Chemosphere 252 (2020) 126461

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Differential responses of gut microbiota of male and female fathead minnow (*Pimephales promelas*) to a short-term environmentally-relevant, aqueous exposure to benzo[*a*]pyrene

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HIGHLIGHTS

- Female and male fathead minnows exhibited significantly different gut microbiota.
- Exposure to BaP altered structures in female gut microbiota, but not in males.
- Exposure to BaP altered predicted functions in gut microbiota of fathead minnow.
- Gut microbiome was more sensitive to a low dose BaP than host's *ahr1* and *cyp1a1*.

ARTICLE INFO

Article history:
Received 14 January 2020
Received in revised form
1 March 2020
Accepted 8 March 2020
Available online 12 March 2020

Handling Editor: Jim Lazorchak

Keywords:
Homeostasis
Next generation sequencing
Freshwater fish
Persistence organics pollution
Molecular ecology

ABSTRACT

In addition to aiding in digestion of food and uptake of nutrients, microbiota in guts of vertebrates are responsible for regulating several beneficial functions, including development of an organism and maintaining homeostasis. However, little is known about effects of exposures to chemicals on structure and function of gut microbiota of fishes. To assess effects of exposure to polycyclic aromatic hydrocarbons (PAHs) on gut microbiota, male and female fathead minnows (*Pimephales promelas*) were exposed to environmentally-relevant concentrations of the legacy PAH benzo[*a*]pyrene (BaP) in water. Measured concentrations of BaP ranged from 2.3×10^{-3} to 1.3 μg L^{-1}. The community of microbiota in the gut were assessed by use of 16S rRNA metagenetics. Exposure to environmentally-relevant aqueous concentrations of BaP did not alter expression levels of mRNA for *cyp1a1*, a "classic" biomarker of exposure to BaP, but resulted in shifts in relative compositions of gut microbiota in females rather than males. Results presented here illustrate that in addition to effects on more well-studied molecular endpoints, relative compositions of the microbiota in guts of fish can also quickly respond to exposure to chemicals, which can provide additional mechanisms for adverse effects on individuals.

1. Introduction

Microbiota in guts of fishes are responsible for regulating a number of beneficial functions, from the development of animals (Phelps et al., 2017) to maintaining energy homeostasis (Butt and Volkoff, 2019). Bacteria line the intestinal tract and in addition to assisting with digestion of food and accumulation of nutrients (Dimitroglou et al., 2011), modulate immune functions (Rolig et al., 2015), and provide structural and functional roles in regulating the intestinal barrier (Pérez et al., 2010). In mammals, dysbiosis, or deviations from a norm, of gut microbiota is associated with a number of deleterious effects, including inflammatory bowel disease, metabolic syndrome and even obesity (Carding et al., 2015; He et al., 2019). While, little is known about the effects of exposures to

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https://doi.org/10.1016/j.chemosphere.2020.126461
0045-6535/

chemicals on structure and function of microbial communities in guts of fishes, dysbiosis of fish gut microbiota is linked to a greater prevalence of stress and disease (Llewellyn et al., 2014).

Dysbiosis of the gut microbiome can be indicative of the health status of the host and potentially reflect the quality of the environment, since gut microbiota can be disturbed by several environmental chemical compounds (i.e. Adamovsky et al., 2018; Bayha et al., 2017; Lloyd et al., 2016). For example, Japanese sea cucumbers (*Apostichopus japonicus*) exposed to Benzo[*a*]pyrene (BaP) exhibited a shift in gut microbiota; exposed sea cucumbers lost bacteria associated with beneficial functions within the host and instead saw growth of alkane-degrading bacteria (Zhao et al., 2019). Enrichment of bacterial taxa associated with hydrocarbon degradation has also been observed in southern flounder (*Paralichthys lethostigma*) exposed to contaminated sediment from the Deepwater Horizon oil spill (Bayha et al., 2017), while African catfish (*Clarias gariepinus*) injected with BaP had reduced levels of *Pseudomonas aeruginosa* (Karami et al., 2012). In mice, it was found that BaP exposure did not alter alpha diversity indices, but overall community structure and relative abundances of certain taxa were affected (Ribière et al., 2016). Also, *in vitro* in a simulated human gut system, bacteria can metabolize BaP to several metabolites (Van de Wiele et al., 2005). Finally, isolates of bacteria from skin of humans can degrade BaP (Sowada et al., 2018). Dysbiosis of communities of bacteria in the gut can ultimately have direct consequences for the host and alter effects of BaP (Levy et al., 2017; Thaiss et al., 2016).

Cross-talk between gut microbiome and modes of toxic action is gaining attention as an added layer for deciphering mechanisms of toxicity. Recently, it has been recognized that the aryl hydrocarbon receptor (AhR) is involved with regulation of host-microbiota communications (Zhang et al., 2017) and that this relationship is bidirectional (Korecka et al., 2016). Several persistent organic pollutants (POPs) are ligands of the AhR and stimulate a signal transduction pathway that results in pleiotropic effects, including upregulation of expression of cytochrome P450 1A (CYP1A), which can, in turn, biotransform those POPs to reactive intermediates (Ortiz-Delgado et al., 2007).

