentrations that were over the limits of detection (≥MDL) but below the limits of quantification (<MQL) were assigned the value considered as the MDL in each case. Concentrations below the MDL were considered to be zero. Gills, kidney and liver samples were from females only. NSAID levels in control fish were below the MDL values given in Table S1.

Figure captions.

Figure 1. Principal component analysis scores plot of the chemical profiles in plasma of roach (male and female) exposed either to a final treated WwTWs effluent or clean water.

Extracts were profiled in -ESI mode by nUPLC-nESI-TOFMS. Quality control (QC) samples were used to monitor the analytical performance of the MS platform. % R2X values are given on the respective components. MC= male control, FC=female, control, FE=female effluent, ME= male effluent.

Figure 2. Effect of effluent exposure on PGE2 and PGF2α concentrations in fish tissues and blood plasma

PGE2 and PGF2α concentrations were measured from the mean (± standard deviation) relative abundance compared to the internal standard. The % reduction in PG concentrations in the effluent-exposed group were determined from the mean values in the control fish which had been normalised to 100% for each tissue type. **p<0.01;***p<0.001;****p<0.0001. n=10 and n=13 for control and exposed male plasma; n=13 and n=16 for control and exposed female plasma; n=12 and n=12 for control and exposed testes; n=10 and n=12 for control and exposed kidney; n=14 and n=12 for control and exposed liver; n=11 and n=10 for control and exposed gills, respectively. PGE2 was not detected in any plasma sample, MDL; method detection limit.

Figure 3. Number and classes of pharmaceuticals detected in the blood plasma and tissues of roach exposed to a WwTW effluent.

NSAIDs, non-steroidal anti-inflammatory drugs. SSRIs, selective serotonin reuptake inhibitors. In total, 31 pharmaceuticals were detected across the different tissues and the identities of 26 were confirmed by pure standards (see Table S4). No pharmaceuticals were detected in samples from control fish.

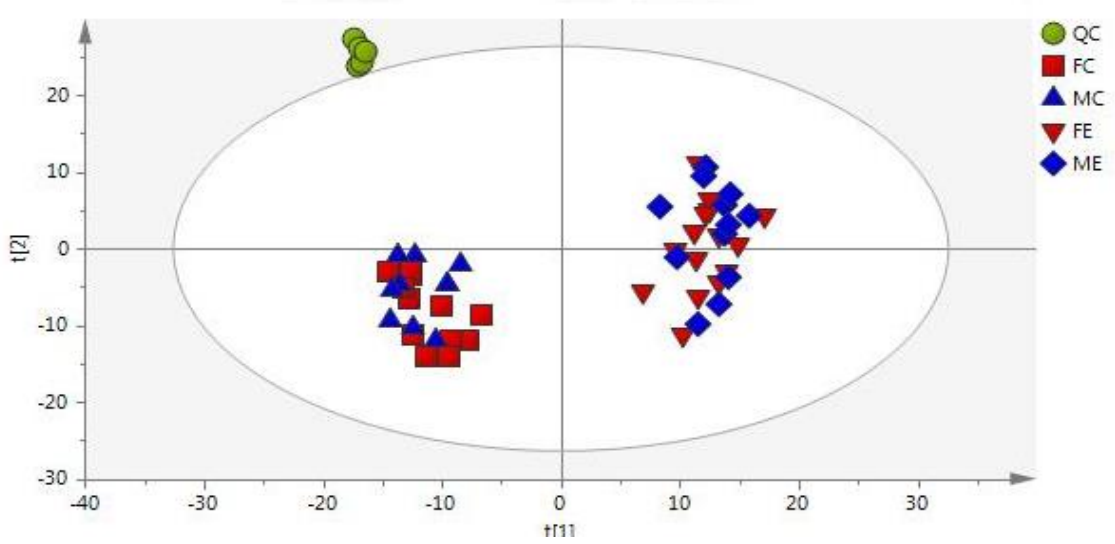

Figure 1.

