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Effects of aqueous fluoxetine exposure on gut microbiome of adult *Pimephales promelas*

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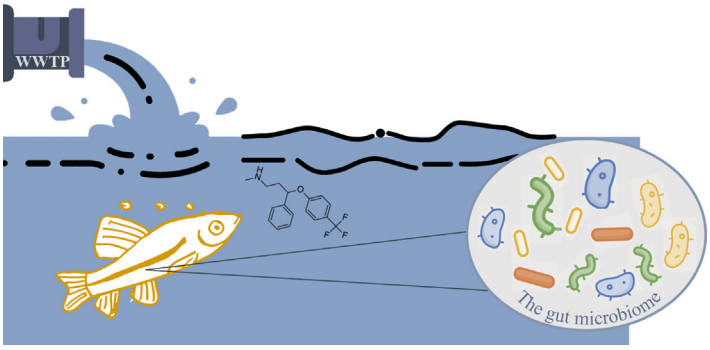
HIGHLIGHTS

- Composition of gut microbiome was altered at highest dose.
- Antimicrobial action of fluoxetine alters fish gut microbiome.
- Sex-specific response of gut microbiome to fluoxetine exposure at taxa-level.
- *Akkermansiaceae* which can regulate host 5-HT was negatively affected by fluoxetine.

GRAPHICAL ABSTRACT



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ABSTRACT

The microbiome of the gut is vital for homeostasis of hosts with its ability to detoxify and activate toxicants, as well as signal to the immune and nervous systems. However, in the field of environmental toxicology, the gut microbiome has only recently been identified as a measurable indicator for exposure to environmental pollutants. Antidepressants found in effluents of wastewater treatment plants and surface waters have been shown to exhibit antibacterial-like properties *in vitro*, where some bacteria are known to express homologous proteins that bind antidepressants in vertebrates. Therefore, it has been hypothesized that exposure to antidepressant drugs might affect gut microbiota of aquatic organisms. In this study, the common antidepressant, fluoxetine, was investigated to determine whether it can modulate the gut microbiome of adult fathead minnows. A 28-day, sub-chronic, static renewal exposure was performed with nominal fluoxetine concentrations of 0.01, 10 or 100 μg/L. Using 16S rRNA amplicon sequencing, shifts among the gut-associated microbiota were observed in individuals exposed to the greatest concentration, with greater effects observed in females. These changes were associated with a decrease in relative proportions of commensal bacteria, which can be important for health of fish including bacteria essential for fatty acid oxidation, and an increase in relative proportions of pathogenic bacteria associated with inflammation. Results demonstrate, for the first time, how antidepressants found in some aquatic environments can influence gut microbiota of fishes.

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1. Introduction

The vertebrate gut microbiome contributes to development and function of the nervous system (Sharon et al., 2016). Thought of as the second brain, the enteric nervous system, embedded in the gastrointestinal lining, holds over 500 million neurons. These gastrointestinal neurons allow for communication between the host and gut microbiota (Carabotti et al., 2015; Sharon et al., 2016). Within the microbiome-gut-brain axis, gut microbiota play important roles in homeostasis of vertebrate hosts, for instance, regulating gastrointestinal functions, immune system function, and modulation of anxiolytic behavior, perception of pain and emotions (Mayer et al., 2014; Sharon et al., 2016). The majority of such studies have focused on mammals, while studies of relationships between gut microbiomes of fishes and neurological events is still lacking (Butt and Volkoff, 2019). However, it has been found that the gut microbiome can influence the hypothalamic-pituitary interrenal (HPI) axis as well as the stress response in fish which in turn can affect immune system function, feeding behaviors and overall homeostasis (Butt and Volkoff, 2019; Davis et al., 2016).

Consumption of antidepressants is continuously increasing worldwide and are now commonly observed in wastewater effluents and downstream of aquatic environments at concentrations of nanograms to micrograms per liter (Ding et al., 2017; Kolpin et al., 2002; Kreke and Dietrich, 2008; Schultz et al., 2011). Among prescription antidepressants, fluoxetine is a model compound for studying the toxicological effects of antidepressants. Fluoxetine (under the trade name Prozac®) is one of the most prominently used Selective Serotonin Reuptake Inhibitors (SSRIs) (Luo et al., 2020). After transformation in the body, less than 10% of fluoxetine is excreted in its unchanged, more lipophilic state, while 20% is excreted as its primary metabolite, norfluoxetine (Silva et al., 2012). Concentrations of fluoxetine have been found to range from 0.012 μg/L in freshwater streams to 0.09 μg/L in wastewater effluent in North America (Brooks et al., 2003; Kolpin et al., 2002; Metcalfe et al., 2010; Silva et al., 2012). Within raw sewage, 0.01 to 3.5 μg/L have been reported (Ding et al., 2017). Fluoxetine has also been detected in tissues of wild fish between 0.02 and 1.58 ng/g, wet mass (wm) (Brooks et al., 2005; Chu and Metcalfe, 2007; Schultz et al., 2010). Bioconcentration of fluoxetine in the Japanese medaka (*Oryzias latipes*) (Paterson and Metcalfe, 2008) and *Daphnia magna* (Ding et al., 2017) leads to concerns for ecotoxicological risks to aquatic animals.

Microbiomes in guts of animals can influence major neurotransmitters. The essential neurotransmitter, serotonin (5-hydroxy-tryptamine, 5-HT), can be regulated by certain enteric microbes (*Turcibacter sanguinis* and *Lactobacillus salivarius*) perhaps stimulating host 5-HT biosynthesis (Fung et al., 2019; Lyte and Brown, 2018; Yano et al., 2015). Recently, several studies have assessed how SSRIs as well as other psychoactive drugs affect gut microbiota of rodents with the aim to elucidate possible effects on humans. Findings include changes in microbial composition and antimicrobial-like properties of fluoxetine on certain bacteria including *T. sanguinis*, *Lactobacillus rhamnosus*, *Escherichia coli* as well as the family *Peptostreptococcaceae* (Cussotto et al., 2018; Fung et al., 2019; Lyte et al., 2019; Sun et al., 2019; Zhang et al., 2021).

There have been multiple studies focusing on toxicological endpoints of SSRIs on aquatic organisms including reproduction, physiology, and behavior. Findings suggest reductions in behaviors related to aggression and anxiety, modulating the predator-prey response, suppression of appetite, and stimulation of the hypothalamic-pituitary-gonadal (HPG) and the HPI axes contributing to reproduction impairments of fish (Brooks et al., 2003; McDonald, 2017; Polverino et al., 2021). Until now, no research has yet been conducted to determine effects of SSRIs on gut microbiomes of fishes.

To investigate how fluoxetine might modulate the microbiome of the gut of the fathead minnow (*Pimephales promelas*), a sub-chronic aqueous exposure of fluoxetine ranging from an environmentally relevant concentration, 0.01 μg/L, to a sub-lethal concentration of 100 μg/L was conducted. The objectives of this study were to: (1) Identify potential differences in the gut microbiome of male and female fathead minnows; (2) Detect effects of fluoxetine on the abundance and diversity of the gut microbiome of fathead minnows and (3) Identify any sex-specific responses of the gut microbiome due to exposure to fluoxetine.

2. Materials and methods

2.1. Fish husbandry, aqueous exposure, and dissection

Adult fathead minnows, approximately 6 months old were obtained from the Aquatic Toxicology Research Facility at the University of Saskatchewan. Five fish were randomly assigned to a 20-L tank, and tanks were randomly assigned a fluoxetine concentration (n = 25 per group, 5 fish per tank, 5 tanks per group). Fluoxetine hydrochloride (ThermoFisher Scientific, Waltham, MA) was dissolved in water to make a stock solution from which working solutions were then made. Nominal exposure concentrations consisted of a control group (0 μg/L), low concentration group (0.01 μg/L), middle concentration group (10 μg/L), and high concentration group (100 μg/L). After one week of acclimation fluoxetine-treated water was added to each tank. Fish were fed twice daily with 2 mg of blood worms and temperatures were consistently monitored (average = 22 °C ± 0.7). Ammonia, pH, nitrites, nitrates, and dissolved oxygen were tested in each tank and light to dark ratio was 16:8. This was a static renewal test where tanks were 2/3rds siphoned daily with replacement of freshly prepared fluoxetine treated water. A 10-milliliter water sample was taken from each tank daily, right after each water-change, for validation of fluoxetine concentrations.

At the end of the exposure, fish were anesthetized in MS-222. Mass and length were measured. Fish were euthanized *via* cervical dislocation. The whole intestinal tract was excised from each fish by use of sterile techniques. Gut contents containing microbes, were gently squeezed out with sterile forceps and discarded, leaving only microbes adhered to the gut tissue for further analyses. Phenotypic sex was determined and recorded. Five fish were discarded because sex was undeterminable by gonadal development. Samples were placed in sterile cryovials and held in liquid nitrogen before being stored at −80 °C. Maintenance of fish was in line with the animal use protocol (Protocol #20090108) approved by the Animal Research Ethics Board at the University of Saskatchewan. Overall fish health was determined following Fulton's condition factor (K) calculated from the mass and length of the fish (Eq. (1)) (Carlander, 1969).

$$K = \frac{\text{Mass}}{\text{Length}^3} \times 100 \quad (1)$$

2.2. Quantification of aqueous fluoxetine

Water samples for the 100 and the 10 μg/L exposure groups were analyzed, and the average concentration of each tank per day was used as our sample size (n = 29, per group). Solid Phase Extraction (SPE) methods were necessary when determining concentrations from the 0.01 μg/L and control groups due to limit of detection levels when running liquid chromatography tandem mass spectrometry (LC-MS). For the 0.01 μg/L and control groups, samples were pooled per tank by day for SPE, where 8 days of the exposure were analyzed (n = 8, per group, Table 1). Stock solutions of fluoxetine and fluoxetine-d5 (ThermoFisher Scientific, Waltham, MA)

Table 1
Mean and standard deviation (SD) of aqueous concentrations of fluoxetine right after each water-change and predicted plasma concentration based on the fish plasma model and linear equation model identified by Margiotta-Casaluci et al. (2014).