Because of its persistence, mode of action, and relative well-studied toxicity, BaP was chosen as a model compound for POPs, and more specifically, for polycyclic aromatic hydrocarbons (PAH). BaP is a ubiquitous PAH that originates from pyrogenic sources, including incomplete combustion of fossil fuels and petrogenic processes such as oil spills (Srogi, 2007). BaP can also suppress the immune system (Carlson et al., 2004a) and impair reproductive capacity (Booc et al., 2014), likely due to formation of reactive metabolites of BaP. BaP can cause adverse outcomes such as lesions, tumors, and developmental defects either through direct interaction with the AhR or due to the formation of reactive metabolites that can form adducts which cause damage to DNA, RNA, or proteins (Beyer et al., 2010; Tuvikene, 1995). Although the deleterious effects of BaP exposure in fish have been well-defined (i.e., Carlson et al., 2004a, 2004b; Costa et al., 2011; Nacci et al., 2002; Phalen et al., 2014), direct effects of BaP on structure and function of the microbiome are poorly studied, particularly in fishes. Therefore, it is conceivable that BaP can modulate bacterial communities in guts of fishes. This could cause adverse outcomes not predicted from more direct mechanisms at potentially lesser concentrations than predictions based on other molecular initiating events.

This study assessed the alteration of gut microbiome resulting from exposure of fathead minnows (*Pimephales promelas*) to environmentally relevant aqueous concentrations of BaP. Specific objectives were to: 1) Characterize the gut microbiome in adult fathead minnows; 2) determine the sex-specific response of gut microbiome to exposure of BaP; 3) characterize effects of BaP on the microbiome in guts of individuals exposed to BaP, relative to that of unexposed controls; and 4) compare shifts in the microbiome to "classic" biomarkers of exposure. To satisfy these objectives, the microbiota in guts of fathead minnows were characterized by use of 16S rRNA metagenetics, after aqueous exposure to environmentally relevant concentrations of BaP for four days.

2. Materials and methods

2.1. Fish husbandry, aqueous exposure, and sampling

Adult fathead minnows of approximately two years of age were obtained from an in-house stock population of the Aquatic Toxicology Research Facility at the University of Saskatchewan. Fish were acclimated at a density of two males and three females per 20-L, flow-through tank in aerated, ammonia-removed, dechlorinated facility water, sourced from the municipal Saskatoon water supply, held at 25 ± 1 °C. Conditions provided approximately three replacements of water per day with a 16h-light:8h-dark photoperiod. Fish were fed larvae of midges (*Chironomidae*), three times daily on a maintenance food ration (2% of their average wet body mass (bm) per day). Water quality conditions were monitored and were: pH 7.6 ± 0.1, dissolved oxygen 6.4 ± 0.2 mg L^{-1}, hardness 131.8 ± 6.8 mg L^{-1} as $CaCO_3$.

After two-weeks of acclimation, fish were exposed to a solvent control (0.02% DMSO), and low, medium, or high concentrations of BaP for four days, with five replicate tanks per exposure (n = 25; 3 females and 2 males per replicate tank). Nominal concentrations of control, low, medium and high doses were set at 0, 1.3, 4.0, and 12.0 μg BaP L^{-1}, which were in accordance with previously reported ranges of aqueous BaP concentrations (Adeniji et al., 2019; CCME , 2010). The experimental unit was individual tanks.

At the end of the exposure, fish were euthanized *via* blunt force trauma to the head. Samples of whole gut, containing both tissues of the fish and microbes, were excised from two females and one male per replicate tank (n = 15) 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 in the sample. Samples were placed in sterile cryovials, and held on ice until transport to a −80 °C freezer. Livers (n = 25; 3 females and 2 males per replicate tank) were removed and immediately placed in sterile cryovials in liquid nitrogen prior to storage at −80 °C. Remaining fish (n = 10; 1 female and 1 male per replicate tank) were taken for chemical and molecular analyses for a separate project (Grimard, *personal communication*). All fish were housed following the animal use protocol (Protocol #20130142) approved by the Animal Research Ethics Board at the University of Saskatchewan.

2.2. Quantification of BaP in exposure media

SGS AXYS Analytical Services Ltd. determined exposure concentrations by means of gas chromatography-mass spectrometry (GC-MS) following C18 solid-phase extraction (SPE) of 0.5- to 1-L water samples. The analysis followed the SGS AXYS method MLA-021 Rev. 12. BaP d-12 was used as the internal standard, the recovery of which ranged from 39 to 81%. Matrix spike samples exhibited recoveries of 101–102%, and lab blanks did not test positively for BaP.

