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The Xenometabolome and Novel Contaminant Markers in Fish Exposed to a Wastewater Treatment Works Effluent

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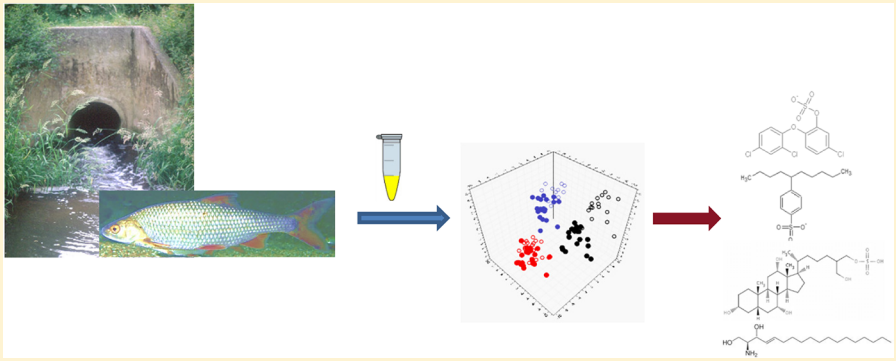
Supporting Information



ABSTRACT: Organisms exposed to wastewater treatment works (WwTW) effluents accumulate complex mixtures of xenobiotics but there is a scarcity of information on the nature and impacts of these chemical mixtures. We applied metabolomics techniques as a novel approach to identify xenobiotics and their metabolites (the xenometabolome) that bioconcentrate in fish exposed to a WwTW effluent. Exposed juvenile rainbow trout (*Oncorhynchus mykiss*) accumulated surfactants, naphthols, chlorinated xylenols, and phenoxyphenols, chlorophenes, resin acids, mefenamic acid, oxybenzone, and steroidal alkaloids in the bile or plasma, and there were perturbations in the plasma concentrations of bile acids and lipids. Exposure of adult roach (*Rutilus rutilus*) to 50% or 100% concentrations of the same effluent resulted in dose-dependent increases in plasma concentrations of xenometabolites as well as cyprinol sulfate and taurocholic acid, lysophospholipids, and a decrease in sphingosine levels (a key component of cell membrane lipids). Our findings reveal the highly complex nature of xenobiotics accumulating in effluent-exposed fish, and the great potential of metabolomics for both identifying plasma marker (bio)chemicals for monitoring exposure to wastewater effluents, and for targeting studies on potential consequent impacts on fish health.

INTRODUCTION

The documented decline of native fish populations in inland waters of many European countries is of concern not only to the fisheries industry but also because of their fundamental role in the ecological functioning of surface waters.[1] Declines have been recorded in migratory species including salmon and eel, as well as nonmigratory fish such as cyprinids and brown trout.[2−4] It has been suggested that freshwater fish may be the most threatened group of vertebrates after the Amphibia.[5] Some of these declines have been linked to a variety of environmental stressors such as a reduction in water quality, loss of habitat, changes in hydrology, or an increase in pathogen-induced diseases.[2−5] It is likely that some environmental stressors may act in combination to impact fish health, for example exposure to some environmental contaminants can depress immune function, thereby increasing fish susceptibility to parasites and pathogenic organisms.[6]

A major source of river pollution arises from the discharges of effluents from wastewater treatment works (WwTWs). In Europe, the Urban Waste Water Treatment Directive controls parameters such as biochemical oxygen demand and plant nutrients affecting the quality of waters downstream of effluent discharges. However many chemicals, including products of human metabolism, pharmaceuticals, and household products, are discharged into rivers via WwTW effluents and can bioaccumulate as complex mixtures in fish.[7] Long-term exposures are likely to affect fish health and this could result in population-level effects. These contaminants include endocrine-disrupting chemicals (EDCs) such as estrogenic chemicals which can feminize male fish causing intersex and a reduction in fertility.[8,9] More recently, EDCs with androgen receptor antagonist activity have also been detected in WwTW effluents which may also impact the reproductive health of fish.[10] Many other contaminants such as anti-inflammatory and lipid-modulating agents, chlorinated bactericides, short-chain

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surfactant molecules, musks, fragrances, and sterols are also present in WwTW effluents but little is known about their accumulation or toxicities in fish inhabiting receiving waters. Furthermore, the discharge of many of these compounds is currently not regulated by the European Union (EU) Water Framework Directive. Importantly, the health effects resulting from exposure to xenobiotic mixtures present in WwTW effluents are likely to be of greater concern in the future with the impacts of climate change.[11] The demands on water resources from a reduction in rainfall and increased water abstraction may result in even lower dilution of effluent in many European catchments and this will in turn increase the environmental pressures on wildlife and impact their sustainability. Already in some UK river sites during low rainfall in the summer, the effluent from WwTW effluents discharges can make up between 50% and 90% of the river flow. In contrast, in catchments that receive increased rainfall, the greater demands on the sewerage treatment system will inevitably lead to discharge of poorly treated sewage into receiving waters via storm overflow systems.[12]

Given this, there is a need for effective tools and technologies to monitor the exposure and the effect of WwTW effluents on fish health. Current approaches use a suite of biomarkers (biochemical measurements) to monitor the effects of chemical exposure. For example, levels of plasma vitellogenin (egg yolk protein) are used for monitoring exposure of male fish to estrogenic contaminants in the ambient water, and upregulation of the cytochrome P4501A or metallothionein gene products are used for assessing exposure to planar chemicals or heavy metals, respectively.[13] However, the current suite of biomarkers available for biomonitoring is likely to detect the effects of only a small proportion of the potentially toxic contaminants present in wastewaters, and we know very little about the health effects of other compounds or their chemical mixtures present in the effluents. Moreover, many of the biomarkers currently used either require sacrifice of the animal or expensive time-consuming analyses. More holistic approaches need to be developed to provide an integrative assessment of the potential health impacts of the whole effluent, and for diagnosing patterns or signatures of exposure to WwTW effluents in fish plasma that do not require sacrifice of the animal.

