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High-resolution mass spectrometry of skin mucus for monitoring physiological impacts and contaminant biotransformation products in fathead minnows exposed to wastewater effluent

J. D. Mosley^{*,†}, D. R. Ekman^{*,†}, J. E. Cavallin^{‡,§}, D. L. Villeneuve^{||}, G. T. Ankley^{||}, and T. W. Collette[†]

[†]National Exposure Research Laboratory, U.S. Environmental Protection Agency, 960 College Station Road, Athens, Georgia 30605, United States

[‡]ORISE Research Participation Program, U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Blvd., Duluth, MN 55804, United States

[§]University of Minnesota-Duluth, Integrated Biosciences Graduate Program, 1035 University Drive, Duluth, MN 55812, United States

^{||}National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, U.S. Environmental Protection Agency, 6201 Congdon Boulevard, Duluth, Minnesota 55804, United States

Abstract

High-resolution mass spectrometry is advantageous for monitoring physiological impacts and contaminant biotransformation products in fish exposed to complex wastewater effluent. We evaluated this technique using skin mucus from male and female fathead minnows (*Pimephales promelas*) exposed to control water or treated wastewater effluent at 5, 20, and 100% levels for 21 d, using an on-site, flow-through system providing real-time exposure. Both sex-specific and non-sex-specific responses were observed in the mucus metabolome, the latter suggesting the induction of general compensatory pathways for xenobiotic exposures. Altogether, 85 statistically significant treatment-dependent metabolite changes were observed out of the 310 total endogenous metabolites that were detected (156 of the 310 were annotated). Partial least squares-regression models revealed strong covariances between the mucus metabolomes and up-regulated hepatic messenger ribonucleic acid (mRNA) transcripts reported previously for these same fish. These

Please contact corresponding author: J. Mosley at Mosley.jonathan@epa.gov or D. Ekman at Ekman.drew@epa.gov.

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DATA AVAILABILITY

Data are available on request.

regression models suggest that mucus metabolomic changes reflected, in part, processes by which the fish biotransformed xenobiotics in the effluent. In keeping with this observation, we detected a phase II transformation product of bisphenol A in the skin mucus of male fish. Collectively, these findings demonstrate the utility of mucus as a minimally invasive matrix for simultaneously assessing exposures and effects of environmentally relevant mixtures of contaminants.

INTRODUCTION

Wastewater treatment plant (WWTP) effluents carry anthropogenic chemicals into surface waters, exposing aquatic organisms to complex mixtures of pharmaceuticals, personal care products, industrial chemicals, and various other contaminants 1–3. The risks posed by these mixtures have historically been evaluated based on the concentrations and, when known, the relevant toxicity of the individual detected contaminants 4. However, this conventional approach has a number of limitations. For example, some contaminants show bioactivity below current detection limits (e.g., steroids) 5. In addition, unknown toxicities may be associated with exposure to contaminant mixtures, and confounding impacts of nonchemical stressors (e.g., low dissolved oxygen, temperature fluctuations) can further complicate the evaluation of potential effects.

Many of the concerns with conventional approaches can be addressed by incorporating biological endpoints into environmental monitoring programs. For example, histopathological evaluations and the observation of apical effects (e.g., reproductive impairment) have recently been used to complement chemical monitoring for assessing adverse impacts of anthropogenic chemicals on fish in the natural environment 6. Unfortunately, these types of biological approaches can be expensive and time-consuming, and many include invasive sampling techniques not suitable for monitoring threatened or endangered species. There is a pressing need for rapid, cost-effective biomonitoring techniques that are nonlethal yet capable of detecting a variety of adverse impacts 7. To address this need, we previously conducted a laboratory study to assess the utility of metabolomics for assessing impacts of chemical exposures using small amounts of fish skin mucus, which can be easily collected in a nonlethal, minimally invasive fashion 8. Our previous study resulted in the detection of a diverse set of low molecular weight endogenous biochemicals (i.e., a metabolome) that could be collected with no discernible adverse impacts on the fish used in the experiments (*Pimephales promelas*, fathead minnows). In the same study, the fathead minnow skin mucus metabolome showed sex-specific differences in both the composition and the relative abundances of metabolites of control fish. Furthermore, the metabolome was sensitive to a flow-through exposure to bisphenol A (10 µg/L) and showed sex specificity in endogenous metabolite response profiles.

