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Novel metabolomic method to assess the effect-based removal efficiency of advanced wastewater treatment techniques

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Abstract

Unprecedented levels of chemicals of anthropogenic origin are currently released into surface waters globally. Wastewater treatment plant effluent has been identified as a major source, containing a broad mixture of pharmaceuticals and consumer chemicals. Therefore, there is a need for implementation of advanced wastewater treatment techniques, such as ozonation and adsorption methods, to reduce the contamination. However, there are conflicting findings on the toxicity of treated effluent and only limited possibilities for assessing the effect-based removal efficiency (EBRE) of different treatment techniques. Here, we describe a metabolomics approach to detect perturbations in fatty acid catabolic pathways as a proxy for biological effects. Metabolites in three fatty acid pathways were analyzed in a common damselfly larva (*Coenagrion hastulatum*) by liquid chromatography coupled to mass spectrometry. The larvae were exposed for one week to either conventionally treated effluent (activated sludge treatment), effluent additionally treated with ozone or effluent additionally treated with biochar filtration and results were compared with those from tap water control exposure. Five lipoxygenase-derived oxylipins (9,10,13-TriHOME, 9,12,13-TriHOME, 9-HODE, 9-HOTrE, and 13-HOTrE) decreased in response to conventionally treated effluent exposure. By using an additional treatment step, oxylipin levels were restored with exception of 9,10,13-TriHOME (ozonated effluent), and 9-HOTrE and 13-HOTrE (effluent filtered with biochar). In conclusion, exposure to wastewater effluent affected fatty acid metabolite levels in damselfly larvae, and a subset of the analyzed metabolites may serve as indicators for biological effects in biota in response to effluent exposure. To that effect, our findings suggest a new metabolomics protocol for assessing EBRE.

Rapid communication

Anthropogenic pollutants, including pharmaceuticals such as lipid regulators, anticonvulsants, and nonsteroidal anti-inflammatory drugs, have been found in aquatic

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environments worldwide (Heberer, 2002; Luo et al., 2014; Ternes et al., 2015; Noguera-Oviedo and Aga, 2016). Many of these pollutants are not removed in conventional wastewater treatment plants (WWTPs) and are therefore discharged into receiving waters (Eggen et al., 2014; Tran et al., 2018; Kümmerer et al., 2019). Pharmaceuticals are bioactive substances that are designed to cause physiological effects in living organisms, and the risks of their adverse effects on ecosystems have increasingly been recognized (German Environment Agency 2016; Ternes 1998). Such effects include feminization of fish caused by endocrine-disrupting compounds (EDCs; Tyler and Jobling 2008) and behavioral alterations in fish exposed to anxiolytics (Brodin et al. 2013). To protect and improve the quality of receiving waters, removal of pharmaceuticals can be enhanced by implementing advanced wastewater treatment techniques, such as biochar or activated carbon adsorption and ozonation (Joss et al. 2008; Weidemann et al. 2018). The efficiency of such techniques has mostly been evaluated in terms of pollutant removal and there are many studies confirming that both techniques remove a broad range of pharmaceuticals from wastewater (Huber et al. 2005; Ternes et al. 2003). However, there is still dispute about the biological effects of ozonated effluents. For instance, some studies have reported reduced toxicity of ozonated WWTP effluents (Bundschuh et al. 2011), whereas others have shown that ozonation can affect gene expression, reproductive success and behavioral endpoints in exposed fish (Pohl et al. 2018).

To address the lack of conclusive findings on biological effects due to WWTP effluent exposure, we hypothesized that metabolomics can be used to assess effect-based removal efficiencies (EBREs). To test this hypothesis, metabolites (oxylipins) in fatty acid catabolic pathways were analyzed using a newly developed metabolomics protocol for damselfly larvae. Oxylipins, including prostaglandins and other eicosanoids, are potent metabolites formed by oxidation of polyunsaturated fatty acids via three enzymatic pathways, i.e., the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways. They regulate numerous metabolic functions, such as reproduction, inflammation and immune cell behavior, in both vertebrates and invertebrates (Heckmann et al. 2008; Yang et al. 2009). Furthermore, oxylipins are responsive to environmental pollution. For example, elevated levels of three oxylipins were found in human lung lavage following air pollution exposure (Gouveia-Figueira et al. 2017) and prostaglandin levels were shown to be altered in fish exposed to WWTP effluent (David et al. 2017). As many common pharmaceuticals are designed to target enzymes responsible for the biosynthesis of oxylipins (Willenberg et al. 2015), they may serve as relevant metabolomic endpoints, in particular for the assessment of EBREs. The use of metabolomic endpoints may provide insights into the mode of action of a pollutant and the health and functional status of an organism at the molecular level. They are also likely to be affected by environmental stressors before changes in other endpoints occur (Bundy et al. 2009; Ekman et al. 2018; Viant 2007).