Genomic approaches, which include transcriptomics, proteomics, and metabolomics (metabolite profiling), are increasingly being used in (eco)toxicological investigations to investigate mechanisms of action of toxic contaminants.[14,15] Metabolomics comprises the profiling of the suite of biochemicals present in an organism at any one time. It is a high-throughput semiquantitative approach to obtaining information on the downstream final products of the genome, referred to as the "metabolome". However, a similar holistic profiling approach can also be used to profile the range of chemical xenobiotics and their metabolites in an organism exposed to environmental contaminants, i.e. the "xenometabolome". In this study, a chemical profiling approach was used to detect chemical contaminants and their metabolites that bioconcentrate in bile and plasma of fish exposed to a WwTW effluent. In the first exposure study, juvenile rainbow trout (*Oncorhynchus mykiss*) were held for 10 days in final effluent, and some individuals were also depurated for varying periods to investigate contaminant clearance following effluent exposure. The results revealed that a complex mixture of contaminants and their metabolites was present in bile and plasma of trout, and many of the contaminants were rapidly eliminated from the fish during depuration. In the course of this study, changes in the concentrations of endogenously derived metabolites were also detected in the trout plasma. A second study, the design of which was informed by the first, was conducted using the same effluent but with sexually mature roach (*Rutilus rutilus*) which is a common cyprinid species in European freshwaters and representative of fish species commonly present in effluent-impacted waters in Europe. A mixed sex population of this species was used to allow for investigations of gender-specific effects of effluent exposure. As cyprinids can metabolize some contaminants more extensively than salmonid species,[16] they were exposed for a longer period to the effluent (28 days) to ensure that metabolite responses to effluent exposure were maximized. Our findings include that using chemical profiling methods to detect changes in plasma chemistry can provision marker (bio)chemicals that could be used to monitor the exposure and impacts of WwTW effluents in fish.

MATERIALS AND METHODS

Fish Exposure. The WwTW chosen for this study received influent from a population equivalent of 142 370 and >99% of the influent was from domestic sources (see Supporting Information (SI) for details). The influent was treated by fine bubble diffusion activated sludge and trickling filters processes. In a previous study, long-term (3.5 years) exposure to this effluent has been shown to cause sex reversal and reduce reproductive fitness of male roach indicating that this effluent can contain endocrine-disrupting chemicals.[17] Female juvenile rainbow trout (*Oncorhynchus mykiss* age 2^+) were obtained from a local fish farm with a mean ± SEM length of 22.8 ± 0.2 cm and weight of 132.9 ± 3.6 g. To investigate the identity of the complex mixture of chemical contaminants and their persistence in effluent-exposed fish, 29 trout were exposed to final undiluted effluent, and a further 31 fish were used as a reference population and held in charcoal-filtered river water abstracted upstream of the effluent discharge point. The fish were held in 1 m^3 plastic tanks (1 tank per treatment) under ambient temperature and photoperiod and were fed commercial trout food until 24 h prior to sampling. The flow rate was 10 L/min and the effluent or river water was continually aerated. After 10 days of exposure, 16 fish from both the effluent tank and the control tank were sampled. The remaining 13 fish in the effluent exposure were transferred to another tank containing charcoal-filtered river water. Six of these fish (along with a similar number of control trout) were sampled after 4 days of depuration (day 14 of the study), and 7 were sampled after 11 days of depuration (day 21 of the study). At the end of the experimental periods, heparinized blood samples were obtained by terminally anesthetizing the fish and sampling for blood from the caudal vein. The samples were centrifuged and the plasma supernatant was removed for analyses. Bile samples were obtained by puncturing the gall bladder with a needle and drawing the bile into a syringe. The hepatosomatic index (HSI) for each fish was calculated as the liver weight/whole body weight × 100.

A further experiment was undertaken with the same WwTW effluent to investigate chemical and metabolite markers of effluent exposure in plasma of sexually mature roach (*Rutilus rutilus*). A mixed-sex population of roach, age 1^+ (one month before spawning and entering their third year) and mean ± SEM length of 10.9 ± 0.2 cm and weight 23.0 ± 1.2 g were exposed in duplicate tanks (20 fish per tank) containing either charcoal- and UV-treated tap water, 50% effluent diluted with

