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Transcriptome signatures of wastewater effluent exposure in larval zebrafish vary with seasonal mixture composition in an effluent-dominated stream

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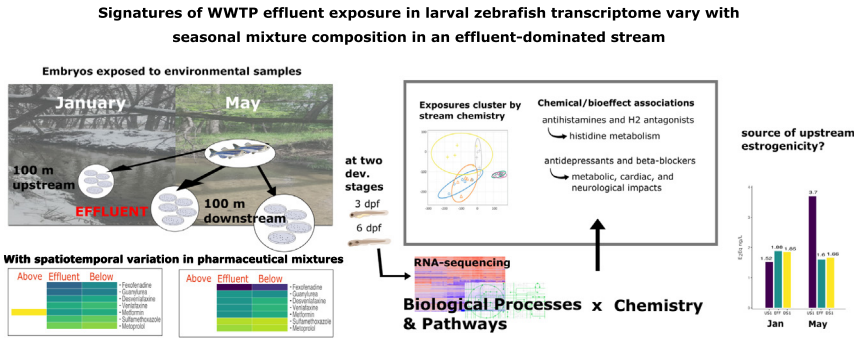
HIGHLIGHTS

- Fish embryos were exposed to seasonal water samples from a WWTP effluent-dominated stream.
- Spatiotemporal change in stream chemistry produced distinct transcriptomic impacts.
- Bioeffects extend beyond pathways related to endocrine system functioning.
- Pathway impacts linked to pharmaceuticals with diverse mechanisms of action.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater treatment plant (WWTP) effluent-dominated streams provide critical habitat for aquatic and terrestrial organisms but also continually expose them to complex mixtures of pharmaceuticals that can potentially impair growth, behavior, and reproduction. Currently, few biomarkers are available that relate to pharmaceutical-specific mechanisms of action. In the experiment reported in this paper, zebrafish (*Danio rerio*) embryos at two developmental stages were exposed to water samples from three sampling sites (0.1 km upstream of the outfall, at the effluent outfall, and 0.1 km below the outfall) during base-flow conditions from two months (January and May) of a temperate-region effluent-dominated stream containing a complex mixture of pharmaceuticals and other contaminants of emerging concern. RNA-sequencing identified potential biological impacts and biomarkers of WWTP effluent exposure that extend past traditional markers of endocrine disruption. Transcriptomics revealed changes to a wide range of biological functions and pathways including cardiac, neurological, visual, metabolic, and signaling pathways. These transcriptomic changes varied by developmental stage and displayed sensitivity to variable chemical composition and concentration

Abbreviations: AOP, Adverse Outcome Pathway; B-H, Benjamini-Hochberg; BLYES, bioluminescent yeast estrogen screen; BP, Biological Process; CEC, contaminant of emerging concern; CTD, Comparative Toxicogenomic Database; DET, differentially expressed transcript; dpf, days post fertilization; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PLS-DA, partial least squares discriminant analysis; PNOEC, predicted no effect concentration; RIN, RNA Integrity Number; USGS, United States Geological Survey; vst, variance stabilizing transformation; WWTP, wastewater treatment plant.

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of effluent, thus indicating a need for stage-specific biomarkers. Some transcripts are known to be associated with genes related to pharmaceuticals that were present in the collected samples. Although traditional biomarkers of endocrine disruption were not enriched in either month, a high estrogenicity signal was detected upstream in May and implicates the presence of unidentified chemical inputs not captured by the targeted chemical analysis. This work reveals associations between bioeffects of exposure, stage of development, and the composition of chemical mixtures in effluent-dominated surface water. The work underscores the importance of measuring effects beyond the endocrine system when assessing the impact of bioactive chemicals in WWTP effluent and identifies a need for non-targeted chemical analysis when bioeffects are not explained by the targeted analysis.

1. Introduction

Wastewater effluent containing complex mixtures of pharmaceuticals and other contaminants of emerging concern (CECs) poses potential threats to aquatic ecosystems worldwide, and small effluent-dominated streams with minimal dilution have increased in number and severity of impact due to urbanization patterns and climate change (Hamdhani et al., 2020; Rice and Westerhoff, 2015; Rice et al., 2013). Although wastewater effluent-dominated streams are often considered to be an arid or semi-arid region phenomena, temperate regions are also affected, particularly with respect to de facto wastewater reuse. Conventional wastewater treatment plants (WWTPs) were not designed to remove CECs, resulting in their frequent detection and wide distribution in surface water including drinking water sources (Bolong et al., 2009; Focazio et al., 2008; Kolpin et al., 2002). Although effluent discharged into small streams may undergo substantial dilution once released into higher volume waterways (Rice and Westerhoff, 2015; Rice et al., 2013), small receiving streams provide important habitat in aquatic and riparian ecosystems (Luthy et al., 2015; Meyer et al., 2007). The continual presence of bioactive chemical inputs can produce exposure conditions in which individual chemical concentrations exceed thresholds documented to harm aquatic organisms (Nilsen et al., 2019; Zhi et al., 2022), particularly during early life stages (Hicken et al., 2011; Wirbisky et al., 2016). However, less is known regarding the potential impacts of chemical mixtures prevalent in streams (Bradley et al., 2017).

Although the chemical contaminants typically found in WWTP effluent represent many use classes, pharmaceuticals warrant heightened scrutiny due to their designed bioactivity and the diversity of their protein targets, most of which are relevant to fish (Gunnarsson et al., 2019). In lab-based exposures with fish, deleterious impacts of exposure to pharmaceuticals have been documented at sublethal concentrations (ng/L-μg/L) (Ford and Fong, 2016) that would otherwise be considered safe by conventional toxicological endpoints (Chen, 2020; Corcoran et al., 2010; Strähle and Grabher, 2010). Impacts include reproductive impairments, altered stress responses, behavioural changes, and decreased disease resilience (Corcoran et al., 2010; Fent et al., 2006; Yan et al., 2018; Yang et al., 2018; Zindler et al., 2020). For example, the insulin-sensitizing diabetes drug, metformin, impairs growth in fish (1000–3000 ng/L) (Jacob et al., 2018; Ussery et al., 2018) and can induce intersex condition in fathead minnow (*Promelas pimephales*) testes (40,000 ng/L) (Niemuth and Klaper, 2015). Furthermore, in three fish species, environmentally relevant concentrations of antidepressants of <1000 ng/L are reported to disrupt stress responses (venlafaxine) (Best et al., 2014), reproductive and predator avoidance behaviors (fluoxetine) (Weinberger and Klaper, 2014), brain monoamine levels (venlafaxine) (Melnyk-Lamont et al., 2014), and diurnal activity patterns (fluoxetine/sertraline/venlafaxine mixtures) (Melvin et al., 2016). Wild fish in effluent-dominated streams may experience both chronic and pulse exposures, both of which have been shown in zebrafish (*Danio rerio*) embryos to cause physiological and behavioural problems later in development (Huang et al., 2017; Parolini et al., 2019; Wilson et al., 2015) and across generations (Martinez et al., 2019; Vera-Chang et al., 2019).

Nevertheless, applying these findings to biomonitoring effluent-dominated surface water in the real world presents significant challenges. Although many sublethal exposure impacts are likely shared, pollution sensitivity can vary substantially by species (Overturf et al., 2015). Furthermore, complex chemical mixtures can induce additive, synergistic, and other kinds of interactive effects that a simple aggregation of effects observed in lab-based exposures to singular chemicals would not capture (Backhaus, 2014; Schoenfuss et al., 2016). In effluent-dominated streams, chemical mixture composition evolves over a downstream gradient and varies spatiotemporally as human use patterns change (Zhi et al., 2020). Wild fish are likely subjected to a variety of chronic and pulse exposure conditions related to their travel patterns and may experience greater vulnerability during reproductive periods, early life stages, and when facing additional stressors (Martyniuk, 2018; Mehdi et al., 2019; Tan et al., 2020). Identifying sublethal impacts and associated biomarkers of exposure to wastewater effluent to monitor in wild fish populations is extremely important as many of these impacts are unlikely to be associated with conventional biomarkers of fish health.

