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Prioritization of Contaminants of Emerging Concern in Wastewater Treatment Plant Discharges using Chemical: Gene Interactions in Caged Fish

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Abstract

We examined whether contaminants present in surface waters could be prioritized for further assessment by linking the presence of specific chemicals to gene expression changes in exposed fish. Fathead minnows were deployed in cages for 2, 4, or 8 days at three locations near two different wastewater treatment plant discharge sites in the Saint Louis Bay, Duluth, MN and one upstream reference site. The biological impact of 51 chemicals detected in the surface water of 133 targeted chemicals was determined using biochemical endpoints, exposure activity ratios for biological and estrogenic responses, known chemical:gene interactions from biological pathways and knowledge bases, and analysis of the co-variance of ovary gene expression with surface water chemistry. Thirty-two chemicals were significantly linked by co-variance with expressed genes. No estrogenic impact on biochemical endpoints was observed in male or female minnows. However, bisphenol A (BPA) was identified by chemical:gene co-variation as the most impactful estrogenic chemical across all exposure sites. This was consistent with identification of estrogenic effects on gene expression, high BPA exposure activity ratios across all test sites, and historical analysis of the study area. Gene expression analysis also indicated the presence of nontargeted chemicals including chemotherapeutics consistent with a local hospital waste stream. Overall impacts on gene expression appeared to related to changes in treatment plant function during rain events. This approach appears useful in examining the impacts of complex mixtures on fish and offers a potential route in linking chemical exposure to adverse outcomes that may reduce population sustainability.

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Keywords

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INTRODUCTION

A growing list of xenobiotics including legacy contaminants (such as metals and persistent organic pollutants) and chemicals of emerging concern (CEC) such as pharmaceuticals, personal care products, flame retardants, industrial chemicals, and pesticides are being released into the environment.^{1,2} Because the potential environmental hazards of a large number of CECs in surface waters have not been well characterized, a number of screening and prioritization approaches have been developed to assess their potential impacts.³⁻⁶ Approaches for assessing CECs impacts on fish health have included determining chemical concentrations in water relative to the amount established as causing a biological effect or Exposure Activity Relationships (EAR)^{7,8}, in vitro assays^{5,6,9,10}, laboratory exposures of fish to field-collected water samples¹¹ and direct exposure of fish to surface water via cages or flow through systems.^{3,12-19} Gene expression changes have been employed to assess effects of complex chemical mixtures on fish, such as the potential of wastewater treatment plant (WWTP) discharge to cause estrogenic effects²⁰, identify differences in exposure sites^{12,13,15,16,19,21}, and infer the potential of specific chemicals to cause effects through the use of prior knowledge of chemical:gene or chemical:protein interactions.^{19,22,23}

A complicating factor in applying existing knowledge to infer impacts of specific chemicals in complex mixtures is the limited availability of chemical:gene interaction data for many chemicals. Existing databases of chemical:gene interactions, such as the Comparative Toxicogenomics Database (CTD)²⁴, STITCH (Search Tool for Interacting Chemicals)²⁵ or Ingenuity Knowledge Base²⁶, are biased towards well studied chemicals and relevant interactions are under-represented for less studied compounds. Since specific chemicals can

induce expression of specific genes in most organisms^{27,28}, the lack of existing chemical:gene interaction data could potentially be overcome by identifying changes in changes in gene expression in exposed caged fish that co-vary with chemical concentrations in exposure water. Using similar logic, Davis et al³ used covariation of metabolomic data from livers of caged fathead minnow and surface water analytical chemistry data to prioritize chemicals for further assessment in surface waters from the Great Lakes. Davis et al³ found that 32% of the 86 detected chemicals could not be significantly linked to the biological effect of metabolite changes and used this to place these chemicals at a low priority for further assessments. Since changes in gene expression and metabolites generally occur earlier and at chemical lower concentrations than apical effects, these approaches could facilitate more rapid analysis of the interact of CECs with the Great lakes ecosystem.

Since gene expression could be used to identify chemicals as well as possible mechanisms of toxicity, we examined whether existing knowledge-based approaches combined with analysis of gene expression covariance with surface water chemistry can be used to link specific chemicals to biological effects. We then examined if gene expression level impacts can be used to prioritize chemicals for further studies. We applied the gene regulatory network inference algorithm Context Likelihood of Relatedness (CLR)²⁹ to infer the ability of a chemical detected in surface water to influence the expression levels of specific genes. CLR has been applied to infer relationships among mixed data sets including gene or protein expression, hormones, and endogenous metabolite profiles.^{30,31} For the current analysis, fathead minnow (*Pimephales promelas*) were deployed in cages for 2, 4 or 8 d near two WWTP discharge sites in the St Louis Bay and at an upstream reference site near Duluth, MN, USA. Rain events occurred during deployment decreasing chemical input from a small WWTP and increasing output from the second. Biological impacts of WWTP discharges were assessed using a combination of approaches including Exposure Activity Ratios, plasma steroid hormone and vitellogenin (VTG, egg yolk protein precursor) assays of both male and female minnows and changes in ovary gene expression. The analysis indicated that many, but not all, of the chemicals measured in surface waters could plausibly have impacted gene expression in caged fathead minnows based on co-variance or previously established relationships. Estrogenic effects on gene expression were detected and could be linked to the presence of bisphenol A (BPA). We expect that further development of these types of approaches will lead to improved assessment of CEC exposure and effects in aquatic systems and could be applied broadly to a diversity of environmental matrices.

MATERIALS AND METHODS

Deployment and Exposure of Fathead Minnows at WWTP sites.

