# Environmental Pollution

**Responses of juvenile fathead minnow (Pimephales promelas) gut microbiome to a chronic dietary exposure of benzo[a]pyrene**

--Manuscript Draft--

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| Abstract:          | The microbiome has been described as an additional host “organ” with well-established beneficial roles. However, the effects of exposures to chemicals on both structure and function of the gut microbiome of fishes are understudied. To determine effects of benzo[a]pyrene (BaP), a model persistent organic pollutant, on structural shifts of gut microbiome in juvenile fathead minnows (Pimephales promelas), fish were exposed ad libitum in the diet to concentrations of 1, 10, 100, or 1,000 µg BaP g⁻¹ food, in addition to a vehicle control, for two weeks. To determine the link between exposure to BaP and changes in the microbial community, concentrations of metabolites of BaP were measured in fish bile and 16S rRNA amplicon sequencing was used to evaluate the microbiome. Exposure to BaP only reduced alpha-diversity at the greatest exposure concentrations. However, it did alter community composition assessed as differential abundance of taxa and reduced network complexity of the microbial community in all exposure groups. Results presented here illustrate that environmentally-relevant concentrations of BaP can alter the diversity of the gut microbiome and community network connectivity. |
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Cover Letter

December 15, 2020

Dear Editor,

Environmental Pollution

We would like to thank the editor and reviewer for the professional and helpful comments. Please find our revised manuscript entitled “Responses of juvenile fathead minnow (Pimephales promelas) gut microbiome to a chronic dietary exposure of benzo[a]pyrene” for your consideration. We have thoughtfully taken into account these comments. Please find a point-by-point response to each comment and actions taken to revise the manuscript in the attached disposition of reviewer comments. We are happy to discuss this with you if necessary.

We look forward to hearing your response to our revisions.

Sincerely,

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John P. Giesy, Ph.D. FRSC, FSETAC, DSAHC
Toxicology Program and Department of Veterinary Biomedical Sciences, University of Saskatchewan
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Disposition of reviewers’ comments on ENVPOL-S-20-02217

Overall Response: The authors thank the anonymous reviewers very much for their valuable comments and suggestions to improve the quality of the manuscript. Careful consideration was given to all of the comments and suggestions and modifications have been made accordingly to the revised manuscript. Our itemized responses to each comment are listed below, along with a clear indication of the revision made to the manuscript and where those revisions occur.

Reviewer #1: This study deals with effects of dietary BaP on gut microbiome composition in fathead minnow. The study is well conducted and very informative. The analysis of microbiome structure and composition is really hard to synthesize but it seems that tools used in the present study are relevant.

The authors may explain why they chose to work on juveniles and not on adults, and which kind of differences could be suspected depending on the age of animals.

Response/Action: Explanation was added (Line 81). Responses of gut microbiome to BaP could be differential between sex and age groups since the gut microbiota of fish is shaped by sex and age. Sex differential differences of fathead minnow were observed in our previous publication (DeBofsky et al. 2020), differential responses of gut microbiota 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. In a controlled environment (constant diet and environment), the morphological changes during development are likely the dominant drivers of changes in the microbiota (Stephens, W. et al, 2016). However, few studies talked about responses of gut microbiome of juveniles to exposure of PAHs.


In the discussion section, I would remove the "dose-dépendent" effect as for me it is not evident that the response is dose-dépendent: the diversity was only modified for the highest dose tested and if considering fig 4, there are substantial differences for

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each dose compared to control but to me not in an aggravation following the increasing concentration.

Response/Action: Removed as suggested.

The graphical abstract is not enough informative, I am not sure to understand that it represents gut microbiome composition with species and networks (I guess) without reading the article.
Response/Action: More information has been added into the graphical abstract (posted below) as suggested.

Reviewer #2: The study, "Responses of juvenile fathead minnow (Pimephales promelas) gut microbiome to a chronic dietary exposure of benzo[a]pyrene", exposed fathead minnows to increasing concentrations of a BaP-dosed food source to observe changes in microbiome communities over the course of a two week period. They found that greater alterations to the microbiome resulted with higher concentrations used in the study. I feel that the information presented in this study is informative and interesting and that it can be used to compare along with other microbiome studies to better understand the role of PAHs, and other contaminants, to alterations in the microbiome in fish species, which is currently not well understood. However, there are some major and minor comments I feel need to be addressed, which are stated below:

I understand that the total PAH concentrations that have been previously reported in the environment can be quite high- in different fish species, prey items, and sediment samples- but concentrations of 1,000 ug BaP/g would, and 100 ug BaP/g, to be frank,
would be more representative of positive controls than measured concentrations found in the environment. I feel that the way the concentrations are presented are a little misleading as written in the materials and methods section by reporting tPAH values and feel that a comparison of the concentrations used in terms of BaP specifically needs to be better justified. I do feel that this is touched upon a little more in the discussion section, but this needs to be clarified more in the materials and methods section, particularly because this higher concentration really seems to be the major driver for the changes observed in the microbiome.

**Response/Action:** We agree. A statement has been added to clarify that BaP was selected as a model compound for PAH (Line 115 - 119).

Line 55- Should add "serves" after "and"

**Response/Action:** Revised as suggested. Line 56.

Lines 77-78- Apostichopus japonicus should be italicized

**Response/Action:** Revised as suggested. Line 78.

Line 81- Sentence should be reworded

**Response/Action:** Revised as suggested. Line 89.

Line 115- I would add "an" before "internal"

**Response/Action:** Revised as suggested. Line 128.

Lines 161-162- Is there an example of another study that could be referenced of conducting the analysis this way?

**Response/Action:** Citation added. Mean or median can be used as imputation value to replace missing data for small-size datasets. Line 178.

Line 178- Remove extra ",." in subtitle to stay consistent throughout manuscript

**Response/Action:** Revised as suggested.

Line 228- Should read "fish" not "fishes" since only referring to one species of fish, the FHM
**Response/Action:** Revised as suggested.

*Line 259- Should read "such as"

**Response/Action:** Revised as suggested. Line 277.

*Line 266- Lower case "Metabolites"

**Response/Action:** Revised as suggested. Line 284.

*Line 289- I would clarify this sentence a little bit to change to wording "growth substrate", possibly, as it sounds like certain taxa are growing on BaP.

**Response/Action:** Revised to avoid ambiguity. Line 307.

I would clarify an approximate age the FHM*s* were in this study. I know you reported them as juvenile in the main body and supplemental materials section, but because maturation can influence the microbiome I think providing this information would allow this data to be more comparable to other studies to better understand how communities change over time, regardless of contaminant.

**Response/Action:** An approximate age of the fish was added. Line 100.

**Reviewer #3: SUMMARY**

DeBofsky et al exposed a common environmental toxicology model organism, the fathead minnow, to Benzo[a]pyrene (BaP) at 3 orders of magnitude differences of concentration in the food (1 ug / g food to 1000 ug / g food). They then measured both (importantly) bile metabolites for products of BaP metabolism, and whole gut microbiome structure, via 16S sequencing. The authors find that, especially at the highest exposure, shifts in alpha and beta diversity metrics support an effect of BaP on microbiome structure.

Strengths of this study are the relatively large sample sizes (though hampered a bit by a very high percentage of dropouts), the fact that BaP bile metabolites were measured directly (the authors should be commended for this), and the potential to compare to their work to a similar experiment with adult fish (though I wish this last were more explicit). I do have some concerns about some of the analyses and conclusions, detailed below.

*This is a well-written manuscript. Thank you for that.*
MAJOR COMMENTS

line 94, and 155-156  The authors state at 155-156 that "No significant differences in measurements were observed among tanks," on these lines, but it's not clear to me here what "measurements" encompasses. One of my questions is whether or not there are tank effects that might be affecting the authors' conclusions. If I play devil's advocate a bit, then it appears that some of the observable effects on microbial community seem to only happen at the highest (1000 ug) exposure, both at the diversity level (alpha diversity, Figure 3a and related metrics) and at the network level. However, if one of three 3 tanks in the 1000ug treatment group (18 samples total) is driving most of these differences, then these results become a bit less exciting. I don't think this is likely to be the case, based on a count of dots away from the cloud in the figure 3A ordination, for example, but it might help to see these samples colored by tank. Do samples cluster non-randomly in their community composition by tank? In general, I think I just need to have a bit more explanation about what the authors did to rule out tank effects beyond this one phrase.

Response/Action: We agree with the reviewer. This should have been better clarified. Fish were randomly assigned to each group (Line 102). There were no significant differences in the calculated indices of alpha- and beta-diversity and body size between tanks within each treatment group. We modified the text to state this (Line 170).

For example:

Observed ASV (Kruskal wallis, pairwise)

| Species  | DNA_0_R2 | DNA_0_R3 | DNA_1_R1 | DNA_1_R2 | DNA_1_R3 | DNA_10_R1 | DNA_10_R2 | DNA_10_R3 | DNA_100_R1 | DNA_100_R2 | DNA_100_R3 | DNA_1000_R1 | DNA_1000_R2 | DNA_1000_R3 |
|----------|----------|----------|----------|----------|----------|-----------|-----------|-----------|------------|-------------|-------------|------------|------------|-------------|-------------|
| p-value  | 0.40     | 0.34     | 0.45     | 0.47     | 0.38     | 0.46      | 0.47      | 0.58      | 0.34       | 0.40        | 0.50        | 0.47       | 0.38       | 0.50        |
| q-value  | 0.87     | 0.78     | 0.82     | 0.78     | 0.88     | 0.78      | 0.89      | 0.91      | 0.78       | 0.88        | 0.91        | 0.89       | 0.78       | 0.91        |

faith_pd indices (Kruskal wallis, pairwise)

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Weighted unifrac distance matrix (Pairwise PERMANOVA)

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Lines 266 and following. The authors attempt to relate bile metabolite concentrations to the literature, which is nice, but also dependent apparently on *maximum* values observed, instead of mean values, which are probably more relevant for justifying that these are "environmentally relevant" exposures. Do these citations support mean values that are in the range of the 1000 ug / g exposure with microbiome effects? **Response/Action:** We agree with the reviewer. This was poor wording on our part. We reported mean values found in the environment but stated maximum. This has been changed to reflect that (Line 288). Concentrations from highly polluted areas still are environmentally relevant and needs more attentions.

