THE ROLE OF MACROINVERTEBRATES AND GUT MICROBIOMES IN FRESHWATER ECOSYSTEM BIOGEOCHEMISTRY AND BACTERIAL COMMUNITY COMPOSITION

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by

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CHAPTER I

GENERAL INTRODUCTION

Freshwater ecosystems serve as habitats for an array of macroinvertebrates and microorganisms (Roberto et al. 2018, Kiersztyn et al. 2019, Koszałka and Jabłońska-Barna 2020). Macroinvertebrates are an integral part of freshwater ecosystems (Covich et al. 1999) and their guts serve as habitats for various microorganisms, including bacteria (Ayayee et al. 2018, Leff 1994, Dilon and Dilon 2004). However, the role of freshwater macroinvertebrates and their gut microbiomes in performing ecosystem functions, including (but not limited to) biogeochemical processes, are relatively less explored than free-living microbiomes (Zeng et al. 2014, Stief et al. 2009). This dissertation explores how macroinvertebrates, along with their gut microbiomes, affect the abundance and dissemination of antibiotic resistance genes (ARGs), nitrogen transformations (specifically, denitrification), and bacterial community composition in freshwater ecosystems.

Microbial Ecology of Freshwater Ecosystems:

Freshwater ecosystems, including streams, lakes, and wetlands, are important for human survival. Despite differences in features and structure of freshwater ecosystems, microbes play a pivotal role in mediating ecosystem functions and maintaining water quality (Zeglin 2015, Mulholland et al. 2008, Findlay 2010, Linz et al. 2018, Carpenter et al. 2011, Baxter et al. 2012, Jiang et al. 2020).

Microorganisms play a variety of roles in carbon and energy flow and nutrient cycling.

Benthic and planktonic algae, cyanobacteria, and diatoms contribute to primary production of freshwater ecosystems (Findlay 2010, Lozano et al. 2020, Brett et al. 2017). Microbes also serve as food for organisms at higher trophic levels and make recalcitrant dietary material, like leaf litter, palatable for macro-organisms (Cummins 1974). In turn, this impacts secondary production, breakdown of organic matter (OM), and overall energy flow (Wymore et al. 2016, Fellows et al. 2006, Wetzel 2001, Findlay 2010).

In addition to OM processing, microorganisms mediate nutrient cycling processes (e.g., denitrification, assimilation, nitrification, nitrogen fixation [Findlay 2010, Yan et al. 2016, Baxter et al. 2012]). Because of advancements in molecular techniques, we can determine the taxonomic composition of communities and can also predict specific functions performed by various microbial taxa which can inform management decisions (Repert et al. 2014, Yan et al. 2016) and evaluation of stressors (Roberto et al. 2019, Van Gray et al. 2020, Finlay et al. 2013). Overall, microorganisms are key to mediating many vital functions in freshwater ecosystems.

Microorganisms are diverse, taxonomically and functionally. Among microorganisms, bacteria are key players in specific processes; they are ubiquitous, highly diverse, abundant, and widely studied in freshwater (Roberto et al. 2019, Ayayee et al. 2018, Zeng et al. 2014, Findlay 2010). Therefore, I focused on bacteria for my dissertation. To understand the roles played by bacteria in ecosystem functions, a combination of conventional and modern techniques, like bacterial cultivation, 16S rRNA gene sequencing, assessing gene abundance and gene expression, is necessary (Debroas et al. 2009). Advancements in molecular techniques have immensely helped researchers gain insights into how bacterial community composition (structure) impacts nutrient cycles (function), including the nitrogen (N) cycle (Fernandez et al. 2016, Baxter et al. 2013, Mason et al. 2021). Use of molecular methods (e.g., 16S rRNA gene

sequencing, quantification of gene abundances) also facilitates assessing bacterial community composition changes in response to human impacts (Roberto et al. 2019, Hosen et al. 2017) as cultivable bacteria only constitute <1% of the entire bacterial community (Hugenholtz 2002). Specifically, such studies help researchers understand whether certain bacterial taxa might be adversely affected by disturbances leading to restriction of ecosystem functions provided by those microorganisms (Van Gray et al. 2020) or functional redundancy within the bacterial community (Louca et al. 2018) facilitates retention of such functions in the ecosystems.

Anthropogenic Impacts on Freshwater Ecosystems:

In addition to harboring a diverse community of macro- and microorganisms, freshwater ecosystems provide important ecosystem services like food, potable water, irrigation water for agriculture, waste disposal, transportation, and recreation. However, freshwater ecosystems are greatly disrupted by anthropogenic disturbances that impact both microorganisms (Roberto et al. 2018, Zhang et al. 2019, Baker and Banfield 2003, Berry et al. 2017) and macroinvertebrates (Boulton 1999, Alonso and Camargo 2013, Lake et al. 2000, Menbohan et al. 2019). These human-mediated disturbances take different forms ranging from disruption of hydrology to influxes of organic (e.g., pharmaceuticals, like antibiotics, organic pollutants) and inorganic (e.g., nutrients, salts, heavy metals) pollutants which alter patterns of existing biogeochemical cycles and bacterial community composition (Van Gray et al. 2020, Mulholland et al. 2008, Roberto et al. 2019, Zhu et al. 2013, Yan et al. 2016, Wymore et al. 2021).

Elevated concentrations of antibiotics and increased abundance of antibiotic resistant bacteria (ARB) and ARGs in our waterways is an emerging public health problem (Prestinaci et al. 2015). In lotic ecosystems, agricultural run-off, sewage and hospital effluents, livestock and aquaculture facilities are major contributors to antibiotic pollution (Pruden et al. 2013, Manaia et

al. 2018). Although wastewater treatment plants (WWTP) were constructed for waste removal from sewage, final WWTP effluents contain high levels of ARB and ARGs that enhance antibiotic resistance in the environment (Manaia et al. 2018, Pruden 2014). Additionally, co-occurrence of metal resistant genes in lotic ecosystems (Roberto et al. 2019) and favorable conditions for horizontal gene transfer (HGT) (Lerner et al. 2017) facilitates increases in abundance of ARB and ARGs. Various components of aquatic ecosystems (e.g., sediment, biofilms, macroinvertebrates) can act as reservoirs for ARGs. Identifying the mechanisms by which ARGs vary in abundances and are dispersed among various freshwater ecosystems components will enhance our knowledge regarding antibiotic resistance in freshwater ecosystems.

Concurrently with antibiotics, nutrients from agricultural run-off and effluents from WWTPs enter aquatic ecosystems. Excessive nutrient loading causes eutrophication that leads to harmful algal blooms capable of releasing toxins and creating “dead zones” (Chislock et al. 2013, Smith et al. 2006, Ansari et al. 2010, Mohamed et al. 2019). Use of nitrogen fertilizers and burning fossil fuels contributes to N overloading in the environment. Bacteria can perform specific processes that convert N from one form to another (Zheng et al. 2020). For example, nitrifiers convert ammonium to nitrate while bacteria performing dissimilatory processes such as dissimilatory nitrate reduction to ammonium (DNRA) and denitrification transform nitrate to ammonium and nitrogenous gases, respectively (Baxter et al. 2012, Daims et al. 2015).

Denitrification can result in two end products: complete denitrification forms di-nitrogen gas while incomplete denitrification produces nitrous oxide (Reay et al. 2012, Beaulieu et al. 2011). Nitrous oxide is a greenhouse gas with a warming potential 310 times higher than carbon dioxide over a 100-year timescale (Nogaro and Burgin, 2014). Numerous studies have

extensively explored denitrification in sediment and biofilms (e.g., Baxter et al. 2012, Baxter et al. 2013, Mulholland et al. 2008, Zhang et al. 2016). However, only in the past decade have freshwater macroinvertebrates been identified as microhabitats for nitrous oxide production via incomplete denitrification (Stief et al. 2009). Thus, further research to understand freshwater macroinvertebrates contribution to the global nitrous oxide budget is needed.

Freshwater Macroinvertebrates and their Gut Microbiome:

Macroinvertebrates are an important part of aquatic ecosystems (Covich et al. 1999) and impact energy flow, nutrient cycles, translocation of nutrients and oxygen, and bacterial assemblages. Freshwater macroinvertebrates can: 1) serve as bioindicators of water quality (Camargo et al. 2011, Camargo 2019), 2) be food sources to higher trophic levels (Wallace and Webster 1996), 3) impact nutrient and oxygen translocation, and 4) alter bacterial community structure and functions directly and indirectly via feeding, excretion, physical movement, bioturbation, and even upon death (carcasses can be nutrient sources for bacteria) (Kristensen 2000, Laverock et al. 2010, Vanni et al. 2002, Wallace and Webster 1996). Additionally, macroinvertebrate bioturbation impacts various biogeochemical transformations, including denitrification (Stief et al. 2009, Foshtomi et al. 2015, An et al. 2021), and alters bacterial community composition (Bertics and Ziebis 2009, Foshtomi et al. 2015, Huang et al. 2016). Given the diverse mechanisms of influencing ecosystem processes and their abundance (Stief et al. 2009, Stief 2013, Covich et al. 1999, Cao et al. 2018), freshwater macroinvertebrates may play a role in biogeochemistry and microbial ecology that is complex to understand.

Freshwater ecosystems include various components, such as sediment substrates, water, wood, fallen leaves, detritus, and macroinvertebrates, that harbor diverse bacterial taxa responsible for mediating numerous ecologically significant processes. Many studies have

examined the interconnections between biogeochemical cycling and bacterial communities including in freshwater ecosystems. However, most focus on sediment, water column and biofilms (e.g., Wakelin et al. 2008, Findlay and Sinsabaugh 2003, Baxter et al. 2012, Hosen et al. 2017, Ren et al. 2017) with many fewer studies incorporating freshwater macroinvertebrates as habitats for bacteria (Stief et al. 2009, Leff et al. 1994, Ayayee et al. 2018).

Bacteria associated with macroinvertebrate guts help the host in various ways, including nutrition, detoxification of secondary plant metabolites, and defense against potentially harmful microbes (Dilon and Dilon 2004, Engel and Moran 2013, Mason et al. 2014, Ayayee et al. 2014). Nutritionally, gut microbes help the hosts in N provisioning, acquiring essential amino acids and degrading secondary metabolites in plant-based food (Mason et al. 2014, Ayayee et al. 2014, Leitão-Gonçalves et al. 2017). However, the impact of the gut microbiome can extend beyond just symbiotic associations with the host. For example, bacteria in macroinvertebrate guts can perform N transformation processes (Stief et al. 2009, Heisterkamp et al. 2010, Horn et al. 2003).

Freshwater macroinvertebrate guts serve as microsites for incomplete denitrification (Stief et al. 2009) like their terrestrial counterparts: earthworms and termites (Horn et al. 2006, Ngugi and Brune 2012). Moreover, as observed in sediment and biofilms, elevated nitrate concentration in waterbodies is positively correlated with animal-associated nitrous oxide emission (Stief et al. 2009, Heisterkamp et al. 2010). This suggests that along with the microbiomes of sediment, biofilms, and water, anthropogenic activities can influence macroinvertebrate gut microbiome mediated processes. In addition to N cycling processes, antibiotic resistance in our waterways is also significantly affected by anthropogenic activities (Roberto et al. 2019, Pruden et al. 2013, Winkworth 2013, Yang et al. 2018). Macroinvertebrate guts are potential hotspots for ARGs due to their conducive environment for horizontal gene

transfer (Shterzer and Mizrahi 2015, Lerner et al. 2017), and ARGs can be further transported within and across ecosystems facilitating their dissemination (Leff et al. 1994, McEwen and Leff 2001). Therefore, macroinvertebrate gut microbiome can impact both, N-transformations, and antibiotic resistance (Leff et al. 1994, Stief et al. 2009). Additionally, different freshwater macroinvertebrate taxa can impact bacterial assemblages within their guts uniquely due to varying gut morphological complexity and physiology as well as food sources (Cummins and Klug 1979, Ayayee et al. 2018). Overall, such studies help us understand how freshwater macroinvertebrates gut microbiomes contribute towards N-transformations and serve as microhabitats for ARGs.

Despite the importance of freshwater macroinvertebrates in mediating ecologically significant processes, mechanisms by which macroinvertebrates and their gut microbiomes influence their immediate environment are not well understood. Moreover, studies in freshwater ecosystems, including streams are scarce; even well documented processes like bioturbation are relatively less investigated using freshwater macroinvertebrates in comparison to their marine counterparts (Zeng et al. 2014, Cariou et al. 2021). Therefore, further research involving freshwater macroinvertebrates and their gut microbiomes will enhance our knowledge regarding their contributions towards ecologically significant processes. Furthermore, using a combination of classical and molecular techniques to examine how bacterial community composition is coupled with N transformation (denitrification) and antibiotic resistance in host-associated and free-living microbiomes concurrently has not been undertaken previously in freshwater ecosystems.

Research Aims and Objectives:

The overall goal of this dissertation is to understand the contribution of freshwater macroinvertebrates towards nitrogen dynamics (denitrification in particular), antibiotic resistance, and the connection of these processes to bacterial community composition and function. Nutrients and pharmaceutical products, like antibiotics, are relatively well studied contaminants and have widespread ill effects in freshwater ecosystems (Kovalakova et al. 2020, Le Moal et al. 2019, Kuwayama et al. 2020, Isaza et al. 2020) so I will focus on two pollutants: N and antibiotics. Specifically, I examined three different processes involving freshwater macroinvertebrates and their gut microbiome: 1) Abundance and dissemination of ARGs, 2) link between functional gene expression and incomplete denitrification, and 3) bioturbation mediated shifts in N dynamics (dissolved inorganic nitrogen and denitrification) and bacterial community composition. The conceptual diagram depicts the connection among the three research chapters (Figure 1).

Chapter II: Antibiotic resistance gene abundance and bacterial community composition in macroinvertebrates of an urban stream

In streams, studies of ARGs have focused on biofilms, sediment, and water columns (e.g., Roberto et al. 2019, Marti et al. 2013, Proia et al. 2016, Pruden et al. 2006). Studies related to antibiotics and how they impact macroinvertebrates have largely focused on ecotoxicological questions (Bundschuh et al. 2017, Martins et al. 2012, Rico et al. 2014, Maul et al. 2006) leaving a knowledge gap regarding the role of macroinvertebrates in harboring and disseminating ARGs in freshwater ecosystems. Given that guts have conditions conducive for gene transfer (Lerner et al. 2017), macroinvertebrate gut microbiomes can serve as hotspots for and be potential vectors of ARGs. Therefore, Chapter II focuses on assessing the role of freshwater macroinvertebrates in harboring and disseminating ARGs.

I examined differences in ARG abundances (*sulI, sulII,* and *tetW*) and bacterial community composition among macroinvertebrate gut, sediment, and water microbiomes in an urban Northeast Ohio stream. Selection of these ARGs was based on the widespread usage of sulfonamide and tetracycline among humans and livestock along with their prior detection at the study sites (Roberto et al. 2019). We tested four hypotheses. First, we hypothesized that because the gut provides a conducive environment for ARG transfer and proliferation, macroinvertebrate gut microbiomes will serve as a more favorable hotspot for ARGs than sediment and water microbiomes. Hotspots are used here to refer to a location with high abundance of ARGs in comparison to its surrounding. It was predicted that ARG abundance in guts will be higher than their immediate environment (sediment and water). Second, based on the differences in their food sources and gut structural and physiological attributes, we hypothesized that macroinvertebrates from different FFGs will vary in ARG abundance. We anticipated that ARG abundance would be different in filter-feeders than predators and omnivores due to differences in gut conditions (Ayayee et al. 2018, Harris 1993, Cummins and Klug 1979). Third, we hypothesized that bacterial community composition will be correlated to ARG abundance as certain bacterial taxa may harbor specific ARGs (Roberto et al. 2019). Fourth, we hypothesized that macroinvertebrates serve as vectors of ARGs as they can transfer bacteria from guts to the immediate environment via defecation.

Chapter III: Denitrification in freshwater crayfish guts: linking gene expression to nitrous oxide emission

Freshwater macroinvertebrate guts are microhabitats for incomplete denitrification due to: 1) optimal denitrifying conditions (Stief et al. 2009) and 2) diminished expression of the nitrous oxide reductase gene (*nosZ*) (Stief et al. 2009). Denitrification has been widely studied in freshwater sediments, biofilms, and water columns (Seitzinger 1990, Arango et al. 2007, Baxter

et al. 2012, Mulholland et al. 2008). However, only a few studies have investigated denitrification in freshwater macroinvertebrate guts (Stief et al. 2009, Stief and Beer 2006) with relatively fewer attempts in connecting denitrification rates to functional gene expression (Stief et al. 2009).

In chapter III, I examined whether crayfish guts are microhabitats for nitrous oxide emission and how much of the nitrous oxide formed via incomplete denitrification accounts for the total nitrogenous gases produced due to denitrification. In this study, I used a readily available model system (crayfish: *Orconectes obscurus*) and modified its gut microbiome by enriching it with denitrifiers possessing or lacking *nosZ* and expression of *narG* and *nosZ* along with denitrification rates were assessed. We hypothesized that freshwater crayfish guts would facilitate incomplete denitrification over complete denitrification as reflected in minimal expression of *nosZ* gene and higher relative production of N_2O (compared to N_2). Furthermore, expression of *nosZ* was anticipated to be less than that of *narG* in crayfish guts enriched with *nosZ* possessing denitrifiers due to unbalanced functional gene expression (Stief et al. 2009, Heisterkamp et al. 2010) resulting in high *narG* to *nosZ* ratio.

Chapter IV: Effect of bioturbation by freshwater invertebrates on denitrification and bacterial community composition

Macroinvertebrates can significantly impact N dynamics and sediment bacterial composition via bioturbation. Distinct macroinvertebrates burrow uniquely where nutrient translocation and oxygen penetration depends on burrow configuration and depths (Kristensen, 2001, Kristensen and Kostka 2005, Hedman et al. 2011). However, our knowledge on the impact

of bioturbating invertebrates on nutrient cycling as and bacterial community composition in freshwater ecosystems is scarce in comparison to their marine counterparts (Zeng et al. 2014).

Therefore, chapter IV focuses on examining the effect of well-structured yet low density U-shaped burrows (formed by *H. bilineata*) and weakly structured high density gallery network burrows (formed by *L. variegatus*) on dissolved inorganic nitrogen (DIN) in water (surface and interstitial), potential denitrification rates, bacterial community composition, and bacterial abundance. We hypothesized that different burrowing modes will result in different bacterial community composition at varying sediment depths, differences in water nutrient chemistry (nitrate and ammonium) and potential denitrification rates. It was anticipated that more oxygen tolerant bacterial taxa and lower denitrification rates (due to increased oxygen penetration) will be observed in microcosms with *L. variegatus* compared to *H. bilineata*.

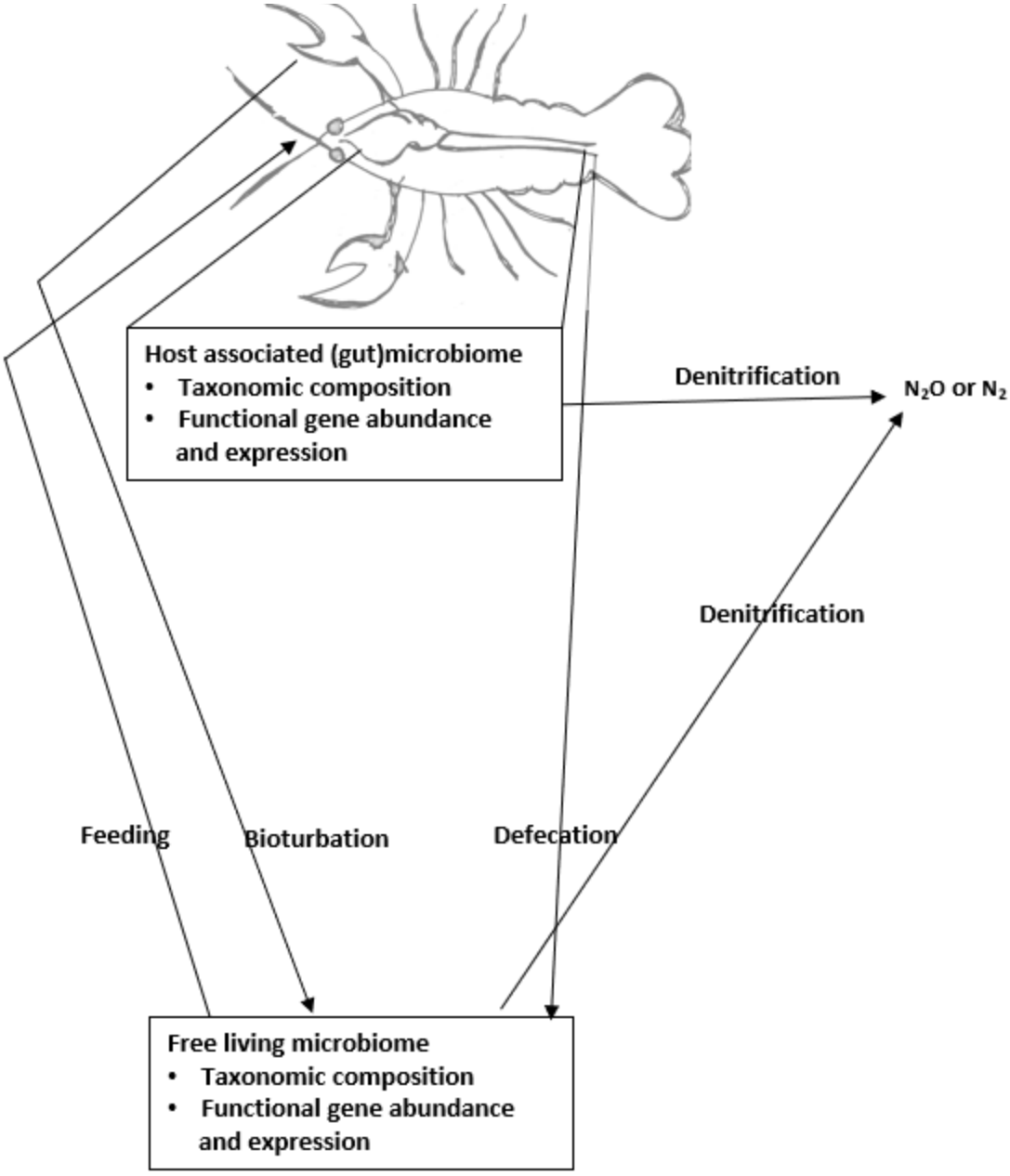

Figure 1. Conceptual diagram depicting the various processes by which host-associated (freshwater macroinvertebrate gut) microbiome and free-living microbiomes interconnect.

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CHAPTER II

ANTIBIOTIC RESISTANCE GENE ABUNDANCE AND BACTERIAL COMMUNITY COMPOSITION IN MACROINVERTEBRATES OF AN URBAN STREAM

ABSTRACT

Increases in antibiotic resistance genes (ARGs) in freshwater ecosystems due to human activities is a matter of global concern. Although researchers have looked at ARG abundance and factors affecting them in biofilms and sediment, the role of freshwater macroinvertebrate gut microbiomes in harboring and disseminating ARGs is unknown. In the current study, we tested whether: 1) macroinvertebrate gut microbiomes served as hotspots for ARGs relative to their immediate environment, 2) ARG abundance in macroinvertebrate guts varied based on feeding groups (FFGs), 3) bacterial community composition was correlated to ARG abundance, and 4) macroinvertebrates were potential vectors of ARGs. Macroinvertebrates, sediment, and water samples were collected from an urban Northeast Ohio stream and two reference streams and abundances of three ARGs (*sulI, sulII* and *tetW)* were determined. In most cases, the macroinvertebrate gut microbiome had higher ARG abundance relative to the free-living microbiomes (sediment and water) implying macroinvertebrate guts may be potential reservoirs

of ARGs. ARG abundances also varied among FFGs. Certain bacterial taxa were significantly correlated with specific ARGs suggesting that they might harbor specific ARGs. Interestingly, freshwater macroinvertebrates did not alter the ARG abundance in their surrounding sediment; perhaps due to the physical movement (i.e., burrowing by *Corbicula* may have dislodged the sediment bacteria and ARGs translocating them to the water column) of the macroinvertebrate taxa used for the experiment. Overall, our study suggests that the macroinvertebrate gut microbiome may serve as favorable habitats for ARGs and enhance their abundance (presumably because of conducive gut conditions).

Keywords

Antibiotic resistance, Freshwater macroinvertebrate guts, Environmental samples, Antibiotic resistance genes, Bacterial community composition, Reservoirs, Vectors

INTRODUCTION

The widespread occurrence of antibiotic resistance genes (ARGs) in freshwater ecosystems, including streams, is an emerging global threat to public health (Prestinaci et al. 2015, Ferri et al. 2017). ARGs may have natural origins (McArthur and Tuckfield 2000) or be introduced into lotic ecosystems from human-mediated sources, like waste-water treatment plants (WWTPs), agricultural lands, livestock areas, aquaculture facilities, and hospitals (Akiyama and Savin 2010, Pruden et al. 2013, Roberto et al. 2019). Anthropogenic disturbance is positively correlated with ARG abundance in streams (Roberto et al. 2019, Pruden et al. 2006, Ouyang et al. 2016), implying that proximity to human-mediated sources increases prevalence of ARGs in the environment. Prolonged exposure to antibiotics may result in selection for antibiotic resistance in resident bacteria (Davies and Davies 2010, Winkworth 2013, Huerta et al. 2013)

contributing to the abundance and distribution of ARGs. Moreover, ARGs can be horizontally transferred among a wide range of bacteria, including distantly related taxa and non-pathogens; thus, facilitating spread of ARGs in the environment (Koonin et al. 2001, Shterzer and Mizrahi 2015, Lerner et al. 2017, Wintersdorff et al. 2016).