Fish used in the study were reproductively mature fathead minnows (5–6 months old) from the US EPA Mid-Continent Ecology Division (Duluth, MN). All procedures involving animals were reviewed and approved by the Animal Care and Use Committee in accordance with Animal Welfare Act and Interagency Research Animal Committee guidelines. Field exposures were performed in September, 2010. Fish were deployed at four sites in the St Louis River near Duluth, MN, USA (SI, Figure S1). One site upstream of the two WWTPs (Fond du Lac, FDL; N 46° 39.517, W 92° 16.951) was chosen as a ‘reference’ location as it

is located near a large forest away from urban, industrial and agricultural input. Two sites were located near the discharge of the Western Lake Superior Sanitary District (WLSSD), a tertiary treatment plant. One (WLSSD-1; N 46° 45.460, W 92° 7.227) was located ~10 m from the discharge, while the second (WLSSD-2; N 46° 45.347, W 92° 7.213) was located further in the main channel, ~100 m from the discharge. A fourth site was located near the discharge of Superior Municipal Treatment Plant, a secondary treatment plant (SMTP; N 46° 43.758, W 92° 4.125). A detailed description of the caging system and deployment can be found in Kahl et al.³² Briefly, three buoys were anchored to the bottom sediment at each site. Three cages of fish, each containing six male and six female adult fathead minnows were attached to a buoy and its anchor, suspending cages at a depth of 1–2 m. The wellmixed water and the open nature of the St Louis Bay made it difficult to obtain separate water samples to determine chemical exposure for each buoy. Therefore, a grab sample of water was obtained near the three buoys at a depth of 1–2 m on exposure days 0, 2, 4, and 8 at a consistent time at each site for chemical exposure analysis. Chemicals were assumed to be well mixed in the surface water at the time of grab sampling. Grab samples were considered to be representative of the surface water at the time of sampling and were used as measure of chemical exposure for all caged fish exposed at that site and time. Each fish exposed at a site was considered an independent exposure replicate due to the well-mixed and open nature of the bay. After 2, 4, or 8 d of exposure, a total of 6 fish from two cages, each from a separate buoy, were transferred into separate buckets containing surface water collected at the appropriate site, and transported to the laboratory (transit time <60 min) for sampling. Animals were not fed during transit nor in the field. Fish were individually anesthetized and euthanized with MS-222 (Argent, Redmond, WA, USA), weighed, and evaluated for any external lesions. Plasma and tissues (liver, gonad) were collected and stored at –80°C until extracted and analyzed.

Analytical Chemistry.

Water samples obtained via grab sampling were analyzed by the US Geological Survey National Water Quality Laboratory (Denver, CO) for 134 organic compounds indicative of industrial, domestic, or agricultural wastewaters, including a suite of 48 pharmaceuticals. The specific chemicals identified were selected as part of the National Water Survey effort including several chemicals known to be endocrineactive (e.g., steroids, alkylphenols, BPA; SI Table S1). The specific procedures used (including quality assurance/quality control measures) are detailed elsewhere.³³

Physiological Endpoints.

Ex vivo steroid production assays were conducted using methods adapted from McMaster et al.³⁴ as described by Villeneuve et al.³⁵ Ex vivo testosterone (T) production was measured for males and females, while ex vivo production of 17 β -estradiol (E2) was measured for females only. Plasma VTG concentrations were determined by enzyme linked immunosorbent assay.³⁵ Plasma T and E2 concentrations were measured by radioimmunoassay.³⁶ All data were analyzed using similar statistical methods. Normality was evaluated using a Kolmogorov-Smirnov test and data were log transformed where necessary to meet assumptions of parametric statistics. Homogeneity of variance was

evaluated using Levene's test. Differences among treatment groups were evaluated by one-way analysis of variance (ANOVA; $p < 0.05$) followed by Duncan's posthoc test ($p < 0.05$).

Transcriptomic Analysis of Caged Fathead minnows.

Ovary samples from four to six biological replicates per deployment site and time were used for RNA extraction. Total RNA was isolated using RNeasy kits following manufacturer's protocol (Qiagen, Valencia, CA, USA) and was DNase treated. RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Total RNA quantity was determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA was stored at -80°C until analyzed. Gene expression was analyzed using custom fathead minnow 15,000 probe arrays (GEO Platform Accession: GPL9248). One μg total RNA was used for all hybridizations. Synthesis of cDNA and cRNA labeling, amplification and hybridization were performed following the manufacturer's protocols (Quick Amp Labeling kit and One-color microarray hybridization protocol) and scanned with a high-resolution microarray scanner (Agilent, Santa Clara, CA, USA). Microarray data were acquired using Agilent Feature Extraction software (Agilent). WLSSD-1 8 d and WLSSD-2 8 d exposures were not used for our analysis due to technical problems with microarrays. Raw microarray data from this study were deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; GSE96678).

Bioinformatics.

Microarray data were imported into GeneSpring GX 14.8 (Agilent, Palo Alto, CA, USA), log₂ transformed and quantile normalized. Normalized data were then used to identify differentially expressed genes (DEG) using a one-way ANOVA followed by pair-wise Tukey tests ($p < 0.05$). The FDL site was the reference to which all other sites were compared. The log₂ transformed and quantile normalized data was examined using Gene Set Enrichment Analysis (GSEA) as implemented in GeneSpring by comparing matching FDL site samples to WWTP site samples (i.e. expression from FDL 2d exposures was compared to SMTP 2d samples) and all GSEA values represent FDL sample/WWTP sample. The analysis used the following gene sets: The Broad Institute curated gene set collection c2.all.v6.0.symbols.gmt which contains gene sets curated from online pathway databases, the biomedical literature, and knowledge of domain experts (<http://software.broadinstitute.org/gsea/msigdb/collections.jsp>). A fathead minnow ovary gene set collection (SI ST9) derived from Villeneuve et al {Villeneuve:2012bx}. GSEA analyses were considered significant if $p\text{-value} < 0.05$ and $q\text{-value} < 0.3$. Ingenuity Pathway Analysis (IPA, Redwood City, CA, USA) was used to do functional analysis on DEG gene lists with fold change expression identified by one-way ANOVA followed by pair-wise Tukey tests ($p < 0.05$) and a cut off of 1.5 for fold change gene expression. The DEG lists were examined for curated "Toxicity Lists" and "Canonical Pathways" using human orthologs as described in Kramer et al.²⁶ Potential chemicals affecting gene expression in exposed fish were identified using the "Upstream Regulator" analysis in IPA and default settings for human annotation.