The development of the adverse outcome pathway (AOP) framework (Ankley et al., 2010) and biomolecule "omics" technologies has enabled sublethal impacts of chemical exposures to be identified from systematic changes along biological pathways at the molecular, cellular, tissue, and organ level. In ecotoxicology, transcriptomics is often used to uncover mechanistic relationships when integrated with phenotypic and other biomolecular data (Ankley et al., 2010; Van Aggelen et al., 2010). Sublethal pathway-based impacts of exposures have been particularly well characterized for endocrine active chemicals, most notably for 17α-ethinylestradiol (EE2) (Ankley et al., 2010; Tan et al., 2020; Van Aggelen et al., 2010), the synthetic estrogen used as birth control (Alcaraz et al., 2021; Martyniuk et al., 2020; Porseryd et al., 2017). "Omics" tools have proved invaluable to the task of assessing sublethal exposure impacts and provide opportunity to expand knowledge of biomarkers associated with specific chemicals and AOPs.

Although transcriptomics has been successfully used to relate bioeffects to surface water gradients in WWTP effluent chemistry (Berninger et al., 2014; Martinović-Weigelt et al., 2014; Zhang et al., 2018), reliable biomarkers of specific pharmaceuticals and pharmaceutical classes have not been established in part because pathway-based impacts of exposure in fish are not well characterized beyond those that involve steroidal reproductive hormones (Corcoran et al., 2010; Overturf et al., 2015; Schmitz et al., 2018). Of 973 currently marketed pharmaceuticals that act on small protein targets, 88 % lack comprehensive ecotoxicity testing data (Gunnarsson et al., 2019). High-throughput in vitro assays used to screen chemicals for specific modes of bioactivity have improved ability to predict and model exposure risks. However, in vitro assays do not reflect whole organism responses and their applicability to fish is limited by their reliance on mammalian targets and cell lines. Without pathway-based genetic biomarkers derived from fish, predicting the net impacts of exposure to chemical mixtures containing pharmaceuticals becomes problematic. Although responses to chemical exposures vary by species, zebrafish embryo and early larval-stage exposures can generate specific toxicological response data that can help identify potential impacts in temperate region native species.

In this study, we evaluated the response of zebrafish embryos exposed to water samples from strategic locations in an effluent-dominated stream and its evolving chemical composition as water flows downstream using RNAseq with the goal to identify biomarkers of effluent exposure beyond traditional stress or estrogenic biomarkers and identify the range of biological impacts that could be monitored in surface water containing wastewater-derived

CECs. Zebrafish embryos at 3 and 6 days post-fertilization (dpf) were exposed to water from select points of an effluent-dominated stream reach. These two developmental stages capture distinct vulnerabilities to environmental insult before and after the onset of free-feeding, which is marked by activation of the cytochrome *p*450 enzymes responsible for metabolizing xenobiotics (Nawaji et al., 2020). Water samples used for exposures in this study were taken in two seasons (winter and spring) and selected from a suite of monthly baseflow samples over a 12-month time period due to their contrasting pharmaceutical profiles. We hypothesized that effluent exposure would reveal biological effects beyond current endocrine system related biomarkers, that gene expression would vary with chemical exposure over two contrasting seasonal conditions, and that disrupted biological pathways would differ by stage of development.

2. Materials and methods

2.1. Study sites

Muddy Creek is a small effluent-dominated stream near North Liberty, Iowa (latitude 41°42′00″, longitude 91°33′46″) that flows into the Iowa River. The stream receives approximately 5300 m^3 of effluent per day from the North Liberty WWTP (FOX Engineering Associates Inc, 2014), which serves an estimated population of 19,500. The WWTP incorporates biological nitrogen and phosphorus removal and has an aerobic membrane bioreactor system, which filters particles >4 nm and eliminates the need for secondary disinfection. Streamflow measured 5 km below the effluent outfall at a U.S. Geological Survey (USGS) gaging station (station 05454090) at the actual time sampling points in January and May 0.03 m^3/s and 0.09 m^3/s, respectively. The stream's 22.5 km^2 drainage area encompasses a mix of suburban (60 %) and agricultural land uses (24.5 %) (Zhi et al., 2020). Three previously established USGS sampling sites were used: (1) 0.1 km above the WWTP outfall (US1; 05454050), (2) the effluent outfall (effluent; 05454051), and (3) 0.1 km below the outfall (DS1; 05454052) (Figs. S.1 and S.2). Further characterization of the field sites, detailed methods for water sampling, and analysis of the monthly chemical data are available in Zhi et al. (2020).

2.2. Water sampling

Monthly 1-l grab samples using the single vertical at centroid-of-flow (VCF) method (U.S. Geological Survey, 2006) were taken in triplicate from each field site over 12 baseflow sampling events between September 2017 and August 2018. Samples were collected at approximately the same time during each collection event (starting at 8:00 AM). Streamflow during monthly sampling events was measured with a flow tracker using established USGS methods (Zhi et al., 2020), and effluent discharge was measured indirectly by subtracting the streamflow measured at US1 from the streamflow measured at DS1. Water quality parameters monitored included water temperature, dissolved oxygen, pH, and conductivity (Zhi et al., 2020). Effluent substantially contributed to Muddy Creek streamflow during the one-year period in which monthly collection events occurred, with a median effluent/DS1 flow ratio of 91 % (range: 55–97 %) (Zhi et al., 2020). Replicate water samples enabled the pairing of chemical analyses and bioeffects data and were used for (1) measurement of 113 pharmaceuticals/degradates and CECs using a previously published USGS method (Furlong et al., 2014), (2) a bioluminescent yeast estrogenicity screen (BLYES) (Sanseverino et al., 2005), and (3) zebrafish embryo exposures (Yang et al., 2007).

2.3. Chemicals and analytical methods

Water samples were analyzed for 113 chemicals (including 109 pharmaceuticals/degradates) at the USGS National Water Quality Lab (NWQL; Denver, Colorado; Table S.1). Chemicals were categorized based on use-categories established by the NWQL and modified to emphasize potential biological activity when relevant to embryonic and larval fish development. Categories included: analgesic-antinflammatory, anti-malarial, antibiotics, antidepressant, antifungal, antihistamines, antiviral, asthma relief, beta blockers, cardiovascular care, corrosion inhibitor, degradates, diabetes care, H2 antagonists, herbicides, neurochemical modulation, over-the-counter (OTC), pesticides, sedatives, steroids, and stimulants (Table S.2). A complete description of the analytical methods and quality assurance/quality control (QA/QC) used to generate the chemical data are fully described in a prior publication (Zhi et al., 2020) and the data are available publically online (Meppelink et al., 2020).

2.4. Animals

Zebrafish embryos were obtained from group spawning events of a wild type 5D zebrafish lab culture (University of Wisconsin-Milwaukee). Adult zebrafish of mixed sexes were housed in a flow-through aquatic system (Aquaneering, San Diego, CA) with recirculating dechlorinated municipal tap water and fed TetraMin flake twice daily. The system was maintained at 27 °C in a 16:8-h light/dark cycle. All procedures were conducted in accordance with animal use and care protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Milwaukee.

2.5. Embryo exposures

Fertilized embryos screened for uniformity in developmental stage progression were used for exposures at 6 h post-fertilization (hpf). Simultaneous 3- and 6-day exposures were performed separately using five replicate petri dishes containing groups of 20 embryos immersed in 30 mL of sample water from each site (pH range: 7.3–7.6). Water samples were stored at −80 °C and thawed prior to use in zebrafish embryo assays. Parafilm-sealed petri dishes were incubated at 28 °C under a 16:8-h light/dark schedule without renewal of the sample water, thus simulating acute developmental exposures with subsequent metabolic transformations.