2.3. 16S rRNA metagenetics

Total DNA was extracted from guts using the DNeasy PowerSoil Kit (Qiagen Inc., Mississauga, ON). Concentrations were measured

by use of a Qubit 4 Fluorometer and dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA). The V3—V4 hypervariable region of the 16S rRNA gene was amplified by use of dual-tagged primer set: forward primer (Bact-341: 5′-CCTACGGGNGGCWGCAG-3′) (Klindworth et al., 2013) and reverse primer (Bact-806: 5′-GGACTACNVGGGTWTCTAAT-3′) (Apprill et al., 2015; Fadrosh et al., 2014). The V3—V4 region was selected based on the sequencing platform, the length of hypervariable regions, and the performance of representing bacterial phyla (Graspeuntner et al., 2018; Lozupone et al., 2013; Osman et al., 2018; Yang et al., 2016). Samples were amplified by use of the Phusion Hot Start II High-Fidelity DNA Polymerase green kit (ThermoFisher Scientific) with a SimpliAmp thermal cycler (ThermoFisher Scientific) under the following conditions: Initial denaturation at 98 °C for 30 s, followed by 25 cycles of 98 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products of samples and negative controls were assessed for size and specificity using electrophoresis on a 1.2% w/v agarose gel and purified using the Qiagen QIAquick PCR Purification Kit (Qiagen Inc.). All purified products were quantified with the Qubit dsDNA HS assay kit and concentrations were adjusted to 1 ng/μL with molecular-grade water. Purified products were pooled at equal concentrations for subsequent sequencing, and libraries were constructed using the NEBNext® DNA Library Prep Master Mix Set for Illumina® kit (New England BioLabs Inc., Whitby, ON). Libraries were quantified prior to sequencing using the NEBNext® Library Quant Kit for Illumina® Phi-X (15%) was spiked in to increase complexity of the library and assess sequencing error. Sequencing was performed on an Illumina® MiSeq sequencer (Illumina, San Diego, CA) using a 2 × 300 bp paired-end chemistry kit.

2.4. Quantitative Reverse-Transcription Real-Time PCR

Liver tissues of individual fish were homogenized in QIAzol lysis reagent (Qiagen Inc.) in a TissueLyser II (Qiagen Inc.) by use of one 5-mm stainless-steel bead (Omni International, Kennesaw GA, USA). Total RNA was extracted in a QIAcube automatic extraction unit (Qiagen Inc.) by use of the RNeasy Plus Universal kit (Qiagen Inc.), following the manufacturer's protocol. The concentration and purity of extracted RNA were assessed by spectral content profiling using a QIAxpert instrument (Qiagen Inc.). RNA samples were stored at −80 °C until further analysis.

Quantitative Reverse-Transcription Real-Time PCR (qPCR) was used to quantify expression of select genes (*cyp1a1, ar, bax*, *esr1*, *vtg*) in liver of individual fish. Primers were designed using Primer-BLAST (Ye et al., 2012) and based on results of previous studies (Supplemental Table 1), and purchased from Thermo-Fisher Scientific (Waltham MA, USA). Complementary DNA (cDNA) was synthesized from 2.5 μg total RNA by use of QuantiNova™ Reverse Transcription kit (Qiagen Inc.). Expressions of targeted genes were measured by use of a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Carlsbad CA, USA) and the QuantiNova™ SYBR Green PCR kit (Qiagen Inc.), following the manufacturers' protocol and including a melt curve to ensure specificity of primers. Reactions were done in duplicates of 10 μL reaction volumes. Relative expressions of target genes were normalized against expression of two housekeeping genes, *ef1* and 18S rRNA (Supplemental Table 1), according to methods described by Simon (2003).

2.5. Bioinformatics

Dual-tagged raw reads were checked for quality and demultiplexed using a custom pipeline with USEARCH v. 11 (Edgar, 2010) and QIIME1 v. 1.9.0 (Caporaso et al., 2010). Demultiplexed sequences were then imported to QIIME2 v. 2019.4 (Boylen et al., 2019). To describe microbial communities, amplicon sequence variants (ASVs) were generated and chimeras were removed using the DADA2 pipeline (Callahan et al., 2016). To remove low quality base pairs at ends of reads, the forward read was truncated to 280 base pairs, while the reverse read was truncated to 230. Additionally, to remove the primer region from each sequence, the first 35 bases were removed. Taxonomy was then assigned in QIIME2 by use of the feature classifier trained against the SILVA 132 reference database (Bokulich et al., 2018; Quast et al., 2013). ASVs that did not align to bacterial kingdoms were discarded as well as sequences that were found fewer than ten times (Bokulich et al., 2013). The majority of ASVs that did not align to bacterial kingdoms were classified as "unknown". On average, 40% of reads assigned to each sample remained after the cleaning process. A full list of reads per sample pre- and post-cleaning can be found in Supplemental Table 2. To reduce biases resulting from differences in sequencing depth, based on a rarefaction graph (Supplemental Fig. 1), the feature table was rarefied at the lowest sequencing depth (7884 sequences per sample). Alpha diversities (Shannon diversity, observed ASVs, Chao1, and Faith phylogenetic distance (PD)), or diversity of prokaryotic communities within samples, and beta diversities (Bray-Curtis dissimilarity, unweighted UniFrac, and weighted UniFrac), or differences between samples, were calculated in QIIME2. Tax4Fun2 was used to predict functional profiles, which use UProC to match microbes with known protein families (Meinicke, 2015), then match those profiles to metabolic pathways by use of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (Kanehisa et al., 2015) based on 16S bacterial sequences (Wemheuer et al., 2018). Data can be accessed at https://dx.doi.org/10.20383/101.0196.