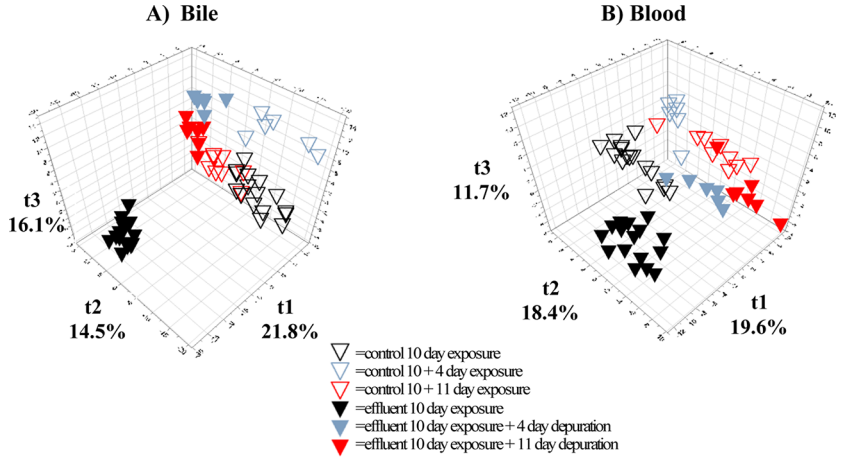

Figure 1. Partial least-squares-discriminant analyses (PLS-DA) scores plots of the chemical profiles of (A) bile and (B) blood of trout exposed either to 100% WwTW effluent or control river water. The samples were profiled in −ESI mode by UPLC-TOFMS. Black, blue, and red open symbols represent 10, 14, and 21 days of exposure for control trout where n = 16, 6, and 9 respectively. Black, blue, and red closed symbols represent 10 day exposure to 100% effluent followed by a depuration in river water for 0, 4, and 11 days where n = 16, 6, and 7, respectively. The percentages of explained variation (R2Y) modeled for the first three latent variables (t1, t2, and t3) are displayed on the related axes.

charcoal- and UV-treated tap water, or 100% effluent for 28 days. Chlorine levels were regularly monitored in the exposure tanks and were not detected. The fish were held in 1 m^3 tanks under ambient temperature and photoperiod and were fed frozen gamma-irradiated brine shrimp (*Artemia* sp.) or bloodworm (*Chironomus* sp.). Feeding was withheld 24 h prior to sampling. The flow rates through each tank were 5 L/min and all tanks were aerated to ensure sufficient oxygen supply. At the end of the exposure period, the fish were terminally anesthetized and the blood sampled and HSI measured as above. All samples were stored at −70 °C until analysis.

Extraction of Bile and Plasma Samples. Bile samples (6 μL) were diluted 50-fold with methanol:water (1:1, v/v). Plasma samples (60 μL roach, 300 μL trout) were deproteinized with a final concentration of 80% ice cold methanol. Samples were spiked with 1 ng of deuterated internal standards, progesterone-d_9 and 17β-estradiol-d_4-sulfate (CDN Isotopes, Quebec, Canada). Plasma extracts were centrifuged (10 000g), the supernatants were evaporated to dryness under vacuum, and the residues were redissolved in 60 or 300 μL of methanol/water (1:1, v/v) for roach and trout samples, respectively. Plasma and bile extracts were filtered using 96-well Strata Protein Precipitation Plates (0.2 μM, Phenomenex, Cheshire, UK) prior to chemical analysis.

Chemical Profiling of Bile and Plasma Samples. Full details of the mass spectrometry and multivariate analyses are given in the SI. Briefly, bile and plasma metabolites were profiled using ultraperformance liquid chromatography time-of-flight mass spectrometry (UPLC-TOFMS). Extracts of bile (7 μL) and plasma (20 μL) were injected onto an Acquity UPLC BEH C18 column (1.0 × 100 mm, particle size 1.7 μm) and chemical components were separated using a water formic acid/acetonitrile gradient. Extracts were analyzed in both positive and negative electrospray ionization (ESI) modes at a mass resolution of 15 000. Spectral peaks were deconvoluted and aligned, and the data sets (retention time × mass spectra signals) were normalized, log transformed, and pareto scaled. The data sets were analyzed using principal component analysis (PCA) to examine clustering of treatment groups, followed by supervised partial least-squares-discriminant analysis (PLS-DA) to identify the class separating differences in the data sets. The explained variation (R2Y) and the predictive ability (Q2Y) parameters of the PLS-DA models were examined to investigate the performance of the models. Discriminative variables were determined using orthogonal projections partial least-squares to latent structures (OPLS) to select signals that influenced for the class separation. The identity of discriminatory chemicals was determined from their accurate mass composition, their isotopic fit, and from fragmentation data obtained from collision-induced dissociation (CID) using quadrupole-TOFMS (Q-TOFMS) analyses and comparison with authentic standards. Additional fragmentation data were also obtained from gas chromatography (GC-MS) analyses of the purified aglycone structures obtained from enzymatic hydrolysis of contaminants in bile or plasma samples (see SI for details).

Statistical Analyses. Non-normally distributed data (determined as such by the Kolmogorov−Smirnov test) were log transformed prior to statistical analysis. The significance of the discriminatory markers of effluent exposure detected in roach plasma was determined using the students t-test together with an estimation for a 5% false discovery rate associated with the multivariate data.[18]

RESULTS AND DISCUSSION

Profiling and Identification of Chemical Contaminants in Bile and Plasma of Trout Exposed to a WwTW Effluent. Bile samples from rainbow trout exposed to river water (controls) or WwTW effluent for 10 days were analyzed by UPLC-TOFMS. PCA of the data sets obtained from either +ESI or −ESI MS modes revealed distinct separation between bile samples from control trout and those exposed to effluent for 10 days, indicating that there were significant changes in the bile chemistry at this time (see SI Figure S1). PLS-DA of the bile +ESI and −ESI data sets resulted in models with good predictivity (Q2Y > 0.52) and a high degree of explained variation (a total R2Y of >0.82) modeled for the first 5 latent variables (Figure 1A for the −ESI data set, and Figure S2 for +ESI data set). Bile samples from control trout held in river water for a further 4 or 11 days after an initial 10 day exposure period clustered separately from those from effluent-exposed trout which had undergone a 4 or 11 day depuration period in