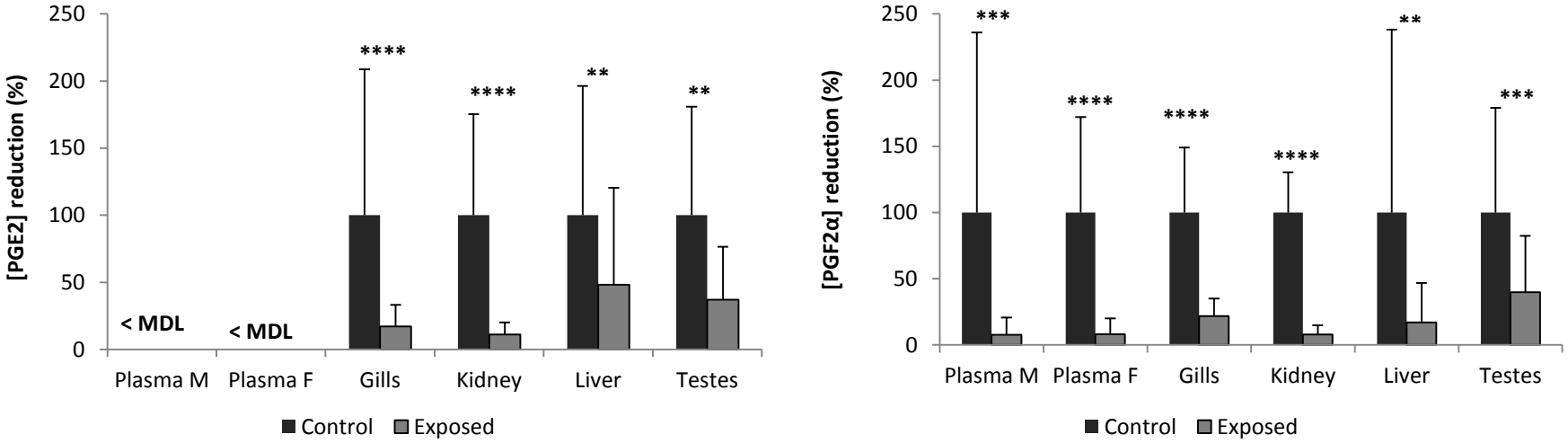
[PGE2] reduction (%)
250
200
150
100
50
0
< MDL
< MDL
****
****
**
**
Plasma M
Plasma F
Gills
Kidney
Liver
Testes
Control
Exposed
[PGF2α] reduction (%)
250
200
150
100
50
0
***
****
****
****
**
***
Plasma M
Plasma F
Gills
Kidney
Liver
Testes
Control
Exposed

Figure 2.

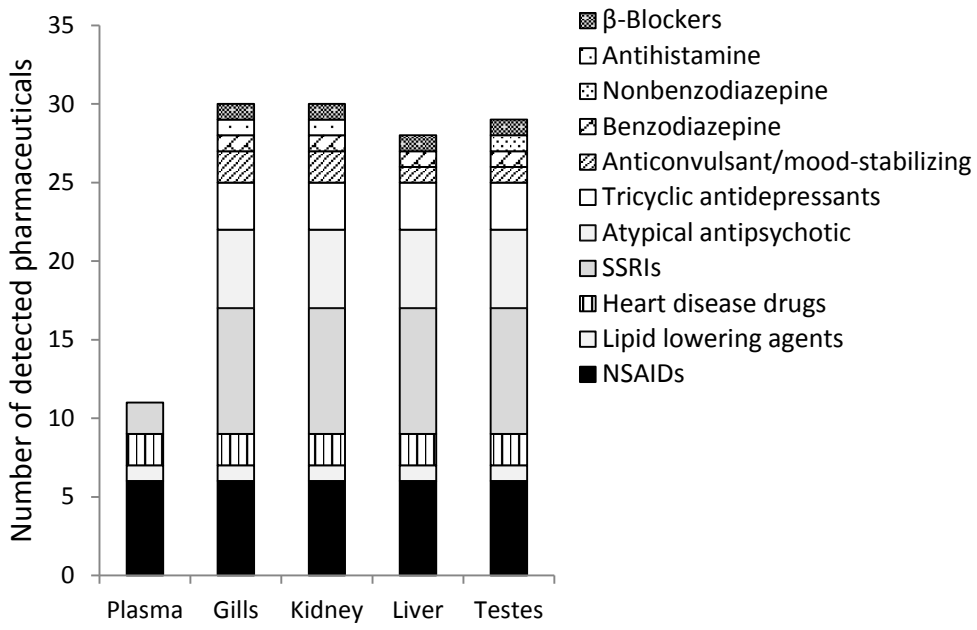
Number of detected pharmaceuticals
35
30
25
20
15
10
5
0
Plasma
Gills
Kidney
Liver
Testes
β-Blockers
Antihistamine
Nonbenzodiazepine
Benzodiazepine
Anticonvulsant/mood-stabilizing
Tricyclic antidepressants
Atypical antipsychotic
SSRIs
Heart disease drugs
Lipid lowering agents
NSAIDs

Figure 3.