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Evaluation of targeted and untargeted effects-based monitoring tools to assess impacts of contaminants of emerging concern on fish in the South Platte River, CO☆

Drew R. Ekman [a, *], Kristen Keteles [b, **], Jon Beihoffer [b], Jenna E. Cavallin [c], Kenneth Dahlin [d], John M. Davis [a], Aaron Jastrow [e], James M. Lazorchak [f], Marc A. Mills [g], Mark Murphy [d], David Nguyen [d], Alan M. Vajda [h], Daniel L. Villeneuve [i], Dana L. Winkelman [j], Timothy W. Collette [a]

[a] U.S. EPA, National Exposure Research Laboratory, 960 College Station Rd., Athens, GA 30605, USA
[b] U.S. EPA, National Enforcement Investigations Center, Denver Federal Center Building 25, E-3, Denver, CO 80225, USA
[c] Badger Technical Services, U.S. EPA, Duluth, MN 55804, USA
[d] U.S. EPA, Region 8, 1595 Wynkoop St., Denver, CO 80202, USA
[e] U.S. EPA, Region 5, Ralph Metcalfe Federal Building, 77 West Jackson Blvd., Chicago, IL 60605, USA
[f] U.S. EPA, National Exposure Research Laboratory, 26 Martin Luther King Blvd., Cincinnati, OH 45268, USA
[g] U.S. EPA, National Risk Management Research Laboratory, 26 Martin Luther King Blvd., Cincinnati, OH 45268, USA
[h] University of Colorado Denver, Department of Integrative Biology, Campus Box 171, P.O. Box 173364, Denver, CO 80217, USA
[i] U.S. EPA, National Health and Environmental Effects Research Laboratory, Duluth, MN 55804, USA
[j] U.S. Geological Survey, Colorado Cooperative Fish and Wildlife Research Unit, Department of Fish, Wildlife and Conservation Biology, 201 J.V.K. Wagar Building, 1484 Campus Delivery, Colorado State University, Fort Collins, CO 80523, USA

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ABSTRACT

Rivers in the arid Western United States face increasing influences from anthropogenic contaminants due to population growth, urbanization, and drought. To better understand and more effectively track the impacts of these contaminants, biologically-based monitoring tools are increasingly being used to complement routine chemical monitoring. This study was initiated to assess the ability of both targeted and untargeted biologically-based monitoring tools to discriminate impacts of two adjacent wastewater treatment plants (WWTPs) on Colorado's South Platte River. A cell-based estrogen assay (*in vitro*, targeted) determined that water samples collected downstream of the larger of the two WWTPs displayed considerable estrogenic activity in its two separate effluent streams. Hepatic vitellogenin mRNA expression (*in vivo*, targeted) and NMR-based metabolomic analyses (*in vivo*, untargeted) from caged male fathead minnows also suggested estrogenic activity downstream of the larger WWTP, but detected significant differences in responses from its two effluent streams. The metabolomic results suggested that these differences were associated with oxidative stress levels. Finally, partial least squares regression was used to explore linkages between the metabolomics responses and the chemical contaminants that were detected at the sites. This analysis, along with univariate statistical approaches, identified significant covariance between the biological endpoints and estrone concentrations, suggesting the importance of this contaminant and recommending increased focus on its presence in the environment. These results underscore the benefits of a combined targeted and untargeted biologically-based monitoring

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* Corresponding author. U.S. EPA, National Exposure Research Laboratory, 960 College Station Rd., Athens, GA 30605, USA.
** Corresponding author. U.S. EPA, National Enforcement Investigations Center, Denver Federal Center Building 25, E-3, Denver, CO 80202 USA.
E-mail addresses: ekman.drew@epa.gov (D.R. Ekman), keteles.kristen@epa.gov (K. Keteles), Beihoffer.jon@epa.gov (J. Beihoffer), cavallin.jenna@epa.gov (J.E. Cavallin), dahlin.kenneth@epa.gov (K. Dahlin), davis.john@epa.gov (J.M. Davis), jastrow.aaron@epa.gov (A. Jastrow), lazorchak.jim@epa.gov (J.M. Lazorchak), mills.marc@epa.gov (M.A. Mills), murphy.mark@epa.gov (M. Murphy), nguyen.david@epa.gov (D. Nguyen), alan.vajda@ucdenver.edu (A.M. Vajda), villeneuve.dan@epa.gov (D.L. Villeneuve), dana.winkelman@colostate.edu (D.L. Winkelman), collette.tim@epa.gov (T.W. Collette).

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strategy when used alongside contaminant monitoring to more effectively assess ecological impacts of exposures to complex mixtures in surface waters.

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1. Introduction

There is growing recognition that chemical monitoring alone does not provide sufficient information to adequately assess the risks from anthropogenic chemicals in the natural environment. This is particularly true for contaminants of emerging concern (CECs), some of which have been shown to cause endocrine disruption in laboratory experiments at levels below their analytical detection limits (Parrott and Blunt, 2005). Many CECs, including widely-used pharmaceuticals and personal care products, are not always effectively removed by wastewater treatment plants (WWTPs) (Ternes, 1998); thus, they occur in the aquatic environment as constituents of complex mixtures whose composition changes over time and location. It is important for risk assessors to better understand the cumulative impacts of these mixtures, and this information cannot be provided by periodic chemical monitoring alone.

The need to understand the biological impacts of CECs is particularly pressing in the arid Western region of the U.S., where the flow in many rivers can be dominated by WWTP effluent (Patten, 1998; Woodling et al., 2006), particularly in years with low snowfall. In these situations, the levels of CECs and other stressors can increase dramatically, potentially producing adverse impacts on fish and other aquatic wildlife. For these reasons, we have undertaken a multi-year integrated biological and chemical field investigation, where fathead minnows (FHM; *Pimephales promelas*) were cage-deployed for five days below and above two urban WWTPs, and also at a reference site in the South Platte River watershed in Colorado, during low flow periods. A primary goal of these studies was to test the efficacy of both targeted and untargeted effects-based monitoring (EBM) tools for characterizing potential ecological impacts. Because the sites were expected to be estrogenic, vitellogenin (vtg) mRNA abundance in the livers of male FHM was examined. We also applied a recombinant *in vitro* bioassay using T47D-KBluc cells to quantify the estrogenic activity of water collected from the various study locations (Wilson et al., 2004). In addition to these targeted approaches focused on estrogenic activity, an untargeted metabolomics analysis that could detect perturbation of a broader range of biochemical pathways was employed.