Inference of Chemical: Gene interactions.

The influence of chemicals detected in surface water on gene expression in caged fish was inferred using the mutual informationbased model, CLR.³⁰ To do this, a data matrix was

created using arrays from all sites (FDL, SMTP, WLSSD-1, and WLSSD-2) with all microarray gene probes after the microarray data was log₂ transformed, quantile normalized, and fold-change values determined relative to the mean of all arrays. Fold-change values were not examined for significance as CLR examines the significance of gene expression relative to the presence and amount of chemical measured in water. Gene expression values from each fish replicate exposed at a specific site/time were combined with water chemistry from the last day of exposure at that site (e.g., all fish exposed for 4d at the SMTP site were paired with the same water chemistry at day 4) in order to compare gene expression with the most recent measurable exposure rather than the average chemical exposure across all exposure days. This comparison was based on our desire to focus on impacts of CECs of concern that do not bioaccumulate to any degree. As a result, we expected that water chemistry from the day of exposure completion would most appropriate for linking to gene expression impacts. Indole and camphor were detected only at FDL, and therefore were not examined in the analysis. The resulting matrix contained data from fathead minnow exposed at 2 sites (FDL and SMTP) for 48, 96 or 192h and at 2 sites (WLSSD-1 and WLSSD-2) for 48 or 96h for a total 56 arrays. A false discovery threshold cutoff of z-score=5 standard deviations over the mean mutual information value of an edge or linkage was used in the analysis. Supporting evidence for chemical:gene interactions determined using CLR was obtained by comparison against known interactions with genes from any species present in the manually curated CTD which aggregates published data on chemical:gene interactions.²⁴

RESULTS AND DISCUSSION

Chemicals Present in Surface Water Near WWTP Discharge Sites.

Among the 134 analytes investigated, 53 were detected in one or more water samples (Figure 1, SI Table S1). There was considerable day-to-day variation in the number and concentrations of chemicals detected. Effluent was diluted to an estimated 20% of original concentration at SMTP and WLSSD-1, which are 5–10 M from the discharge sites, and to 5% of the original effluent concentration at the WLSSD-2 site, 200M from the discharge site. Numbers of chemicals detected at sites ranged from 8 to 34 at SMTP, 21 to 40 at WLSSD-1, and 15–25 at WLSSD-2. Sites nearest WWTP discharges (SMTP and WLSSD-1) had similar chemical profiles, consisting of sterols present at an average mass of 40.7% of the total ug/L levels of chemical detected in water samples, followed by flame retardants (30.2%), pharmaceuticals (9.9%), alkylphenols (9.6%), personal care products (5.8%), a plasticizer (BPA, 3.4%), pesticides (0.7%), flavors and fragrances (1.3%), industrial aromatics (0.7%), solvent (0.3%), PAHs (0.5%), and hormones (0.03%).

Rain events on the day preceding water sampling for SMTP 4d and WLSSD-1 4d are likely to have impacted discharges causing a decrease in chemical input before SMTP day 4 sampling and an increase before WLSSD-1 day 4 sampling. The ratios of the total ug/L chemical detected on day 2, 4 and 8 relative to day 0 for SMTP was 1.074, 0.31 and 0.28 respectively (SI ST1). The ratios for chemicals input into WLSSD-1 on days 2 and 4 were 1.57 and 4.47. For comparison, the total amount of chemicals detected at the FTP reference site varied by only 8% over the 2–8 d sampling period. As SMTP facility is relatively small, rain fall would have diverted input to the facilities combined sewer overflow system for later

treatment thereby reducing the amount of chemicals discharged prior to water sampling. WLSSD is a large facility capable with no combined sewer overflow system. Large rain events at WLSSD likely increased the volume of effluent discharge and consequently increased the amount of chemicals detected in water.

Despite the apparent variability in chemistry caused by rain events, several chemicals, including BPA, were present continuously over the days sampled. Consistent with dilution away from the effluent discharge site, chemical concentrations decreased substantially from WLSSD-1 to the distal WLSSD-2 site where total chemical concentrations were an average of 33% (SD= 17%) of those at WLSSD-1. While not pristine, the upstream reference site (FDL), not associated with a WWTP discharge, consistently had the fewest detectable analytes, varying from three to eight chemicals. The study design was limited to analysis of grab samples near caged fish at the start of exposures and end of exposures. While grab samples enable determination of water chemistry at the time a sample is taken, it is likely that some chemicals are missed as they have dispersed prior to sampling. Other approaches such as composite water sampling strategies can compensate for this although chemical concentrations are then averaged over the time a composite sample is made.¹⁸

Potential of Surface Water to Cause Estrogenic Effects at Effluent Discharge Sites.

Effluent from WLSSD and/or the surface water near the effluent discharge site (WLSSD-1) has been consistently demonstrated to be estrogenic.^{9,14,22,37} In the present study, BPA and alkylphenols, known endocrine active chemicals, and estrone, which converts to E2 in fathead minnow,³⁸ were detected in one or more samples. As these chemicals were the most abundant endocrine-active chemicals, we examined whether, given the concentration of the chemicals detected in surface water, it was plausible that biological or estrogenic effects could in caged fish occur given a constant exposure. Exposure Activity Ratios for in vivo estrogenic effects were used to indicate if the exposure concentration in surface water exceeded the amount of chemical needed to significantly activate a biological response.⁹ Here, we used the lowest observable effect concentration (LOEC) for in vivo studies published in the literature found to cause 1. Any biological effect as an indication of a threshold for a change in gene expression, or 2. An estrogenic effect, defined as increased plasma VTG in males fathead minnows, as the activity thresholds above which effects might be expected. While these studies often exceeded this study in length of exposure, the analysis does provide support as to whether or not enough chemical is in the surface water to cause effects given an appropriate length of exposure.