Gut microbiomes harbor a plethora of bacteria, including those that carry ARGs (Lerner et al. 2017, Ji et al. 2012). An enclosed system like the gut facilitates exchange and proliferation of ARGs because of highly diverse bacterial communities, proximity of host and donor cells, and constant nutrient supply from food (Flint 1994, Lerner et al. 2017, Shterzer and Mizrahi 2015). This suggests that gut microbiomes may serve as 'hotspots' in which ARGs are stable and abundant components of the metagenome. Given that ARGs are of concern in stream ecosystems and macroinvertebrates are an integral compartment (Covich et al. 1999), potentially the gut microbiome of macroinvertebrates enhances occurrence of ARGs. Furthermore, ARGs within the guts of freshwater macroinvertebrates can be transferred to other habitats (Leff et al. 1994) via feces (Leff and Leff 2000, McEwen and Leff 2001). In some instances, adult terrestrial macroinvertebrates carry ARGs present in the guts of their aquatic larval forms; thus, enhancing the chances of cross-ecosystem transfer of ARGs as some antibiotic resistance bacteria (ARB) are retained upon metamorphosis (McEwen and Leff 2001). Collectively this evidence suggests that freshwater macroinvertebrate guts may be hotspots for ARG proliferation and persistence and be potential vectors for dispersal.

Freshwater macroinvertebrates vary in modes of feeding and this is reflected in classification into functional feeding groups (FFGs, Cummins and Klug 1979, Harris 1993). The gut microbiome differs among FFGs, likely due to differences in food as well as gut structure and physiology (Ayayee et al. 2018, Pechal and Benbow 2016, Cummins and Klug 1979, Harris

1993). Therefore, specific gut conditions (e.g., pH, structural complexity) and differences in food-associated bacterial communities may be crucial in creating differences in gut microbiomes among FFGs. In turn, these differences in microbiome composition may subsequently impact ARG abundance as certain bacterial taxa are more likely to harbor specific ARGs (Roberto et al. 2019, Balcázar et al., 2015).

Given the possibility that the gut microbiome plays an important role in dissemination and prevalence of ARGs, we examined differences in abundance of three ARGs, which convey resistance to tetracycline (***tet****W*) and sulfonamide (***sul****I* and ***sul****II*), and bacterial community composition of gut and free-living microbiomes in an urban Northeast Ohio stream. Selection of these ARGs was based on the widespread usage of sulfonamide and tetracycline among humans and livestock and their prevalence in Ohio waterways (Bernot et al. 2016). Moreover, these ARGs were previously detected at the study sites (Roberto et al. 2019). A conceptual diagram for this study depicts the role of macroinvertebrate gut microbiomes in harboring and disseminating ARGs (Figure 2).

We tested four hypotheses. First, we hypothesized that because the gut provides a conducive environment for ARG transfer and proliferation, macroinvertebrate gut microbiomes will serve as a hotspot for ARGs compared to sediment and water microbiomes. Here, hotspots are defined as a location with high abundance of ARGs in comparison to surroundings. Second, based on the differences in their food sources and gut structural and physiological attributes, we hypothesized that macroinvertebrates from different FFGs will vary in ARG abundance. We anticipated that ARG abundance would be different in filter-feeders than predators and omnivores due to differences in gut conditions and food sources (Ayayee et al. 2018, Harris 1993, Cummins and Klug 1979). Third, we hypothesized that bacterial community composition

will be correlated to ARG abundance as certain bacterial taxa may harbor specific ARGs (Roberto et al. 2019). Fourth, we hypothesized that macroinvertebrates serve as vectors of ARGs as they can transfer bacteria from guts to the immediate environment via defecation.

METHODS

Study site: Tinker's Creek (TC), is a stream in the Cuyahoga River Watershed, was selected because of the large number of WWTPs and longitudinal differences in land use (Tertuliani et al. 2008) and documented patterns of differences in bacterial community composition and ARGs (Roberto et al. 2018, Roberto et al. 2019). Two forested streams, Yellow Creek (4^{th} order stream) and Furnace Run (3^{rd} order stream), served as reference sites (1 site per stream) relative to five sites along TC. Sampling sites at TC had WWTPs located upstream and did not meet the biological criteria for attainment according to the Ohio EPA (OAC Rule 3745-1-07; Ohio Environmental Protection Agency, 2003). Among the five sites along TC, agriculture was the predominant land use pattern at the two most upstream sites (TC site 1 and 2) while the remaining three sites (TC site 3, 4 and 5) located downstream had more urbanized (commercial/residential/industrial) land usage (Roberto et al. 2019).

Samples were collected in June or July 2020 and temperature, conductivity, and pH were measured in triplicate using a Hqd/IntelliCAL Rugged Field kit (Hach Company, Loveland, CO, USA). To determine dissolved inorganic nitrogen, water samples collected at each site were filtered using 0.45 µm pore size sterile syringe filters (Whatman, Sigma-Aldrich, St. Louis, MO USA) and frozen at -20 ℃ upon return to the laboratory. Total ammonium nitrogen (TAN-N) (i.e., unionized ammonia NH_3 and the ammonium ion, NH_4^+) was assessed using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA,USA) via the indophenol blue method (Grasshoff et al. 1983, Aminot et al. 1997). Water nitrate

concentrations were analyzed using a Dionex ICS-2100 Ion chromatograph (Thermo Fisher Scientific, Waltham, MA, USA). This system detects inorganic anion concentrations using a hydroxide-selective anion-exchange column (IonPac AS19).

Sample Collection:

To collect water and benthic samples (sediment or rocks), three transects (to serve as replicates; n=3) were established at each site. Sediment samples were scooped up using a plastic container to a depth of about 5 cm and rocks (sufficient to provide ~130 cm^2 surface area) were picked up by hand depending on the dominant substrate type at each site. Water samples were collected in acid washed, autoclaved Nalgene bottles for DNA extraction and nutrient analysis. Three samples of each type were collected along each transect, pooled, and transported on ice back to the laboratory, and stored at -80 ℃ for DNA extraction.

Sampling of macroinvertebrates used three strategies: 1) picking up by hand from the benthos (for mollusks), 2) turning over rocks, and 3) using a D-frame dip net and kicking its surrounding substrate to dislodge macroinvertebrates. Three stream reaches (~ 20m each, 10m apart) were established at each site; each reach had one transect for benthic and water sample collection as described above. Each reach served as a replicate (N=3), and approximately ten different locations within each reach were sampled haphazardly to collect enough macroinvertebrates for DNA extraction. All samples were sorted according to their morphospecies in the field, placed on ice, transferred to the laboratory, and frozen at -80 ℃ until DNA extraction. Voucher specimen of each morphospecies was stored in 80% ethanol for identification (at the family level) using a dissecting microscope according to Merritt and Cummins (1996). Further, macroinvertebrates were assigned to functional feeding groups according to Cummins and Klug (1979). In this study, the functional feeding groups obtained in

sufficient quantities for further analysis were filter-feeders (families: *Cyrenidae, Hydropsychidae, Chironomidae* [putatively *Chironomus plumosus* a known filter feeder, note chironomids can belong to different FFGs, Stief et al. 2009]), scrapers (family: *Psephenidae*), predators (families: *Lestidae*, *Gerridae, Dugesiidae*), and omnivores (family: *Cambaridae*).

DNA extraction and Molecular Analysis- DNA samples were divided into two parts, one for qPCR and the other for sequencing, and stored at -80 ℃ after extraction. Water samples (250 ml from each site) were filtered through 0.2 µm pore size filters (Whatman, Sigma-Aldrich, St. Louis, MO USA), and DNA was extracted from filters using the Qiagen DNAeasy Powersoil Kit (Germantown, MD, USA) according to the manufacturer's protocol. Biofilms on rocks were scrubbed from the entire surface area using a sterile toothbrush by placing the rock in a known volume of sterile water. Thereafter, the water was filtered on a 0.2 µm filter (Whatman, Sigma-Aldrich, St. Louis, MO USA) and the filter was used for DNA extraction as above.

Macroinvertebrates were rinsed in 10% bleach (30 s) and sterile deionized water (1 min) to remove microbes present on the exoskeleton. Guts were removed from macroinvertebrates that were large enough for dissection and the guts were processed for DNA extraction. For small macroinvertebrates (< =1cm in total body length), the head capsule and appendages were removed and then the entire body was used for DNA extraction (Hammer et al. 2015, Ayayee et al. 2018). DNA extraction was performed as above.

To quantify ARG abundance, quantitative polymerase chain reaction (qPCR) was performed with a Stratagene Agilent Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA USA) using SYBR® Green PCR PerfeCTa (Quanta Biosciences, Inc. Gaithersburg, MD USA) to measure the abundances of three antibiotic resistance genes (tetracycline [***tet****W*] and sulfonamide [***sul****I* and ***sul****II*]) along with 16S rRNA genes. The reaction volume of each reaction

was 20 µL and contained 0.4 µM of each primer and 2 µL of the respective template DNA. Details of the primers used are in Roberto et al. (2019). Thermal cycling conditions were: 1 cycle at 95 °C for 3 min followed by 41 cycles at 95 °C for 60s, annealing temperature (58 °C [***tet****W* and 16S], 68 °C [***sul****I*], 60 °C [***sul****II*]) for 1min, and 72 °C for 1 min. Each run had one no-template controls (that did not generate a product) for checking contamination and the specificity of the PCR products were confirmed by generating a dissociation curve. Standard curves were generated from serial dilutions of linearized plasmid DNA containing cloned inserts of genes to calculate gene copy number using the PREXCEL-Q method (Gallup and Ackermann, 2006, Hargreaves et al., 2013). All gene copy numbers obtained via qPCR were expressed as gene copies per gram sediment/macroinvertebrate gut dry weight.

To determine bacterial community composition, subsamples of extracted DNA were checked for the presence of 16S rRNA gene via PCR (as in Roberto et al. 2019, Ayayee et al. 2018) and sent to the Molecular and Cellular Imaging Center of The Ohio State University for library preparation along with sequencing of the hypervariable V4-V5 region (Sun et al. 2013) using Illumina Mi-Seq Next Generation Sequencing.

Microcosm experiment: To examine the role of macroinvertebrates as vectors of ARGs, a laboratory microcosm experiment was conducted. A site (TC site 1) with low sediment and high overall macroinvertebrate ARG abundances (based on qPCR results) was chosen for this experiment. There were three treatments (done in triplicate): 1) autoclaved sediment and site water devoid of macroinvertebrates, 2) autoclaved sediment and site water with macroinvertebrates from the same site, 3) unautoclaved sediment and site water with macroinvertebrates from the same site. The first treatment served as a control, the second treatment was to assess how ARGs harbored by the macroinvertebrates performed in an

environment without competition from other bacteria, while the final treatment was to assess the performance of macroinvertebrate harbored ARGs in an environment with competition from other bacteria.

Microcosms consisted of 1L plastic beakers filled with 250mL of sediment and 500ml of site water. Microcosms were aerated using Phipps and Bird 6-paddle stirrers at 20 rpm (Leff et al. 1994, Leff and Leff 2000). Crayfish and clams (*Corbicula*) were chosen for the experiment due to their abundance at the site. Each treatment microcosm with macroinvertebrates had 3 clams and 2 crayfish (abundances per square meter at the study site: clam = ~150 and crayfish = ~ 5). In addition, macroinvertebrate abundances in the microcosms were consistent with that used by Leff and Leff (2000). After one week, macroinvertebrates and sediment were collected, DNA extraction and qPCR of the ARGs (*sulI*, *sulII* and *tetW*) was performed as above.

All qPCR related statistical analyses were performed using R (ver. 3.3.0; R Core Team 2016). Data were transformed using logarithmic (log base 10) transformation and Tukey's HSD was used for post-hoc analysis. For data that did not meet the assumptions of normality and heteroscedasticity after transformation, a non-parametric test, Kruskal-Wallis, followed by Dunn's test was used to identify significant differences (Roberto et al. 2019).

For examining differences in ARG abundances among macroinvertebrate taxa and their immediate environmental samples at a particular site (transects were treated as replicates), a one-way ANOVA was run with samples (macroinvertebrate taxa, sediment, and water) as the independent variable (transects served as replicates; n = 3). Similar analysis was run for all the three ARGs of interest (*sulI*, *sulII* and *tetW*) separately where the ARG abundances were expressed relative to 16S rRNA gene copy numbers. Only microbiomes (macroinvertebrate taxa, sediment, and water) in which ARGs were detected were used for analyses.

For comparison of FFGs, macroinvertebrates across all sites were grouped into 3 functional feeding groups and averages of each FFG were used for comparison across sites. The 3 FFGs considered for analyses were: filter-feeders, predators, and omnivores. A linear model was used to assess significant differences with functional feeding groups as the independent variable, and the analysis was run for *sulI, sulII* and *tetW* separately where the ARG abundances were expressed relative to 16S rRNA gene copies. Only those FFGs were included whose members were sampled at two or more sites.

For the microcosm experiment, the role of macroinvertebrates in transferring ARGs from their guts to sediments was assessed using a one-way ANOVA with sediment treatment types as the independent variable. Similar analysis was run for *sulI, sulII* and *tetW* separately where the ARG abundances were expressed as gene copy numbers/ g sediment dry weight. Genes that were not detected in certain treatments were omitted from analyses. The abundance of *sulI* and *tetW* genes were assessed for significance using a two-way ANOVA for the recovered macroinvertebrates (*sulII* was omitted as it was not detected). Respective treatments and macroinvertebrate types served as independent variables while the ARG abundances were expressed as gene copy numbers/g macroinvertebrate gut weight. In instances where the interaction between treatment and macroinvertebrate type was not significant, one-way ANOVA was run to assess significant differences across treatments and macroinvertebrate types individually (Van Gray et al. 2020).

Bioinformatics and bacterial community composition analyses: To examine bacterial community composition, QIIME 2 (version 2019.7.0) microbiome bioinformatics pipeline (accessed via Docker Desktop 3.1.0. build: 51484) was used for processing sequence data and performing amplicon sequence variant (ASV) clustering. Use of ASVs does not involve arbitrary

dissimilarity thresholds like OTU methods and detects differences as little as one nucleotide. Thus, ASV methods improve microbial data resolution, and have additional benefits of being reusable across studies (Eren et al. 2015, Needham et al. 2017, Callahan et al. 2017). In order to perform ASV clustering, briefly, cudapet (https://github.com/qiime2/q2-cutadapt, Martin 2011) was used to discard the primer sequences and then remaining sequences with no further truncation of the forward and reverse reads for combining the paired-end reads. Then the DADA2 algorithm (Callahan et al. 2016) was applied for denoising and sequencing induced error correction of the remaining sequences, and finally RDP database (Knight et al. 2018) was used to assign taxonomy to representative bacterial sequences. Final filtered ASVs resulted in 15271 unique sequences which were then rarefied to a depth of the lowest number of sequences according to the datasets used for analyses. Samples retaining < 650 rarefied sequences and sequences pertaining to unknown taxonomic affiliations were omitted from further analysis. A bacterial community data matrix was exported as an excel file for further downstream processing in R statistical environment (v3.3.1; R Core Team, 2016).

The rarefied ASV abundance data was then Hellinger transformed to perform a redundancy analysis (RDA) to assess how sampled microbiomes (macroinvertebrate taxa, sediment, and water) at respective sites differed in bacterial community composition (using R package vegan, Oksanen et al. 2013, and tidyverse). RDAs are useful in visualizing how much variation in a set of measured variables is explained by explanatory variables (Pally and Shankar 2016). The significance test was determined using 999 permutations (Mason et al. 2021). Furthermore, the differences in taxonomic composition among the sampled host-associated and free-living microbiomes at each individual site was depicted at the family level using stacked bar plots. Relative abundance was based on the average of the three replicates (from which DNA

was extracted [see methods]). Dominant bacterial families (relative abundance > 1%) were considered and the cut-off of ">1% of relative abundance" was chosen for: 1) easy interpretation of data and 2) 1% is widely accepted as the threshold for dominant taxa (Roberto et al. 2018, Nyirabuhoro et al. 2020). Thereafter, for each site, significant differences in relative abundances of dominant families among the microbiomes were assessed using generalized linear models (GLM). The same approach was used for analysis of taxonomic differences in FFGs at the phylum level.

To identify whether relative abundance of bacterial families and the ARG relative abundance profile were correlated to each other, a Spearman's rank correlation was performed with Hellinger transformed ASV data. The direction (as implied by the correlation coefficient) and significance ($P<0.05$) of the relationship between the above-mentioned variables were identified, results were obtained in a matrix form, and depicted as a heatmap. The univariate p-values were corrected using Benjamini- Hochberg (B-H) procedure to reduce the false discovery rate. The dominant bacterial families (> 1% relative abundance) across all sites were used in place of doing separate analyses for individual sites because the families overlapped among the sites and sample types. Similar analysis was done to identify correlation between physicochemical parameters and the ARG abundance profile.

RESULTS

Among the sulfonamide resistance genes, abundance of *sulI* (relative to 16S rRNA gene abundance) was significantly different among macroinvertebrate taxa, sediment, and water at four sites: TC- 1 ($P < 0.001$; Figure 3 A), TC- 2 ($P < 0.001$; Figure 3 B), and TC- 4 ($P < 0.01$; Figure 3 D). At these sites, macroinvertebrate gut microbiomes had higher *sulI*:16S abundance than free-living microbiomes. For TC- site 3 ($P = 0.07$; Figure 3 C), TC site 5 ($P = 0.41$; Figure 3

E) and sites at Yellow Creek (P = 0.38; Figure 3 F) and Furnace Run (P = 0.08; Figure 3 G) there were no significant differences among microbiomes.

Like *sulI*, abundance of *sulII* (relative to the 16S rRNA gene) was significantly different among macroinvertebrate taxa, sediment, and water at sites TC- 1 (P < 0.001; Figure 4 A), TC- 2 (P < 0.001; Figure 4 B), TC- 3 (P < 0.01; Figure 4 C), and TC- 4 (P < 0.001; Figure 4 D). At each of these four sites, macroinvertebrate gut microbiomes had higher relative gene (*sulII:*16S rRNA) abundance than free-living microbiomes. There were no significant differences among the microbiomes in relative *sulII* abundance at Furnace Run (P = 0.37; Figure 4 E). TC site 5 and Yellow Creek was omitted from analysis since *sulII* was not detected in the sampled macroinvertebrates.

Abundance of the tetracycline gene (*tetW* relative to 16S rRNA gene) was significantly different among macroinvertebrate taxa, sediment, and water at site TC-1 (P < 0.001; Figure 5 A) macroinvertebrate gut microbiomes had higher relative gene (*tetW:*16S rRNA) abundance than free-living microbiomes. However, at sites TC-2 (P<0.01; Figure 5B), TC-5 (P < 0.01; Figure 5D), and Furnace Run (P< 0.05; Figure 5 E), water samples had highest *tetW* relative abundance. TC-site 3 had no significant differences among the microbiomes (P= 0.07; Figure 5C). TC site 4 was omitted from analysis as sampled macroinvertebrate microbiomes did not amplify for all the three replicates while Yellow Creek was excluded from analysis as free living microbiomes did not amplify for all the three replicates.

Macroinvertebrates in different FFGs differed significantly in *sulI* (P < 0.001; Figure 6 A) and *tetW* (P < 0.01; Figure 6 C) relative abundances but did not differ significantly for *sulII* (P = 0.36; Figure 6 B). Omnivores (only *Cambaridae*) had significantly higher *sulI:* 16S rRNA copies than filter feeder and predators while filter feeders had significantly higher *tetW:* 16S

rRNA copies than omnivores and predators. Only the FFGs which had representative macroinvertebrates in more than one site were used for analysis.

For the physicochemical parameters that were measured, TC-4 had highest nitrate concentration and temperature, TC-2 had highest pH, TC-5 had highest ammonium concentration while TC-3 had highest conductivity (Table 1). Additionally, the P values and Spearman's correlation coefficients for the correlations between the physicochemical parameters and relative ARG abundances are shown in Table 2. For *sulI*, ammonium and pH were positively correlated while temperature was negatively correlated. For *sulII*, ammonium concentration, conductivity and temperature were significant and negatively correlated. Nitrate concentration and temperature were negatively while pH was positively correlated with *tetW*.

Redundancy analysis (RDA) revealed significant differences between macroinvertebrate gut and free-living microbiomes (replication = 3 if not mentioned otherwise; microbiomes served as the predictor variable) for each site at ASV and family levels. Based on the ordination plots, bacterial community composition among sampled microbiomes (macroinvertebrate taxa, sediment, and water) was distinct (Figure 7 A-G; TC-site 1 [$P<0.001$], TC-site 2 [$P<0.001$], TC-site 3 [$P<0.01$], TC-site 4 [$P<0.01$], TC-site 5 [$P<0.05$; *Cambaridae*, n=2], Yellow Creek site [$P<0.001$] and Furnace Run [$P<0.001$]). At TC-sites 1, 3, 4 and the reference sites, there was clear separation between the macroinvertebrate taxa, sediment, and water bacterial communities. However, at TC site 2 there was overlap between sediment and water samples. At TC site 5, there was overlap between sediment and water samples along with some degree of clustering among the macroinvertebrate taxa and sediment.

The RDA results for the functional feeding groups were significant at ASV ($P<0.001$) and phylum ($P<0.05$) levels (Figure 8 A and B respectively). At the ASV level, the different

functional feeding groups depicted clear separation in bacterial community composition from one another while at the phylum level there was some clustering among the FFGs.

The bacterial community was composed of 37 phyla and 220 families. Across all sites, the phyla contributing most to community composition were Proteobacteria, Bacteroidetes, and Actinobacteria. The differences in the relative abundances of the families (> 1% relative abundance) among the sampled microbiomes at each site are shown in Figure 9 (A-G). Common families in each phylum were: Proteobacteria (*Sphingomonadaceae, Rhodobacteraceae, Rhodobiaceae, Burkholderiaceae, Comamonadaceae, Xanthomonadaceae*), Bacteroidetes (*Chitinophagaceae, Saprospiraceae, Flavobacteriaceae, Cytophagaceae),* Actinobacteria (*Microbacteriacea*), Verrucomicrobia *(*family: *Verrucomicrobiaceae*), and Planctomycetes *(*family: *Planctomycetaceae*). In contrast to these commonly detected groups, certain bacterial taxa were dominant only at particular sites (e.g., *Acidimicrobiaceae* was dominant only at the reference sites, *Intrasporangiaceae* only at the TC sites, and *Neisseriaceae* [macroinvertebrate guts] only at the downstream TC sites). Additionally, *Trueperaceae* was only dominant in TC sites 2 and 3 (sediment), *Ignavibacteriaceae* at TC site 1(sediment), and *Alcaligenaceae* was dominant only in Yellow Creek. Downstream sites TC-4 and 5 had relatively more similar taxonomic composition (Figure 9 D and E).

Additionally, some site specifics trends in the dominant bacterial taxa are mentioned below. For TC-site 1, *Intrasporangiaceae* and *Microbacteraceae* were only dominant in water samples, also Flavobacteraceae had highest relative abundance in water samples. Comamonadaceae was highest in sediment while *Sphingomonadaceae* and *Chitinophagaceae* had highest relative abundances in *Lestidae.* For TC-site 2, *Intrasporangiaceae* and *Microbacteraceae* were only dominant in water samples. *Chitinophagaceae* had highest relative

abundance in sediment while *Sphingomonadaceae* had highest relative abundance in *Lestidae*. At TC-site 3, *Intrasporangiaceae* and *Microbacteraceae* were only dominant in water sample while *Rhodobacteraceae* and *Sphingomonadaceae* had highest relative abundance in sediment and *Chitinophagaceae* had highest relative abundance in *Lestidae*. In case of TC-site 4, *Intrasporangiaceae* and *Microbacteraceae* were only dominant in water sample while *Chitinophagaceae* and *Sphingomonadaceae* had highest relative abundances in *Dugesiidae* and sediment, respectively. Similar to TC-site 4, *Intrasporangiaceae* and *Microbacteraceae* were only dominant in water sample while *Chitinophagaceae* and *Sphingomonadaceae* had highest relative abundances in *Dugesiidae* and sediment, respectively for TC-site 5. Moreover, *Neisseriaceae* was only dominant in macroinvertebrates sampled from TC -sites 4 and 5. At both, Yellow Creek and Furnace Run, *Microbacteraceae* was only dominant in water sample while *Flavobacteriaceae* had highest relative abundance in the same. Specifically, at Yellow Creek, *Porphyromonadaceae, Desulfobacteraceae,* and *Sphingomonadaceae* had highest relative abundances for *Hydropsychidae, Gerridae,* and sediment, respectively while at Furnace Run, *Sphingomonadaceae* and *Cytophagaceae* had highest relative abundance in *Psephenidae* and *Chironomidae* had highest relative abundance for *Enterobacteriaceae*.