Group	Nominal exposure concentrations (μg/L)	Aqueous concentration (mean ± SD, μg/L)	Predicted plasma concentrations (μg/L)
High	100	90.1 ± 8.8 (n = 29)	1201.3
Middle	10	8.0 ± 0.9 (n = 29)	29.9
Low	0.01	0.006 ± 0.002 (n = 8)	0.02
Control	0	0.002 ± 0.001 (n = 8)	0.006

were made in HPLC grade methanol (Fisher Scientific) at 100 mg/L. A seven-point calibration curve ranging from 0.5 to 500 μg/L and spiked with 50 μg/L fluoxetine-d5 was used for quantification by isotope dilution (linearity >0.999 for all analyses). The 10 and 100 μg/L exposure solutions were sub-sampled (1 mL) directly into LC vials and spiked with fluoxetine-d5 at a target concentration of 50 μg/L for direct injection analysis. Control and 0.01 μg/L exposure solutions were sub-sampled (40 mL) into 45 mL falcon tubes, spiked with fluoxetine-d5, and extracted using SPE OASIS™ HLB cartridges (6 cc, Waters Corporation, Milford, MA). After pre-conditioning with methanol followed by water, 40 mL of water samples were drawn through at ≈5 mL min^{-1}, cartridges were then vacuum dried, and eluted with 2 × 3 mL fractions of methanol (combined). Extracts were evaporated to dryness under nitrogen in a water bath at 40 °C, and reconstituted in 0.5 mL of 50:50 MeOH-H_2O into amber LC vials. Fluoxetine was quantified by use of a Vanquish UHPLC and Q-ExactiveTM HF Quadrupole-Orbitrap™ mass spectrometer (Thermo-Fisher). LC separation was achieved with a Kinetex 1.7 μm Biphenyl LC column (100 × 2.1 mm) (Phenomenex, Torrance, CA) by gradient elution with 95% water + 5% methanol (A) and 100% methanol (B), both containing 0.1% formic acid at a flow rate of 0.2 mL min^{-1} and column temperature of 40 °C. The gradient method started at 10% B, ramping linearly to 100% B over 7 min, held for 1.5 min, and returning to starting conditions for column re-equilibration between 8.5 and 11 min. Further details on LC-MS parameters can be found in in the SI Text S1.

2.3. Extraction of bacterial DNA and 16S rRNA metagenomics

DNA was extracted from whole intestines using the DNeasy PowerSoil Kit (Qiagen Inc., Mississauga, ON). Concentrations of DNA were measured using a Qubit 4 Fluorometer and dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA). The V3-V4 hypervariable region of the bacterial 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR) with dual-tagged primers, 341F (5′-tag-CCTACGGGNGGCWGCAG-3′) and 806R (5′-tag-GGACTACNVGGGTWTCTAAT-3′). PCR products were checked by agarose gel electrophoresis and no bands were observed for blank controls. Thirteen samples were discarded due to PCR amplification failure. Products were then purified using the QIAquick 96 PCR Purification (Qiagen Inc., Mississauga, ON) following manufactures instructions. Library preparation and next generation sequencing was conducted following procedures previously published by DeBofsky et al. (2020).

2.4. Bioinformatics

Bioinformatic analyses were conducted under QIIME2 v. 2020.10 environment (Bolyen et al., 2019). After demultiplexing, amplicon sequence variants (ASV) were denoised and extracted using DADA2 (Callahan et al., 2016). Taxonomy was annotated against SILVA 132 reference database (Bokulich et al., 2018). All unassigned and nonbacterial ASVs were then removed, and rarefaction was performed where sampling depth (11,949 sequences per sample) was decided based on the ability to maximize the depth threshold while minimizing sample loss. Nine samples were removed due to low sequencing depth. After rigorous quality control, 73 samples were retained for further analyses, including 31 males and 42 females. Of the males, there were 8 in the control group, 8 in the low group, 6 in the middle group and 9 in the high group. Of the females there were 11 within the control group, 11 in the low group, 9 in the middle group and 11 in the high group. Metadata for samples are presented in SI Table S1.

2.5. Statistics

All statistical analyses were conducted in R Statistical Language v 4.0.3 (R core team, 2020). Normal distribution and equal variance were first determined by use of a quantile-quantile plot, residuals *vs* fitted plots, Shapiro-Wilk test and a Levene's test. To determine fitness of fish, sexes were separated, and K was identified (Eq. (1)) for each fish. If the assumption of a normal distribution was met, an analysis of variance (ANOVA) was used to test condition factor to exposure concentrations of fluoxetine as well as tank number. If normal distribution was not met, data was log transformed before running a Levene's test to determine equal variance and an ANOVA was then performed.

For parametric testing, a two-sided Student's *t*-test was applied when evaluating differences between sexes and an ANOVA with a Tukey's HSD was performed to evaluate differences between exposure groups. If equal variance was not met while still normally distributed, a Welch's *t*-test was run for sex differences. The non-parametric tests performed when normality was not met included a Wilcoxon rank sums test used to compare differences in alpha diversity between sexes and a Kruskal-Wallis test with a Dunn's post-hoc test used for alpha diversity between exposure groups. To compare beta diversity metrics for both sex and exposure group, a multivariate analysis of variance with permutation (PERMANOVA) was conducted along with testing for homogeneity of multivariate dispersion (PERMDSIP) (Borcard et al., 2011). Because sex was found to explain alpha and beta diversity, analyses comparing exposure groups were conducted by separating the sexes. To determine differential abundance of taxa, a linear discriminant analysis (LDA) effect size (LEfSe) was performed (Segata et al., 2011). Neighborhood selection relationships between ASVs were constructed by the SPIEC-EASI package (Kurtz et al., 2015). Correlations between relative abundance of ASV and aqueous concentration of fluoxetine were confirmed to be robust if the adjusted false discovery rate (FDR) was statistically significant ($P_{FDR} < 0.05$). The network was displayed and analyzed with Cytoscape V3 (Otasek et al., 2019).

3. Results

3.1. Aqueous concentration of fluoxetine and fitness of fish

Daily, mean aqueous concentrations after each water change and 24 h after each renewal are summarized in Table 1. Measurements of fluoxetine in water confirmed that concentrations consistently decreased between renewal periods (24 h). Mean (SD) pH of water was 7.9 (0.2) throughout the exposure. Condition factors of both female (ANOVA test, $p > 0.05$; $n = 48$, SI Fig. S1A) and male (ANOVA test, $p > 0.05$; $n = 34$, SI Fig. S1B) fish were not significantly affected by exposure to fluoxetine. Thirteen samples were lost in the PCR process and sex was undetermined for 5 of the fish leaving us with 48 females and 34 males for the condition factor analysis.

3.2. Host sex shaped gut microbiome

Female and male fish exhibited distinct compositions of gut microbial communities. In total, 386 ASVs were recovered for both female ($n = 42$) and male fishes ($n = 31$), with 5.2 million sequenced reads. 77.7% of reads survived after quality check, filtering, merging and non-chimeric cleaning (SI Table S1). Rarefaction at an even sequencing depth of 11,125 sequences per sample retained 377 ASVs (SI Fig. S2). *Fusobacteria*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes* dominated the gut microbiome of fathead minnow (SI Fig. S3). Alpha-diversities (Faith's Phylogenetic Diversity and Shannon diversity) of the gut microbiomes of females were greater than those of males (Wilcoxon one-tailed signed rank test, $p < 0.01$, Fig. 1A and B). Beta-diversities (unweighted and weighted UniFracs distances) found significant separations between male and females (PERMANOVA, unweighted UniFrac distance, $F = 11.96$, $p = 0.001$, Fig. 1C; weighted UniFrac distance, $F = 4.25$, $p = 0.006$, Fig. 1D). Dispersion of unweighted UniFrac distances was heterogeneous while weighted was homogenous between female and male fish (unweighted UniFrac distance; PERMDSIP test, $F = 7.45$, $P_{perm} = 0.008$, weighted UniFrac distance; PERMDISP test, $F = 0.03$, $P_{perm} = 0.86$). Fifty-two bacterial families were enriched in female fish, while three families were enriched in male fish (Fig. 1E, LEfSe test, $p < 0.05$ and $\log_{10}$ transformed LDA score > 2). Within the top 10 most abundant families, *Barnesiellaceae*, *Chitinibacteraceae*, *Rubritaleaceae*, *Shewanellaceae*, and *Vibrionaceae*, were