BPA had in vivo Exposure Activity Ratios from 4.2 to 33 indicating that BPA would likely affect gene expression across all WWTP sites, as well as one reference site sample, FDL day 2 (see SI ST2a for more details). BPA was not at concentrations sufficient to induce plasma VTG levels in males in 4d at any site but was high enough to cause VTG induction if longer periods of 164d were considered.^{39,40} Estrone EARs were below that needed to cause a biological response (male aggression after a 21 d).⁴¹ No site had estrone EARs sufficient to cause estrogenic effects (increase plasma VTG in males after 4d and 21d exposures).^{39,41} 4-Nonylphenol exceeded the EAR for biological responses (increased plasma E2 in females after 42d exposure) only in the WLSSD-1 day 4 sample.⁴² EARs for 4-Nonylphenol were

too low for an effects based on an increase in VTG in males after 7d.⁴³ Other alkyl phenols were detected, however their summed EARs was no sufficient to predict that estrogenic of biological effects would occur (SI Table S2a).^{45,46}

Since LOECs from in vivo studies often had longer exposure periods, constant concentrations and variable exposure conditions than found in this study, Exposure Activity Ratios were also developed based on each chemicals ability to activate an in vitro human Estrogen Receptor (ER alpha) assay (ATG_ERa_TRANS_up) to 50% of its maximum activity (AC50) as found in the ToxCast Database (<http://actor.epa.gov/dashboard>; SI Table S2b). While ER alphas from fathead minnow may behave differently than the human ER alpha, the assay provides an indication of the estrogenic potential of the water.⁶ Using a 0.01 EAR as a threshold for activating the ER alpha assay, BPA was predicted to be at sufficient concentrations to cause an estrogenic effect at all WWTP sites (samples SMTP 0d and 2d; WLSSD1 0, 2 and 4d; WLSSD2 0, 2 and 4d). Using the ER alpha assay, estrone ERAs were high enough to cause estrogenic effects in WLSSD-1 2 and 4d and WLSSD-2 2d samples. 4-Nonylphenol concentrations were not high enough to cause an estrogenic effect based on the ER alpha assay EAR.

The combination of in vivo and in vitro assay EAR analysis of water chemistry indicated that 4-nonylphenol, BPA, and estrone were at concentrations high enough to biologically affect caged fathead minnows. 4-Nonylphenol had the potential to cause a biological response at WLSSD-1 on day 4 (most likely due to a rain event), but was not at levels known to cause apical estrogenic effects or known to activate the ER alpha assay in vitro. Estrone levels at WWTP exposure sites were not predicted to cause apical effects, however, WLSSD-1 4d, WLSSD-2 0d and WLSSD-2 4d were predicted to have estrogenic activity by the ER in vitro assay. BPA was present in all WWTP sites at levels known to cause gene expression responses and activate an in vitro ER assay, but not at levels where observable estrogenic effects would be expected during the exposure periods used here. Based on our prior work with BPA, gene expression effects were expected to be affected at much lower concentrations than apical biological effects examined here. We have found that BPA exposure affects ovary gene expression at 1,000-fold lower concentrations than plasma VTG in females.³⁹ In the absence of an apical effect during the deployment periods we would still expect observable changes in gene expression.

Impacts of Surface Water on Physiological Endpoints of Deployed Fish.

Few significant effects were observed at the physiological level. Neither plasma E2 nor plasma VTG (data not shown) concentrations in females differed significantly from those of fish caged at the FDL site. Significant effects were observed in female ex vivo (ovarian) E2 production, most notably at the SMTP site, but trends among sites and over time were not consistent (data not shown). Plasma T and ex vivo (testicular) T production were not significantly impacted in males (data not shown). Among the endpoints examined, only plasma E2 concentrations in males were consistently affected. They were elevated in males deployed at WLSSD-1 across all time points (SI Figure S2). Increased levels of plasma E2 were likely due to conversion of estrone in surface waters to E2 in males.³⁸ The lack of change in estrogen responsive endpoints was consistent with the concentrations of 4-

nonylphenol, BPA, and estrone being too low to cause estrogenic effects such as an increase in plasma VTG levels in males during the exposures periods used in this study. Alternatively, antagonistic effects of other chemicals present in the mixture could mask or slow down appearance of apical effects.

Effect of Surface Waters on Ovary Gene Expression in Caged Fish.

The strategy outlined in Figure 2 was used to investigate exposure effects on caged female fathead minnows using gene expression in ovaries. Ovaries were used as target organ in this study because of the ability to detect impacts on steroidogenesis and functions related to reproduction. Functional impacts on gene expression were determined using GSEA, canonical pathway analysis and toxicity list analysis and human ortholog gene symbols with the exception of a gene set collection targeting fathead minnow genes involved in ovary function (SI Tables S3-S5, S8). Overall, as was expected given the variation in chemicals detected in surface waters, the number and type of DEGs, enriched gene sets, canonical pathways and enriched toxicity lists were variable with sample sites and exposure times. Due to the poorly characterized nature of the fathead minnow genome, many of these approaches rely on relating human functional annotations to the fathead minnow where functions may not be conserved, therefore conclusions should be drawn with caution.