Here we present results from these analyses for the field study conducted in September 2013. The exposure scenario from this study provided a rich opportunity to evaluate these EBM approaches for assessing the impacts of CECs. For example, one of the WWTPs serves a relatively smaller population and also uses biological nutrient removal prior to discharge. The other WWTP serves a much larger population and employs less advanced treatment technology (at the time this study was conducted). In addition, this WWTP discharges using two separate outfalls that receive different levels of influent and also utilize different levels of treatment. Our deployments were designed to test whether these EBM tools would discriminate biological activities associated with these different treatment scenarios.

In addition to carrying out EBM, chemical monitoring was conducted using water collected at all of these sites. Analyses for CECs (pharmaceuticals, personal care products, pesticides, etc.), common wastewater indicators, steroid hormones, and inorganic chemicals were performed. In addition, we measured a group of water quality parameters, which, if out of normal ranges, could serve as non-chemical stressors for fish (e.g., pH, temperature, dissolved oxygen, etc.). Finally, partial least squares (PLS) regression was used to determine the extent of covariance between these chemical and water quality measurements and the metabolite profiles. This approach is effective at prioritizing stressors according to the extent to which they are eliciting biological responses at a given site (Davis et al., 2016). Such information could help inform a wide range of environmental monitoring programs, and may allow improved decision making regarding the prioritization of contaminant monitoring and remediation efforts.

2. Materials and methods

2.1. Study design

Two WWTPs that release effluent into the South Platte River were selected for this study (Fig. 1). Both WWTPs (W1 and W2) used primary and secondary treatment (activated sludge), solids removal, and disinfection with chlorine. Site W1 received approximately 18 million gallons per day (MGD) and has aerobic nitrifying trickle filters and a de-nitrification process for nutrient removal. W2 received approximately 100 MGD and has two treatment complexes (South and North). At the time of this study, the North complex (W2-N) received twice the amount of influent as the South complex (W2-S). W2-N treated influent nitrogen using nitrification whereas W2-S employed ammonification.

To assess fish responses to the effluents from these WWTPs, sexually-mature (6-8 month-old) male fathead minnows (*Pimephales promelas*) were deployed in cages in proximity of the two WWTPs, and at a relatively unimpacted reference site (REF). Specifically, after acclimation (approximately 48hrs. following overnight shipment from the U.S. EPA Aquatic Research Laboratory in Cincinnati, OH), fish were deployed at W1, W2-UP, W2-N, W2-S, and REF using PVC cages (15 fish per cage; one cage per site) with mesh on both ends (Fig. 1). Note that previous chemical and hydrologic analyses at these sites have shown that very little mixing of the effluents occurs between W2-N and W2-S, and that REF was located in a large, relatively unimpacted, creek that flows into the South Platte (Fig. 1). There are no major WWTPs upstream of REF, and no evidence of estrogenic endocrine disruption at this site (Woodling et al., 2006); thus it was used as the “control” for evaluating the impact from the WWTPs in that it takes into account any stresses on the fish as a result of being cage-deployed, and it most closely reproduces the (non-chemical) field conditions of the various test sites. Deployments occurred from September 5th through September 10th, 2013 during seasonal low-flow conditions. Flow data at each site including the percent contribution to total river flow from the W2-N and W2-S effluent streams (data for the W1 percent contributions were not available) are provided in the Supporting Information (Table S1). A storm event occurred on the last day of the deployment. However, fish were removed from the sites and the five-day grab samples were taken prior to increases in flow.

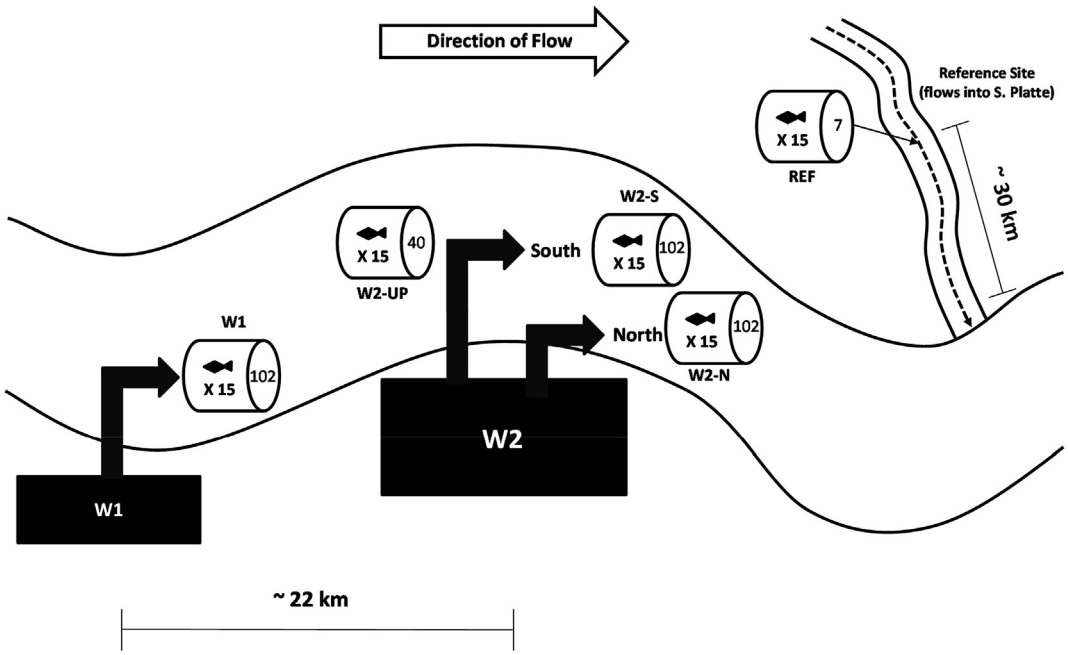

Fig. 1. Relative site locations for adult male fathead minnow deployments and water sample collections near adjacent wastewater treatment plants (W1 and W2) on the South Platte River in CO (USA). Cylinders represent submerged cages, each containing 15 males. Acronyms (defined in the text) for the various locations appear above or below each cylinder. The number of chemicals detected at each site (mean of day 0 and day 5 grab samples) is indicated within the base of each cylinder.

