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ORIGINAL ARTICLE

Freshwater mussels and host fish gut microbe community composition shifts after agricultural contaminant exposure

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Abstract

Aims: We examined the effects of a mixture of contaminants found in agricultural watersheds on the gut microbiota and physiology of both the freshwater mussel *Lampsilis cardium*, and *L. cardium* host fish *Micropterus salmoides*.

Methods and results: *Lampsilis cardium* and *M. salmoides* were exposed to three concentrations of agricultural contaminants for 60 days (observing behaviour daily) before being sampled for gut microbiota analyses. DNA was extracted from the gut samples, amplified via PCR, and sequenced using the Illumina Mi-Seq platform. Only *L. cardium* guts had differing microbiota across treatments, with an increase in potentially pathogenic *Aeromonas*. We also provide novel evidence of a core microbiota within *L. cardium* and *M. salmoides*. In terms of physiology, female *L. cardium* exhibited a decrease in movement and marsupial gill display in contaminant exposures.

Conclusions: Exposure to contaminants from agricultural watersheds may affect population recruitment within freshwater mussel communities over time. Specifically, increased pathogenic micro-organisms and altered behaviour can reduce the likelihood of glochidia dispersal.

Significance and impact of the study: This study supports emerging research that contaminants found in agricultural watersheds may be a factor in freshwater mussel population declines. It also provides novel evidence that unionids have a core gut microbiota.

KEYWORDS

behaviour, contaminants, freshwater mussel, host fish, metagenomics, sequencing

INTRODUCTION

Micro-organisms living in the sediments and water column drive the basic functions of life. Micro-organisms (hereafter referred to as microbes) reduce and oxidize molecules such as carbon, nitrogen and phosphorus to make compounds bioavailable for use by the local aquatic biota (Gougoulias et al., 2014; Jacoby et al., 2017; Schloter et al., 2018). Microbes are also a vital part of the food web in many environments and act as an important carbon, energy and food source to freshwater mussels (Langdon & Newell, 1990; Nichols & Garling, 2000), and to a variety of organisms lower on the food webs such as zooplankton (Sherr & Sherr, 1988). Aquatic environments require a stable microbial community to perform biological functions and to maintain food web stability. However, studies have shown that contaminants may be altering microbial communities within aquatic environments (Rosi et al., 2018;

TABLE 1 Measured and nominal concentrations of contaminants used to study the effects of contaminants of emerging concern commonly found in agricultural environments on *Lampsilis cardium* and host fish *Micropterus salmoides*. All values are in ng/L

	Bromacil		Estrone		Metolachlor	
Treatment	**Measured**	**Nominal**	**Measured**	**Nominal**	**Measured**	**Nominal**
Low	25 ± 0	8	50 ± 0	1	50 ± 17	11.78
Medium	216 ± 53	76	50 ± 0	15	372 ± 55	117.8
High	2048 ± 451	763	98 ± 77	147	3645 ± 363	1178.01
CON-EtOH	25 ± 0		50 ± 0		5 ± 0	
CON-H_2O	25 ± 0		50 ± 0		5 ± 1	

Abbreviations: TBEP, tributoxyethyl phosphate, DEET, N,N- diethyl-meta-toluamide.

Vasileiadis et al., 2015), which may have impacts further up the food chain for freshwater mussels and aquatic organisms at all trophic levels.

Contaminants of emerging concern (CECs) are widespread pollutants that may influence aquatic biota, but whose effects are relatively unknown (EPA, 2016). Microbial communities in areas affected by pharmaceutical CECs, for example, antihistamines like cimetidine and diphenhydramine, are altered when compared to those in drug-free environments and may provide mussels with a lower variety of microbes to select from (Rosi et al., 2018). Triclosan, an antibacterial agent commonly found in soaps and toothpaste, has also been found to alter the makeup of operational taxonomic units (OTUs) (where OTUs are defined in Chen et al., 2013) in bacterial communities in contaminated waterways (Drury et al., 2013). Once a habitat has been contaminated, organisms that are able to use Triclosan as a carbon source, or are resistant to it, will thrive and become the most abundant group in that area (Drury et al., 2013; Hay et al., 2001). Pharmaceutical mixtures have also been tested that reduced algal biomass in exposed waterways, and changed the overall microbial community composition (Rosi-Marshall et al., 2013). Herbicides were noted to negatively affect a microbial community's ability to recover after being exposed to the chemical in riverine environments (Pesce et al., 2006), and can help some microbial species outcompete others that were initially dominant before exposure (Perotti et al., 2003). Exposure to herbicides such as glyphosate, glufosinate and dicamba was able to increase antibiotic resistance genes within affected bacteria, and also positively select for herbicide-resistant bacteria, altering the microbial community over time (Liao et al., 2021). Glyphosate herbicides have also been noted to eventually disrupt the gut microbiota of rodents after continuous exposure (Hu et al., 2021). The herbicide, metolachlor, specifically has been shown to decrease methanotrophic soil bacteria (Seghers et al., 2003). These microbial community changes can become problematic when considering that organisms, such as freshwater mussels (family Unionidae; hereafter referred to as unionids) rely on microbial communities as an important food source (Nichols & Garling, 2000; Rosa et al., 2015; Ward & Shumway, 2004). In fact, it has been suggested that unionids are able to selectively retain bacteria from the surrounding water column (Weingarten et al., 2019), with identified differences from gut microbiota occurring across species and locations (Higgins et al., 2021; McCauley et al., 2021). Altering this food source increases the likelihood that imperilled organisms such as unionids may lose an important source of nutrition, which could have negative biological consequences.