GSEA provides a different analysis from traditional pathway enrichment approaches and can identify phenotype changes that traditional gene enrichment approaches may not be able to detect. GSEA indicated that fathead minnows deployed to the WWTP related experienced greater stress the longer they were exposed at a site. After 2d of exposure at the SMTP site, gene sets were down regulated in comparison to FDL samples for innate immune response, genes down regulated in response to mercaptopurine and high doses of methotrexate (also predicted by upstream regulator analysis, see SI S3), and up regulated in NUP98/HOXA9 fusion cancer (SI Table S8). At 4d, SMTP samples were down regulated relative to FDL samples for a gene set important in oocyte maturation. At 8d, SMTP samples were up regulated relative to FDL samples for ERK1/2 MAPK signaling, a signaling pathway important in ovary function, growth factor signaling and cancer signaling. At the WLSSD1 effluent discharge site, ovary gene expression in fish exposed for 2d was down regulated relative to FDL samples for a gene set that is down regulated in response cancer. At 4d, WLSSD1 samples were down regulated relative to FDL samples for a set of genes important in oocyte maturation. Two-hundred meters from the effluent discharge site, after 2d of exposure, WLSSD2 samples were down regulated relative to FDL for response to progesterone gene set, the prion disease gene set which contains many stress response pathways, the Netrin 1 signaling gene set which guides axon development and is a biomarker for ovarian cancer, a complement cascade gene set with genes for innate immune response, targets of NUP98/HOXA9 fusion gene set with genes involved cancer, and resistance to BCL2 inhibitor gene set with genes up regulated in cancer cells. After 4d exposure, WLSSD-2 samples were down regulated in gene sets for NOD1/2 signaling (innate immune signaling pathway), signaling by TGF Beta receptor complex which is involved in ovarian signaling and development and cell proliferation, and the gene set containing genes down regulated in multiple myelomas. WLSSD-2 4d was also up regulated

for the EIF4 pathway gene set, a translational regulator that is induced by chemical stress and cancer.

Based on GSEA, fish deployed near WWTP discharge sites (SMTP and WLSSD-1), were initially depressed in signaling pathways and innate immune response then progressed to impaired oocyte maturation processes that could ultimately affect their reproduction. After 8d exposure at SMTP, fish appeared to have recovered from impacts on oocyte maturation possibly due to dilution of chemicals due to rain events as the total ug/L of chemicals on day 4 and 8 were 28% and 31% of the total measured on day 2. WLSSD-1 deployed fish were stressed with impaired oocyte maturation processes at after 4 d exposure. The work of Cavallin et al.¹¹ suggests that longer exposures at the concentrations experienced at day 4 at WLSSD-1 (0.328 ug/L BPA) and WLSSD-2 (0.312 ug/L BPA) could have adverse impacts on fecundity. Cavallin et al.¹¹ found that 21d exposure to 100% effluent with an average BPA concentration of 0.9 ug/L significantly reduced fathead minnow fecundity while 20% diluted WLSSD effluent increased cumulative fecundity. Based on relative concentrations of 3-beta-coprostanol, a compound used as a sewage tracer, the concentration of effluent that fish were exposed to during the 2d deployment at WLSSD-1 was approximately a 10% dilution of the 100% effluent used in Cavallin et al.¹¹ After rain events, the 4d deployment was exposed to approximately a 37% dilution of effluent compared to the 100% WLSSD effluent. As a result, fecundity could be impacted if effluent exposure remained high at WLSSD-1. However it is likely that the concentration of effluent in water would return to pre-rain event levels of below 20%, which have been found increase fecundity in fathead minnow.¹¹

The total number of DEG and enriched canonical pathways detected in exposed fish did not increase with increasing numbers of chemicals detected on the day that exposures were concluded or with the total number of different chemicals detected during an exposure period. While a number of different signaling pathways and human toxicity lists related to liver necrosis, Renal necrosis, LXR/RXR Activation, and cancer were enriched, no pathways related to estrogen signaling were enriched at any exposure site or time (SI Tables S3-S5). DEGs from females exposed at the SMTP site (2 and 8 d exposures) and WLSSD-1 site (2 d exposure) were significantly enriched ($p < 0.05$) for the human pathway “cellular effects of sildenafil (Viagra)” suggesting that the pharmaceutical sildenafil, not included in the chemical analysis, may have been present in effluent discharges (SI Tables S3, S4).

Chemical Impacts supported by Upstream Regulator Analysis of Differentially Expressed Genes.

Knowledge on what genes are expressed in response to a specific chemical exposure can be useful in identifying chemical(s) that an animal may have been exposed to. The Ingenuity Knowledge Base is a causal network linking drug and chemical interactions to gene expression and apical effects in humans that enables prediction of potential upstream regulators of gene expression.²⁷ Vertebrates have many common biological and regulatory pathways, so we examined whether DEG of fathead minnow were similar enough to genes in the Ingenuity Knowledge Base to enable upstream regulator analysis in order to provide supporting evidence of chemical impacts on exposed fathead minnows. Since the approach

is limited to chemicals and genes present in the Knowledge Base, conclusions could not be drawn for many chemicals detected in the surface water as they were not represented in the Ingenuity Knowledge Base. Despite this limitation, several chemicals were identified as potential regulators of the gene expression patterns found in exposed fish (Figure 3; SI Table S6). The total number of potential chemical regulators predicted from DEGs of fish from each site/exposure period increased with the number of days of exposure (Pearson $p=0.853$) and the total number of different chemicals measured over an exposure period ($p=0.694$). These observations are consistent with increasing biological effects, as measured by gene expression changes, with increasing exposure to more chemicals over longer periods.