2.2. Sample collection

After five days of exposure, the cages were removed, and the fish were euthanized by anesthetization with tricaine methanesulfonate (MS-222) followed by rapid decapitation. Lengths and wet weights were measured, then livers were collected, split into two equal portions, and flash-frozen. One portion was placed into an Eppendorf Safe-Lock tube and stored at −80 °C for metabolomics. The other portion was placed into an RNAse free tube and stored at −80 °C for vtg mRNA analysis. Water samples were collected both at the time of cage deployment (day 0) and at the time of cage removal (day 5) for measurement of CECs, steroids, and metals (191 compounds analyzed; see Supporting Information) and for use with the T47D-KBluc cell assay. Contaminant concentrations reported in these water samples (Table S2) as well as those used for identifying relationships to biological endpoints (e.g., partial least squares regression analysis described below) are averages of detections from these two sampling times. All water samples were stored at 4 °C prior to analysis. A Hydrolab MS5 Multiparameter Mini Sonde (OTT Hydromet) was used at each site to measure conductivity, dissolved oxygen, temperature, and pH. Flow was determined using United States Geological Survey (USGS) stream gauge stations that were in close proximity to each deployment site.

2.3. T47D-KBluc estrogen receptor transactivation assay

To measure relative estrogenic activity of water collected at each time point at each site, water samples were assayed using a recombinant T47D-KBluc cell line that was developed by Wilson et al. (2004) and has previously been used to measure total estrogenic activity of complex mixtures (Wehmas et al., 2011). Use of this method for the current study is described in Supporting Information. Differences in mean values across sites were evaluated for statistical significance by one-way analysis of variance (ANOVA) with a post-hoc Tukey's test (Minitab v16.2.4, Minitab Inc.).

2.4. vtg mRNA expression analyses

Liver fragments were evaluated using qPCR to determine changes in hepatic vtg mRNA expression associated with effluent exposure. Methods for total RNA extraction and measurement of vtg mRNA amplicons are described in Supporting Information. Differences in mean values across sites were evaluated for statistical significance by ANOVA on these log-transformed data with a post-hoc Tukey's test.

2.5. ^{1}H-NMR-based metabolomics

To profile changes in relative abundances of endogenous hepatic metabolites associated with WWTP contaminant exposure, liver fragments (75 fragments in total (5 sites × 15 fish per site)) were extracted using a previously described method (Ekman et al., 2012) which produces both a polar and nonpolar (lipophilic) extract for each liver sample (only data from the polar extracts are reported here). The polar extracts were dried under nitrogen and reconstituted in 600 μL of 0.1 M sodium phosphate buffered deuterium oxide (pH 7.4) that contained 50 μM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP). The reconstituted samples were then analyzed by NMR spectroscopy at 21.5 °C on a JEOL Eclipse+ 500 MHZ spectrometer (500.16 MHz, ^{1}H) using a 5 mm triple resonance broadband probe and a 1D NOESY with observed presaturation pulse sequence. Acquired spectra were processed as described by Davis et al. (2013) and as reported in Supporting Information. Principal component analysis (PCA; not shown) was used to provide an overall assessment of the data (SIMCA-13.0, Umetrics Inc.) and to identify outliers with a Hotelling's T^2 test at the 95% confidence interval (Jackson, 1991; Wikstrom et al., 1998). Four fish (two from the reference site (REF) and two from the site upstream of W2 (W2-UP)) were thus identified and subsequently removed from the dataset. Investigations into potential causes for this outlier behavior revealed that the liver from two of these fish (one from REF and one from W2-UP) were the smallest (by wet weight)

among the sample set and produced particularly low spectral intensities. Spectra for the remaining two outliers, for unexplained reasons, showed unusually high levels of lactate and creatine.

To identify endogenous metabolites that were statistically different for the fish deployed at a given site, compared to those fish deployed at the reference site, we generated t-test-filtered difference spectra based on the binned and normalized NMR spectra. Details on this approach, including procedures for controlling for false positives, have been reported previously (Ekman et al., 2012; Collette et al., 2010) and are described in Supporting Information. For significantly different bins, we identified metabolite peaks using Chenomx NMR Suite 7.0 (Chenomx Inc.) and previously published metabolite chemical shift values (Ekman et al., 2007; Teng et al., 2009; Wishart et al., 2009). To further evaluate impacts of exposures on male FHMs, we calculated total intensity values for the t-test-filtered difference spectra by summing the absolute values of the magnitudes for all significantly different bins (see Supporting Information). This provided a single metric that integrated the breadth of metabolites that were altered by an exposure and also the magnitude of such alterations. Comparing impacts across field sites (with impact at each site determined relative to the REF site) provided a relative benchmark for the extent that profiles were perturbed by the exposures. A one-way ANOVA with a posthoc Tukey's test assessed whether these values differed among sampling sites.

2.6. Comparing metabolite changes and chemical concentrations using partial least squares regression

We used partial least-squares (PLS) regression (SIMCA-13.0) to assess the relationship between changes in endogenous metabolite profiles and contaminant concentrations (mean values for day 0 and day 5 grab samples) in the water samples, to both identify those contaminants that significantly covary with metabolite changes in the liver and to screen out those contaminants that did not (Davis et al., 2016). Modeling details for the current application are provided in Supporting Information, and are similar to those previously published (Davis et al., 2016, 2017).