GLM was performed for the families shown in Figure 9 (A-G) to detect significant differences among the microbiomes. Based on these results, among the dominant families that were common at all sites, *Chitinophagaceae* (specifically, *Lestidae, Dugesiidae, Hydropsychidae*), *Cytophagaceae* (specifically, *Psephenidae*) had higher relative abundances in macroinvertebrate guts while relative abundance of *Flavobacteriaceae, Rhodobacteraceae, Sphingomonadaceae* (exceptions *Lestidae* and *Psephenidae*), and *Comamonadaceae* was higher in the free-living than gut microbiomes. In addition, *Planctomycetaceae, Neisseriaceae,*

Methylophilaceae, and *Rhodobiaceae* had higher relative abundances in macroinvertebrate guts compared to free-living microbiomes at the downstream sites in (TC- sites 4 and 5) while families *Hyphomicrobiaceae, Methylophilaceae, Sinobacteraceae, Verrucomicrobiaceae, Xanthomonadaceae,* and *Burkholderiaceae* had higher relative abundance in free-living microbiomes compared to the host-associated microbiomes at most of the TC sites. For both the reference sites, families *Methylophilaceae* (specifically, high in *Psephenidae* and sediment for Furnace Run and Yellow Creek, respectively), *Xanthomonadaceae* (highest in macroinvertebrate guts and sediment for Furnace Run and Yellow Creek, respectively) and *Verrucomicrobiaceae* (highest in sediment) differed significantly in relative abundances among host-associated and free-living microbiomes. Interestingly, *Desulfobacteraceae* was the only family with higher than 1% relative abundance for *Gerridae* sampled at the Yellow Creek site. Families not mentioned above either had greater than 1% relative abundance only for a single microbiome (e.g., *Microbacteraceae* in water) or were not significantly different among the host-associated and free-living microbiomes.

For the FFGs, differences in relative abundance among the dominant phyla (>1% relative abundance) are shown in Figure 10. There was considerable overlap among the dominant phyla where Proteobacteria and Bacteroidetes had the highest relative abundances in all FFGs. However, phylum Acidobacteria was not among the dominant phyla in omnivores (only *Cambaridae*) while Planctomycetes was dominant only in filter-feeders and omnivores.

Out of the 90 dominant (> 1% relative abundance) bacterial families that were common and abundant across sites, relative abundances of only 6 (after FDR correction) were found to be significantly correlated with at least one of the two sulfonamide ARGs while none were correlated to *tetW*. According to the heatmap (Figure 11 A), significant positive correlations was

found between family *GpIV* and abundance of *sulI* and *sulII*. All the remaining families reported in Figure 11 A were only positively correlated to *sulI* relative abundance and their corresponding corrected P values are shown in Figure 11 B.

When examining potential transfer of ARGs by macroinvertebrates in experimental microcosms, there were no significant differences in abundance of ARGs in the sediment among treatments with and without macroinvertebrates (*sulI*: P = 0.23, *sulII*: P = 0.36 and *tetW*: P = 0.19 [Figure 12 A:C]). In contrast, there were significant differences in gut microbiomes for *sulI* ($P<0.05$; Figure 13 A) between the treatments with the autoclaved and unautoclaved sediment. Specifically, macroinvertebrates in the unautoclaved treatment had higher gene copies of *sulI* than in the autoclaved treatment. For *tetW*, the two macroinvertebrate types differed ($P < 0.01$; Figure 13 B) where *Cambaridae* (crayfish) had higher *tetW* gene copies than *Corbicula* (clams). No significant ($P>0.05$) interaction effect between treatment and macroinvertebrate type was detected for *sulI* or *tetW*. *sulII* was not detected in any of the macroinvertebrates sampled.

DISCUSSION

A variety of factors, such as physiochemical conditions of the environment, abundance of metal resistant genes, watershed land use patterns, and bacterial community composition, are related to abundance of ARGs in freshwater ecosystems (Roberto et al. 2019, Winkworth 2013, Pal et al. 2015). In this study, we introduce another potential driving factor of ARG abundance: macroinvertebrate gut microbiomes. Freshwater macroinvertebrate gut microbiomes may be reservoirs for ARGs relative to their surroundings. Additionally, this work suggests that FFGs can impact ARG abundances and certain bacterial taxa may harbor specific ARGs. However, we did not find evidence that freshwater macroinvertebrates serve as vectors of ARGs. This was surprising because macroinvertebrates used in the study had higher ARG abundance in

comparison to sediment and macroinvertebrates have been shown to transfer gut bacteria via defecation (McEwen and Leff 2001).

Any habitat that harbors high abundance of ARGs in comparison to the surrounding environment can be termed as a "hotspot" of ARGs. Guts have conducive environments for horizontal gene transfer (HGT) (Shterzer and Mizrahi 2015, Lerner et al. 2017, Chong et al. 2020) which may facilitate ARG spread and abundance. The current study demonstrated that freshwater macroinvertebrate gut microbiomes can serve as hotspots for ARGs in comparison to their free-living counterparts in lotic ecosystems, presumably due to favorable gut conditions. An important step in understanding overall antibiotic resistance in any ecosystem is to identify potential reservoirs for ARGs (Arnold et al. 2016) and our results demonstrate that freshwater macroinvertebrate guts can serve that purpose. Although we observed site specific variation in ARG abundance among microbiomes (with most sites exhibiting higher ARG abundance in host-associated than free-living microbiomes), further research in multiple streams is necessary to draw generalized conclusions. Additionally, for some of the sites (TC-2, TC-5, and Furnace Run) we observed that water samples had highest relative *tetW* abundance. Although our study lacks data about the concentration of tetracycline in these sites during our sampling, it is possible that high concentration of tetracycline in water which came from agricultural and WWTP sources during our sampling may have increased *tetW* relative abundance in the water than the other microbiomes. Moreover, pristine sites are known to harbor ARGs (McArthur and Tuckfield 2000), therefore, to completely understand what might have caused increased *tetW* relative abundance in Furnace Run (forested site) water samples, further investigation is necessary.

In terms of bacterial community composition, abundance of families *Chitinophagaceae*, *Comamonadaceae, Burkholderaceae, Verrucomicrobiaceae,* and *Rhodobacteraceae* across all

sites was in accordance with the results of Roberto et al. 2018 (assessed bacterial community composition in biofilms in the same sites). Overall, the prevalence of several dominant Proteobacterial and non-Proteobacterial families in the macroinvertebrate gut microbiome is consistent with the observations of the study by Marti et al. (2018) which investigated antibiotic resistance in fish guts. Additionally, similarly to Ayayee et al. (2018), bacterial families like *Enetrobacteraceae, Ruminococcaceae,* and *Chitinophagaceae* (at certain TC sites) were more abundant in macroinvertebrates while *Microbacteriacea, Comamonadaceae, Flavobacteriaceae* and *Burkholaderiaceae, Saprospiraceae* were dominant in free-living microbiomes. Additionally, in this study *Neisseriaceae* was dominant in macroinvertebrate gut microbiome in downstream sites (TC 4 and 5). Sorting of bacteria due to gut morphological and physiological conditions may result in families common in free-living microbiomes to be reduced or eliminated from macroinvertebrate guts (Ayayee et al. 2018, Cummins and Klug 1979).

ARG abundance may be connected to variation in bacterial assemblages because certain bacterial taxa have a higher probability of harboring specific ARGs (Roberto et al. 2019, Li et al. 2015, Balcázar et al. 2015, Zhou et al. 2017). To examine the relationship of bacterial community composition to ARG abundances, we examined the correlation between bacterial community composition and the ARG profile at the bacterial family level. Positive significant correlations with multiple ARGs indicate co-occurrence and cross resistance (Li et al. 2015, Summers 2002). However, in our study only one family (*GpIV*) was significantly correlated to both the sulfonamide genes, and no correlation was found between the *tetW* relative abundance and bacterial families. This suggests that other potential factors (e.g., antibiotic concentration, concentration of metal resistance genes [Wang et al. 2014, Roberto et al. 2019]) may be influencing bacterial community composition and ARG abundance at these sites. The

concentration of antibiotics is known to have a positive relationship with corresponding ARGs (Wang et al. 2014) while Roberto et al (2019) observed that multiple families were related to both ARGs and metal resistance genes simultaneously. Therefore, to fully understand the correlation between ARG abundance and bacterial community composition, a future study including other stressors (e.g., concentrations of heavy metals, antibiotics, and metal resistance genes) is necessary.

In this study, bacterial community composition and ARG abundances differed among FFGs (more appropriately, taxa specific as only limited members of different FFGs were used). The separation of the omnivore gut bacterial community composition from other FFGs is consistent with the findings reported by Ayayee et al. (2018). Additionally, according to Ayayee et al. (2018) gut microbiomes differed among FFGs consistently and independently from differences among sites. This suggests that similarities in gut morphophysiological conditions within members of a FFG is more important than stream conditions in predicting gut bacterial community composition. However, it is important to note that like Ayayee et al. (2018), we only had *Cambaridae* as a representative for omnivores so generalized interpretation of our results to all omnivores is not appropriate. Differences in gut morpho-physiological conditions and dietary materials can result in variation in bacterial community composition among FFGs (Ayayee et al. 2018, Cummins and Klug 1979, Pechal and Benbow 2016). For example, guts of crayfish are structurally complex, and pH may range from 4.8-7 (Dorn and Wojdak 2004, Brown 1995) while filter-feeders are usually characterized by straight tube guts (Ayayee et al. 2018, Cummins and Klug 1979). However, members of *Chironomidae* are known to house bacteria in their enlarged hindguts (Cummins and Klug 1979). Presumably bacterial community composition, in turn, influences ARG abundance in different FFGs. Additionally, high *tetW* relative abundance in

filter feeders maybe associated with the dietary materials they consumed from the water column which had high *tetW* relative abundance in some sampled sites (e.g.: TC-site 1). However, it must be noted that sediment and water samples used were not independent of each other as we did not look at the particular dietary materials consumed by respective taxa but the whole microbiome (sediment, water). Moreover, it must be noted that we had a limited sample size in this study and future experiments with a greater number of representatives from different FFGs will be necessary to understand how FFGs affect ARG abundance and bacterial community composition.

Environmental factors, including physicochemical conditions, play an important role in shaping the bacterial community composition and ARG abundances in streams (Zhou et al. 2017, Pruden et al. 2006, Garner et al. 2016, Roberto et al. 2018). We know that increased anthropogenic activities alter stream physicochemical conditions (Mulholand et al. 2008, Roberto et al. 2018) and, in the current study, certain physicochemical variables were correlated with the ARG profile. Prior research at these sites has also shown that dissolved nitrate and ammonium concentration, temperature, and conductivity correlated with ARG abundance in biofilms (Roberto et al. 2019).

One of the other roles of freshwater macroinvertebrates examined in this study was whether they can be vectors of ARGs. We showed that macroinvertebrate gut microbiomes serve as reservoirs of ARGs, however, macroinvertebrates were not able to disseminate and significantly alter the ARG abundances in sediment. This was unexpected as prior research reported that freshwater macroinvertebrates can disseminate their gut bacteria to their immediate environment (McEwen and Leff 2001). Although it must be noted that in the study by McEwen and Leff (2001) the extent of gut colonization and subsequent dissemination via feces varied

among bacterial species. Additionally, one of the invertebrates used in this study was *Corbicula*; burrowing animals are capable of dislodging sediment and transferring bacteria to the water column (Leff and Leff 2000). Therefore, further experimentation with an array of macroinvertebrates is necessary to fully assess how ARG abundance and bacterial community composition (guts and free-living microbiome) are interconnected. Tetracyclines are a broad-spectrum antibiotic widely used for various purposes like treating human and animal infections (Xu et al. 2021). Additionally, they have long aquatic half life which makes them more persistent and harder to degrade in the environment leading higher accumulation (Chee-Sanford et al. 2009, Lu et al. 2018). These factors may have resulted in high *tetW* abundance in control microcosm sediments.

CONCLUSIONS

This study documented for the first time that freshwater macroinvertebrate guts may serve as potential reservoirs for ARGs. We also found that there were differences in relative ARG abundance among the gut microbiomes of different FFGs (specifically, at the taxa scale). Additionally, we found that only a few bacterial taxa were correlated with sulfonamide genes which may suggest that specific bacterial taxa are more likely to harbor specific ARGs; however, further research with information about additional stressors (e.g., antibiotic concentration, metal resistance genes) is necessary to fully understand how bacterial community composition and ARG abundances are related. Although the current study demonstrated that at most of the sites freshwater macroinvertebrate guts have higher ARG abundance than their free-living counterparts, to draw generalizations about how host (gut) associated and free-living microbiomes vary in ARG abundance in lotic ecosystems, future studies need to sample macroinvertebrate and free-living microbiomes in multiple streams. Additionally, given that

anthropogenic impacts affect ARGs in stream biofilms, another possible future study can look at the role of human activities (e.g., watershed land use patterns, effect of stressors like metal resistance genes, concentration of antibiotics and metals) on ARG abundances in host-associated microbiome

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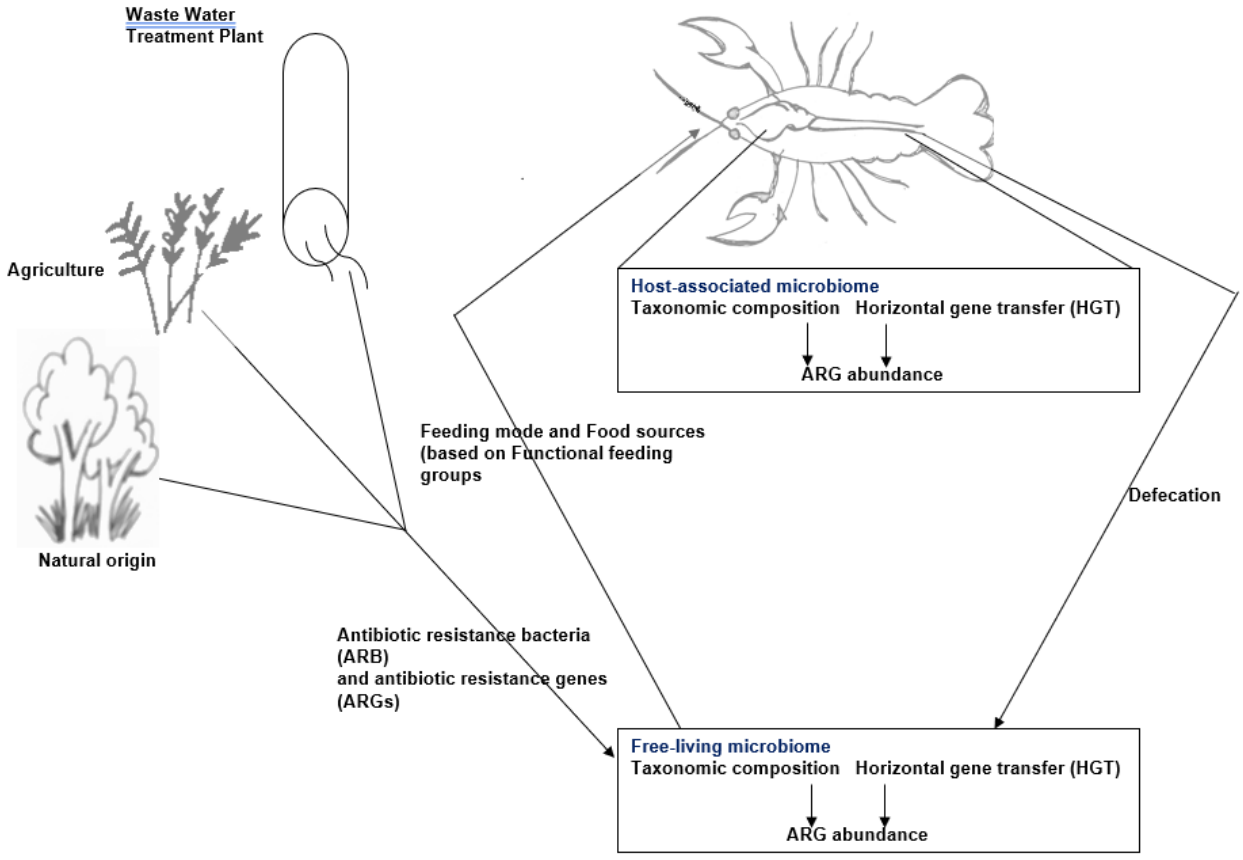


Figure 2. Conceptual diagram showing the possible role of macroinvertebrate microbiome in harboring and disseminating ARGs. The arrows indicate different things like flow paths and mechanisms. Difference in ARG abundance among microbiomes and functional feeding groups (vis feeding) were tested in hypotheses 1 and 2, respectively. Correlation between bacterial community composition and ARG abundance was tested in hypothesis 3 while hypothesis 4 tested whether macroinvertebrates can be vectors of ARGs (dissemination via defecation).

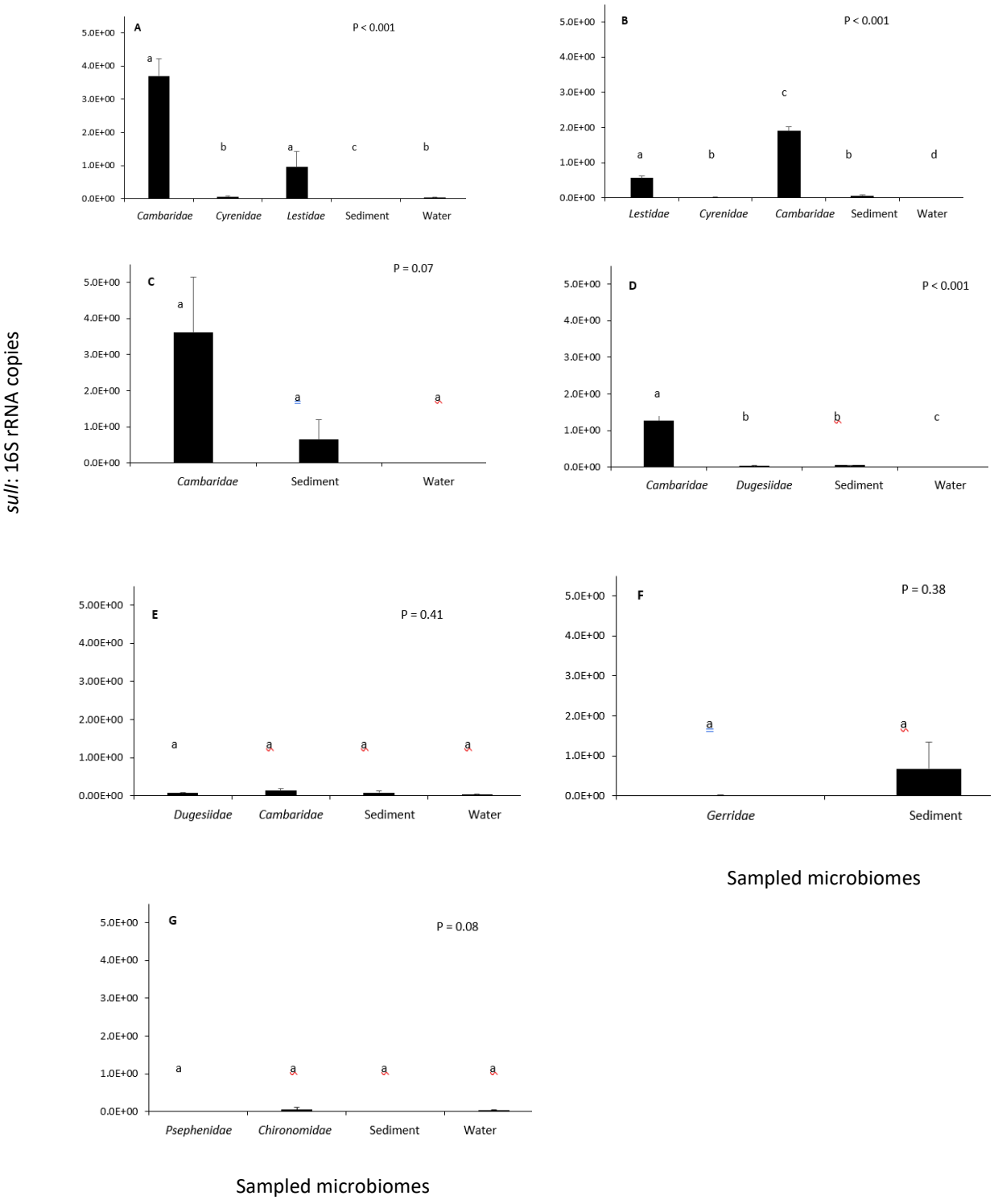


Figure 3. Differences in *sulI* abundance relative to 16S r RNA gene among sampled microbiomes. Specifically, at Tinker's Creek: site 1 (A), site 2 (B), site 3 (C), site 4 (D), site 5 (E); Yellow Creek (F), and Furnace Run (G). Values (untransformed data) are mean and standard errors (n=3). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences.

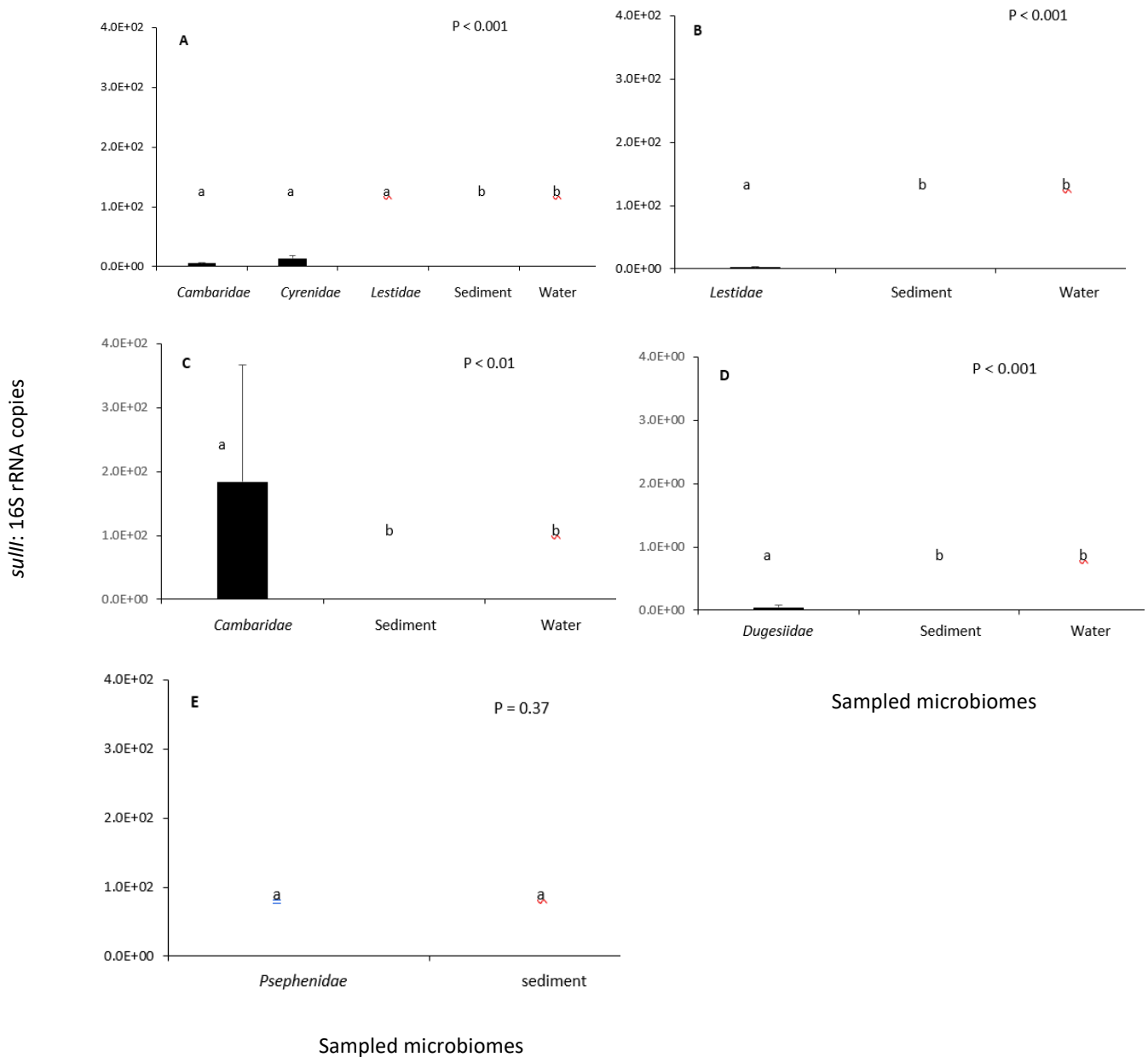


Figure 4. Differences in *sulII* abundance relative to 16S r RNA gene among sampled microbiomes. Specifically, at Tinker's Creek site 1: (A), site 2 (B), site 3 (C), site 4 (D); and Furnace Run (E). Values (untransformed data) are mean and standard errors (n=3). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences. (Note change in Y-axis maximum for D)

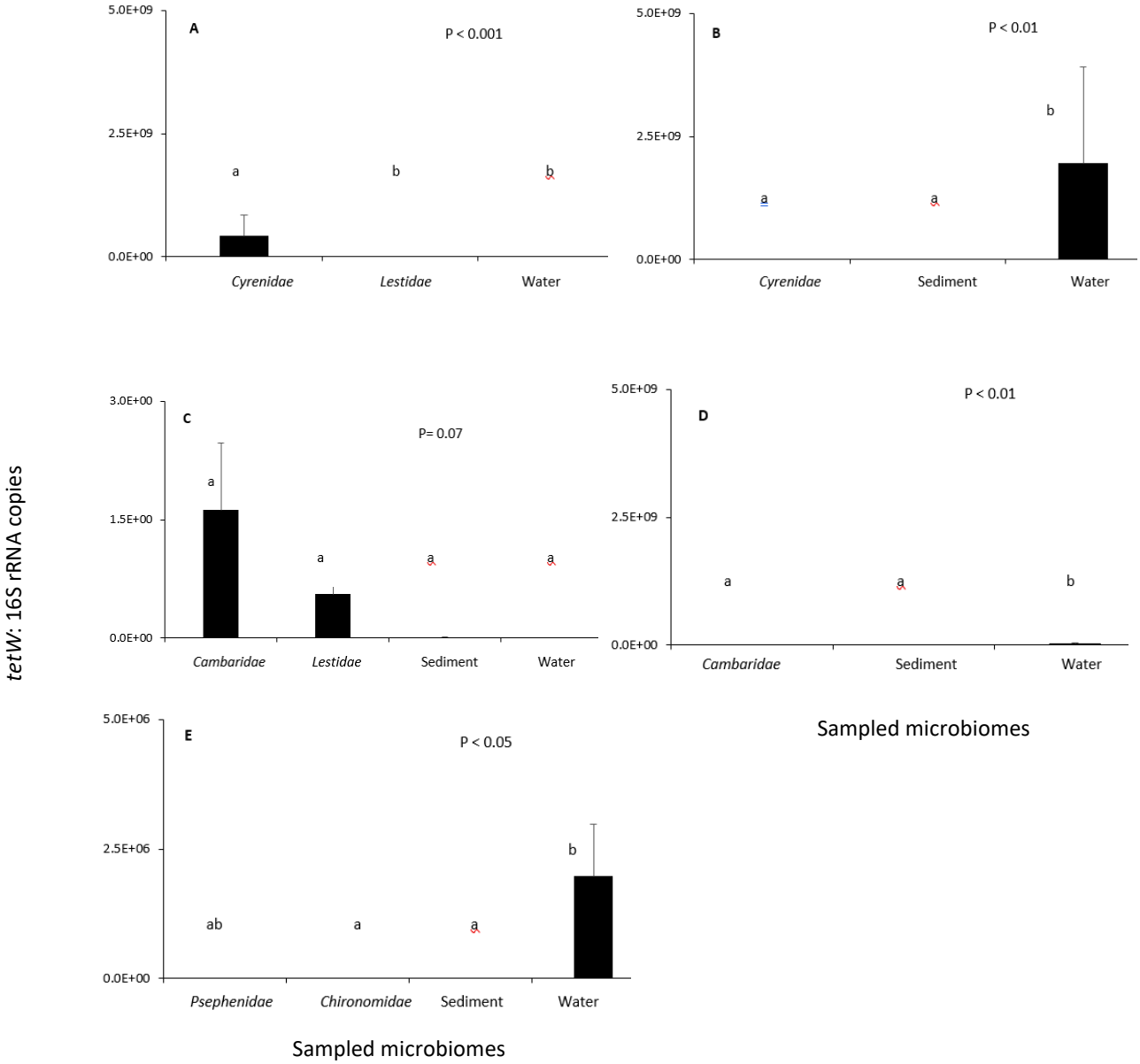


Figure 5. Differences in *tetW* abundance relative to 16S r RNA gene among sampled microbiomes. Specifically, at Tinker's Creek: site 1 (A), site 2 (B), site 3 (C), site 5 (D), and Furnace Run (E); Values (untransformed data) are mean and standard errors (n=3). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences. (Note change in Y-axis maximum for C and E).

