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Title

RNA-seq reveals potential gene biomarkers in fathead minnows (*Pimephales promelas*) for exposure to treated wastewater effluent

Authors

Peter G. Schumann[a], Emma B. Meade[a], Hui Zhi[b], Gregory H. LeFevre[b], Dana W. Kolpin[c], Shannon M. Meppelink[c], Luke R. Iwanowicz[d], Rachael F. Lane[e], Angela Schmoldt[f], Olaf Mueller[f], Rebecca D. Klaper[a, f, *]

[a]University of Wisconsin-Milwaukee, Milwaukee, WI USA
[b]University of Iowa, Iowa City, IA USA
[c]U.S. Geological Survey, Iowa City, IA USA
[d]U.S. Geological Survey, Kearneysville, WV USA
[e]U.S. Geological Survey, Lawrence, KS USA
[f]Great Lakes Genomics Center, Milwaukee, WI, USA

*Corresponding Author:
rklaper@uwm.edu; Phone: 414-382-1713; School of Freshwater Sciences, University of Milwaukee, Milwaukee WI, United States

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Abstract

Discharged wastewater treatment plant (WWTP) effluent greatly contributes to the generation of complex mixtures of contaminants of emerging concern (CECs) in aquatic environments which often contain neuropharmaceuticals and other emerging contaminants that may impact neurological function. However, there is a paucity of knowledge on the neurological impacts of these exposures to aquatic organisms. In this study, caged fathead minnows (*Pimephales promelas*) were exposed *in situ* in a temperate-region effluent-dominated stream (i.e., Muddy Creek) in Coralville, Iowa, USA upstream and downstream of a WWTP effluent outfall. The pharmaceutical composition of Muddy Creek was recently characterized by our team and revealed many compounds there were at a low microgram to high nanogram per liter concentration. Total RNA sequencing analysis on brain tissues revealed 280 gene isoforms that were significantly differentially expressed in male fish and 293 gene isoforms in female fish between the upstream and downstream site. Only 66 (13%) of such gene isoforms overlapped amongst male and female fish, demonstrating sex-dependent impacts on neuronal gene

expression. By using a systems biology approach paired with functional enrichment analyses, we identified several potential novel gene biomarkers for treated effluent exposure that could be used to expand monitoring of environmental effects with respect to complex CEC mixtures. Lastly, when comparing the results of this study to those that relied on a single-compound approach, there was relatively little overlap in terms of gene-specific effects. This discovery brings into question the application of single-compound exposures in accurately characterizing environmental risks of complex mixtures and for gene biomarker identification.

Environmental Significance

Wastewater treatment plants (WWTPs) greatly contribute to the formation of complex mixtures of contaminants of emerging concern (CECs) in aquatic environments. The biological and ecological consequences of these mixtures are poorly understood, which hinders the development of effective chemical mixture regulations. Biomarkers that correlate with exposure to these mixtures are needed to better characterize their undesired impacts. By using an *in-situ* stream exposure with *Pimephales promelas*, we were able to identify potential gene biomarkers for exposure to treated effluent that could help improve effects-based monitoring with regards to complex chemical mixtures. Additionally, the results of this study revealed discrepancies in previously reported gene-specific impacts from single-compound exposures, bringing into question their environmental relevance.

1. Introduction

It is estimated that there are over 900 streams composed of at least 50% effluent in the United States (U.S.), and it is generally assumed that these “effluent-dominated” streams are primarily found in arid regions. However, the existence of these effluent-dominated streams extends into temperate regions as well and their prevalence is projected to increase overtime due to increased urbanization and climate change driving more frequent and intense drought patterns (1). Contaminants of emerging concern (CECs) have been known to be ineffectively

removed during wastewater treatment (2) and emitted into aquatic systems as environmental contaminants since the 1960s and 1970s (3,4). An example of such CECs are pharmaceuticals and personal care products (PPCPs), which are increasingly being detected in surface waters around the world (5), and there is a general lack of understanding of their total ecological impacts. Environmental concentrations generally range from single ng/L to 1000s of ng/L (6–11).

Neuropharmaceuticals are almost always amongst the suite of CECs most frequently detected in aquatic systems around the world (5). From 2015 to 2018, 13.2% of adults in the US reported using an antidepressant within the last 30 days, and the overall use of psychiatric drugs is projected to continue to increase (12). Although the use of neuropharmaceuticals such as antidepressants or antianxiety medications is widely considered safe for humans, they have been shown to cause deleterious effects in fish. This includes disruption of normal reproductive and predator avoidance behaviors (13), disruption of brain monoamine levels (14), disruption of neurogenesis (15,16), and transgenerational alteration of stress responses (17,18). Consequently, these individual, sublethal impacts are implicated in or directly relate to the alteration of ecologically important behaviors that pertain to individual fitness such as mating, predator escape responses, and feeding. These behavioral alterations could lead to population declines and ultimately have severe impacts on ecosystems (19).

The current standard approach to assessing sublethal impacts of neuropharmaceuticals is to use single-compound exposures at environmentally relevant or higher concentrations. These laboratory-controlled exposure scenarios can be useful for identifying toxicants and elucidating their potential adverse outcome pathways, but they likely fail to accurately reflect the true impact of these compounds in the real world as these compounds invariably occur as part of a complex mixture that contains other neuropharmaceuticals, other CECs (i.e., per- and polyfluoroalkyl substance (PFAS), anticorrosives, tire leachate chemicals), and other contaminants in general

(20). Complex chemical mixtures create a major challenge in ecotoxicology with regards to establishing cause-and-effect relationships. A contributing factor to this challenge is that the environmental fate of chemical mixtures resulting from effluent discharge is highly dynamic depending on the composition of the influent, which fluctuates with patterns of human use (10), as well as on multiple environmental factors that lead to differential attenuation (21). In turn, the environmental risk of these contaminants is left largely under characterized, which hampers the development of CEC mixture regulations (22).

Recently, our team characterized the pharmaceutical composition and estrogenicity of a temperate-region effluent-dominated stream (i.e., Muddy Creek) near Coralville, Iowa, USA (10,23,24) using water samples collected during monthly baseflow conditions from September 2017 to August 2018. A large portion of the CECs routinely detected in WWTP effluent and receiving waters (including at the Muddy Creek site) are neuroactive pharmaceuticals (25–27). In this follow-up, a field exposure study was conducted to capture the impacts of complex chemical mixtures on the biochemical pathways related to neurological function and other important systems. Transcriptomes of the brain tissue of fathead minnows (*Pimephales promelas*, FHMs) were analyzed after a 96 h *in-situ* stream exposure via RNA-sequencing.

To better contextualize the observed transcriptomic impacts, we employed a systems biology approach through network analyses. Network analyses have been proven to aid in the identification of biological pathways impacted by chemical exposure (28–30) as well as in the identification of biologically motivated candidate biomarker genes of specific phenotypic states (31–34). Although we did not directly characterize phenotypic impacts of the stream exposure in FHMs, phenotypic information can be inferred through network analyses by relying on the use of reverse causal reasoning (35). Reverse causal reasoning is a method used to support the construction of computational models to help evaluate and predict chemical toxicity (36,37). Therefore, this approach falls in line with the current push towards more computationally based

toxicity assessments (38). By applying a systems biology approach to our brain-tissue-specific molecular dataset, it was possible to identify potential gene biomarkers that were significantly correlated with exposure to WWTP effluent and with neurologically relevant functions and disease states.

The objective of this work was to investigate the biological and environmental relevance of gene expression changes in the brain tissue of FHMs exposed to treated effluent in a real-world scenario using transcriptomics. This information could be used to help predict potential neurological impacts and expand the range of existing biomarkers used in effects-based water monitoring. Additionally, the gene expression impacts reported in previous single-compound exposure studies, using various pharmaceuticals that were found in Muddy Creek, were compared to the impacts observed from this real-world exposure. In so doing, the environmental relevance of those genes was brought into question as well as the current standard approach for ecotoxicology assessments. This is especially relevant with regards to genes identified in this work that could potentially be used as indicators for exposure to environmental CEC mixtures.

2. Materials and Methods

2.1 Study site

Muddy Creek is a small effluent-dominated stream in Coralville, Iowa (latitude 41°42′00″, longitude 91°33′46″) that flows into the Iowa River. Within its 22.5 km^2 drainage area, land uses are primarily suburban (60%) with minor agricultural land uses (pasture/hay and cultivated crops, 24.5%) (10). Muddy Creek receives approximately 5,300 m^3 of effluent per day from the North Liberty WWTP (39), which serves the second fastest growing population in Iowa (est. pop. 19,501) (40). In a 2008 expansion, the WWTP underwent construction of a membrane bioreactor facility, which filters particles > 0.004 μm and eliminated the need for secondary disinfection. The WWTP also incorporates biological nitrogen and phosphorus removal (39).