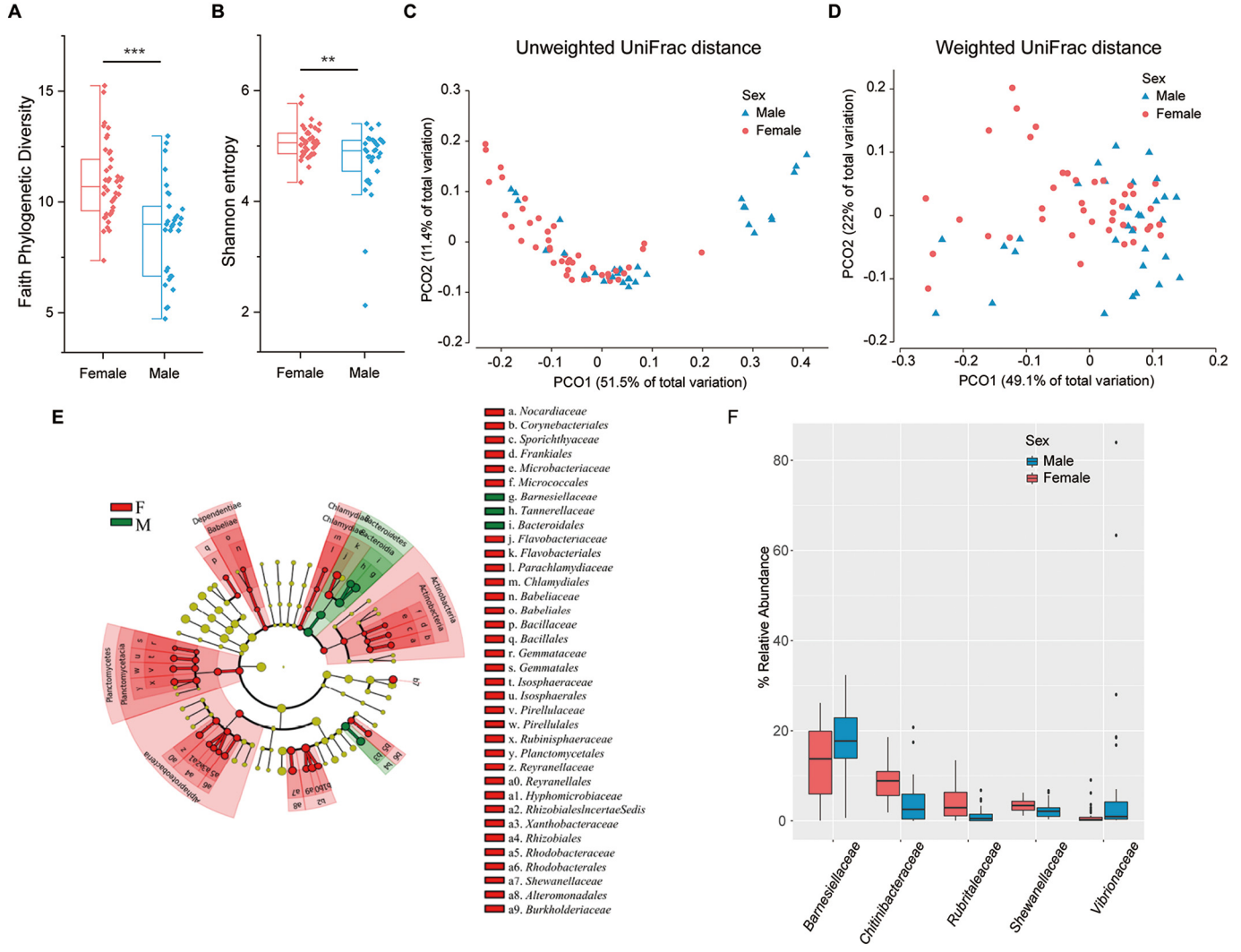

Fig. 1. Alpha diversity matrices comparing female ($n = 42$) and male ($n = 31$) fathead minnows: (A) Faith Phylogenetic Diversity; $p = 6.127e-06$ and (B) Shannon Diversity Index; $p = 0.02$. (C) Unweighted UniFrac and (D) weighted UniFrac depicting the distances in microbial composition between female and male fish; Unweighted PERMANOVA; F = 11.96, $p = 0.001$, Weighted PERMANOVA; F = 4.25, $p = 0.006$. (E) Cladogram of taxonomic levels from a LEfSe analysis. Taxa in red are differentially and greater expressed in female fish and those in green are greater expressed in males. (F) Boxplot depicting relative abundant families found to be differentially expressed in females *versus* males. Significant correlations: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. F, female; M, male. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly differentially abundant between male and female fish (Fig. 1F). Female fish hosted a more complex microbial network than male fish where females had, 209 nodes, 562 edges and 5378 neighbors and males had, 171 nodes, 339 edges and an average of 4238 neighbors (Neighborhood Selection Network analysis, Fig. 2A and Table S2). Microbial Network of males contained fewer nodes per cluster corresponding to lower alpha diversity than compared to females.

3.3. Female gut microbiome response to fluoxetine exposure

Fluoxetine caused marginal effects on select metrics of alpha diversity in female fish within the high exposure group. Faith PD was elevated in the high (100 μg/L) compared to the middle (10 μg/L) exposed group ($p < 0.05$). No significant difference was observed between the control group and the high group, nor between the control group and the low and middle exposure groups when observing alpha diversity metrics (Fig. 3A & B).

Exposure to aqueous fluoxetine significantly altered the relative percent composition of the gut microbiome of female fish (PERMANOVA test: unweighted distances, F = 4.39, $p = 0.001$; weighted distances: F = 5.38, $p = 0.001$, Fig. 3C & D). Beta diversity of the high exposure group was significantly different from that of all other groups (Table S3). No significant differential beta diversity matrices were found between control, low, and middle exposure groups within female fish. Five family level taxa were negatively associated with an increase in fluoxetine concentration while fifteen were positively correlated (Fig. 4A). Several bacterial families were significantly different when comparing each exposure group to the control group (LEfSe test). *Akkermansiaceae*, *Peptostreptococcaceae* and *Barnesiellaceae* show a significant decrease in abundance in the high group while there was a significant increase in *Rubritaleaceae*, *Chitinibacteraceae*, *Shewanellaceae*, *Flavobacteriaceae*, and *Aeromondaceae* in the high group compared to the control group (Fig. 4 B & C). Abundance of *Akkermansiaceae* was also significantly less in the low group compared to the control group. Classes in the largest cluster of the female gut microbial network were significantly correlated with increasing concentrations of fluoxetine (Fig. 2A), which is comparable with the heatmap of family level taxa (Fig. 4A).

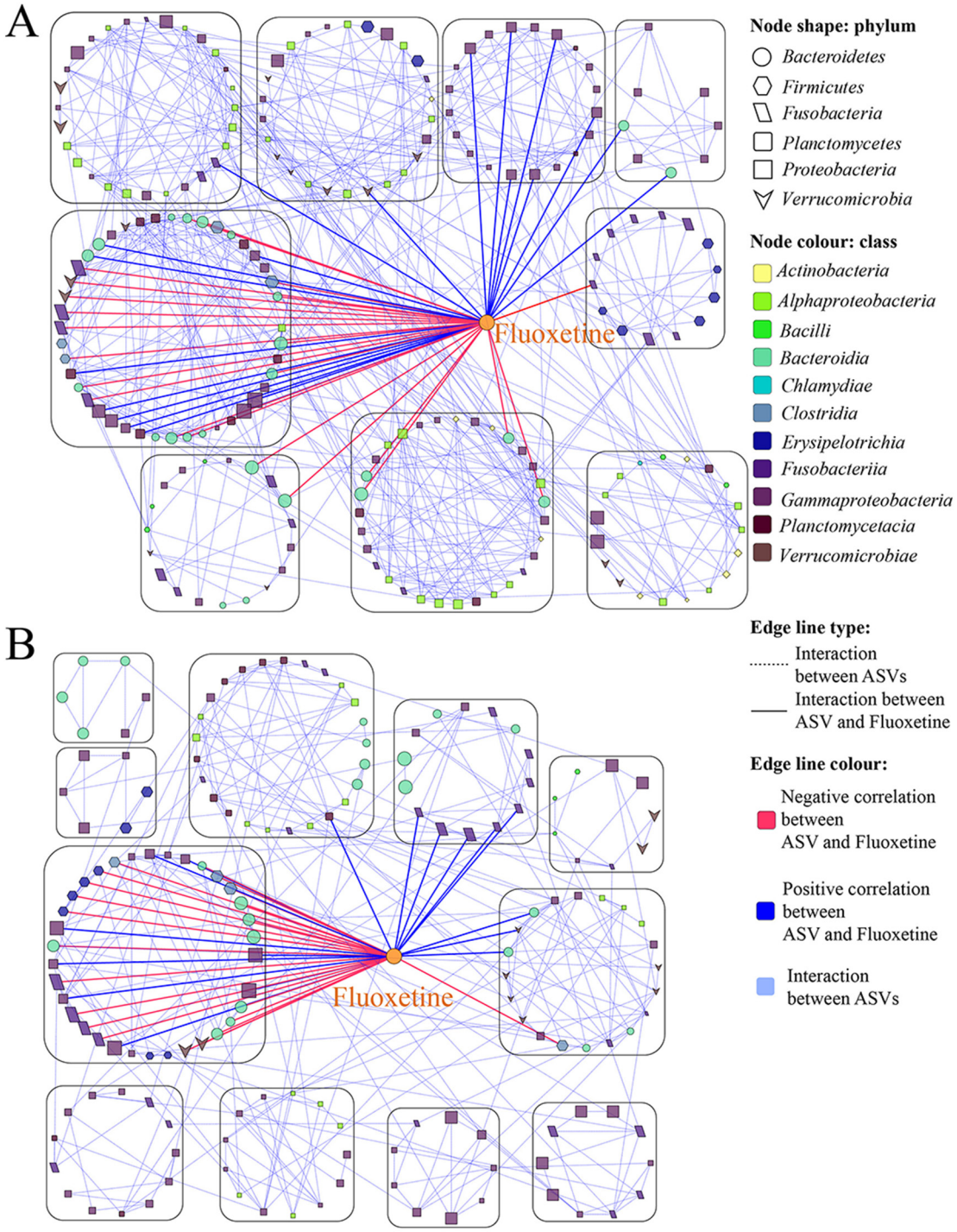

Fig. 2. Neighborhood Selection Network analyses indicating an increase of fluoxetine to be significantly correlated to major clusters of class level taxa. Clusters from (A) female fish contain more nodes and thus higher alpha diversity. Clusters from (B) male fish hold less nodes representing less abundance of class level taxa. Red lines indicate a negative correlation between fluoxetine and abundance of classes while dark blue lines indicate a positive correlation. Light blue lines represent an interaction between nodes. Shapes of nodes indicate phylum level while colour indicates what the class of node is. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Male gut microbiome response to fluoxetine exposure