Lines 197-199. The authors report that 33 (22%) of samples were dropped because of low sequencing depth, and 34 (23%) of samples were dropped because fish were sexually differentiating over the course of the experiment (which, especially in light of their prior work showing sex differences in microbiome-shifting exposures, is prudent). Unfortunately, this is a large number of samples to throw out. Fortunately, the authors retain reasonable sample sizes after tossing this large number of samples. However, this does raise the concern that some differences in microbiome could be due to sexual maturation that wasn't detected yet, as the authors’ own prior work suggests a strong difference between adult male and female gut microbiomes. At minimum, for comparison to other studies, what are the DPH ages of these juveniles in this experiment (is this reported somewhere I missed)? Were there tank effects related to which fish were removed because of sexual maturation? **Response/Action:** We agree with the reviewer. We added an age of the fish in the methods section (Line 100). There were no tank effects of the fish that were removed, but these fish were outliers relative to the rest of the fish in the tanks. We added a statement to the methods indicating that we looked for sexual maturity of the fish during dissection (Line 122). Elaborative quality control was applied since host variables can confound gut microbiota studies. At least in a controlled environment (constant diet and environment), the morphological changes during development are
likely the dominant drivers of changes in the microbiota (Stephens, W. et al, 2016). We expected a few samples might be discarded as outliers during the experiment design.


*Given the authors have done a related experiment in adult animals (DeBofsky et al 2020), I was expecting more of a comparison to that prior work in the Discussion (beyond the existing dominant phyla comparison). What do we learn from this work that is different or adds to this adult work, with respect to exposure effects?*

**Response**: We agree. In the earlier paper, we had a limited sample size and a very small range of concentrations. Because we saw an effect, we believed it worthwhile to examine the effects at a higher concentration. Additionally, in that paper, an aqueous exposure route was used. Because the solubility of PAHs (and in particular BaP) is extremely low, it is unlikely fish would be exposed in this manner. Utilization of a dietary exposure route represents a more realistic route of exposure, since fish would likely encounter PAHs in the sediment or *via* their diet. Finally, by using juveniles, we attempted to eliminate the potential for sex to be a driving factor as was shown in our previous paper. We did see some sexual maturation in our fish over the course of the experiment, but overall, the majority of our fish did not exhibit primary or secondary sexual characteristics. Explanation was added (Line 93).

*Since fish were exposed *ad libitum* in the diet, what is the chance that exposure through the skin/gills/etc. became a significant route of exposure? This might be irrelevant, but since the authors bring up that exposure route for BaP (line 68) results in different distribution of body burden, and because they previously found exposure effects on microbiome from water exposure (in female adult fish), I’m wondering if they ever measured BaP in the water. Acknowledging that they measured bile metabolites, I still wonder if it’s possible the effect is a complex combination of dietary and inadvertent water exposure.*

**Response/Action**: This is a good point, however, since the tanks were siphoned daily, and since the solubility of BaP is very low in water, it is unlikely that BaP built up on the water. We did add a statement about siphoning the tanks daily (Line 106).

*Line 205. I love that the authors measured bile metabolites directly. Since the authors do have these individual fish bile BaP metabolites, I would like to see this*
The correlation value is not actually reported or plotted. They only report here a weak p-value. Same for line 211 and ASVs. Table 1 does not consider this metabolite data, unfortunately. Also, are these alpha diversity results (and associated significance estimates) calculated from the rarefied ASV table?

Response/Action: We have now reported the correlation value for Shannon and ASVs (Line 224 and 230). The alpha diversity results are calculated from the rarefied ASV table (Line 160).

Line 215 and methods. Can the authors at least somewhere briefly justify why they are using an unweighted Unifrac metric for beta diversity? Since all of Figure 2 and Figure 3B highlight differences in abundances of key differentially abundant taxa, it seems odd to use an abundance-naïve distance metric for beta diversity comparisons.

Response/Action: The reason why we used unweighted Unifrac is that the changes of low abundant or rare species played important roles in the structure changes of gut microbiota exposed to BaP. We are showing the plot below for CAP with weighted Unifrac. We also didn’t see much for statistical differences when using weighted Unifrac.

Line 218. I'm surprised the 100 ug exposure group did not differ from controls. I'm sorry, but I don't understand this explanation for the lack of beta-diversity differences with controls: "since that group exhibited smaller variation of microbial composition
than did other groups." How was this measured (variation) and how would this contribute to fewer differences with controls?

Response/Action: Thank you for the comment. We reran the Pairwise PERMANOVA in QIIME2 instead of Adonis2 in R. The 100 µg BaP g⁻¹ exposure group also exhibited significantly distinctive community structures than that of the controls. The stats have been updated (Line 234-237).

Line 252 and Figure S4. I find this figure hard to draw any conclusion from, and certainly the correlation and best fit line are misleading. There are 5 data points, and if I remove the control (arbitrarily set to be 1e-3 ug BaP / g food), then there is no meaningful negative correlation among BaP exposure concentrations (from 1 to 1000 ug BaP / g food). This figure is also lacking proper units.

Response/Action: Thank you for the comment. Figure S4 was removed. Parameters of network were summarized in SI Table S4. Explanation was added (Line 271).

MINOR COMMENTS

Line 25: what is metagenetics?

Response/Action: For clarity, just changed it to amplicon sequencing (Line 25).

Abstract, Line 27-28: I think the authors should qualify these results and explain which were true for all exposure concentrations vs. only for the highest concentration.

Response/Action: Revised as suggested (Line 28).

Line 47: A citation or two would be appropriate, given you state there have been "considerable research" in mammals.

Response/Action: Added (Line 47).

Line 81: remove extra "of"

Response/Action: Removed as suggested.

line 102. Perhaps this is hidden in the refs, but I don't understand how sediment concentrations of PAHs are relevant to the justification for choosing BaP exposure values.
Response: The thought is that fish could be exposed to such high concentrations of PAHs via incidental ingestion.

Line 147: perhaps this is in the referenced paper, but could the authors add how taxonomy was assigned, as that seems key to many of the analyses looking at differential abundance of taxa and inferred pathways.

Response/Action: Added as suggested (Line 154-156).

Line 148: please include parameters for PICURSt2 or state that defaults were used, for reproducibility.
Response/Action: Added (Line 165).

Line 161: I'm a little concerned about assigning the average value to missing bile data. At minimum, I think it would be helpful to summarize here how many missing values there were.
Response: This is hard to say. With the control/low concentration groups, the majority of the bile data was “empty” as the values were below the LOD, particularly for OH-BaP, which is already not present in high abundance. In general, we had fewer values for the low concentrations. For the higher concentrations, it was generally between one and three samples that were averaged.

Line 164. How were "larger masses and/or length" defined in removing outlier fish
Response/Action: Revised to avoid ambiguity (Line 180).

Line 213. Figure 3C does not support this point.
Response/Action: This was a typo. Changed to Fig. 3A (Line 232).

Line 237. This is a pet peeve of mine with PICRUST, and I know the authors put predicted in this section title, but can the authors change this to state these are *inferred* MetaCyc pathways. There is no shotgun sequencing in this paper.
Response/Action: Revised as suggested (Line 255).

Line 263. Can you easily compare this apparent discrepancy to Narrowe et al, who you cite as using fathead minnows and who also used juvenile fish?
Response: We cannot compare our results with Narrowe’s paper since relative abundances of phyla were not apparent in the results of that paper.

Line 266. Pattern --> Patterns

Response/Action: Revised (Line 286).

Line 689. "Letters denote statistical significance within metabolite groups." What letters? What metabolite groups? Also, what are asterisks for?

Response: Fixed. This was a typo. Asterisks stand for significance of correlations. Line 709.

Line 701. Should probably include the statistical test used here, as well.

Response/Action: Added (Line 720).

Figure 4. This is a grumpy comment, but I can’t say I really get much from this figure. All I see is that E looks different, but we expected that based on decreased alpha diversity, too. What are you trying to demonstrate, here? I thought it was more about the alteration of the network. In that case, you might consider a layout where each panel is the same nodes in the same layout, and the edges are drawn differently in each panel depending on presence, and/or nodes are greyed out if removed from that network. That might better demonstrate the shift in the network structure. This also might be too unwieldy. I’d say only address this if another reviewer independently is equally as grumpy about these hairball figures. Otherwise, feel free to ignore this comment.

Response/Action: It’s a challenge to present complex network of gut microbiome. Figure 4 was simplified by ThematicMap App (http://apps.cytoscape.org/apps/thematicmap) and updated for better biological understanding.
Responses of juvenile fathead minnow (*Pimephales promelas*) gut microbiome to a chronic dietary exposure of benzo[a]pyrene

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ABSTRACT

The microbiome has been described as an additional host “organ” with well-established beneficial roles. However, the effects of exposures to chemicals on both structure and function of the gut microbiome of fishes are understudied. To determine effects of benzo[a]pyrene (BaP), a model persistent organic pollutant, on structural shifts of gut microbiome in juvenile fathead minnows (*Pimephales promelas*), fish were exposed *ad libitum* in the diet to concentrations of 1, 10, 100, or 1,000 µg BaP g⁻¹ food, in addition to a vehicle control, for two weeks. To determine the link between exposure to BaP and changes in the microbial community, concentrations of metabolites of BaP were measured in fish bile and 16S rRNA amplicon sequencing was used to evaluate the microbiome. Exposure to BaP only reduced alpha-diversity at the greatest exposure concentrations. However, it did alter community composition assessed as differential abundance of taxa and reduced network complexity of the microbial community in all exposure groups. Results presented here illustrate that environmentally-relevant concentrations of BaP can alter the diversity of the gut microbiome and community network connectivity.

KEYWORDS: homeostasis; Next-generation sequencing; persistent organic pollutants; fish; metagenomics; 16s rRNA metagenetics

Main findings:

Environmentally-relevant concentrations of BaP can alter the diversity of the gut microbiome and community network connectivity *via* dietary exposure route.
1. Introduction

The gut microbiome is a crucial component of an animal host and is responsible for a number of important biological processes, including energy and nutrient cycling (Dimitroglou et al., 2011), regulation of intestinal barrier functions (Pérez et al., 2010), and modulation of the immune system (Rilig et al., 2015). Disturbance of structure of the gut microbiome is associated with several harmful effects, including inflammatory bowel disease, metabolic syndromes, stress, and disease (Carding et al., 2015; He et al., 2019; Llewellyn et al., 2014). Although considerable research efforts to understand links between xenobiotics and gut microbiome have been conducted in mammals (i.e., Breton et al. 2013; Lefever et al., 2016; Ribière et al., 2016), the effects of toxicants on gut microbial community structure and function in fish are largely unknown.