2.6. Statistics

Statistical analyses were performed using R Statistical Language v. 3.6.1 (R Core Team, 2013). For all statistical tests, assumptions of normality and equal variance were tested by visual inspection using a Q-Q plot, a Shapiro-Wilk test, and a Levene's test (Borcard et al., 2011). If these conditions were met, a two-sided Student's t-test was used to compare sex effects, and analysis of variance (ANOVA) was used to assess exposure effects. If conditions were not met, Welch's *t*-test was used when comparing sex effects and the Kruskal-Wallis test was used when comparing exposures (Dalgaard, 2006). Shannon diversity values were cubed to obtain normality of data for the purpose of statistical analysis. Dunnett's tests were used to compare exposure groups to the solvent control using the DescTools package in R (Signorell, 2019). A nested ANOVA design was used to investigate effects of exposure within sex for alpha diversity, but since sex appeared to drive the separation of alpha diversity ($p < 0.001$) whereas exposure did not ($p > 0.05$), the two sexes were analyzed separately to assess effects of BaP exposure on species composition and alpha diversity of gut microbiota. Unless otherwise noted, statistics were calculated using vegan statistical package (Oksanen et al., 2019) and visualized with gglot2 (Wickham, 2016).

Statistical Analysis of Metagenomic Profile (STAMP) bioinformatics software v. 2.1.3 was used to visualize significantly different bacterial taxa at the family level and KEGG pathways based on sex and exposure (Parks et al., 2014). Beta diversity was analyzed with the phyloseq package (McMurdie and Holmes, 2013), by use of the feature table and rooted tree generated in QIIME2. Principal coordinate analysis (PCoA) plots were generated with Bray-Curtis dissimilarities by use of an agglomerated feature table to combine taxa of the same genus and reduce complexity of ordination. To determine statistical differences in the Bray-Curtis dissimilarities

between males and females, the Adonis2 test was performed using the vegan statistical package in R (Oksanen et al., 2019). First a nested Adonis design was used with exposure nested in sex, but because the results returned a significant interaction between the variables ($F = 5.74$, $p < 0.001$), the sexes were analyzed and plotted separately to assess exposure effects. The significant interaction implied that ordination of samples based on treatment was not the same between male and female fish. Gut microbial community differences based on exposure were calculated using the agglomerated taxa table using the pairwise. adonis2 function in R (Martinez Arbizu, 2019). To correct for multiple comparisons, a Benjamin-Hochberg False Discovery Rate adjustment was used.

For gene expression analyses, only females in the highest exposure group were selected for qPCR analyses. Gene expression results from qPCR were tested for statistical significance using a one-way ANOVA (Wickham, 2016). In all cases, results were considered significant at a significance level of $\alpha < 0.05$.

3. Results

3.1. Concentrations of BaP in water

Measured concentrations of BaP in water were 0.83 ng L^{-1} (solvent control), 32 ng L^{-1} (low), 88 ng L^{-1} (medium), and 1338 ng L^{-1} (high). These values were less than nominal concentrations of 0, 1300, 4,000, and 12,000 ng BaP L^{-1}. The disparity likely resulted from BaP binding to the plastic materials in the exposure apparatus.

3.2. Composition and sex differences of gut microbiota of fathead minnows

Metagenetics of 16s rRNA revealed the composition of gut microbiome of fathead minnows exposed to BaP under controlled laboratory conditions. A total of 9346 ASVs were identified, but after contingency-based filtering, 2715 non-singleton ASVs of 123 unique genera of bacteria among 58 samples (23 males, 35 females) remained. The dominant phyla in the gut of fathead minnows (% ± S.E.) were *Proteobacteria* (63% ± 3%), *Fusobacteria* (18% ± 2%), *Bacteroidetes* (8% ± 1%), *Firmicutes* (7% ± 2%), *Spirochaetes* (1.7% ± 0.04%), and *Planctomycetes* (1.4% ± 0.04%).

Female and male fathead minnows exhibited significantly different microflora in their guts. Microbiota in guts of females exhibited greater biodiversity than microbiomes of males (Shannon diversity: $t = 5.77$, $p < 0.001$; Chao1: $t = 6.45$, $p < 0.001$; Faith PD: $t = 7.44$, $p < 0.001$; observed ASVs: $t = 6.59$, $p < 0.001$); Table 1). Of the 19 most abundant families (Fig. 1), ten families exhibited significantly greater abundances in guts of female relative to males (Fig. 2A; Welch's *t*-test, corrected $p < 0.05$). *Vibrionaceae* was the only abundant family significantly more abundant in guts of male fish relative to females ($p < 0.001$). Community structure of microbiomes in guts of fish were significantly different between male and female fish (Adonis test: $F = 21.15$, $p < 0.001$). This separation was also supported by results of PCoA, with the first axis explaining 46.1% of the variation due to sex (Fig. 2B). In total, 232 pathways within predicted metagenomic functional profiles were significantly different between male and female fish (Supplemental Table 4). For instance, fatty acid biosynthesis (fatty acid metabolism and fatty acid degradation) was enriched in female fish (adjusted corrected $p < 0.001$) and bacterial chemotaxis (bacterial invasion of epithelial cells) was enriched in males (adjusted $p < 0.001$).