Chemical exposure has also been noted to alter unionid reproductive behaviour. Exposure to fluoxetine led to an increase in the production of nonviable glochidia and an increase in lure displays from females, while males experienced an increase in spermatozeugmata release (Bringolf et al., 2010). Fluoxetine effects on unionid behaviour led to the discovery that exposure can also increase unionid foot protrusion, while also supporting previous results concerning the increase in female luring (Hazelton et al., 2013). However, differing results were identified with synthetic oestrogen exposure (17α-ethinylestradiol) where female lure display was reduced and no effects to foot protrusion were identified (Leonard et al., 2014). Any alterations to luring behaviour and glochidia viability immediately affect reproduction as females may prematurely lure fish, a potential required host (Haag, 2012) and release nonviable glochidia due to exposure effects, which could affect population replacement as fewer glochidia will have a chance to become juveniles. Alternatively, reduced luring implies that even if glochidia are viable they may not have a chance to develop into juveniles as there are no required host fish present. Previous research has only examined some of these behavioural changes after single exposure studies. Naturally, unionids are exposed to mixtures of chemicals, and combined exposure effects on reproductive behaviour are currently unknown.

Unionids have a complex life history, including a parasitic life stage where larvae are dependent on host fish for nutrients to develop from a larval stage (i.e. glochidium) into a juvenile (Haag, 2012), therefore, it is necessary

TBEP		4-Nonylphenol		Atrazine		DEET		Bisphenol A	
Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal
658 ± 481	127	50 ± 0	13	97 ± 29	24	123 ± 59	14	50	4
2850 ± 1090	1267	50 ± 0	130	848 ± 160	243	432 ± 82	137	50	40
14,797 ± 8397	12,671	195 ± 352	1300	8892 ± 961	2431	2129 ± 1073	1372	422 ± 466	398
957 ± 1113		50 ± 0		5		113 ± 117		91.4 ± 62	
654 ± 1802		50 ± 0		8 ± 10		85 ± 214		124 ± 269	

that hosts are alive, healthy and available for the parasitic life stage to occur. One important indicator of fish health is the gut microbiota, with many fish having a core microbiota that rarely changes (Tarnecki et al., 2017). Liu et al. (2016) have performed research suggesting that the plasticizer bisphenol A (BPA), a CEC ubiquitous in watersheds including agricultural locations (Elliott et al., 2018), can alter the gut microbiota of the zebrafish (*Danio rerio*), but recommended further research be performed to confirm results. BPA exposure has also changed the gut microbial community within mice (*Mus musculus*) to match that of high fat and sucrose diets (Lai et al., 2016) and can alter the natural gut microbiota (Javurek et al., 2016). Alteration of the gut microbiota can negatively impact an organism's behaviour as heavy evidence suggests links between gut health and mental health, with behavioural shifts occurring after gut microbiota alterations (Heijtz et al., 2011; Neufeld et al., 2011). CECs commonly found in agricultural watersheds are of special concern as agricultural CECs often have a seasonal presence depending on when chemicals (such as herbicides and pesticides) are being used. These seasonal presences can coincide with important events such as fish spawning and glochidia release, causing an increase in exposure during sensitive time points. The seasonal presence of CECs is also problematic as the exact concentration of CECs within water can be difficult to quantify, and therefore, the combined effects of agricultural CECs can be difficult to predict (Baldwin et al., 2016). Previous research has identified that feeding patterns and stress can affect the gut microbiota of fish (Bolnick et al., 2014; Cahill, 1990; Zha et al., 2018). Alternatively, research has also shown that an altered gut microbiota can elicit a stress response in fish (Zha et al., 2018). Fish responses to chemical stressors can lead to altered gill mucus levels and loss of gill epithelial cells (Sun et al., 2020). As unionids are reliant on the gills of healthy host fish for glochidial survival, fish stress responses from an altered gut microbiota due to CEC exposure could be harmful for unionid survival. Stress responses have also been noted in humans (Dinan & Cryan, 2012), and mice (Sudo et al., 2004), however, a similar effect has not yet been noted in unionids.

It remains unclear what effects CEC mixtures have on the gut microbiota of unionids and unionid host fish, and what responses may occur from exposure that could influence fitness leading to reduced reproduction and population decline. This study attempts to determine the influence of a representative mixture of CECs found in agriculturally dominated watersheds on gut microbial communities of *Lampsilis cardium* and *L. cardium* host *Micropterus salmoides*, and whether altered gut microbiota could induce behavioural effects for *L. cardium*. It is predicted that exposure to higher concentrations of a mixture of CECs will alter the profile of microbes found within the guts of *L. cardium* and *M. salmoides*, and will lead to altered physiological functions.

MATERIALS AND METHODS

Sampling and 60 day vivarium trial

Lampsilis cardium (hand collected from the Grand River in Lyons, MI, USA in early July 2017) and *M. salmoides* (obtained from the Stoney Creek Fish Hatchery, Grant, MI, USA in June 2017) were acclimated in aerated tanks and two living streams, respectively, within the Central Michigan University Vivarium. *L. cardium* and *M. salmoides* were then moved to five separate recirculating systems ($n = 20$ *L. cardium* and $n = 25$ *M. salmoides* per system) with one system holding a different experimental treatment (low, medium, high) or control (water [CON-H_2O] [carbon filtered, dechlorinated] and ethanol [CON-EtOH] [solvent control, 0.5 μl EtOH/L]). The treatments were low (0.1× medium), medium (ecologically relevant based on Elliott et al., 2018), or high (10× medium) concentrations of a mixture of CEC commonly found in agricultural watersheds that was formulated based on previous fieldwork from the United States Fish and Wildlife Service (Elliott et al., 2018; Table 1), and made at the

United States Geological Survey (USGS) National Water Quality Lab (Dr. Edward Furlong; USGS National Water Quality Laboratory, Denver CO; Table 1). The CON-EtOH was needed to evaluate if ethanol, used as a solvent to dissolve the CECs, contributed to the effects seen (ASTM International, 2006). The treatments and controls were in systems that each contained 30–3 L tanks with 25 *M. salmoides* and 20 *L. cardium*. Tank pairings are outlined in Table S1.