3. Results and discussion

3.1. In vitro and In vivo targeted assays for detecting estrogenic activity

Using the T47D-KBluc cell bioassay, the water samples collected from the South Platte River were shown to possess considerable estrogenic activity. Immediately downstream of W1, approximately 15 ng EE2-EQ/L of estrogenic activity was detected (Fig. 2). Estrogenic activity was significantly less at the site upstream of W2 (W2-UP), which was located approximately 22 km downstream from W1, suggesting significant contaminant attenuation due to dilution, partitioning out of the dissolved fraction, or chemical transformation downstream from the source (W1). However, downstream of W2, estrogenic activity rose considerably, nearly doubling that observed immediately downstream of W1. Total estrogenic activity detected in water collected downstream of W1 and W2 was relatively high, falling at the upper range of values reported by Conley et al. in their assessment of *in vitro*-based estrogenic activity of 35 streams across the United States (Conley et al., 2017). Furthermore, the activities detected at W1 and W2 (in the range of 15–30 ng EE2-EQ), exceed levels at which health effects have been observed by other researchers (Brand et al., 2013; Jarošova et al., 2014) as well as EE2 concentrations that have caused adverse effects on fish in both laboratory and field settings. However, it remains unclear whether specific conditions within the South Platte

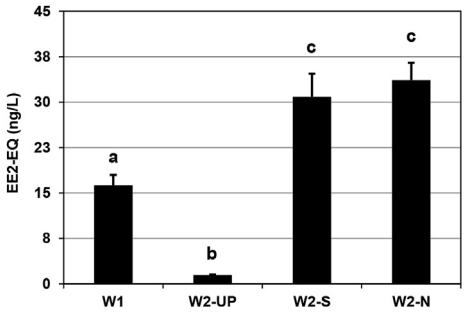

Fig. 2. T47D-KBluc assay results showing average values (mean ± SE) for estrogenic activity, expressed as 17α-ethynylestradiol equivalents (ng/L), for days one and five of the exposure. Bars with different letters indicate significant differences based on ANOVA with posthoc Tukey's test, $p < 0.05$. Acronyms for site designations (W1, W2-UP, W2-N, W2-S) are defined in the text. The reference site (REF) showed no activity.

River system may moderate some of these effects. For example, it is expected that fish in this river have the ability to migrate in and out of regions of highest estrogenic activity and that the activity will vary at different times of the year, for example due to dilution from spring snow melt. Additional characterization of seasonal and spatial variation in estrogenic activity and migration patterns of resident fish would help to define the duration and periods of their life-cycle when they may be exposed to potentially adverse levels of estrogenic activity. Understanding such exposure dynamics are particularly important since current evidence suggests that many of these impacts may be reversible assuming exposure occurs outside of critical development windows (Baumann et al., 2014). Moreover, based on the *in vitro* bioassay alone, it is unclear exactly which compounds are contributing to the estrogenic activity and whether they are bioavailable and may be either rapidly metabolized, or conversely, bioconcentrated.

In vitro characterization of total estrogenic activity was complemented with an *in vivo* measure of estrogenic activity, by determining vtg mRNA induction in livers of male FHM that were caged for 5 days at the study sites (Fig. 3). These *in vivo* results were broadly in-line with the *in vitro* data, with significant induction observed in males caged downstream of W2, but not immediately upstream (W2-UP). Comparing both sets of data suggests that the *in vitro* estrogenic activity of the grab samples (Fig. 2) is an overestimate of the *in vivo*-relevant dose experienced by males in the field (Fig. 3). Flick et al. (2014) reported that induction of vtg mRNA expression could be reliably detected at EE2 concentrations ranging

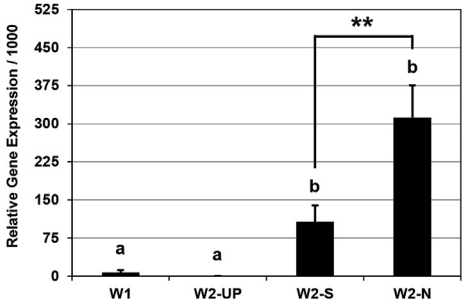

Fig. 3. Average vitellogenin (vtg) mRNA expression (mean ± SE) for fish deployed at each exposure site. Values shown are relative to measured 18S mRNA levels. Bars with different letters indicate significant differences based on ANOVA with posthoc Tukey's test, $p < 0.05$, and including all four sites. The double-asterisk (**) indicates a p-value < 0.01 for a Student's *t*-test comparing only sites W2-S and W2-N. Acronyms for site designations (W1, W2-UP, W2-N, W2-S) are defined in the text. The relative mean vtg mRNA expression for the reference site (REF) was 7.28 and thus not visible using the y-axis scale shown.

from 5 ng/L to 100 ng/L after 2, 4, or 7 d of exposure. Our analysis (Fig. 3) suggests that estrogenic activity is at or near the lower end of this range (in EE2 equivalents) for the W1 site, with a two-class Student's t-test comparing W1 and REF yielding a p-value of 0.092. Therefore, the 15 ng/L EE2-equivalents estimated for W1 using the T47D-KBluc cell bioassay (Fig. 2) suggests that these *in vitro* results overestimate estrogenic activity when compared to *in vivo* responses, but likely by no more than an order of magnitude. This discrepancy may be due, in part, to use of grab samples (which are more susceptible to extreme events) for the *in vitro* bioassay, whereas the fish were exposed continuously for 5 d. It cannot be determined whether the apparent discrepancy reflects this difference, or, perhaps, other factors such as a lack of bioavailability for some of the active compounds, metabolism and excretion of estrogenic compounds by the fish, or some combination of these. Finally, while the levels of estrogenic activity indicated by both *in vitro* and *in vivo* measurements are of some concern, treatment technology upgrades that have been implemented at W2 since the present study (addition of denitrification) are expected to decrease the estrogenic activity of receiving waters considerably.

3.2. Evidence of estrogenic impacts in the hepatic metabolome

The two targeted biological assays clearly establish that estrogenic activity is associated with water from these sites. Given this, we analyzed the NMR-detectable metabolite changes in the same livers used for the vtg mRNA analysis with regard to metabolites identified in a previous study as responding to estrogens (Ekman et al., 2008). Toward this end, we generated *t*-test filtered difference spectra that retained only those metabolites that show statistically significant changes at the various impacted sites (W1, W2-UP, W2-N, W2-S) relative to the reference site (REF). Fig. 4 presents these difference spectra for the field sites, along with a difference spectrum that was constructed (in the same fashion, using matched controls) from a 4-day laboratory exposure of male FHM to 83 ng/L of the model estrogen 17α-ethynylestradiol (EE2). It is evident from Fig. 4 that the difference spectra from fish caged at the W2 downstream sites (W2-N and W2-S) exhibit many of the same features observed for the lab study with EE2. For example, with the lab-based exposure, we observed elevations in alanine (Ala) and glutamate (Glu), which were correlated with increases in plasma vitellogenin (VTG) (Ekman et al., 2008). The elevation in these two metabolites was not unexpected given that they are the two most abundant amino acids used in the biosynthesis of FHM VTG, and that the liver is the primary site of VTG production (Parks et al., 1999). Thus, elevation of Ala and Glu for the fish at W2-N and W2-S (and to a lesser extent W1) in the current field study was interpreted largely as a requirement to meet the demand to produce VTG.