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Table 1. Contaminant Markers of Effluent Exposure and Metabolite Perturbations Detected in Bile or Plasma of Trout Exposed for 10 Days to a WwTW Effluent and Following a 4-Day Depuration Period

		bile		plasma	
class of contaminant or metabolite	chemical identity	fold change after 10 days of effluent exposure[b] (mean ± SE)	% decrease after an additional 4 days depuration	fold change after 10 days of effluent exposure[b] (mean ± SE)	% decrease after an additional 4 days depuration[c]
linear alkylbenzene sulfonic acid (LAS) anionic surfactants	C5−C10 sulfophenyl carboxylates	57 ± 12−1101 ± 120	77−100		
	monohydroxy C10-sulfophenyl carboxylate	377 ± 10	100		
	dihydroxy C10-LAS glucuronide	97 ± 24[¶]	100		
	C13-LAS			8 ± 1	85
nonylphenol ethoxylate (NP*n*EO) surfactants[a]	nonylphenol glucuronide	21 ± 2	82	87 ± 14	100
	NP1EO−NP6EO glucuronides	13 ± 2−1890 ± 123	68−100		
alcohol ethoxylate (A*n*EO) nonionic surfactants[a]	dodecanol glucuronide	195 ± 6	80		
	dodecanol 1EO−5EO glucuronides	107 ± 5−1174 ± 61	95−100		
	tridecanol glucuronide	398 ± 14	88		
	tridecanoic acid glucuronide	175 ± 7	94		
	tridecanol 1EO−7EO glucuronides	73 ± 16−2313 ± 92	73−100		
	tetradecanol glucuronide	155 ± 6	100		
	tetradecanol 1EO−8EO glucuronides	96 ± 32−2997 ± 179	81−100		
	tetradecanol 3EO−7EO			4 ± 2−188 ± 34	50−100
	pentadecanol glucuronide	118 ± 15	100		
	pentadecanol 1EO−8EO glucuronides	181 ± 18−2623 ± 173	74−100		
	pentadecanol 4EO−7EO			89 ± 15−302 ± 28	100
alcohol ethoxycarboxylates (A*n*ECs)[a]	dodecanol 5EC	27 ± 7	59		
	tridecanol 4EC−7EC	859 ± 64−2183 ± 114	26−100		
	tetradecanol 4EC−7EC (bile includes 8EC)	111 ± 7−390 ± 24	11−96	32 ± 12−359 ± 41	100
	pentadecanol 4EC−7EC (bile includes 8EC−10EC)	79 ± 8−621 ± 20	8−100	41 ± 12−90 ± 5	100
aromatic hydrocarbons	1-naphthol/2-naphthol glucuronides	189 ± 7	75		
chlorinated phenols	dichlorophenol glucuronide	237 ± 10	89		
	trichlorophenol glucuronide	233 ± 17	100		
	trichlorophenol sulfate			24 ± 5	100
chlorinated xylenols	chloroxylenol glucuronide	104 ± 28	92	91 ± 7	100
	chloroxylenol sulfate			6 ± 3	100
	methoxy chloroxylenol glucuronide	293 ± 13	93	29 ± 2	100
	methoxy chloroxylenol sulfate			73 ± 5	100
	methoxy dichloroxylenol glucuronide	341 ± 18	68		
	dichloroxylenol glucuronide	353 ± 22	95		
chlorophenes	chlorophene glucuronide	1558 ± 346	96	54 ± 4	100
	methoxy chlorophene glucuronide	206 ± 50	97		
	chlorophene sulfate			13 ± 4	100
chlorinated phenoxyphenols	triclosan glucuronide	46 ± 6	85	47 ± 3	100
	triclosan sulfate			370 ± 38	89
	diclosan glucuronide	122 ± 6	93		
	diclosan sulfate			28 ± 3	100
pharmaceutical	mefenamic acid glucuronide	122 ± 3	100		
sunscreen product	oxybenzone glucuronide	325 ± 14	100		
resin acids	mixture of resin acids glucuronides	7 ± 1	94		
steroidal alkaloids	solanidine			85 ± 9	nd
	dihydrosolanidine			38 ± 7	nd
bile acids	taurocholic acid			10 ± 5	88
	taurochenodeoxycholic acid			9 ± 4	92
	cyprinol sulfate			12 ± 9	76
lipids	sphingosine			0.5 ± 0	38[c]

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Table 1. continued

class of contaminant or metabolite	chemical identity	bile		plasma	
		fold change after 10 days of effluent exposure[b] (mean ± SE)	% decrease after an additional 4 days depuration	fold change after 10 days of effluent exposure[b] (mean ± SE)	% decrease after an additional 4 days depuration[c]
	2-methylbutryolcarnitine*			3 ± 0.4	65
	lysophosphatidylethanolamine (16:0)			2 ± 0	38

[a] *n*EO where *n* = number of ethoxymers in the ethoxylate chain, and *n*EC where *n* = number of ethoxymers in the ethoxycarboxylate chain. [b] Fold change was calculated from the relative concentrations (normalized to the maximum spectral area in each sample) of M − H, M + H ion, or M + Na adduct in blood and bile of effluent-exposed trout compared with the values in control trout held in river water. [c] All metabolites decreased in concentration with the exception of sphingosine, where levels increased by 38% after a 4 day depuration period. After 11 days depuration, the elimination of all contaminants was between 90 and 100% compared to levels in non-depurated fish. Full details of MS identification of compounds are given in Supporting Information Tables S1 and S2. * = compound identity putative.

river water following the 10 days of effluent treatment. Analysis of the PLS-DA models was undertaken by omitting one replicate in turn from each treatment class, remodelling the data sets and testing classification of the test replicate. This revealed 100% accuracy of classification between the 10 day control and effluent exposure data sets, and >90% accuracy between the data sets from depurated trout and those of their respective controls. These data indicate that the changes in bile chemistry as a result of a 10-day exposure to the WwTW effluent were still apparent after a subsequent 11-day depuration period.