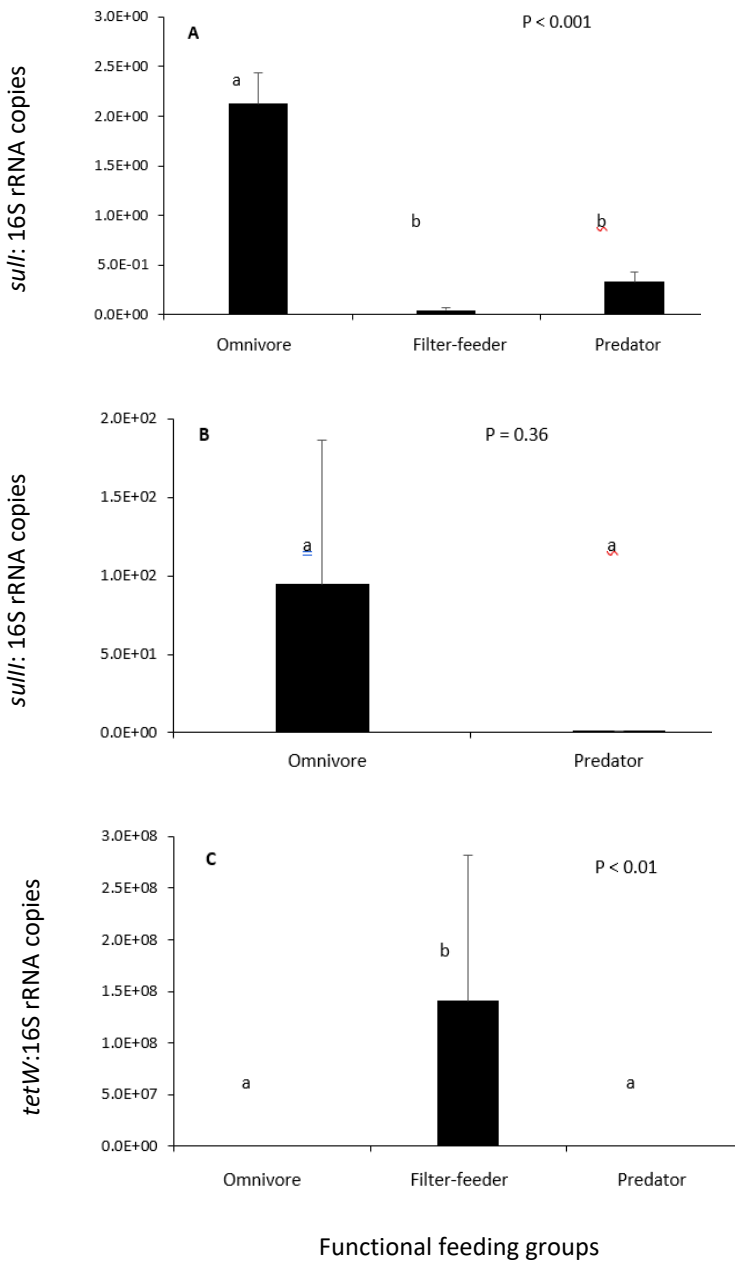


Figure 6. Differences in *sulI* (A), *sulII* (B), and *tetW* (C) abundances relative to 16S rRNA gene among freshwater macroinvertebrate functional feeding groups (averaged across sampling sites; untransformed data). Values are mean and standard errors. Lower case letters directly above bar graph indicate significant (letters differ) or no significant (letters are the same) differences.

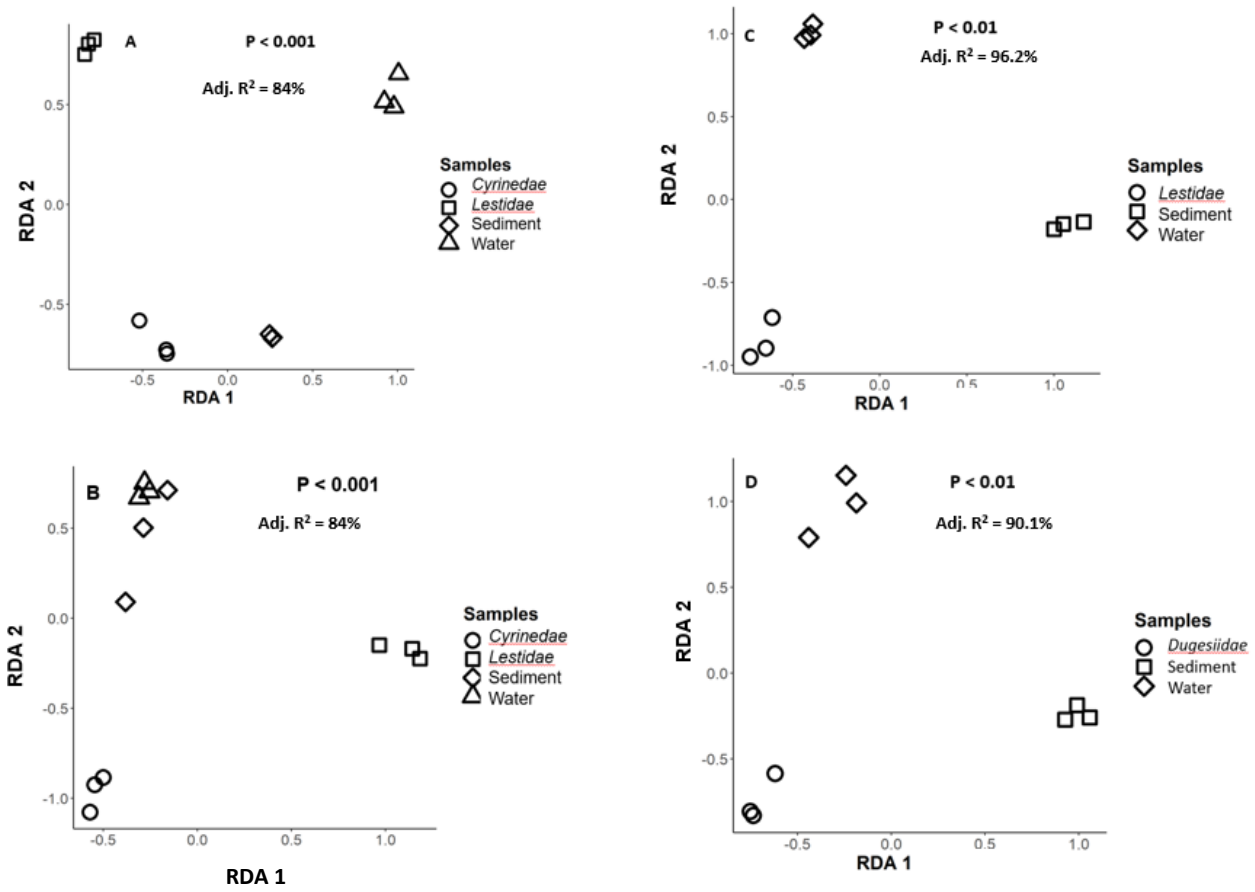


Figure 7 A. RDA plots at family level depicting differences in bacterial community composition among different microbiomes sampled at TC- sites: 1 (A), 2 (B), 3 (C), and 4 (D).

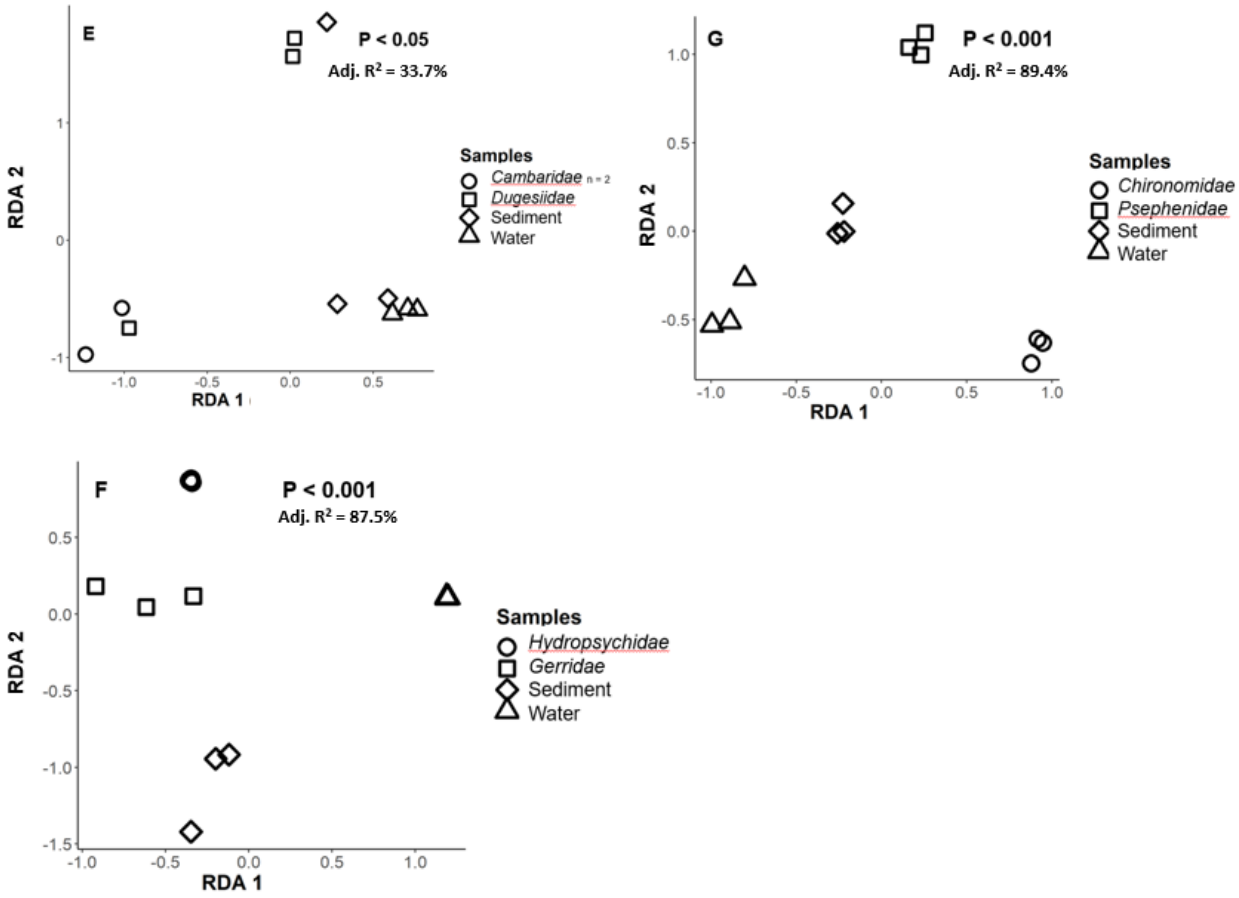


Figure 7 B. RDA plots at family level depicting differences in bacterial community composition among different microbiomes sampled at TC- site 5 (E), Yellow Creek (F), and Furnace Run (G).

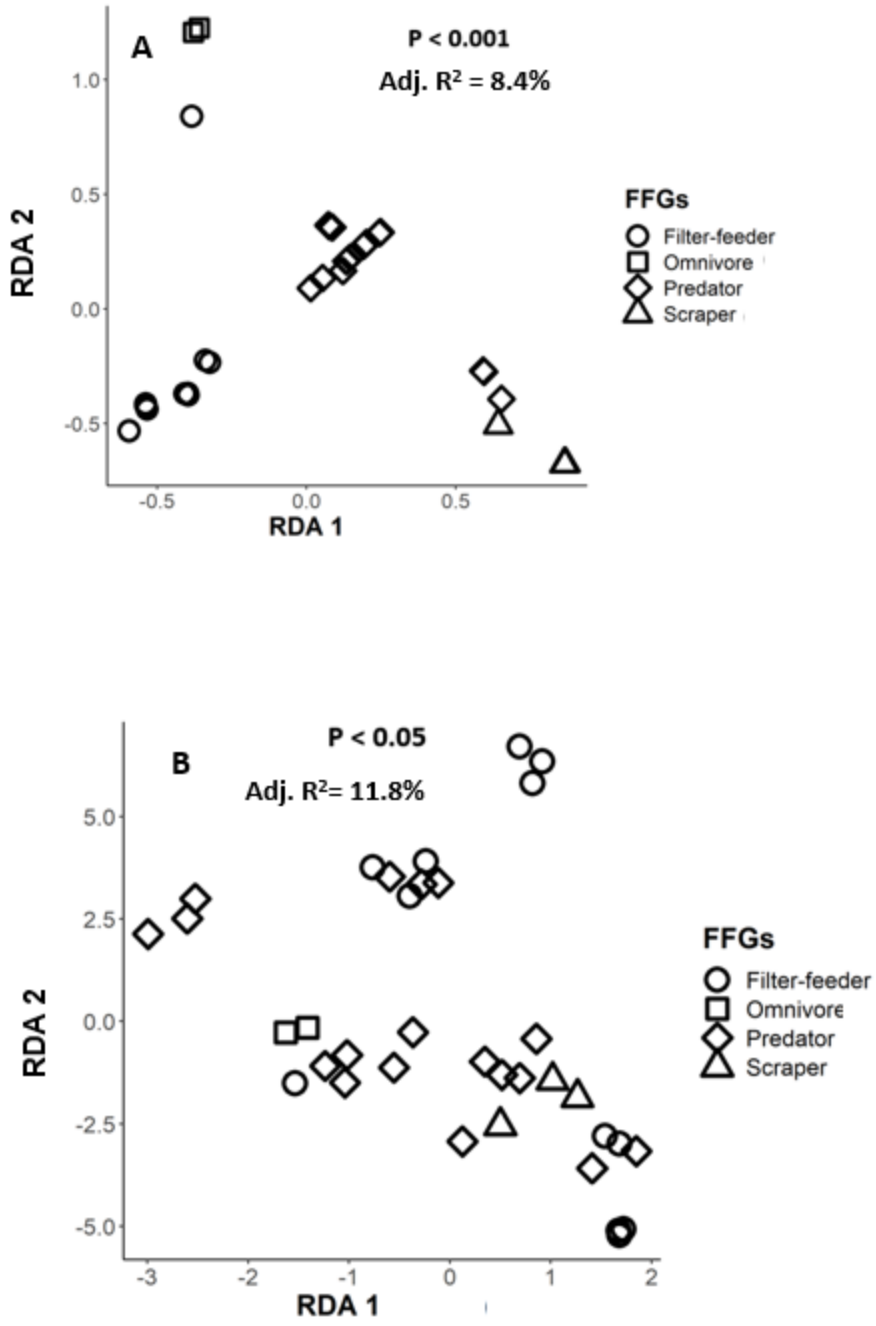


Figure 8. RDA plots on ASV level (A), and phylum level (B) depicting differences in bacterial community composition across different functional feeding groups.

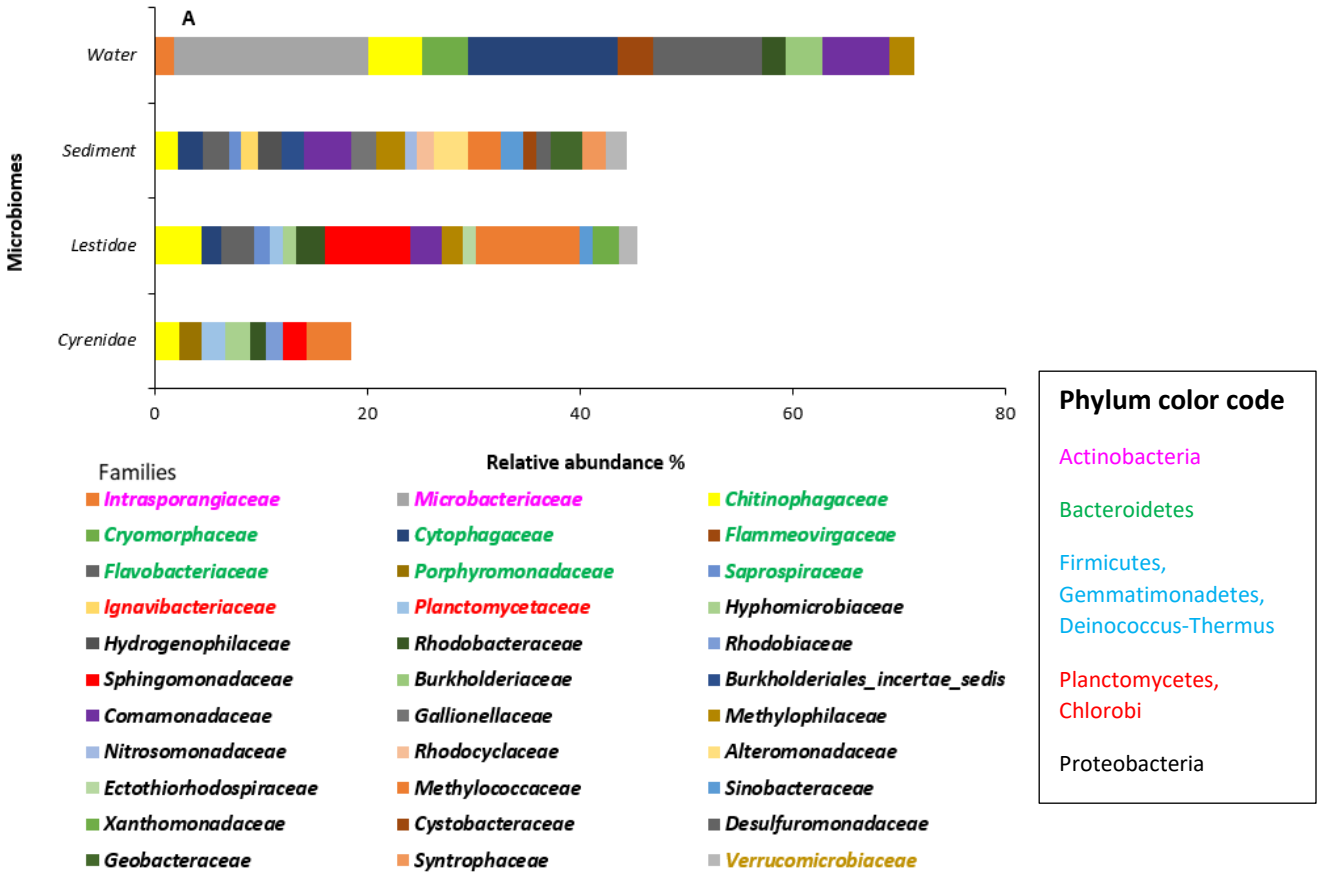


Figure 9 A. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for TC-site 1 (A)

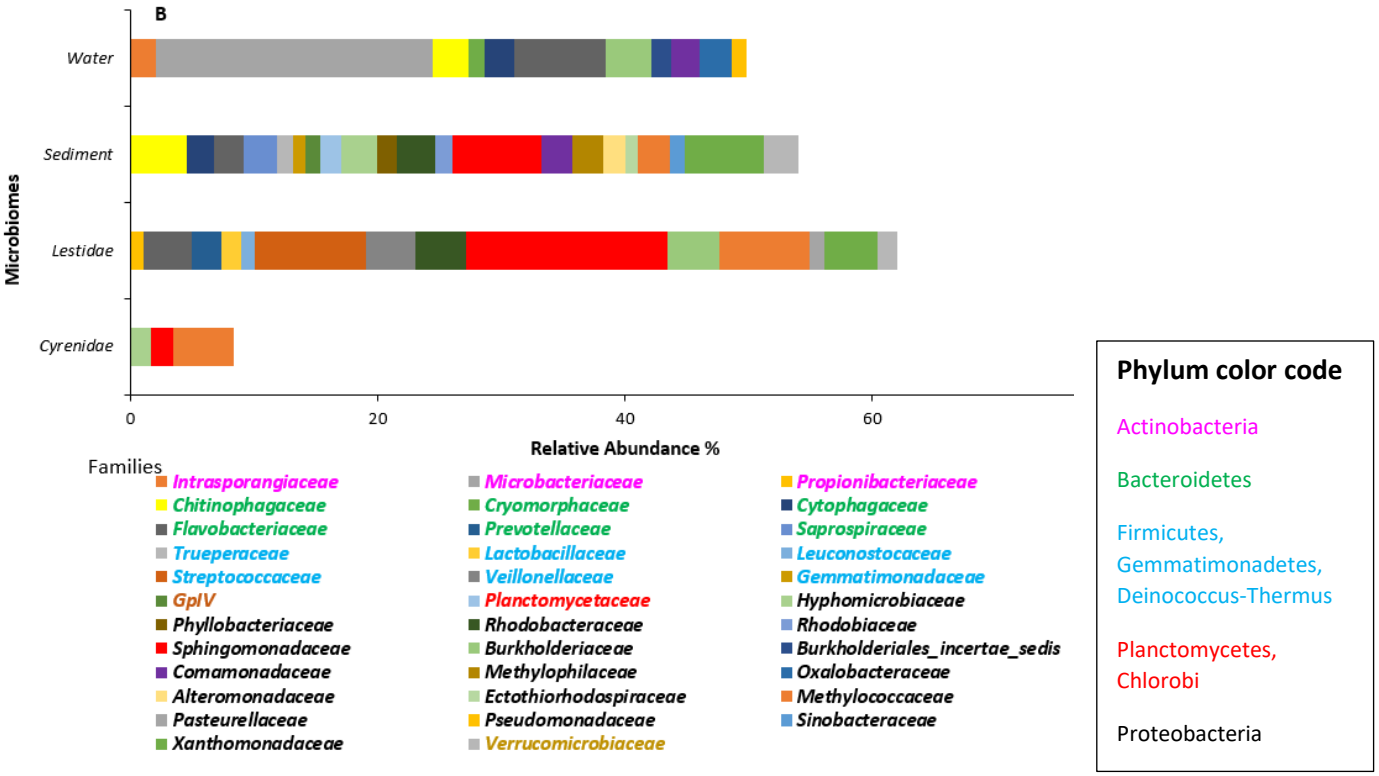


Figure 9 B. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for TC-site 2 (B).