Northern damselfly larvae (*Coenagrion hastulatum*) were used as an invertebrate model species in this investigation because of their wide distribution and ecological importance. More specifically, damselfly larvae play an important role in aquatic food webs, preying on small invertebrates and serving as prey for larger invertebrates and vertebrates (Jonsson et al. 2014). Moreover, damselfly larvae have previously been used to detect effects of exposure to pollutants (e.g., antihistamines, anxiolytics and pesticides) on a wide range of endpoints,

such as life history traits, behavior and physiology (Brodin et al. 2014; Finotello et al. 2017; Jonsson et al. 2014; Sniegula et al. 2017).

The aim of this study was to assess EBREs of different wastewater treatment technologies using a new metabolomics protocol. Damselfly larvae were exposed to conventionally treated effluent (activated sludge treatment without N-removal), effluent additionally treated with ozone or effluent additionally treated with biochar filtration, and oxylipin profiles of exposed larvae were measured by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Northern damselfly larvae were collected in August 2017 at Lake Nydalasjön in Umeå, Sweden and taken to a laboratory at Umeå University. They were introduced into individual aquaria (10×10 cm), and subsequently exposed to WWTP effluents for seven days (see Supplementary Material). The exposure groups were as follows: i) conventionally treated WWTP effluent (E, $n = 21$), ii) effluent treated with ozone (E+O, $n = 21$), iii) effluent treated with biochar (E+B, $n = 22$), and iv) tap water control (C, $n = 18$).

Final effluent was obtained at a WWTP and additionally treated with either ozone or a biochar adsorbent in-lab. Ozonation (5.5 mg/L ozone, 15 min residence time) was carried out in a column reactor using O₃ generated from O₂ gas by an EXT120-T Ultra ozone generator (Longevity Resources, Sidney, Canada). Biochar treatment was performed by adding 20 g biochar to 2.5 L effluent, followed by manually shaking for 5 min and a final filtration step. Biochar was produced from tomato residues by torrefaction, a slow pyrolysis operated under mild conditions (Nordin et al. 2013), at 260 °C for three hours in a rotating furnace (described by Weidemann and Lundin 2015).

To assess EBRE, the effect of exposure to differently treated WWTP effluents on relative concentrations of oxylipins in damselfly larvae was examined. Tissue extractions were performed according to Jonsson et al. (2014), followed by oxylipin analysis using a LC-MS/MS method (Yang et al. 2009). For the extraction of oxylipins, internal standards were added to the damselfly larvae and homogenization was performed using 1.5 mL acetonitrile and zirconium beads added to each sample with shaking for 4 min at 42000 oscillations per minute using a Mini Beadbeater (Biospec. Bartlesville, USA). Samples were centrifuged at 14 000 revolutions per minute for 10 min, the supernatant was withdrawn, and then samples were re-extracted using 1.5 mL acetonitrile following the above described procedure. The supernatants were combined, evaporated to dryness and reconstituted in 100 µL of methanol. A total of 87 oxylipins were analyzed in the samples using LC-MS/MS (Yang et al. 2009).

Student's *t*-test was used to detect significant differences ($p < 0.05$) in relative oxylipin concentrations between the exposure and control groups.