Three previously established U.S. Geological Survey (USGS) sampling sites were used for caged fathead minnow exposures: (1) 0.1 km above the WWTP outfall (US1; USGS 05454050), (2) 0.1 km below the outfall (DS1; USGS 05454052) and (3) at a USGS gaging station (DS2; USGS 05454090) located 5.1 km downstream from the outfall (**Fig. S.1**). Further characterization of the field sites are published elsewhere.(10,21,23,41) Only fish from sites US1 and DS1 were used in the present study to simplify the assessment of the impacts of exposure to effluent-derived CEC mixtures.

2.2 Water sampling

Stream water samples were collected 3 times per day at each field site using the single vertical at centroid-of-flow (VCF) method as described in "Collection of Water Samples" (Section 4.1.3A) of the USGS National Field Manual for the Collection of Water-Quality Data. These samples were analyzed for 14 pharmaceuticals and pharmaceutical degradates (**Fig. 1**). One sample was taken midway through the exposure (July 16) at each field site and was analyzed for 113 pharmaceuticals/degradates and other CECs (data available online at https://nwis.waterdata.usgs.gov). Sampling methods, quality assurance/quality control (QA/QC), and analysis are fully described in a prior publication (42).

In addition to the analysis of the aforementioned chemicals, total estrogenicity of water sample extracts (43) was determined using the bioluminescent yeast estrogen screen (BLYES) as previously described (44,45), but with minor modifications detailed in the ESI (section S1.1 †). The detection limit for this assay was 0.18 ng/L $E_2Eq_{(BLYES)}$. These extracts were derived from a single sampling event that occurred during the approximate midpoint of the exposure period at each of the FHM exposure sites (US1, DS1, and DS2) in addition to one sample taken at the site of the effluent outfall.

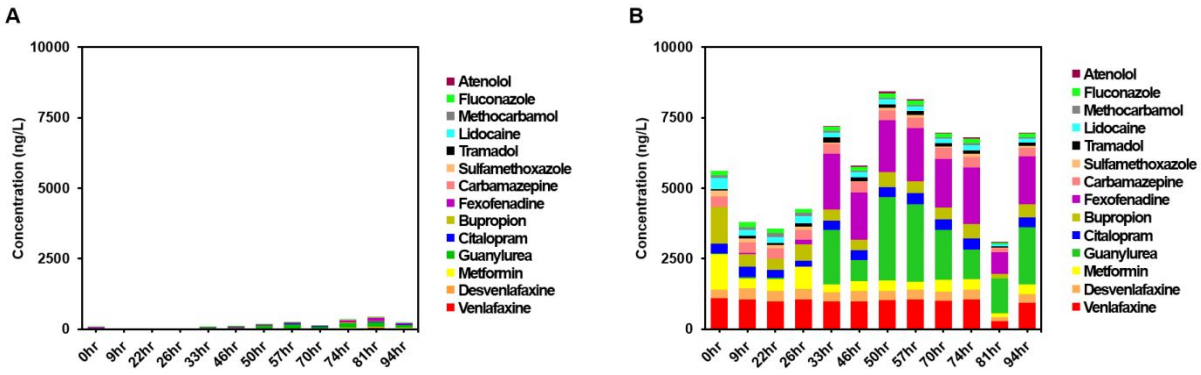


Fig. 1 Stacked bar graph showing the concentrations of the 14 most common pharmaceuticals and pharmaceutical degradates in found Muddy Creek during the 96 h in-stream exposure at (**A**) the site 100m upstream of the WWTP effluent outfall (US1) and (**B**) 100m downstream of the outfall (DS1).

2.3 Caged-fish exposure

Three cages each containing six male and six female FHMs were deployed using modified mesh minnow traps above and below the WWTP effluent outfall of Muddy Creek (sites US1, DS1, and DS2) for a 4-day exposure (96 h) in July 2019 (10AM 7/14 to 10AM 7/17) during baseflow conditions (**Fig. S.2, S.3**). Previous studies have shown significant bioaccumulation of pharmaceuticals in fish under this acute exposure timeframe (46,47). The fathead minnows used in this study were obtained in April 2019 from Aquatic Research Organisms, Inc. (Hampton, New Hampshire). The fish were shipped overnight in oxygen-saturated water to the University of Wisconsin-Milwaukee at four months old and housed as a single cohort of mixed sexes at 20°C with a 16:8-hour light:dark photoperiod and fed TetraMin flake and blood worms twice daily. The fish selected for the caged exposure were 6-months old, reproductively mature, and displayed secondary sex characteristics (male colors, dorsal pad, and tubercules; female ovipositor and abdomen shape) (48). The fish were retrieved after 96 h deployment in the stream and anesthetized in buffered tricaine methanesulfonate (MS- 222; Syndel, Fernale, WA). After length (nose to caudal fin) and wet weights were recorded, the brain tissue was collected from each fish and preserved in RNAlater (Sigma) at 4°C for two days and subsequently stored at −20°C.

2.4 RNA sequencing

Total RNA was isolated from 31 female FHM brains and 33 males (64 total samples) using the standard protocol for Direct-zol RNA MiniPrep (Zymo Research, R2051). A different number of males and females was used due to a lack of extracted RNA samples that met quality criteria described below. Whole FHM brains 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 and RNA integrity measured on an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). Only samples with a 260/280 ratio 1.8-2.0 and 260/230 ratio 2.0-2.2 and RIN >7 were used for sequencing. RNA was quantified on a Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, MA).

RNA Sequencing libraries were prepared using Illumina TruSeq Stranded mRNA sample preparation kit (Illumina, RS-122-2102) and IDT for Illumina – Tru Seq RNA UD Indexes (Illumina, 20022371) following standard protocol, using 1µg of total RNA. Libraries were sequenced on an Illumina NovaSeq6000 (paired-end 150 bp reads).

2.5 Read mapping and differential expression analysis

The raw sequence data had a total genomic yield surpassing 6.884 billion paired-end reads, a median per-sample yield of 39.22 million fragments, and a standard deviation of 37.13 million fragments. The sequence data was then quality-assessed using FastQC v0.11.5, with no apparent base-calling errors needing to be removed (49). Illumina TruSeq 3'-anchored primers were clipped using Cutadapt v1.18, and the resulting quality-controlled data was pseudoaligned and sample-quantified against the GCF_016745375.1 GenBank release of the FHM reference genome using Kallisto v0.45.0 (50,51). This resulted in 40,476 mapped features within the female dataset and 27,252 features within the male dataset.

DESeq2 was used within R v3.5.3 to perform analysis of differential expression between the control, upstream, and downstream samples (52). Genes were considered differentially expressed at a Benjamini-Hochberg adjusted p-value of less than 0.05. The resulting tables of

differentially expressed genes (DEGs) were re-annotated with the GenBank reference information and relationally joined with Kallisto sample quantification counts using custom tooling. High-throughput parallelization of Kallisto was achieved using a compute cluster leveraging the Slurm job scheduler, while all other steps were completed using a high-performance local workstation employing GNU Parallel (53,54). RNA-seq data are available in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) under accession number GSE180719. The RNA-seq results were validated using RT-qPCR (see ESI, Spreadsheet †).

2.6 Data processing

Prior to network analysis, the DaMiRseq package was used within R v4.0.2 to filter and normalize US1 vs. DS1 estimated count data (55) on a sex-separate basis. Gene expression can vary significantly based on biological sex in both human (56) and fish brains (57). Therefore, it was important to account for this phenomenon in our data analysis. Estimated count data from Kallisto was first filtered to remove genes with less than 10 counts across 50% of samples. 160 genes were removed for males and 7510 were removed for females. There were more genes removed from the female dataset due to the overall greater amount of successfully mapped genes (40,476 features) than in the male dataset (27,252 features). The data was then normalized to library size using the variance stabilizing transformation (VST) method. Hypervariant genes with all sample "class" coefficients of variance greater than three were also removed (90 in the female dataset and 27 in the male dataset). After filtering and normalization, 27,065 genes remained in the male dataset and 32,876 genes remained in the female dataset. Additionally, a sample-by-sample mean absolute gene expression correlation was used as a threshold to remove potential sample outliers. All female and male samples were retained at a correlation threshold of 0.85.

The MixOmics package was used in R to aid in visualizing the distribution of the VST normalized count data among site and cage variables through a partial least squares-discrimination analysis (PLS-DA) (58). Again, male and female data were processed separately

to eliminate the contribution of sex differences on observed variability. PLS-DA revealed a batch effect captured by cage differences (i.e., a potential cage effect). This variation due to cage differences was corrected for using the removeBatchEffect function available through the limma package (59) (**Fig. S.4a, S.4b**).