To further advance approaches for environmental monitoring, Cavallin et al. 9 exposed adult fathead minnows to treated effluent from the Western Lake Superior Sanitary District WWTP, which flows into the St. Louis River at Duluth (MN, USA) and for which previous studies have reported estrogenic activity 10, 11. The experiments in the Cavallin et al. study 9 were conducted using an on-site, flow-through system providing continuous exposure to either control water or the final treated effluent (5, 20, or 100%) under real-time WWTP

operating conditions. Cavallin et al. reported the results of this exposure for a suite of targeted molecular- and apical-level responses designed to assess impacts on fish reproduction 9, and also linked apical impacts (e.g., decreased fecundity) to targeted molecular endpoints to provide meaningful insights into the biological responses of the exposed organisms.

In the present study we report the use of untargeted high-resolution mass spectrometry (MS)-based metabolomics with skin mucus collected from the fish exposed as part of the Cavallin et al. study 9, to evaluate the utility of high-resolution MS for further elucidating biological impacts of the complex effluent. We evaluated both the sex-specific and non-sex-specific nature of the responses with regard to impacts on biochemical pathways and attempted to determine whether compensatory mechanisms resulting from 21 d of exposure could be observed. Furthermore, although animals from this particular experiment were euthanized at the end of the exposure period (to collect tissues and fluids for other endpoint measurements), we used the study to demonstrate the potential of skin mucus as a sample matrix for minimally invasive monitoring of xenobiotics as well as their biotransformation products (i.e., contaminants that were both bioavailable and biotransformed).

MATERIALS AND METHODS

Fish Exposure

The exposure system employed in the present study has been described previously 9. Briefly, sexually mature (5–6 mo old) fathead minnows were acclimated for 9 d in control sand-filtered (10 μ m), ultraviolet (UV)-treated Lake Superior water at the Duluth US Environmental Protection Agency (USEPA) laboratory and subsequently transported as established pairs to the exposure unit at the Western Lake Superior Sanitary District WWTP. The breeding pairs were assigned to exposure tanks in a stratified, random manner and allowed to acclimate for an additional 12 d. A 21-d effluent exposure with 3 treatment levels (5, 20, and 100%) was conducted. Intermediate treatment levels of effluent concentrations were achieved by dilution with Lake Superior water and were chosen to correspond with estimated dilutions found at increasing distances from the point of discharge into the receiving water (20% $\approx$ 5–10 m away; 5% $\approx$ 200 m away) 12. Four replicate tanks, with 3 spawning pairs of fathead minnows separated by mesh dividers, were assigned for controls (Lake Superior water only) and each effluent treatment level, resulting in a sample size of n = 12 pairs (12 male, 12 female) per treatment.

Sample Collection

After 21 d of effluent exposure, fish in all treatments were anesthetized with buffered tricaine methanesulfonate (MS-222; 100 mg/L buffered with 200 mg NaHCO₃/L; Finquel, Argent). Fish were weighed and skin mucus was sampled by placing each fish on its side and laying a small piece (20 $\times$ 5 mm) of precut glass-fiber filter paper (GE Healthcare Life Sciences) on the side of the fish, from the caudal peduncle to the head and blotting gently. The filter paper was placed into a 2.0-mL microfuge tube (Eppendorf), flash-frozen in liquid nitrogen, and stored at –80 °C until extraction. All laboratory procedures involving animals were reviewed and approved by the USEPA's Mid-Continent Ecology Division Animal Care

and Use Committee in accordance with Animal Welfare Act regulations and Interagency Research Animal Committee guidelines.

Extraction of Mucus Samples

Metabolites were extracted from the glass-fiber filter strips by the addition of 400 μL of ice-cold methanol:water (10:1,v/v) to each well in 96-well Captiva Non-Drip 0.2- μm filter plates (Agilent Technologies) and transferring the filter strips with forceps into their respective wells. After 10 min, the samples were mixed and then centrifuged at 3000 g for 10 min at 4 $^{\circ}\text{C}$. The collected filtrate was dried using a vacuum concentrator (Thermo Scientific), and residues were reconstituted in 150 μL of water:acetonitrile (19:1,v/v). Samples were processed in a randomized fashion.