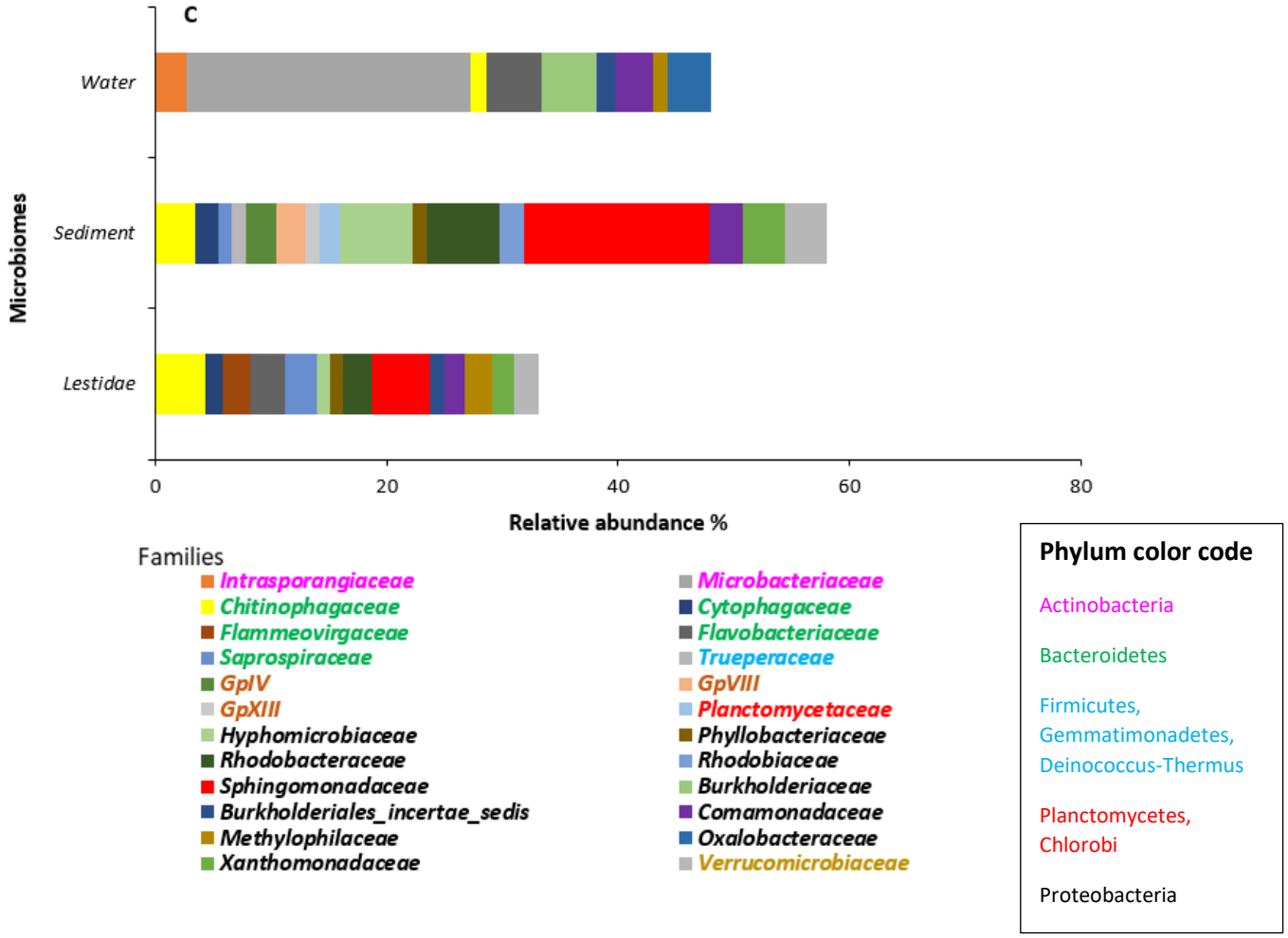


Figure 9 C. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for TC-site 3 (C)

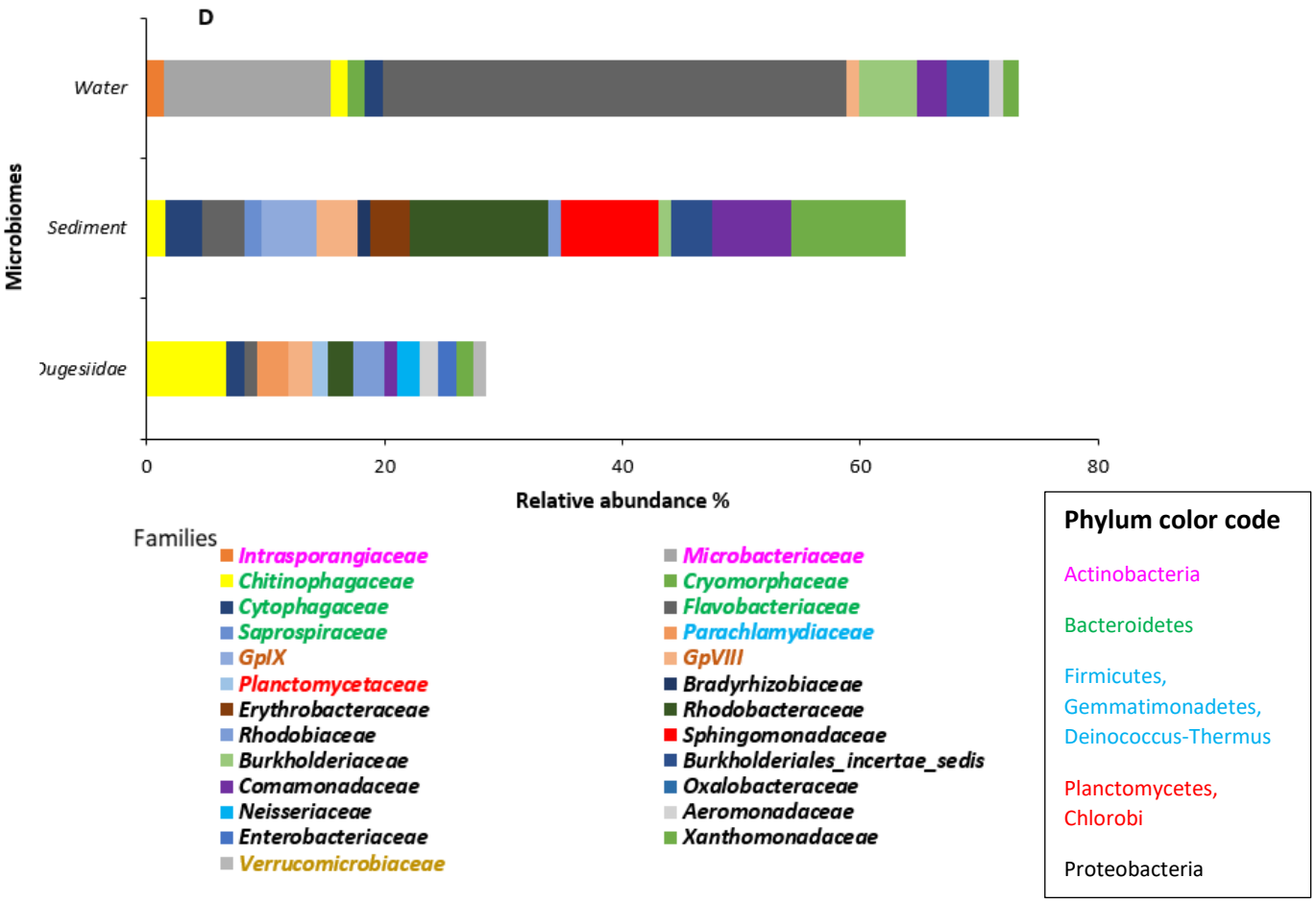


Figure 9 D. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for TC-site 4 (D)

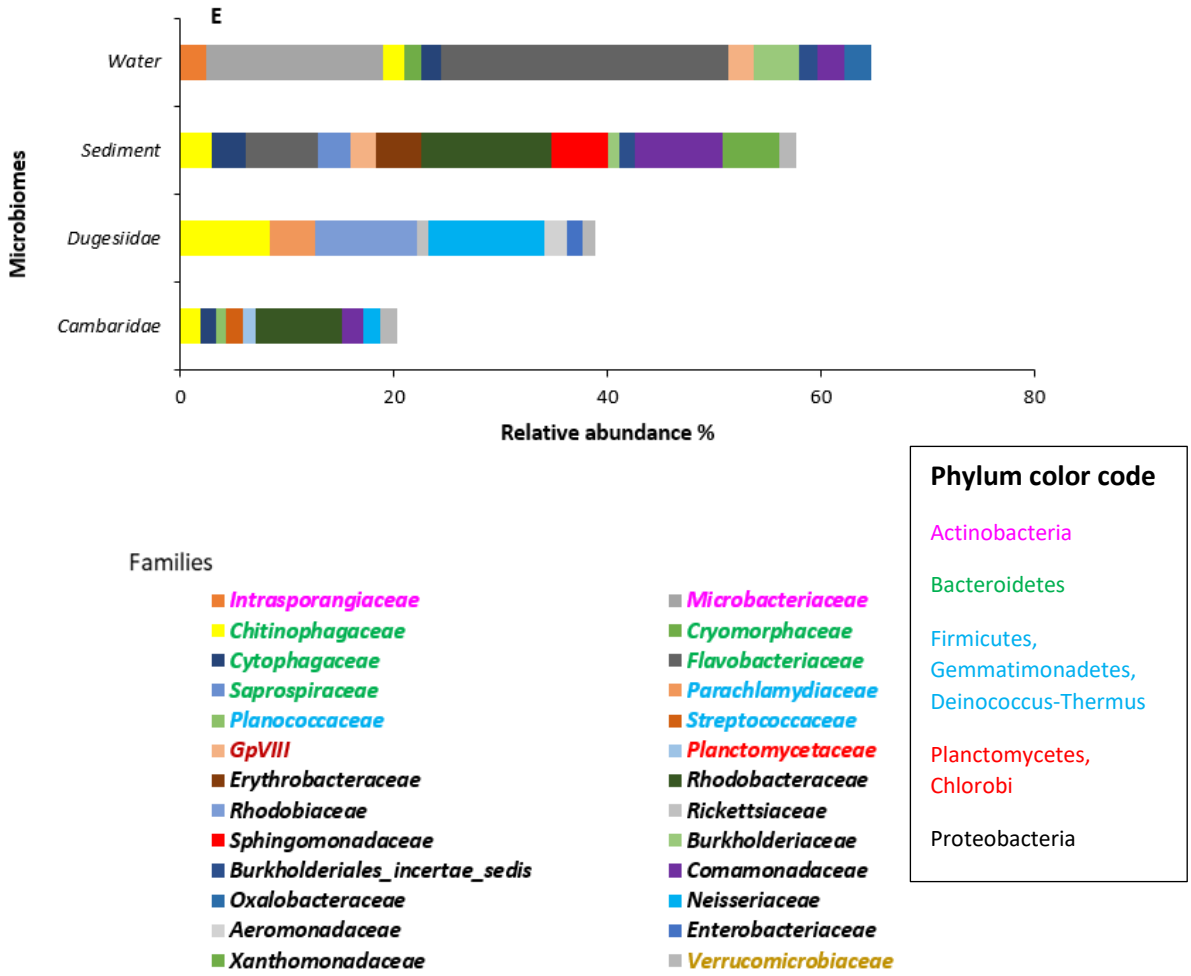


Figure 9 E. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for TC-site 5 (E)

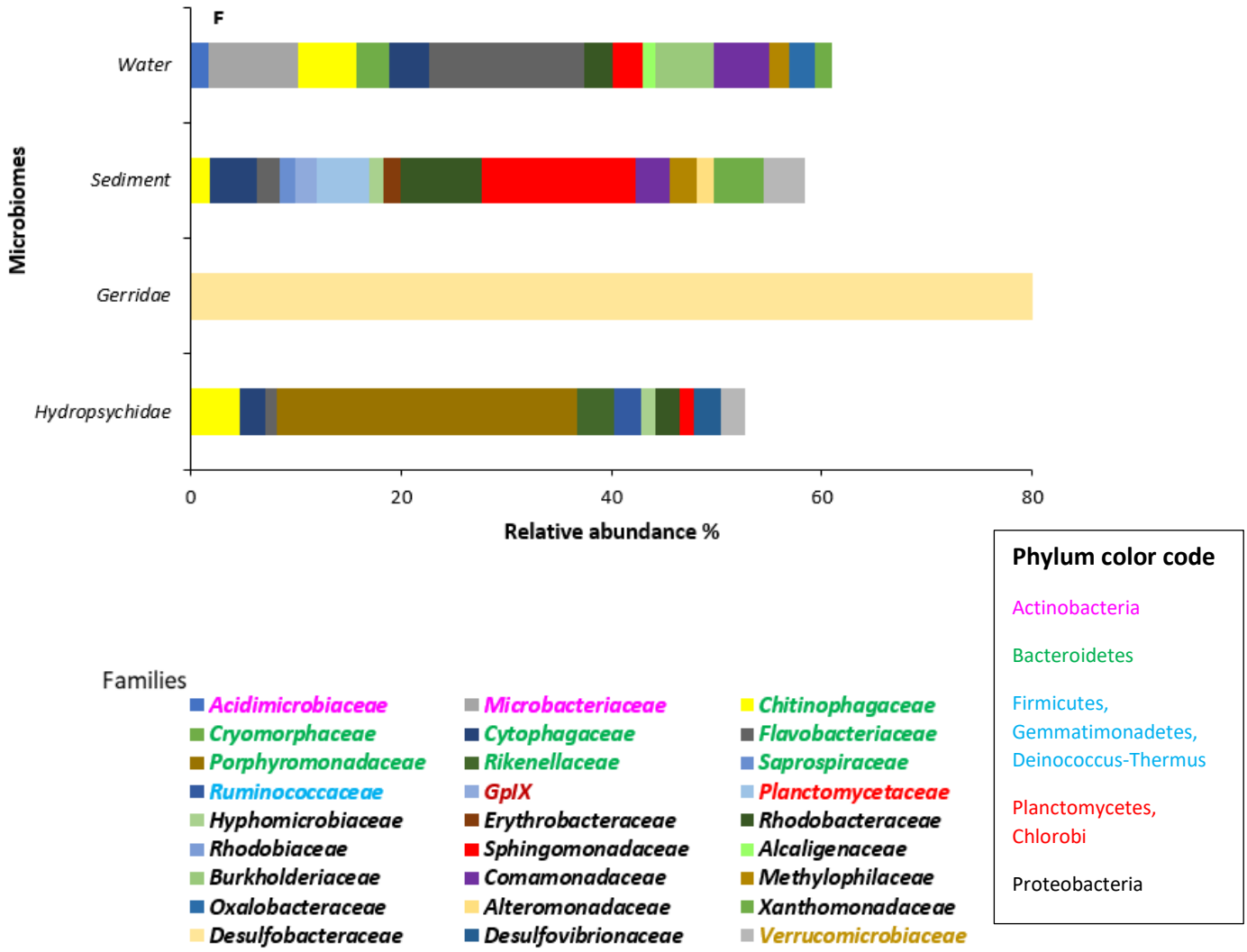


Figure 9 F. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for Yellow Creek (F).

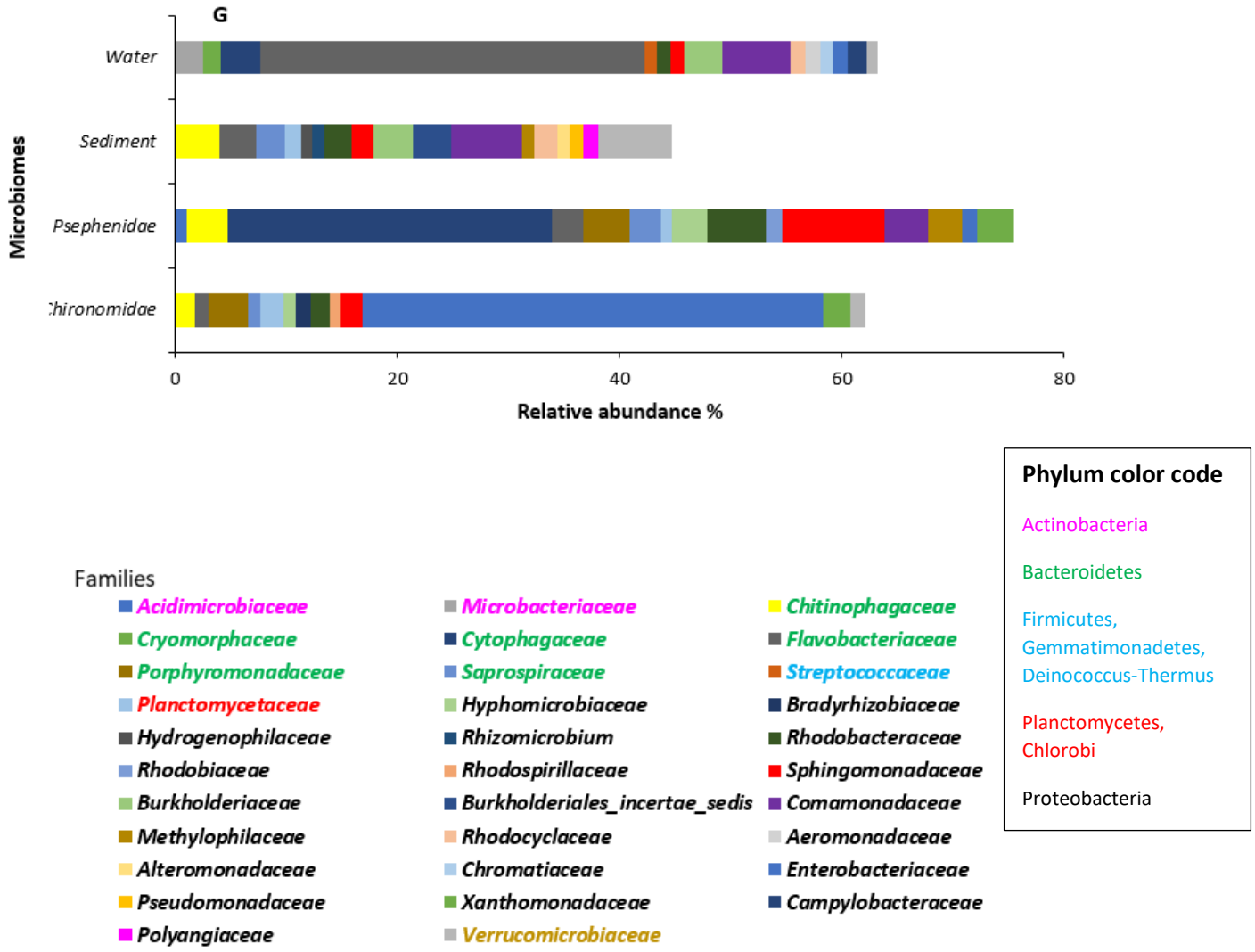


Figure 9 G. Relative abundance (% total) of all families (>1%) across different microbiomes (relevant macroinvertebrate taxa, sediment and water) for Furnace Run (G).

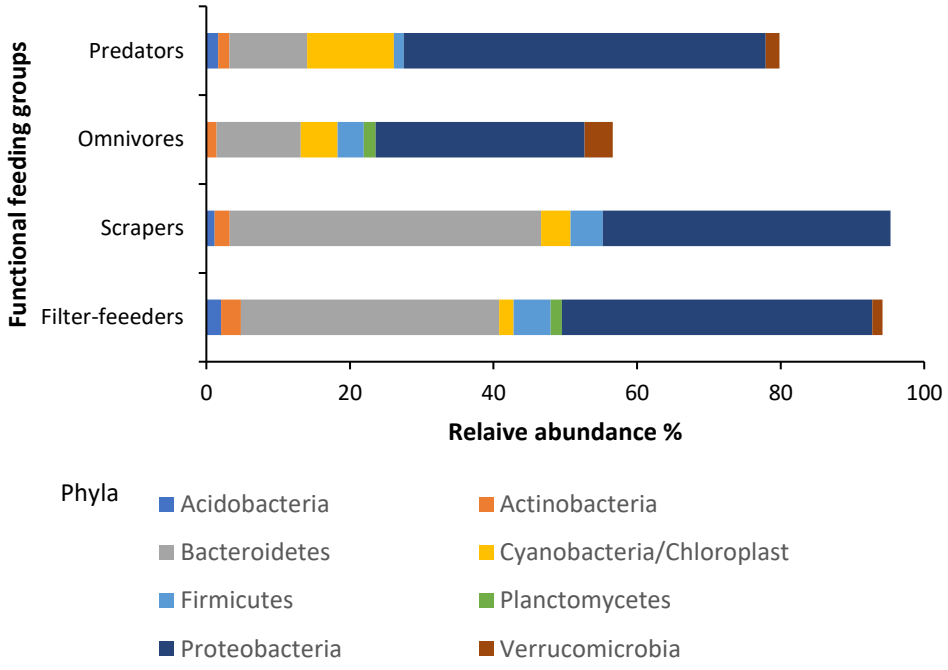
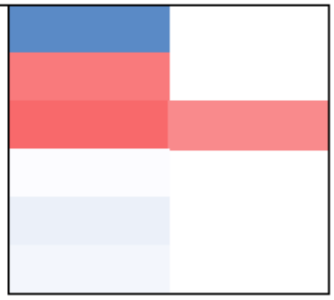


Figure 10. Relative abundance (% total) of all phyla (>1%) in different functional feeding groups.

A

Family	*sulI*	*sulII*
Burkholderiales_incertae_sedis		
Comamonadaceae		
GpIV		
GpIX		
GpVIII		
Trueperaceae		



B

Family	*sulI*	*sulII*
Burkholderiales_incertae_sedis	0.0008	
Comamonadaceae	0.004	
GpIV	0.004	0.01
GpIX	0.03	
GpVIII	0.04	
Trueperaceae	0.03	

Figure 11. Spearman's rank correlation coefficients (A) of the bacterial families that significantly correlated ($P < 0.05$) with relative ARG abundance and FDR corrected P values (B) for the same. In the heatmap shades of red indicate high while shades of blue refer to low correlation coefficients values.

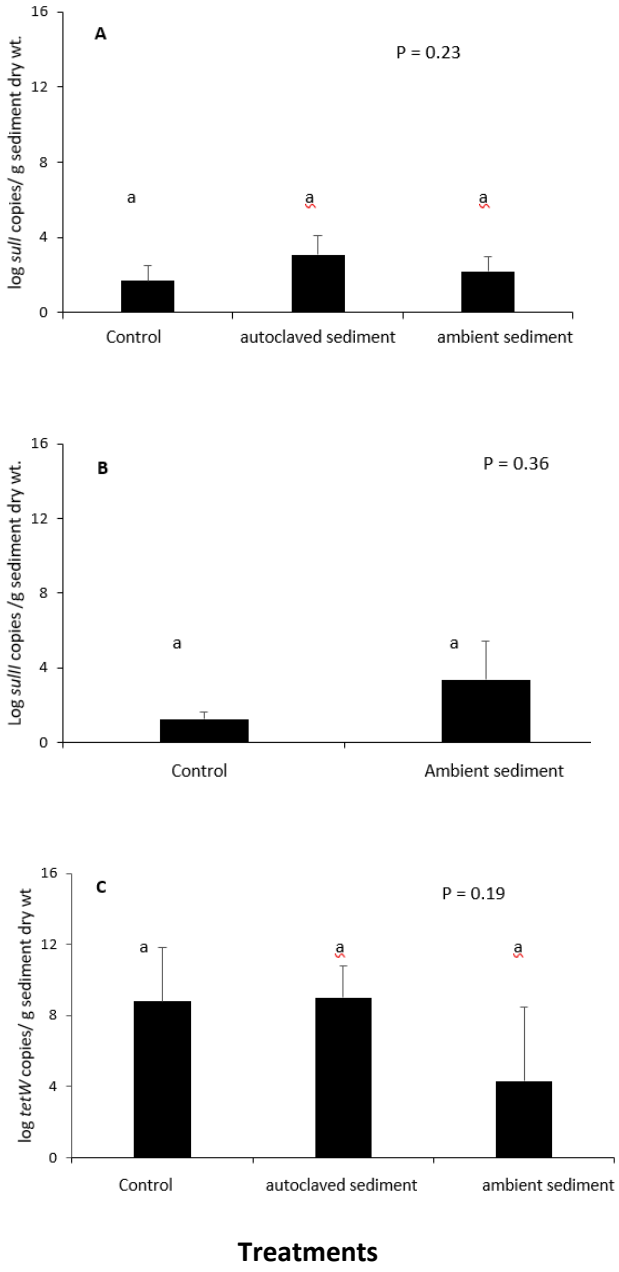


Figure 12. Differences between treatments (sediment samples) in case of *sulI* (A), *sulII* (B), and *tetW* (C) for the microcosm experiment. Values are means and standard errors (data log base 10 transformed; n=3).