Six chemicals measured in water were predicted to have affected gene expression based on upstream regulator analysis. Benzo(a)pyrene was identified as a regulator only in WLSSD-1 4d exposed fish, consistent with analytical detection at this site/sampling time. The identification of E2 as a potential regulator of genes is consistent with the analytical detection of estrone in surface water during exposures with the exception of SMTP 4d (Figure 3). Prediction of E2 as a regulator is also consistent with the widespread presence of BPA, an estrogen receptor agonist in fathead minnow.⁴⁴ BPA was identified as a regulator where EARs were greater than 10 and no estrone was present (WLSSD-2 4d). Cholesterol, detected at all sites, was a predicted upstream regulator of DEGs only in twoSMTP exposures.

4-Nonylphenol was identified as a potential regulator in fish from SMTP 2d and WLSSD-2 2d exposures. While 4-nonylphenol was not detected at these sites, the prediction was consistent with the presence of other alkylphenols (4-nonylphenol diethoxylate, 4-nonylphenol monoethoxylate, 4-tert-ctylphenol, 4-tert-octylphenol diethoxylate) found in SMTP day 2 samples and 4-tert-octylphenol at WLSSD-2 day 2 samples (Figure 1). However, the analysis did not detect impacts of alkylphenols on fish exposed at WLSSD-1 for 2d and 4d despite the presence of alkylphenols at concentrations equal to, or greater than, those found in SMTP and WLSSD-2 water samples where impacts on gene expression were found. Celecoxib was predicted as an upstream regulator in fish exposed at the SMTP site for 8d, although it was only detected in WLSSD-2 day 2 water samples. While the phytosterol beta-stigmastanol detected in surface waters was not a predicted regulator of gene expression, the structurally similar chemical stigmastanol was a predicted regulator in WLSSD-1 4d exposures where beta-stigmastanol was in a high concentration. Overall, 4 of the 6 chemicals that were detected in water and identified in upstream regulator analysis could be linked to an appropriate exposure by the same or similar class of chemicals, indicating that this approach may provide useful support that a chemical has impacted gene expression. The reason that two chemicals could not be linked to an appropriate exposure may be the fact that the fish experienced continuous exposure to chemicals which may not be well represented by the water grab samples.

Upstream regulator analysis of DEGs from exposed fish also indicated that chemicals below the detection limit of the instrumental analysis may be present and impacting gene expression, including the pesticide chlorpyrifos, androgenic hormones, and estrogenic hormones. The identification of estrogenic hormones as upstream regulators (e.g., progesterone and the synthetic estrogen diethylstilbestrol) is limited by the confounded

effects of BPA and estrone in surface waters. For example, the presence of diethylstilbestrol is suspect and likely due to estrogenic signals as it has been banned for human use in the US, although it is still used as medication for incontinence in dogs.⁴⁵

Several therapeutics were identified as potential upstream regulators in two or more exposure periods in at least one site (Figure 3). Fish near the SMTP outfall were predicted to be impacted by a large number of compounds related to medical waste streams such as the chemotherapeutic cisplatin, which has been detected in hospital wastewater and can pass through wastewater treatment systems.⁴⁶ This is consistent with the processing of an untreated local hospital waste stream by Superior Municipal Treatment Plant which is then discharged at the SMTP site. The observation that few are detected at the WLSSD-1 is consistent with WLSSD facility requiring pretreatment of hospital waste prior to treatment by WLSSD. Interestingly, many of the predicted chemotherapeutics to impact expression are used in treatment of ovarian cancer, consistent with analysis of gene expression in fathead minnow ovaries. Dexamethasone, an anti-inflammatory steroid, was predicted to be present in all exposure periods at SMTP and has been previously detected in surface waters.^{47,48} Tretinoin (all-trans retinoic acid) was identified as potentially impacting gene expression at all WWTP discharge exposure sites. The presence of tretinoin, used in the treatment of acne, keratosis, and skin aging and a known wastewater effluent contaminant,⁴⁹ is also consistent with enrichment of the Retinoic acid Mediated Apoptosis Signaling pathway in DEG from fish exposed at the SMTP site for 2d and the LXR/RXR activation pathway in DEG from fish exposed at the SMTP site for 2 and 8d (SI table S3).

Support for Specific Chemical Effects Based on Known Chemical:Gene Interactions.

A rich database of specific chemical:gene interactions has been distilled from the literature in the CTD, STITCH and other databases.^{24, 25} We used CTD to examine whether or not expression of any of the DEGs associated with the different exposure sites/durations have previously been documented to be affected by chemicals detected at the same place/time. The use of CTD was limited by the number of chemicals and species for which gene associations were available at the time of analysis. For example, CTD had curated data for 39 of 51 detected chemicals and many of the detected chemicals associated with genes in CTD had fewer than 100 gene interactions. Of the 39 chemicals for which CTD had gene data, 26 have been documented as affecting expression of the same or orthologous genes that were differentially expressed in fish exposed at different sites (Figure 4).

CTD analyses also indicated 25% to 33% of all DEGs for each exposure site/duration were matched to genes in CTD with E2 interactions, consistent with the prediction of E2 as an upstream regulator and BPA present at levels sufficient to impact gene expression. For example, 35.4% of DEGs from exposure sites associated with benzo(a)pyrene in CTD are also affected by E2. Similar overlaps were found for carbamazepine, pentachlorophenol, and caffeine where 56.6%, 36.2%, and 51.9%, respectively, of exposure site DEGs found to be associated with one of the chemicals in CTD were also found to be affected by E2. Therefore, to avoid confounding E2 activation of gene expression with that of other chemicals, genes known to be affected by E2 in CTD were subtracted from those affected by specific chemicals in CTD. For example, subtraction of DEGs associated with E2 in CTD

reduced the average number of DEGs found in CTD per site for benzo(a)pyrene from 127 to 34. After adjustment for E2-related effects, DEGs from exposure sites were associated with 19 of 39 detected chemicals for which CTD expression data existed (Figure 4).

Support for Specific Chemical Impacts by Inference of Chemical:Gene Interactions from Water Chemistry and Gene Expression Changes.