2.7 Weighted gene co-expression network analysis (WGCNA)

A weighted gene co-expression network analysis was conducted using the WGCNA package in R (60) for both male and female upstream vs. downstream DEGs. Prior to network construction, it was ensured that the filtered and normalized transcriptomic datasets (containing 27,065 genes for males and 32,876 genes for females) had detectable expression levels in all biological replicates. A soft threshold procedure was used to transform calculated gene expression correlations into an adjacency matrix. It is generally accepted that a soft-thresholding power with an R^2 value greater than 0.8 is sufficient in approximating scale-free topology (61). Therefore, we chose threshold parameters (six for males and eight for females) that satisfied this criterion while preserving adequate mean connectivity (**Fig. S.5**). The correlational adjacency matrix was then used to generate a topological overlap matrix (TOM). Using this TOM, a complete linkage clustering function organized genes into a network that was then cut using the dynamic tree cut algorithm with a deep split of 2 and a minimum module size of 50. Modules are defined as densely interconnected clusters of genes and are assigned a unique color identifier (61). By correlating module eigengene (ME) values (i.e., the first principal component of the module) to each exposure site, several modules were identified that significantly correlated ($p<0.05$) to fish exposed at the US1 or DS1 site.

2.8 Functional enrichment analysis of WGCNA modules

Modules that were considered significantly correlated with an exposure site were used in functional enrichment analyses using g:Profiler (62). Genes in these significantly correlated modules were found to be over-enriched in functional groups and biological pathways, which were

identified using the custom g:SCS significance threshold and a corrected p-value cutoff of 0.05. At the time of this study, the g:Profiler database did not contain the FHM genome annotations. Therefore, we utilized the zebrafish (*Danio rerio*) genome annotation database to assign known functions, pathways, phenotypes, and disease conditions to the module gene clusters. Specifically, we utilized the following data sources: Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome (REAC), and Human Phenotype Ontology (HP). This functional analysis was also performed on the lists of significant DEGs that were identified using DESeq2 in the US1 versus DS1 comparisons in males, females, and their shared genes.

2.9 PPI and co-expression networks to identify hub genes

To help identify potential biomarkers with neurological relevance as indicators for effluent exposure, we screened modules that were significantly correlated with both the downstream treatment group and with neurological terms (as determined by functional analysis) for candidate hub genes. Genes that had both a module membership (MM) correlation absolute value of > 0.8 and a gene significance (GS) correlation absolute value > 0.5 were considered as candidate hub genes. With MM defined as the correlation of the gene expression profile with the module eigengene and GS defined as the absolute value of the correlation of the gene expression profile with the treatment group (61), which in this case, is the DS1 exposure. Duplicates that resulted from gene isoforms represented by the same gene symbol and met the criteria of |cor. MM| > 0.8 and |cor. GS| > 0.5 were removed.

The degree of gene interconnectivity in significant modules was assessed using STRING (https://string-db.org/) to help identify key hub genes through the generation of protein-protein interaction (PPI) networks. PPI networks were generated according to the latest STRING database (63) using WGCNA modules of interest. Node pairs with a combined score ≥ 0.4 were selected, and these results were then visualized using Cytoscape software v3.8.2. Molecular Complex Detection (MCODE) (64) was utilized within Cytoscape to identify

densely interconnected genes (i.e., molecular complexes) within the PPI network. The cutoff value was set as: node score cutoff = 0.2, K-Core = 2, and degree cutoff = 2. The highest scoring molecular complexes were selected for assessing the degree of overlap with the GS and MM correlation thresholds.

3. Results and Discussion

3.1 WGCNA modules yielded functional terms related to abnormal neurological phenotypes

WGCNA is by design an unsupervised analysis — it does not use predefined gene sets (60). By first assessing the biological context of the genes that comprise this transcriptomic dataset, we can better infer the significance of genes that were differentially expressed between the US1 and DS1 exposure sites and the significance of genes highly correlated to these DEGs. Functional enrichment analyses on identified WGCNA modules allowed for a more concerted determination of the biological context of the FHM brain-derived transcriptome. This revealed gene clusters in both the male and female datasets that were significantly correlated to a difference in DS1 and US1 exposures and have been found in abnormal neurological phenotypes in other organisms in the literature. Network analysis identified 41 gene modules in the female dataset and 54 modules in the male dataset (**Fig. 2**). 9 modules were significantly correlated with a difference in DS1 and US1 in females and 12 modules in males (**Table 1**). Functional enrichment analyses were performed separately on each of these 21 modules. Of these modules, the female MEturquoise module and the male MEred module were selected for further analysis as they had the most terms related to abnormal neurological function. The male MEred module showed significant enrichment ($p_{adj} < 0.05$) in 79 GO terms, 3 REAC terms, and 17 HP terms (**Fig. 3a**). The female MEturquoise module showed a similar number of terms with 87 GO terms, 2 KEGG pathways, 1 REAC terms, and 12 HP terms (**Fig. 3b**).

Both modules exhibited significant enrichment in terms related to seizure-like states as described by the HP database. Additionally, the male MEred module had several GO terms specifically related to neuronal cellular components (e.g., synapse, neuron projection, synaptic vesicle, axon, etc.). Comparatively, enriched terms related to translation (e.g., RNA binding, translation regulator activity, regulation of mRNA splicing, via spliceosome, etc.) and development (e.g., anatomical structure development, nervous system development, multicellular organism development) were more common in the female MEturquoise module (see ESI, Spreadsheet †). It is possible that terms describing development are related to adult neurogenesis, which can be stimulated in response to physical stressors (65) or endocrine disruption (66). The female MEturquoise module also contained a KEGG term for the MAPK signaling pathway, which was not significantly enriched in the male MEred module.

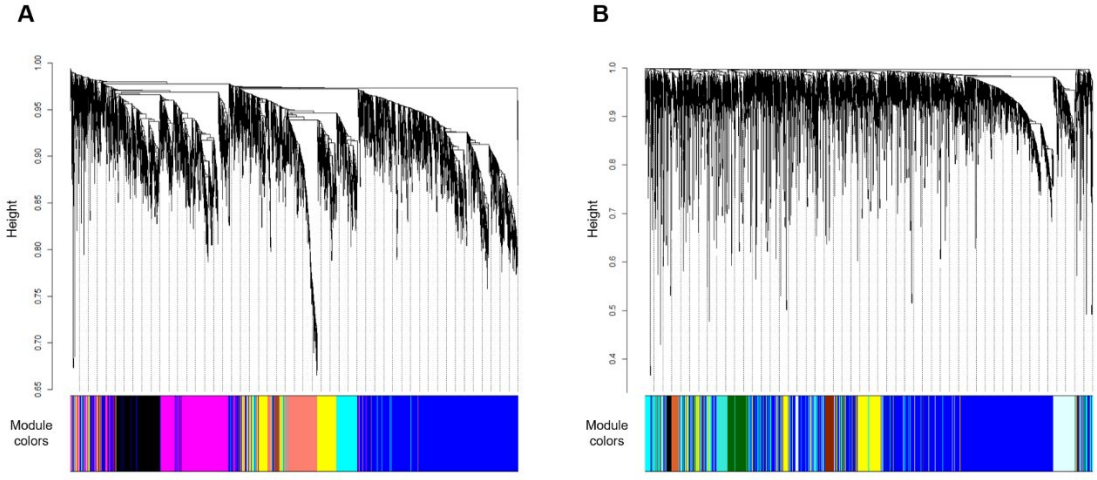
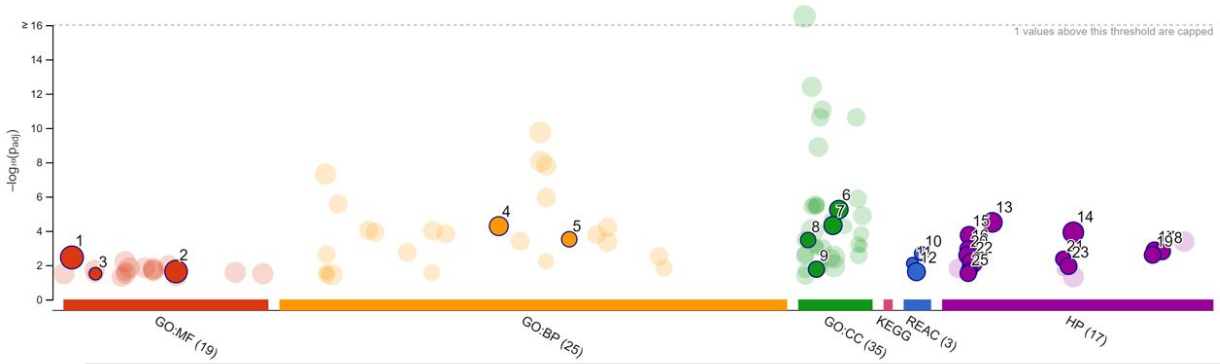


Fig. 2 Cluster dendrograms generated by weighted gene co-expression network analysis (WGCNA) of gene co-expression networks for (**A**) male fathead minnows (*Pimephales promelas*) and (**B**) female fathead minnows (*Pimephales promelas*) datasets. Genes are clustered into modules represented by uniquie color identifiers (bar below each graph) as determined by the dynamic tree cut algorithm. Each module is defined by high inter-gene correlations determined through normalized count data.