Although periodic renewal of exposure water can help maintain initial chemical concentrations, a static exposure design was used to reduce handling of embryos and accommodate our limited supply of sample water. In addition to these considerations, the known chemistry of the water samples did not include compounds with high volatility or compounds known to experience significant abiotic attenuation (through sorption or photolysis). Microbial degradation of pharmaceuticals, while possible, was not expected to appreciably affect chemical mixture composition as biodegradation had minimal contribution to the attenuation of chemical mixtures in Muddy Creek samples in a 14-day bench study (Zhi et al., 2021).

Upon completion, surviving larval fish from each replicate dish were pooled into 1.5 mL tubes and snap frozen in liquid nitrogen for RNA extraction. Pooled samples exposed to the January and May 2018 waters from the US1, effluent, and DS1 sites were selected for RNA sequencing in this study. The selection of January and May sampling events was based on prior published work (Zhi et al., 2020) at this site in which these months captured seasonal pharmaceutical use patterns with higher concentrations of antibiotics in January and higher antihistamines in May (Table S.3). The site above the outfall (US1) was used as a point of comparison to assess the relative impact of wastewater effluent exposure at the effluent and DS1 site. Using an upstream field site as a reference rather than a municipal tap water control effectively limits variability in gene expression associated with the ambient conditions common to all sites. Significant changes at the effluent and DS1 sites compared to the US1 are thus more likely to reflect the characteristics of the effluent rather than difference between the basal water chemistry of the stream and a more “pristine” municipal source.

2.6. RNA sequencing

2.6.1. RNA extraction, library prep, and sequencing

Total RNA was isolated using standard protocol for Direct-zol RNA MiniPrep (Zymo Research, R2051). Whole embryos were homogenized in TRIzol with a pestle in a microfuge tube, and RNA was purified on Zymo-Spin IIC columns. Sample purity was assessed with a NanoDrop

spectrophotometer (Thermo Fisher Scientific, Waltham, MA) with acceptable wavelength ratios of 1.8–2.0 for 260/280 nm and 2.0–2.2 for 260/230 nm. RNA integrity (RIN) was measured on an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA), and samples with a RIN > 7 were used. RNA was quantified on a Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Two samples (January US1 3 dpf and May US1 6 dpf) had RIN scores below 7 and were not used for RNA sequencing. Libraries were prepared using Illumina TruSeq Stranded mRNA sample preparation kit (Illumina, RS-122-2102) and IDT for Illumina – TruSeq RNA UD Indexes (Illumina, 20022371) following standard protocol, using 200 ng of total RNA. Libraries were sequenced on an Illumina NovaSeq6000 (paired-end 150 bp reads).

2.7. Processing of RNAseq data

The total genomic yield surpassed 2.104 billion paired-end reads with a median per-sample yield of 51 million fragments and a population standard deviation of 14 million fragments. Sequence data were quality-assessed using FastQC v0.11.5 (Andrews, 2010), and sequencing adapters were clipped using Cutadapt v1.18. The resulting quality-controlled data were pseudoaligned and sample-quantified against the GRCz11 Ensembl release of the zebrafish reference transcriptome using Kallisto v0.45.0 (Bray et al., 2016; Martin, 2011).

DaMiRseq was used to filter and normalize raw count data (Chiesa et al., 2018). Transcripts were removed if they had fewer than 10 counts across 70 % of samples or were hypervariant (coefficient of variance threshold of 3). Raw counts were normalized to library size using variance stabilizing transformation (vst), which reduces the dependence of the mean on variance. DESeq2 was used to perform analysis of differential expression between the upstream baseline (US1) and the effluent and DS1 samples (Love et al., 2014). Resulting tables of differentially expressed transcripts (DETs) were re-annotated with Ensembl reference information and relationally joined with Kallisto sample quantification counts using custom tooling. Transcripts differentially expressed at the effluent and DS1 sites (versus US1) were considered significant at a Benjamini-Hochberg (B-H) adjusted p-value < 0.01 and $|\log_2$ fold change$| > 1$. RNA-seq data are available in the National Center for Biotechnology Information's Gene Expression Omnibus under accession number GSE179335.

2.8. PLS-DA and functional enrichment

MixOmics (Rohart et al., 2017) was used for partial least squares-discriminant analysis (PLS-DA) to determine the relative influence of month, developmental stage, and site on the overall transcriptome. PLS-DA was performed over all exposures to compare the influence of month and developmental stage and performed separately over the 3 and 6 dpf exposures to compare the influence of month and site.

Overrepresentation analysis was conducted on DETs from each comparison using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa and Goto, 2000) and Gene Ontology (GO) annotations from the Gene Ontology Consortium (Ashburner et al., 2000; Carbon et al., 2021). GO terms and KEGG pathways overrepresented among significant DETs (versus US1) were identified using clusterProfiler (Yu et al., 2012), which employed hypergeometric enrichment tests with a B-H adjusted p-value < 0.05 to control for multiple testing and an FDR < 0.1.

2.9. Comparative toxicogenomic database enrichments

Enrichments of genes associated with specific chemicals detected in the Muddy Creek field sites were conducted using the manually curated Comparative Toxicogenomic Database (CTD), which provides integration of chemical-gene-disease interactions across 16,300 environmental chemicals and 51,300 genes from 600 species (Davis et al., 2020). Of the 113 chemicals measured in the USGS method, 65 were detected in the January and May Muddy Creek water samples. Chemical-gene interactions were available in CTD for 28 of these chemicals and were pulled for all vertebrate organisms and filtered for interaction effects on gene expression (Table S.4) (Davis et al., 2009). Enrichment of CTD gene targets associated with each chemical within a set of DETs was determined using Fisher's exact tests with Benjamini-Hochberg adjusted p-values.

Although chemical-gene interactions specific to fish were available for 18 of chemicals detected in the January and May Muddy Creek sampling, a general vertebrate query was used to capture a broader set of chemical-gene interactions with potential relevance to zebrafish based on presence of an orthologous gene target. Pharmaceutical targets tend to be highly conserved across vertebrates, and drug target orthologs are particularly common in fish. Zebrafish have drug target orthologs for approximately 95 % of pharmaceuticals registered before 2006 (Gunnarsson et al., 2019).

2.10. Bioluminescent yeast estrogenicity

Water samples were shipped on ice to USGS Organic Geochemistry Research Lab (OGRL; Lawrence, KS) for solid phase extraction prior to estrogenicity analysis at the Eastern Ecological Science Center (Kearneysville, WV). Total estrogenicity of sample extracts (Romanok et al., 2018) was determined using the bioluminescent yeast estrogen screen (BLYES) as previously described (Ciparis et al., 2012; Sanseverino et al., 2005), but with minor modifications detailed in the Supporting Information. The detection limit for this assay was 0.18 ng/L $E_2Eq_{(BLYES)}$ and the data are available online (Meppelink et al., 2020).

3. Results and discussion

3.1. Transcriptome profiles differ with shifting effluent chemistry across exposure months and relate to known pharmaceutical targets

The influence of WWTP effluent on gene expression was evident in both the January and May monthly sampling at each developmental stage. Minimal dilution of the effluent at the DS1 site (>80 % effluent in both months) (Zhi et al., 2020) produced effluent and DS1 sites with similar chemical profiles and similar patterns in gene expression (Zhi et al., 2020). Developmental stage was a strong determinant of the transcriptome profile among all samples (Fig. 1a). Similarity between the effluent and DS1 sites was evident in PLS-DA performed separately on the 3 and 6 dpf exposures where the effluent and DS1 sites of each month were more similar to each other than to the US1 site (Fig. 1b and c).