Similarly, PCA of the data sets of plasma profiles revealed that in −ESI MS mode there was a clear separation between plasma samples from control trout and those exposed to effluent for 10 days (Figure S3). PLS-DA models of both the plasma data sets from either −ESI analyses (Figure 1B) or +ESI analyses (Figure S4) revealed separation between plasma samples from control trout and those exposed to effluent for 10 days, and also between samples from fish depurated for 4 and 11 days after effluent exposure and their respective controls at these time points (for both PLS-DA models, the total R2Y > 0.86 and Q2Y > 0.50 for the first 6 latent variables).

The discriminatory chemicals that were responsible for class separation were extracted from the S-Plot of the OPLS models of bile and plasma data sets according to Wiklund et al.,[19] and their identities were determined by MS fragmentation. Bile samples from the control and effluent-exposed trout (10 day exposure) contained 94 (−ESI mode) and 71 (+ESI mode) MS signals (not including ion adducts) that were increased in the effluent-exposed fish. Plasma samples from the same fish contained 36 (−ESI mode) and 41 (+ESI mode) signals of compounds that differed between the control and effluent treatments after 10 days exposure. MS analysis revealed that most of the compounds that increased in bile and plasma of fish after exposure to effluent were xenobiotics (see Table 1 for a summary of the unequivocally identified chemical contaminants in trout bile, and Tables S1 and S2 for a full list of chemical markers and Q-TOFMS and GC-MS details of identified structures in trout bile and plasma).

Many of the contaminants detected in trout bile and plasma were derived from commonly used surfactants. A series of sulfophenyl alkyl monocarboxylates (SPCs) were detected in bile and, depending on the alkyl chain length, their concentrations were 57−1101 fold times higher in effluent-exposed fish compared to controls. SPCs can be derived from the carboxylation and chain shortening of parent C10−C13 linear alkylbenzene sulfonic acids (LAS) during wastewater treatment and this has been reported to result in SPCs with alkyl chain lengths of C7−C11.[20] In the trout bile C5−C10-SPCs were detected, and in addition monohydroxylated C10-SPC, indicating further chain shortening and oxidation of LAS contaminants either during wastewater treatment or in the fish itself. A glucuronide conjugate of a dihydroxylated C10-LAS metabolite was detected in the bile, and the C13-LAS surfactant was detected in plasma indicating that the parent LAS surfactants were also taken up and bioconcentrate in the fish.

Nonionic surfactants including nonylphenol ethoxylates (NP*n*EOs, *n* = number of ethoxymers) and alcohol ethoxylates (AEOs) were predominant markers of effluent exposure in fish bile and plasma. A series of NP1EO−NP6EO homologues, as well as NP itself, were detected as glucuronide conjugates in the bile. NP and short-chain NPEOs are formed from the degradation of long-chain polyethoxylates surfactants in WwTWs and the short-chain biodegradation products are bioavailable to fish.[21] Glucuronide metabolites of AEOs (A*x*EO*n*; where *x* = carbon chain length of C12−C15; *n* = 0−8) were identified in trout bile, and UPLC-TOFMS analysis revealed that each AEO homologue contained a mixture of isomers with similar elemental compositions suggesting it comprised a mixture of branched and linear alkyl chains. C14EOs and C15EOs were also detected as the free nonconjugated AEOs in the plasma (Table 1). Linear and branched-chain AEOs are widely used in household and industrial detergents. In household products, the alkyl composition range is predominantly C12−C15 with an average ethoxymer distribution of 3−8EOs[22] (although homologues containing up to 18EOs are used in industrial products), and the accumulation of a similar distribution of AEOs in trout bile and plasma may indicate the presence of poorly degraded domestic residues in the effluent. Alkyl polyethoxycarboxylates (AE*n*Cs where *A* = alkyl groups and *n* = number of ethoxy units plus a terminal CH_2COOH moiety) were detected in both bile and plasma. AE*n*Cs could be formed from omega oxidation of the ethoxylate group of the parent AEO during wastewater treatment,[23] however the major aerobic biodegradation pathway of AEOs is via central cleavage of the molecule to form polyethylene glycols and the free fatty alcohol.[24] AE*n*Cs are also used as anionic surfactants in the textile industry and in household and personal care products, and thus may be an additional source of detergent-derived contaminants in WwTWs effluents.

A variety of phenolic xenobiotics, including 1-naphthol, 2-naphthol, dichlorophenol, trichlorophenol, chloroxylenol, dichloroxylenol, chlorophene, diclosan, and triclosan, were identified in bile from effluent-exposed trout, and these were predominantly conjugated to glucuronic acid. Many of these chlorinated contaminants were detected as either the