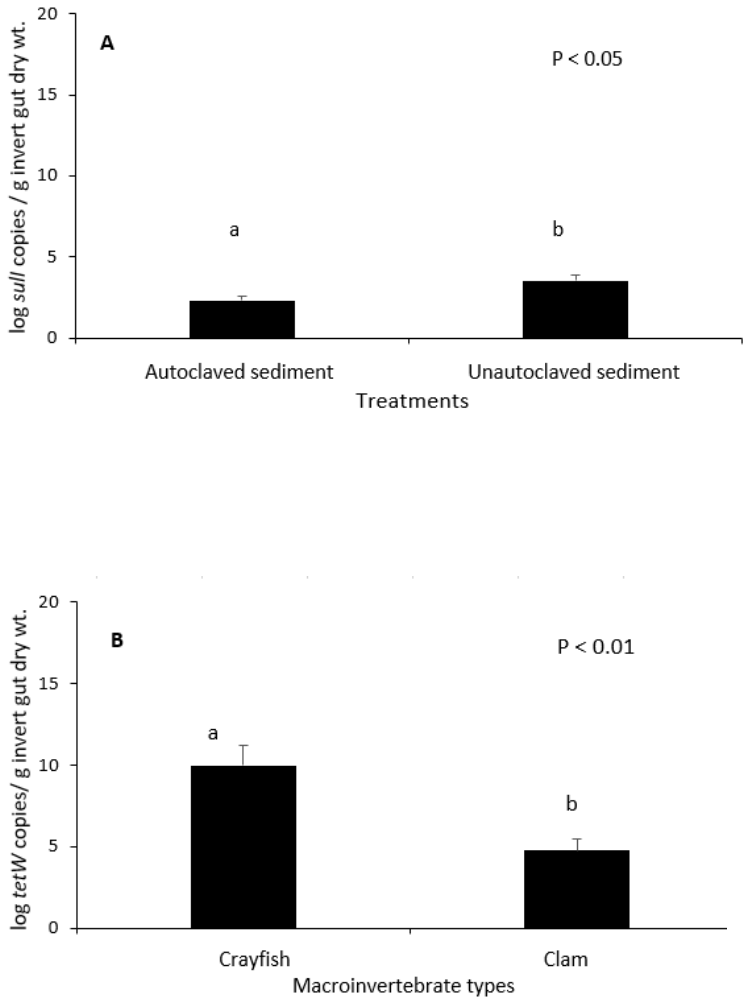


Figure 13. Significant differences between treatments (macroinvertebrate guts) in case of *sulI* (A) and invertebrate types in case of *tetW* (B) for the microcosm experiment. Values are means and standard errors (data log base 10 transformed).

Table 1. Means of physicochemical parameters measured across different sites

Site	Nitrate concentration (mg/L)	Ammonium concentration (mg/L)	Temperature (℃)	pH	Conductivity (µs/cm)
TC-site 1	0.08	0.06	19.4	7.6	608
TC-site 2	2.8	0.08	19.4	7.9	688
TC-site 3	7.63	0.05	20.5	7.8	886
TC-site 4	9.4	0.04	22.9	7.6	779
TC-site 5	4.7	0.11	21.8	7.6	786
Yellow Creek	0.47	0.06	22.4	7.4	879
Furnace Run	0.35	0.04	22.6	7.6	815

Table 2. P values (FDR corrected) and Spearman's correlation coefficients of physicochemical factors that were significantly related to ARG abundance

Physiochemical parameters	***sulI***	***sulII***	***tetW***
nitrate concentration (mg/L)			P =0.009; -0.17
ammonium concentration (mg/L)	P= 0.003; 0.20	P= 0.0006; -0.22	
temperature (℃)	P= 0.01; -0.16	P= 0.0005; -0.23	P= 0.0007; -0.22
pH	P= 0.003; 0.21		P = 0.000004; 0.30
Conductivity (µs/ cm)		P = 0.00001; -0.29	

CHAPTER III

DENITRIFICATION IN FRESHWATER CRAYFISH GUTS: LINKING GENE EXPRESSION TO NITROUS OXIDE EMISSION

ABSTRACT

Freshwater macroinvertebrate guts are microhabitats for incomplete denitrification; yet we do not know what controls the proportion of nitrogenous gases produced. In this study, we hypothesized that freshwater crayfish guts would facilitate incomplete denitrification over complete denitrification as reflected in minimal expression of *nosZ* gene and higher relative production of N_2O (compared to N_2). After reduction in gut bacterial load to facilitate colonization, gut microbiomes were modified by enriching with denitrifier isolates possessing (*S. marcescens)* or lacking *nosZ* (*P. chlororaphis*). Thereafter, denitrification rates and functional gene expression (*narG* and *nosZ*) were assessed. Compared to controls (with no added bacteria), potential total denitrification rate was significantly higher and incomplete denitrification was significantly lower in crayfish guts enriched with *S. marcescens* demonstrating that complete denitrification occurred in the gut. Additionally, extant gut bacteria were capable of denitrification in the absence of ingestion of bacteria. We did not find a significant relationship between functional gene expression (*narG* and *nosZ*) and denitrification rates. Expression of *narG* by non-denitrifying dissimilatory nitrate reducers and shifts in concentration of nitrate or

nitrite may have resulted in an inconsistent relationship between functional gene expression and denitrification rates. Overall, our results demonstrated that both, complete and incomplete denitrification occur in crayfish guts and the species of denitrifier may play an important role in determining the relative production of N_2O (compared to N_2) and *nosZ* expression.

Keywords

Incomplete denitrification, Crayfish, Nitrous oxide, Gene expression, denitrifying bacteria.

INTRODUCTION

Denitrification is ecologically significant in streams as it facilitates removal of reactive nitrogen and mitigates downstream eutrophication (Seitzinger 1988; Mulholland et al. 2008). However, nitrous oxide produced via incomplete denitrification is a major greenhouse gas (Stief et al. 2009, Tian et al. 2020) whereas complete denitrification results in production of dinitrogen (Tomasek et al. 2019, Phillipot et al. 2013). Environmental drivers of denitrification include anoxia, nitrate concentration, and the organic matter pool (Knowles 1982; Cavigelli and Robertson 2000; Dodds and Kemp 2000). In addition to physicochemical factors, denitrification rate depends on denitrifier abundance (Baxter et al. 2012, Van Gray et al. 2020) and expression of relevant functional genes (Wallenstein et al. 2006, Hong et al. 2019).

Macroinvertebrate gut microbiomes are diverse and capable of performing nitrogen (N) transformations, including denitrification (Dillon and Dillon 2004, Stief et al. 2009, Horn et al. 2006). Along with earthworms (Horn et al. 2003) and termites (Ngugi et al. 2012), aquatic macroinvertebrates (Stief et al. 2009, Heisterkamp et al. 2016) release nitrous oxide via incomplete denitrification. Dominance of incomplete over complete denitrification varies among

freshwater macroinvertebrate taxa (Stief et al. 2009, Wust et al. 2009). For example, nitrous oxide produced via incomplete denitrification in *Chironomus plumosus* accounted for 43-68% of the total nitrogenous gas flux while in *Ephemera danica* it was 15-29% (Stief et al. 2009). In spite of these variations among macroinvertebrate taxa, the in-vivo yield of nitrous oxide via incomplete denitrification in guts was consistently higher than in sediments (Stief et al. 2009, Heisterkamp et al. 2010, Seitzinger et al. 1988, Horn et al. 2006). Yet, studies on whether incomplete or complete denitrification is dominant in freshwater macroinvertebrates are limited to a few model organisms (Stief et al. 2009, Heisterkamp et al. 2010). Additionally, although relatively high in-vivo nitrous oxide emission compared to in-situ (undisturbed) sediment was reported in several freshwater macroinvertebrates belonging to different functional feeding groups, the specific contribution of incomplete (N_2O) and complete denitrification (N_2) processes to the nitrogenous gases produced via denitrification was not evaluated (Stief et al. 2009). Thus, although we have evidence suggesting that incomplete denitrification can be dominant in freshwater macroinvertebrate guts, to draw generalities regarding which of the two denitrification processes (incomplete and complete) is dominant we need to assess relative N_2O yield in other model organisms.

Macroinvertebrate gut conditions (specifically, anoxia or low oxygen concentrations, ample supply of organic carbon from food, and availability of nitrate) facilitate in-vivo denitrification (Horn et al. 2003, Stief and Beer 2006, Heisterkamp et al. 2016). Prior studies have concluded that animal-associated denitrification within the gut occurs due to activation of denitrifiers ingested with food (Stief et al. 2009, Heisterkamp et al. 2016, Horn et al. 2006, Matthies et al. 1999). Empty guts and dissected gut epithelia cleared of food particles have undetectable or very low rates of denitrification compared to whole guts containing ingested

materials (Stief et al. 2009, Ihssen et al. 2003). Additionally, earthworms fed with soil treated with antibiotics had negligible denitrification rates (Matthies et al. 1999) implying that the main source of nitrous oxide production in animal guts are ingested denitrifiers. Moreover, denitrifiers isolated from macroinvertebrate guts were phylogenetically related to those from food sources suggesting that denitrifying bacteria within the guts were derived from ingested material (Horn et al. 2006, Stief et al. 2009). However, abundance of indigenous gut bacteria is very low in earthworms (Horn et al. 2006, Karsten and Drake 1997) which might have influenced these results. Moreover, only few model aquatic macroinvertebrates have been used in detailed studies; hence, limiting the extent to which generalities can be drawn regarding the role of extant gut bacteria in host-associated denitrification (Stief et al. 2009).

Environmental drivers control denitrification as modulated by expression of denitrification functional genes (Philippot et al. 2011). The four oxidoreductase genes, *narGHI* (encodes nitrate reductase), *nirk* or *nirS* (encodes nitrite reductase), *norB* or *norC* (encodes nitric oxide reductase), and *nosZ* (encodes nitrous oxide reductase), are induced sequentially under optimal denitrifying conditions (Heisterkamp et al. 2016, Horn et al. 2006). Nitrous oxide reductase activity is more responsive to factors affecting denitrification, like oxygen availability, C:N ratio and pH (Tiedje 1988), than other oxidoreductases (e.g., nitrate reductase). Therefore, fluctuations in gut physicochemical conditions (e.g., availability of nitrate, organic C, pH) can adversely affect nitrous oxide reductase activation by suppressing expression of *nosZ* in gut denitrifiers; thus, facilitating incomplete denitrification in aquatic macroinvertebrates (Stief et al. 2009, Heisterkamp et al. 2010). Additionally, the sudden transition of ingested denitrifiers into anoxic guts from a relatively oxic environment creates a disparity in gene expression (initial genes expressed more than later ones) within the denitrification reaction chain (Hatig and Zumft

1999; Philippot et al. 2001, Stief et al. 2009, Drake et al. 2006) causing *nosZ* to remain unexpressed or minimally expressed which results in higher N_2O:N_2 ratios (Heisterkamp et al. 2010, Stief et al. 2009).

The aim of this study was to assess whether crayfish guts are microhabitats for nitrous oxide emission and how much of the nitrous oxide formed via incomplete denitrification accounts for the total nitrogenous gases produced due to denitrification. In this study, we used a readily available model system (crayfish: *Orconectes obscurus*) and modified its gut microbiome by enriching it with denitrifiers possessing or lacking *nosZ* and expression of *narG* and *nosZ* along with denitrification rates were assessed. We hypothesized that freshwater crayfish guts would facilitate incomplete denitrification over complete denitrification as reflected in minimal expression of *nosZ* gene and higher relative production of N_2O (compared to N_2). Furthermore, expression of *nosZ* was anticipated to be less than that of *narG* in crayfish guts enriched with *nosZ* possessing denitrifiers due to unbalanced functional gene expression (Stief et al. 2009, Heisterkamp et al. 2010) resulting in high *narG* to *nosZ* ratio.

METHODS

Experimental design: The experiment was conducted as depicted in Figure 14. Briefly, crayfish (*Orconectes obscurus*), the model animal for this study, were collected in the field and reared in the laboratory under sterile conditions with the objective of obtaining crayfish with reduced gut bacterial load by feeding them sterile food mixed with antibiotics. Subsequently, as described below, the crayfish were reinoculated with specific denitrifiers. Denitrifiers were isolated from the guts of the crayfish and characterized using *nosZ* and 16S rRNA genes before further experimentation.

Experiments were done to assess crayfish gut denitrification rates and links to expression of denitrification functional genes using denitrifier isolates with or without *nosZ* to build the host gut flora. The rationale of the experiment was to examine the shift in proportion of nitrous oxide to di-nitrogen produced via denitrification in crayfish guts due to presence or absence of the nitrous oxide reductase gene (*nosZ*) in the denitrifiers ingested by the host. Additionally, the expression of the nitrate reductase gene (*narG*) was compared to *nosZ* to test how induction of the first (*narG*) and last gene (*nosZ*) in denitrification varied within crayfish guts across different treatments (crayfish fed denitrifier isolates with or without *nosZ*).

Treatment and control groups were replicated thrice, and crayfish (3 per tank) in the respective treatment groups were fed sterile food combined with denitrifier isolates (with or without *nosZ*) while in controls crayfish were fed only sterile food. After building the gut flora, denitrification rates and functional gene expression (for *narG* and *nosZ*) in the crayfish guts were assessed as described below.

Crayfish collection: Crayfish (*Orconectes obscurus*) were collected from the West Branch of the Mahoning River (WBMR; a 4th order stream) at Jenning's Woods, Ravenna, OH, U.S.A., a stream that has been used in prior microbial ecology research (Ayayee et al. 2018, Rubin and Leff 2007, Olapade and Leff 2005). The stream is relatively unimpacted by anthropogenic disturbance and sediment is comprised primarily of cobble, sand and gravel making it a good habitat for crayfish that are abundant at this site (Clark et al. 2008). Additionally, preliminary experiments showed that crayfish from the stream had high denitrification rates making them suitable models for isolating denitrifiers and conducting the experiment.

A D-frame dip net was placed on the stream bottom and substrate was kicked vigorously to flush out crayfish. Crayfish were put in plastic containers (3-4 crayfish per container) along with stream water and transported back to the lab. About 10 crayfish were immediately used for denitrifier isolation while the rest were used for rearing in the lab for further experimentation.

Denitrifier isolation and characterization: Denitrifier isolation was carried out under aseptic and anaerobic conditions. Guts of four crayfish were dissected out, pooled, homogenized, and then the homogenates were serially diluted until 10^{-3} then plated on nitrate agar plates (prepared according to Brower et al. 2013). The plates were incubated in anaerobic boxes (BD GasPak™ EZ container systems) with an EZ GasPak sachet at 27ºC for 24-72 hours (bacteria only capable of N_2O reduction are excluded by this method). After incubation, distinct colonies (according to morphological characteristics) were identified and streaked to isolation on nitrate agar plates then grown under anaerobic conditions. Five morphologically distinct isolates were inoculated in nitrate broth containing Durham tubes and incubated at 27ºC for 24-72 hours, and the isolates that formed gas bubbles were further verified by a nitrate reduction test. The isolates were stored in 50% glycerol at -80ºC for future experimentation and Sanger sequencing.

DNA was extracted from the isolates as described in Brower et al. (2013), after growing them in nitrate broth tubes for 72 hours, using a Quick DNA Bacterial Miniprep kit (ZymoResearch, Irvine, CA, U.S.A). DNA extracted from the isolates were checked for the presence of *nosZ* via Polymerase chain reaction (PCR) (primers mentioned below) where *Pseudomonas aeruginosa* (ATCC number BAA-47; GenBank accession number AE004091) served as positive control. Based on PCR results, all denitrifier isolates obtained possessed *nosZ*. Therefore, *Pseudomonas chlororaphis* (ATCC 43928) was served as the denitrifier without the *nosZ* gene (Zhang et al. 2019; Sigman et al. 2001). PCR was also performed on the 16S rRNA

gene using universal primers 27F and 1492 R (Frank et al. 2008), and PCR products were sent to UKHC Genomic Core Laboratory at Lexington, Kentucky, USA for Sanger sequencing. The 16S rRNA gene sequences (in Fastq format) were merged and compared with other microorganisms using Basic Local Alignment Search Tool (BLAST) program of the NCBI database to identify the isolates taxonomically. Out of the four isolates, two were putatively identified as *Citrobacter freundii*, and *Citrobacter braakii* while one as *Serratia marcescens* (the remaining one isolate did not work). Prior work by Brower (2013) reported isolation of 10 taxonomically distinct denitrifier isolates from sediment using the same technique. Abundance of *nosZ* copy numbers showed that the *S. marcescens* isolate had the highest copy numbers and it was selected for further experimentation.

Growth curve for denitrifier isolates: To feed the crayfish the same number of initial cells, a growth curve was done for both *S. marcescens* and *P. chlororaphis*. All inoculations were performed aseptically where *S. marcescens* and *P. chlororaphis* were inoculated in nitrate and nutrient broths respectively and uninoculated broths served as controls. All inoculated and control tubes were replicated thrice, kept at room temperature, and their O.D. values were measured at 600 nm every 4 hours starting from 0 hour as the first time point using a spectrophotometer until the O.D. values were ~ 0.2.

Crayfish rearing and Antibiotic treatment: Sterile 10L glass aquaria with lids were used for rearing crayfish in the laboratory and were filled with autoclaved artificial stream water (prepared according to Ghosh and Leff 2013). Ciprofloxacin (150 µg/ml concentration), an antibiotic shown to effectively reduce gut bacterial load of insects (Yuval et al. 2010; Yosef et al. 2010), was administered to the crayfish through food for the purpose of reducing their gut bacteria.

To determine the success of reduction of the gut flora, every 2 to 3 days, two crayfish were taken randomly from the rearing tanks, and their guts were dissected out. Bacterial abundance was monitored based on colony forming units (CFU), cell counts based on microscopy, and DNA extraction using Qiagen DNeasy PowerSoil extraction kits (Qiagen Inc., Germantown, MD, USA) to check 16S rRNA gene copy numbers via quantitative polymerase chain reaction (qPCR) using the protocol and primers as described in Roberto et al. 2019. CFU numbers were based on growth on nitrate and nutrient agar plates (following the protocols above) and DAPI staining (according to Ghosh and Leff 2013). All the above procedures were replicated thrice and once the overall gut bacterial load was reduced sufficiently (at least < 20 times from the control); the crayfish were subjected to further experimentation. Two crayfish were used immediately after bringing them back from the field during the initial collection to serve as controls. Potentially rearing in the laboratory without antibiotic treatment could also result in reduced bacterial ingestion but may not impact extant gut flora.

Relative to controls, after six days of antibiotic treatment the gut bacterial load declined by ~30 times (colony counts on nutrient agar plates), ~2.1 times (colonies on nitrate agar plates), ~37 times (for DAPI count), and ~1.8 times (for 16S gene copy numbers). After 6 days the bacterial load started to increase based on a preliminary experiment. Therefore, it was determined that 6 days of antibiotic treatment would be administered before further experimentation.

Building crayfish gut flora: Crayfish were placed in aquaria using the protocol described above; nine aquaria were used for three treatments (control, denitrifier isolate with *nosZ*, and denitrifier isolate without *nosZ*) with four crayfish in each aquarium. Crayfish were starved for a period of 3-4 days to ensure that they would feed on the agar strips. Agar plates with fish flakes

were prepared as above; agar strips were cut from the prepared plates, and then inoculated with a denitrifier isolate maintaining aseptic conditions throughout. Briefly, each isolate was grown in medium for 10 and 12 hours for *S. marcescens* (nitrate broth, with *nosZ*) and *P. chlororaphis* (nutrient broth, without *nosZ*) respectively. 10µL of culture was spread on to the food Petri plates, using a bent glass rod, and then approximately same size agar strips were cut out for feeding purpose. The inoculated food (either with *S. marcescens* or *P. chlororaphis)* was fed to crayfish while the control group was fed with uninoculated agar strips. Once feeding was initiated, the crayfish were fed ad libitum over a period of 72 hours.

Denitrification rate measurements: After 72 hours of feeding, 2 crayfish from each aquarium were used for measuring denitrification rates. Briefly, each crayfish was placed in a 400 mL mason jar (autoclaved prior to use) with enough autoclaved artificial stream water (~10mL) to keep the environment moist. The mason jars were made air-tight using a lid with rubber septa that facilitated headspace gas sampling. 10% v/v acetylene was injected to the headspace of one jar (to measure potential total denitrification) whereas acetylene was not added to the other jar (to measure incomplete denitrification). Subsequently, 5mL of headspace gas was collected after 15 min, and thereafter headspace gas samples were obtained hourly for 4 hours. After removal of headspace gas samples, jars under the acetylene treatment extracted headspace gas was replenished by an equal volume of gas (containing a mixture of 90% air and 10% acetylene) while for ones without acetylene an equal volume of air was injected. Samples were stored in pre-evacuated 10mL glass vials made air-tight with butyl rubber septa and aluminum crimp seal and nitrous oxide was measured using a Shimadzu GC-2014 Gas Chromatograph (Shimadzu Corporation, Columbia, MD).

Molecular analysis: Crayfish used in the above-mentioned experiment were put in RNA later solution (Sigma-Aldrich, St. Louis, MO, U.S.A.) separately and frozen at -80℃ for RNA extraction. RNA was extracted from the frozen crayfish guts (after dissection under aseptic conditions) using RNeasy PowerMicrobiome Kit (Qiagen Inc., Germantown, MD, USA), and checked for DNA contamination by performing a PCR to detect the amplification of the 16S rRNA gene. After obtaining RNA without DNA contamination, RNA was reverse transcribed to form cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, U.S.A.). Each 20µL reaction consisted, 5µL of 5X iScript reaction mix, 1µL of iScript reverse transcriptase, 7µL of nuclease free water, and 8µL of respective RNA template, and conversion to cDNA was performed according to the manufacturer's protocol. Converting mRNA to cDNA is beneficial because it helps to convert the easily degradable RNA to a more stable form which facilitates performing further downstream processes like Reverse Transcribed Quantitative Polymerase Chain Reaction (RT-qPCR). RT-qPCR was done with the cDNA to check the expression (i.e., transcript copy numbers) of the genes *narG* and *nosZ* using a Stratagene MX3005P Real-time PCR System (Agilent Technologies, Santa Clara, CA, USA). The primers used were *nosZ*1-qPCR F, *nosZ*1-qPCR R (Henry et al. 2006), *narG*1960-F, and *narG* 2650-R (Philippot et al. 2002) for *nosZ* and *narG* respectively. Thermal conditions were based on as mentioned in Stief et al. 2009.

Statistical analyses: All analyses were performed using the statistical software R (ver. 3.3.0; R Core Team 2016). Data was used untransformed or was log transformed as required to meet homoscedasticity and normality assumptions. Tukey's HSD was used for post-hoc analysis for pairwise comparison if the null hypothesis was rejected.

A one-way ANOVA was performed to assess significant differences for potential total denitrification rates (expressed as μg N_2O/g crayfish weight/hour, based on acetylene block treatment) across treatments (control, denitrifier with *nosZ: S. marcescens*, and denitrifier without *nosZ: P. chlororaphis*). Similar analyses were performed for nitrous oxide produced via incomplete denitrification (from treatments without acetylene block) and nitrous oxide formed from complete denitrification (in treatments with acetylene block) that was calculated by subtracting total nitrous oxide produced via incomplete denitrification from potential total denitrification (Heisterkamp et al. 2010, [expressed as μg N_2O/g crayfish weight/hour]).

Furthermore, the ratio between incomplete and potential denitrification rate ([N_2O/(N_2O+N_2); specifically, ratio between N_2O produced via incomplete and potential denitrification]; Phillipot et al. 2011, Stief et al. 2009) was analyzed using one-way ANOVA with treatments as independent variables. The ratio between the nitrogenous gases was assessed as it is indicative of whether complete or incomplete denitrification is predominant.

For gene expression, a one-way ANOVA was done to examine differences in absolute *nosZ* transcript copy numbers (expressed as transcript copies/g crayfish gut weight) where treatments served as independent variables. Similar analyses were performed for *narG* expression. Additionally, for assessing the relationship between gene expression and denitrification rates, linear regressions were performed with potential denitrification (N_2O+N_2) and incomplete denitrification rates (N_2O) as dependent while *nosZ* transcripts served as independent variables for all the three treatments separately. Similar analyses were also performed using *narG* transcript copies. Shapiro-Wilk test was performed to assess the assumptions of normality.

Additionally, to examine differences between *narG* and *nosZ* expression in *S. marcescens* treatments (with and without acetylene block), a one-way ANOVA was done. Only *S. marcescens* was selected for this analysis as it possesses *nosZ* and the *narG* to *nosZ* ratio was assessed only for this treatment.

RESULTS

Total potential denitrification rate in crayfish guts (expressed as µg N_2O/g crayfish weight/hour) was significantly different among treatments ($P<0.05$; Figure 15 A). Treatments with the denitrifier isolate possessing *nosZ* (*S. marcescens*) had significantly higher (~2.3 times) rates than controls. The *S. marcescens* treatment had ~1.4 times higher potential total denitrification rates than *P. chlororaphis* treatment but the difference was not statistically significant.

Potential complete denitrification (expressed as µg N_2O/g crayfish weight/hour [see methods for calculation]) also differed significantly among treatments ($P<0.05$; Figure 15 B). Crayfish that were fed the isolate possessing *nosZ* (*S. marcescens)* produced significantly higher (~ 4.7 times) dinitrogen compared to controls. In contrast, although incomplete denitrification was ~1.9 times higher in the *P. chlororaphis* treatment compared to the control and *S. marcescens* treatment, no significant difference among treatments was observed (Figure 15 C; $P = 0.21$).

The $N_2O/N_2+ N_2O$ ratio (relative N_2O production) was significantly different among treatments ($P < 0.01$; Figure 16) where the treatment with *S. marcescens* had significantly lower N_2O proportion (~3 times) than control and *P. chlororaphis* treatments. Control and the treatment with *P. chlororaphis* did not differ.