Adapted from ASTM protocols (ASTM International, 2006), carbon filtered dechlorinated water within each control and treatment was renewed daily (100%) at 07:00, 12:00, 16:00 and 20:00, and the chemicals for the respective treatments were added to the holding tanks to maintain treatment concentrations (Table S2). Behaviour was also examined daily (males: $n = 5$ except for CON-H_2O [$n = 4$], females: $n = 5$ except for CON-EtOH [$n = 4$]), where organisms were observed over the course of a collective 15-min time period, and key behaviours were noted onto a checklist. *L. cardium* were fed 0.5 ml of algal mix per individual (i.e. 1 ml per 3 L tank) twice a week using a mixture of *Nannochloropsis* spp. and Shellfish diet (Reed Mariculture) as per Wang et al. (2007). *M. salmoides* were fed blood worms (~9–10 g) and brine shrimp (~3 g) (Hikari Bio-Pure, Hayward CA, USA) dissolved in dechlorinated water (25 ml beaker) twice per week with a disposable transfer pipette ad libitum to minimize any contributions to excess ammonia within tanks that could be toxic to unionid glochidia (Wang et al., 2007).

Preliminary and takedown data collection

To obtain preliminary microbiota data (sometimes referred to as microbiome see Berg et al., 2020) on *L. cardium* and *M. salmoides* dissections were performed to gather tissue for gut microbiota analysis on three samples per species after the short acclimation period within the vivarium mentioned earlier prior to CEC exposure. To maintain a sterile environment, ethanol (95%) was used to wipe down all tools, gloves and surfaces used during the dissections of each organism. The gut of *L. cardium* has a mucus-like consistency, and therefore, after the stomach was cut open the sample was scooped out with a sterile microspatula and placed into a sterile 1.7 ml microcentrifuge tube. Gut samples for *M. salmoides* were solid and a small snip of the gut was able to be obtained and placed in a sterile 1.7 ml microcentrifuge tube. Gut samples from both *L. cardium* and *M. salmoides* filled microcentrifuge tubes to ~50 µl and were housed in a −20°C freezer (Maloy et al., 2009). Data from preliminary samples were used to determine the different microbiota present in the guts before exposing *L. cardium* and *M. salmoides* to contaminants, which helped to identify changes in the gut bacterial community post-exposure. Dissections were performed on *L. cardium* and *M. salmoides* on days zero (preliminary), and 60 (end), with 60 days chosen as a final endpoint to allow adequate time for gut microbial and behavioural shifts. Environmental air controls ($n = 3$) were also obtained during preliminary and end gut dissections to account for any bacteria within the air that may contaminate the samples (Napoli et al., 2012) by opening a tube to the air during dissections and running analyses on the tube as if it contained a sample.

DNA extraction

DNA was extracted from the gut samples to examine the microbial community. Extractions were performed on three samples being used for *M. salmoides* from the beginning ($n = 3$), three samples per system used from the end ($n = 15$) dissections of the trial and three samples being used for *L. cardium* from the beginning of the trial ($n = 3$) with three samples being used from each system from the end dissection ($n = 15$). Samples were removed from the −20°C freezer and thawed before extracting DNA using the Qiagen DNeasy Blood & Tissue kit (Qiagen LLC), as outlined in Maloy et al. (2009) under a laminar flow hood. To increase DNA yields, samples were concentrated using the DNA Clean and Concentrator™ Kit (Zymo Research). The resulting DNA concentration of each extraction was quantified using a *Qubit*® Fluorometer (Thermofisher Scientific™).

DNA amplification and sequencing

To attain sufficient concentrations of DNA for sequencing (>1 ng µl^{-1} of DNA), polymerase chain reactions (PCRs) were run on all of the samples. The 16 s rRNA gene (27F and 1492R primers) was amplified using a high-fidelity DNA polymerase, Q5® DNA polymerase (New England Biolabs®, Inc.) with PCR conditions described in Hengy et al. (2017), with the exception of using 39 cycles. The samples were visualized with gel electrophoresis to verify the correct PCR product size. Final concentrations of DNA within each PCR reaction were quantified using a *Qubit*® Fluorometer. The resulting DNA was then sequenced using an Illumina MiSeq platform (paired end 2 × 250 bp format) at Michigan State University's Research Technology Support Facility to generate amplicons of the V4 16S rRNA region (Kozich et al., 2013). Raw reads have been deposited to NCBI GenBank SRA: BioProject ID PRJNA804100.

Data analysis

DNA sequencing reads were analysed using Mothur version 1.41.3 (Schloss et al., 2009). The Mothur MiSeq SOP (Kozich et al., 2013) was used with some modification (Horton et al., 2018). The reference database used was SILVA (v. 128), and any sequences that were not able to be identified from the SILVA reference database were run individually on BLAST (Madden, 2002), with only those OTUs of a 98% similarity or higher being chosen from BLAST. Chimeric fragments were identified and then removed, using UCHIME (Edgar et al., 2011). Finally, samples were normalized and subsampled using Mothur.