A total of 24 oxylipins derived from four fatty acids (arachidonic acid, linoleic acid [LA], alpha-linolenic acid [ALA] and eicosapentaenoic acid [EPA]) via two enzymatic pathways (LOX and CYP) were detected in the samples (Table S1 in the Supplementary Material). Among these, 16 were present in more than 75% of samples of at least one exposure group, and hence included in the statistical analysis. Of these 16 oxylipins, 10 were not significantly different among the groups. Five LOX-derived oxylipins (9,10,13-TriHOME,

9,12,13-TriHOME, 9-HODE, 9-HOTrE and 13-HOTrE) were significantly lower in larvae exposed to conventionally treated effluent compared to the control (Figure 1 a–e). Fewer oxylipins were affected by additional effluent treatments. One oxylipin, 9,10,13-TriHOME, was significantly lower in larvae exposed to ozonated effluent compared to the control. Two oxylipins, 9-HOTrE and 13-HOTrE, were significantly lower in larvae exposed to effluent treated with biochar. Additionally, 14,15-DiHETE (Figure 1f), formed via the CYP pathway, was significantly lower in the biochar exposure group but not affected by any other treatment. Overall, our results suggested that further treatment by ozonation or biochar filtration reduces the biological effects, as reported in other studies (Reungoat et al. 2010). In conclusion, in terms of EBREs, lower effect was observed for both ozonation and biochar filtration compared to conventional WWTP effluent, i.e., these two treatment techniques had similar removal efficiencies of pollutants, such as pharmaceuticals affecting oxylipin pathways.

Oxylipins have prior to this not been detected in damselfly larvae. Hence, the biological role of these metabolites and their underlying biochemical pathways in this species remains to be investigated. However, in another invertebrate, *Daphnia magna*, several oxylipins, including 9-HODE, 13-HODE, 9-HOTrE and 13-HOTrE, were shown to increase following single exposure to different psychiatric drugs (Garreta-Lara et al. 2018), whereas in the present study decreased levels were observed for the same compounds. We speculate that effluent exposure led to LOX inhibition by pharmaceuticals targeting LOX enzymes, which, in turn, resulted in decreased levels of five oxylipins. Another explanation may be decreased precursor fatty acid levels due to pollutant-induced stress. Finotello et al. (2017) showed that exposure to a pyrethroid pesticide led to lower fat content and reduced levels of two essential and two precursor fatty acids in damselfly larvae. Pollutant-induced stress requires more energy for maintaining physiological integrity (Van Praet et al. 2014), which in turn may lead to lower fatty acid levels and suppressed oxylipin synthesis.

To gain a better understanding of the affected metabolic pathways in damselfly larvae, analyses of other physiological endpoints, such as fatty acids and other metabolites related to available energy reserves, are needed. Inhibitor experiments, similar to studies performed in other species (Knight et al. 1999), may provide valuable insights into oxylipin biosynthesis in damselfly larvae. To verify enzymatic activity, chiral LC-MS/MS analysis of monohydroxy metabolites, such as 9-HODE and 13-HODE, can also be used since it enables identification of (S) (enzymatic) and (R) (non-enzymatic) enantiomers (Garreta-Lara et al. 2018). In the present study, control group exposure was carried out in tap water instead of lake water to minimize uncertainty due to a potential presence of pollutants in the lake water. Future studies should address the effect of tap water in comparison to lake water. Additionally, metabolite levels following lab-exposure should be compared to levels of naïve individuals.

Because little is known about the fatty acid metabolism of damselfly larvae, effects of altered oxylipin levels on individuals and populations are difficult to predict. However, owing to their important physiological functions in other invertebrates, disruption of oxylipin biosynthesis is likely to have severe consequences (Heckmann et al. 2008). Considering the current knowledge gap, this study represents a first important step towards

using oxylipins as a proxy for biological effects, a method that can be used to address mixture effects of WWTP effluent and EBREs of various wastewater treatment techniques.

In conclusion, oxylipins belonging to the LOX and COX pathways were detected in the larvae of the Northern damselfly, an organism considered a suitable target species for EBRE assessment owing to their ecological importance. We showed that exposure of damselfly larvae to WWTP effluent led to disruption of the biosynthesis of a subset of these fatty acid metabolites. Additional effluent clean-up by either ozonation or biochar filtration resulted in restored levels of some metabolites. The results suggested that metabolomics, in particular the fatty acid cascade as physiological endpoints, may be a useful tool for evaluating the effect-based removal efficiencies of wastewater treatment technologies.