Complex interactions between the host microbiome and xenobiotics can vary by route of exposure. In fish, due to partitioning and bioaccumulation, exposure to environmental toxicants can occur via multiple routes and transport of persistent organic pollutants (POPs) tend to accumulate in food chains (Schlenk et al., 2008; Wang and Wang, 2006). POPs can be taken up through the gut, skin, and gill, and can ultimately have deleterious effects on fish in freshwater ecosystems. The mucosal layers of the skin, gill, and gut all contain microbiomes that provide protective barriers for fish defense against pathogens (Salinas and Magadán, 2017) and serve as an intermediary in the metabolism pathway of some toxicants (Adamovsky et al., 2018).

Benzo[a]pyrene (BaP) is a promising model polycyclic aromatic hydrocarbon (PAH) to study the potential effects of toxicants on the gut microbiome because much is known about its effects in the host but little on the microbiome. BaP originates from sources such as the incomplete combustion of fossil fuels and oil spills (Srogi, 2007) and has well-characterized
deleterious effects in fishes (Carlson et al., 2004a; Costa et al., 2011; Nacci et al., 2002; Phalen et al., 2014). BaP up-regulates the expression of cytochrome P450 1A (CYP1A), which results in the biotransformation of BaP to reactive intermediates (Ortiz-Delgado et al., 2007). Adverse outcomes of exposure to BaP include the development of lesions and tumors, as well as suppression of immune function (Beyer et al., 2010; Carlson et al., 2004b; Tuvikene, 1995). Conjugated products from phase II metabolism of BaP often end up in the bile of exposed fish (Nishimoto et al., 1992). In fish, routes of exposure to BaP are primarily through ingestion with food, incidental ingestion of sediment, dermal contact, and via ventilation across the gills (Mccarthy et al., 2003; Nichols et al., 1996; Snyder et al., 2015; Tuvikene, 1995). Route of exposure is a critical component of the distribution of BaP. For example, aqueous exposure of BaP in rainbow trout (*Oncorhynchus mykiss*) results in detectable BaP throughout the body, while dietary exposure mainly results in accumulation of BaP in the bile and intestine (Sandvik et al., 1998).

Effects of BaP on the structure and function of the gut microbiome of fishes are not well studied. Aqueous exposures of adult fathead minnows to small concentrations of BaP resulted in an enrichment of taxa associated with hydrocarbon degradation and community compositional shifts (DeBofsky et al., 2020a), and aqueous exposure of Japanese sea cucumbers (*Apostichopus japonicus*) to BaP resulted in fewer bacteria associated with beneficial functions within the host accompanied by an increase in alkane-degrading bacteria (Zhao et al., 2019). BaP exposure also induced dysbiosis of the microbiome and inflammation of adult western mosquitofish and zebrafish (Xie et al., 2020). Furthermore, concentrations of PAHs in dorsal muscle after an oil spill correlated with gut community composition in walleye (*Sander vitreus*) and with several families of bacteria within the gut microbiome of other species of wild fish (DeBofsky et al.,
However, since the gut microbiome of fishes may be shaped by the morphological changes during development, at least in a controlled environment (Yan et al., 2016), there still a knowledge gap of the effects of BaP on gut microbiomes of juveniles.

This study assessed effects of the gut microbiome in juvenile fathead minnows to dietary BaP exposure. A limited duration exposure via the diet can directly deliver BaP into the intestines at comparably greater concentrations than via aqueous routes. Specific objectives of this study were to: 1) Characterize the fathead minnow gut microbiome in juvenile fathead minnows; 2) Measure bile metabolites resulting from exposure to BaP; 3) Characterize effects of BaP on the microbiome in guts of fish exposed to BaP, relative to that of unexposed controls; 4) Compare shifts in the microbiome to measured concentrations of BaP metabolites in the bile. To satisfy these objectives, the microbiome in guts of fathead minnows were characterized using 16S rRNA metabarcoding after dietary exposure to BaP for two weeks.

2. Materials and methods

2.1. Fish husbandry, dietary exposure, and sampling

Juvenile fathead minnows of approximately 2.5 months of age were obtained from an in-house stock population of the Aquatic Toxicology Research Facility at the University of Saskatchewan. After a one-week acclimation, fish were randomly assigned to each group (n = 10 fishes per tank; 3 tanks per group) and were exposed to a solvent control (0.02% methanol, the solvent carrier for BaP), or nominal concentrations of 1, 10, 100, or 1,000 µg BaP g⁻¹ in food (dry mass, dm) for two weeks. Food was prepared by adding a solution of BaP to the food and allowing the methanol to evaporate. Tanks were siphoned daily to remove excess food and waste. Nominal concentrations were based on environmentally-relevant, albeit extreme,
concentrations of PAHs found in fish prey at contaminated sites. For instance, in Norway, concentrations in tissues of the common limpet (*Patella vulgata*), a marine mollusk, were observed to be as great as 15 µg PAHs g$^{-1}$ (Knutzen and Sortland, 1982) while 303 µg PAHs g$^{-1}$ has been measured in the tissue of mussels from the French coast (Claisse, 1989). In sediments, concentrations as great as 142 µg PAHs g$^{-1}$ in Puget Sound (Malins et al., 1987), and 7,283 µg PAHs g$^{-1}$ have been reported in weathered creosote-contaminated sediment in Eagle Harbor, Washington (Neff et al., 2005). Thus dietary exposures could be as great as these concentrations (Silva et al., 2008). BaP was chosen as a model compound for PAH due to its persistence, mode of action, and relatively well-studied background. Additionally, because PAHs are rapidly metabolized, utilizing high concentrations would allow for accumulation of comparable concentrations in the bile as would be found in contaminated sites (Ohiozebau et al., 2016). At the end of the exposure, fish were euthanized *via* blunt force. Whole-body mass and total length were measured prior to dissection. Samples of whole gut, containing both tissues of the fish and adherent microbes, were excised from all fish. Gallbladders were also removed for quantification of BaP metabolites. If fish showed development of gonads, sex was recorded. Samples were placed in sterile cryovials, and held in liquid nitrogen until storage in a -80 °C freezer. All fish were maintained following the animal use protocol (Protocol #20090108) approved by the Animal Research Ethics Board at the University of Saskatchewan. The detailed methods for fish husbandry are described in the Supporting Information (SI) Text S1.

2.2. Quantification of BaP in food

To quantify BaP in food, an internal calibration and isotope dilution were used to quantify BaP in samples using an eight-point calibration curve between 0.5 and 500 ng BaP mL$^{-1}$, each containing 100 ng mL$^{-1}$ with BaP-d12. Pressurized liquid extraction was conducted to extract the
target compounds. A blank cell (no fish food) was also loaded and extracted to serve as an extraction blank. Quantification of BaP was done by GC-QE-Orbitrap mass spectrometer system (Q Exactive GC, Thermo Scientific, Mississauga, ON) with a Thermo RSH autosampler and a TRACE 1310 GC with a heated split/splitless injector running in splitless mode. For detailed information about the extraction and instrumental analysis methods, please refer to the method section of SI Text S2.

2.3. Relative quantification of metabolites of BaP in bile

A semi-quantitative method was applied due to the lack of available standards for Gluc and SO$_4$ metabolites of BaP. Concentrations of mono-hydroxylated benzo[a]pyrene (OH-BaP) were quantified directly with the use of analytical standards and external calibration. Semi-quantification of OH-BaP-O-glucuronide (BaP-Gluc), and sulfate-BaP (BaP-SO4) was conducted using a relative response factor approach. Detailed methods for quantification can be found in the SI Text S3. Instrument detection limits of OH-BaP were determined using the lowest calibration standard (0.3 ng mL$^{-1}$) estimated as 3x and 10x the signal-to-noise ratio for the limit of detection (LOD) and limit of quantification (LOQ), respectively. Detection limits for BaP-Gluc and BaP-SO4 had to be estimated from OH-BaP using the reported response factors, as was done for the bile concentrations (SI Table S1). Given the lack of matched isotopically-labeled standards and the extrapolation of averaged response factors based on a small number of representative bile samples, this data should be treated as semi-quantitative. However, it provides information on the relative concentrations of the major metabolite classes.

2.4. 16S rRNA metabarcoding and bioinformatics

Total DNA was isolated from intestines using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Mississauga, ON). PCR, construction of the sequencing library, next-generation sequencing, and
Bioinformatics were performed as described in DeBofsky et al. (2020a). Taxonomy was assigned in QIIME2 by use of the feature classifier trained against the SILVA 132 reference database (Bokulich et al., 2018; Quast et al., 2013). On average, 69% of demultiplexed reads survived through the cleaning process. In total, 99% of the cleaned reads aligned to bacteria. A full list of reads per sample pre- and post-cleaning can be found in SI Table S2. To avoid biases resulting from differences in sequencing depth, based on a rarefaction curve (SI Fig. S1), the feature table was rarefied at a depth of 13,133 sequences per sample. Alpha-diversities (Shannon diversity and observed ASVs), or diversity within samples, and beta-diversities (unweighted UniFrac) (Lozupone and Knight, 2005), or differences between samples, were calculated in QIIME2 (Bolyen et al., 2019). PICRUSt2 (Douglas et al., 2019) was used to predict functional abundances of MetaCyc pathways (Caspi et al., 2017) based on 16S rRNA gene sequences, using default parameters. Data can be accessed at https://dx.doi.org/10.20383/101.0247.

2.5. Statistics

Statistical analyses were performed using the R Statistical Language v. 3.6.1 (R Core Team, 2013). Unless otherwise noted, statistics were calculated using vegan v. 2.5-6 (Oksanen et al., 2019). The distribution of variables was checked and compared between groups following previous pipelines (DeBofsky et al., 2020). No significant differences in body size, or calculated indices of alpha- and beta-diversity were observed between tanks within each treatment group.