3.3. Sex-specific responses of fathead minnows to BaP

Exposure to BaP did not result in significant differences in alpha diversities (Chao1, number of ASVs, Shannon, Faith Phylogenetic Distance) in male or female fish ($p > 0.05$; Table 1). In female fish, the calculated alpha diversity metrics did increase in the high concentration exposure group, but the results were not statistically significant. Based on results obtained with the Dunnett's test, in

Table 1
Shannon diversity index, number of observed amplicon sequence variants (ASVs), Chao1, and Faith Phylogenetic Distance (PD) are presented for male and female fish at the different exposure levels (mean ± standard error, sample size n = 7–10 for females, 4–8 for males).

Sex	Male				Female			
Treatment	Solvent	Low	Medium	High	Solvent	Low	Medium	High
Shannon	3.16 ± 0.35	3.15 ± 0.21	2.36 ± 0.57	3.07 ± 0.61	4.18 ± 0.24	4.03 ± 0.32	4.20 ± 0.25	4.36 ± 0.28
Observed ASVs	44.38 ± 7.34	32.67 ± 3.37	29.20 ± 7.12	38.00 ± 7.38	60.67 ± 4.48	62.40 ± 6.48	71.86 ± 4.38	80.44 ± 8.45
Chao1	45.01 ± 7.65	32.83 ± 3.42	29.20 ± 7.12	38.00 ± 7.38	60.70 ± 4.49	62.40 ± 6.48	72.26 ± 4.43	80.82 ± 8.55
Faith PD	6.48 ± 0.71	5.12 ± 0.42	5.39 ± 0.62	6.12 ± 0.67	8.45 ± 0.57	8.69 ± 0.62	9.78 ± 0.43	10.10 ± 0.69

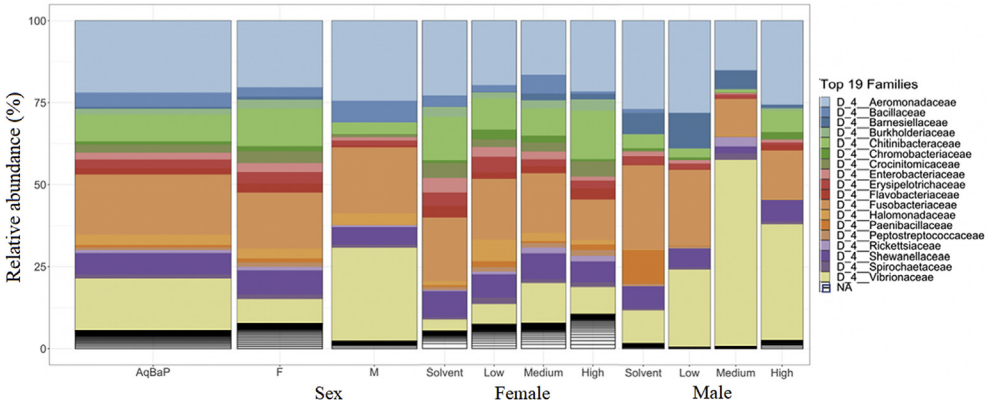

Fig. 1. Relative proportions of 19 most abundant bacterial families. The first bar shows relative proportions of these taxa for all fish within the study. The second 2 bars show female and male fish, respectively. The final bars show proportions of taxa for females and males, exposed to each concentration of BaP, respectively.

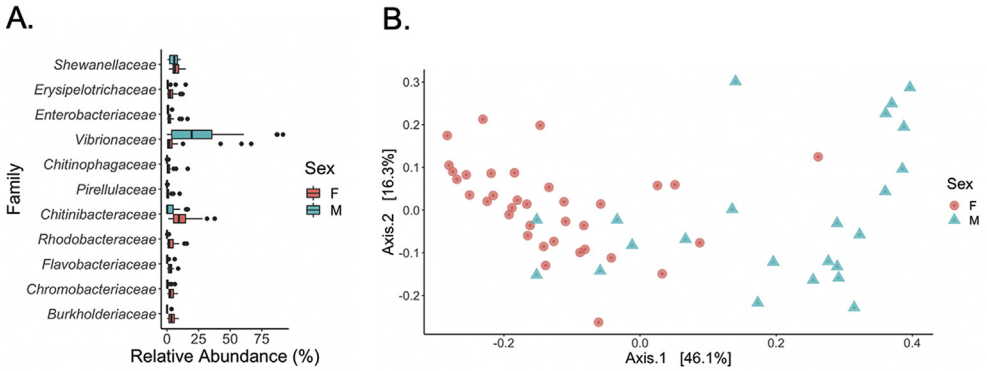

Fig. 2. (A.) Relative abundances (%) of bacterial families that were significantly different between male and female fish ($p < 0.05$). Females are shown in red and males are shown in blue. (B.) Ordination of bacterial communities based on sex (PCoA with Bray-Curtis dissimilarity matrix). Females are shown with red circles and males are shown with blue triangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

female fish, only three bacterial families were present in statistically greater abundances relative to that of the solvent control (Fig. 3A): *Isosphaeraceae* (absent in solvent control, but 0.53% of total reads in the highest exposure group, adjusted $p = 0.04$), *Rubritaleaceae* (4.1× more in the highest exposure group relative to the control, adjusted $p = 0.03$) and *Xanthobacteraceae* (absent in solvent control, but 0.05% of total reads in fish exposed to the greatest concentration of BaP; adjusted $p = 0.04$). In male fish (Fig. 3B), *Vibrionaceae* were enriched in the two greatest exposure groups relative to that in the solvent control, but only statistically significant in the medium group relative to control (adjusted $p = 0.02$).