To overcome the lack of evidence linking exposure of specific chemicals to discrete changes in gene expression in fathead minnows, we inferred specific chemical interactions with genes by relating variations in surface water chemistry to variations in gene expression levels in ovaries of exposed fish. The algorithm CLR uses mutual information as a metric to detect statistical dependence between two variables. But unlike correlation, it does not assume linearity, continuity, or other specific properties of dependence. Since single large grab samples of surface water were used to measure chemicals in water surrounding exposed fish at each site/exposure time, a single water chemistry profile was used for all fish replicates collected for a specific site/exposure time. The use of the same chemistry profile for all microarray data sets from a specific site/time raises the potential of bias in the CLR analysis. However, Faith et al.²⁹ demonstrated that technical replicate microarrays from the same experimental conditions are valuable in the reconstruction of networks using CLR which supports the use of a common chemistry profile for replicate fish arrays. Another potential limitation of this study design is that only 10 different sites/exposure periods are present with a total of 56 arrays of gene expression from independent replicate fish. We determined that 56 arrays were likely to be suitable for CLR analysis since Faith et al.²⁹ found that as few as 60 microarrays representing different conditions provided sufficient data to infer gene networks with high recall and precision using CLR. Using CLR, 32 of 51 detected chemicals were associated with 88 different DEGs when exposures from all sites and times were considered and where the significance of associations for all interactions was greater than, or equal to, a z-score of 5 standard deviations over the mean influence interaction (Figure 4, SI Table S7).

Based on CLR analysis, naphthalenes influenced expression of the most genes followed by 4-nonylphenol, BPA, and p-cresol suggesting that these chemicals could have impacts on gene expression at exposure sites where the chemicals were detected. The high number of genes associated with naphthalene is consistent with in vitro EAR predictions where naphthalene is present at greater than 0.13 EAR for activation of a TP53 activation assay in ToxCast (APR_HepG2_p53Act_72h_up; SI Table S10). For comparison, 4-nonylphenol and BPA had 0.01 to 0.02 EARs, although the in vitro assay and sensitivity was different. In addition, naphthalene has a high log bioconcentration value of 2.54 suggesting that it could bioaccumulate over time and cause greater effects than other chemicals.⁵⁰ The relatively high EAR at two sites combined with a large bioconcentration factor may have contributed to the association of naphthalene with many genes. Literature support was found for five of six CLR BPA:gene associations in fathead minnow and other species; pabn1 in fathead minnow,³⁹ atf3 in rat,⁵¹ foxm1 in zebrafish,³⁹ tspo in zebrafish,^{39,52} and os9 in humans.⁵³ Literature support was also found for two of 37 CLR naphthalene:gene associations; smad7 in rat⁵⁴ and eef2l2 in fathead minnow.⁵⁵ Since literature support was not limited to fathead minnow, care should be taken when extrapolating function across species as gene function

may not be conserved. CLR based chemical:gene associations were supported by chemical:gene associations found in the CTD. Twelve of the 32 chemicals linked to DEG changes by CLR were also identified in CTD as potentially impacting DEGs from the caged fish (Figure 2). No CTD support was available for the 20 remaining chemicals as CTD contained too few gene interactions (100 or less) for each chemical.

Integration of Analyses to Prioritize Chemical Impacts and Biological effects in Caged Fish Deployed Near WWTP Outfall Sites.

Prioritization in this study was an effort to rank the chemicals detected in surface water by the biological effects, principally gene expression effects in this study, that they cause in order to reduce the number chemicals that need to be further investigated more thoroughly. The combination of multiple lines of evidence is likely to yield a better prioritization approach than one specific line of evidence. Therefore, we combined information on EARs, enriched pathways, GSEA, upstream regulator analysis, CLR chemical:gene associations, CTD chemical DEG associations, and changes in physiological endpoints with analytical measurements at each site to better understand potential chemical effects at the study sites/times (Figure 5). For example, naphthalene was detected only at SMTP in day 8 water samples of the WWTP sites, so CLR associations were restricted to fish exposed at the SMTP site during day 8 since naphthalene could only affect expression at that site and exposure.

Multiple lines of evidence indicated that many, but not all, of the chemicals detected in the surface water could be linked to impacts on the exposed fish. Thirty-nine of the 51 chemicals detected had evidence of causing biological effects based on analysis of gene expression in ovaries of caged fish using known interactions and/or inference approaches. Fourteen chemicals had two or more lines of evidence for causing biological effects in caged fathead minnows. Nineteen chemicals had evidence of biological impacts based only on CLR analysis. Six chemicals had evidence of biological impacts based on CTD analyses alone. However, CTD data alone do not strongly support chemical impacts since the gene associations may be from species other than fathead minnow and may also be affected by other chemicals. Twelve of the 51 chemicals could not be associated with gene expression changes in ovaries of fathead minnows. Prioritization of chemicals using evidence of impacting gene expression would place 12 chemicals as low priority for further examination of potential impacts on fish health in the St Louis Bay.