The gut microbiome of male fish was not significantly altered following aqueous exposure to fluoxetine as females. Alpha diversity (Faith PD and Shannon Diversity indices) of the microbial communities in guts of males was not significantly different among treatments (Kruskal-Wallis test, $p > 0.05$, Fig. 5A & B). However, fluoxetine did significantly alter the matrix of weighted UniFrac distances among exposure groups (PERMANOVA, $F = 7.14$, $p = 0.001$, Fig. 5C). No significant differences were identified for Unweighted UniFrac distances for male fish (PERMANOVA, $F = 1.41$, $p = 0.218$, Fig. 5D). Pairwise PERMANOVAs indicate the significant differences in the weighted UniFrac were between the high exposure group and all other groups as well as between the middle exposure group and all other groups (Table S3). Homogeneity of multivariate dispersion was found significant for the weighted UniFrac between control and high as well as low and high exposure groups (PERMDISP, $p = 0.01$, Table S3).

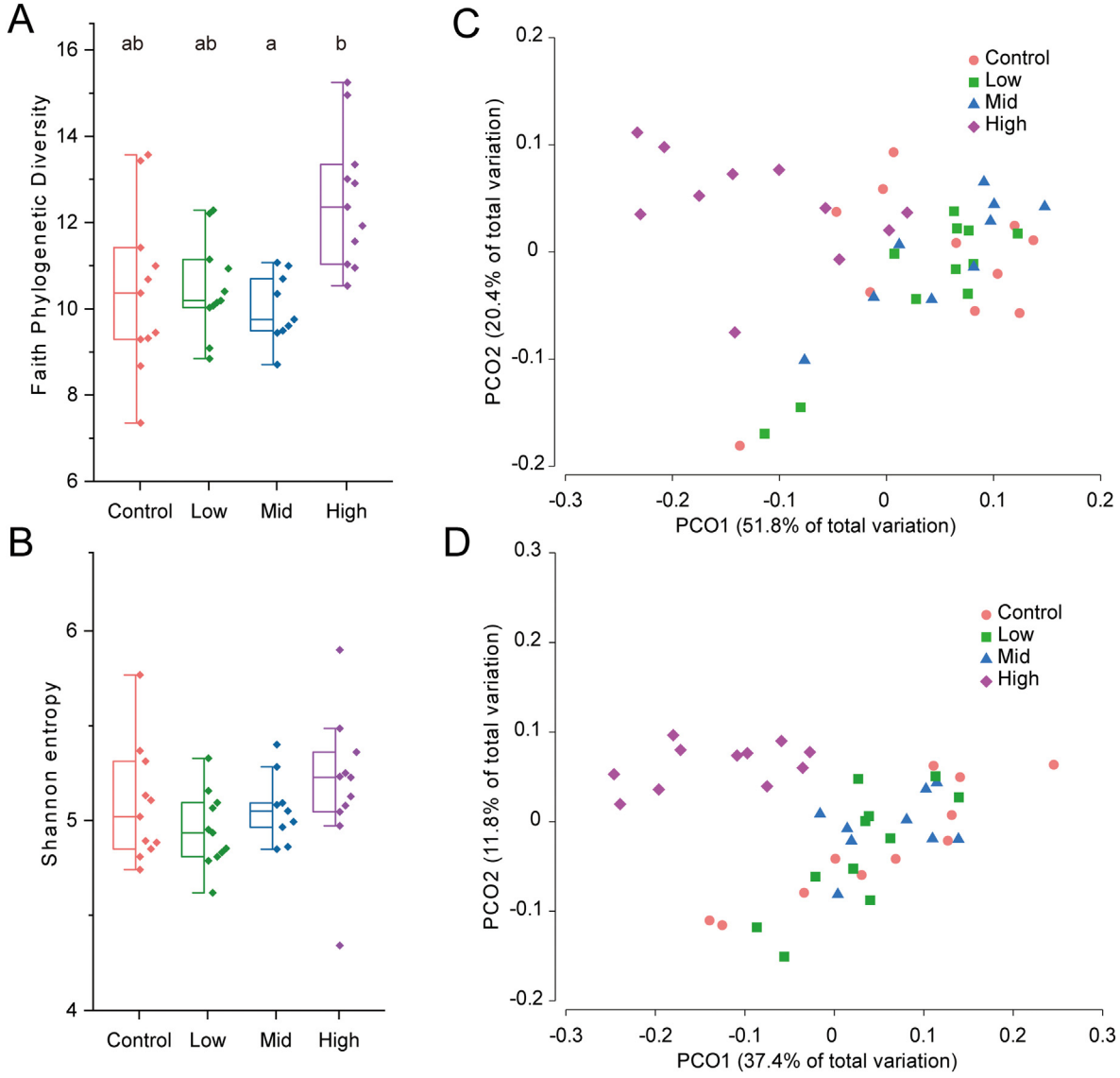

Fig. 3. Comparison of alpha diversity matrices and beta diversities of female gut microbiomes between groups. (A) Faith Phylogenetic Diversity; (B) Shannon Diversity Index; (C) weighted UniFrac distance; (D) Unweighted UniFrac distance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Proportions of thirteen abundant families in the male gut microbiome were significantly correlated with aqueous concentrations of fluoxetine, five being negatively correlated and eight being positively correlated (Fig. 6A). A LEfSe analysis found that within the top ten most relative abundant families, seven were differentially abundant when comparing each group separately to that of the control. *Akkermansiaceae*, *Erysipelotrichaceae* and *Peptostreptococcaceae* were more abundant in controls compared to the high exposure group while *Vibrionaceae* was more abundant in the high exposure group compared to the control (Fig. 6B & C). *Tannerellaceae* and *Akkermansiaceae* were more abundant in the middle group compared to the control and *Shewanellaceae* was more abundant in low exposure group compared to control. The Neighborhood Selection Analysis revealed classes of taxa in the largest cluster in male fish correlated with an increase in fluoxetine (Fig. 2B).

4. Discussion

The present study focused on whether fluoxetine can affect the gut microbiome of the fathead minnow in a sub-chronic aqueous exposure. Structures of male and female gut microbiomes were found to be significantly distinct while fluoxetine caused changes in community structure of both sexes when exposed to the greatest concentration. Concentrations to which fish were exposed during this study that were close to the environmental quality standard value of interest for fluoxetine (0.01 μg/L) (European Commission, 2000) did not significantly alter the alpha-diversity and overall composition of gut microbiome. However, the high exposure concentration (100 μg/L) significantly altered alpha and beta diversity of the gut microbiome. The high exposure level was predicted to cause a plasma concentration of 1201.3 μg/L (Table 1; Predicted Plasma Concentrations), which is greater than the human therapeutic range (H_TPC, 91–302 μg/L) (Margiotta-Casaluci et al., 2014; Pan et al., 2018).

Although the concentration of fluoxetine found to affect the gut microbiome in this study was substantially greater than concentrations observed in aquatic environments and greater than the H_TPC, there is still relevance for understanding how this data can be used for cross-species extrapolation to predict similar effects on the evolutionarily conserved molecular targets in both fish and mammals (Margiotta-Casaluci et al., 2014; Rand-Weaver et al., 2013). Results of previous studies have revealed that fluoxetine can cause anxiolytic behavior, appetite suppression, reproduction impairments and modulation of the predator-prey response in fish