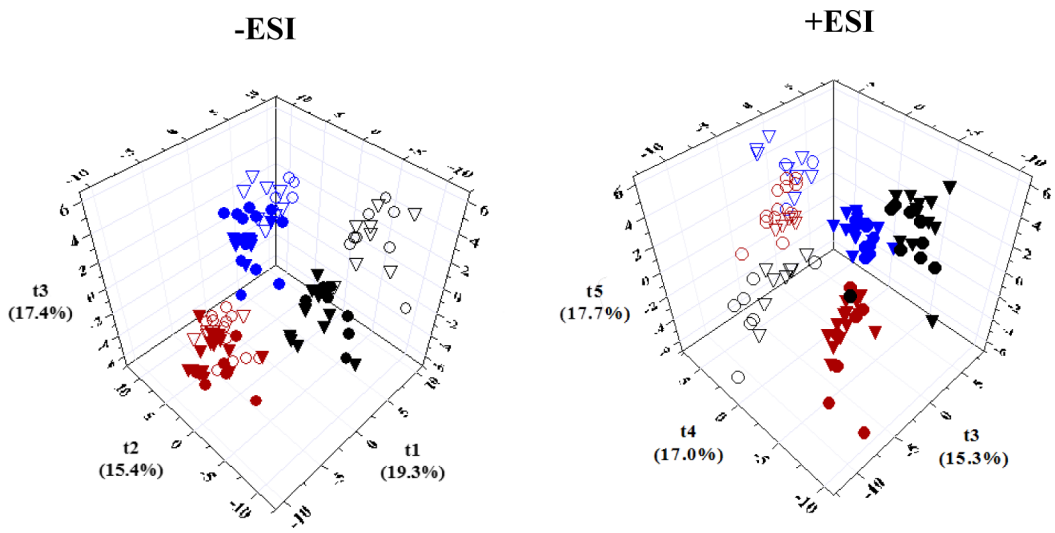

Figure 2. Partial least-squares-discriminant analyses (PLS-DA) scores plots of the chemical profiles of blood samples from roach exposed to two concentrations of WwTW effluent or a clean water control. The samples were profiled in either +ESI or −ESI mode by UPLC-TOFMS. Fish were kept in duplicate tanks (tank 1: circle, tank 2: triangle). Control river water exposure comprised n = 15 females, n = 21 males; 50% effluent exposures n = 12 females, n = 20 males; 100% effluent exposures n = 17 females, n = 20 males. Female roach: (open black symbols ○/▽), (open blue ○/▽), and (open red ○/▽); and male roach: (black closed symbols ●/▼), (blue closed ●/▼), and (red closed ●/▼) represent 0%, 50%, and 100% effluent exposure, respectively. The percentages of explained variation (R2Y) modeled for three of the latent variables are displayed on the axes.

glucuronide or sulfate conjugates in the plasma too. Chlorinated phenols and xylenols are commonly used antiseptics or disinfectants, and triclosan is an antibacterial agent used in household products.[25] Chlorophene, chloroxylenol, and triclosan have previously been reported in bile of contaminated fish[26,27] and are widely detected in surface waters and effluents,[28] and show antiandrogenic activity in in vitro assays.[27] Other classes of xenobiotics detected in the trout samples included a mixture of glucuronide conjugates of isomeric resin acids in the bile, which were separated by GC-MS and were identified from authentic standards as pimaric, isopimaric, abietic, and neoabietic acids. Resin acids are tricyclic diterpenes that occur naturally in the resin of tree wood and bark and are transferred to process waters during pulping operations, and they have been detected in bile of fish captured in the downstream receiving waters.[29] In bile samples the glucuronide conjugates of the nonsteroidal anti-inflammatory pharmaceutical, mefenamic acid, and the sunscreen agent oxybenzone were detected. The steroidal alkaloids, solanidine and dihydrosolanide, which may arise from the metabolism of potato products, were also detected in plasma samples.[30]

This study, using a chemical profiling approach based on ESI, will only detect highly abundant ionizable metabolites and, without the use of sample preparation methodologies, less abundant compounds such as steroids would be subjected to ion suppression and not detected.[31] Likewise nonpolar compounds such as alkanes would not be detected using ESI methods. Some differences were observed between profiles of the contaminants in the trout bile compared with those in the plasma, and this was likely due to the higher concentration of contaminants in the bile compared with blood allowing detection of more contaminants in the bile samples. Nevertheless, this analysis of the more highly abundant xenobiotics present in the biofluids reveals that fish are exposed to a diverse group of chemical contaminants present in wastewaters. Many of the glucuronide metabolites of xenobiotics are likely to be formed by conjugation of the parent compound within the fish itself as glucuronide conjugates formed by human metabolism (for instance the glucuronide conjugate of mefenamic acid) can be readily hydrolyzed by bacteria during the WwTWs resulting in the detection of the parent compound in the final effluent.[32] Glucuronide conjugates are mainly excreted via the bile of fish, and depuration studies revealed that with a few exceptions, 68−100% of the xenobiotic concentrations were eliminated from the bile after 4 days in clean water and 90−100% were lost after 11 days (Table 1). The elimination of the majority of the xenobiotic load during depuration is in accordance with the PLS-DA models of chemical profiles in the bile and plasma, which revealed decreased chemical space between controls and depurated fish compared with the nondepurated fish (Figure 1). The health impacts of the presence of such a complex mixture of xenobiotics circulating in trout plasma are unknown. The majority of xenobiotics were not persistent in the trout biofluids, and it is possible that as many of the contaminants are lost rapidly from the fish during depuration, that they exert little, if any, toxicity to the fish. However, it is possible that exposure to such a variety of contaminants that are substrates for glucuronosyl transferase enzymes expressed in liver and other tissues of the fish could disrupt the metabolism and excretion of endogenous metabolites such as steroids and bile pigments.