Table 1 Weighted gene co-expression network analysis (WGCNA) modules that were found to be significantly correlated with the DS1 exposure. Here, Pearson correlation coefficient values were calculated with respect to the DS1 site. Bolded modules are those that were found to be both significantly correlated with exposure and have interesting neurological functionalizations.

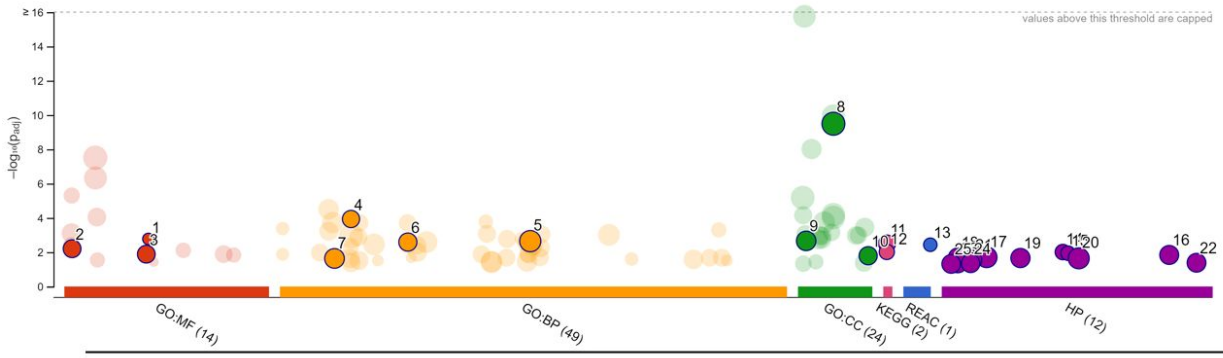
Sex	Module	# of Genes	Corr. Coeff.	p-value

Female	MEmediumpurple3	58	-0.58	3.80E-03
	MEdarkgreen	355	-0.71	1.67E-04
	MEturquoise	**2579**	**-0.55**	**6.64E-03**
	MEyellowgreen	82	0.62	1.69E-03
	MEorangered4	65	0.68	3.41E-04
	MEred	1647	0.96	5.06E-13
	MEsteelblue	111	-0.44	3.60E-02
	MEdarkturquoise	347	-0.42	4.85E-02
	MEdarkgrey	294	0.66	6.31E-04
Male	MEblack	977	-0.55	4.23E-03
	MEcyan	523	-0.41	4.40E-02
	MEdarkgrey	250	-0.62	9.60E-04
	MEdarkolivegreen	178	-0.49	1.29E-02
	MEdarkslateblue	92	0.44	2.75E-02
	MEgreenyellow	666	0.93	1.63E-11
	MEivory	118	0.55	4.09E-03
	MEpaleturquoise	181	-0.44	2.64E-02
	MEpalevioletred3	63	0.40	4.52E-02
	MEpink	886	-0.61	1.09E-03
	MEred	**1250**	**0.58**	**2.41E-03**
	MEsaddlebrown	199	-0.66	3.18E-04



Module	ID	Source	Term Name	Term ID	p_{adj}
	1	GO:MF	catalytic activity	GO:0003824	3.56E-03
	2	GO:MF	ion binding	GO:0043167	2.36E-02
	3	GO:MF	beta-catenin binding	GO:0008013	3.18E-02
	4	GO:BP	establishment of protein localization	GO:0045184	5.29E-05
	5	GO:BP	regulation of vesicle-mediated transport	GO:0060627	3.09E-04
	6	GO:CC	synapse	GO:0045202	5.72E-06
	7	GO:CC	neuron projection	GO:0043005	4.82E-05
	8	GO:CC	synaptic vesicle	GO:0008021	3.42E-04
	9	GO:CC	axon	GO:0030424	1.73E-02
	10	REAC	RAB geranylgeranylation	REAC:R-DRE-8873719	2.11E-03
	11	REAC	GABA synthesis, release, reuptake and degradation	REAC:R-DRE-888590	7.80E-03
	12	REAC	Membrane Trafficking	REAC:R-DRE-199991	2.42E-02
MEred	13	HP	Involuntary movements	HP:0004305	3.19E-05
	14	HP	Abnormal central motor function	HP:0011442	1.22E-04
	15	HP	Generalized-onset seizure	HP:0002197	1.81E-04
	16	HP	Generalized non-motor (absence) seizure	HP:0002121	1.14E-03
	17	HP	Myoclonic seizure	HP:0032794	1.35E-03
	18	HP	Non-motor seizure	HP:0033259	1.58E-03
	19	HP	Generalized-onset motor seizure	HP:0032677	2.52E-03
	20	HP	Bilateral tonic-clonic seizure	HP:0002069	2.65E-03
	21	HP	Atonic seizure	HP:0010819	4.33E-03
	22	HP	Upper motor neuron dysfunction	HP:0002493	7.45E-03
	23	HP	Dialeptic seizure	HP:0011146	1.13E-02
	24	HP	Intellectual disability, profound	HP:0002187	2.23E-02
	25	HP	Generalized myoclonic seizure	HP:0002123	3.03E-02

Fig. 3a A subset of 25 of the most informative functional terms from the g:Profiler ORA (g:GOSt) of the male *P. promelas* MEred module are shown here in a Manhattan plot with corresponding numerical identifiers. GO:MF = molecular function, GO:BP = biological process, GO:CC = cellular component. Functional terms are grouped along the x-axis and color-coded by data sources. The y-axis shows the adjusted enrichment p-values in negative Log_{10} scale. The circle sizes correspond to the term size (i.e., terms with more gene intersections have larger circles). The term location on the x-axis is fixed and terms from the same subtrees are located closer to each other. The ORA resulted in 19 GO:MF terms, 25 GO:BP terms, 35 GO:CC terms, 0 KEGG terms, 3 REAC terms, and 17 HP terms that were significantly (p_{adj}<0.05) enriched.



Module	ID	Source	Term Name	Term ID	p_{adj}
	1	GO:MF	translation initiation factor binding	GO:0031369	1.68E-03
	2	GO:MF	actin binding	GO:0003779	6.27E-03
	3	GO:MF	GTPase regulator activity	GO:0030695	1.30E-02
	4	GO:BP	negative regulation of signal transduction	GO:0009968	1.16E-04
	5	GO:BP	system development	GO:0048731	2.26E-03
	6	GO:BP	actin cytoskeleton organization	GO:0030036	2.57E-03
	7	GO:BP	nervous system development	GO:0007399	2.31E-02
	8	GO:CC	intracellular membrane-bounded organelle	GO:0043231	3.22E-10
	9	GO:CC	cytoskeleton	GO:0005856	2.18E-03
	10	GO:CC	transferase complex	GO:1990234	1.60E-02
	11	KEGG	MAPK signaling pathway	KEGG:04010	3.14E-03
	12	KEGG	Spliceosome	KEGG:03040	9.63E-03
MEturquoise	13	REAC	mRNA 3'-end processing	REAC:R-DRE-72187	3.67E-03
	14	HP	Atonic seizure	HP:0010819	9.78E-03
	15	HP	Typical absence seizure	HP:0011147	1.14E-02
	16	HP	Thin corpus callosum	HP:0033725	1.44E-02
	17	HP	Abnormal muscle tone	HP:0003808	1.98E-02
	18	HP	Gait disturbance	HP:0001288	2.10E-02
	19	HP	Atrophy/Degeneration affecting the central nervous system	HP:0007367	2.19E-02
	20	HP	Abnormal muscle physiology	HP:0011804	2.28E-02
	21	HP	Abnormal cerebral white matter morphology	HP:0002500	2.84E-02
	22	HP	Abnormal emotion/affect behavior	HP:0100851	4.12E-02
	23	HP	Spasticity	HP:0001257	4.38E-02
	24	HP	Sleep disturbance	HP:0002360	4.49E-02
	25	HP	Autistic behavior	HP:0000729	4.83E-02

Fig. 3b A subset of 25 of the most informative terms from the g:Profiler ORA (g:GOSt) of the female *P. promelas* MEturquoise module are shown here in a Manhattan plot with corresponding numerical identifiers. The ORA resulted in 14 GO:MF terms, 49 GO:BP terms, 24 GO:CC terms, 2 KEGG terms, 1 REAC term, and 12 HP terms that were significantly (p_{adj}<0.05) enriched.