Condition factor was calculated as (Equation 1).

\[
\text{Condition Factor (K)} = \frac{\text{Mass (g)}}{\text{Standard Length (mm)}^3} \times 100
\]  

(1)

To normalize data, concentrations of BaP metabolites were log_{10}-transformed; to account for the presence of zeros in this data set, an arbitrary value of 0.0001 was given to these zero values. In some cases, volumes of bile were too small to obtain a sufficient response, which resulted in
an N/A for those samples. To retain as much microbiome data as possible, empty bile values were assigned an average value from their treatment group (Raghunathan, 2004). Outlier values were removed based on the following criteria: fish showing sexual differentiation or fish of statistically greater masses and/or length in each treatment group. Differentially abundant bacterial taxa and MetaCyc pathways were calculated using an ANOVA-Like Differential Expression tool (ALDEx2) v 1.18.0 (Fernandes et al., 2014). ALDEx2 transforms the data using Aitchison’s centered log-ratio (CLR). Additional Spearman correlations were also computed using CLR-transformed abundances of taxa and MetaCyc pathways. Differences among community compositions based on unweighted Unifrac distances were assessed using adonis2 (Oksanen et al., 2019), and the pairwise.adonis2 or PERMANOVA with Bonferroni p-value adjustment (Martinez Arbizu, 2019). A Constrained Analysis of Principal Coordinates (CAP) was conducted using the capscale function in vegan to ordinate the data and view the clusters of samples. Significant BaP metabolites contributing to the ordination were assessed using an ANOVA. Association networks of abundant amplicon sequence variants (ASVs) within treatment groups were inferred using SparCC (Friedman and Alm, 2012) with 100 bootstraps to assign p-values. Networks were displayed and analyzed with Cytoscape v. 3.8.0 (Shannon et al., 2003).

3. Results

3.1. Concentrations of BaP in food and bile metabolites

Concentrations of metabolites in bile confirmed exposure of fish to BaP. Measured concentrations of BaP in food were close to the nominal concentrations (within 20% relative difference, SI Table S3). Concentrations of bile metabolites (log_{10}-transformed) were
significantly different among exposures (Fig. 1). BaP via dietary route was rapidly metabolized by juvenile fathead minnows. OH-BaP metabolites, initial oxidation of BaP, had a significantly greater concentration in fish fed 100 and 1,000 µg BaP g⁻¹ in the diet, relative to other groups (Dunnett’s test, p < 0.001). Sulfate conjugated metabolite (BaP-SO₄) dominated the bile metabolites. Condition factor of fish was not significantly impacted by exposure to BaP (SI Fig. S2).

3.2. Gut microbiome of juvenile fathead minnows

Gut microbiome of juvenile fathead minnows was dominated by three phyla, for instance, *Fusobacteria* (which included mainly *Fusobacteriia*), *Proteobacteria* (which included mainly *Gammaproteobacteria*), and *Bacteroidetes* (which included mainly *Bacteroidia*) (Fig. 2A, B). The dominant families (± standard error) were *Fusobacteriaceae* (65% ± 1%), *Aeromonadaceae* (19% ± 1%), *Pseudomonadaceae* (3% ± 0.3%), and *Flavobacteriaceae* (3% ± 0.3%) (Fig. 2C). A total of 1,309 non-singleton ASVs of 64 unique genera of bacteria among 83 samples (control: n = 17; 1 µg BaP g⁻¹ food: n = 16; 10 µg BaP g⁻¹ food: n = 16; 100 µg BaP g⁻¹ food: n = 16; 1,000 µg BaP g⁻¹ food: n = 18) remained after filtering. Filtering removed 67 total samples of the 150 total samples; 33 samples were removed due to low sequencing depth and 34 samples were removed due to sexual differentiation over the course of the exposure.

3.3. Dietary BaP exposure altered composition of gut microbiome

Alpha-diversity indices of gut microbiome decreased with increasing BaP doses. Shannon diversity, which accounts for both evenness and abundance of species present, was marginally different among exposures (Kruskal-Wallis chi-squared = 9.2, p = 0.06), but the Shannon
diversity value for the 1,000 µg BaP g⁻¹ exposure group was significantly less than that of the controls (Dunnett’s test, *p* = 0.03; Table 1). Overall, there was an inverse correlation (*r* = -0.22, *p* = 0.09) between Shannon diversity and log₁₀-transformed of concentrations of BaP-SO₄ metabolites (lgBaP-SO₄). The number of observed ASVs was also significantly inversely proportional to exposure concentrations (ANOVA, *F* = 8.93, *p* < 0.001), with the communities in fish fed 1,000 µg BaP g⁻¹ having significantly fewer ASVs than the control group (Dunnett’s test, *p* < 0.001; Table 1). There was also an overall inverse trend between the number of ASVs as a function of log₁₀-transformed concentrations of BaP-SO₄ (*r* = -0.36, *p* = 0.004).

Community structure of the gut microbiomes were significantly different dependent upon exposure to BaP (Fig. 3A; PERMANOVA test (Adonis2): *F* = 4.14, *p* = 0.001). The 1, 10, 100 and 1,000 µg BaP g⁻¹ exposure groups all exhibited significantly distinctive community structures than that of the controls (Pairwise PERMANOVA on unweighted Unifrac distances: 1 µg BaP g⁻¹ vs. control, pseudo-*F* = 3.30, adjusted *p* = 0.001; 10 µg BaP g⁻¹ vs. control, pseudo-*F* = 3.80, adjusted *p* = 0.001; 100 µg BaP g⁻¹ vs. control, pseudo-*F* = 1.95, adjusted *p* = 0.041; 1,000 µg BaP g⁻¹ vs. control, pseudo-*F* = 4.46, adjusted *p* = 0.001). The BaP metabolites used in the dbRDA exhibited a distinct driver of the exposure groupings in the logical direction of the metabolite vectors, visually indicating that the metabolites were defining the groupings (Fig. 3A). OH-BaP and BaP-SO₄ were significantly constrained the ordination (*F* = 3.00 and 2.64, *p* = 0.003 and 0.005, for OH-BaP and BaP-SO₄, respectively).

Exposure to BaP altered families with lesser abundances rather than the dominant bacterial families. Based on exposure groups, several relative abundances of families were significantly different among exposure groups (Fig. 2C). Barnesiellaceae (*p* = 0.03), Rubritaleaceae (*p* < 0.001), Xanthomonadaceae (*p* = 0.02), Weeksellaceae (*p* = 0.01), and Chromobacteriaceae (*p* = 0.001).
0.007) were all significantly less in fish of 1,000 µg BaP g⁻¹ group relative to the control group. Several relative abundances of families (CLR-transformed) were significantly correlated with lgBaP-SO₄. Relative abundances of families that included Bacteroidaceae, Barnesiellaceae, and Chromobacteriaceae were significantly negatively correlated with lgBaP-SO₄. While Brevinemataceae, Caulobacteraceae, Microbacteriaceae, Erysipelotrichaceae, Chitinibacteraceae, and Moraxellacea significantly positively correlated families with lgBaP-SO₄ (Fig. 3B).

3.4. Dietary BaP exposure altered predicted microbial functional pathways

In total, based on Spearman correlations, 13 inferred MetaCyc pathways were also significantly correlated with lgBaP-SO₄. Seven pathways [superpathway of polyamine biosynthesis III, chitin derivatives degradation, mannan degradation, norspermidine biosynthesis, 3-phenylpropanoate degradation, superpathway of hexitol degradation (bacteria), and teichoic acid (poly-glycerol) biosynthesis] were negatively correlated with lgBaP-SO₄. Six pathways [coenzyme M biosynthesis I, methanogenesis from H2 and CO2, 7-(3-amino-3-carboxypropyl)-wyosine biosynthesis, factor 420 biosynthesis, methanogenesis from acetate, and tetrahydromethanopterin biosynthesis] were positively correlated with concentrations of lgBaP-SO₄ (Fig. 3C).

3.5. Dietary BaP exposure altered association networks of gut microbiome

Dietary exposure to BaP altered the association network of gut microbiomes in fathead minnows. Network structure reveals clusters of microbial associations for each dosage group (Association network at family level, Fig. 4; network at genus level, SI Fig. S3). Network
analysis revealed a reduction in the complexity of community structures of gut microbiome in the exposure groups. The major clutters of microbial associations were disrupted in the highest dosage group (Fig. 4E). Number of nodes, heterogeneity, and centralization of treatment groups were less than those of the control group (Table S4).

4. Discussion

The dominant bacterial phyla of gut microbiome in juvenile fathead minnows are consistent with those of other freshwater fishes, which indicates that gut microbiome have conserved biological function among fishes. Dominance of *Fusobacteria* and *Proteobacteria* has been observed not only in other studies utilizing fathead minnows (DeBofsky et al., 2020; Narrowe et al., 2015), but also in other fish species, such as zebrafish (*Danio rerio*) (Roeselers et al., 2011) and common carp (*Cyprinus carpio*) (Li et al., 2013). Minor differences occurred between the dominant taxa reported in this study and those reported previously for the fathead minnow (DeBofsky et al., 2020). In this study, *Fusobacteria* was dominant (65%), whereas we previously reported the dominance of *Proteobacteria* (63%). This disparity could have resulted from maturation of these fish, since age or sexual differentiation are driving factors in bacterial community composition (Org et al., 2016; Stephens et al., 2015; Wong et al., 2015).

Patterns between metabolites of BaP and exposure doses measured in this study were also consistent with those found in contaminated sites around the world. Concentrations of biliary metabolites, expressed as BaP equivalents, have been found at concentrations of 193 ng ml\(^{-1}\) in *Alepocephalus rostratus*, a deep-sea fish, in the Mediterranean Sea (Escartín and Porte, 1999). Mean sum concentrations of PAH metabolites in bile of lake whitefish (*Coregonus clupeaformis*) in the Athabasca River at Fort McKay in the oil sands region of Canada were 8,100 ng ml\(^{-1}\) (Ohiozebau et al., 2016). In this study, mean concentrations of sum BaP metabolites expressed as
ng ml⁻¹ in bile of fish exposed to 1,000 ug BaP g⁻¹ was 517 ng ml⁻¹. Therefore, concentrations of BaP fed to fathead minnows resulted in concentrations of metabolites in bile that are in the range of concentrations observed in fishes at moderately contaminated sites. Measuring biliary metabolites reflects recent exposure to PAHs; incorporating concentrations of BaP metabolites into this study allows for comparison to environmental monitoring studies of PAHs, where this is a common practice (Ohiozebau et al., 2016).

The results of this study revealed that a dietary exposure to BaP at environmentally-relevant concentrations has significant effects on the fathead minnow gut microbiome. BaP might be altering the gut microbiome directly or since BaP is a ligand of the aryl hydrocarbon receptor (AhR), via modulation of pathways associated with the AhR (Ortiz-Delgado et al., 2007). The AhR regulates host-microbiome communications (Zhang et al., 2017) in a bidirectional manner (Korecka et al., 2016). Exposure to BaP not only altered the composition of certain low abundant taxa and overall beta-diversity, but also changed network connectivity of those taxa.