The proportion of DETs shared between the effluent and DS1 sites within a month and developmental stage also signals the influence of effluent exposure and likely reflects the high proportion of effluent to DS1 streamflow in both months: 89 % in January and 80 % in May (Table S.5) (Meppelink et al., 2020). In the January 3 and 6 dpf exposures, 40–42 % of DETs were shared between the effluent and DS1 sites. In the May exposures, 76–82 % of DETs were shared between these sites (Fig. 1e). All DETs shared between sites within the same month and developmental stage had consistent fold change directions (positive or negative). Although 37–46 % of DETs from January exposures were shared with a corresponding May exposure, few had the same fold change direction: approximately 100 in the 3 dpf exposures and fewer than 10 in the 6 dpf exposures (Fig. 1f). The comparatively few DETs shared between months at the same site and developmental stage highlights the likely influence of seasonal differences in stream chemistry.

Prior studies characterizing the spatial and temporal dynamics of complex pharmaceutical/degradate mixtures in Muddy Creek have identified seasonal patterns in mixture composition related to attenuation processes and human use patterns (Zhi et al., 2020; Zhi et al., 2021). For example, measured representations of the antihistamine fexofenadine were significantly higher in the effluent and at the sites below during warm months (May–October; water temperature at US1 > 10 °C), although the opposite was true for metformin (Zhi et al., 2021). The chemical profiles of the January and May Muddy Creek water samples used in this study reflect some of these seasonal patterns. Compared to January, total concentrations of H2 antagonists (suppress gastric acid production by blocking H2 receptors; 917 ng/L) and antivirals (841 ng/L)

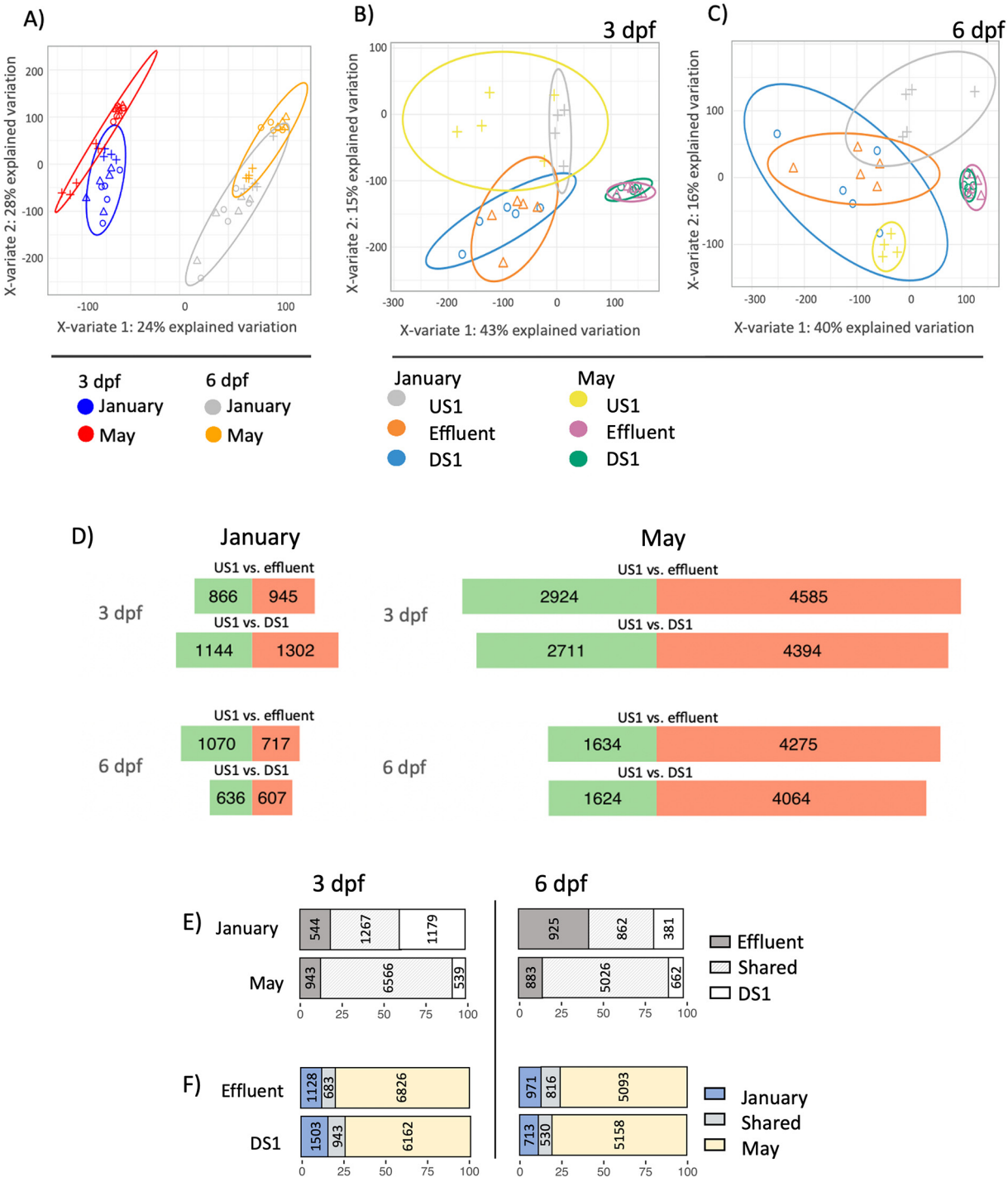


Fig. 1. Partial least squares discriminant analysis (PLS-DA) of January and May samples exposed to water from the effluent, DS1, and US1 sites at 3 dpf and 6 dpf. Ovals represent 95 % confidence intervals. Sites: + = US1, △ = effluent, and ○ = DS1.
(A) PLS-DA using samples grouped by month of exposure and developmental stage: Jan 3 dpf (n = 14), Jan 6 dpf (n = 15), May 3 dpf, (n = 15), May 6 dpf (n = 14).
(B) PLS-DA of 3 dpf samples classified by month and site: Jan 3 dpf US1 (n = 4), Jan 3 dpf effluent (n = 5), Jan 3 dpf DS1 (n = 5), May 3 dpf US1 (n = 5), May 3 dpf effluent (n = 5), May 3 dpf DS1 (n = 5).
(C) PLS-DA of 6 dpf samples classified by month and site: Jan 6 dpf US1 (n = 5), Jan 6 dpf effluent (n = 5), Jan 6 dpf DS1 (n = 5), May 6 dpf US1 (n = 4), May 6 dpf effluent (n = 5), May 6 dpf DS1 (n = 5).
(D) The total number of upregulated (red) and downregulated (green) transcripts with significant differential expression (DETs) from the US1 baseline are represented for each month, developmental stage, and site. DETs were defined as protein-coding transcripts with $|\log_2$ fold change$| > 1$ and adjusted p-value < 0.01.
(E) Number and percent of DETs shared between and unique to the effluent and DS1 sites in each month and developmental stage.
(F) Number and percent of DETs shared between and unique to January and May at both developmental stages and sites.

were three times higher in the May effluent, and antidepressants (4152 ng/L) and antihistamines (3838 ng/L) were 30 and 36 % higher, respectively. In January, effluent concentrations of antibiotics (992 ng/L) were three times higher than in May and the total concentration of beta-blockers was 28 % higher (980 ng/L). Methyl-1H-benzotriazole, a corrosion inhibitor, was also three times higher in January (713 ng/L) (Fig. 2; Tables S.2, S.3).