Microbial OTUs were then used to evaluate the diversity within each sample. Mothur was also used to obtain alpha diversity measures such as Shannon's diversity index, Good's coverage index and Chao1 where singletons and doubletons were included in the analyses. Initial alpha diversity analyses were performed with approximately 6000 OTUs. Shannon's diversity index, where 0 implies no diversity as only one species is present, examines the evenness and abundance of microbial taxa within each control and treatment, which helps to identify microbial resilience within treatments. Good's coverage index (Good, 1953) determines what percentage of sequences that were potentially present in the sample were analysed, which helps to identify how thorough the sequencing analyses were. Chao1 (Chao, 1984) estimates the abundance of OTUs using singletons and doubletons to calculate potential total OTUs present as compared to the OTUs that were identified, and is used as another supplemental method to identify how thorough the sequencing analyses were. All alpha diversity (i.e. diversity within treatment) measures were analysed with a one-way analysis of variance test (ANOVA) to identify any differences among treatments, controls and time points (i.e. preliminary and day 60) after ensuring that all data met assumptions of normality and homogeneity of variance. Beta diversity (i.e. diversity across treatments) analyses were run using the statistical software R (version 3.4.3; R Core Team, 2013) with all singletons and doubletons removed to minimize the inclusion of incorrectly identified OTUs (Horton et al., 2018). After singletons and doubletons were removed, 1540 OTUs were used in beta diversity analyses. OTUs identified within the air control data were subtracted from the dataset, and from all analyses to remove OTUs that may be present from air contamination (Parris et al., 2016). All samples were subsampled based on the smallest number of sequences identified in a sample to normalize the data. Initial examinations were performed with exploratory non-metric multidimensional scaling analyses (NMDS) to identify any differences among controls and treatments in the gut microbial communities of both *L. cardium* and *M. salmoides* and compare treatments after 60 days of exposure to preliminary conditions. Multi-response permutation procedures (MRPP) were then used to determine if differences among microbial communities within each control and treatment were significant ($p < 0.05$). Families and genera were also compared, examining groups that made up 80% of taxa within the gut microbiota of both *L. cardium* and *M. salmoides* (Parris et al., 2016; Vaidya et al., 2013).

For physiological data analysis, we used a repeated measures test to examine behavioural differences for *L. cardium* across all treatments to account for data collection from the same *L. cardium* through time to determine if behaviour was altered by exposure to our mixture of CECs.

RESULTS

Shannon's diversity index, measuring relative abundance and evenness of the species present within the guts of each sample was not significantly different across (comparing treatments) or within (comparing across species) systems when combining data for both *L. cardium* and *M. salmoides* (ANOVA: $F = 1.494$, $p = 0.203$). The range of Shannon's diversity index scores for *L. cardium* was between 1.52 and 3.16 while the range for *M. salmoides* varied from 1.54 to 1.87 (Table 2). Good's coverage index, measuring what percentage of total species in the samples were identified was also not significantly different among or within treatments (ANOVA: $F = 0.715$, $p = 0.713$; Table 2). Most values were around 0.999 ± 0.0006 implying that approximately 99.9% of all potential microbial sequences within the sample were identified (Table S3). There were no significant differences in Chao1, measuring total relative abundance of species in each sample, either (ANOVA: $F = 0.715$, $p = 0.713$; Table 2), however, with the exception of the water control and preliminary samples *L. cardium* have lower Chao1 values than *M. salmoides* (Table 2).

Lampsilis cardium gut microbiota

Overall, *L. cardium* had 693 OTUs not identified within the preliminary samples and only detected in the end samples. A total of 1076 OTUs were found in *L. cardium* across the entire experiment, which were considered in an NMDS (Figure 1a). An MRPP on the microbial OTUs present within the guts of *L. cardium*, shows significant differences in the gut microbial communities across

controls and treatments, with variation among individuals occurring within controls and treatments ($T = -0.62$, $A = 0.11$, $p = 0.044$). The medium treatment had the most unique changes to microbial taxonomy when compared to the other treatments (low and high), controls and preliminary samples (Figures 2 and 3). Specifically, the medium treatment had a higher relative abundance of *Rhodobacteraceae* and *Aeromonadacae* (Figure 2). The medium treatment also had the largest number of unique OTUs identified at 210, as opposed to the low treatment that had only 16 OTUs (Table 3). Each control and treatment had unique OTUs, however, there was overlap in OTUs across controls and treatments (Figure 4). *Clostridiaceae*, *Enterobacteriaceae* and *Rhodobacteraceae* were the main families identified within all treatments and controls, potentially implying at their inclusion in the core gut microbiota. *Enterobacteriaceae* was the only microbial family that was identified in the top five most commonly identified microbial families from all six sampling groups (preliminary, CON-H_2O, CON-EtOH, low, medium and high) after ordering OTUs by similar taxa (Table 3). There were also 23 OTUs found in only CON-H_2O and CON-EtOH systems.

Micropterus salmoides gut microbiota

There were a total of 364 OTUs that appeared in the end samples for CON-H_2O, CON-EtOH, low, medium and high treatments for *M. salmoides* but not in the preliminary sample (Figure 3). Overall, *M. salmoides* had 578 OTUs identified throughout the entire experiment. *M. salmoides* had no significantly different microbial groups identified among controls and treatments, however there was variation among individual OTUs ($T = 0.18$, $A = -0.048$, $p = 0.74$) at the family (Figure 2) and genus

TABLE 2 Alpha diversity measures (Shannon's diversity, Good's coverage index and Chao1) of the guts of *Lampsilis cardium* and *Micropterus salmoides* exposed to an agricultural mixture of contaminants of emerging concern for 60 days

Species	Treatment	Shannon's diversity	Good's coverage	Chao1
Lampsilis cardium	Preliminary	2.37 ± 0.33	0.99 ± 0.00030	511.33 ± 324.91
	CON-H_2O	2.38 ± 0.52	0.99 ± 0.00020	550.01 ± 619.29
	CON-EtOH	3.16 ± 1.13	0.99 ± 0.00080	166.50 ± 38.89
	Low	2.54 ± 0.41	0.99 ± 0.00030	153.71 ± 34.34
	Medium	1.52 ± 1.36	0.99 ± 0.00040	154.3 ± 31.53
	High	2.37 ± 0.77	0.99 ± 0.00050	143.71 ± 14.92
Micropterus salmoides	Preliminary	1.7 ± 0.32	0.99 ± 0.00020	317.16 ± 83.18
	CON-H_2O	1.87 ± 0.27	0.99 ± 0.0029	402.78 ± 69.43
	CON-EtOH	1.81 ± 0.34	0.99 ± 0.00040	275.45 ± 128.73
	Low	1.58 ± 0.63	0.99 ± 0.0011	1041.51 ± 1549.36
	Medium	1.73 ± 0.61	0.99 ± 0.000050	384.1 ± 249.52
	High	1.54 ± 0.31	0.99 ± 0.00020	190.25 ± 37.35