Due to exposure to BaP, there were taxa that were significantly enriched, signifying that certain taxa might be able to use BaP as energy resources. Altered conditions, both in the microbial community and within the host, might allow pathogenic taxa to proliferate (Fig. 3C).

The family Caulobacteraceae has been observed in soils contaminated with crude and diesel oils (Bell et al., 2011; Yergeau et al., 2012) and is capable of degradation of aromatic compounds (Nierman et al., 2001). Enrichment of the family Microbacteriaceae has similarly been associated with contaminated soil sites (Bell et al., 2011; Jacques et al., 2007). Increased abundance of the family Erysipelotrichaceae was also observed in mice that were exposed via the diet to BaP (Ribière et al., 2016) and via drinking water to heavy metals (Breton et al., 2013). Studies in humans have related abundance of Erysipelotrichaceae with colorectal cancer (Chen
et al., 2012), a particularly interesting finding since BaP is carcinogenic (Gelboin, 1980).

Increases in abundances of bacteria in the family Moraxellaceae, which contains several opportunistic pathogens (Austin and Austin, 2016), is associated with increased stress in fish (Boutin et al., 2013). Furthermore, these same taxa are capable of degrading BaP when isolated from human skin (Sowada et al., 2014).

Analyses of individual taxa also revealed several taxa that were negatively correlated with the BaP-SO$_4$ metabolite that might be associated with direct deleterious effects on the physiology of the host. The presence of the family Bacteroidaceae in the gut of a host is considered mutualistic, with both the bacteria and the host benefiting from the interaction (Bäckhed et al., 2005). Bacteroidaceae are in part responsible for the production of digestive enzymes and the digestion of polysaccharides (Bäckhed et al., 2005; Ikeda-Ohtsubo et al., 2018; Thomas et al., 2011) and is involved in regulation of the immune system (Hiippala et al., 2018). Mice exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), another AhR modulator, exhibited lesser abundances of several families of Bacteroidetes (Lefever et al., 2016). These results suggest that the reduction in Bacteroidaceae, and possibly Barnesiellaceae, another member of the order Bacteroidales, might be associated with deleterious effects caused by exposure to BaP, including impairment of immune function (Carlson et al., 2004b; Reynaud and Deschaux, 2006).

Several MetaCyc pathways were significantly associated with concentrations of BaP-SO$_4$. One pathway, chitin derivatives degradation, which had a negative relationship with BaP-SO$_4$, is associated with Bacteroidaceae (Olsen et al., 1996). Inability to degrade chitin might result in lesser ability to obtain nutrients from food for proper health or defend against pathogens (Ringø et al., 2012). The same holds true for the reduction in the mannan degradation pathway, where reduced ability to degrade mannan might result in increased susceptibility to pathogens.
(Dimitroglou et al., 2009; Torrecillas et al., 2012), and reduction of teichoic acid (poly-glycerol) biosynthesis, which is involved in activating the innate immune response (Bron et al., 2012; Hoseinifar et al., 2015). Pathways that increased in relation to concentrations of BaP-SO$_4$ in bile included methanogenesis from H$_2$ and CO$_2$ and methanogenesis from acetate. Anaerobic degradation of hydrocarbons to methane and CO$_2$ requires H$_2$ and CO$_2$ utilizing bacteria as well as acetate-utilizing methanogenic bacteria (Chang et al., 2005; Zengler et al., 1999). It is therefore plausible that the exposure results in an enrichment of bacteria capable of degrading hydrocarbons. While these pathways are informative, it should be noted that they are predictive and not confirmed with functional transcriptomic analysis.

The reduction in network complexity was an unexpected result of exposure to BaP. Reduction in the number of nodes represents fewer taxa present in those association networks, while a reduction in the number of edges reflects fewer associations among those nodes (Friedman and Alm, 2012). Although interactions between microbes have not been well-explored (Hunt and Ward, 2015), ecological network responses to anthropogenic perturbation are not new (Elmqvist et al., 2003; Power et al., 1996; Vinebrooke et al., 2004). Loss of co-occurrence of taxa within a microbial community might result in altered interactions among taxa, which can change function of certain taxa or allow proliferation of others (Karimi et al., 2017). At greater concentrations of BaP in the diet, numbers of associations with other taxa decreased, meaning that the abundance of those taxa was independent of other taxa (Karimi et al., 2017). The greater the number of edges, the greater the complexity of the system (Tylianakis et al., 2009), and a large degree of connectedness can be considered a shared ecological niche within the community (Karimi et al., 2017). A reduction in network complexity has been observed in terrestrial systems with high concentrations of air pollution (Karimi et al., 2016) and at a chlor-alkali tailings dump (Zappelini
et al., 2015). Losses of nodes, or taxa, and edges, or those connections, signifies the breakdown of the ecological niche. Therefore, loss of community structure in the microbiome could be an indicator of exposure to a toxicant (Derocles et al., 2018).

5. Conclusion

Overall, this study revealed that chronic exposure to BaP in the diet significantly altered the community structure of gut microbiome of fathead minnows. The measurement of concentrations of metabolites of BaP in bile was more closely related to effects on individual and community responses and more thoroughly explained diversity than did nominal exposure concentrations in the diet. Several taxa associated with health and hydrocarbon degradation were significantly correlated with measured metabolite concentrations. Community compositions shifted and network associations were drastically altered based on the exposures. These results highlight the need for future work to determine mechanistic causes of community compositional differences and ultimately how gut microbial changes may interplay with host adverse outcomes.

Acknowledgments

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Appendix A. Supporting Information

**Text S1**, Fish husbandry. **Text S2**, Quantification of BaP in food. **Text S3**, Relative quantification of metabolites of BaP in bile. **Table S1**, Limit of quantification (LOQ) and limit of detection (LOD) of OH-BaP, BaP-Gluc, and BaP-SO4. **Table S2**, The counts of sequenced, filtered, denoised, merged, non-chimeric, and bacteria only reads for each sample. **Table S3**, Concentrations of benzo[a]pyrene (BaP) in food and metabolites of BaP in bile. Reported as mean ± standard errors (SE). **Table S4**, Parameters of co-association gut microbial network. **Fig. S1**, Rarefaction curve of Shannon Diversity values across sequencing depths. **Fig. S2**, Condition factor of fish exposed to BaP within each exposure group. Exposure did not significantly affect the condition factors of these fish. **Fig. S3**, Association networks assembled at the level of genera for (A) Control, (B) 1, (C) 10, (D) 100, and (E) 1000 µg BaP g⁻¹.

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Figure 1. Concentrations of BaP metabolites (OH-BaP, BaP-Gluc, BaP-SO$_4$, and the sum of all metabolites) (ng g$^{-1}$) from bile (± S.E.) on a log$_{10}$-transformed. Letters denote statistical significance within metabolite groups. KW test, Kruskal–Wallis one-way analysis of variance.

Fig. 2. Relative abundances of more abundant bacterial (A) phyla, (B) class, and (C) families in guts of juvenile fathead minnows, both pooled and based on exposures. KW test, Kruskal–Wallis one-way analysis of variance.

Fig. 3. Responses of the gut microbiome in fathead minnows to dietary exposure of BaP.

(A) Constrained Analysis of Principal Coordinates (CAP) of the different exposure groups constrained by the vectors of the measured metabolite concentrations, using unweighted Unifrac distances. lgBaP-OH, log$_{10}$-transformed of concentrations of OH-BaP metabolite; lgBaP-Gluc, log$_{10}$-transformed of concentrations of BaP-Gluc metabolite; lgBaP-SO$_4$, log$_{10}$-transformed of concentrations of BaP-SO$_4$ metabolite. (B) Correlation plot of families that are significantly correlated (Spearman correlation, p < 0.05) with lgBaP-SO$_4$. Significance of correlations: ***, p < 0.001; **, p < 0.01. (C) MetaCyc pathways that are significantly correlated (Spearman correlation, p < 0.05) with lgBaP-SO$_4$. Various pathways are shown relative to their correlation coefficients ($\rho$).

Figure 4. Association networks of microbial taxa at the class level relative to exposure groups for the (A) control, (B) 1, (C) 10, (D) 100, and (E) 1,000 µg BaP g$^{-1}$ exposure
groups. Associations were generated by SparCC with 100 bootstraps to assign p-values.

The associations were filtered to include only correlations with a correlation $\rho > 0.7$ and a ‘two-tailed’ $p$-value < 0.01. Only correlations with $\rho > 0.50$ and $p < 0.05$ (two-tailed) were included. Association networks of microbial ASVs were summarized by ThematicMap App (http://apps.cytoscape.org/apps/thematicmap) at class level. Networks with genus labels are presented in SI Fig. S3.
Table 1. Mean values and standard error for Shannon Diversity Index and observed number of amplicon sequence variants (ASVs) for each exposure group. Asterisks denote statistical differences from the control groups, as calculated with Dunnett’s tests.

<table>
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<tr>
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**Highlights:**

- Dominant phyla of gut microbiome are consistent with those of other freshwater fishes
- BaP metabolites and exposure doses were consistent with those found in contaminated sites
- Dietary BaP exposure has significant, dose-dependent effects on the fish gut microbiome
- Dietary BaP exposure altered association networks of gut microbiome
Graphical abstract
Responses of juvenile fathead minnow (*Pimephales promelas*) gut microbiome to a chronic dietary exposure of benzo[a]pyrene

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The microbiome has been described as an additional host “organ” with well-established beneficial roles. However, the effects of exposures to chemicals on both structure and function of the gut microbiome of fishes are understudied. To determine effects of benzo[a]pyrene (BaP), a model persistent organic pollutant, on structural shifts of gut microbiome in juvenile fathead minnows (*Pimephales promelas*), fish were exposed *ad libitum* in the diet to concentrations of 1, 10, 100, or 1,000 µg BaP g⁻¹ food, in addition to a vehicle control, for two weeks. To determine the link between exposure to BaP and changes in the microbial community, concentrations of metabolites of BaP were measured in fish bile and 16S rRNA amplicon sequencing was used to evaluate the microbiome. Exposure to BaP only reduced alpha-diversity at the greatest exposure concentrations. However, it did alter community composition assessed as differential abundance of taxa and reduced network complexity of the microbial community in all exposure groups. Results presented here illustrate that environmentally-relevant concentrations of BaP can alter the diversity of the gut microbiome and community network connectivity.