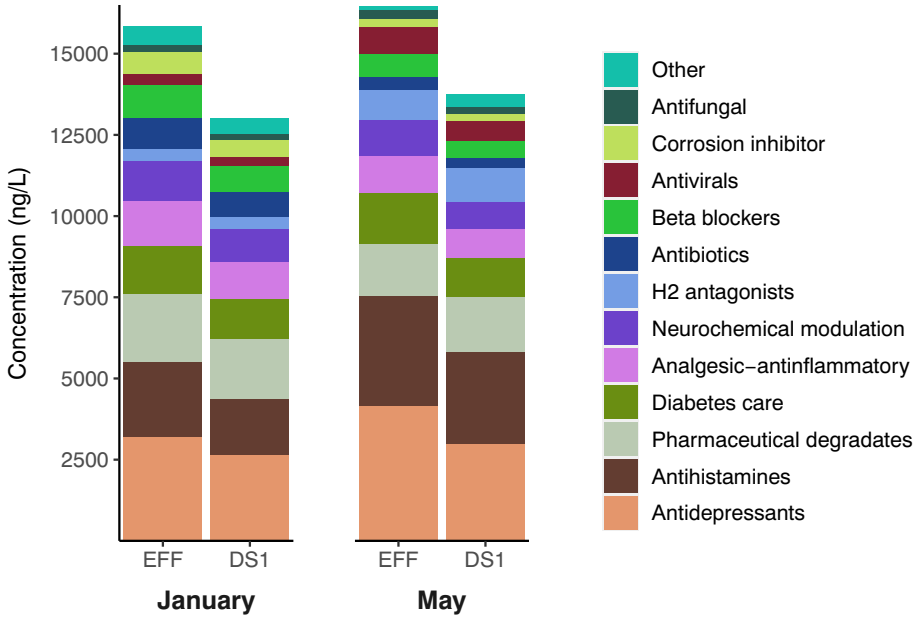


Fig. 2. Concentrations of chemicals detected in January and May in the effluent (EFF; 05454051) and at the site 100 m below the effluent outfall (DS1; 05454052) are aggregated by category. See Supplemental Information for complete chemical composition data (Table S.2, Table S.3).

The enrichment of CTD gene targets within DET sets from each exposure indicates a greater influence in May of most chemicals with significant enrichments. Gene targets of antidepressants and other neuro-pharmaceuticals were overrepresented in May, and not January (except for carbamazepine), suggesting potential for greater impacts across neuro-pharmaceutical pathways in May (Table 1). As CTD chemical gene targets cover a variety of biological responses and are not specifically limited to pathways related to pharmaceutical mechanisms of action, the significance of their enrichments is suggestive of influence but not descriptive of specific pathways. However, significant enrichments in multiple pharmaceuticals within the same class suggest there may be impacts related to pathways and targets shared between those chemicals.

3.2. Significant differences in expression signatures across months reflect differences in chemical mixtures

Impacts related to glycolysis, the musculoskeletal system, and cardiac functioning were indicated in both months with 19 Gene Ontology Biological Process (GO:BP) enrichments and 4 KEGG pathways shared between exposure months of the same developmental stage (Tables S.6-S.7). However, many other biological processes and pathways were uniquely or primarily enriched in exposures to May water samples and likely reflect influence of specific chemical mixtures at higher concentrations relative to January. Unique KEGG pathways and biological process Gene Ontology (GO:BP) terms included adrenergic signaling in cardiomyocytes, visual system functions, neurological development, the MAPK, FoxO and PPAR signaling pathways, and histidine metabolism (Fig. 3).

Overlapping enrichments accounted for 36 % of unique GO:BP terms enriched in the January exposures but only 13 % in the May exposures, which yielded a greater quantity and functional range of enrichments (195 versus 53 GO:BP terms; Fig. 3; Table S.6). KEGG pathways and GO:BP terms involving the musculoskeletal system, heart, cell adhesion, metabolism, and other non-specific impacts to embryo and larval development were overrepresented in both months and developmental stages. However, these shared enrichments likely represent a subset of the shared response to wastewater effluent exposure, and the lack of enrichments in January does not preclude impacts in many of the other pathways enriched in May. Because the baseline chemical conditions at US1 likely differed in the two months beyond what were measured in the USGS method, it is possible that the relatively fewer number of enrichments in January are a consequence of global differences between US1 and effluent chemistry that resulted a stronger bioeffects gradient in May.

Table 1
Chemicals and associated genes from the Comparative Toxicogenomic Database (CTD) with significant overrepresentation in sets of differentially expressed genes from larval zebrafish (*Danio rerio*) exposed to water samples from Muddy Creek in Coralville, Iowa (latitude 41°42′00″, longitude 91°33′46″). Differential expression of transcripts at the effluent (EFF) and downstream site (100 m below the effluent outfall; DS1) was determined relative to an upstream reference (100 m above the effluent outfall; US1) using a log2 fold change threshold of 1 and adjusted *p*-value < 0.01. The number of differentially expressed genes (as human orthologs) is shown in parentheses for each exposure.

	Chemical	CTD gene set	Jan				May			
			3 dpf		6 dpf		3 dpf		6 dpf	
			EFF (1071)	DS1 (1519)	EFF (1122)	DS1 (863)	EFF (4179)	DS1 (4032)	EFF (3083)	DS1 (2981)
Antidepressant	Fluoxetine	314	31	46	30	24	116	112	98*	95*
	Venlafaxine	159	15	28	19	13	76*	77*	59*	59*
Neurochemical Modulation	Carbamazepine	1781	173*	212	153	129	574	545	419	421
	Diazepam	68	9	13	15*	10	37*	36*	32*	32*
	Gabapentin	53	4	6	5	3	28*	26*	22*	19
Analgesic-Antinflammatory	Lidocaine	66	9	8	9	3	27	26	25*	24
Beta blocker	Propranolol	89	3	8	13	5	35	36	30	32*
OTCOTC	Dextromethorphan	32	1	3	4	3	16	16	16*	14
	Omeprazole	324	23	33	20	14	119	121*	92	86

* Significant enrichment of CTD gene sets within an exposure is denoted with an asterisk (B-H adjusted *p*-value < 0.05).

Contrast between the US1 reference and the effluent and DS1 sites was most pronounced in May with at least three times as many DETs (versus US1) at each site and developmental stage compared to January (Fig. 1d). It is noteworthy that >95 % of enriched GO:BP terms in the 3 and 6 dpf May exposures resulted from upregulated transcripts and 100 % of GO:BP enrichments in January resulted from downregulated transcripts (Table S.6). Imbalance between up and downregulated enrichments may relate to the distribution of fold change values among statistically significant DETs below the fold change cutoff (1) used in this study (adjusted p-value < 0.01, |log2 FC| > 1). The bulk of January DETs excluded from analysis due to low fold change values were upregulated, whereas most DETs with low fold changes below the cutoff in May were downregulated (Fig. S.5).

The dysregulation of cardiac processes across month and developmental stage is consistent with the presence of beta-blockers. In the May 3 dpf exposure, 54 transcripts were impacted in the adrenergic signaling in cardiomyocytes pathway, including ion membrane transporters, the actin-myosin crossbridge, and a β2-adrenergic receptor (*adrb2b*) involved in regulation of heart rate (Fig. S.3b). Adrenergic signaling receptors are involved in a wide variety of biological processes including heart contractions, lipolysis (breakdown of triglycerides), blood flow, and regulation of metabolism (Massarsky et al., 2011), which involve interaction with the FoxO and MAPK pathways, both disrupted in the May 6 dpf exposures. Although few studies have examined sublethal impacts of exposure to beta-blockers on fish, propranolol (80 ng/L) is reported to reduce larval zebrafish heart rate (Finn et al., 2012) and atenolol (2 ng/L) can block epinephrine-stimulated glucose production in trout hepatocytes (Ings et al., 2012). Total concentrations of the three beta blockers measured at the Muddy Creek effluent site (metoprolol, atenolol, and propanolol) exceed these levels by orders of magnitude (i.e., 980 ng/L in January and 701 ng/L detected in May) (Table S.3). Although enrichment of the adrenergic signaling pathway was unique to the May exposures, the enrichment of the cardiac muscle contraction pathway in the January exposures involved many of the same transcripts (Fig. S.4).