3.5 Reverse causal reasoning implicates deleterious neurological responses to short-term effluent exposure

This study relies on the use of reverse causal reasoning to contextualize the transcriptomic impacts of treated effluent exposure in fish. By using several omics databases to predict functionalization of gene clusters containing differentially expressed genes in response to effluent exposure, we found significant enrichments to abnormal neurological phenotypes relating to seizure-like states as defined by the HP database in the male MEred and female MEturquoise modules (**Fig. 3a**, **3b**). Although we did not directly measure any neurological

impacts in exposed fish to confirm these results, this information is useful in the prediction of potential neurological outcomes of exposure to treated effluent.

There were several DEGs identified in both the MEred and MEturquoise modules that contributed to the enrichment of the seizure-like terms. This included *gabrg2* (gamma-aminobutyric acid receptor subunit gamma-2), *maptb* (microtubule-associated protein tau b), and *opa1* (OPA1 mitochondrial dynamin like GTPase). The contribution of *gabrg2* and *maptb* to the enrichment in abnormal neurological phenotypes is supported by previous studies that report the human orthologs of these genes play crucial roles in various forms of human neurological disease such as epilepsy and multiple forms of neurodegeneration (67,68). Similarly, orthologs of *opa1* have been associated with optic atrophy (69) in humans and was identified as a key gene for normal mitochondrial metabolism in developing zebrafish (70).

It is important to emphasize that we did not directly characterize any neurological phenotypes in the exposed FHMs. In fact, some of the human neurological diseases associated with this transcriptomic dataset can often present overlapping pathophysiologies (71). Epilepsy and neurodegeneration, for instance, exhibit a bidirectional molecular mechanism of development (72). Therefore, this inherent ambiguity in the biological relevance of these observed transcriptomic impacts prevents attributing these effects to any specific neurological disorder, rather that these impacts indicate a potential deleterious neurological outcome. Characterizing specific neurological outcomes of exposure to CEC mixtures is a research area of interest, as it has been previously suggested that there may be an association of exposure to mixtures of psychoactive pharmaceuticals found in the environment to the development of various human neurological disorders (73). This is a severely understudied topic, and the results from this study support the need for further investigation in this direction.

3.2 Module hub genes were identified as candidate biomarkers for effluent exposure

We identified several potential gene biomarkers in the MEred and MEturquoise modules that could provide ways to monitor effluent exposure. Based on the functional enrichment data, many of these genes may also be useful in characterizing off-target neurological effects of CEC mixtures. 27 genes in the MEred module and 53 genes in the MEturquoise module met the hub gene criteria of |cor. MM| > 0.8 and |cor. GS| > 0.5 (**Fig. 4**). Hub genes are important to consider for biomarker analysis as they are defined as genes with the greatest correlation with the candidate modules and with effluent exposure. Only 6 of the 59 (unique) DEGs shared between both male and female FHMs met the WGCNA hub gene criteria. A complete list of these genes along with lists of hub genes from other modules can be found in the ESI (Spreadsheet †).

The molecular complexes identified by MCODE within the module protein-protein interaction (PPI) networks were then compared to the genes that met the WGCNA hub gene criteria (**Fig. 5a, 5b**). PPI networks are useful for assessing the degree of inter-gene connectivity in terms of their biological function. Molecular complexes identified by MCODE are found within the core of the PPI network, and so the genes that describe these molecular complexes are more likely to play important roles in the abnormal neurological processes described by the module functional enrichment analysis. Therefore, hub genes also identified by MCODE are well supported as candidate gene biomarkers for effluent exposure that may also be useful in characterizing neurological effects.

For the female MEturquoise module, only two genes were found to be shared between these two methods: *nob1* and *cct2*. Each of these genes are predicted to be involved in protein processing. For the male MEred module, we found no genes shared by the MCODE and WGCNA analyses. However, it is important not to rely solely on the MCODE and WGCNA overlaps, as PPI networks are experimentally based and can prone to error (74). Hub genes that were also differentially expressed ($p_{adj} < 0.05$) between US1 and DS1 are similarly important to consider. Three of the male MEred hub genes were differentially expressed:

LOC120494102, *edil3b*, and *hnrnpm*. *edil3b* is predicted to have calcium ion binding activity, and *hnrnpm* is predicted to have mRNA binding activity. *LOC120494102* is a tripartite motif-containing protein 5-like that currently has an uncharacterized function.

When analyzing modules that had much greater correlations with DS1 — MEgreenyellow (corr. coeff. = 0.93, p = 1.63E-11) for males and MEred (corr. coeff. = 0.96, p = 5.06E-13) for females — there were more hub genes identified that were also found in the MCODE clusters and were found to be significantly differentially expressed (see **Fig. S.6a** and **Fig. S.6b**). Nearly all (13/14) of the genes found to overlap in these modules are predicted to code for heat shock proteins. Despite that these two modules showed very little significant ($p_{adj} < 0.05$) enrichment in terms that were explicitly related to neurological function, they remain important for consideration as potential gene biomarkers. These hub genes defined modules that exhibited significant enrichment in terms related to transcription and translation, and so they may simply lie further upstream of more complex neurological pathways identified in the male MEred and female MEturquoise modules. **Table 2** provides a summary of some of these identified candidate gene biomarkers.

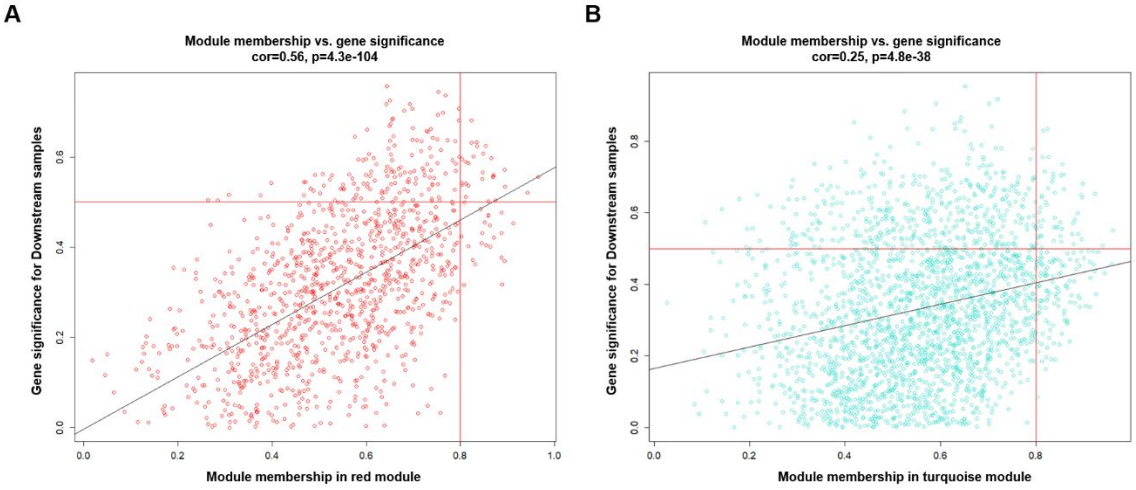


Fig. 4 Scatter plots displying the correlation of module membership (MM) and gene signifcance (GS) for genes contained within the (**A**) male fathead minnow MEred and (**B**) female fathead minnow MEturquoise. Red lines represent thresholds for hub gene candidacy (|MM|>0.8 and |GS|>0.5). 27 genes

met the hub gene criteria in the MEred module, and 53 genes met the hub gene criteria in the MEturquoise module.