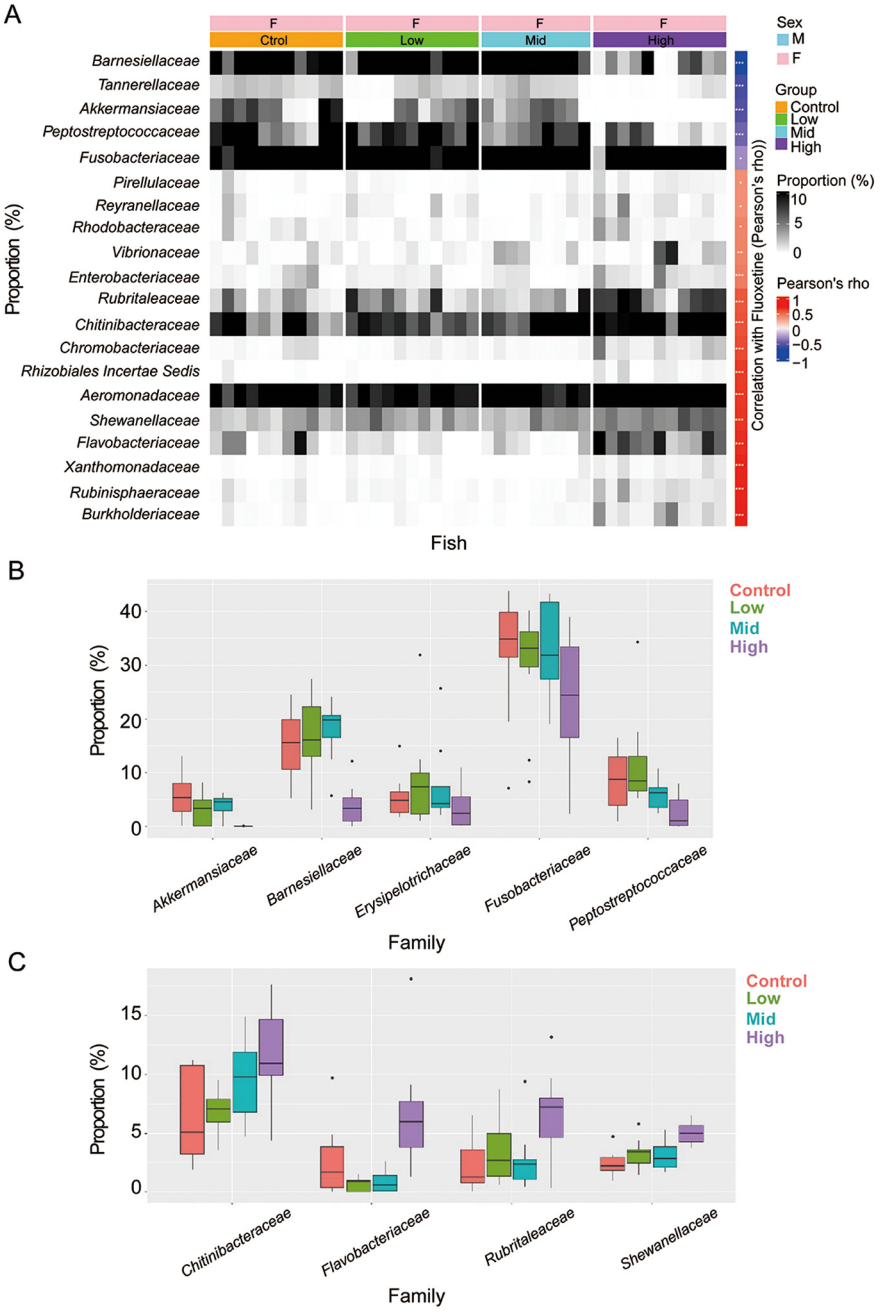

Fig. 4. (A) Heatmap of abundant family proportions of female gut microbiomes and their Pearson's correlation ecoefficiencies (rho) with exposure dosage. Abundance of family level taxa are illustrated by the greyscale where black indicates higher abundance and white indicates lower abundance. Significant level: * < 0.05, ** < 0.01, *** < 0.001. (B) Families within the top 10 most abundant in female fish that were significantly reduced in the highest dose compared to controls, (C) those, that were significantly more abundant in the highest dose compared to controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Lister et al., 2009; McDonald, 2017; Mennigen et al., 2010; Pelli and Connaughton, 2015; Weinberger and Klaper, 2014). However, like this study, some of the adverse effects identified in those exposures occurred at concentrations greater than those typically observed in the environment and exposed for relatively short durations of less than 35 days. It also demonstrates a plausible mechanism of action for SSRIs to cause changes in the microbiome of vertebrates *via* potentially binding to the homologous transporter proteins expressed by some bacteria cells (Lyte et al., 2019).

Abundant phyla identified in this study were consistent with other studies done on fathead minnow, including the two most prominent phyla *Fusobacteria* and *Proteobacteria* to be dominant within both males and females (Bridges et al., 2018; Debofsky et al., 2021; DeBofsky et al., 2020; Narrowe et al., 2015). *Bacteroidetes* and *Firmicutes*, the third and fourth most dominant phyla within fathead minnow in this study, are also prevalent in other fish gut microbiomes including rainbow trout, pinfish, silver perch, mummichog, and black sea bass (Colston and Jackson, 2016; Givens et al., 2015). Tryptophan metabolism pathways have been found to be enriched in these four dominant phyla in the fish gut (Kaur et al., 2019), which suggests that the fish gut microbiome can produce neuroactive compounds influencing the gut-brain axis (Bastiaanssen et al., 2020). Sex of the host shapes composition of the gut microbiome, where, lower alpha diversity in males has been reported in fish, mice, and humans (de la Cuesta-Zuluaga et al., 2019; DeBofsky et al., 2020; Li et al., 2016; Yurkovetskiy et al., 2013). Within families enriched in the female fish gut

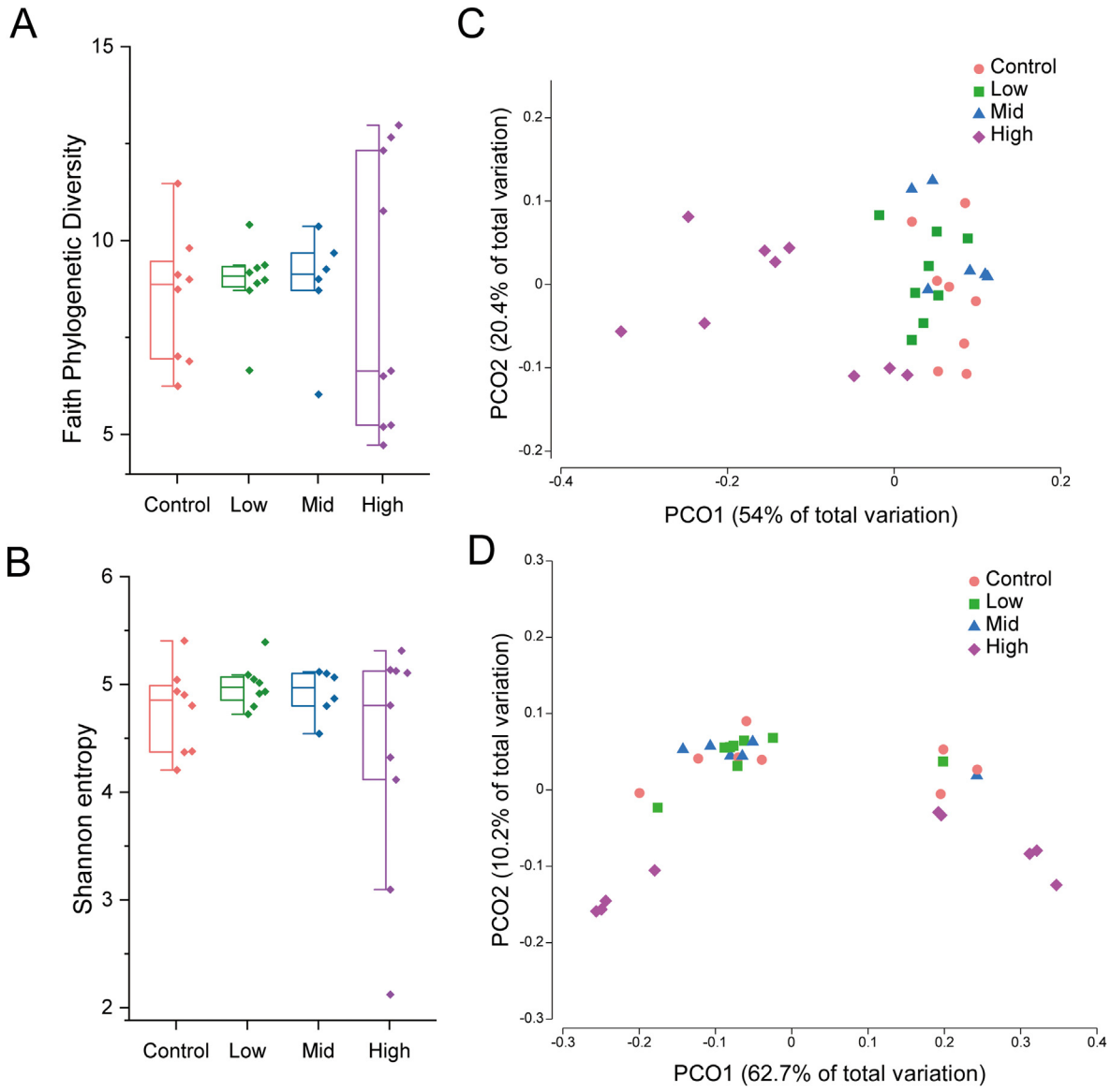

Fig. 5. Comparison of alpha diversity matrices and beta diversities of male gut microbiomes between groups. (A) Faith Phylogenetic Diversity; (B) Shannon Diversity Index; (C) weighted UniFrac distance; (D) Unweighted UniFrac distance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microbiome in this study, *Burkholderiaceae* and *Bacillaceae* are predicted to have a substantial capability to metabolize tryptophan within the vertebrate gut, as well as *Pseudomonadaceae* (Kaur et al., 2019), found in both sexes.