Chemical Profiling of Mucus Samples

A full description of the liquid chromatography (LC) and MS parameters is given in the Supplemental Data. Briefly, mucus extracts were profiled using an LC coupled to a Q-Exactive orbitrap mass spectrometer (Thermo Fisher Scientific). Spectral peaks were deconvoluted and aligned with the XCMS package in R, an open source framework for processing and visualizing chromatographically separated mass spectral data 13, 14. Isotopic features and ion adducts were annotated with the CAMERA package in R 15. Parameters used to extract and annotate mass spectral features are given in the Supplemental Data, Table S1. Prior to statistical analysis, the processed dataset was mined for features that were detected in at least half of the control samples (heretofore referred to as “endogenous metabolites”). Other features were denoted as exogenous and were not used in the statistical analyses. The intensities of features from the endogenous metabolites were then normalized to unit total integrated intensity for each processed spectrum, resulting in a data matrix of 310 endogenous metabolites. The full details of methods used to mine for endogenous metabolites are given in the Supplemental Data. Determination of xenobiotic transformation products was conducted manually on the raw (unprocessed) data files using the Xcalibur software (Thermo Fisher Scientific), guided by the detected chemicals in the wastewater effluent and the literature.

Statistical Analyses

Multivariate data analysis was performed using SIMCA-P+ (Ver 13.0, Umetrics). Data were mean-centered and scaled to unit variance. Principal component analysis (PCA) models were constructed with the endogenous metabolite data to assess the overall structure of the data and to identify potential outliers (no outliers were observed). The PCA score values were tested for normality (determined by the Kolmogorov–Smirnov test) and equal variance (determined by the Bartlett test) prior to conducting analysis of variance (ANOVA). Significant class separations in PCA scores plots were determined by ANOVA followed by Tukey's honest significant difference test. Nonparametric tests (Kruskal–Wallis) were used when assumptions of normality or equality of variance were not met. For all tests, class separations were considered significant at $p = 0.05$. In addition, partial least squares-regression models were constructed to identify possible covariance between the mucus metabolites (X-variables) and the various biological endpoints measured by Cavallin et al. 9, each used individually as a single Y-variable per model 16, 17. Targeted data from Cavallin

et al. 9 were log-transformed, mean-centered, and scaled to unit variance. Based on variable importance in projection (VIP) values, we determined which endogenous metabolites best explained the variability in the targeted biological endpoints 18. Values ≥ 1 for VIP scores indicate metabolites with greater than average importance to the model. Endogenous metabolites with the largest VIP scores (i.e., the top 10%) are the most relevant for explaining the Y-variable.

Student's *t* test was used to determine significant changes between control and effluent treatments, with separate *t* tests conducted for each metabolite, sex, and effluent treatment. Strimmer's method was used for false discovery rate corrections 19. Responses that passed at the level of ≤ 0.1 for the resulting *q*-values were considered significant within a treatment group and sex.

Metabolite Annotation

The open source Python program *mummichog* (Ver 1.0) was used to identify impacted metabolic pathways from mass spectral features without a priori annotation of metabolites 20. This package, which also suggests the identity of detected metabolites based on the occurrence of their exact masses with those of endogenous metabolites in shared metabolic pathways, aided in metabolite annotation. However, the endogenous metabolites proposed by *mummichog* were subjected to further scrutiny to increase confidence in their identities. Specifically, the highest confidence in metabolite annotation (i.e., metabolomics standard initiative [MSI] level I) was achieved by matching exact mass and retention times to authentic standards 21. Where no laboratory reference standards were available, metabolite annotation was determined from the exact mass composition, from the goodness of isotopic fit for the predicted molecular formula using Compound Discoverer™ (Ver 2.0, Thermo Fisher Scientific), and from MS/MS fragmentation matching with databases (Massbank, METLIN, and mzCloud), qualifying for annotation at MSI level II, or the newer designation by Schymanski et al. as a “probable structure” 21, 22. Where no library fragmentation data were available for matching, the unequivocal chemical formula was reported. For endogenous metabolites lacking a chemical formula, the accurate molecular mass was given based on the calculated isotopic features and ion adducts.