These consistent lab and field findings suggest that the concerted hepatic elevation of Ala and Glu may be a useful indicator for estrogenic exposure in male FHM. These findings are also encouraging as they suggest that metabolomic responses determined in laboratory exposures with model chemicals may also be detected in field studies that involve exposures to complex mixtures containing chemicals with the previously studied biological mode-of-action (MOA). This is a major consideration for the successful application of metabolomics to real-world exposure scenarios. To further explore this potential, we examined the intensity difference observed for the alanine (Ala) resonance in the *t*-test filtered difference spectra (Fig. 4). Ala was chosen because the resonance for its methyl peak is narrow (a doublet), and occurs in a relatively uncrowded region of the NMR spectrum, making it more convenient for this application than Glu. This analysis was conducted for all of the field sites for this present study, as well as for the aforementioned lab study, which in addition to the 83 ng/L EE2 exposure also included an exposure to 7 ng/L EE2. The trend in Ala enhancement observed for these field sites (Fig. 5) is similar to that for the vtg mRNA results which were observed using a portion of the same livers (Fig. 3). In addition, the Ala elevation for the field site with the highest estrogenic response (W2-N) is on par with that which we observed for 7 ng/L of EE2 from our earlier lab study. Interestingly, we also conducted a mesocosm male-FHM exposure study with 5, 10, and 20 ng/L of EE2 in conjunction with an earlier (2012) pilot study that included most of these same sites (W1, W2-UP, W2-N, and REF) (Schwindt et al., 2014). For that study, vtg mRNA induction was measured such that the response in the field deployments could be compared directly to those from the mesocosm. That comparison suggested that the estrogenic response at W2-N in 2012 was on par with ~5 ng/L of EE2. The general consistency of all of these findings further support the utility of Ala elevation as an indicator of estrogenic activity, particularly when coupled with an elevation of Glu and perhaps other metabolites

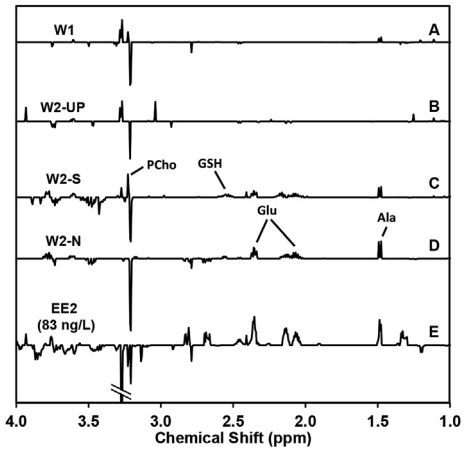

Fig. 4. Average difference spectra (A, B, C, D) generated to determine specific changes in liver metabolite profiles and to assess the overall impacts of the exposures (impacted field site minus the reference field site (REF)). Peaks above the baseline indicate metabolites that increased relative to the REF fish, and those below the baseline indicate metabolites that decreased. Only those peak abundances that were found to be significantly different from those of the REF fish are shown (*t*-test, $p < 0.05$). The results of an identical analysis conducted for a laboratory exposure to 83 ng/L of 17α-ethynylestradiol reported by Ekman et al. (Ekman et al., 2008) is shown (E) for comparison. PCho, phosphocholine; GSH, glutathione; Glu, glutamate; Ala, Alanine. Acronyms for site designations (W1, W2-UP, W2-N, W2-S) are defined in the text.

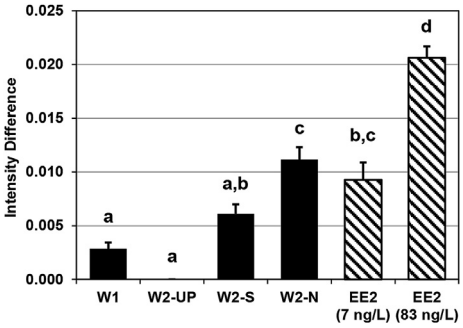

Fig. 5. Average intensity difference values (mean ± SE) of alanine for each site (or lab exposure), relative to the reference site (REF) (or lab controls). Bars with different letters indicate significant differences based on ANOVA with posthoc Tukey's test $p < 0.05$. Acronyms for site designations (W1, W2-UP, W2-N, W2-S) are defined in the text.

involved in VTG synthesis. Additionally, while not included in the current study, lipids required for VTG synthesis (e.g., phosphatidylcholine) have also been shown to be highly impacted in fish exposed to estrogens (Ekman et al., 2009), and thus when used in conjunction with changes in Ala and Glu, could offer additional validation for the presence of estrogens in complex mixtures. However, further studies (including exposures to a variety of estrogens and contaminants that display other modes-of-action) will be required to determine the robustness of these metabolites, given the many roles they play in other aspects of fish biochemistry.

3.3. Untargeted metabolomics to inform non-estrogenic responses

While the difference spectra are useful for identifying specific metabolites whose relative abundances are changing upon exposure, they can also be used to estimate the overall impact of the exposures on the whole measurable metabolome. This can be achieved by summing the absolute intensities of all of the peaks in each difference spectrum. This analysis was conducted for the current dataset to enable comparison to the results of the targeted assays (that only measure estrogenic activity). Consistent with these targeted assays, the total intensity differences (relative to the reference site) from metabolomics demonstrate that the impacts at W1 and W2-UP are considerably less than those from the W2-S and W2-N sites (Fig. 6). However, this analysis also demonstrates that, as opposed to the vtg mRNA results, the impact on the hepatic metabolome from exposure to W2-N does not exceed that from W2-S, suggesting that the fish are experiencing non-estrogenic impacts at W2-S that exceed those experienced by the fish at W2-N. The difference spectra can be further used in an untargeted manner to determine the specific metabolites that were impacted at W2-S relative to W2-N and W2-UP in order to explore any non-estrogenic effects that were unique (or more severe) at W2-S. In this way, we identified a significant increase in total hepatic glutathione (excluding any glutathione bound to cellular macromolecules) for the fish deployed at W2-S (Fig. 4C). Glutathione plays a crucial role in cellular defense, scavenging free radicals and other reactive oxygen species and aiding in xenobiotic detoxification and excretion. Its increase may represent a compensatory response in the fish deployed at W2-S, commensurate with what has been observed in other fish species and bivalves exposed to oxidative stressors (Doyotte et al., 1997; Zhang et al., 2004).