Profiling and Identification of Chemical Contaminants in Plasma of Roach Exposed to a WwTW Effluent. To further investigate disruption of the endogenously derived metabolome as a result of effluent exposure, adult roach were exposed in duplicate tanks to water (controls), 50% or 100% WwTW effluent. After 28 days of exposure, plasma was sampled and metabolomic analyses performed as described for the above trout studies. PLS-DA of plasma from either sex revealed a clear separation between samples from 0%, 50%, or 100% effluent exposed roach in the ESI data sets (Figure 2). For any one treatment there was no difference in the clustering of samples from duplicate tanks which indicated that the effects due to effluent treatment or sex outweighed any differences due to tank effects. However, there was a clear difference between the plasma metabolome of male and female fish in control tanks

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Table 2. Contaminant Markers and Metabolite Perturbations Identified in Plasma of Roach Exposed to 50% or 100% WwTW Effluent Concentrations for 28 Days[a]

observed ion (m/z)	RT (min)	putative formula	theoretical mass of ion	Δ ppm	chemical identity	gender	control vs 50% effluent: mean fold change	control vs 50% effluent: p-value	control vs 100% effluent: mean fold change	control vs 100% effluent: p-value
478.2991	8.2	$C_{27}H_{44}NO_4S$	478.2991	0.0	solanidine sulfate $[M + H]^+$	F	470.2	**1.29×10^{-7}**	987.7	**3.54×10^{-9}**
						M	349.3	**3.72×10^{-12}**	767.4	**3.72×10^{-12}**
480.3146	8.3	$C_{27}H_{46}NO_4S$	480.3148	−0.4	dihydrosolanidine sulfate $[M + H]^+$	F	193.2	**1.60×10^{-5}**	421.0	**1.17×10^{-7}**
						M	161.5	**4.06×10^{-6}**	417.4	**2.80×10^{-11}**
264.9937	8.6	$C_9H_{10}O_5SCl$	264.9937	0.0	methoxy chloroxylenol sulfate $[M - H]^-$	F	7.1	**2.40×10^{-2}**	67.4	**1.71×10^{-9}**
						M	7.6	**2.10×10^{-2}**	59.7	**1.01×10^{-6}**
366.9002	15.4	$C_{12}H_6O_5SCl_3$	366.9002	0.0	triclosan sulfate $[M - H]^-$	F	363.6	**5.59×10^{-7}**	855.1	**1.71×10^{-9}**
						M	565.1	**8.18×10^{-11}**	1377.7	**9.94×10^{-13}**
325.1837	22.20	$C_{18}H_{29}O_3S$	325.1837	0.0	C12-LAS $[M - H]^-$	F	2.0[b]	**4.00×10^{-3}**	5.0[b]	**1.12×10^{-8}**
						M	1.8[b]	**1.42×10^{-5}**	2.6[b]	**3.48×10^{-9}**
339.1994	23.38	$C_{19}H_{31}O_3S$	339.1994	0.0	C13-LAS $[M - H]^-$	F	7.3[b]	**4.31×10^{-6}**	10.6[b]	**5.76×10^{-6}**
						M	3.5[b]	**3.41×10^{-8}**	10.4[b]	**1.69×10^{-7}**
514.2841	8.8	$C_{26}H_{44}NO_7S$	514.2838	0.5	taurocholic acid $[M - H]^-$	F	3.0[b]	6.30×10^{-2}	5.6[b]	**2.00×10^{-3}**
						M	2.3[b]	6.13×10^{-1}	3.8[b]	1.57×10^{-1}
531 0.2997	11.5	$C_{27}H_{47}O_8S$	531.2992	0.9	cyprinol sulfate $[M - H]^-$	F	2.3[b]	**1.32×10^{-4}**	2.5[b]	**5.56×10^{-5}**
						M	1.4[b]	1.68×10^{-1}	1.7[b]	**8.00×10^{-3}**
282.2796	20.5	$C_{18}H_{36}NO$	282.2797	−0.3	sphingosine $[M + H - H_2O]^+$	F	0.7[b]	8.00×10^{-2}	0.5[b]	**2.23×10^{-5}**
						M	0.9[b]	5.43×10^{-1}	0.5[b]	**1.71×10^{-6}**
452.2777	20.64	$C_{21}H_{43}NO_7P$	452.2777	0.0	Lyso PE 16:1 $[M + H]^+$	F	1.3[b]	8.70×10^{-2}	1.9[b]	**6.07×10^{-5}**
						M	1.0[b]	5.97×10^{-1}	1.3[b]	**2.10×10^{-2}**
494.3252	20.80	$C_{24}H_{49}NO_7P$	494.3247	1.0	Lyso PC 16:1 $[M + H]^+$	F	1.3[b]	8.70×10^{-2}	2.0[b]	**4.27×10^{-6}**
						M	1.2[b]	1.28×10^{-1}	1.7[b]	**5.55×10^{-5}**
478.2934	21.32	$C_{23}H_{45}NO_7P$	478.2934	0.0	Lyso PE 18:2 $[M + H]^+$	F	1.3[b]	**2.70×10^{-2}**	2.1[b]	**1.80×10^{-6}**
						M	1.2[b]	9.30×10^{-2}	1.4[b]	**2.80×10^{-2}**
520.3402	21047	$C_{26}H_{51}NO_7P$	520.3403	−0.2	Lyso PC 18:2 $[M + H]^+$	F	1.2[b]	3.30×10^{-1}	1.8[b]	**4.14×10^{-4}**
						M	1.1[b]	3.58×10^{-1}	1.1[b]	**7.00×10^{-2}**

[a]Lyso PC = lysophosphatidylcholine. Lyso PE = lysophosphatidylethanolamine. *F* = female. *M* = male. Full list of lysophospholipid species where plasma levels increased after effluent exposure are given in Table S3. Mean fold change calculated from relative concentrations (normalized to the maximum spectral area in each sample) of the compound in plasma from roach exposed to either 50% or 100% effluent for 28 days compared with the levels in control roach held in clean water (n = 16−20 fish for each gender). p-value was calculated from t-test between the control and the effluent exposure, and values in bold are statistically significant with a false discovery rate <5%. [b]Concentrations above the limit of detection were detected in plasma of the control roach.

and also in the effluent treatments of the +ESI data set. The structures of the metabolites that were identified as discriminatory signals between the plasma metabolomes from control and effluent-treated fish are given in Table 2. The predominant xenobiotics detected in the plasma of roach (both sexes) exposed to either 50 or 100% effluent were C12 and C13 LAS surfactants, as well as metabolites of triclosan, chloroxylenol, and solanidine, and these were also some of the predominant xenobiotic contaminants detected previously in the trout plasma. It was likely that other xenobiotics were also present in roach plasma, but they were not detected as the sample size analyzed on column was 2-fold lower than previous studies with trout.