KEYWORDS: homeostasis; Next-generation sequencing; persistent organic pollutants; fish; metagenomics; 16s rRNA metagenetics

Main findings:

Environmentally-relevant concentrations of BaP can alter the diversity of the gut microbiome and community network connectivity *via* dietary exposure route.
1. Introduction

The gut microbiome is a crucial component of an animal host and is responsible for a number of important biological processes, including energy and nutrient cycling (Dimitroglou et al., 2011), regulation of intestinal barrier functions (Pérez et al., 2010), and modulation of the immune system (Roling et al., 2015). Disturbance of structure of the gut microbiome is associated with several harmful effects, including inflammatory bowel disease, metabolic syndromes, stress, and disease (Carding et al., 2015; He et al., 2019; Llewellyn et al., 2014). Although considerable research efforts to understand links between xenobiotics and gut microbiome have been conducted in mammals (i.e., Breton et al. 2013; Lefever et al., 2016; Ribiére et al., 2016), the effects of toxicants on gut microbial community structure and function in fish are largely unknown.

Complex interactions between the host microbiome and xenobiotics can vary by route of exposure. In fish, due to partitioning and bioaccumulation, exposure to environmental toxicants can occur via multiple routes and transport of persistent organic pollutants (POPs) tend to accumulate in food chains (Schlenk et al., 2008; Wang and Wang, 2006). POPs can be taken up through the gut, skin, and gill, and can ultimately have deleterious effects on fish in freshwater ecosystems. The mucosal layers of the skin, gill, and gut all contain microbiomes that provide protective barriers for fish defense against pathogens (Salinas and Magadán, 2017) and serve as an intermediary in the metabolism pathway of some toxicants (Adamovsky et al., 2018).

Benzo[a]pyrene (BaP) is a promising model polycyclic aromatic hydrocarbon (PAH) to study the potential effects of toxicants on the gut microbiome because much is known about its effects in the host but little on the microbiome. BaP originates from sources such as the incomplete combustion of fossil fuels and oil spills (Srogi, 2007) and has well-characterized
deleterious effects in fishes (Carlson et al., 2004a; Costa et al., 2011; Nacci et al., 2002; Phalen et al., 2014). BaP up-regulates the expression of cytochrome P450 1A (CYP1A), which results in the biotransformation of BaP to reactive intermediates (Ortiz-Delgado et al., 2007). Adverse outcomes of exposure to BaP include the development of lesions and tumors, as well as suppression of immune function (Beyer et al., 2010; Carlson et al., 2004b; Tuvikene, 1995). Conjugated products from phase II metabolism of BaP often end up in the bile of exposed fish (Nishimoto et al., 1992). In fish, routes of exposure to BaP are primarily through ingestion with food, incidental ingestion of sediment, dermal contact, and via ventilation across the gills (Mccarthy et al., 2003; Nichols et al., 1996; Snyder et al., 2015; Tuvikene, 1995). Route of exposure is a critical component of the distribution of BaP. For example, aqueous exposure of BaP in rainbow trout (Oncorhynchus mykiss) results in detectable BaP throughout the body, while dietary exposure mainly results in accumulation of BaP in the bile and intestine (Sandvik et al., 1998).

Effects of BaP on the structure and function of the gut microbiome of fishes are not well studied. Aqueous exposures of adult fathead minnows to small concentrations of BaP resulted in an enrichment of taxa associated with hydrocarbon degradation and community compositional shifts (DeBofsky et al., 2020a), and aqueous exposure of Japanese sea cucumbers (Apostichopus japonicus) to BaP resulted in fewer bacteria associated with beneficial functions within the host accompanied by an increase in alkane-degrading bacteria (Zhao et al., 2019). BaP exposure also induced dysbiosis of the microbiome and inflammation of adult western mosquitofish and zebrafish (Xie et al., 2020). Furthermore, concentrations of PAHs in dorsal muscle after an oil spill correlated with gut community composition in walleye (Sander vitreus) and with several families of bacteria within the gut microbiome of other species of wild fish (DeBofsky et al.,
However, since the gut microbiome of fishes may be shaped by the morphological changes during development, at least in a controlled environment (Yan et al., 2016), there still a knowledge gap of the effects of BaP on gut microbiomes of juveniles. This study assessed effects of the gut microbiome in juvenile fathead minnows to dietary BaP exposure. A limited duration exposure via the diet can directly deliver BaP into the intestines at comparably greater concentrations than via aqueous routes. Specific objectives of this study were to: 1) Characterize the fathead minnow gut microbiome in juvenile fathead minnows; 2) Measure bile metabolites resulting from exposure to BaP; 3) Characterize effects of BaP on the microbiome in guts of fish exposed to BaP, relative to that of unexposed controls; 4) Compare shifts in the microbiome to measured concentrations of BaP metabolites in the bile. To satisfy these objectives, the microbiome in guts of fathead minnows were characterized using 16S rRNA metabarcoding after dietary exposure to BaP for two weeks.

2. Materials and methods

2.1. Fish husbandry, dietary exposure, and sampling

Juvenile fathead minnows of approximately 2.5 months of age were obtained from an in-house stock population of the Aquatic Toxicology Research Facility at the University of Saskatchewan. After a one-week acclimation, fish were randomly assigned to each group (n = 10 fishes per tank; 3 tanks per group) and were exposed to a solvent control (0.02% methanol, the solvent carrier for BaP), or nominal concentrations of 1, 10, 100, or 1,000 µg BaP g⁻¹ in food (dry mass, dm) for two weeks. Food was prepared by adding a solution of BaP to the food and allowing the methanol to evaporate. Tanks were siphoned daily to remove excess food and waste. Nominal concentrations were based on environmentally-relevant, albeit extreme,
concentrations of PAHs found in fish prey at contaminated sites. For instance, in Norway, concentrations in tissues of the common limpet (*Patella vulgata*), a marine mollusk, were observed to be as great as 15 μg PAHs g⁻¹ (Knutzen and Sortland, 1982) while 303 μg PAHs g⁻¹ has been measured in the tissue of mussels from the French coast (Claissé, 1989). In sediments, concentrations as great as 142 μg PAHs g⁻¹ in Puget Sound (Malins et al., 1987), and 7,283 μg PAHs g⁻¹ have been reported in weathered creosote-contaminated sediment in Eagle Harbor, Washington (Neff et al., 2005). Thus dietary exposures could be as great as these concentrations (Silva et al., 2008). BaP was chosen as a model compound for PAH due to its persistence, mode of action, and relatively well-studied background. Additionally, because PAHs are rapidly metabolized, utilizing high concentrations would allow for accumulation of comparable concentrations in the bile as would be found in contaminated sites (Ohiozebau et al., 2016). At the end of the exposure, fish were euthanized via blunt force. Whole-body mass and total length were measured prior to dissection. Samples of whole gut, containing both tissues of the fish and adherent microbes, were excised from all fish. Gallbladders were also removed for quantification of BaP metabolites. If fish showed development of gonads, sex was recorded. Samples were placed in sterile cryovials, and held in liquid nitrogen until storage in a -80 °C freezer. All fish were maintained following the animal use protocol (Protocol #20090108) approved by the Animal Research Ethics Board at the University of Saskatchewan. The detailed methods for fish husbandry are described in the Supporting Information (SI) Text S1.

2.2. Quantification of BaP in food

To quantify BaP in food, an internal calibration and isotope dilution were used to quantify BaP in samples using an eight-point calibration curve between 0.5 and 500 ng BaP mL⁻¹, each containing 100 ng mL⁻¹ with BaP-d12. Pressurized liquid extraction was conducted to extract the
target compounds. A blank cell (no fish food) was also loaded and extracted to serve as an
extraction blank. Quantification of BaP was done by GC-QE-Orbitrap mass spectrometer system
(Q Exactive GC, Thermo Scientific, Mississauga, ON) with a Thermo RSH autosampler and a
TRACE 1310 GC with a heated split/splitless injector running in splitless mode. For detailed
information about the extraction and instrumental analysis methods, please refer to the method
section of SI Text S2.

2.3. Relative quantification of metabolites of BaP in bile

A semi-quantitative method was applied due to the lack of available standards for Gluc and
SO₄ metabolites of BaP. Concentrations of mono-hydroxylated benzo[a]pyrene (OH-BaP) were
quantified directly with the use of analytical standards and external calibration. Semi-
quantification of OH-BaP-O-glucuronide (BaP-Gluc), and sulfate-BaP (BaP-SO₄) was
conducted using a relative response factor approach. Detailed methods for quantification can be
found in the SI Text S3. Instrument detection limits of OH-BaP were determined using the
lowest calibration standard (0.3 ng mL⁻¹) estimated as 3x and 10x the signal-to-noise ratio for the
limit of detection (LOD) and limit of quantification (LOQ), respectively. Detection limits for
BaP-Gluc and BaP-SO₄ had to be estimated from OH-BaP using the reported response factors,
as was done for the bile concentrations (SI Table S1). Given the lack of matched isotopically-
labeled standards and the extrapolation of averaged response factors based on a small number of
representative bile samples, this data should be treated as semi-quantitative. However, it provides
information on the relative concentrations of the major metabolite classes.

2.4. 16S rRNA metabarcoding and bioinformatics

Total DNA was isolated from intestines using the AllPrep DNA/RNA Mini Kit (Qiagen Inc.,
Mississauga, ON). PCR, construction of the sequencing library, next-generation sequencing, and
bioinformatics were performed as described in DeBofsky et al. (2020a). Taxonomy was assigned in QIIME2 by use of the feature classifier trained against the SILVA 132 reference database (Bokulich et al., 2018; Quast et al., 2013). On average, 69% of demultiplexed reads survived through the cleaning process. In total, 99% of the cleaned reads aligned to bacteria. A full list of reads per sample pre- and post-cleaning can be found in SI Table S2. To avoid biases resulting from differences in sequencing depth, based on a rarefaction curve (SI Fig. S1), the feature table was rarefied at a depth of 13,133 sequences per sample. Alpha-diversities (Shannon diversity and observed ASVs), or diversity within samples, and beta-diversities (unweighted UniFrac) (Lozupone and Knight, 2005), or differences between samples, were calculated in QIIME2 (Bolyen et al., 2019). PICRUSt2 (Douglas et al., 2019) was used to predict functional abundances of MetaCyc pathways (Caspi et al., 2017) based on 16S rRNA gene sequences, using default parameters. Data can be accessed at https://dx.doi.org/10.20383/101.0247.