Further examination of the difference spectra revealed that phosphocholine (Fig. 4C) was also increased in the W2-S fish. A recent NMR-based metabolomic analysis of oxidative stress in wild fish collected from mercury-contaminated surface waters also reported an increase in hepatic phosphocholine, which the authors suggested was a result of phosphatidylcholine degradation following hepatic membrane disruption (Brandao et al., 2015). Moreover, the authors reported that this increase in hepatic phosphocholine occurred with a concomitant increase in glutathione as well as various enzymatic oxidative stress markers. This further suggests that oxidative stress pathways may be responsible for the additional impacts detected in the livers of the fish deployed at the W2-S site. In hopes of elucidating these and other responses, investigations for subsequent years of this multi-year project will employ metabolomic analysis of additional biological matrices (e.g., plasma and skin mucus) as well as high-resolution mass spectrometry to identify additional pathways that may be affected. In addition, assays targeting specific responses other than estrogenic activity (e.g., oxidative stress) may be employed to follow-up on hypotheses generated by the metabolomics results.

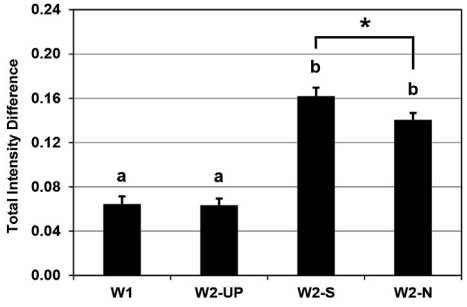

Fig. 6. Total intensity difference values (mean ± SE) for each site. Total intensities were generated by summing the absolute values of metabolite peak changes for each comparison (impacted field site minus the reference field site (REF)). Bars with different letters indicate significant differences based on ANOVA with posthoc Tukey's test, $p < 0.05$, and including all four sites. The asterisk (*) indicates a p-value < 0.05 for a Student's t-test comparing only sites W2-S and W2-N. Acronyms for site designations (W1, W2-UP, W2-N, W2-S) are defined in the text.

3.4. Relating metabolome impacts to contaminant concentrations

The complexity of contaminant mixtures, and thus the likelihood of multiple interacting MOAs impacting fish and other biota, requires the use of new approaches for evaluating the relationships between such mixtures and the biological responses they produce. As a first attempt at discerning the most relevant contaminants across all of these sites, we employed a partial least squares (PLS) regression approach recently developed to assess covariance in contaminant measurements and metabolomics responses in fish deployed at impacted sites across the Great Lakes Basin (Davis et al., 2016). As described by Davis et al. (2016), this approach relies on the assumption that anthropogenic chemicals whose abundances do not co-vary with endogenous metabolite changes (across sites with a gradient of contamination) are likely not responsible for the molecular events that drive the observed biological changes. Furthermore, the approach assumes that the strength of the co-variance can be used as a coarse estimate (e.g., low, medium or high) of the chemical's contribution to the biological response, relative to other detected chemicals. For the present study, when applying this method, 122 stressors were included in the initial PLS global model. This included 90 anthropogenic organic chemicals (wastewater indicators, pharmaceuticals, steroids, etc.), 28 inorganic chemicals, and four water quality parameters (e.g., pH, temperature, dissolved oxygen, and conductivity) (Table S2). After a final optimized model was created, 58 stressors were eliminated due to a lack of co-variance with the endogenous metabolite changes observed across the sites. The remaining 66 stressors were then sorted according to their strength of co-variance as measured by the Q^2Y parameter (Eriksson et al., 2008). The top 40 stressors ranked according to their Q^2Y value, all of which are organic contaminants, are shown in Table S3. Interestingly, strong covariance is observed for thiabendazole, malathion, and 1,4-dichlorobenzene – three pesticides that are known to produce a variety of responses in fish (including oxidative stress) (Escher et al., 2013). Their covariance with the metabolomics responses recommends further investigation with regard to their importance at these sites, and to other pesticides that are potentially present but were not detected or included in the analytical schedule that was employed.

Not surprisingly, significant covariance was also observed for contaminants regarded as indicators of wastewater effluent such as acetaminophen, ibuprofen, and caffeine. These compounds, while typically not of high relevance from a toxicity standpoint at the concentrations detected, are routinely ranked highly by the PLS approach due to their strong covariance with other potentially more harmful components in WWTP effluents (Davis et al., 2016). Interestingly, water parameters known to produce significant stress

on aquatic biota (e.g., dissolved oxygen and temperature) did show covariance with the metabolite changes, but were not among the 40 with the highest Q^2Y values, emphasizing that CECs were likely the primary stressors at these sites at the time of sampling, at least in relation to the hepatic metabolome.

We expected that the PLS approach would also identify correlations with estrogenic compounds given the magnitude of responses we saw in the targeted estrogenic assays (T47D-KBluc and vtg mRNA). Indeed, we saw a strong covariance (i.e., ranked among the top ten by Q^2Y values) between the metabolomics responses and the measured concentrations of 17β-estradiol, the primary female sex hormone. While the concentration of 17β-estradiol measured at W2-N and W2-S would not be expected to induce vtg mRNA expression, the presence of additional estrogens could, in combination, produce such responses. For example, we identified significant covariance with nonylphenol diethoxylate and nonylphenol monoethoxylate (two relatively weak estrogens), and also with estrone, an estrogen found endogenously in humans (and many other vertebrates) and routinely measured at WWTP-influenced sites (Dammann et al., 2011).