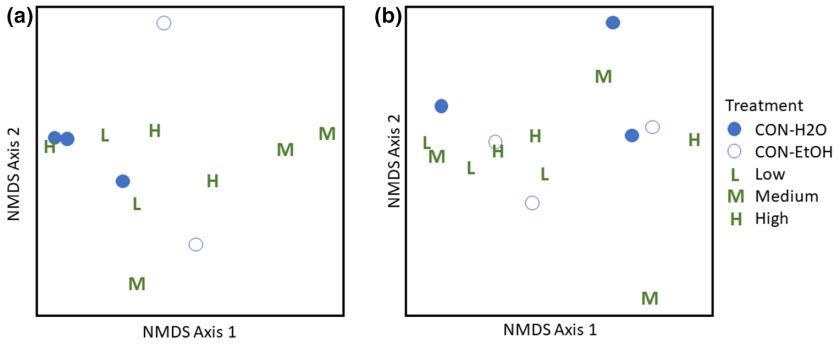

FIGURE 1 Non-metric multidimensional scaling (NMDS) analysis depicting the similarities of bacterial communities based on operational taxonomic unit results found within the guts of (a) *Lampsilis cardium*, and (b) *Micropterus salmoides* exposed to low (0.1× medium), medium (ecologically relevant), high (10× medium), CON-EtOH and CON-H_2O treatments of agricultural contaminants of emerging concern.

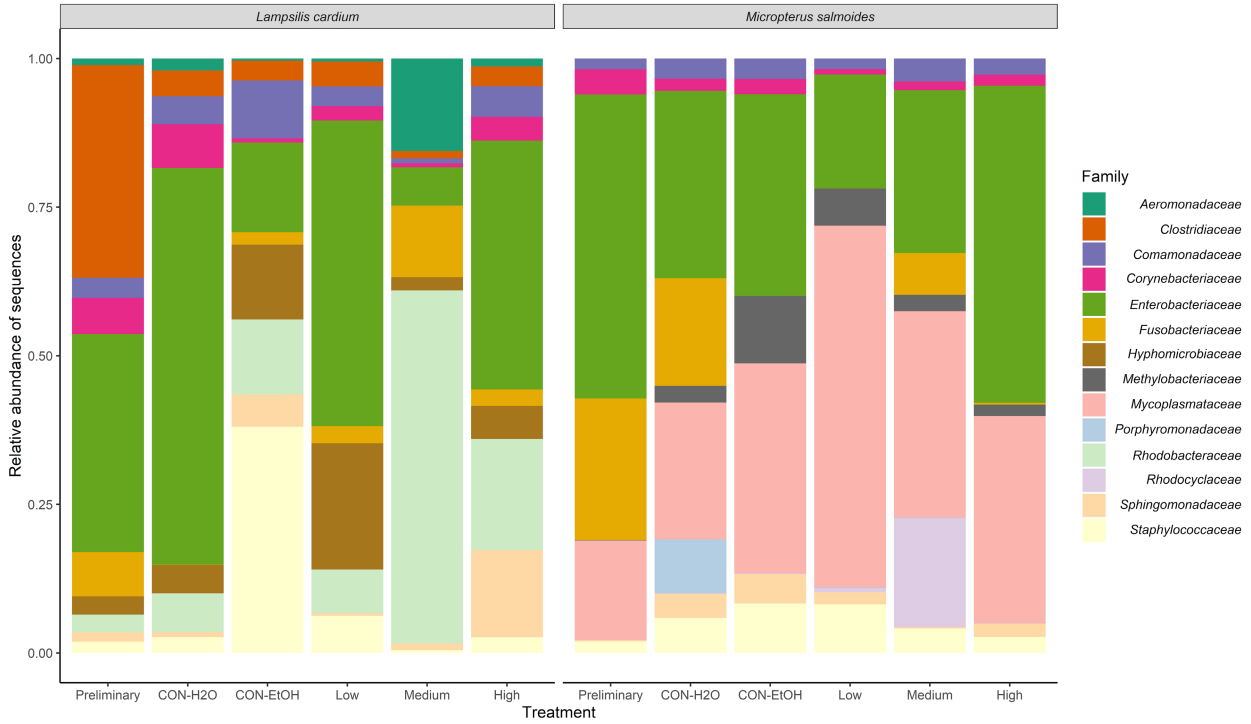

FIGURE 2 Top ten families identified within bacterial communities obtained from operational taxonomic unit results found within the guts of *Lampsilis cardium* and *Micropterus salmoides* exposed to low (0.1× medium), medium (ecologically relevant), high (10× medium), CON-EtOH, CON-H_2O and preliminary sampling groups of agricultural contaminants of emerging concern.