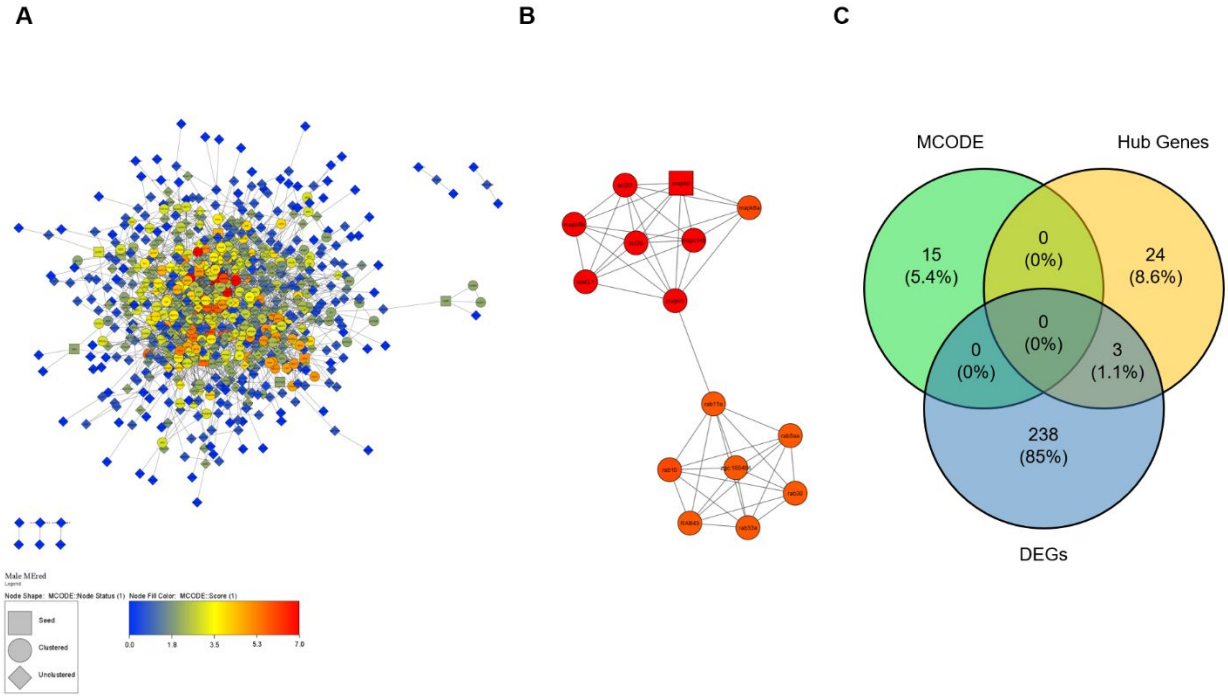


Fig. 5a (**A**) Protein-protein interaction (PPI) network of genes in the male fathead minnow MEred module. Node colors are scaled by molecular complex detection (MCODE) scores where green nodes are less interconnected and red nodes are highly interconnected. (**B**) Highest scoring (i.e., most densely interconnected) molecular complex as identified by MCODE. (**C**) Venn diagram of genes identified by MCODE clustering, genes that met the WGCNA hub gene criteria (|MM|>0.8 and |GS|>0.5) and genes that were found to be significantly differentially expressed (DEGs). No genes were identified that met each of these criteria for this module. However, three hub genes were differentially expressed: *LOC120494102, edil3b, and hnrnpm*.

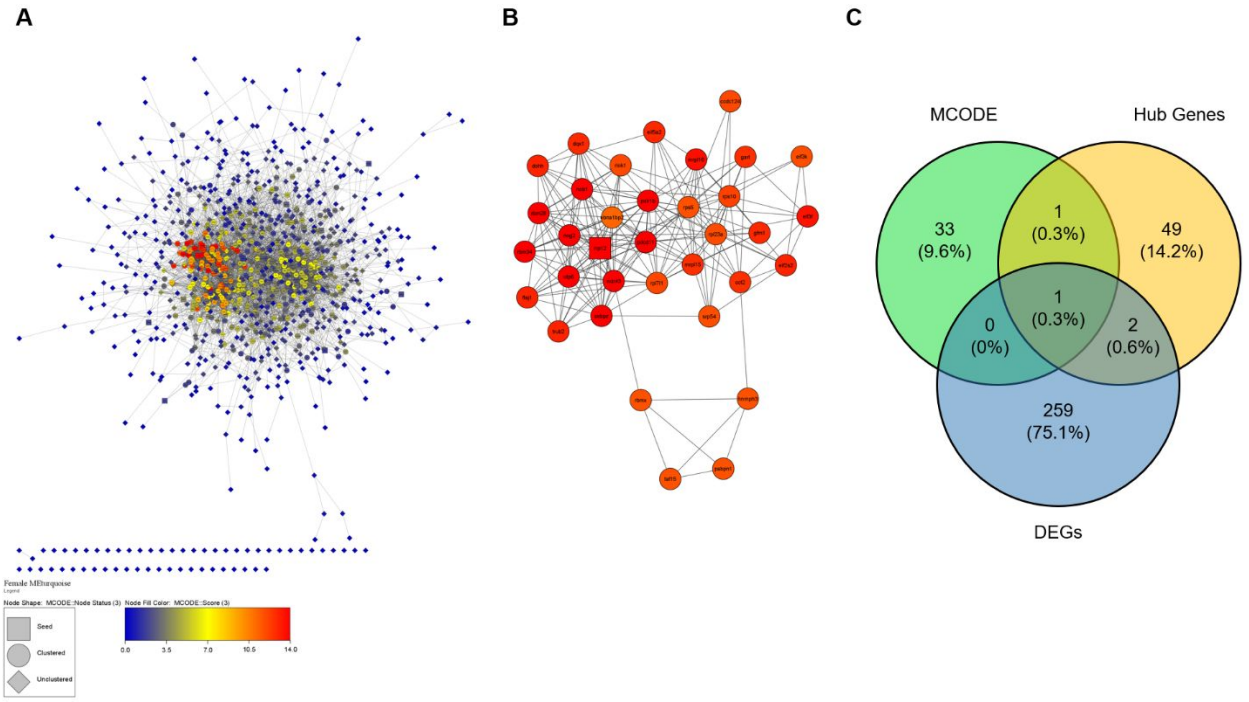


Fig. 5b (**A**) Protein-protein interaction (PPI) network of genes in the female fathead minnow MEturquoise module. Node colors are scaled by molecular complex detection (MCODE) scores where green nodes are less interconnected and red nodes are highly interconnected. (**B**) Highest scoring (i.e., most densely interconnected) molecular complex as identified by MCODE. (**C**) Venn diagram of genes identified by MCODE clustering, genes that met the WGCNA hub gene criteria (|MM|>0.8 and |GS|>0.5) and genes that were found to be significantly differentially expressed (DEGs). Only 1 gene met all these criteria: *nob1*.

Table 2 Summary of 20 candidate gene biomarkers for effluent exposure and associated information on Log_2(Fold Change) with p_{adj}-value in parentheses from DESeq2 analysis, WGCNA module membership, hub gene status, and molecular complex status as determined by MCODE. Log_2(Fold Change) values are bolded if p_{adj}-value < 0.05. Potential gene biomarkers are not limited to this list.

Gene	Name	Log_2(Fold Change) in Males	Log_2(Fold Change) in Females	Male WGCNA Module	Female WGCNA Module	Male WGCNA Hub Gene	Female WGCNA Hub Gene	Male Molecular Complex (MCODE)	Female Molecular Complex (MCODE)
snap91a	synaptosome associated protein 91a	**-0.50 (0.002)**	**-0.39 (0.032)**	MEgreenyellow	MEred	NO	NO	NO	NO
nsun2	NOP2/Sun RNA methyltransferase 2	**0.36 (0.006)**	**0.33 (0.036)**	MEgreenyellow	NO	NO	NO	NO	NO
setb	SET nuclear proto-oncogene b	**0.50 (0.016)**	**0.52 (0.001)**	NO	MEred	NO	NO	NO	NO
cry3a	cryptochrome circadian regulator 3a	**-0.67 (0.047)**	**-0.61 (0.037)**	NO	MEturquoise	NO	NO	NO	NO

ctnnd2b	catenin (cadherin-associated protein), delta 2b	**-0.81 (0.002)**	**-0.95 (0.018)**	NO	MEred	NO	NO	NO	NO
gabrg2	gamma-aminobutyric acid type A receptor subunit gamma2	**-0.24 (0.049)**	0.55 (0.069)	MEred	MEturquoise	NO	NO	NO	NO
maptb	microtubule-associated protein tau b	0.77 (0.067)	**0.91 (0.003)**	MEred	MEturquoise	NO	NO	NO	NO
opa1	OPA1 mitochondrial dynamin like GTPase	-0.17 (0.138)	**-0.22 (0.020)**	MEred	MEturquoise	YES	NO	NO	NO
nob1	NIN1 (RPN12) binding protein 1 homolog	**-0.37 (9.12E-05)**	**-0.30 (0.021)**	NO	MEturquoise	NO	YES	NO	YES
cct2	chaperonin containing TCP1, subunit 2 (beta)	0.09 (0.863)	0.23 (0.266)	NO	MEturquoise	NO	YES	NO	YES
hsp70.3	heat shock cognate 70-kd protein, tandem duplicate 3	-1.08 (0.007)	-0.90 (0.003)	NO	MEred	NO	YES	YES	YES
hspa4a	heat shock protein 4a	**0.62 (2.94E-04)**	**0.53 (6.05E-04)**	MEgreenyellow	MEred	YES	YES	YES	YES
dnajb1a	DnaJ heat shock protein family (Hsp40) member B1a	**0.52 (3.53E-04)**	**0.50 (0.031)**	MEgreenyellow	MEred	YES	YES	YES	YES
hspa5	heat shock protein 5	**0.75 (0.001)**	**0.64 (0.019)**	MEgreenyellow	MEred	YES	YES	YES	YES
dnajb1b	DnaJ heat shock protein family (Hsp40) member B1b	**0.83 (0.002)**	0.61 (0.140)	MEgreenyellow	NO	YES	NO	YES	NO
fkbp4	FKBP prolyl isomerase 4	**0.36 (0.013)**	0.33 (0.059)	MEgreenyellow	MEred	YES	YES	YES	NO
dnaja1	DnaJ heat shock protein family (Hsp40) member A1	**0.35 (0.003)**	0.22 (0.196)	MEgreenyellow	MEred	YES	NO	YES	NO
hsp90aa1.2	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	**0.97 (5.61E-05)**	**0.96 (9.53E-06)**	MEgreenyellow	MEred	YES	YES	YES	YES

serpinh1b	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1b	**1.52 (1.07E-05)**	**1.31 (0.001)**	MEgreenyellow MEred	YES	NO	YES	NO
hsf1	heat shock transcription factor 1	**0.46 (3.35E-04)**	**0.39 (0.007)**	MEgreenyellow MEred	NO	YES	YES	YES