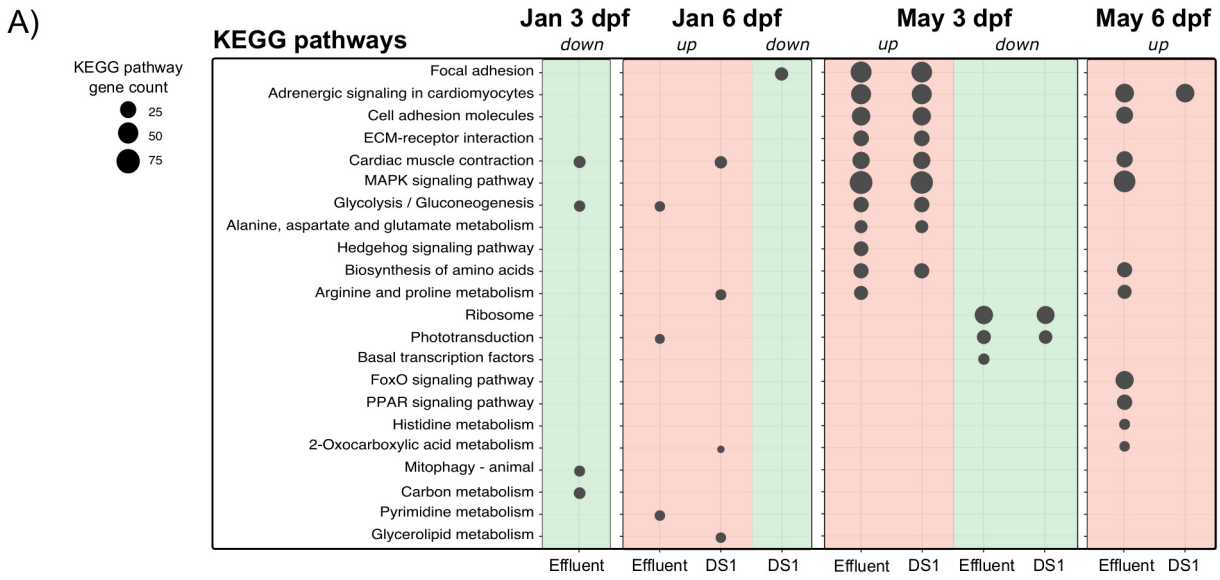
The antidepressant venlafaxine has been shown to impact many interconnected pathways and biological processes and was one of the top pharmaceuticals measured in Muddy Creek with effluent concentrations at 1240 ng/L (January) and 1550 ng/L (May) (Table S.2). Venlafaxine is known to disrupt neurological development (Thompson et al., 2017), MAPK signaling (Shen et al., 2017), metabolism (Best et al., 2014), stress response, locomotor activity (Melvin, 2017), and adrenergic signaling (Ings et al., 2012; Thompson et al., 2017). Although the exact mechanism of action on the MAPK pathway is unclear, the direct impact of venlafaxine may result from upregulation of brain derived neurotrophic factor (BDNF), which initiates a phosphorylation cascade that reaches the MAPK pathway (Shen et al., 2017). Impacts to larval fish have been documented at low concentrations; an 80-h exposure of larval zebrafish to just 80 ng/L of venlafaxine was sufficient to increase embryonic malformations, including loss of pigmentation (Rodrigues et al., 2020), which may involve changes in beta-adrenergic signaling (Xu and Xie, 2011).

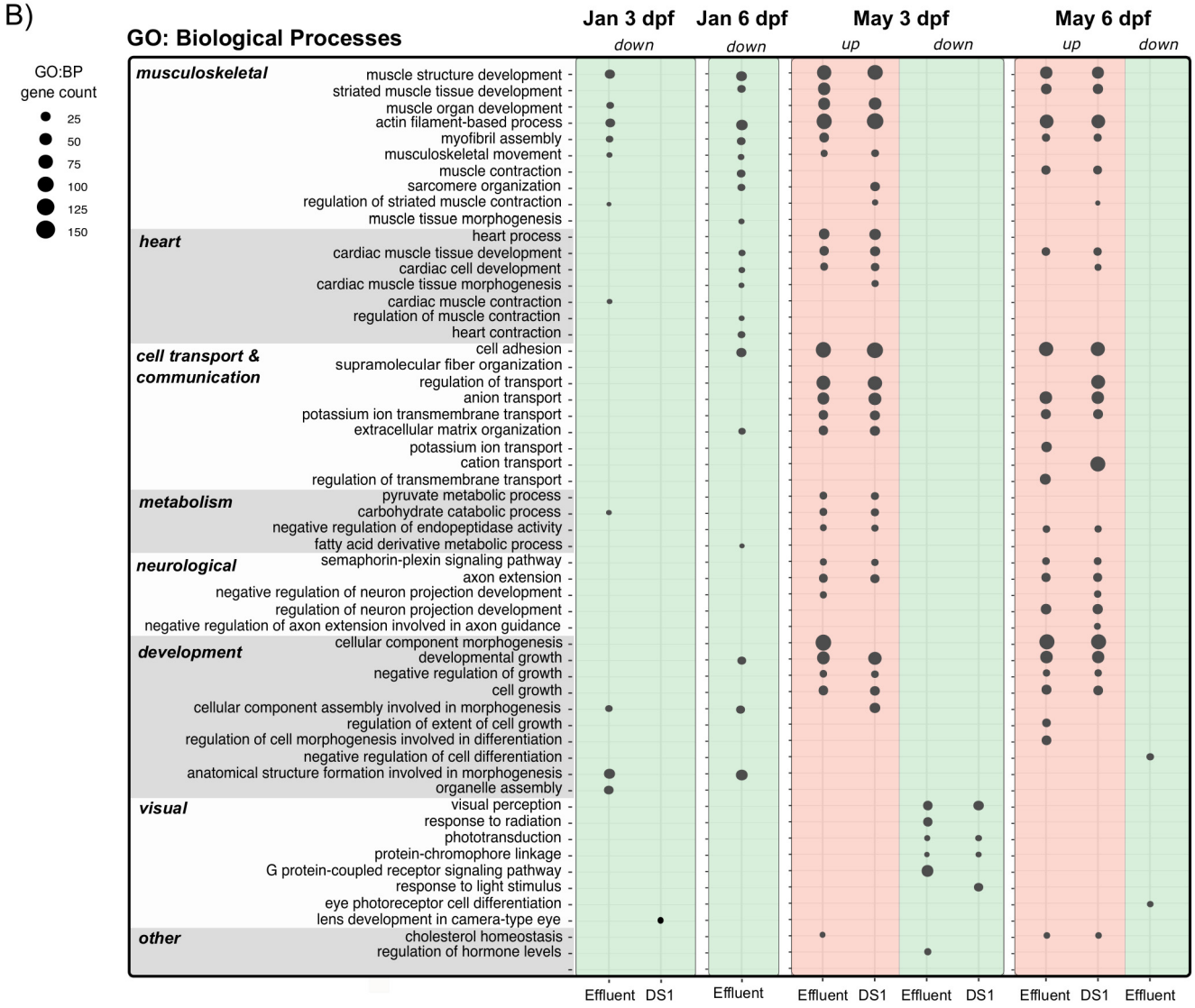
Despite the relatively high concentrations of venlafaxine and other antidepressants in the water samples from both months, disruptions to neurological processes, the MAPK signaling pathway and the adrenergic signaling pathways occurred in May, but not January. Complex mixtures of chemicals in wastewater effluent can induce interactive, compensatory, and other difficult-to-predict biological responses in exposed organisms that span many intersecting biological pathways. The lack of an antidepressant signature in January highlights the complexity of predicting bioeffects from exposures to complex mixtures of bioactive chemicals. Although exposure to field samples might not reveal pathway-based biomarkers associated with specific pharmaceuticals, they do generate signals of the in-situ conditions.