Exposure to BaP significantly altered beta diversities in female gut microbiota, but not in males. The highest exposure group showed significant separation from the solvent group (Adonis2 test: $F = 2.32$, $p = 0.04$); the medium exposure group approached significance separation from the solvent group (Adonis2 test: $F = 1.97$, $p = 0.06$). Clustering in a PCoA also showed these differences, with the solvent control group clustering separately from communities in guts of fish exposed to the highest and medium

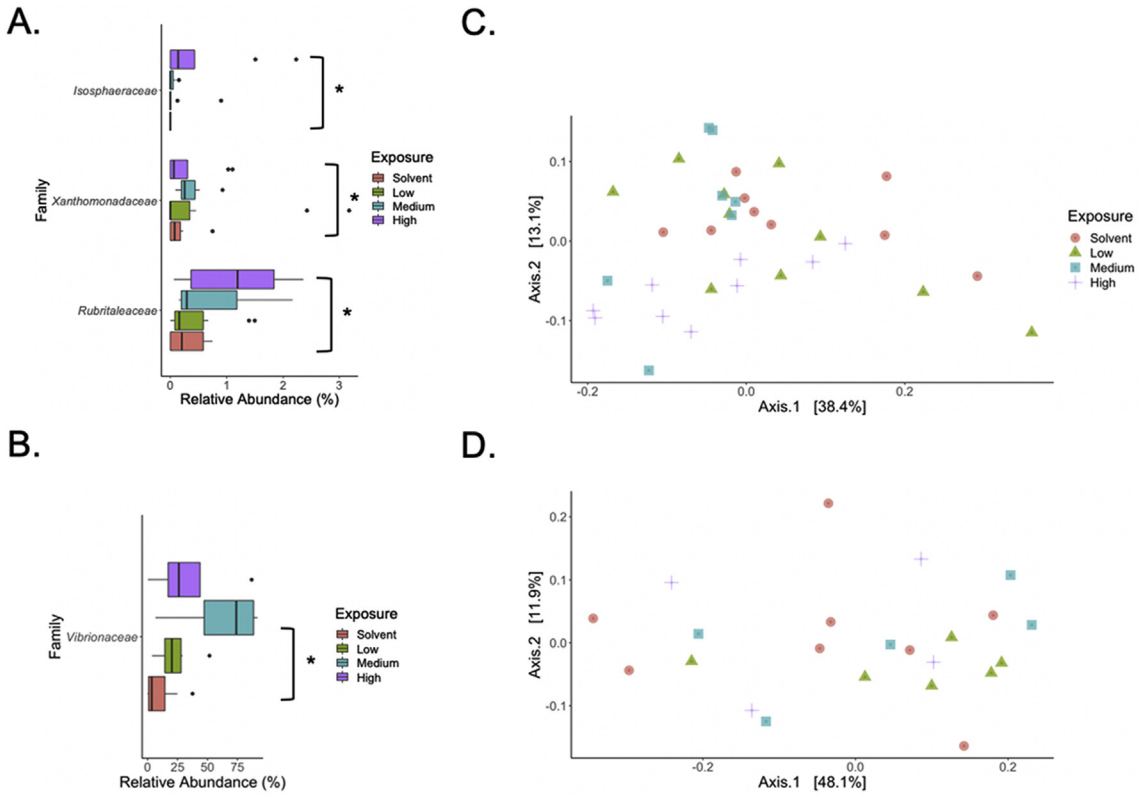

Fig. 3. (A). Relative abundances (%) of bacterial families that are significantly different in exposure groups relative to the solvent control ($p < 0.05$) in female fish. (B). Ordination of bacterial communities based on exposure (PCoA with Bray-Curtis dissimilarity matrix) in female fish. (C). Relative abundances (%) of bacterial families that are significantly different in exposure groups relative to the solvent control ($p < 0.05$) in male fish. (D). Ordination of bacterial communities based on exposure (PCoA with Bray-Curtis dissimilarity matrix) in male fish. In the relative abundance plots, solvent control is shown in red, low in green, medium in blue, and high in purple. In each PCoA, the solvent control group is shown in red circles, the low group in green triangles, the medium group in blue squares, and the highest group in purple crosses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