3.3 Exposure to WWTP effluent altered gene expression in a sex-dependent manner

We observed a substantial degree of differences in the male and female FHM transcriptomic datasets with regards to the significantly (p_{adj}<0.05) differentially expressed genes (DEGs). Only 66 (13%) of the identified DEGs overlapped amongst females and males (**Fig. 6**). With this observation, it could be argued that sex-specific gene biomarkers for toxicological endpoints are needed.

The differential expression analysis between the US1 and DS1 samples revealed that of the more than 40,000 genes successfully measured in the female FHM brain tissue, only 293 gene isoforms were found to be significantly differentially expressed. In contrast, just over 27,000 genes were measured in the male FHM brain samples and yet resulted in 280 DEGs, a similar number to the female FHM results. The pattern of differential expression observed in the transcriptomic results were validated using RT-qPCR (**Fig. S.7**).

Each of the shared DEGs showed identical fold change directions and in general had similar magnitudes for males and females. There were a few notable exceptions of genes that had greater fold changes in one sex over the other. This included *LOC120491996*, which codes for heat shock protein HSP 90-alpha 1 and was found to have a Log_2(Fold Change) value nearly 3-fold greater in females (-2.3) than in males (-0.83). The top seven downregulated genes all had greater magnitudes in males than in females—nearly all which code for either heat shock proteins or zinc-finger proteins, except for *rsrp1*, a human ortholog of which has been shown to mediate spliceosome assembly (75). There were also two notable genes that had greater

upregulation magnitudes in females – *kdm6bb* and *ssr1*. *kdm6bb* enables histone binding and demethylase activity. *ssr1* is involved in the cellular response to estrogen stimulus. Sex-dependent impacts in response to PPCP exposure is a commonly reported phenomenon in ecotoxicology with respect to many of the neuropharmaceuticals (76–79) and endocrine disrupting compounds (EDCs) (80) that were detected at the DS1 exposure site. Sex-dependent impacts have also been shown in studies that utilize chemical mixtures (81).

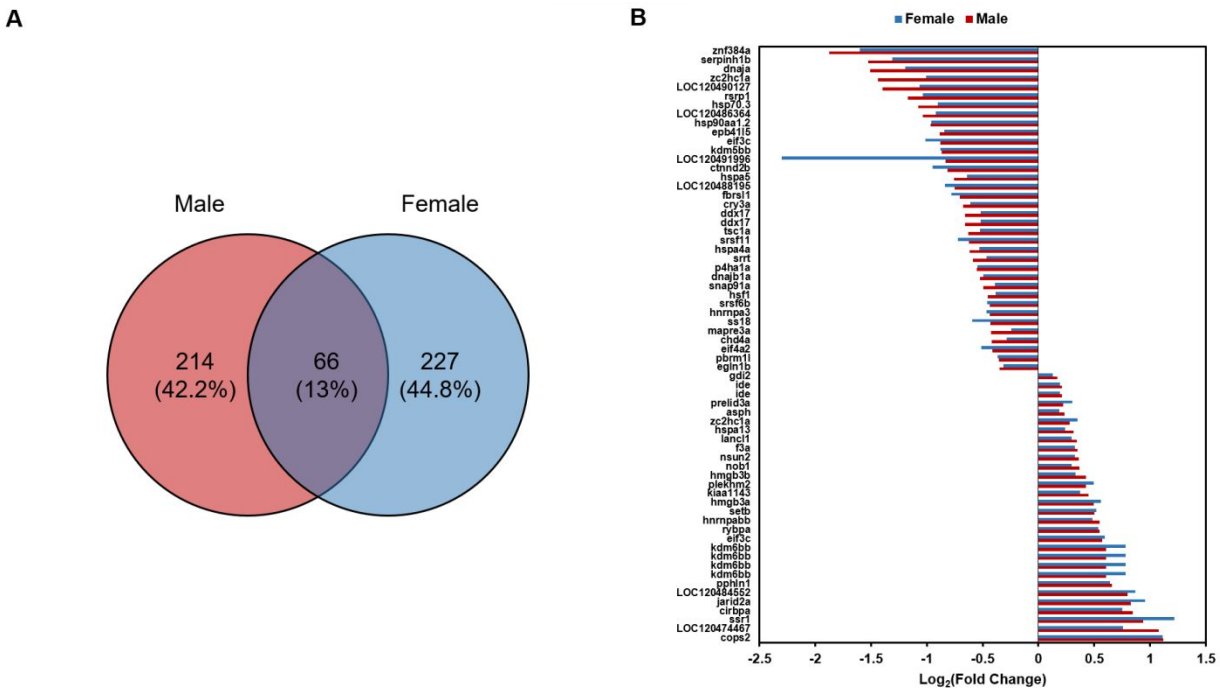


Fig. 6 (**A**) Venn diagram of the 507 gene isoforms that were found to be significantly (p_{adj}<0.05) differentially expressed between US1 and DS1 exposures in male and female fathead minnows (*P. promelas*). Only 66 (13%) of these genes were found to be shared amongst males and females. (**B**) Bar graph of Log_2(Fold Change) values for the 66 differentially expressed gene isoforms shared amongst male and female fathead minnows. Female results are represented in blue and male results are represented in red. The majority of the differentially expressed genes (DEGs) share a comparable magnitude of expression and direction.

3.4 Similarities between the male and female FHM transcriptomic impacts

Despite the sex-differences observed in the gene expression patterns resulting from effluent exposure, shared gene expression impacts remain valuable with regards to their potential use as biomarkers — namely for the sake of efficiency for effects-based monitoring efforts. Of the key hub genes identified in the modules with more neurological functionalization

(MEred and MEturquoise), only *nob1* was significantly ($p_{adj} < 0.05$) differentially expressed in both male and female FHMs (**Fig. 5b**).

Many of the similarities between males and females seem to be due to heat shock proteins or genes with histone modification or DNA/RNA methylation functions. There is significant interest in the field of neuroscience in understanding the relationship between epigenetics and neurological disease (i.e., the discipline of "neuroepigenetics") (82). *nsun2* and *kdm5bb* are examples of such genes that were also identified as shared DEGs. Human orthologs of these genes were implicated in intellectual disability and developmental disorders (83,84). Other genes that have known epigenetic functions and were found to be differentially expressed in response to WWTP effluent exposure in both male and female FHMs include: *jarid2a*, *kdm6bb*, *pphln1*, and *setb*. Transcriptomic impacts on genes with DNA/RNA methylation functions indicate that there may have been more specific biological impacts observed if there had been a longer exposure period as these can alter the expression of underlying genes (85).

Given that these genes also commonly met the WGCNA hub gene criteria and found within PPI molecular complexes, it is possible that they are involved in regulating the expression of other shared DEGs identified within the same WGCNA modules. Many of these other DEGs have functions more specifically related to neurological function and changes in the expression of their human orthologs are also associated with various human neurological diseases and disorders. This included *snap91a* (synaptosome associated protein 91a), *cry3a* (cryptochrome circadian regulator 3a), and *ctnnd2b* (catenin [cadherin-associated protein], delta 2b), which were downregulated in our dataset (**Table 2**). Human orthologs of these genes have been implicated in various neurological disorders and disease states such as Alzheimer's Disease (86), familial advanced sleep phase disorder (87), autism (88), schizophrenia (89), and mild intellectual disability (90,91).

3.6 Gene specific impacts of single-compound exposures are not reflected in real-world exposure scenarios

We found that the specific transcriptional impacts observed via single-compound exposures using the highly concentrated neuroactive compounds found at Muddy Creek were not well reflected in the transcriptional impacts observed through a real-world exposure scenario. Given the complexity of the effluent composition, it was beyond the scope of the current study to determine which compounds may have played the largest role in the observed outcomes. Studies that include an analysis of CEC tissue concentrations would be useful in future areas of research to help make this determination. Moreover, it is likely that there were additional compounds present in Muddy Creek that were not detected in this targeted analysis. These data gaps could be filled by employing non-targeted analysis approaches to probe potentially overlooked chemicals as detection technologies like high-resolution mass spectrometry (HRMS) continue to grow (92,93). However, it remains important to compare the results of this study to those of single-compound exposure studies to help assess their environmental relevancy.