In general, there might be two major direct routes of interaction between fluoxetine and gut microbiota, including, antimicrobial potential of fluoxetine and the interaction with metabolite pathways generating neuroactive compounds (Chait et al., 2020; Zhang et al., 2021). Results of this study suggest antimicrobial action of fluoxetine might have caused significant alteration of gut microbiomes of both females and males, which resulted in changes in structures of the microbial community in guts of fish exposed to the highest concentration. Relative proportions of *Akkermansiaceae* and *Peptostreptococcaceae* observed here were negatively correlated with aqueous concentrations of fluoxetine, consistent with the antimicrobial activity of antidepressants found in previous studies (Chait et al., 2020; Zhang et al., 2021). It is hypothesized that the proposed antimicrobial capabilities of SSRIs are a result of binding to homologous serotonin reuptake transporters on bacterial cells. *In vitro* colonization of certain bacteria, such as *Lactobacilli* and *E. coli*, were found to be inhibited when exposed to fluoxetine due to its ability to block a biogenic amine transporter found on some bacterial cells, homologous to that of the serotonin reuptake transporter (SERT) in vertebrates (Cussotto et al., 2018; Lyte and Brown, 2018).

The representative bacterial species in human gut microbiomes are sensitive to non-antibiotic drugs, such as antidepressants (Maier et al., 2018), which suggests that alterations of gut microbiota by fluoxetine might influence fitness of the host. Importantly, changes in presence of *Akkermansia muciniphila*, in the family *Akkermansiaceae* found to be negatively affected by fluoxetine in this study, can adversely affect host health. An increase in *A. muciniphila* is linked to fatty acid oxidation while a decrease could be associated with inflammatory markers, metabolic alterations, and potential disease progression (Schneeberger et al., 2015; Sivixay et al., 2021). Importantly, *Akkermansiaceae* can regulate host serotonin through an outer membrane protein, Amuc_1100 (Wang et al., 2021).

Due to the distinct composition of gut microbiome between female and male fish, response to fluoxetine exposure presented sex-specific patterns at each taxa-level. *Clostridiales* which might have transporters similar to that of SERT (Fung et al., 2019), was identified here to be

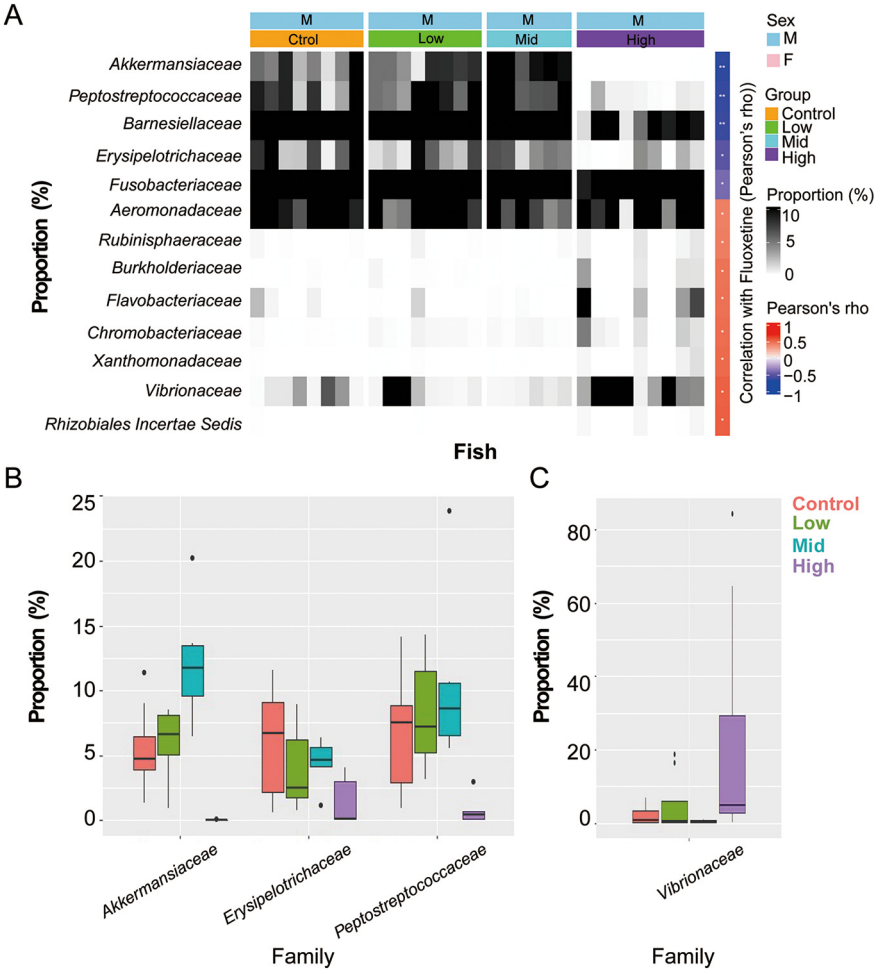

Fig. 6. (A) Heatmap of abundant family proportions of male gut microbiomes and their Pearson's correlation ecoefficiencies (rho) with exposure dosage. Abundance of family level taxa are illustrated by the greyscale where black indicates higher abundance and white indicates lower abundance. Significant level: * < 0.05, ** < 0.01, *** < 0.001. (B) Families within the top 10 most abundant in male fish that were significantly reduced in the highest dose compared to controls, (C) those that were significantly more abundant in the highest dose compared to controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly less abundant in females exposed to the greatest concentration of fluoxetine than that of controls. Abundances of Proteobacterial families: *Shewanellaceae* and *Aeromondaceae* in females and *Vibrionaceae* in males were positively correlated with concentrations of fluoxetine and have been found to be associated with anxiety, inflammation, and pathogenesis, respectively (Colwell and Grimes, 1984; De Palma et al., 2017; Reid et al., 2008; Song et al., 2016). When exposed to the greatest concentration of fluoxetine, there were several more families altered in abundance in females compared to males. The greater effect of fluoxetine on females could be a result of sex-hormones playing a role in the microbial makeup. It has been identified that the gut microbiomes of males and females might be affected differently by pharmaceuticals, where females have been found to hold more microbial genes that are antibiotic-resistant compared to males (Sinha et al., 2019).

Due to continuous release of pharmaceuticals into aquatic environments and their ability to bioconcentrate (Pan et al., 2018), chronic and multigenerational studies at environmental relevant concentrations would be of value (Polverino et al., 2021; Silva et al., 2015; Tan et al., 2020). A future chronic study should be conducted at environmentally relevant concentrations to reveal whether the presence of fluoxetine may perturb the gut microbiome of fish long-term. Work should be done addressing tissue and blood concentrations, which would also help interpret effects of fluoxetine across vertebrate species that hold conserved molecular pharmaceutical targets (Rand-Weaver et al., 2013). Finally, functional capabilities of gut microbiota perturbed by fluoxetine through integrated multi-omics techniques would be valuable to further understand the connection between the microbiome and its host.

5. Conclusions

Shifts in dominant taxa in the gut microbiomes of fathead minnows were observed in individuals exposed to the greatest concentration of fluoxetine (100 μg/L). The lowest (0.01 μg/L), which was more representative of environmentally relevant concentrations, and middle concentration (10 μg/L) did not significantly alter the gut microbiomes of fathead minnows. This study demonstrated that fluoxetine can affect the gut microbiome, yet at concentrations greater than observed in the environment. Due to evolutionarily conserved transporter proteins in vertebrates and microbiota, it can be predicted that certain gut microbiota in other vertebrates may be impacted by fluoxetine as well. Future long-term studies will help determine if fluoxetine, at environmental relevant concentrations, can affect the gut microbiome of fish. A reduction in several commensal bacteria and an increase in pathogenic and inflammatory related taxa indicate that host health may be perturbed long term. The gut microbiome plays a crucial role in host immune and nervous systems; thus, it is pertinent to establish an understanding of microbiota colonizing the gut and their functional capabilities to better understand how host homeostasis is affected through xenobiotic exposure.

Abbreviations

ASV	amplicon sequence variants
FDR	false discovery rate
HPG	hypothalamic-pituitary-gonadal
HPI	hypothalamic–pituitary–interrenal
LDA	linear discriminant analysis
LEfSe	linear discriminant analysis effect size
PERMANOVA	multivariate analysis of variance with permutation
PERMDSIP	homogeneity of multivariate dispersion
SERT	Serotonin Reuptake Transporter
SSRI	Selective Serotonin Reuptake Inhibitors

CRediT authorship contribution statement

Alana Weber: Conceptualization, Investigation, Methodology, Data curation, Visualization, Writing – original draft. **Yuwei Xie:** Conceptualization, Methodology, Supervision, Visualization, Validation, Writing – review & editing. **Jonathan K. Challis:** Resources, Data curation, Writing – review & editing. **Abigail DeBofsky:** Methodology, Data curation, Writing – review & editing. **Phillip J. Ankley:** Resources, Writing – review & editing. **Markus Hecker:** Resources. **Paul Jones:** Resources. **John P. Giesy:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing.

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

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Supplementary information for

Effects of Aqueous Fluoxetine on Gut Microbiome of Adult *Pimephales promelas*

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Table S1 Table showing reads per sample before and after filtering, denoising, merging and removable of chimeras.

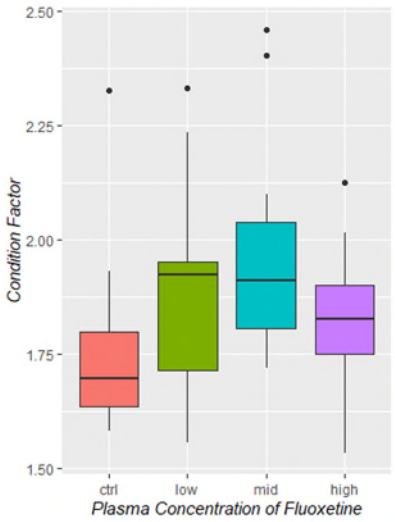
Table S2 Summary of statistics from the Neighborhood Selection Network analyzer for male and female fish.