RESULTS AND DISCUSSION

Responses to effluent exposure on the skin mucus metabolome

Based on the PCA scores plot derived from skin mucus metabolomic data from all exposed and control fish, the largest source of variation was from differences between males and females (Figure 1), which is consistent with our previous findings 8. However, beyond this sex-based separation, the mucus metabolome of fish exposed to the 100% effluent treatment showed separation from controls for both sexes (Figure 1), and also some discernable impact from the diluted effluent in the females (but not the males). To focus on these impacts from the treatments, we removed the confounding effects because of sex by constructing separate PCA models built for males and females. Separate ANOVA analysis of the PC1 and PC2 scores values from these 2 sex-specific models (Supplemental Data, Figures S1 and S2) demonstrates that, indeed, the 100% treatment class separates from controls along the PC2

axis for both males and females. However, there was no statistically significant separation along either PC1 or PC2 for either of the diluted effluent treatments for either sex. Thus, from PCA analysis, significant impacts of the exposure are evident, but only in the 100% effluent treatments, regardless of sex.

From an earlier study of shorter duration conducted near the WWTP discharge, greater impacts were observed in male versus female fathead minnows 10. Specifically, the hepatic metabolome of caged male fish was significantly impacted after just 2 d of exposure to the effluent receiving water, whereas females did not show significant changes. The authors also reported significant estrogenicity in the receiving waters as measured with an in vitro cell bioassay using T47D-KBluc cells, which detects estrogen receptor activation 10. In another study, Martinovi et al. measured high, albeit variable, levels of estrogenicity in the Western Lake Superior Sanitary District effluent using a rainbow trout estrogen receptor in vitro binding assay 11. For the present exposures, Cavallin et al. reported estrogenic activity (1.5–18.9 ng/L 17 α -ethynylestradiol equivalents) in the effluent, using the T47D-KBluc cell bioassay 9. Plasma vitellogenin (VTG) levels were also measured after 21 d of exposure 9. This is an egg-yolk precursor protein that occurs naturally in the plasma of female fish, but is normally absent in male fish. However, its production can be induced in males by exposure to an estrogenic chemical. As a result, VTG induction in male fish has become a sensitive and widely used bioindicator of estrogenic exposure 23. Significant increases in plasma VTG levels were observed in the male fish for the present exposures, as reported by Cavallin et al., but no change was measured for the females 9. Interestingly, PCA of the mucus metabolomic data (Figure 1 and Supplemental Data, Figures S1 and S2) indicated that the females were impacted to the same degree as the males, suggesting that effects other than estrogenicity may be relevant for our exposure conditions (e.g., duration, effluent concentration, effluent composition).

Impacts of effluent exposures on metabolic pathways

To investigate metabolic pathway responses in the skin mucus related to the exposure, we first used *t* tests (with false discovery rate corrections) to determine which metabolites were significantly impacted by the various effluent treatment levels (relative to controls) for each sex (Supplemental Data, Table S2). Briefly, 85 endogenous metabolites varied significantly between at least one of the treatment levels and the controls within a given sex. For the 100% effluent exposures, 56 metabolite changes were measured in the males, and 43 in the females. (Note that 17 of these changes were observed in both males and females, giving a combined total of 82 unique metabolite responses at the 100% effluent treatment level for both sexes relative to controls). For females exposed to 20% effluent, 17 metabolites varied significantly relative to controls, whereas none varied significantly at the 20% level for males. No metabolites were significantly altered in the 5% treatment for either sex. Of the 17 exposure-related endogenous metabolite responses measured at intermediate levels, only 3 did not also significantly change at the 100% effluent level, and these 3 remained unidentified (Supplemental Data, Tables S2 and S3). Thus, we limited further pathway analyses to the 100% effluent treatment level for both sexes.

Next, we built biological activity models for males and females separately using the *mummichog* program to identify which metabolic pathways were impacted by the 100% effluent exposure. The *mummichog* program facilitates functional interpretation of metabolic pathways and networks directly from mass spectral features (i.e., exact masses of isotopic features and ion adducts) 20. Specifically, we entered a list of m/z features (proton adduct ions of endogenous metabolites) that significantly varied with treatment level (relative to controls; determined by prior statistical analysis with *t* tests) into *mummichog*, along with a list of all m/z features detected by the mass spectrometer for a given ionization mode. Including the full list of m/z features (both those changing in response to the exposure and those that did not) provided a reference for all potential pathways present, whereas the list of significantly changed metabolites allowed for determination of those pathways that were significantly impacted because of the exposure. Thus, we were able to glean biological context from our data while bypassing prior metabolite annotation, which is arguably the biggest challenge in mass spectrometric-based metabolomics 21, 22. The resulting positive ionization and negative ionization models were merged to give a global predictive network of biological pathway activities relevant to the exposure (Supplemental Data, Figures S3 and S4 for males and females, respectively). Following verification of the metabolite identities suggested by *mummichog* (described previously), 156 endogenous metabolites were annotated. Of these, 1 was annotated at the MSI I level, 38 were annotated at the MSI II level, and 117 were annotated with an unequivocal molecular formula.