Neuroactive pharmaceuticals like venlafaxine, carbamazepine, citalopram, and gabapentin were some of the most concentrated pharmaceuticals detected at DS1 (**Fig. 1**) with high nanogram to low microgram per liter levels. Although this does not represent a comprehensive comparative analysis, there were only a few notable overlaps regarding alterations of gene expression found between the results from the Muddy Creek exposure and single-compound exposures with regards to these neuropharmaceuticals. This comparison suggests that the complex interaction of the various chemical components of the effluent-dominated stream water could lead to different transcriptomic impacts than laboratory-based exposure studies that rely on individual compounds. Importantly, the single-compound studies examined here were often not performed in a tissue-specific manner or utilized total RNA-sequencing as was done in this

study. Additionally, many of these comparisons were made across species or across species at different developmental stages due to a dearth of information on gene expression impacts from PPCP exposure in adult FHMs. Regardless, these results bring into question how we currently assess true environmental risk of neuropharmaceuticals and other PPCPs using gene expression data. The similarities that were observed related more broadly to biological processes and functions (**Table 3**). The following sections describe some of these similarities and differences found between various single-compound exposure studies using neuropharmaceuticals and the Muddy Creek exposure.

Carbamazepine

We found that carbamazepine impacted the expression of genes found within the glutamate and gamma-aminobutyric acid (GABA) neurotransmitter system in developing zebrafish (*D. rerio*) (94). None of the genes of interest from this previous study were significantly impacted in our results (see ESI, Spreadsheet †). However, *gabrg2*, a GABA receptor subunit, was both differentially expressed and clustered within MEred and MEturquoise. Additionally, *ctnnd2b* was differentially expressed and is known to have a peripheral relationship to the GABA system (91). The impact of effluent exposure on this system is also supported by the REAC term, “GABA synthesis, release, reuptake and degradation”, which was significantly enriched in the male MEred module (**Fig. 3a**). Other studies suggest that genes within the FK506-binding protein (FKBP) family could serve as potential gene biomarkers for exposure to carbamazepine in the sea bream (*Sparus aurata*) (95) as well as for fluoxetine, sertraline (96), and paroxetine (28) in zebrafish. However, these studies report significant increases in expression of *fkbp5*, whereas our results show a significant increase in expression of its co-chaperone, *fkbp4*. Thus, our data supports the relationship between exposure to neuropharmaceuticals and impacts on the FKBP family, in general.

Gabapentin

One study by He et al. (2019) revealed transcriptomic impacts of exposure to an environmentally relevant concentration of gabapentin (0.1 µg/L) that related to the MAPK signaling pathway in zebrafish (*Danio rerio*) embryos (96 hpf) (97). This pathway was found significantly enriched in the female MEturquoise module (**Fig. 3b**). Additionally, *maptb*, a gene associated with this pathway according to KEGG was also found to be differentially expressed (p_{adj} = 0.003) in female FHMs. The other significantly impacted pathways identified in this previous study were not reflected in our results. When comparing the DEGs associated with the enriched pathways identified in this previous study to those identified in the Muddy Creek exposure, there were only 2 out of 67 that were shared between these studies — *hsp70.3* and *tsc1a*.

Venlafaxine

Some exposure studies using venlafaxine report an impact on the regulation of the SNARE binding complex in adult FHMs (98) and in rats (99). *snap91a*, a gene associated with SNARE binding activity, was significantly downregulated and found in gene modules that were highly correlated with DS1 exposure in both male and female FHMs. Costa et al. (2021) examined transcriptional impacts in the brain tissue of the European seabass (*Dicentrarchus labrax*) after a 21-day exposure to 1 µg/L venlafaxine, which revealed over 1200 DEGs (100). Despite DS1 having a similar average concentration of venlafaxine (**Table 3**), only three genes were differentially expressed in response to both the Muddy Creek and the 1 µg/L venlafaxine exposure—*selenbp1*, *gapdh*, and *serp2*.

Citalopram

It has been suggested that genes involved in histone modification (specifically in modifying levels of H3K27me3) can be activated in humans when treated for major depressive episodes using citalopram (101). These histone modifications were found to lead to increased expression of *BDNF* (102), which was not found to be differentially expressed in this study. However, as

discussed previously, several histone modifying genes were found to be differentially expressed in response to effluent exposure.

Additional Compounds

It is important to note that we decided to focus on potential neurological impacts based on the high concentrations of neuropharmaceuticals detected at Muddy Creek. However, the role of different chemical classes is likely to play a significant role in the observed transcriptomic impacts as well. For instance, there is substantial evidence to support the role of endocrine disrupting compounds (EDCs) in causing neurotoxic effects in fish (80). The bioluminescent yeast estrogen screen (BLYES) results showed that the DS1 site had the highest E_2Eq measurements (1.04 ng/L) of any sampling site within Muddy Creek (**Table S.2**). It is possible that other compounds, such as EDCs, could be interacting with these neuropharmaceuticals and limiting the overlap in the observed gene-specific outcomes of exposure. Chemical interactions within dynamic, environmental mixtures remain poorly understood, especially with regards to their biological significance (103,104).

Table 3 Summary of shared biological impacts found between the Muddy Creek (MC) exposure and various single-compound exposure studies with regards to four different neuroactive pharmaceuticals: venlafaxine, citalopram, carbamazepine, and gabapentin.

Pharmaceutical	MC Conc.	Lab Conc.	Organism	Shared Biological Impacts	Reference
Venlafaxine	955 ng L^{-1}	1 µg L^{-1}	*Dicentrarchrus labrax*	Generalized neurotransmission; selenbp1, gapdh, and serp2 (3 out of 1250 DEGs)	Costa et al. 2021
		50 µg L^{-1}	*Pimephales promelas*	Neural system development	Thomas et al. 2012
		10 mg/kg i.p.	Adult male Sprague–Dawley rats	Regulation of the SNARE binding complex	Milanese et al. 2013
Citalopram	314 ng L^{-1}	10 - 60 mg per day for 8 weeks	Human	Histone modification	Belzeaux et al. 2018

Carbamazepine	342 ng L^{-1}	1 - 100 µg L^{-1}	*Danio rerio*	Gamma-aminobutyric acid (GABA) neurotransmitter system	Chen et al. 2020
		7 µg L^{-1}	*Sparus aurata*	FK506-binding protein (FKBP) family	Hampel et al. 2017
Gabapentin	272 ng L^{-1}	0.1 and 10 µg L^{-1}	*Danio rerio*	MAPK signaling pathway; *hsp70.3* and *tsc1a* (2 out of 67 DEGs in enriched pathways)	He et al. 2019

4. Conclusions

Treated wastewater effluent discharged into aquatic environments yields extremely complex chemical mixtures of CECs. Regarding pharmaceuticals, bioaccumulation has been shown to increase linearly with dose and duration (105). Despite using a relatively short-term exposure (96 h), we observed significant gene expression changes under this scenario. This lends support for the use of this short-term, *in-situ* exposure approach in effects-based monitoring. Few studies have assessed the neurological impacts of complex chemical mixtures on aquatic organisms (103,104,106), especially those that contain high levels of human pharmaceuticals from various drug classes (107). Here, we were able to describe some of the potential neurological impacts on FHMs according to various functional databases by analyzing the resulting transcriptomes of FHM brain tissue after a 96-h exposure within an effluent-dominated stream. In so doing, we identified genes that were significantly differentially expressed between the sites upstream from those downstream of a WWTP effluent outfall. Many of these gene expression impacts were found to be sex specific. Additionally, there were very few genes identified in this study that were also shown to be significantly impacted in single-compound exposure studies, indicating potential limitations of status-quo chemical risk assessment approaches with regards to gene expression impacts. Several genes were identified to serve as hub genes in molecular networks that were found to be significantly enriched in functional terms related to abnormal neurological function (**Table 2**). Through further validation, these genes offer a path forward in investigating potential gene biomarkers for

effluent exposure and predicting neurological effects in fish. The use of these candidate gene biomarkers could aid in better characterizing the environmental risks of complex chemical mixtures and concomitantly improve effects-based monitoring efforts (108).

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

All laboratory procedures involving fish were performed in accordance with the Animal Welfare Act and the University of Wisconsin – Milwaukee Institutional Animal Care and Use Committee (IACUC) guidelines and procedures.

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Footnote

† Electronic supplementary information (ESI) available. See ###############