For the preliminary results it was observed that approximately mean potential denitrification, potential complete denitrification, and incomplete denitrification rates were 0.007, 0.003, and 0.004, respectively (expressed as µg N_2O/g crayfish weight/hour). Based on this the $N_2O/N_2+ N_2O$ ratio (relative N_2O production) was ~62% (Figure 16 B).

For both potential total and incomplete denitrification assays, *nosZ* expression (expressed as transcript copy numbers/ g crayfish wt.) was significantly different among treatments ($P<0.001$; Figure 17 A and $P<0.001$; Figure 17 B, respectively) and absolute transcript copies was significantly higher in crayfish treated with *S. marcescens* than control and *P. chlororaphis* treatments. With acetylene block, absolute transcript copies numbers of *narG* exhibited significant differences among treatments ($P < 0.01$; Figure 18 A) where crayfish fed with *S. marcescens* had significantly higher transcript copies than the other two treatments. In the absence of acetylene block, *narG* transcript copies (absolute) were not significantly different ($P = 0.11$; Figure 18 B) among treatments.

Linear regression did not reveal any significant relationship between potential total denitrification rates and *narG* transcript copy numbers for control, treatment with *S. marcescens,* or treatment with *P. chlororaphis* ($P = 0.71$; Figure 19 A, $P = 0.84$; Figure 19B, 0.29; Figure 19 C, respectively). Similarly, no significant relationship was found between incomplete denitrification rates and *narG* transcript copy numbers for control, treatment with *S. marcescens* or treatment with *P. chlororaphis* ($P = 0.057$; Figure 20 A, $P = 0.45$; Figure 20 B, $P = 0.45$; Figure 20 C, respectively).

Likewise, there were no significant relationships between potential total denitrification rates and *nosZ* expression for control, treatment with *S. marcescens,* or treatment with *P. chlororaphis* ($P = 0.38$; Figure 19 D, $P = 0.07$; Figure 19 E, $P = 0.1$; Figure 19 F, respectively).

Incomplete denitrification rates and *nosZ* transcript copy numbers too did not reveal significant relationships for control, treatment with *S. marcescens*, or treatment with *P. chlororaphis* (P = 0.11; Figure 20 D, P = 0.68; Figure 20 E, P = 0.7; Figure 20 F, respectively).

Expression of *narG* was significantly higher than *nosZ* (P < 0.01; Figure 21 A) in *S. marcescens* treatments with acetylene block. However, expression of *narG* and *nosZ* was not significantly different in the absence of acetylene block (P = 0.59; Figure 21 B). The *narG* to *nosZ* ratio (absolute transcript copies) with and without acetylene block were $9.14x10^4$ and 4.39, respectively.

DISCUSSION

Incomplete denitrification produces nitrous oxide which is a powerful greenhouse gas with the potential to destroy the ozone layer (Forster et al. 2007). Guts of terrestrial (Ngugi et al. 2012, Horn et al. 2006, Drake et al. 2003) and freshwater macroinvertebrates (Svenningsen et al. 2012, Stief et al. 2009) serve as microhabitats for denitrification. In this study, we tested whether crayfish guts facilitated incomplete over complete denitrification resulting in diminished *nosZ* expression. Moreover, we assessed the contribution of nitrous oxide produced via incomplete denitrification to the total nitrogenous gases produced. To accomplish the objective, we used a novel approach where the gut microbiome of crayfish was modified by enriching it with denitrifiers possessing (*S. marcescens*) or lacking (*P. chlororaphis*) *nosZ*.

Our results demonstrated that crayfish guts facilitate both, complete and incomplete denitrification and that expression of *nosZ* may not be fully diminished. Potential complete denitrification was detected in all treatments and was significantly higher in crayfish guts enriched with *S. marcescens* that bears the *nosZ* gene. Use of a bacterium with the ability for

complete denitrification demonstrates that if the microbiome has the genetic potential for complete denitrification that it may occur under some circumstances.

Crayfish with native gut flora (see preliminary results in methods) and the control of our experiment exhibited more incomplete denitrification relative to potential total denitrification suggesting that nitrous oxide is the dominant end product of denitrification in crayfish guts under some circumstances. This suggests that the outcome is dependent on bacterial community composition. Generally, a sudden shift of denitrifiers (mostly facultative anaerobes) from relatively oxic to anoxic conditions leads to unbalanced gene expression (Stief et al. 2009, Heisterkamp et al. 2010, Firestone et al. 1980). However, our knowledge regarding the changes in gene expression in response to shifting external conditions (transition from oxic to anoxic environment) for specific denitrifier taxa is limited (Lycus et al. 2017). Some denitrifying taxa have smooth transitions from relatively oxic to anoxic conditions and can express the complete set of denitrification functional genes. However, in some cases, species (e.g., *P. denitrificans*) may prefer to adopt a "bet-hedging" strategy where the entire set of denitrification functional gene is not expressed resulting in unbalanced functional gene expression (Hassan et al. 2014, Hassan et al. 2016). This strategy enhances the chances of survival for bacterial populations in new environments and is ideal for short-term anoxic conditions (Lycus et al. 2017). Therefore, it is possible that unlike *S. marcescens*, most of the ingested transient denitrifiers in the environment are unable to produce the entire denitrification proteome resulting in reduced *nosZ* production and enhanced incomplete denitrification rates.

Although enriching the crayfish gut microbiome with *S. marcescens* (possessing *nosZ*) resulted in lower relative N_2O (than N_2) production, the overall contribution of nitrous oxide produced by incomplete denitrification to the nitrogenous gases was almost 20%. Similar values

were reported in *E. danica* (15-29%) by Stief et al. (2009). Despite di-nitrogen gas being the dominant end product of denitrification in the above-mentioned cases, these observations are of particular ecological significance because a nitrous oxide yield of 20% is very high in comparison to 1% reported in ambient aquatic sediment (Stief et al. 2009, Heisterkamp. et al. 2010, Seitzinger et al. 1988). The unbalanced *nosZ* expression while moving from relatively oxic environment of the surroundings compared to the highly anoxic gut environment was proposed to be the main cause of such elevated N_2O yields (Stief et al. 2009, Horn et al. 2006). In accordance with prior studies (Stief et al. 2009, Heisterkamp. et al. 2010), our results too suggest that irrespective of the dominant end product of denitrification (dinitrogen or nitrous oxide), macroinvertebrate guts can be potential microhabitats for nitrous oxide emission compared to their surroundings.

Previous studies suggested that in comparison to ingested denitrifiers, gut resident bacteria do not play a major role in production of nitrogenous gases via denitrification (Stief et al. 2009, Heisterkamp et al. 2016, Horn et al. 2006). However, in the current study, despite the crayfish in the control treatment being administered antibiotics (to reduce gut bacterial load) and fed sterile food during the experiment (the overall process lasting for almost 15 days), we observed detectable complete and incomplete denitrification. This contradicts what was proposed for freshwater macroinvertebrates (Stief et al. 2009) and earthworms (Horn et al. 2006) and suggests that extant gut bacterial have a role in crayfish gut denitrification. However, it must be noted that only a few freshwater macroinvertebrate taxa have been subjected to such detailed studies making it hard to generalize that only ingested denitrifiers contribute to gut denitrification (Stief et al. 2009). Additionally, for earthworms, it is known that the abundance of indigenous gut bacteria is very low (Karsten and Drake 1997). Therefore, based on the

abundance of gut resident bacteria (specifically, denitrifiers), the contribution of extant denitrifying bacteria to macroinvertebrate gut denitrification rates may vary. However, we cannot extrapolate this observation to the field scale as macroinvertebrates in their original habitats do not have reduced gut bacterial load and constantly consume transient gut denitrifiers via food. Therefore, if transient denitrifiers outcompete extant denitrifiers then the contribution of the indigenous denitrifying taxa may not be quantitatively significant. Since bacteria from exoskeleton and lab environment can also be ingested and be present in the guts further confirmatory experiments (like mentioned in Stief et al. 2009) will be needed to confirm the contribution of extant bacteria to crayfish gut denitrification

We observed that expression of *narG* was higher than *nosZ* in crayfish guts enriched with *S. marcescens*. There are two plausible explanations for this observation: 1) *narG* transcripts of other extant non-denitrifying dissimilatory nitrate reducers (e.g., bacteria performing dissimilatory nitrate reduction to ammonia) in the gut were contributing to the high *narG* transcripts and 2) expression of *narG* and *nosZ* was unbalanced resulting in reduced transcript copies of *nosZ*. We do not have evidence that *nosZ* expression was unbalanced in our study since we did not see high nitrous oxide production via incomplete denitrification in *S. marcescens* treatments. Although one might speculate that high *narG* to *nosZ* ratio implies unbalanced *nosZ* expression, the result must be interpreted with caution as the transcript copy numbers were absolute. Assessing the relative gene expression (*narG* or *nosZ* to a housekeeping gene, or16S rRNA) could better explain whether denitrification functional genes were unbalanced or not within the crayfish guts. The significantly low expression of *nosZ* than *narG* in the presence of acetylene block could be attributed to the effect of acetylene on nitrous oxide reductase enzyme activity that subsequently suppressed *nosZ* expression to prevent conversion of N_2O to N_2

(Saleh-lakha et al. 2009). Moreover, it is possible that some of the extant gut bacteria that were non-denitrifying dissimilatory nitrate reducers (bacteria performing DNRA) also contributed to the *narG* transcripts (Stief et al. 2009).

We observed no significant relationship between functional gene expression (both, *narG* and *nosZ*) and denitrification rates (however, low sample size maybe the most plausible explanation for impacting the robustness of this analysis). This is consistent with prior studies where no relationship between denitrification functional gene abundance or expression (including *nosZ*) and denitrification rates were detected in soils (Chen et al. 2015, Dandie et al.2011, Tomasek et al. 2017). Understanding the relationship between gene expression and denitrification can be complex. An array of other factors (e.g., nitrate and organic carbon availability, abundance, and expression of other denitrification functional genes) can influence denitrification rates and functional gene expression (Tiedje 1988). For example, providing additional nitrate increases denitrification rates in macroinvertebrate guts (Stief et al. 2009, Horn et al. 2006). Additionally, if the abundance of denitrifiers in the guts is not rate limiting for the denitrification enzymes, then even upon enrichment with denitrifying bacteria there may be no consistent relationship between functional gene expression and denitrification rates (Dandie et al.2011).

Owing to increasing anthropogenic impacts worldwide (Tomasek et al. 2017, Halpern et al. 2019), and the role of nitrate in enhancing N_2O emission (Wang et al. 2013, Stief et al. 2009), it is important to identify microsites for N_2O production. Based on previous studies (Stief et al. 2009, Heisterkamp et al. 2010, Horn et al. 2006) and our findings, macroinvertebrate guts are favorable microenvironments for nitrous oxide production via incomplete denitrification in comparison to the relatively oxic free-living microbiome. At a larger scale, abundance of

macroinvertebrate impacts N_2O production in the environment (Meyer et al. 2008, Stief et al. 2009, Megalhaes et al. 2005). Therefore, aquatic ecosystems with high numbers of macroinvertebrates that facilitate incomplete over complete denitrification in their guts may contribute to the global nitrous oxide budget. Moreover, we know that nitrate can increase denitrification rates; therefore, future studies should focus on designing field scale experiments to assess the contribution of the dominant macroinvertebrate gut microbiomes to nitrous oxide production in freshwater ecosystems facing risks of nitrate overloading.

CONCLUSIONS

Enriching the gut microbiome enriched with denitrifier isolate possessing *nosZ* significantly reduced the proportion of nitrous oxide produced in crayfish guts implying that the denitrifying taxa may have a role in determining the dominant end product of denitrification in macroinvertebrate guts and both complete and incomplete denitrification can occur in crayfish guts. Additionally, our results suggests that extant bacteria may play a role in denitrification within freshwater macroinvertebrate guts although further experimentation is necessary to confirm this. However, extrapolating these results to field scale is not appropriate as our model organism had reduced bacterial load and was administered with only two types of denitrifiers. We did not find significant relationships between denitrification rates and functional gene expression, perhaps because other factors might be simultaneously affecting functional gene expression and denitrification rates within the guts (e.g., nitrate and nitrite concentration). Nevertheless, in accordance with prior studies we showed that crayfish guts can be potential microhabitats for nitrous oxide emission. Future work needs to focus on assessing the contribution of macroinvertebrate gut microbiomes (specifically, the taxa that facilitate incomplete over complete denitrification in their guts) to N_2O emission in aquatic ecosystems.

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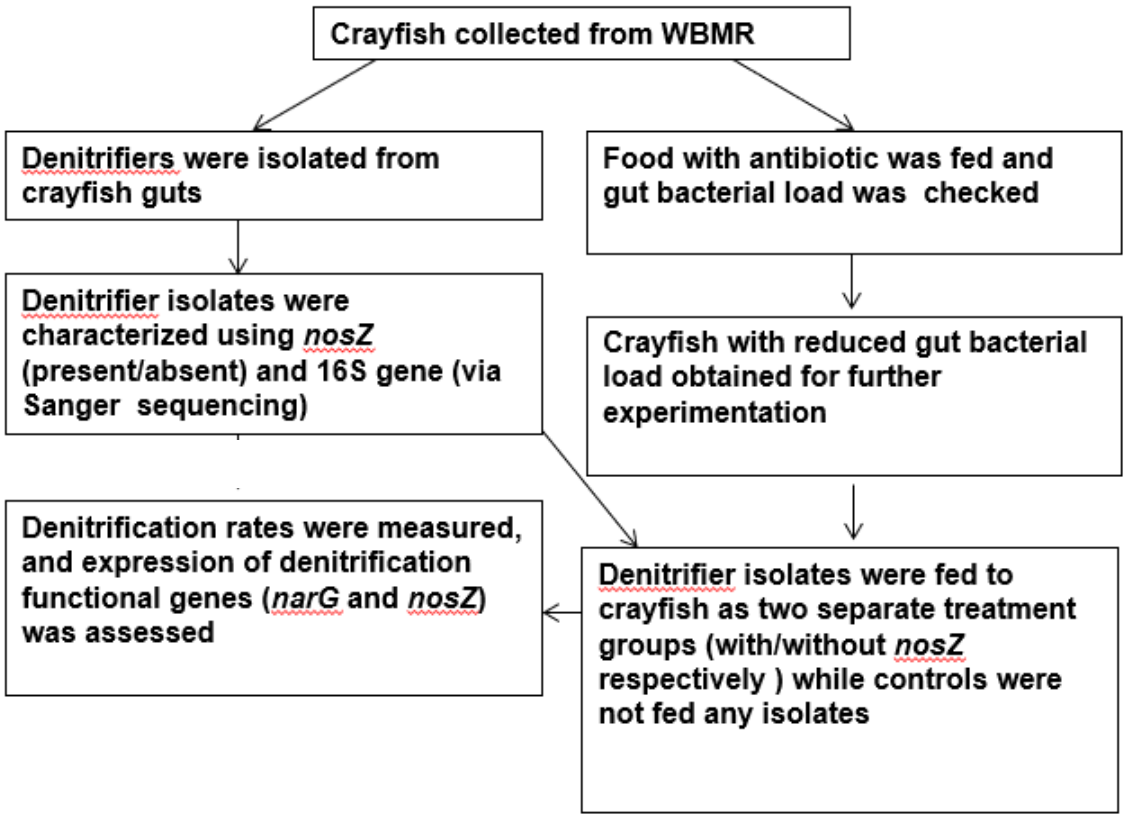

Figure 14. Diagrammatic representation of the experimental design.

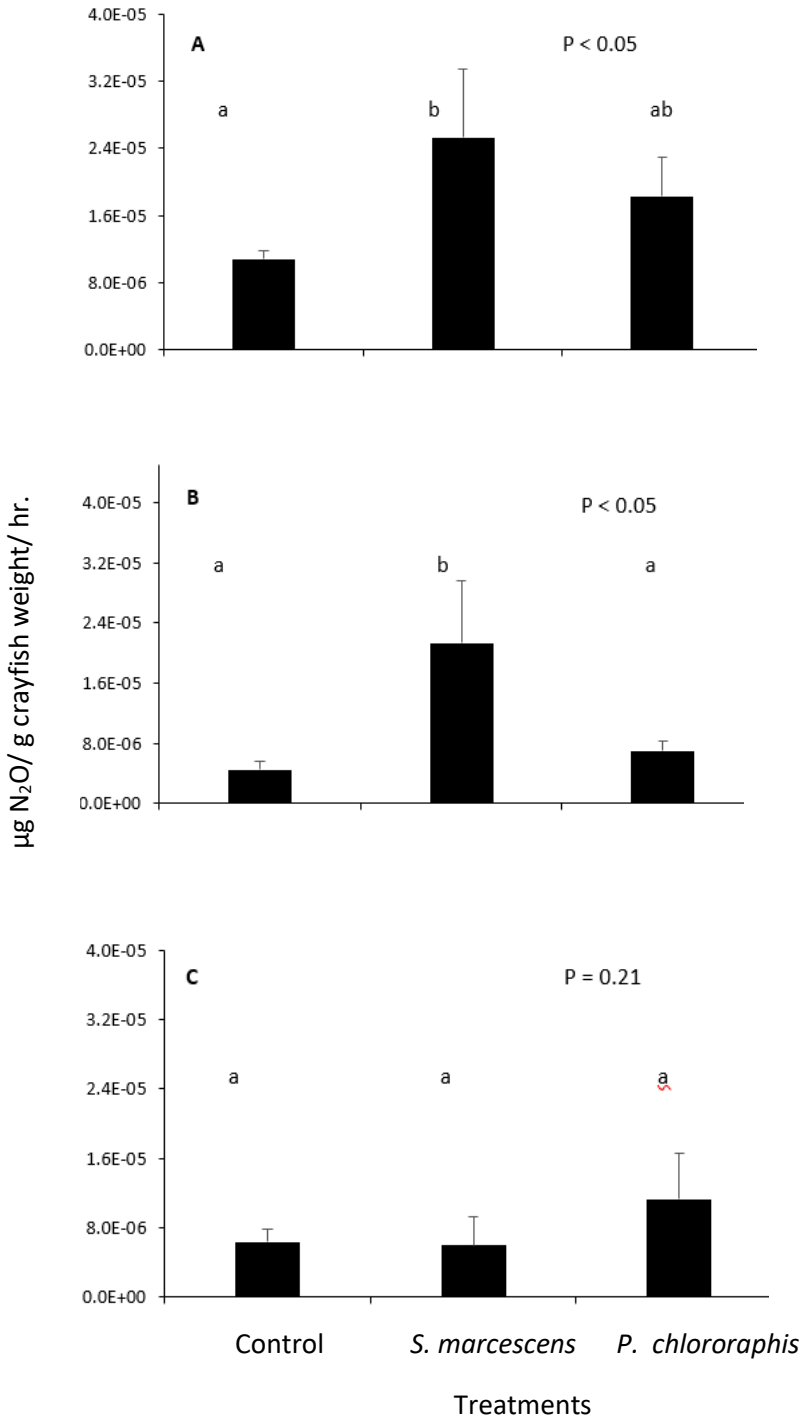

Figure 15. Differences in potential total (A), potential complete (B) and incomplete (C) denitrification rates in crayfish guts among treatments. Values are means are standard deviations (untransformed data, n=3). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences.

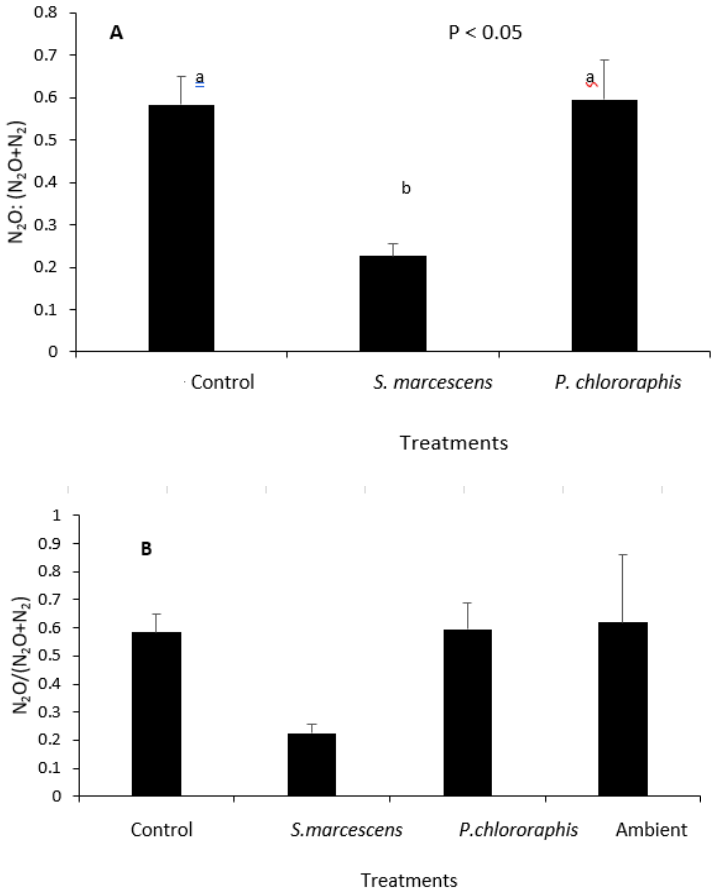

Figure 16. Difference in $N_2O/N_2+ N_2O$ ratio. A) Significant differences for $N_2O/N_2+ N_2O$ ratio across treatments. Values are means and standard errors (n=3, untransformed data). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences. B) Depicts comparison (untransformed data) of N_2O proportion (as in A) with crayfish in actual environment (ambient) where values are means and standard deviations.

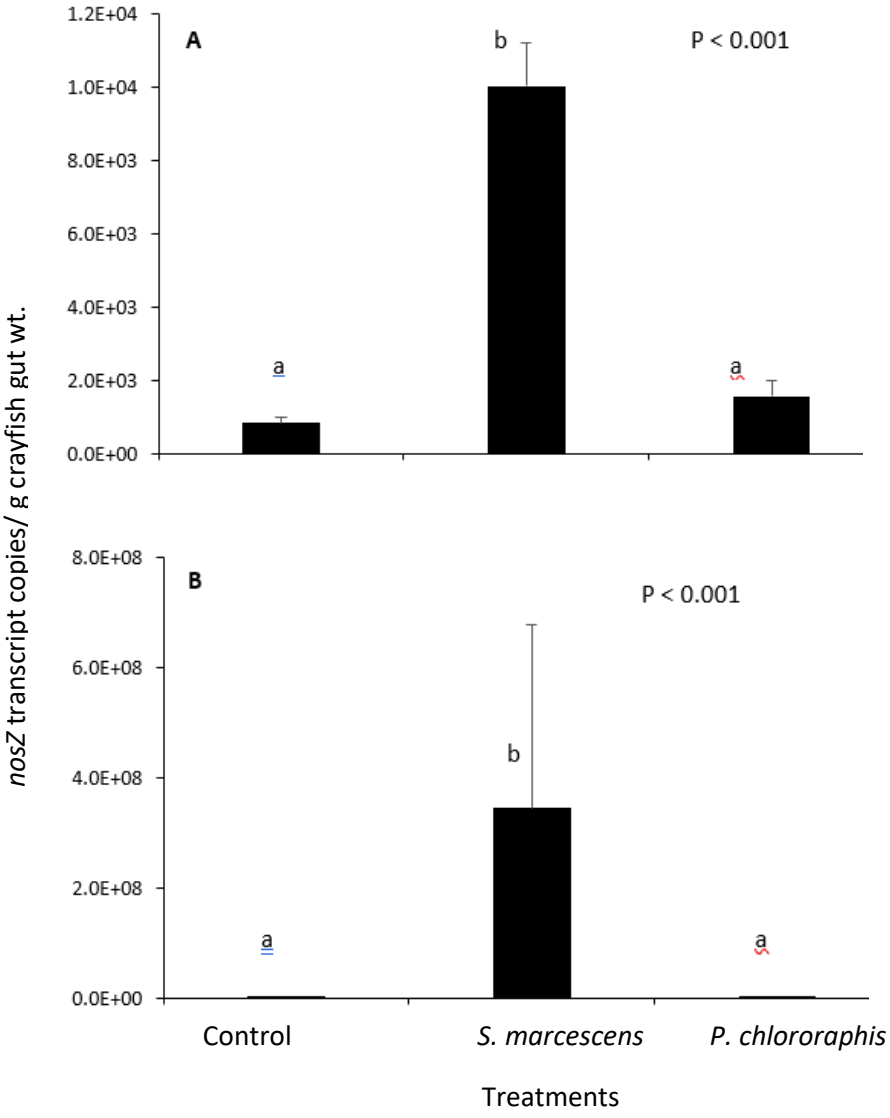

Figure 17. Differences in absolute transcript copy numbers of *nosZ* for with acetylene block (A) and without acetylene block (B) among treatments. Values are means and standard errors (n = 3; untransformed data). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences.