Interestingly, the visual system was also the target of transcriptomic changes. Impacts occurred primarily in the May 3 dpf exposures through enrichment of the phototransduction KEGG pathway and several other visual GO:BP terms among downregulated DETs (Fig. 3). Impaired eye development in fish necessarily impacts neurological functioning, behavior and metabolic processes, and thus may later impair growth, reproduction, and survival (Chen, 2020). Many chemicals found in wastewater effluent, including progestins (Bridges et al., 2019; Shi et al., 2019a), antidepressants (Huang et al., 2019; Huang et al., 2020), flame-retardants (Shi et al., 2019b), and pesticides (Dawar et al., 2016; Ranjani et al., 2020), have been associated with visual system disruptions including altered gene expression in the phototransduction cascade. In the 3 dpf May exposure, transcripts were downregulated across the phototransduction cascade and included light-sensitive pigments, transducins involved in G-protein signaling, Ca^{2+} and Na^{+} voltage-gated channel proteins, and rhodopsin kinases that deactivate phototransduction in dark conditions (Fig. S.3a). In total, 71 unique genes were enriched among vision-related biological processes at the effluent site and 61 at DS1.

In the 6 dpf May exposure, the overrepresentation of histidine metabolism may relate to the influence of antihistamines, which are inverse agonists of the H1 histamine receptor, and to H2 antagonists, both of which were detected at higher concentrations in May compared to January. In fish, the synthesis of histamine through histidine metabolism primarily occurs within histaminergic neurons in the hypothalamus. The histaminergic system in zebrafish has been shown to play a role in modulating swimming behavior (Peitsaro et al., 2003), arousal (Sundvik et al., 2011), and behaviors important in establishing social hierarchies (e.g., aggression and boldness) (Reichmann et al., 2020; Sundvik et al., 2021). Both H1 and H2 histamine receptors (*hrh1* and *hrh2*) are expressed early in zebrafish development, with *hrh1* peaking at 5–7 dpf. Unlike most antihistamines that can only indirectly block histamine production through inverse agonism of the H1 histamine receptor (*hrh1*) (Church and Church, 2011), diphenhydramine also has been shown to reduce allergic response by inhibiting production of histamine in the first place by downregulating the rate limiting enzyme that synthesizes histamine, histidine decarboxylase (Hdc) (Mizuguchi et al., 2016). Notably, there were significant negative fold changes in *hdc* expression ranging from −2.5 to −1.9 in the May effluent and DS1 exposures at developmental stages but no significant differential expression of the gene in January. Diphenhydramine was the only antihistamine detected with the ability to downregulate *hdc* expression (Mizuguchi et al., 2016). Diphenhydramine concentrations at the effluent and DS1 sites were 113 and 81 ng/L in January and 150 and 109 ng/L in May (Table S.2).

The LC50 of diphenhydramine is high in fish (692 mg/L for 3 dpf zebrafish and 262 mg/L for 6 dpf) (Kristofco et al., 2015), and as a result antihistamines are often considered to pose low risk to aquatic vertebrates. However, sublethal impacts on behavior have been documented at much lower concentrations of diphenhydramine, possibly owing to its inhibition of serotonin reuptake (Wong et al., 2005). For example, an LOEC of 5.6 μg/L was established for reduced feeding rate in fathead minnows (Berninger et al., 2011). More recently, the histaminergic system has drawn attention for the role it plays in modulating aggression and other behaviors important in establishing social hierarchies in zebrafish (Reichmann et al., 2020; Sundvik et al., 2021). *Hdc* was shown to be upregulated in dominant zebrafish, along with histamine receptors *hrh1* and *hrh2* (Filby et al., 2010). Elevated *hdc* expression was maintained in adult offspring of dominant (male and female) zebrafish pairings in a transgenerational study that identified inherited dominant and subordinate behaviors (Sundvik et al., 2011). In addition to the production of histamine for allergic responses, *hdc* also plays a role during embryonic brain development where histamine regulates the number of hypocretin/orexin neurons that are hypothesized to eventually regulate the number of mast cells producing histamine in adults (Panula et al., 2014). The expression of *hdc* in larval zebrafish thus may contribute to the plasticity of the histaminergic system in the brain later in adulthood. Finally, the histamine/H1 receptor axis is now also known to play an essential role during cardiac development in larval zebrafish in promoting cardiomyocyte differentiation through activation of the ERK 1/2-STAT3 pathway (Zhu et al., 2020).


A)
KEGG pathways
Jan 3 dpf
Jan 6 dpf
May 3 dpf
May 6 dpf
down
up
down
up
down
up
KEGG pathway gene count
25
50
75
Focal adhesion
Adrenergic signaling in cardiomyocytes
Cell adhesion molecules
ECM-receptor interaction
Cardiac muscle contraction
MAPK signaling pathway
Glycolysis / Gluconeogenesis
Alanine, aspartate and glutamate metabolism
Hedgehog signaling pathway
Biosynthesis of amino acids
Arginine and proline metabolism
Ribosome
Phototransduction
Basal transcription factors
FoxO signaling pathway
PPAR signaling pathway
Histidine metabolism
2-Oxocarboxylic acid metabolism
Mitophagy - animal
Carbon metabolism
Pyrimidine metabolism
Glycerolipid metabolism
Effluent
Effluent
DS1
DS1
Effluent
DS1
Effluent
DS1
Effluent
DS1


B)
GO: Biological Processes
Jan 3 dpf
Jan 6 dpf
May 3 dpf
May 6 dpf
down
down
up
down
up
down
GO:BP gene count
25
50
75
100
125
150
musculoskeletal
muscle structure development
striated muscle tissue development
muscle organ development
actin filament-based process
myofibril assembly
musculoskeletal movement
muscle contraction
sarcomere organization
regulation of striated muscle contraction
muscle tissue morphogenesis
heart
heart process
cardiac muscle tissue development
cardiac cell development
cardiac muscle tissue morphogenesis
cardiac muscle contraction
regulation of muscle contraction
heart contraction
cell transport & communication
cell adhesion
supramolecular fiber organization
regulation of transport
anion transport
potassium ion transmembrane transport
extracellular matrix organization
potassium ion transport
cation transport
regulation of transmembrane transport
metabolism
pyruvate metabolic process
carbohydrate catabolic process
negative regulation of endopeptidase activity
fatty acid derivative metabolic process
neurological
semaphorin-plexin signaling pathway
axon extension
negative regulation of neuron projection development
regulation of neuron projection development
negative regulation of axon extension involved in axon guidance
development
cellular component morphogenesis
developmental growth
negative regulation of growth
cell growth
cellular component assembly involved in morphogenesis
regulation of extent of cell growth
regulation of cell morphogenesis involved in differentiation
negative regulation of cell differentiation
anatomical structure formation involved in morphogenesis
organelle assembly
visual
visual perception
response to radiation
phototransduction
protein-chromophore linkage
G protein-coupled receptor signaling pathway
response to light stimulus
eye photoreceptor cell differentiation
lens development in camera-type eye
other
cholesterol homeostasis
regulation of hormone levels
Effluent
DS1
Effluent
Effluent
DS1
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DS1
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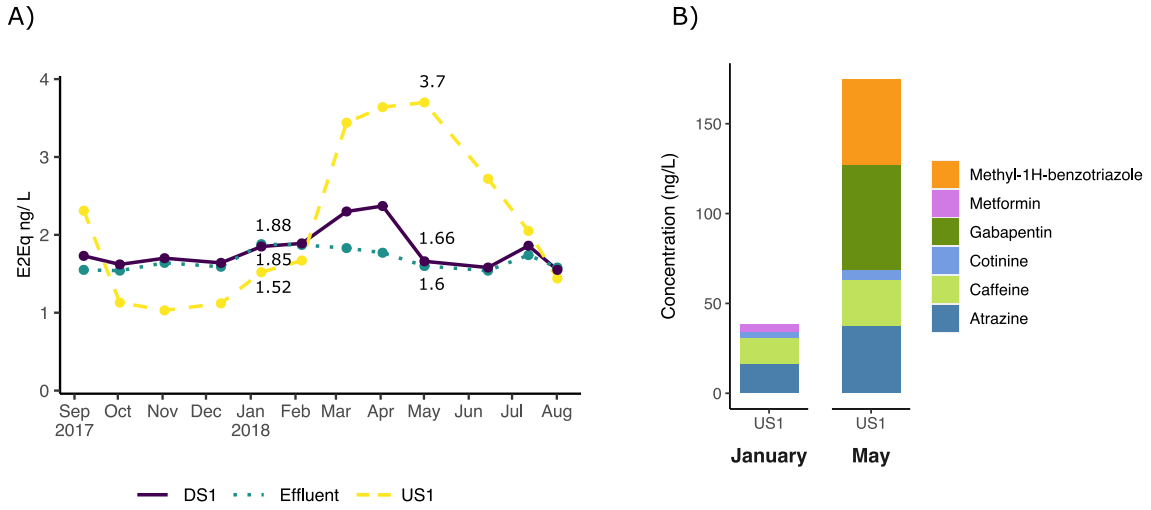