The supplementary material contains further information on the exposure set-up, normalized peak areas of all oxylipins detected in the samples (Table S1) and a list of oxylipins included in the analysis but not detected in any of the samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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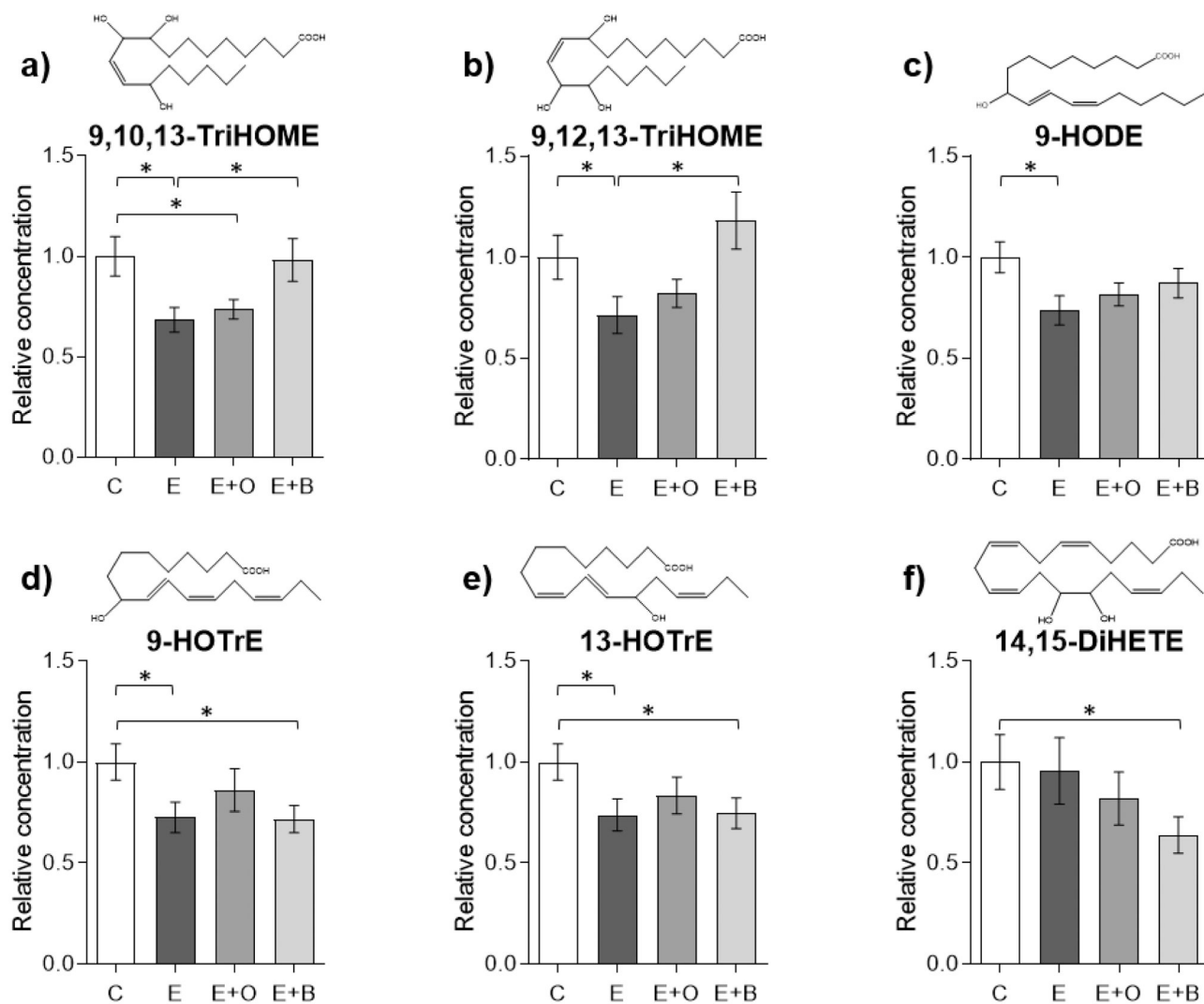


Figure 1.

Relative oxylipin concentrations in damselfly larvae exposed to tap water (control, C), conventionally treated effluent (E), effluent additionally treated with ozone (E+O) and effluent additionally treated with biochar filtration (E+B) (* $p < 0.05$). a)-e): LOX-derived oxylipins (9,10,13-TriHOME, 9,12,13-TriHOME, 9-HODE, 9-HOTrE and 13-HOTrE) were significantly lower in larvae exposed to conventionally treated effluent compared to the control. f) CYP-derived 14,15-DiHETE was significantly lower in the biochar exposure group but not affected by any other treatment.