The bioactivity networks revealed significant overlap between male and female metabolite changes in the 100% effluent exposure. We found that viewing these networks (Supplemental Data, Figures S3 and S4) while comparing the sex-specific metabolite changes (Supplemental Data, Table S3) was helpful in visualizing similarities between the male and female biological responses to the exposure. For example, 17 endogenous metabolites (which are in bold face in the Supplemental Data, Table S3) significantly varied in both sexes exposed to 100% effluent with the same direction (and comparable magnitudes) of change. Seven of these 17 metabolites appeared in the bioactivity network for both males and females (Supplemental Data, Figures S3 and S4, respectively). The percentage of change data (calculated separately from the *mummichog* models) for these 7 metabolites, which were all annotated at the MSI II level except for isoleucine (MSI level I), are presented in Figure 2. Interestingly, relative changes of the nucleotides uridine monophosphate (UMP; 324.0359 amu) and cytidine monophosphate (CMP; 323.0519 amu) were similar with respect to each other and with respect to the sexes, albeit to varying degrees at the intermediate exposure levels. Specifically, both metabolites, which are involved in pyrimidine metabolism, were significantly up-regulated at the 100% effluent level for both sexes, but only CMP was significantly up-regulated at the 20% level, and only in the female fish. These nucleotides have been shown to be important in energy metabolism, based on metabolic parameters measured in muscle, blood serum, and liver (i.e., levels of lactate, glucose, and several metabolic enzymes) during exercise in rats treated with CMP/UMP 24. Other metabolite changes included fructose-6-phosphate (F6P; 260.0296 amu), sedoheptulose-7-phosphate (S7P; 290.0402 amu), and uridine diphosphate glucose (UDP-Glc; 566.0565 amu), which were up-regulated to some degree in all exposure groups, and significantly so in the 100% effluent in both sexes. A search of the Kyoto

Encyclopedia of Genes and Genomes reference pathways reveals that these metabolites are involved in various pentose and glucuronate pathways 25–27. Additional relevant pathways were also determined by use of the *mummichog* package. According to these analyses, a total of 52 pathways were significantly altered (males: 45; females: 47; adjusted *p* value 0.1), and 42 of these were similarly impacted for both sexes (Supplemental Data, Table S4). This considerable overlap between the sexes suggests that males and females responded similarly to the 100% effluent exposure at the time of mucus collection (21 d).

The overlap between the observed biological impacts in male and female fish also suggests that the metabolomic responses were not predominantly related to estrogenic impacts after 21 d of exposure. This result can be compared with the analyses of Cavallin et al. 9, who tracked fecundity (egg production) for these same fish daily throughout the exposure. Although the authors measured a significant reduction in fecundity in fish exposed to the 100% effluent during the first 14 d of the experiment, by the third week, egg production had recovered to control levels. Given that the measured level of effluent estrogenicity did not decline during this period 9, the resumption of normal egg production in the third week suggests that compensation to the effluent contaminants may have occurred. In light of this, the metabolic pathway changes measured in the skin mucus samples (collected at day 21) from both males and females may reflect mechanisms induced to maintain homeostasis. Thus the impacted metabolic pathways identified in the present study warrant further investigation as potential candidates for long-term indicators of exposure to WWTP effluent.

Prediction hepatic gene expression with skim mucus metabolome changes

Targeted hepatic gene expression analyses conducted by Cavallin et al. 9 provide further evidence that compensatory mechanisms are likely contributing to observed metabolomic responses in the test fish. Specifically, up-regulation of gene transcripts was reported for 2 cytochrome P450 (CYP) isozymes associated with oxidative metabolism of xenobiotics (*cyp1a1* and *cyp3a*) and a uridine diphosphate (UDP)-glucuronosyltransferase (*ugt1a1*) in the male livers in response to the effluent exposure 9. Up-regulation of 2 of these genes (*cyp1a1* and *ugt1a1*) was also observed in female livers 9. Given the roles of these genes in general xenobiotic metabolism, their up-regulation may indicate adaptive or compensatory responses to the continual exposure to the complex effluent. For instance, *cyp1a1* codes for an enzyme that is utilized in phase I transformations of xenobiotics such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls in fish 7. The general lack of sex-specificity in up-regulation of these genes likely reflects the requisite needs of the fish to metabolize contaminants to maintain homeostasis, regardless of sex.