Fig. 4. (A) Calculated estrogen equivalents (E_2Eq in nanograms per liter; relative to 17 β-estradiol) of extracts from monthly water samples collected between September 2017–August 2018 in Muddy Creek in Coralville, Iowa (latitude 41°42′00″, longitude 91°33′46″). Sampling sites were US1 (100 m above the wastewater treatment plant effluent outfall; 05454050), EFF (at the effluent outfall; 05454051), DS1 (100 m below the effluent outfall; 05454052), and DS2 (5 km below the effluent outfall; 05454090) (Meppelink et al., 2020).
(B) Concentrations of individual chemicals detected at US1 in January and May 2018 (Table S.2).

3.3. Estrogenicity still an important signal identified by transcripts but with unknown origins

Notable estrogenicity was observed at all three sites during monthly collections. This included an estrogenic signal with unknown origins detected by the BLYES assay in May at US1 (Fig. 4a). The greatest estrogenic measurement of 3.7 ng/L $E_2Eq_{(BLYES)}$ occurred at the US1 site in May, the highest at any of the Muddy Creek field sites over the 12-month study period. This measurement was more than double the estrogenicity of US1 in January at 1.52 $E_2Eq_{(BLYES)}$ ng/L, which had the lowest measured estrogenicity among stream sample exposures used for RNA sequencing. Estrogenicity was most variable at US1, which had the highest and lowest E_2Eq values of all sites over the 12-month sampling period (1.03 E_2Eq ng/L in November 2017 and 3.7 ng/L in May 2018) (Fig. 4a). In contrast, estrogenicity measurements below US1 ranged from 1.54 to 1.88 $E_2Eq_{(BLYES)}$ ng/L at the effluent and from 1.55 to 2.37 $E_2Eq_{(BLYES)}$ ng/L at DS1 (Fig. 4a). The January and May samples thus reflect contrasting estrogenic stream gradients with comparable estrogenicity among the three sites in January but substantially higher estrogenicity at US1 (compared to the other two sites) in May.

Current predicted no effect concentrations (PNOECs) range from 0.1 to 0.73 ng/L $E_2Eq_{(BLYES)}$ (Laurenson et al., 2014; Wu et al., 2014) thus identifying the baseline estrogenicity of Muddy Creek as high and of biological significance. PLS-DA suggests that exposure to the high estrogenicity signal at US1 in May could have affected 6 dpf larvae more than 3 dpf larvae. The transcriptomes of January and May US1 samples overlapped in PLS-DA of the 3 dpf samples but were completely distinct in PLS-DA of the 6 dpf samples (Fig. 1b and c).

Four pharmaceuticals were detected at US1 in both months, and the corrosion inhibitor methyl-1H-benzotriazole was detected in May (Fig. 4c; Table S.2) (Zhi et al., 2020). These detections occurred at levels far below those seen at the EFF and DS1 sites and below levels that could explain a strong estrogenicity signal. It is likely, therefore, that other chemicals (outside of the 113 pharmaceutical and non-pharmaceutical compounds measured) were present and varied at the US1 site. Neonicotinoid pesticides were detected at US1 in a 2021 study; however, concentrations at US1 were consistently lower than at EFF and DS1 over a year of biweekly measurements (Webb et al., 2021). A 2022 study reported high concentrations of 26 pharmaceuticals at the US1 site in Muddy Creek in February 2018 (including four not measured in the chemical methods for 109 pharmaceuticals) (Wilkinson et al., 2022). Measurements at US1 were consistently higher than at EFF, and measurements were even higher in a site further upstream. These data suggest the occurrence of pulse chemical inputs that originate above the effluent outfall. Thus, the high estrogenicity signal at US1 likely indicates the presence of chemicals not captured through the targeted analysis. Strategic non-targeted chemical analysis could help resolve cases in which distinct bioeffects occur but are not explained by the chemicals detected in a targeted analysis, which would be particularly useful for field reference sites (Kumar et al., 2021).

4. Conclusions

Variation in chemical signatures across two months (i.e., January and May) is recapitulated in gene expression, and even with the estrogenic input observed at the US1 reference site, the transcriptome still reveals key relationships between pathways and processes relevant to understanding the environmental effects of chemical exposures. Although the enrichments shared between the two months related to general processes like musculoskeletal and cardiac development, possibly representing broad compensatory responses to chemical mixture exposures, other functional enrichments uniquely associated with the January and May effluent (e.g., histidine metabolism, eye development, neurological function, and MAPK signaling) may also reflect chemicals that were in higher concentrations in each of those time periods. Exposure to environmental water

Fig. 3. Statistically significant (adjusted p-value < 0.05) overrepresentation of biological pathways and processes among differentially expressed transcripts (DETs; $|\log_2$ fold change$| > 1$ and adjusted p-value < 0.01) up- and downregulated at the effluent and DS1 sites (relative to US1, the upstream reference) in larval zebrafish (*Danio rerio*) exposures to January and May water samples from Muddy Creek in Coralville, IA (latitude 41°42′00″, longitude 91°33′46″). Overrepresentation analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000) and Gene Ontology Biological Pathways (GO:BP) (Ashburner et al., 2000) was performed in clusterProfiler (R v4.0.1) (Yu et al., 2012).
(A) Enriched KEGG pathways from each set of DETs (versus US1).
(B) Enriched GO:BP terms from each set of DETs (versus US1) were selected to represent functional contrasts and similarities between months. On display are 25 of 53 unique GO:BP terms enriched in January exposures and 46 of 195 unique GO:BP terms enriched in May exposures.

samples from effluent-dominated streams approximates some of the complexity of biological responses to wastewater effluent in the real world. As demonstrated in this study, transcriptome-based bioeffects span many biological pathways beyond the endocrine system suggesting that consequences of exposure may continue to impact a range of biological systems in later stages of development. Further research is needed to characterize these pathway-based responses to effluent exposure and explore potential for related physiological impacts at multiple life stages.

CRediT authorship contribution statement

Emma B. Meade: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Project administration. **Luke R. Iwanowicz:** Conceptualization, Methodology, Resources, Investigation, Writing – review & editing. **Nicklaus Neureuther:** Methodology, Investigation. **Gregory H. LeFevre:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Dana W. Kolpin:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Hui Zhi:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Shannon M. Meppelink:** Investigation, Data curation. **Rachael F. Lane:** Validation, Resources. **Angela Schmoldt:** Investigation, Resources. **Aurash Mohaimani:** Software, Formal analysis, Data curation. **Olaf Mueller:** Software, Formal analysis, Data curation. **Rebecca D. Klaper:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159069.

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