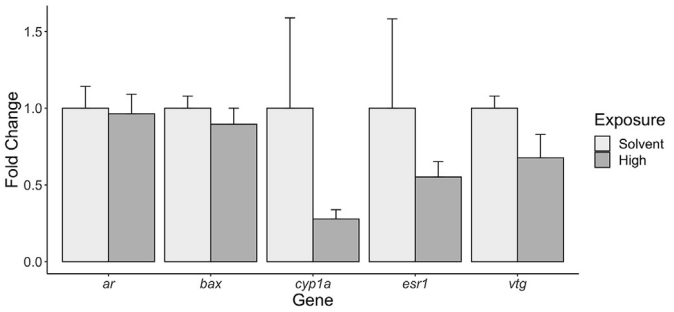

Fig. 4. Relative fold change in expressions of androgen receptor (ar), BCL2 Associated X, Apoptosis Regulator (bax), Cytochrome P450, family 1, subfamily A, polypeptide 1 (cyp1a1), estrogen receptor alpha (esr1), and vitellogenin (vtg) in female fish after four days of exposure to BaP (n = 4–5). Solvent control fish are shown in light grey while the highest exposure group are shown in dark grey. No significant differences between the groups were observed ($p > 0.05$).

concentrations of BaP (Fig. 3C), with the first axis explaining 38.4% of the variation. There were no significant differences among exposure groups for the male fish ($p > 0.05$; Fig. 3D), although the first axis did explain 48.1% of the variation, suggesting another variable was contributing to this separation.

While not statistically significant ($p = 0.08$), the predicted polycyclic aromatic hydrocarbon degradation pathway did increase from the solvent group (0.008% ± 0.001%) to the highest exposure group (0.02% ± 0.004%) in female fish. KEGG pathway analysis predicted no pathways with differential enrichment between BaP exposure groups in male fish.

3.4. Host gene expression analyses

Analysis of differential expression of genes revealed minimal effects of a short-term exposure on the relative gene expression of genes in female fathead minnows exposed to the highest concentration. Abundances of transcripts of all measured genes was reduced relative to the controls; however, none of these results were statistically significant (Fig. 4).

4. Discussion

Results of this study confirmed that the community compositions of gut microbiota in fathead minnows were consistent with other freshwater fish, which indicates that gut microbiota have conserved biological function among fishes. Dominance of *Proteobacteria* has been found in mummichogs (*Fundulus heteroclitus*) (Givens et al., 2015), rainbow trout (*Oncorhynchus mykiss*) (Desai et al., 2012), and fathead minnows (Narrowe et al., 2015). *Fusobacteria*, *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, and *Planctomycetes* are also commonly found phyla in guts of fishes (Colston and Jackson, 2016). Furthermore, at the family level, consistent with Narrowe et al. (2015), *Aeromonadaceae, Shewanellaceae, Flavobacteriaceae,* and *Fusobacteriaceae*, were dominant in guts of both male and female fathead minnows. In addition to these families, Roeselers et al. (2011) found *Vibrionaceae, Burkholderiaceae*, and *Enterobacteriaceae* as core members of the microbiome in guts of zebrafish, which is consistent with the results of this study.

Within the animal kingdom, sex is a major determinant of the microbiome, which was supported in this study. Compositions of the bacterial community were significantly different between males and females. Significantly greater alpha diversity of microbiota in guts of females, relative to that of male fish as well as significant separation between communities of males and females based on beta diversity metrics, indicates that sex is a major driver in determining microbial composition. This finding in intestinal bacterial communities of fish is consistent with results of other studies that found significantly greater diversity of bacterial communities in guts of wild, female, largemouth bronze gudgeon (*Coreius guichenoti*) (Li et al., 2016), and sex-dependent responses to diet in three-spined stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*) (Bolnick et al., 2014). Differences in microbiomes of mice have been observed (Org et al., 2016; Yurkovetskiy et al., 2013), where microbiota in guts of female mice also have a greater alpha diversity value than do male mice. Furthermore, this pattern has also been observed in humans (Gao et al., 2018; Ying et al., 2015).

KEGG pathway analysis also predicted significant differences between microbiota in male and female fish. Fatty acid pathways were enriched in females relative to males; enrichment of fatty acid metabolism has been seen in microbiota of female relative to male mice (Davis et al., 2017), which might be attributed to hormonal differences (Decsi and Kennedy, 2011). In male fish, enrichment of bacterial chemotaxis and bacterial invasion of epithelial cells might be indicative of the BaP exposure inducing susceptibility of pathogen attack, as discussed below (Carlson et al., 2004b). Differences in microbial communities between guts of males and females are believed to result from hormonal regulation, driven by androgen receptor activity (Markle et al., 2013), with the cyclic nature of estrogen cycles maintaining a greater microbial diversity (Yurkovetskiy et al., 2013). Furthermore, disparities in growth and behavior between males and females might also regulate this pattern (Bolnick et al., 2014; Jašarević et al., 2016; Schnorr et al., 2014).

The gut microbiome was more sensitive to a four-day environmentally relevant BaP exposure than host gene expression (*ahr1* and *cyp1a1* genes). In addition to differences between male and female fathead minnows, this study revealed that following exposure to a small concentration of aqueous BaP for a duration as short as four days, the microbiome was significantly altered, even when gene expression did not reflect this change. It was expected that a four-day exposure to BaP would result in induction of *cyp1a1*, since binding of BaP to the AhR induces expression of *cyp1a1* (Shimizu et al., 2000), but it is likely that the concentration of BaP in the water was too low to stimulate this change. Similar patterns of sensitivity were observed in earthworms (*Eisenia fetida*), where microbial groups were more sensitive to triclosan than enzyme activity within the organisms (Ma et al., 2017).