Having identified a strong correlation to 17β-estradiol in the PLS results, we then evaluated the strength of this contaminant's covariance with the targeted *in vitro* and *in vivo* estrogen assays (i.e., T47D-KBluc and vtg mRNA, respectively) as well as with the intensity differences for Ala measured in the livers of the fish at each site, using simple linear regression. This analysis revealed strong relationships between the 17β-estradiol concentrations measured at the sites and all three of these biological indicators (Table S4). For example, the best-fit line relating vtg mRNA expression to 17β-estradiol concentrations across all sites gave an R^2-value of 0.696. Perhaps not surprisingly, T47D-KBluc, the *in vitro* assay which measures transactivation of the estrogen receptor, showed an even stronger correlation to 17β-estradiol with an R^2-value 0.996. The R^2-value measured for the relationship between 17β-estradiol and Ala was 0.890.

We also evaluated the extent of correlation with these three endpoints for estrone. There is growing concern within the environmental community with regard to risks associated with the presence of estrone at sites impacted by WWTPs (Dammann et al., 2011; Cavallin et al., 2016). Estrone is often measured at higher levels than other estrogens at sites that receive WWTP effluent, but, due to its lower potency, it has not been given as much attention as 17β-estradiol and the synthetic estrogen EE2 (Dammann et al., 2011). Our chemical analysis found estrone to be the most abundant among those estrogens measured (Table S2), occurring, in fact, at levels reported in laboratory studies to impact plasma VTG, secondary sex characteristics, plasma 17β-estradiol, and hepatic vtg mRNA (Dammann et al., 2011; Ankley et al., 2017). In the same fashion as the linear regression conducted for 17β-estradiol, we evaluated the estrone concentration data relative to the results of the targeted assays and the Ala responses (Table S4). This analysis for vtg mRNA expression, estrogenic activity in the T47D-KBluc assay, and Ala intensities produced R^2-values of 0.924, 0.872, and 0.991 respectively. These results add to the likely (although often overlooked) importance of estrone in surface waters and thus recommend its careful monitoring along with other estrogens at such sites. Indeed, careful monitoring of estrone is of increasing importance given results recently published by Ankley et al. suggesting that estrone may undergo enzymatic conversion to 17β-estradiol in fish after uptake (Ankley et al., 2017). Given the greater potency observed for 17β-estradiol relative to estrone in fish (Dammann et al., 2011), and the observation that estrone is often found at higher concentrations in contaminated surface waters, increased consideration to the presence of estrone in surface waters appears to be warranted.

3.5. Conclusions

Clearly, these findings argue for expanding the use of targeted biological assays to closely monitor surface waters for estrogenic activity. Furthermore, the development and application of untargeted tools (such as metabolomics) is also critical given the complexity of anthropogenic mixtures and the variety of effects they may produce. Indeed, the results of the current study recommend the complementary use of both types of tools to assess risks, particularly given that urbanization and extreme climate events place increasing, and often unpredictable, stresses on aquatic ecosystems. It will no doubt be informative to further evaluate findings from these tools in subsequent years of this study, as planned WWTP upgrades are implemented and other anticipated (or unanticipated) changes occur.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.04.054.

References

Ankley, G.T., Feifarek, D., Blackwell, B.R., Cavallin, J.E., Jensen, K.M., Kahl, M.D., Poole, S., Randolph, E., Saari, T., Villeneuve, D.L., 2017. Reevaluating the significance of estrone as an environmental estrogen. Environ. Sci. Technol. 51 (8), 4705–4713.

Baumann, L., Knorr, S., Keiter, S., Rehberger, K., Volz, S., Schiller, V., Fenske, M., Holbech, H., Segner, H., Braunbeck, T., 2014. Reversibility of endocrine disruption in zebrafish (*Danio rerio*) after discontinued exposure to the estrogen 17α-ethinylestradiol. Toxicol. Appl. Pharmacol. 278 (3), 230–237.

Brand, W., de Jongh, C.M., van der Linden, S.C., Mennes, W., Puijker, L.M., van Leeuwen, C.J., van Wezel, A.P., Schriks, M., Heringa, M.B., 2013. Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays. Environ. Int. 55, 109–118.

Brandao, F., Cappello, T., Raimundo, J., Santos, M.A., Maisano, M., Mauceri, A., Pacheco, M., Pereira, P., 2015. Unravelling the mechanisms of mercury hepatotoxicity in wild fish (*Liza aurata*) through a triad approach: bioaccumulation, metabolomic profiles and oxidative stress. Metallomics 7, 1352–1363.

Cavallin, J.E., Jensen, K.M., Kahl, M.D., Villeneuve, D.L., Lee, K.E., Schroeder, A.L., Mayasich, J., Eid, E.P., Nelson, K.R., Milsk, R.Y., Blackwell, B.R., Berninger, J.P., LaLone, C.A., Blanksma, C., Jicha, T., Elonen, C., Johnson, R., Ankley, G.T., 2016. Pathway-based approaches for assessment of real-time exposure to an estrogenic wastewater treatment plant effluent on fathead minnow reproduction. Environ. Toxicol. Chem. 35 (3), 702–716.

Collette, T.W., Teng, Q., jensen, K.M., Kahl, M.D., Makynen, E.A., Durhan, E.J., Villeneuve, D.L., Martinovic-Weigelt, D., Ankley, G.T., Ekman, D.R., 2010. Impacts of an anti-androgen and an androgen/anti-androgen mixture on the metabolite profile of male fathead minnow urine. Environ. Sci. Technol. 44 (17), 6881–6886.