We employed a partial least squares-regression approach, as described by Davis et al. 28, to explore covariances between the metabolomic responses and the gene expression results and to investigate the likelihood that these responses reflect, at least in part, the involvement of xenobiotic metabolism pathways. This analysis revealed a strong covariance between the *cyp1a1* messenger ribonucleic acid (mRNA) expression levels and normalized abundances of endogenous metabolites in mucus from both sexes (Figure 3 and Supplemental Data, Table S5). Indeed, the regression line for a graph of predicted versus measured values (black, solid) is close to a 1 to 1 ratio (red, dotted) for both sexes, and the adjusted R^2 values

are 0.87 and 0.80 for females and males, respectively (Figure 3). Of the endpoints measured by Cavallin et al. 9, the *cyp1a1* gene expression data was the best predicted by the mucus metabolome for males and females (Figure 3 and Supplemental Data, Table S5). More than 100 endogenous metabolites covaried significantly with *cyp1a1* in both sexes (males, 127; females, 115; VIP ≥ 1); of these, 73 were important in both models. In fact, many of the endogenous metabolites showing similar patterns of differential abundance among treatments in both males and females (Figure 2) were among the 10% of most strongly covarying metabolites in these partial least squares models. For the partial least squares model built with female data, 5 of the 7 metabolites from Figure 2, including isoleucine, 4-hydroxy-glutamic acid (OH-Glu), UMP, UDP-Glc, and F6P, were among the top 10%. For the male model, those among the top 10% were OH-Glu, UMP, CMP, UDP-Glc, F6P, and S7P. Furthermore, all 7 metabolites from Figure 2 had VIP scores ≥ 1 for both male and female partial least squares models that were built with the mucus metabolome and *cyp1a1* gene expression datasets. Thus these metabolites were among the most relevant to the model prediction of *cyp1a1* gene expression levels in both male and female fish.

To further explore these gene-metabolite relationships, we also built models for *cyp3a* mRNA expression with data from both sexes. These models revealed that *cyp3a* covaried with the male metabolome (adjusted $R^2 = 0.74$; Supplemental Data, Figure S5) but not with that of the females (Supplemental Data, Table S5). The CYP3A isozyme is especially prominent in drug metabolism 29. We found that 127 metabolites were relevant (VIP scores ≥ 1) to the *cyp3a* model with the males. Interestingly, the *vtg* gene, which codes for the VTG protein, did not covary with either the male or female mucus metabolomes. Likewise, plasma VTG concentrations reported by Cavallin et al. 9 did not covary with the metabolome. It would be intriguing to measure the mucus metabolomic responses at earlier time points in the exposure (e.g., at 7 and 14 d). For instance, earlier metabolomic responses could elucidate impacts relevant to other endpoints (e.g., VTG induction). However, after 21 d of exposure to the WWTP effluent, these results suggest that the mucus metabolome may reflect the activities of xenobiotic metabolism pathways more than it reflects responses to estrogenic chemicals in the effluent.

Glucuronidation mediated by UDP-glucuronyl transferases (UDPGTs) is a well-studied mechanism for facilitating the excretion of lipophilic xenobiotic compounds 30. For example, a recent study with zebrafish reported that glucuronidation of several phenolic aglycones, such as bisphenol A (BPA), is accomplished by the UDPGT encoded by *ugt1a1* 31. Furthermore, Wang et al. reported that the *ugt1a1* isoform, expressed in both the liver and the gills, was the most active member of the *ugt1* family with respect to BPA glucuronidation, and that no other family of genes was involved in this reaction. Because up-regulation of *ugt1a1* in the fathead minnows in the present study only occurred at the highest effluent concentration, partial least squares models were not utilized to explore effluent concentration-based covariance between the metabolomics dataset and *ugt1a1* expression. Instead, a Pearson's correlation test was performed to investigate the relationship between the UDP-Glc mucus metabolite and *ugt1a1* in both males and females at the highest effluent concentration; a significantly positive correlation for both sexes was found (males: $r = 0.30$, $n = 48$, $p < 0.05$; females: $r = 0.33$, $n = 47$, $p < 0.05$). Note that the required substrate for glucuronidation in teleost fish is uridine 5'-diphosphoglucuronic acid 7, which the Kyoto

Encyclopedia of Genes and Genomes shows is directly converted to UDP-Glc within the pentose and glucuronate interconversion pathway [25–27]. This relationship, along with the involvement of the pentose and glucuronate interconversion pathway (proposed in the present study by *mummichog*; Supplemental Data, Table S4), as well as the induction of *ugt1a1* reported by Cavallin et al. 9, suggests the possibility of detecting UDPGT-mediated xenobiotic transformation products in the skin mucus.