Table S3 Pairwise PERMANOVA and PERMDISP of Weighted and Unweighted UniFrac distances to determine significant differences of gut microorganisms between exposure groups within female or male fish. Bolded and starred p values indicate significance ($p < 0.05$).

Text S1: Detailed LC-MS parameters for water concentration verification

Samples were ionized by positive mode heated electrospray ionization (HESI) with the following source parameters: sheath gas flow = 20; aux gas flow = 5; sweep gas flow = 1; aux gas heater = 300 ºC; spray voltage = 3.5 kV; S-lens RF = 60; capillary temperature = 350 ºC. A targeted-SIM and PRM (collision energy = 20) method at 60,000 resolution, AGC target = $1x10^6$, and max injection time = 100 ms was used to monitor [M+H]+ precursor and transition ions of fluoxetine (m/z 310.141 → 148.112) and fluoxetine-d5 (m/z 315.173 → 153.143). Precursor and product ions were used for quantification and confirmation, respectively.

A


2.50
2.25
2.00
1.75
1.50
Condition Factor
ctrl
low
mid
high
Plasma Concentration of Fluoxetine

B

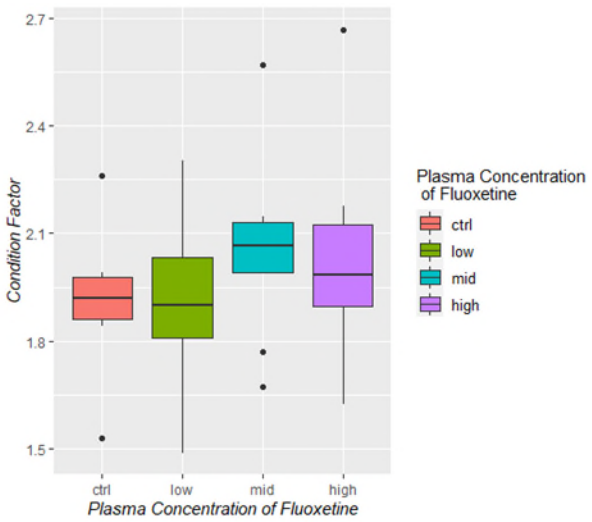

2.7
2.4
2.1
1.8
1.5
Condition Factor
ctrl
low
mid
high
Plasma Concentration of Fluoxetine
Plasma Concentration of Fluoxetine
ctrl
low
mid
high

Figure S1.

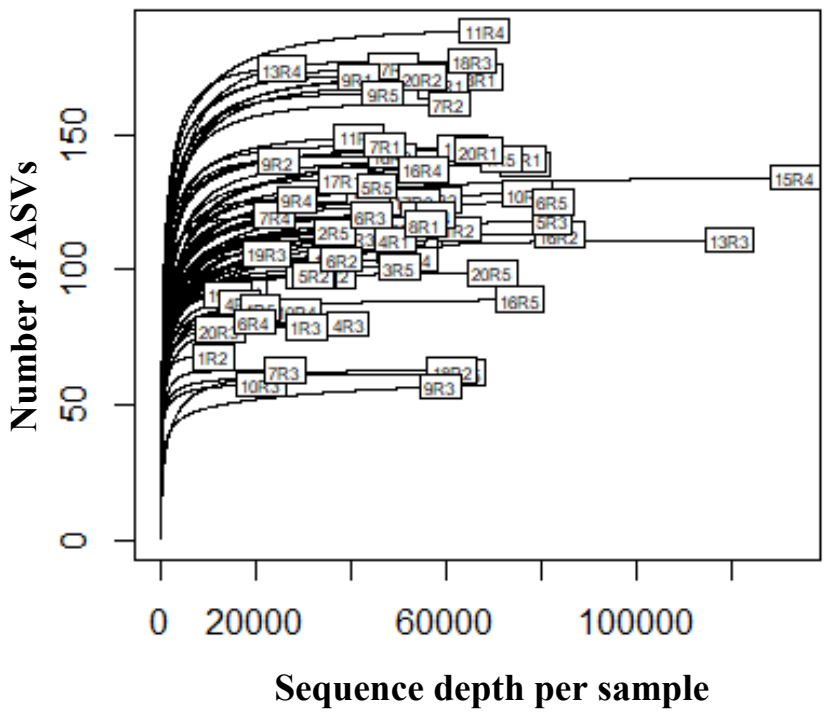

Number of ASVs
0
50
100
150
0
20000
60000
100000
Sequence depth per sample
11R4
13R4
18R3
20R2
9R5
7R2
20R1
15R4
9R2
16R4
5R5
9R4
6R5
6R3
5R3
2R5
8R1
13R3
19R3
6R2
3R5
20R5
5R2
16R5
6R4
1R3
4R3
1R2
7R3
9R3

Figure S2.

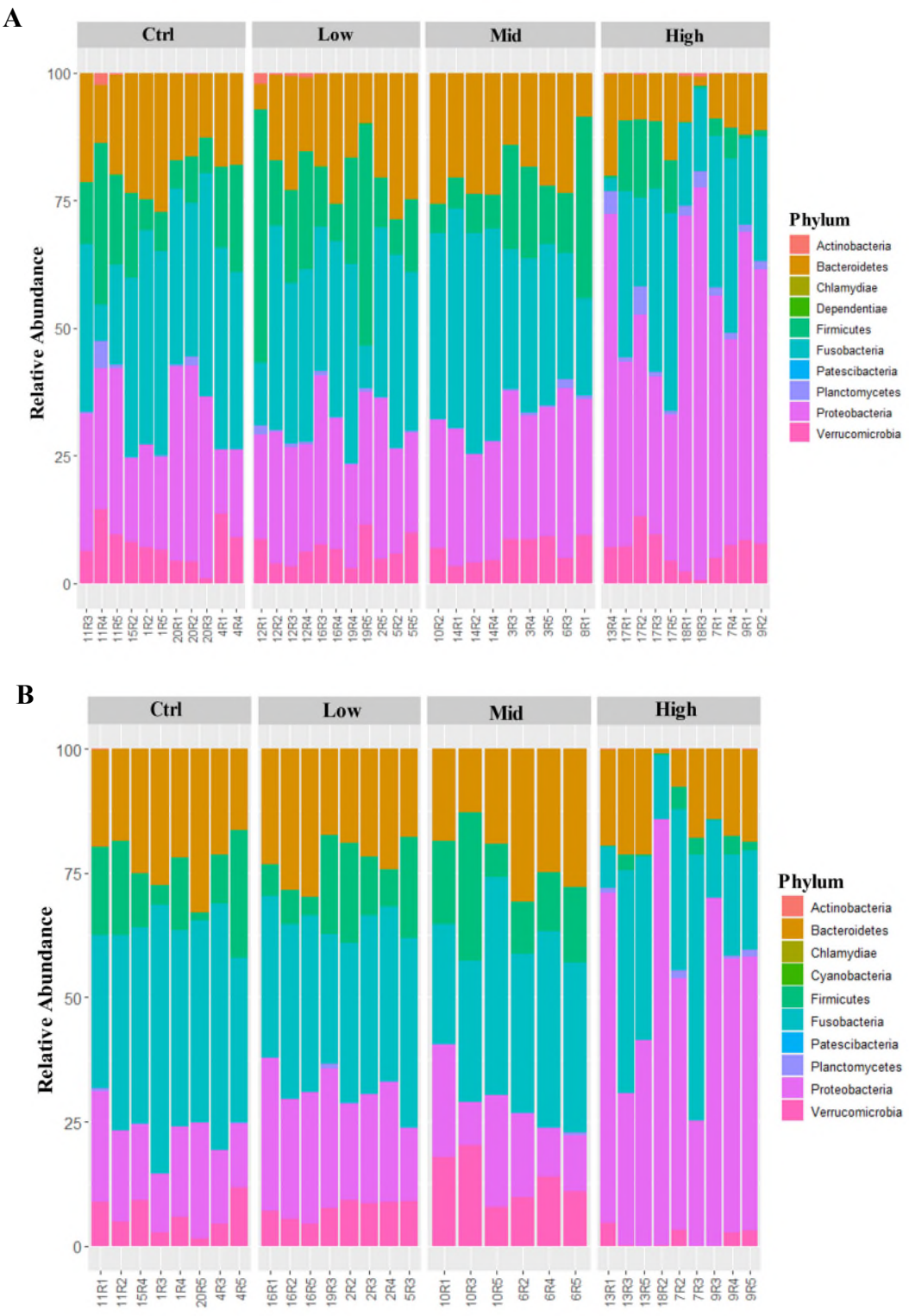


Figure S3: Relative abundances of Phyla in guts of (A) females and (B) males.

Table S1: Table showing reads per sample before and after filtering, denoising, merging and removable of chimeras.