Profiling of the trout plasma samples revealed changes in endogenously derived metabolites as a result of effluent exposure. These included 9−12 fold increases in the plasma bile acids, and 3- and 2-fold increases in methylbutyrylcarnitine and lysophosphatidylethanolamine concentrations, respectively, and a 2-fold decrease in sphingosine concentrations indicating possible disruption of lipid pathways as a result of xenobiotic exposure (Table 1). Bile acids that increased in trout in response to effluent exposure included cyprinol sulfate which, although not usually reported in trout, was present in the plasma and bile samples, albeit in much lower (10-fold less) concentrations compared with taurocholic acid. In roach, similar to the trout exposure, the bile acids taurocholic acid and cyprinol sulfate were prominent markers of effluent exposure in the plasma. In addition, a 2-fold decrease in sphingosine and up to 2-fold increases in lysophospholipid concentrations were also confirmed in the effluent-exposed roach (Table 2; the full lysophospholipid list is given in Table S3). Sphingosine is an important second messenger in many cells and can inhibit protein kinase C enzymes, mobilize calcium ions from internal stores, and alter cell proliferation.[33] It is also a metabolite in the biosynthesis of sphingolipids which are structural components of cell membranes and modulate many processes including cell−cell communication and cellular adhesion. Alterations in sphingolipid metabolism have been reported as a result of mycotoxin exposure, and it is possible that either exposure to environmental chemicals or the potential presence of mycotoxins in the effluent could have disrupted sphingosine metabolism in the fish.[33]

The concentrations of taurocholic acid and cyprinol sulfate increased by 4−5 fold in females and 2−3 fold in males exposed to 100% effluent, and increases in plasma bile acid concentrations were also observed in both sexes exposed to 50% effluent (Table 2). In mammals, increases in concentrations of plasma bile acids are indications of liver damage and

toxicity,[34] and it is possible that the exposure to a mixture of xenobiotics, many of which are metabolized in the liver prior to transport to the bile, may have resulted in liver injury and increased release of bile acids into the plasma. It is possible that the detection of a variety of surfactant molecules, some nonionic containing up to 8EO units, as well as anionic structures may result in damage to cell membranes in a variety of tissues including the liver.[35] The hepatosomatic index of the roach was significantly increased by exposure to undiluted effluent (Table S4) which may indicate inflammation and potential injury of the liver, or alternatively may be a result of increased vitellogenin synthesis in response to estrogens present in the effluent.[36] However, changes in plasma bile acid composition could also be a result of modulation of bile acid synthesis and metabolism, or of enterohepatic recirculation as a compensatory response to liver changes or inflammation.

In mammalian systems there is now much evidence that bile acids act as regulatory molecules and can activate a number of nuclear receptors including the farnesoid X receptor, the pregnane X receptor, and the vitamin D receptor[37] as well as activating selected G protein receptors and a number of other signaling pathways in liver cells. As a result, bile acids can play a role in lipid and carbohydrate metabolism and homeostasis. For instance, exposure of rodents to bile acids has been shown to decrease lysophosphatidylcholine and sphingomyelin levels in liver tissues.[38] In our study, significant decreases in plasma sphingosine levels and increased concentrations of lysophospholipid (LPE, LPC) species were observed in the plasma metabolome of roach exposed to undiluted effluent, and it was possible that the alterations in the lipid profiles in these fish were associated with the increased concentrations of bile acids in the circulatory system resulting in disruption of sphingolipid and phospholipid homeostasis. However, it is also possible that xenobiotics detected in the fish plasma also resulted in disruption of lipid homeostasis. An analysis of the correlation matrix comparing concentrations of xenobiotics with the concentrations of endogenously derived marker metabolites of effluent exposure in the same fish revealed significant correlations with many of the chemical contaminants detected in the plasma (Tables S5A, B). Hence, further work is needed to determine the causes of disruption lipid and bile acid metabolism and the potential linkage with liver injury as a result of effluent exposure.

The identities of the xenobiotic and metabolite markers of effluent exposure were the same in both sexes of roach. No significant differences were detected between the males and females in the plasma concentrations of markers, however the fold increase in concentrations of bile acids and lysophospholipids were generally higher in females than males indicating possible gender differences in the metabolomic response to exposure to contaminants in the effluent (Table 2).

This study reveals the complex nature of xenobiotics accumulating in fish exposed to a domestic WwTW effluent, and importantly the potential of studies on the exposome to identify biochemical markers (and pathways) altered signaling for effects on health status. Our findings show that exposure to WwTW effluents can result in disruption of bile acid and lipid homeostasis with probable consequences for cellular signaling and maintenance of cell membrane integrity. The further development of trace analytical methodologies will allow profiling of less abundant components of the metabolome, including hormones, alongside the primary metabolites and the xenometabolome. (Bio)chemical profiling approaches of the nature reported here offer an exciting, noninvasive prospect for future application to assessing the risk to fish of complex chemical mixtures exposures in surface waters.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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