Monitoring phase II metabolism of contaminants in skin mucus

Numerous studies have shown the utility of measuring xenobiotic chemicals in the biofluids of fish exposed to WWTP effluent as a means to determine which chemicals are bioavailable, and thus potentially harmful 1–3. One advantage of MS-based metabolomics is the ability to detect both endogenous and exogenous (i.e., xenobiotic) chemicals in the available biological matrix, including those with low abundances 32. Indeed, xenobiotic metabolites have frequently been measured with this technique in biofluids such as fish bile and plasma 3, as well as rat urine 33, but have not commonly (if ever) been reported using fish skin mucus. As discussed in the section *Predicting hepatic gene expression with skin mucus metabolome changes*, the up-regulation of *ugt1a1* in the present study is indicative of phase II transformations of several xenobiotics, including BPA, which is nearly ubiquitous in the environment and was detected in the effluent by Cavallin et al. 9. In recent years, BPA has been the subject of significant environmental and human health concern 34, 35, and thus reference standards for BPA transformation products, including the glucuronide BPA-GlcA, are readily available. Given the findings of a potential link between the skin mucus metabolome and UDPGT activity, the targeted identification of BPA-GlcA was investigated to evaluate the utility of skin mucus for environmental monitoring of transformation products of priority contaminants.

Dilution-dependent differences in the abundance of BPA-GlcA were indeed detected in the skin mucus of males exposed to the WWTP effluent (Figure 4). BPA-GlcA was detected in the 100% effluent- and 20% effluent-exposed males, but it was not detected in the 5% effluent- or Lake Superior water control-exposed fish. It was also not detected in the skin mucus of any female fish in the present study, not even the females that shared the same aquaria with males in which the highest levels of BPA-GlcA were detected. Chemical analysis of the 100% effluent from this exposure had revealed the mean presence of BPA at $0.68 \pm 0.30 \mu\text{g/L}$ (with no detection above the reporting limit of $0.04 \mu\text{g/L}$ in the Lake Superior water control water during the 21 d of exposure) 9. A recent review of environmental concentrations of BPA in several countries, including the United States, estimated an upper limit at the 95th percentile of $8.2 \mu\text{g/L}$ for surface waters 34, more than 10 times the average concentration found in the present study. Thus the exposure to BPA within a complex mixture of contaminants present in the WWTP effluent had a measureable impact at environmentally relevant concentrations, as indicated by the detection of a known biotransformation product of BPA in the skin mucus of exposed male fathead minnows. The presence of BPA-GlcA in the skin mucus is strong evidence that BPA is being metabolized and excreted by males in response to the effluent exposure.

The detection of BPA-GlcA in effluent-exposed males—but not females—is noteworthy, because it suggests there may be differences between the sexes in response to BPA exposure within a complex mixture. Interestingly, sex-dependent BPA metabolism pathway differences have been reported for humans, based on a different distribution of BPA metabolites in urine 36. Specifically, BPA-GlcA was the predominant metabolite in urine from men, whereas BPA-GlcA and BPA-sulfate were equally abundant in urine from women 36. It is possible that similar sex differences exist in fish, although we did not detect BPA-sulfate or BPA-GlcA in female fish skin mucus samples. Alternatively, our inability to detect BPA conjugates in the skin mucus of the females may be because of a lesser excretion of BPA conjugates than in males.

Finally, using the contaminant detections reported by Cavallin et al. 9, we searched the dataset for additional glucuronidated transformation products present in the skin mucus. This resulted in the tentative identification of 3 additional transformation products (Supplemental Data, Table S6). The associated parent compounds (1,7-dimethylxanthine, cotinine, and triclosan) represent a group of contaminants commonly detected in WWTP effluents and other anthropogenically influenced surface waters 37. Although it was beyond the scope of the present study to investigate their contributions to any of the metabolomics responses, their tentative identification provides further evidence of the utility of skin mucus not only for determining health impacts in fish exposed to complex contaminant mixtures (via endogenous metabolite profiling), but also for the nonlethal monitoring of the contaminants themselves, with particular regard to those that are biotransformed, thus establishing their bioavailability. Clearly, not all bioavailable contaminants are of high concern (e.g., 1,7-dimethylxanthine is a metabolite of caffeine that generally displays low toxicity). However, bioavailability facilitates toxicity, making the detection of transformed contaminants in skin mucus valuable evidence in evaluating the components of complex mixtures for potential ecosystem impacts.