Sex-specific responses to contaminant exposure are well-documented in the field of ecotoxicology. For example, Vega-López et al. (2007) found that CYP1A ethoxyresorufin *O*-deethylase activity and alcohol dehydrogenase in male, Chapultepec splitfin fish (*Girardinichthys viviparous*) was more up-regulated than females when exposed to polychlorinated biphenyls. Sex is also a significant factor in understanding toxicokinetics following exposure to xenobiotics (Gochfeld, 2017). Specifically, in regards to BaP, Booc et al. (2014) reported reproductive functions are more impaired in male fish than in female fish. Males are also more susceptible to the pathogen attack from bacteria, viruses, and parasites than females (Galligan and Fish, 2015; Thornton et al., 2018), and since BaP is an immunosuppressant (Carlson et al., 2004b), it is conceivable that males would be more impacted by these effects.

In the context of this study, differences in responses of the microbial communities in guts of male and female fish to BaP might be linked to microbial degradation of BaP within guts of female fish and immune impairment in male fish. Although BaP did not result in significant changes in alpha diversity of the microbiota in guts of either sex, community composition was significantly different in

the highest two exposure groups relative to the control in female fish. Female fish in the highest exposure group had increased levels of *Xanthobacteraceae* associated with hydrocarbon degradation (Oren, 2014). Enrichment of the PAH degradation pathway in female fish along with bacterial taxa associated with PAH degradation support the hypothesis that bacteria are degrading BaP in female fish. To test this hypothesis that specific strains of bacteria present in the guts of these fish can degrade BaP, *Xanthobacteraceae* would need to be cultured and exposed to BaP, similar to the study by Sowada et al. (2018), where bacteria cultures from human skin were exposed to BaP and degradation of the BaP was assessed. This would also allow for confirmation that PAH degrading pathways are enriched using transcriptomics rather than the predictive analysis of Tax4Fun2. However, the potential exists that *Xanthobacteraceae* does not interact with BaP in the same manner outside the body, as was the case with *P. aeruginosa* cultured from African catfish (Karami et al., 2012).

In this study, in guts of males exposed to the greatest concentration of BaP, the microbiome was enriched with *Vibrionaceae*, a bacterial family associated with disease in fish (Colwell and Grimes, 1984). Although not significantly correlated in this study, relative abundance of *Vibrio* can be negatively correlated with *Aeromonas* growth and result in an increase in neutrophil recruiting (Rolig et al., 2015). Neutrophils release cytokines that recruit immune cells and mediate a proinflammatory response to an infection; therefore, neutrophils are imperative for host defense (Harvie and Huttenlocher, 2015; Rolig et al., 2015).

Results of this study highlight the need to incorporate the microbiome as well as ecological theory, particularly in regards to differences between males and females, into our understanding of the link between chemical exposure, the microbiome, and the host organism (Adamovsky et al., 2018; Jašarević et al., 2016). Toxicants are capable of purturbing the microbiome at environmentally relevant concentrations, which may result in deleterious effects on the host, or it may provide protection (Dietert and Silbergeld, 2015). Either way, these rapid shifts in the microbiota may be useful as an early-warning sign of exposure. Limitations of this study include a small range of concentrations used to elucidate effects, the use of a single time point, and the use of Tax4Fun2. It should be noted that Tax4Fun2, while informative, is predictive and might not reflect actual functioning of the bacteria within the fish (Evariste et al., 2019), nor should it replace transcriptomic studies.

5. Conclusions

This study revealed that short-term low-dose BaP exposure significantly altered the community structure of gut microbiota in female, but not male, fathead minnows. Taxonomic analyses revealed minimal effects of BaP exposure on male fish, but no changes were observed in predictive KEGG pathway analysis. In female fish, the exposure revealed the potential for enrichment of hydrocarbon-degrading taxa and therefore functionality. Gene expression did not reveal any impacts of BaP on internal processes within the fish. More work is needed to elucidate the mechanisms behind changing community composition and to better understand the relationships between BaP exposure, the bacteria, and the effect on the host.

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.

CRediT authorship contribution statement

Abigail DeBofsky: Investigation, Methodology, Data curation, Writing - original draft. **Yuwei Xie:** Conceptualization, Methodology, Validation, Writing - review & editing. **Chelsea Grimard:** Resources, Data curation. **Alper James Alcaraz:** Resources, Data curation. **Markus Brinkmann:** Writing - review & editing. **Markus Hecker:** Funding acquisition, Writing - review & editing. **John P. Giesy:** Conceptualization, Project administration, Funding acquisition, Writing - review & editing.

Acknowledgements

Funding was provided by “Next generation solutions to ensure healthy water resources for future generations” funded by the Global Water Futures program, Canada First Research Excellence Fund (#419205) and the GenomeCanada EcoToxChip project. Dr. Brinkmann was also supported through the Global Water Futures program. Profs Giesy and Hecker were supported by the Canada Research Chairs Program of the Natural Sciences and Engineering Research Council of Canada (NSERC). Prof. Giesy was also supported by a distinguished professor in residence by the Environmental Science Department of Baylor Uniersity, Waco, Texas.

AppendixA. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126461.

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