Conley, J.M., Evans, N., Cardon, M.C., Rosenblum, L., Iwanowicz, L.R., Hartig, P.C., Schenck, K.M., Bradley, P.M., Wilson, V.S., 2017. Occurrence and in vitro bioactivity of estrogen, androgen, and glucocorticoid compounds in a nationwide

screen of United States stream waters. Environ. Sci. Technol. 51 (9), 4781–4791.
Dammann, A.A., Shappell, N.W., Bartell, S.E., Schoenfuss, H.L., 2011. Comparing biological effects and potencies of estrone and 17β-estradiol in mature fathead minnows, *Pimephales promelas*. Aquat. Toxicol. 105, 559–568.
Davis, J.M., Collette, T.W., Villeneuve, D.L., Cavallin, J.E., Teng, Q., Jensen, K.M., Kahl, M.D., Mayasich, J.M., Ankley, G.T., Ekman, D.R., 2013. Field-based approach for assessing the impact of treated pulp and paper mill effluent on endogenous metabolites of fathead minnows (*Pimephales promelas*). Environ. Sci. Technol. 47, 10628–10636.
Davis, J.M., Ekman, D.R., Teng, Q., Ankley, G.T., Berninger, J.P., Cavallin, J.E., Jensen, K.M., Kahl, M.D., Schroeder, A.L., Villeneuve, D.L., Jorgenson, Z.G., Lee, K.E., Collette, T.W., 2016. Linking field-based metabolomics and chemical analyses to prioritize contaminants of emerging concern in the Great Lakes Basin. Environ. Toxicol. Chem. 35 (10), 2493–2502.
Davis, J.M., Ekman, D.R., Skelton, D.M., LaLone, C.A., Ankley, G.T., Cavallin, J.E., Villeneuve, D.L., Collette, T.W., 2017. Metabolomics for informing adverse outcome pathways: androgen receptor activation and the pharmaceutical spironolactone. Aquat. Toxicol. 184, 103–115.
Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M., Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. Aquat. Toxicol. 39, 93–110.
Ekman, D.R., Teng, Q., Jensen, K.M., Martinovic, D., Villeneuve, D.L., Ankley, G.T., Collette, T.W., 2007. NMR analysis of male fathead minnow urinary metabolites: a potential approach for studying impacts of chemical exposures. Aquat. Toxicol. 85 (2), 104–112.
Ekman, D.R., Teng, Q., Villeneuve, D.L., Kahl, M.D., Jensen, K.M., Durhan, E.J., Ankley, G.T., Collette, T.W., 2009. Profiling lipid metabolites yields unique information on sex- and time-dependent responses of fathead minnows (*Pimephales promelas*) exposed to 17α-ethynylestradiol. Metabolomics 5, 22–32.
Ekman, D.R., Teng, Q., Villeneuve, D.L., Kahl, M.D., Jensen, K.M., Durhan, E.J., Ankley, G.T., Collette, T.W., 2008. Investigating compensation and recovery of fathead minnow (Pimephales promelas) exposed to 17α-ethynilestradiol with metabolite profiling. Environ. Sci. Technol. 42, 4188–4194.
Ekman, D.R., Hartig, P.C., Cardon, M., Skelton, D.M., Teng, Q., Durhan, E.J., Jensen, K.M., Kahl, M.D., Villeneuve, D.L., Gray, L.E., Collette, T.W., Ankley, G.T., 2012. Metabolite profiling and a transcriptional activation assay provide direct evidence of androgen receptor antagonism by bisphenol-A in fish. Environ. Sci. Technol. 46 (17), 9673–9680.
Eriksson, L., Trygg, J., Wold, S., 2008. CV-ANOVA for significance testing of PLS and OPLS® models. J. Chemom. 22, 594–600.
Escher, B.I., van Daele, C., Dutt, M., Tang, J.Y.M., Altenburger, R., 2013. Most oxidative stress response in water samples comes from unknown chemicals: the need for effect-based water quality trigger values. Environ. Sci. Technol. 47, 7002–7011.
Flick, R.W., Bencic, D.C., See, M.J., Biales, A.D., 2014. Sensitivity of the vitellogenin assay to diagnose exposure of fathead minnows to 17α-ethynylestradiol. Aquat. Toxicol. 152, 353–360.
Jackson, J.E., 1991. A User's Guide to Principal Components. John Wiley, New York.
Jarošova, B., Bláha, L., Giesy, J.P., Hilscherová, K., 2014. What level of estrogenic activity determined by in vitro assays in municipal waste waters can be considered as safe? Environ. Int. 64, 98–109.
Parks, L.G., Cheek, A.O., Denslow, N.D., Heppell, S.A., McLachlan, J.A., LeBlanc, G.A., Sullivan, C.V., 1999. Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. Comp. Biochem. Physiol. 123C, 113–125.
Parrott, J.L., Blunt, B.R., 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20 (2), 131–141.
Patten, D.T., 1998. Riparian ecosystems of semi-arid North America: diversity and human impacts. Wetlands 18 (4), 498–512.
Schwindt, A.R., Winkelman, D.L., Keteles, K., Murphy, M., Vajda, A.M., 2014. An environmental oestrogen disrupts fish population dynamics through direct and transgenerational effects on survival and fecundity. J. Appl. Ecol. 51, 582–591.
Teng, Q., Huang, W.L., Collette, T.W., Ekman, D.R., Tan, C., 2009. A direct cell quenching method for cell-culture based metabolomics. Metabolomics 5 (2), 199–208.
Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.
Wehmas, L.C., Cavallin, J.E., Durhan, E.J., Kahl, M.D., Martinovic, D., Mayasich, J., Tuominen, T., Villeneuve, D.L., Ankley, G.T., 2011. Screening complex effluents for estrogenic activity with the T47D-KBluc cell bioassay: assay optimization and comparison with in vivo responses in fish. Environ. Toxicol. Chem. 30 (2), 439–445.
Wikstrom, C., Albano, C., Eriksson, L., Friden, H., Johansson, E., Nordhal, A., Rannar, S., Sandberg, M., Kettaneh-Wold, N., Wold, S., 1998. Multivariate process and quality monitoring applied to an electrolysis process - Part I. Process supervision with multivariate control charts. Chemom. Intell. Lab. Syst. 42, 221–231.
Wilson, V.S., Bobseine, K., Gray Jr., L.E., 2004. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicol. Sci. 81, 69–77.
Wishart, D.S., Knox, C., Guo, A.C., Eisner, R., Young, N., Gautam, B., Hau, D.D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J.A., Lim, E., Sobsey, C.A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li, L., Vogel, H.J., Forsythe, I., 2009. HMDB: a knowledgebase for the human metabolome. Nucleic Acids 37 (Suppl. 1), D603–D610.
Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O., Vajda, A.M., 2006. Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams. Comp. Biochem. Physiol. Part C 144, 10–15.
Zhang, J.F., Wang, X.R., Guo, H.Y., Wu, J.C., Xue, Y.O., 2004. Effects of water-soluble fractions of diesel oil on the antioxidant defences of the goldfish *Carassius auratus*. Ecotox. Environ. Saf. 58, 110–116.