Davis et al.³ applied a different, but related, approach to prioritize CECs based on biological effects on caged fathead minnows across 17 different sites in five different watersheds in the Great Lakes, including samples from some of the same fish at the four sites analyzed in this study. In that study, a partial least squares analysis identified chemicals detected in surface water whose concentrations co-varied with liver metabolomics profiles of caged fathead minnows from 17 sites. Fifty-nine of 86 detected chemicals were found to significantly co-vary with the concentration of a specific endogenous metabolite in male or female livers, enabling further assessment of CECs on which to focus in terms of biological impacts. Transcriptomic and metabolomic based approaches are likely to be complementary in that they likely would reveal both common and different chemical effects. Despite differences in

numbers of sites (3 vs 17), tissue examined (ovary vs liver), biological endpoint (gene expression vs metabolomics), and analysis (a mutual information network inference algorithm vs a partial least-squares regression), the two analyses had 20 chemicals in common that were associated with a change in gene expression or metabolite levels (excluding those with only CTD support) of the 51 detected in surface water samples examined in both studies. The two approaches also identified different chemical associations, resulting in different prioritizations (e.g., 12 chemicals were linked to ovary gene expression changes but not to liver metabolomic interactions and 9 chemicals were linked to metabolite changes but not to gene expression changes). This was not surprising as biological responses are often tissue and biological pathway specific. An essential element in the usefulness of these tools in assessing impacts of CECs will be causal linkage of measured biological effects to apical outcomes that impact fish health. These approaches could also be helped through experimentation with other statistical tools such as correlation models⁵⁶

One advantage of transcriptomics compared to chemical analysis of the surface waters is the ability test hypotheses on how chemicals detected in water may functionally affect fish exposed to that water. Because of previous studies indicating that effluent discharges into the St Louis Bay were estrogenic, we tested the hypothesis that chemicals introduced by effluent discharge into the St. Louis Bay had estrogenic effects on caged fish exposed to these discharges. No significant effect was observed on physiological endpoints related to estrogenic effects. While no enrichment in the estrogen receptor signaling pathway was found, estrogenic effects were observed on gene expression in exposed fish based on identification of E2 as an upstream regulator of gene expression and identification of DEG known to be regulated by E2 in CTD at all sites. Similar effects were seen by Cavallin et al¹¹ where 100% WLSSD effluent caused VTG induction in males, but exposures to 20% dilution (similar to that found at WLSSD-1) and 5% dilution (similar to WLSSD-2) did not yield significant changes in VTG although reproduction at 21d was effected. Those data are consistent with results of this study that suggest an estrogenic signal is present, but is not at levels sufficient to cause overt VTG induction or reproductive effects at the concentrations found in surface waters and with the exposure times used.

4-Nonylphenol, BPA, and estrone had several lines of evidence to support their role(s) in causing biological impacts and estrogenic responses in caged fish. All three chemicals were present in at least one site in sufficient concentrations to cause a biological response or an estrogenic effect. BPA was present at all sites at concentrations known to activate human ER alpha in in vitro assays, cause estrogenic effects (VTG induction in male fathead minnow livers) over long periods (164 d) and was identified as changing gene expression in exposed fish by upstream regulator analysis, CLR inferred interactions, and through identifying DEGs known to be responsive to BPA in the CTD. Estrone was present at two sites in sufficient quantities to activate in vitro ER alpha assays, identified as affecting gene expression in exposed fish by upstream regulator analysis (as E2), and linked to gene expression changes by CLR and CTD analyses. 4-Nonylphenol was present at one site (WLSSD-1 day 4) at 13% of that known to cause estrogenic effects over 7 d, was identified by upstream regulator analysis as affecting gene expression (although at a site different from where it was analytically detected), and CLR inferred interactions and CTD analysis. The

alkylphenols 4-nonylphenol monoethoxylate and 4-tert-octylphenol diethoxylate were detected at the sites where 4-nonylphenol was implicated as a chemical regulator and may contribute to estrogenic effects based on linkage of changes in gene expression to chemical levels via CLR. The lack of observable apical estrogenic effects is consistent with the LOECs for estrogenic effects which were generally for exposures longer than 4 d as well as the results of Cavallin et al.¹¹ Overall, this evidence supports the hypothesis that effluent inputs at the sites are estrogenic, and also indicates that BPA is likely the most prevalent estrogenic chemical across the sites.

This work demonstrates that transcriptomics-based approaches can be useful in assessing the potential of contaminants in surface waters to cause biological effects, both in terms of overall impacts and individual chemicals in complex mixtures. Use of fathead minnow as a biosensor for biologically active chemicals and stressors provides a first tier analytical method which can inform what likely compounds might be present in an environmental sample and causing effects. This could then be followed up through targeted quantitative chemical and hazard analysis. Analysis of the DEGs in caged fish using known chemical:gene interactions in the Ingenuity Knowledge Base, and enriched biological pathways suggest that the caged fish were exposed and responded to many other chemicals that were not targeted by the accompanying analytical chemistry. This information may be useful in identifying additional analytes for future monitoring. We also envision that this approach could also be useful in understanding chemical mixture interactions on animals especially at low concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Heat map of chemicals detected at St Louis River sampling sites near wastewater treatment plant discharges.

Figure 2.
Flow chart of chemical and gene expression analysis.

Figure 3.

Identification of chemicals that are potential upstream regulators of gene expression in caged fathead minnow exposed near wastewater treatment plant discharges. Differentially expressed genes at each site were analyzed for significant enrichment of genes whose expression is known to be regulated by specific chemicals as upstream regulators using Ingenuity Pathway Analysis (Qiagen, Redwood City, CA). Asterisks denote samples where a chemical was analytically detected. P values represent the significance of the upstream regulator analysis.

Figure 4.

Chemical interactions with genes in caged fish across St Louis River Waste Water Treatment Plant discharge sites. Chemical:gene CLR = the number of genes significantly associated with a chemical across all exposure sites using the algorithm Context Likelihood of Relatedness (CLR). Chemical:gene in CTD = number of CLR inferred interactions found in published literature. DEG in CTD = number of differentially expressed genes detected in fish at each site also known to be affected by a chemical in the Toxicogenomics Database (CTD, ctdbase.org); used as an indirect measure of the potential impact of chemicals at exposures sites. CTD data? = genes known to be effected by a chemical in CTD for comparison to differentially expressed genes in caged fish. Estradiol = number of estradiol associated genes in CTD that were also present in differentially expressed genes in caged fish.

Figure 5.
Site specific impacts based on integration of biological effects with analytical chemistry at each site.