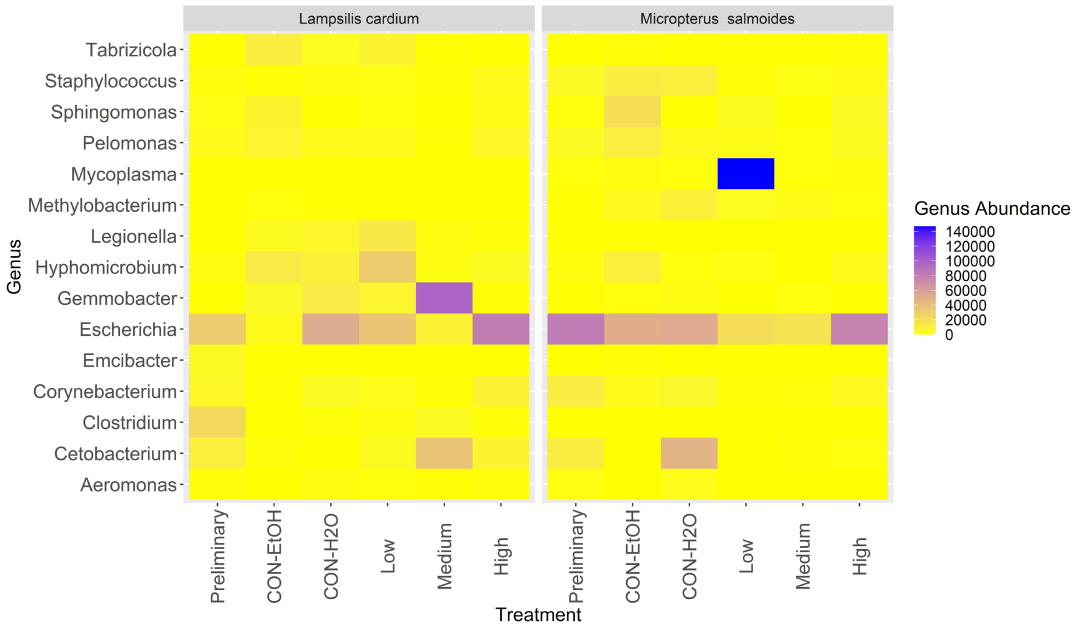

FIGURE 3 Heat map depicting the top 80% of genera identified within bacterial communities obtained from operational taxonomic unit results found within the guts of *Lampsilis cardium* and *Micropterus salmoides* exposed to low (0.1× medium), medium (ecologically relevant), high (10× medium), CON-EtOH, CON-H_2O and preliminary sampling groups of agricultural contaminants of emerging concern.

(Figure 3) levels. Even though there were no significant differences in beta diversity (Figure 1b), each control and treatment had its own unique OTUs with CON-H_2O having the most OTUs (n = 82) and CON-EtOH having the least (n = 35) (Table 3). *Mycoplasmataceae*, *Enterobacteriaceae* and *Firmicutes* were seen in all of the top five OTUs within each treatment for *M. salmoides* (Table 3).

TABLE 3 Unique and average (±standard deviation) operational taxonomic units found within the guts of *Lampsilis cardium* and *Micropterus salmoides* exposed to an agricultural mixture of contaminants of emerging concern for 60 days

Species	Treatment	Unique OTU	Average OTU	Dominant families				
				1st dominant	2nd dominant	3rd dominant	4th dominant	5th dominant
Lampsilis cardium	Preliminary	75	154 ± 38.3	Streptococcaceae	Legionellaceae	Acetobacteraceae	Aeromonadaceae	Planctomycetaceae
	CON-H_2O	56	139 ± 17.1	Enterobacteriaceae	Corynebacteriaceae	Rhodobacteraceae	Hyphomicrobiaceae	Clostridiaceae
	CON-EtOH	196	290 ± 239	Streptococcaceae	Enterobacteriaceae	Hyphomicrobiaceae	Caulobacteraceae	Chromatiaceae
	Low	16	116.5 ± 10.6	Enterobacteriaceae	Hyphomicrobiaceae	Legionellaceae	Streptococcaceae	Halothiobacillaceae
	Medium	210	179.3 ± 159.9	Rhodobacteraceae	Aeromonadaceae	Fusobacteriaceae	Enterobacteriaceae	Hyphomicrobiaceae
	High	34	109 ± 75.7	Enterobacteriaceae	Sphingomonadaceae	Rhodobacteraceae	Legionellaceae	Rhodobacteraceae
Micropterus salmoides	Preliminary	69	114 ± 19.9	Enterobacteriaceae	Fusobacteriaceae	Mycoplasmataceae	Corynebacteriaceae	Erysipelotrichaceae
	CON-H_2O	82	98.3 ± 30.9	Enterobacteriaceae	Mycoplasmataceae	Fusobacteriaceae	Porphyromonadaceae	Staphylococcaceae
	CON-EtOH	35	81 ± 8.5	Mycoplasmataceae	Enterobacteriaceae	Methylobacteriaceae	Staphylococcaceae	Rhodobacteraceae
	Low	46	91.3 ± 31.2	Mycoplasmataceae	Enterobacteriaceae	Staphylococcaceae	Methylobacteriaceae	Sphingomonadaceae
	Medium	66	93.3 ± 19	Mycoplasmataceae	Enterobacteriaceae	Rhodocyclaceae	Chromobacteriaceae	Fusobacteriaceae
	High	61	89.7 ± 8.7	Enterobacteriaceae	Mycoplasmataceae	Staphylococcaceae	Comamonadaceae	Sphingomonadaceae

Abbreviation: OTU, operational taxonomic unit.

Behavioural changes

Female *L. cardium* had differences in behaviour for movement ($p = 0.015$), and marsupial gill displays ($p = 0.033$) with less movement and less marsupial gill displaying occurring in the high and medium agricultural CEC treatments than within the other controls and treatments (CON-H_2O, CON-EtOH, AL) for both behaviours. Early glochidial release was not quantified during the experiment, therefore it remains unknown if the lack of marsupial gill display signified early release or not. Male *L. cardium* had no behavioural differences over the course of the 60 day exposures (valve opening $p = 0.066$, filtering $p = 0.057$, movement $p = 0.050$), although they could be ecologically relevant. Female *L. cardium* had no differences in behaviour for valve opening ($p = 0.181$), luring ($p = 0.288$) and filtering ($p = 0.211$). The repeated measures test showed that exposure time was seen to have the greatest effect and was significant ($p < 0.01$) across all controls and treatments.