2.5. Statistics

Statistical analyses were performed using the R Statistical Language v. 3.6.1 (R Core Team, 2013). Unless otherwise noted, statistics were calculated using vegan v. 2.5-6 (Oksanen et al., 2019). The distribution of variables was checked and compared between groups following previous pipelines (DeBofsky et al., 2020). No significant differences in body size, or calculated indices of alpha- and beta-diversity were observed between tanks within each treatment group. Condition factor was calculated as (Equation 1).

\[
\text{Condition Factor} (K) = \frac{\text{Mass} (g)}{\text{Standard Length} (mm)^3} \times 100
\]  

(1)

To normalize data, concentrations of BaP metabolites were log_{10}-transformed; to account for the presence of zeros in this data set, an arbitrary value of 0.0001 was given to these zero values. In some cases, volumes of bile were too small to obtain a sufficient response, which resulted in
an N/A for those samples. To retain as much microbiome data as possible, empty bile values were assigned an average value from their treatment group (Raghunathan, 2004). Outlier values were removed based on the following criteria: fish showing sexual differentiation or fish of statistically greater masses and/or length in each treatment group. Differentially abundant bacterial taxa and MetaCyc pathways were calculated using an ANOVA-Like Differential Expression tool (ALDEx2) v 1.18.0 (Fernandes et al., 2014). ALDEx2 transforms the data using Aitchison’s centered log-ratio (CLR). Additional Spearman correlations were also computed using CLR-transformed abundances of taxa and MetaCyc pathways. Differences among community compositions based on unweighted Unifrac distances were assessed using adonis2 (Oksanen et al., 2019), and the pairwise.adonis2 or PERMANOVA with Bonferroni p-value adjustment (Martinez Arbizu, 2019). A Constrained Analysis of Principal Coordinates (CAP) was conducted using the capscale function in vegan to ordinate the data and view the clusters of samples. Significant BaP metabolites contributing to the ordination were assessed using an ANOVA. Association networks of abundant amplicon sequence variants (ASVs) within treatment groups were inferred using SparCC (Friedman and Alm, 2012) with 100 bootstraps to assign p-values. Networks were displayed and analyzed with Cytoscape v. 3.8.0 (Shannon et al., 2003).

3. Results

3.1. Concentrations of BaP in food and bile metabolites

Concentrations of metabolites in bile confirmed exposure of fish to BaP. Measured concentrations of BaP in food were close to the nominal concentrations (within 20% relative difference, SI Table S3). Concentrations of bile metabolites (log10-transformed) were
significantly different among exposures (Fig. 1). BaP via dietary route was rapidly metabolized by juvenile fathead minnows. OH-BaP metabolites, initial oxidation of BaP, had a significantly greater concentration in fish fed 100 and 1,000 µg BaP g⁻¹ in the diet, relative to other groups (Dunnett’s test, p < 0.001). Sulfate conjugated metabolite (BaP-SO₄) dominated the bile metabolites. Condition factor of fish was not significantly impacted by exposure to BaP (SI Fig. S2).

3.2. Gut microbiome of juvenile fathead minnows

Gut microbiome of juvenile fathead minnows was dominated by three phyla, for instance, *Fusobacteria* (which included mainly *Fusobacteriia*), *Proteobacteria* (which included mainly *Gammaproteobacteria*), and *Bacteroidetes* (which included mainly *Bacteroidia*) (Fig. 2A, B). The dominant families (± standard error) were *Fusobacteriaceae* (65% ± 1%), *Aeromonadaceae* (19% ± 1%), *Pseudomonadaceae* (3% ± 0.3%), and *Flavobacteriaceae* (3% ± 0.3%) (Fig. 2C). A total of 1,309 non-singleton ASVs of 64 unique genera of bacteria among 83 samples (control: n = 17; 1 µg BaP g⁻¹ food: n = 16; 10 µg BaP g⁻¹ food: n = 16; 100 µg BaP g⁻¹ food: n = 16; 1,000 µg BaP g⁻¹ food: n = 18) remained after filtering. Filtering removed 67 total samples of the 150 total samples; 33 samples were removed due to low sequencing depth and 34 samples were removed due to sexual differentiation over the course of the exposure.

3.3. Dietary BaP exposure altered composition of gut microbiome

Alpha-diversity indices of gut microbiome decreased with increasing BaP doses. Shannon diversity, which accounts for both evenness and abundance of species present, was marginally different among exposures (Kruskal-Wallis chi-squared = 9.2, p = 0.06), but the Shannon
diversity value for the 1,000 µg BaP g$^{-1}$ exposure group was significantly less than that of the controls (Dunnett’s test, $p = 0.03$; Table 1). Overall, there was an inverse correlation ($r = -0.22, p = 0.09$) between Shannon diversity and log$_{10}$-transformed of concentrations of BaP-SO$_4$ metabolites (lgBaP-SO$_4$). The number of observed ASVs was also significantly inversely proportional to exposure concentrations (ANOVA, $F = 8.93, p < 0.001$), with the communities in fish fed 1,000 µg BaP g$^{-1}$ having significantly fewer ASVs than the control group (Dunnett’s test, $p < 0.001$; Table 1). There was also an overall inverse trend between the number of ASVs as a function of log$_{10}$-transformed concentrations of BaP-SO$_4$ ($r = -0.36, p = 0.004$).

Community structure of the gut microbiomes were significantly different dependent upon exposure to BaP (Fig. 3A; PERMANOVA test (Adonis2): $F = 4.14, p = 0.001$). The 1, 10, 100 and 1,000 µg BaP g$^{-1}$ exposure groups all exhibited significantly distinctive community structures than that of the controls (Pairwise PERMANOVA on unweighted Unifrac distances: 1 µg BaP g$^{-1}$ vs. control, pseudo-$F = 3.30$, adjusted $p = 0.001$; 10 µg BaP g$^{-1}$ vs. control, pseudo-$F = 3.80$, adjusted $p = 0.001$; 100 µg BaP g$^{-1}$ vs. control, pseudo-$F = 1.95$, adjusted $p = 0.041$; 1,000 µg BaP g$^{-1}$ vs. control, pseudo-$F = 4.46$, adjusted $p = 0.001$). The BaP metabolites used in the dbRDA exhibited a distinct driver of the exposure groupings in the logical direction of the metabolite vectors, visually indicating that the metabolites were defining the groupings (Fig. 3A). OH-BaP and BaP-SO$_4$ were significantly constrained the ordination ($F = 3.00$ and 2.64, $p = 0.003$ and 0.005, for OH-BaP and BaP-SO$_4$, respectively).

Exposure to BaP altered families with lesser abundances rather than the dominant bacterial families. Based on exposure groups, several relative abundances of families were significantly different among exposure groups (Fig. 2C). Barnesiellaceae ($p = 0.03$), Rubritaleaceae ($p < 0.001$), Xanthomonadaceae ($p = 0.02$), Weeksellaceae ($p = 0.01$), and Chromobacteriaceae ($p =$
0.007) were all significantly less in fish of 1,000 µg BaP g\(^{-1}\) group relative to the control group. Several relative abundances of families (CLR-transformed) were significantly correlated with lgBaP-SO\(_4\). Relative abundances of families that included \textit{Bacteroidaceae, Barnesiellaceae,} and \textit{Chromobacteriaceae} were significantly negatively correlated with lgBaP-SO\(_4\). While \textit{Brevinema\textit{taceae, Caulobacteraceae, Microbacteriaceae, Erysipelotrichaceae, Chitinibacteriaceae,} and Moraxellacea} significantly positively correlated families with lgBaP-SO\(_4\) (Fig. 3B).

3.4. Dietary BaP exposure altered predicted microbial functional pathways

In total, based on Spearman correlations, 13 inferred MetaCyc pathways were also significantly correlated with lgBaP-SO\(_4\). Seven pathways [superpathway of polyamine biosynthesis III, chitin derivatives degradation, mannan degradation, norspermidine biosynthesis, 3-phenylpropanoate degradation, superpathway of hexitol degradation (bacteria), and teichoic acid (poly-glycerol) biosynthesis] were negatively correlated with lgBaP-SO\(_4\). Six pathways [coenzyme M biosynthesis I, methanogenesis from H\(_2\) and CO\(_2\), 7-(3-amino-3-carboxypropyl)-wyosine biosynthesis, factor 420 biosynthesis, methanogenesis from acetate, and tetrahydromethanopterin biosynthesis] were positively correlated with concentrations of lgBaP-SO\(_4\) (Fig. 3C).

3.5. Dietary BaP exposure altered association networks of gut microbiome

Dietary exposure to BaP altered the association network of gut microbiomes in fathead minnows. Network structure reveals clusters of microbial associations for each dosage group (Association network at family level, Fig. 4; network at genus level, SI Fig. S3). Network
analysis revealed a reduction in the complexity of community structures of gut microbiome in the exposure groups. The major clutters of microbial associations were disrupted in the highest dosage group (Fig. 4E). Number of nodes, heterogeneity, and centralization of treatment groups were less than those of the control group (Table S4).

4. Discussion

The dominant bacterial phyla of gut microbiome in juvenile fathead minnows are consistent with those of other freshwater fishes, which indicates that gut microbiome have conserved biological function among fishes. Dominance of Fusobacteria and Proteobacteria has been observed not only in other studies utilizing fathead minnows (DeBofsky et al., 2020; Narrowe et al., 2015), but also in other fish species, such as zebrafish (Danio rerio) (Roeselers et al., 2011) and common carp (Cyprinus carpio) (Li et al., 2013). Minor differences occurred between the dominant taxa reported in this study and those reported previously for the fathead minnow (DeBofsky et al., 2020). In this study, Fusobacteria was dominant (65%), whereas we previously reported the dominance of Proteobacteria (63%). This disparity could have resulted from maturation of these fish, since age or sexual differentiation are driving factors in bacterial community composition (Org et al., 2016; Stephens et al., 2015; Wong et al., 2015).