Considering our findings, as well as the results reported by Cavallin et al. 9, a somewhat complex biological picture emerges with respect to the fishes' responses to the effluent exposure. The mucus metabolome reflected impacts in both males and females after 21 d of exposure to an effluent historically recognized as (and measured in the present study to be) estrogenic. However, much of the metabolomic data suggest that effects at the 100% effluent level were not sex-specific, and not obviously indicative of an estrogenic response. Furthermore, the strong covariance between the mucus metabolomic responses and hepatic *cyp1a1* gene expression levels (and a lack of covariance with VTG) suggests that the skin mucus metabolome in both sexes reflected, to some extent, shared xenobiotic biotransformation pathways. Indeed, although estrogens present in the effluent might have caused transient apical effects, after 21 d of exposure more general responses, likely reflecting compensatory mechanisms, appeared to dominate the metabolomic changes. On the other hand, the detection of BPA-GlcA in the skin mucus of exposed male (but not female) fathead minnows, as well as measured impacts at the 20% effluent level in the females (but not in the males), demonstrates that there is some degree of sex specificity in the responses to the effluent exposure.

CONCLUSIONS

The ability to use skin mucus samples to detect biological effects of complex chemical exposures, along with information on contaminants and their biotransformation products, provides a useful new approach for conducting environmental assessments with fish. Skin mucus, which can be readily collected in a low-cost and minimally invasive manner, also provides the opportunity to collect data on endangered or threatened species, which cannot be sampled in a destructive manner. Using modern high-resolution MS, we have demonstrated that metabolomic analysis of skin mucus can reveal responses from exposures to complex mixtures in an untargeted fashion, providing information on multiple impacted biochemical pathways. Furthermore, data collected from the same sample can also be mined for biotransformed anthropogenic chemicals, suggesting which contaminants are bioavailable and potentially eliciting biological responses. In the present study, we investigated glucuronide conjugates of chemicals detected in the effluent as a proof-of-concept, but this aspect of the analysis can be expanded in an untargeted fashion (similar to the endogenous metabolite profiling) to measure transformation products of unknown or unsuspected pollutants in the skin mucus. Thus the present study offers compelling evidence for the utility of this approach in expanding current efforts focused on the monitoring and surveillance of surface waters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Mean scores plot of the principal component analysis model built with the skin mucus metabolome dataset from male (left side) and female (right side) fish exposed to treatment levels of wastewater treatment plant effluent at 100% effluent (red, diamonds), 20% effluent (purple, circles), 5% effluent (green, triangles), and Lake Superior water only (controls; blue, squares). Error bars correspond to the standard error of the means.

Figure 2.

Percentage of change bar plots for a selection of 7 annotated endogenous metabolites responding to the 21-d exposure to wastewater treatment plant effluent. The standard error of the means is used to generate error bars for percentage of change relative to controls at the 100% effluent (red), 20% effluent (purple), and 5% effluent levels (green). Ile = isoleucine; OH-Glu = 4-hydroxy-glutamic acid; UMP = uridine monophosphate; CMP = cytidine monophosphate; UDP-Glc = uridine diphosphate glucose; F6P = fructose-6-phosphate; S7P = sedoheptulose-7-phosphate.

Figure 3.

Observed versus predicted graphs for the partial least squares-regression models built with the mucus metabolome dataset and the relative hepatic mRNA expression levels for *cyp1a1* in female (A) and male (B) fish. The linear regression line from the model (black, solid) and the 1:1 line (red, dotted) are shown. The key is the same as that for Figure 1.

Figure 4.

(**A**) The metabolomics standards initiative level I-confirmed structure of the glucuronidated transformation product of bisphenol A (BPA-GlcA) detected in (male) fish skin mucus. (**B**) Box-and-whisker plots of the integrated intensities for BPA-GlcA measured in male fish exposed to wastewater treatment plant effluent. LSW = Lake Superior water.