DISCUSSION

This study provides evidence that CEC found in agricultural watersheds can impact the gut microbiota of *L. cardium* and *M. salmoides*. We were able to identify specific taxonomic changes within the gut microbiota at both the family and genus level when exposed to a mixture of agricultural CECs, however we also saw similar taxa across each treatment within both unionid and host fish species. While this supports evidence that core microbiota exist for *M. salmoides*, it uniquely suggests the presence of one for *L. cardium*. We also noted behavioural differences with the female *L. cardium* where decreases in movement and marsupial gill displays occurred after exposure to the medium and high agriculture contaminant exposures.

Lampsilis cardium

We provided evidence that supports the idea that core microbes exist within unionids. Core microbes have been identified in fish and mussel species from similar origins that were able to remain constant in dynamic and new environments (Aceves et al., 2018; Parris et al., 2016). It is unclear if a core microbiota in unionids would contribute to a stable gut microbiota and aid in increasing their overall health as observed in fish (Tarnecki et al., 2017). In the case of the *L. cardium* used in our experiment, *Clostridiaceae*, *Enterobacteriaceae* and *Rhodobacteraceae* have the potential to be part of the core microbiota as they were identified within all samples including all treatments, controls and preliminary guts. *Clostridiaceae* is a very commonly found

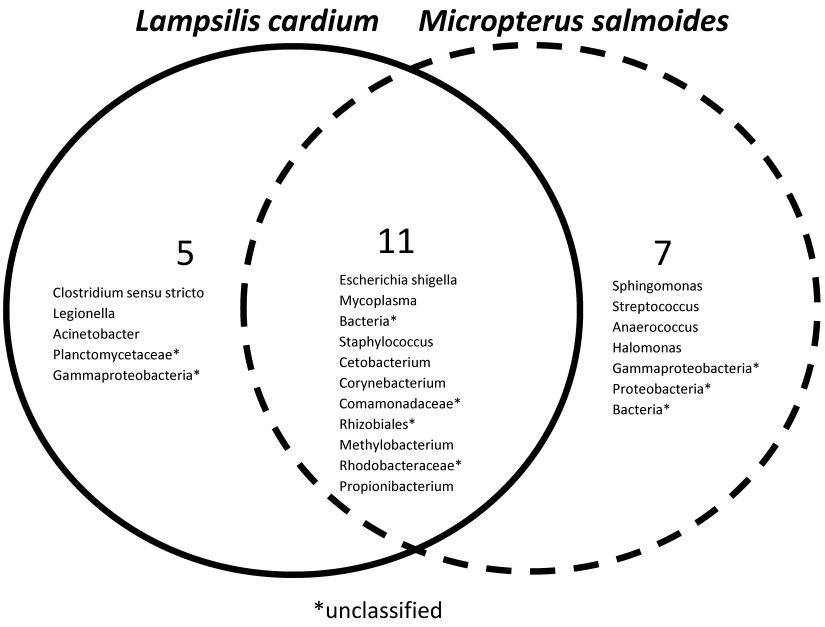

FIGURE 4 Venn diagram depicting bacterial taxa identified within the gut microbiota of all treatments and controls for both *Lampsilis cardium* (left) and *Micropterus salmoides* (right) after exposure to a mixture of chemicals found in agricultural environments. Taxa listed in the middle appeared in the guts of every tested sample from both species.

bacteria within guts, and is commonly identified within human faeces (Guo et al., 2020). *Enterobacteriaceae* is another common gut microbe, that have been to known to act as residents in the human gut (Martinson et al., 2019). *Rhodobacteraceae* has also been previously identified as a gut microbe as *Rhodobacteraceae* was observed before in the guts of shrimp (Huang et al., 2022; Imaizumi et al., 2021). *Clostridiaceae* (specifically *Clostridium*) had an overall decrease in relative abundance from the preliminary sample to any of the treatments, and was the lowest in the guts of *L. cardium* exposed to the medium concentration of agricultural contaminants. It is possible that *Clostridiaceae* was outcompeted during the course of the experiment by one or more of the 693 OTUs that developed during the 60 days. Previous research has shown that *Clostridium* species can be outcompeted in the gut environments (Reeves et al., 2012). Other research has shown that *Clostridiaceae* abundance can change based on diet (Bermingham et al., 2017), and metabolic parameters such as glucose and insulin levels (Lecomte et al., 2015). It is possible that some of the other OTUs which developed were able to outcompete *Clostridium* in the laboratory environment due to the dietary change that occurred from *L. cardium* eating a natural diet to a laboratory diet made up of an algal mix (Bermingham et al., 2017), potentially shifting what nutrients *L. cardium* was digesting as their diet changed.

While not part of the core microbiota, *Aeromonadaceae* was a dominant family in the medium agricultural CEC treatment and is of note due to its potential pathogenic nature to unionids. There has been some research showing that some *Aeromonas* species are pathogenic to zebra mussels (*Dreissena polymorpha*) (Maki et al., 1998), an invasive bivalve to North America, and certain species have been known to affect fish health, by darkening their scales and creating lesions (Shayo et al., 2012; Thune et al., 1993). Therefore, it appears that our medium mixture of agricultural CECs, which represents ecologically relevant levels present in agricultural regions, was able to provide conditions for potentially pathogenic *Aeromonas* species to thrive. *Aeromonas* can have a tolerance threshold to chemicals, however, which may explain why *Aeromonas* did not have a higher relative abundance within the high treatment (Goñi-Urriza et al., 2000). These results also support the idea that agricultural CECs such as atrazine and metolachlor may alter the growth of bacterial species (Seghers et al., 2003), and encourage microbes that can harm unionids and decrease unionid (e.g. *L. cardium*) health over time including contributing to die off events (Richard et al., 2021). Currently, the function of many bacterial species for mussel health remains unknown, therefore we suggest the possibility that alterations from the original gut microbiota pre-exposure could be impactful to unionid health. We also emphasize that chemical exposure could lead to an increase in harmful bacteria and provide an additional stressor that allows such harmful bacteria to further erode mussel health contributing to mass unionid die-offs (Henley et al., 2019; Richard et al., 2021).