Patterns between metabolites of BaP and exposure doses measured in this study were also consistent with those found in contaminated sites around the world. Concentrations of biliary metabolites, expressed as BaP equivalents, have been found at concentrations of 193 ng ml$^{-1}$ in Alepocephalus rostratus, a deep-sea fish, in the Mediterranean Sea (Escartín and Porte, 1999). Mean sum concentrations of PAH metabolites in bile of lake whitefish (Coregonus clupeaformis) in the Athabasca River at Fort McKay in the oil sands region of Canada were 8,100 ng ml$^{-1}$ (Ohiozebau et al., 2016). In this study, mean concentrations of sum BaP metabolites expressed as
ng ml$^{-1}$ in bile of fish exposed to 1,000 ug BaP g$^{-1}$ was 517 ng ml$^{-1}$. Therefore, concentrations of BaP fed to fathead minnows resulted in concentrations of metabolites in bile that are in the range of concentrations observed in fishes at moderately contaminated sites. Measuring biliary metabolites reflects recent exposure to PAHs; incorporating concentrations of BaP metabolites into this study allows for comparison to environmental monitoring studies of PAHs, where this is a common practice (Ohiozebau et al., 2016).

The results of this study revealed that a dietary exposure to BaP at environmentally-relevant concentrations has significant effects on the fathead minnow gut microbiome. BaP might be altering the gut microbiome directly or since BaP is a ligand of the aryl hydrocarbon receptor (AhR), via modulation of pathways associated with the AhR (Ortiz-Delgado et al., 2007). The AhR regulates host-microbiome communications (Zhang et al., 2017) in a bidirectional manner (Korecka et al., 2016). Exposure to BaP not only altered the composition of certain low abundant taxa and overall beta-diversity, but also changed network connectivity of those taxa.

Due to exposure to BaP, there were taxa that were significantly enriched, signifying that certain taxa might be able to use BaP as energy resources. Altered conditions, both in the microbial community and within the host, might allow pathogenic taxa to proliferate (Fig. 3C).

The family Caulobacteraceae has been observed in soils contaminated with crude and diesel oils (Bell et al., 2011; Yergeau et al., 2012) and is capable of degradation of aromatic compounds (Nierman et al., 2001). Enrichment of the family Microbacteriaceae has similarly been associated with contaminated soil sites (Bell et al., 2011; Jacques et al., 2007). Increased abundance of the family Erysipelotrichaceae was also observed in mice that were exposed via the diet to BaP (Ribière et al., 2016) and via drinking water to heavy metals (Breton et al., 2013). Studies in humans have related abundance of Erysipelotrichaceae with colorectal cancer (Chen
et al., 2012), a particularly interesting finding since BaP is carcinogenic (Gelboin, 1980). Increases in abundances of bacteria in the family *Moraxellaceae*, which contains several opportunistic pathogens (Austin and Austin, 2016), is associated with increased stress in fish (Boutin et al., 2013). Furthermore, these same taxa are capable of degrading BaP when isolated from human skin (Sowada et al., 2014).

Analyses of individual taxa also revealed several taxa that were negatively correlated with the BaP-SO₄ metabolite that might be associated with direct deleterious effects on the physiology of the host. The presence of the family *Bacteroidaceae* in the gut of a host is considered mutualistic, with both the bacteria and the host benefiting from the interaction (Bäckhed et al., 2005). *Bacteroidaceae* are in part responsible for the production of digestive enzymes and the digestion of polysaccharides (Bäckhed et al., 2005; Ikeda-Ohtsubo et al., 2018; Thomas et al., 2011) and is involved in regulation of the immune system (Hiippala et al., 2018). Mice exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), another AhR modulator, exhibited lesser abundances of several families of Bacteroidetes (Lefever et al., 2016). These results suggest that the reduction in *Bacteroidaceae*, and possibly *Barnesiellaceae*, another member of the order Bacteroidales, might be associated with deleterious effects caused by exposure to BaP, including impairment of immune function (Carlson et al., 2004b; Reynaud and Deschaux, 2006).

Several MetaCyc pathways were significantly associated with concentrations of BaP-SO₄. One pathway, chitin derivatives degradation, which had a negative relationship with BaP-SO₄, is associated with *Bacteroidaceae* (Olsen et al., 1996). Inability to degrade chitin might result in lesser ability to obtain nutrients from food for proper health or defend against pathogens (Ringø et al., 2012). The same holds true for the reduction in the mannan degradation pathway, where reduced ability to degrade mannan might result in increased susceptibility to pathogens.
and reduction of teichoic acid (poly-glycerol) biosynthesis, which is involved in activating the innate immune response (Bron et al., 2012; Hoseinifar et al., 2015). Pathways that increased in relation to concentrations of BaP-SO\(_4\) in bile included methanogenesis from H\(_2\) and CO\(_2\) and methanogenesis from acetate. Anaerobic degradation of hydrocarbons to methane and CO\(_2\) requires H\(_2\) and CO\(_2\) utilizing bacteria as well as acetate-utilizing methanogenic bacteria (Chang et al., 2005; Zengler et al., 1999). It is therefore plausible that the exposure results in an enrichment of bacteria capable of degrading hydrocarbons. While these pathways are informative, it should be noted that they are predictive and not confirmed with functional transcriptomic analysis.

The reduction in network complexity was an unexpected result of exposure to BaP. Reduction in the number of nodes represents fewer taxa present in those association networks, while a reduction in the number of edges reflects fewer associations among those nodes (Friedman and Alm, 2012). Although interactions between microbes have not been well-explored (Hunt and Ward, 2015), ecological network responses to anthropogenic perturbation are not new (Elmqvist et al., 2003; Power et al., 1996; Vinebrooke et al., 2004). Loss of co-occurrence of taxa within a microbial community might result in altered interactions among taxa, which can change function of certain taxa or allow proliferation of others (Karimi et al., 2017). At greater concentrations of BaP in the diet, numbers of associations with other taxa decreased, meaning that the abundance of those taxa was independent of other taxa (Karimi et al., 2017). The greater the number of edges, the greater the complexity of the system (Tylianakis et al., 2009), and a large degree of connectedness can be considered a shared ecological niche within the community (Karimi et al., 2017). A reduction in network complexity has been observed in terrestrial systems with high concentrations of air pollution (Karimi et al., 2016) and at a chlor-alkali tailings dump (Zappelini
et al., 2015). Losses of nodes, or taxa, and edges, or those connections, signifies the breakdown of the ecological niche. Therefore, loss of community structure in the microbiome could be an indicator of exposure to a toxicant (Derocles et al., 2018).

5. Conclusion

Overall, this study revealed that chronic exposure to BaP in the diet significantly altered the community structure of gut microbiome of fathead minnows. The measurement of concentrations of metabolites of BaP in bile was more closely related to effects on individual and community responses and more thoroughly explained diversity than did nominal exposure concentrations in the diet. Several taxa associated with health and hydrocarbon degradation were significantly correlated with measured metabolite concentrations. Community compositions shifted and network associations were drastically altered based on the exposures. These results highlight the need for future work to determine mechanistic causes of community compositional differences and ultimately how gut microbial changes may interplay with host adverse outcomes.

Acknowledgments

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Appendix A. Supporting Information

Text S1, Fish husbandry. Text S2, Quantification of BaP in food. Text S3, Relative quantification of metabolites of BaP in bile. Table S1. Limit of quantification (LOQ) and limit of detection (LOD) of OH-BaP, BaP-Gluc, and BaP-SO4. Table S2. The counts of sequenced, filtered, denoised, merged, non-chimeric, and bacteria only reads for each sample. Table S3. Concentrations of benzo[a]pyrene (BaP) in food and metabolites of BaP in bile. Reported as mean ± standard errors (SE). Table S4. Parameters of co-association gut microbial network. Fig. S1. Rarefaction curve of Shannon Diversity values across sequencing depths. Fig. S2. Condition factor of fish exposed to BaP within each exposure group. Exposure did not significantly affect the condition factors of these fish. Fig. S3. Association networks assembled at the level of genera for (A) Control, (B) 1, (C) 10, (D) 100, and (E) 1000 µg BaP g⁻¹. Fig.

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**Figure caption**

**Fig. 1.** Concentrations of BaP metabolites (OH-BaP, BaP-Gluc, BaP-SO₄, and the sum of all metabolites) (ng g⁻¹) from bile (± S.E.) on a log₁₀-transformed. Letters denote statistical significance within metabolite groups. KW test, Kruskal–Wallis one-way analysis of variance.

**Fig. 2.** Relative abundances of more abundant bacterial (A) phyla, (B) class, and (C) families in guts of juvenile fathead minnows, both pooled and based on exposures. KW test, Kruskal–Wallis one-way analysis of variance.

**Fig. 3.** Responses of the gut microbiome in fathead minnows to dietary exposure of BaP. (A) Constrained Analysis of Principal Coordinates (CAP) of the different exposure groups constrained by the vectors of the measured metabolite concentrations, using unweighted Unifrac distances. lgBaP-OH, log₁₀-transformed of concentrations of OH-BaP metabolite; lgBaP-Gluc, log₁₀-transformed of concentrations of BaP-Gluc metabolite; lgBaP-SO₄, log₁₀-transformed of concentrations of BaP-SO₄ metabolite. (B) Correlation plot of families that are significantly correlated (Spearman correlation, p < 0.05) with lgBaP-SO₄. Significance of correlations: ***, p < 0.001; **, p < 0.01. (C) MetaCyc pathways that are significantly correlated (Spearman correlation, p < 0.05) with lgBaP-SO₄. Various pathways are shown relative to their correlation coefficients (ρ).

**Figure 4.** Association networks of microbial taxa at the class level relative to exposure groups for the (A) control, (B) 1, (C) 10, (D) 100, and (E) 1,000 µg BaP g⁻¹ exposure
groups. Associations were generated by SparCC with 100 bootstraps to assign p-values.

The associations were filtered to include only correlations with a correlation \( \rho > 0.7 \) and a ‘two-tailed’ p-value < 0.01. Only correlations with \( \rho > 0.50 \) and p < 0.05 (two-tailed) were included. Association networks of microbial ASVs were summarized by ThematicMap App (http://apps.cytoscape.org/apps/thematicmap) at class level. Networks with genus labels are presented in SI Fig. S3.
Table 1. Mean values and standard error for Shannon Diversity Index and observed number of amplicon sequence variants (ASVs) for each exposure group. Asterisks denote statistical differences from the control groups, as calculated with Dunnett’s tests.

<table>
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<th>Indices</th>
<th>Exposure (µg g⁻¹)</th>
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Conflict of Interest

The authors declare no competing financial interest.
CRediT author statement

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