Fish	Sex	Group	Tank	input	filtered	% of input passed filter	denoised	merged	% of input merged	non-chimeric	% of input non-chimeric
10R2	F	mid	10	108046	94679	87.63	93925	92106	85.25	83333	77.13
11R3	F	ctrl	11	64056	56303	87.9	55652	53740	83.9	47062	73.47
11R4	F	ctrl	11	85331	73265	85.86	72779	72093	84.49	71669	83.99
11R5	F	ctrl	11	82650	72023	87.14	71624	70494	85.29	68829	83.28
12R1	F	low	12	91713	79911	87.13	79587	79090	86.24	78461	85.55
12R2	F	low	12	72499	64053	88.35	63379	61684	85.08	54701	75.45
12R3	F	low	12	60670	53625	88.39	53380	53040	87.42	52710	86.88
12R4	F	low	12	49037	43129	87.95	42618	41007	83.62	37176	75.81
13R4	F	high	13	31527	26856	85.18	26654	26339	83.54	26253	83.27
14R1	F	mid	14	97706	85531	87.54	85006	83548	85.51	78720	80.57
14R2	F	mid	14	78301	69450	88.7	68930	67006	85.57	59677	76.21
14R4	F	mid	14	70557	62375	88.4	61831	60343	85.52	55397	78.51
15R2	F	ctrl	15	50619	44255	87.43	43754	42689	84.33	41745	82.47
16R3	F	low	16	61802	54324	87.9	53863	52783	85.41	50630	81.92
16R4	F	low	16	75500	65916	87.31	65438	63839	84.55	58246	77.15
17R1	F	high	17	50453	42407	84.05	42223	41862	82.97	41651	82.55
17R2	F	high	17	60410	52725	87.28	52400	51688	85.56	51332	84.97
17R3	F	high	17	75858	65995	87	65370	63221	83.34	57451	75.73
17R5	F	high	17	76433	67061	87.74	66541	65126	85.21	61027	79.84
18R1	F	high	18	73400	62661	85.37	62322	61756	84.14	60783	82.81
18R3	F	high	18	79479	68590	86.3	68362	67860	85.38	67281	84.65
19R4	F	low	19	36195	31825	87.93	31657	31453	86.9	31329	86.56
19R5	F	low	19	18164	15945	87.78	15817	15675	86.3	15621	86
1R2	F	ctrl	1	18627	16427	88.19	15957	14788	79.39	11949	64.15
1R5	F	ctrl	1	97595	86349	88.48	85644	83424	85.48	74640	76.48
20R1	F	ctrl	20	87092	76155	87.44	75701	74195	85.19	68872	79.08
20R2	F	ctrl	20	72803	63040	86.59	62462	60579	83.21	56229	77.23
20R3	F	ctrl	20	15207	13283	87.35	13135	12914	84.92	12696	83.49
2R5	F	low	2	46761	40719	87.08	40579	40338	86.26	40235	86.04
3R3	F	mid	3	57417	49084	85.49	48791	48296	84.11	48112	83.79
3R4	F	mid	3	72308	63499	87.82	62970	61311	84.79	56380	77.97
3R5	F	mid	3	76008	67313	88.56	66614	64235	84.51	55284	72.73
4R1	F	ctrl	4	68788	59910	87.09	59535	58634	85.24	56760	82.51
4R4	F	ctrl	4	23046	20298	88.08	20009	19577	84.95	19151	83.1
5R2	F	low	5	44198	38988	88.21	38628	37632	85.14	34817	78.78
5R5	F	low	5	62594	54411	86.93	53958	52480	83.84	48906	78.13
6R3	F	mid	6	59402	51610	86.88	51168	50209	84.52	48775	82.11
7R1	F	high	7	56125	49083	87.45	48891	48611	86.61	48393	86.22
7R4	F	high	7	31429	27712	88.17	27377	26416	84.05	24681	78.53
8R1	F	mid	8	77411	67395	87.06	66838	64964	83.92	59648	77.05
9R1	F	high	9	50976	44191	86.69	43874	43174	84.69	41996	82.38
9R2	F	high	9	30780	26409	85.8	26146	25606	83.19	24897	80.89
10R1	M	mid	10	23771	20814	87.56	20530	19716	82.94	18758	78.91
10R3	M	mid	10	27899	24375	87.37	24045	23342	83.67	22411	80.33
10R5	M	mid	10	76565	66718	87.14	66104	64527	84.28	59375	77.55

11R1	M	ctrl	11	54800	48121	87.81	47650	46386	84.65	44548	81.29
11R2	M	ctrl	11	84409	74539	88.31	74044	72392	85.76	66451	78.73
13R1	M	high	13	78187	68495	87.6	68211	67781	86.69	67272	86.04
13R3	M	high	13	196660	174047	88.5	173145	169770	86.33	123743	62.92
13R5	M	high	13	89009	79274	89.06	79021	77855	87.47	65108	73.15
15R4	M	ctrl	15	191025	167242	87.55	166531	163825	85.76	150610	78.84
16R1	M	low	16	42038	36686	87.27	36289	35355	84.1	33201	78.98
16R2	M	low	16	119429	105377	88.23	104800	102440	85.77	87388	73.17
16R5	M	low	16	94265	83666	88.76	83375	82609	87.63	79180	84
18R2	M	high	18	70899	63398	89.42	63112	62618	88.32	61455	86.68
19R3	M	low	19	30764	27044	87.91	26642	25675	83.46	23977	77.94
1R3	M	ctrl	1	50154	44317	88.36	43691	41737	83.22	31741	63.29
1R4	M	ctrl	1	73271	64818	88.46	64186	62061	84.7	54731	74.7
20R5	M	ctrl	20	107156	94950	88.61	94247	92192	86.04	73735	68.81
2R2	M	low	2	63308	55316	87.38	54856	53556	84.6	50439	79.67
2R3	M	low	2	64461	56831	88.16	56105	53918	83.64	45813	71.07
2R4	M	low	2	81520	71685	87.94	71106	69473	85.22	63744	78.19
4R3	M	ctrl	4	54228	47743	88.04	47371	46059	84.94	39619	73.06
4R5	M	ctrl	4	29312	25802	88.03	25516	24817	84.66	23876	81.45
5R3	M	low	5	117491	103372	87.98	102665	100337	85.4	89798	76.43
6R2	M	mid	6	51814	45631	88.07	45386	44834	86.53	44062	85.04
6R4	M	mid	6	26873	23655	88.03	23449	23060	85.81	22574	84
6R5	M	mid	6	111762	98300	87.95	97853	96427	86.28	93335	83.51
7R2	M	high	7	73769	64416	87.32	64002	63207	85.68	61451	83.3
7R3	M	high	7	35098	30972	88.24	30635	29862	85.08	26283	74.88
9R3	M	high	9	74355	65801	88.5	65612	65039	87.47	59640	80.21
9R4	M	high	9	33822	29696	87.8	29461	29157	86.21	28824	85.22
9R5	M	high	9	58023	49996	86.17	49601	48730	83.98	47120	81.21

Table S2: Summary of statistics from the Neighborhood Selection Network analyzer for male and female fish.

Network analysis Summary of Statistics	Females	Males
Number of nodes	209	171
Number of edges	562	339
Avg. number of neighbors	5,378	4,238
Network diameter	8	11
Network radius	5	6
Characteristic path length	3.52	3.949
Clustering coefficient	0.16	0.206
Network density	0.026	0.027
Network heterogeneity	0.673	0.792
Network centralization	0.187	0.221
Connected components	1	12
Analysis time (sec)	0.019	0.045

Table S3: Pairwise PERMANOVA and PERMDISP of Weighted and Unweighted UniFrac distances to determine significant differences of gut microorganisms between exposure groups within female or male fish. Bolded and starred p values indicate significance ($p < 0.05$).

Host Sex	Group	Weighted UniFrac		Unweighted UniFrac	
		PERMANOVA	**PERMDISP**	**PERMANOVA**	**PERMDISP**
Female	**Ctrl-Low**	F = 0.68, $p = 0.52$	$p = 0.76$	F = 1.06, $p = 0.34$	$p = 0.28$
	Ctrl-Mid	F = 0.76, $p = 0.56$	$p = 0.37$	F = 1.13, $p = 0.27$	***p* = 0.006***
	Ctrl-High	**F = 8.16, *p* = 0.002***	$p = 0.52$	**F = 6.36, *p* = 0.001***	$p = 0.31$
	Low-Mid	F = 0.88, $p = 0.40$	$p = 0.57$	F = 1.7, $p = 0.10$	***p* = 0.0008***
	Low-High	**F = 9.35, *p* = 0.001***	$p = 0.32$	**F = 7.28, *p* = 0.001***	$p = 0.80$
	Mid-High	**F = 9.56, *p* = 0.001***	$p = 0.078$	**F = 9.43, *p* = 0.001***	$p = 0.08$
Male	**Ctrl-Low**	F = 1.5, $p = 0.23$	$p = 0.99$	F = 0.92, $p = 0.396$	$p = 0.19$
	Ctrl-Mid	**F = 3.2, *p* = 0.041***	$p = 0.8$	F = 0.65, $p = 0.53$	$p = 0.12$
	Ctrl-High	**F = 10.0, *p* = 0.001***	***p* = 0.01***	F = 1.55, $p = 0.183$	***p* = 0.04***
	Low-Mid	**F = 2.6, *p* = 0.049***	$p = 0.81$	F = 0.62, $p = 0.661$	$p = 0.52$
	Low-High	**F = 7.7, *p* = 0.002***	***p* = 0.01***	F = 1.98, $p = 0.136$	***p* = 0.006***
	Mid-High	**F = 10.3, *p* = 0.001***	$p = 0.05$	F = 1.7, $p = 0.187$	***p* = 0.009***