Micropterus salmoides

Our experiment also provides support that a core microbiota may exist within fish as well. Core microbiota within humans are believed to be important to assist in overall health and boost the immune system (Turnbaugh et al., 2007). It is believed that a stable gut microbiota within fish will also aid in increasing their overall health

(Tarnecki et al., 2017). As *M. salmoides* are an important host fish for *L. cardium*, their health is extremely important to maintain to allow them to act as healthy hosts for glochidia. We noted evidence of *Mycoplasmataceae* and *Enterobacteriaceae* appearing within the guts of fish from all treatments. *Mycoplasmataceae* has been identified within fish guts before (Llewellyn et al., 2016; Ofek et al., 2021), as has *Enterobacteriaceae* (Ray et al., 2012). The gut microbiota of fish can change slightly in new habitats, but are usually stable once the core microbiota has been established (Egerton et al., 2018; Roeselers et al., 2011). The results here confirm some stability within the microbiota as the experimental conditions did not have a large impact on beta diversity. However, we did still see some differences, such as with *Fusobacteriaceae*, and genera such as *Cetobacterium*. On average *Fusobacteriaceae*, specifically *Cetobacterium*, changed the most among treatments from the top 10 most dominant taxa within the guts of *M. salmoides*. *Cetobacterium* is a common taxa within fish guts (Dulski et al., 2018) and has been identified as an anaerobic bacteria in the intestinal tract of fish (Tsuchiya et al., 2008). There is a chance that bacterial shifts such as these could have health implications for fish and unionid species which rely on them for reproduction.

Behavioural effects

Behaviour (marsupial gill display and movement) was altered throughout the course of our experiment, with *L. cardium* in the medium and high experiments. Typically, mussels will display their marsupial gills when they contain mature glochidia and will use a lure to draw a host fish towards the gills (Barnhart et al., 2008). In the case of our experiment, the *L. cardium* in the high and medium treatments were luring the same amount as the other controls and treatments but showing their marsupial gills less. Unionids that display this behaviour could be expending energy attempting to lure a fish in when they did not have any glochidia to release. Allocating unnecessary resources towards luring in a host fish before glochidia are developed increases the chances of not having enough resources left for reproductive functions, which decreases the likelihood that the female unionid will be able to add to the next generation of the *L. cardium* population. While not significant, there was more movement and less filtering for male *L. cardium* within the high treatment than when compared to the other controls and treatments as well. The gut microbiota of unionids was seen to shift significantly after exposure to our treatments. Research concerning the effects of altered gut microbiota on mice have shown that gut bacteria can influence behaviour. Additions of bacteria typically found in submissive mice into germ free mice led to the development of submissive behaviours (Agranyoni et al., 2021). Links between behaviour and the gut microbiota have been found in humans (Dinan et al., 2015), and we believe it may exist in unionids as well. Therefore we propose that exposure to our chemical mixture altered the gut microbiota of female *L. cardium*, which may have caused shifts in behaviour. Altered gut microbiota have been identified in mice to affect mental health and induce depression (Zheng et al., 2016), and in zebrafish differences in gut microbiota can lead to changes in serotonin levels and shoaling behaviour (Borrelli et al., 2016). Borrelli et al. (2016) attributed this altered behaviour in zebrafish to an increase in overall *Firmicutes*. This draws parallels to our experiment where we noticed that *Firmicutes* species were commonly seen across all tested gut samples, and that we had some behavioural differences. While further research is needed to identify if specific bacteria can alter unionid behaviour, our experiment provides the first piece of evidence in support of this statement.

Limitations and future unionid gut microbe studies

We have shown evidence of core microbiota in host fish and unionids but additional investigations, with larger sample sizes, would test the ubiquity and the influence of changes in the core microbiota and in turn elucidate ecological effects and help with the conservation of unionids. Our research provides further evidence of the complex effects contaminants may have on aquatic taxa health. Considering shifts in bacterial composition with varying chemical exposures using a standard protocol, as per this study, could expand on the importance of unionid and host fish gut microbiota. Although we have provided strong evidence of core microbiota within both fish and unionids, a larger sample size is needed for future research as per Aceves et al. (2018). Our smaller sample size may have led to us incorrectly observing non-significant differences in behaviour that would be seen as significant across a larger sample size, and therefore would be of concern in a larger natural unionid population. Also, our smaller sample size also made it difficult to perform more in-depth microbial and behavioural analyses to identify key bacteria that may influence specific behaviours. In addition, this study only focused on one region (V4) of the 16S rRNA gene, and this does limit the taxonomic resolution of the data, which should be considered in future studies. Identifying the influence of contaminants across

multiple unionids species would also aid in determination of whether contaminants may be contributing to broad-scale mussel decline.

We have shown that a mixture of CECs found in agricultural waterways can alter local microbiota within *L. cardium* and *M. salmoides*, and allow potentially pathogenic bacteria to thrive. Our study has also provided evidence that both unionids and fish may have a core microbiota, as the microbial community remained stable within their guts post exposure. Finally, we were able to provide evidence that behaviour may affect the gut microbiota of unionids, with filtering and marsupial gill display frequencies changing in female *L. cardium*. Our experiment has provided support that exposure to chemicals commonly found in agricultural environments may alter the gut microbiota of both unionids and fish, and affect behaviour, however, it has also highlighted several research areas that must be expanded upon. It is possible to identify potentially problematic bacteria through similar studies that are specifically responsible for reduced luring in females, which could lead to population reduction in the future. This study has shown that it is important to be aware of potential pathogenic microbes that may develop overtime after exposure to agricultural chemicals, and to monitor the microbial communities for compositional changes that may affect both unionids and their host fish.

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CONFLICT OF INTEREST

There is no conflict of interest declared.

DATA AVAILABILITY STATEMENT

Raw reads have been deposited to NCBI GenBank SRA: BioProject ID PRJNA804100.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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