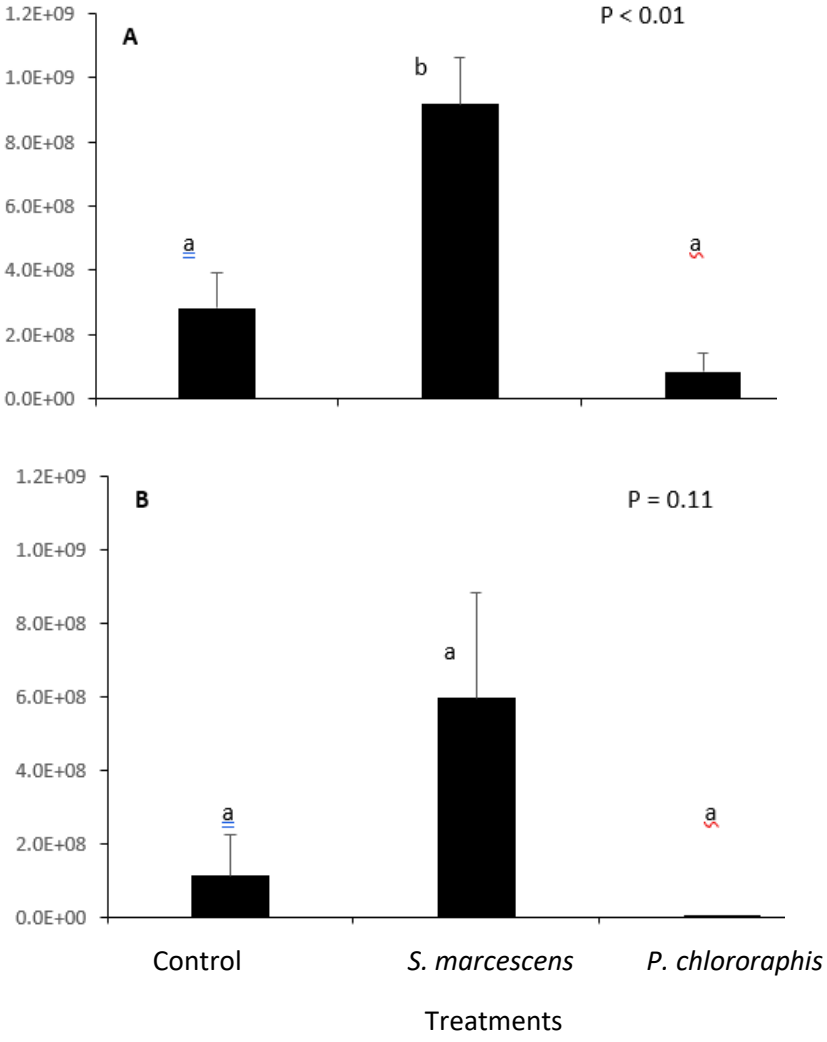

Figure 18. Differences in absolute transcript copy numbers of *narG* for with acetylene block (A) and without acetylene block (B) among treatments. Values are means and standard errors (n = 3; untransformed data). Lower case letters directly above bar graphs indicate significant (letters differ) or no significant (letters are the same) differences

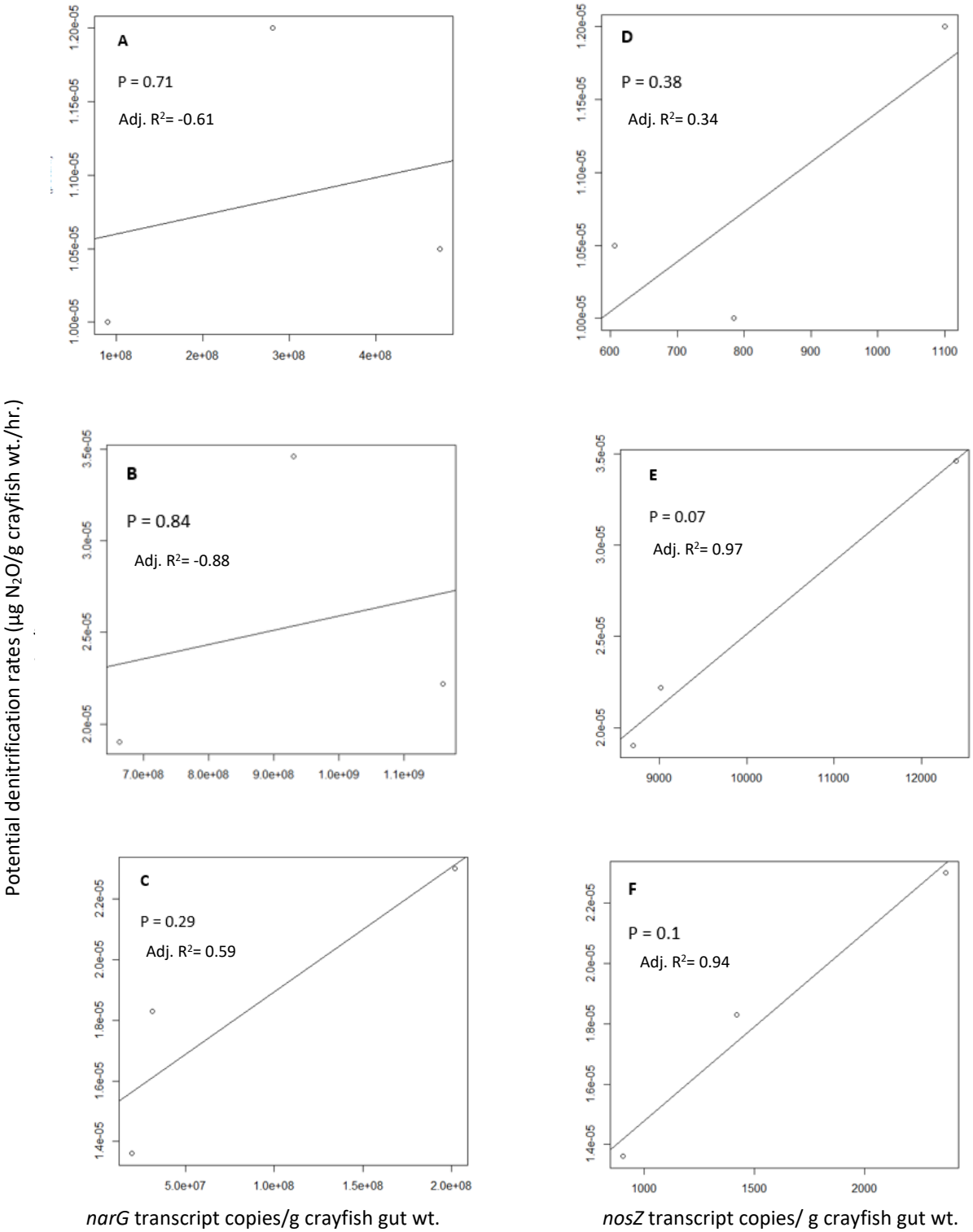

Figure 19. Scatter plot for linear regression depicting the relationship of potential total denitrification rates with *narG* and *nosZ* transcript copies. Depicts the relationship (n =3; untransformed data) for control (A), *S. marcescens* (B), and *P. chlororaphis* (C) while with *nosZ* for control (D), *S. marcescens* (E), and *P. chlororaphis* (F).

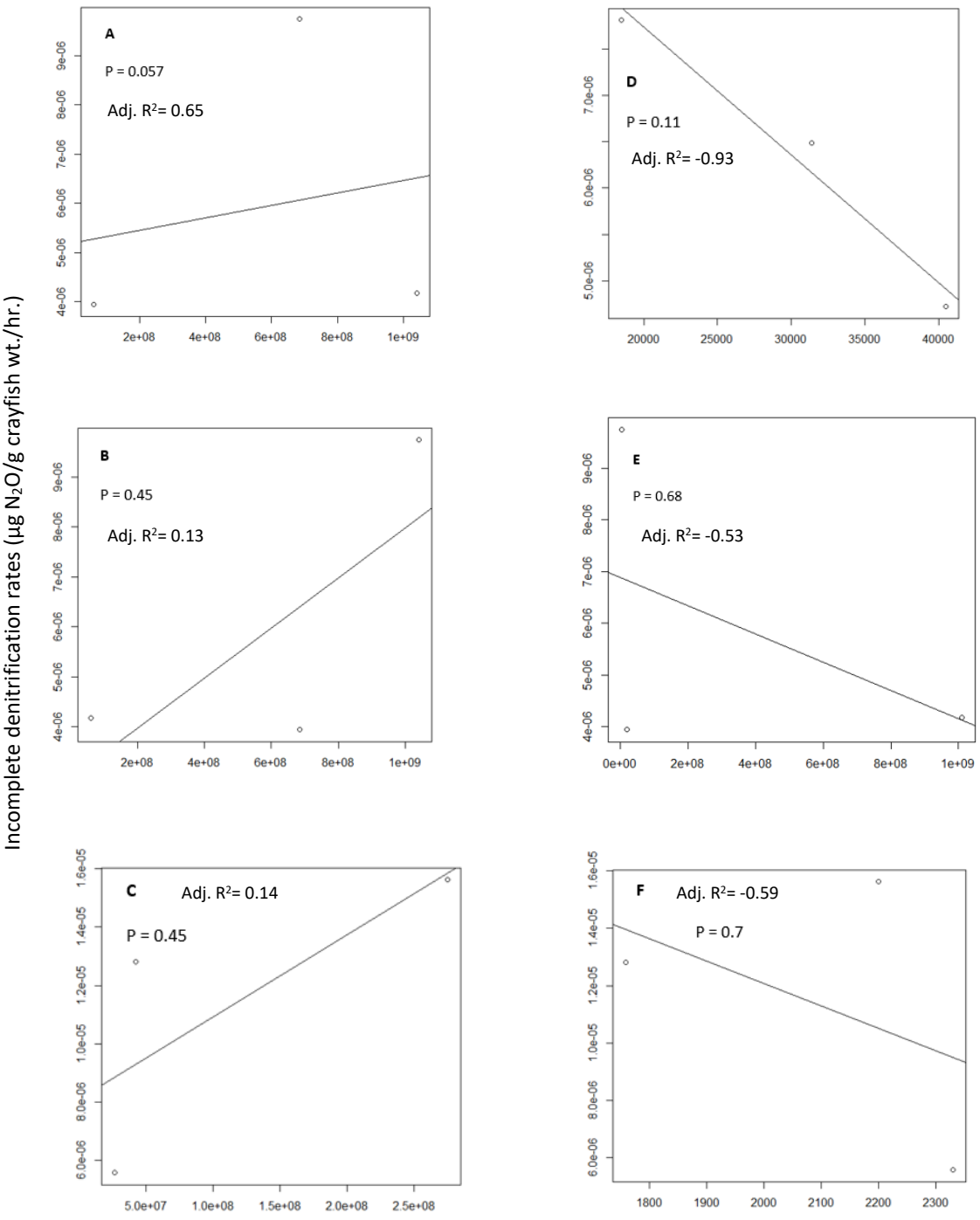

Figure 20. Scatter plot for linear regression depicting the relationship of incomplete denitrification rates with *narG* and *nosZ* transcript copies. Depicts the relationship (n =3; untransformed data) for control (A), *S. marcescens* (B), and *P. chlororaphis* (C) while with *nosZ* for control (D), *S. marcescens* (E), and *P. chlororaphis* (F).

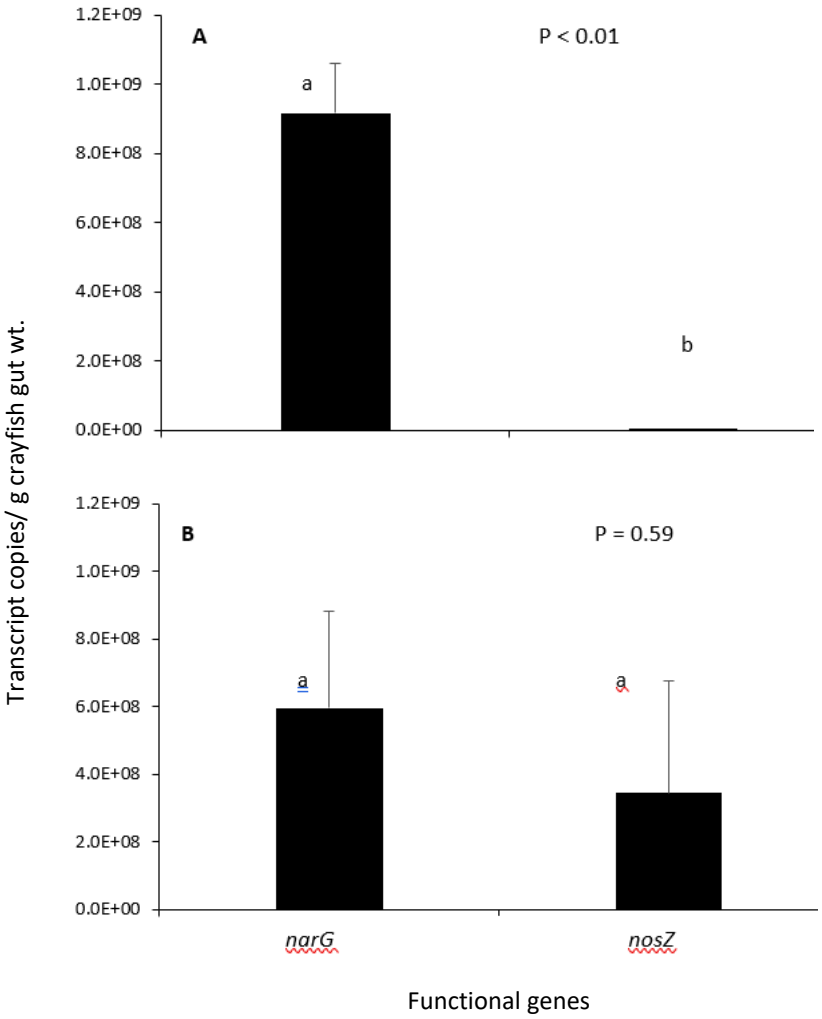

Figure 21. Differences between *narG* and *nosZ* transcript copies (absolute) in *S. marcescens* treatments with acetylene block (A) and without acetylene block (B). Values are means and standard errors (n = 3; data untransformed).

CHAPTER IV

EFFECT OF BIOTURBATION BY FRESHWATER INVERTEBRATES ON DENITRIFICATION AND BACTERIAL COMMUNITY COMPOSITION

ABSTRACT

Burrowing bioturbators impact dissolved inorganic nitrogen (DIN) chemistry and denitrification in freshwater ecosystems. However, only a few studies in freshwater ecosystems have attempted to relate bioturbation mediated changes in DIN to shifts in bacterial community composition. *Hexagenia bilineata* (mayfly nymph) and *Lumbriculus variegatus* (annelid worm) were used as model bioturbators in laboratory microcosms to compare how their varying burrow configuration and depths alter DIN, potential denitrification rates, bacterial abundance, and sediment bacterial community composition. We hypothesized that distinct burrowing modes will result in differencess in bacterial community composition at varying sediment depths, water nutrient chemistry (nitrate and ammonium) and potential denitrification rates. In general, relative to control, *H. bilineata* treatments had lower nitrate and higher ammonium concentrations. Overall, bacterial community composition differed among treatments at fine (amplicon sequence variant [ASV]) but not at coarse (phylum, family) taxonomic levels. Specifically, *L. variegatus*

microcosms had distinct sediment bacterial community composition relative to controls. Although the bacterial community composition was altered there was no difference in denitrification rate and bacterial abundance perhaps due to functional redundancy within the bacterial community. At the ASV level, *L. variegatus* treatments showed higher relative abundance of *Methylococcaceae* **(**aerobe) and lower relative abundances of Firmicutes (anaerobe) compared to *H. bilineata*. Overall, the study suggest that burrowing mode is potentially an important driver of sediment bacterial community composition and changes in DIN and *L. variegatus* may favor success of aerobes (presumably because of increased oxygen penetration) relative to *H. bilineata*.

Keywords

Bioturbation, Freshwater macroinvertebrates, Burrowing, Bacterial community composition, Nitrogen cycle, Denitrification

INTRODUCTION

Increasing anthropogenic activities have resulted in excess nitrogen in freshwater ecosystems. Denitrification is an ecologically significant process capable of removing nitrate from freshwater ecosystems by transforming it to nitrogenous gases. Various environmental factors, such as nitrate concentration, anoxia, and availability of organic matter, are key drivers of denitrification (Knowles 1982, Korom 1992, Inwood et al. 2007). Attributes of the microbial community that perform denitrification, such as taxonomic composition, abundance, and activity, may also influence denitrification rates (Zumft 1997, Baxter et al. 2012, Xu et al. 2018). In turn, environmental drivers and their interrelationships may be influenced by activity of macro-organisms that alter both the environment and the microbial community.

Bioturbation, a process by which animals restructure sediment via physical movement, plays a crucial role in biogeochemistry (Meysman et al. 2006, Mermillod-Blondin 2011, Anschutz et al. 2012, Gilbertson et al. 2012, Kristensen et al. 2012). Specifically, burrowing animals alter sediment nutrient dynamics, directly during burrow-building, excretion, and feeding, and indirectly when burrows collapse or burrowers die (Kristensen et al. 2012, Vanni et al. 2002). Thus, burrowers act as ecosystem engineers that facilitate biogeochemical transformations by translocation of solutes and gases between water and sediments. Denitrification can be significantly enhanced via bioturbation due to creation of microsites with gradients of oxygen and dissolved inorganic nitrogen (Gilbert et al. 1998, Svensson et al. 2001, Callier et al. 2006)**.**

Along with physicochemical conditions, denitrification is influenced by bacterial community composition (Cao et al. 2008, Peralta et al. 2010, Tomasek et al. 2017). Burrowers are capable of modifying sediment bacterial community composition via physical restructuring and irrigation at specific depths (Krantzberg 1985, Kristensen 2000). In general, bioturbation associated bacterial community shifts have primarily been studied in marine ecosystems (e.g., Laverock et al. 2010, Foshtomi et al. 2015, Li et al. 2019). Similar studies in freshwater ecosystems (see Svennson et al. 1996, Zeng et al. 2014) help us draw generalizations on the effect of bioturbation on sediment bacterial community structure and function.

Macroinvertebrates use a range of strategies to create burrows; nutrient translocation and oxygen penetration depends on the mode of burrowing (Kristensen, 2001, Kristensen and Kostka 2005, Hedman et al. 2011). The two bioturbators selected for this study have distinct modes of burrow formation. Nymphs of the mayfly *Hexagenia bilineata* dig U-shaped burrows (Fremling 1989) in the upper 5 cm of the sediment, preferably constituted of clay, silt, and sand (Edsall

2001). These nymphs constantly pump water into their burrows while filter feeding, which causes oxygen and solutes from the surface water to interact with the burrow sediment. On the other hand, *Lumbriculus variegatus* (annelid worm) builds gallery network burrows where numerous burrows are interconnected and typically found in the upper 8 cm of the sediment (McCall and Tevesz 1982, Boekar et al. 2016, Work et al. 2002), thus, providing a greater surface area for sediment and water column interaction than U-shaped burrows (Mermillod-Blondin and Rosenberg 2006). Additionally, U-shaped yet low density burrows (formed by *H. bilineata*) are more stable than weakly structured, high density gallery network burrows (formed by *L. variegatus*).

In this study, we examined the effect of *H. bilineata* and *L. variegatus* burrows on DIN, potential denitrification rates, functional gene abundance, and sediment bacterial community composition. We hypothesized that distinct burrowing modes will result in shifts in bacterial community composition at varying sediment depths, water nutrient chemistry (nitrate and ammonium) and potential denitrification rates. Specifically, we predicted that numerous fine structured burrows constructed by *L. variegatus* would create more aerobic microsites with increased oxygen and nutrient penetration; thus, exposing more sediment surface area to the surface water oxygen at increased sediment depths. Hence, more oxygen tolerant bacterial taxa and lower denitrification rates (due to increased oxygen penetration) were predicted to occur in microcosms with *L. variegatus* compared to *H. bilineata*.

METHODS

Study organisms- Two bioturbators with different modes of burrowing were used for the experiment: *H. bilineata* (mayfly nymph), a U – shaped burrower (The Reel Thing, Green Bay, WI, USA) and *L. variegatus* (worm), a gallery network burrower (Carolina Biological Supply,

Burlington, NC, USA). After arrival, invertebrates were held for 24 H in plastic containers containing equal parts deionized water (invertebrates were shipped in deionized water) and study site water and were gently aerated to allow them to acclimate to laboratory conditions. For simplicity in the text below, we refer to *H. bilineata* as "mayfly" and *L. variegatus* as "worm".

Study site- Sediment and water were obtained from The Observatory Wetlands at Kent State University (Kent, OH, USA) for use in laboratory microcosms. The wetland was constructed to control flooding during heavy storm events and covers an area of 2.49 ha. Preliminary experiments demonstrated that the sediment is conducive for burrowing by the invertebrates used in this study (T. Michael, personal communication). Sediment consisted of 39.2% coarse sand, 15.8% medium grain sand, 13.8% fine sand, 17.35% very fine sand, and 3.5% silt/clay (based on sieving). Before transferring into microcosms, sediment was sieved through a 1mm (mesh pore size) sieve to remove any other macroinvertebrates.

Microcosm set up and experimental design – Microcosms consisted of transparent polyvinyl chloride (PVC) pipes (20 cm high with 4.5 cm inner diameter) that were sealed tightly at the bottom with polyethylene caps. Sieved sediment was placed into microcosms to a depth of 10 cm and allowed to settle for 24 H. Then the microcosms were topped off with site water (5 cm), and further allowed to settle over a period of 48 H before addition of invertebrates. Gentle aeration was provided by syringe needles attached to aquarium air pumps via plastic tubing. Microcosms were loosely covered to minimize evaporation.

Two mayfly nymphs (weight and length ranged from 0.24-0.26 g and 2.45-2.65 cm, respectively) and ten worms (weight and length ranged from 0.1- 0.12 g and 1.8-2 cm, respectively) were added to their respective microcosms (N=3). The number of invertebrates added was based on the numbers in which they are generally found in their natural habitats

(Cook 1969, Lawrence et al. 1982, Reynoldson et al. 1989, Bachteram et al. 2005, Blankson and Klerks, 2016). Controls (N=3) contained no macroinvertebrates.

Microcosms were incubated for 7 days at room temperature. Water lost due to evaporation was replenished with deionized water daily. On days 2, 4, and 6, 30 ml of surface water was gently removed from each microcosm using a syringe with a needle. On these same days, interstitial water was extracted using Eijkelkamp Rhizon Soil Moisture Samplers (5cm tubes from Forestry Suppliers, Jackson, MS, USA) from two depths (5 and 10 cm) in the sediment. The amount of water extracted was replenished by the same volume of site water. Water samples were frozen at -20 ℃ for nutrient analysis after filtering using 0.45 μm pore size sterile syringe filters (Whatman, Sigma-Aldrich, St. Louis, MO USA).

Total ammonium nitrogen (TAN-N) (i.e., unionized ammonia NH_3 and the ammonium ion, NH_4^+), based on the indophenol blue method (Aminot et al. 1997), was assessed using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA,USA). Water nitrate concentrations were analyzed using a Dionex ICS-2100 ion chromatograph (Thermo Fischer Scientific, Waltham, MA, USA). This system detects inorganic anion concentrations using a hydroxide-selective anion-exchange column (IonPac AS19).

At the end of the experiment, surface water was gently and completely removed using a syringe. Then, a core extruder was used to slowly extrude the sediment core from each microcosm. As the sediment core was extruded, it was sliced at four different depths (2, 4, 6, and 8 cm) from the surface water-sediment interface. Individual aseptic scalpels were used to prevent cross-contamination. Sediment core slices were homogenized and immediately subdivided into 3 parts. One part was frozen at -80 ℃ for future DNA extraction, one part was preserved in 4%

paraformaldehyde with 0.1% tetrasodium pyrophosphate (stored at 4 ℃) for bacterial enumeration, and the remaining part was used to measure denitrification potential.

Contribution of excretion towards nutrient concentrations – To assess macroinvertebrate excretion, additional microcosms were constructed as above. However, rather than using site water and sediment, they contained autoclaved (to control for bacteria mediated N transformations) site water and sand. Water collection and analysis was performed as mentioned above, but interstitial water could not be collected at 10 cm depth in sufficient quantity for DIN analysis. Macroinvertebrates were observed to have reduced ability to burrow in this substrate.

Potential denitrification rate measurement- Each sediment slice for denitrification was subdivided. Half was used for measuring dry weight and ash free dry mass (AFDM) while the other half was used to measure potential denitrification rate using the acetylene block technique. For determining dry weight, samples were weighed and dried for 24 H at 60℃. Then samples were heated at 500℃ for 6 H to determine AFDM.

For measuring the potential denitrification rate, site water was amended with chloramphenicol to obtain a final concentration of 1mM (Royer et al. 2004, Baxter et al. 2013). Then 10 g of sediment from each respective subsample was mixed with 20 ml of site water to form a slurry. The slurry was put into 100-ml glass vials that were flushed with dinitrogen for 5 minutes and sealed with a butyl rubber septum and aluminium crimp seal. Subsequently, 10% v/v acetylene was injected to the headspace of the vials. Headspace gas samples (5 mL) were taken on an hourly basis for 3 H for nitrous oxide measurements. Gas samples were stored in pre-evacuated 10ml air-tight glass vials and analyzed with a Shimadzu GC-2014 Gas Chromatograph. Extracted headspace gas was replenished by an equal volume of gas (containing a mixture of 90% nitrogen and 10% acetylene). Potential denitrification rates were calculated

(Royer et al. 2004, Ayayee et al. 2020) from linear increase over time (slope) and expressed after standardizing with sediment weights or microcosm area.

Bacterial enumeration- Subsamples preserved for bacterial enumeration were sonicated (using an ultrasonic cleaner, model 2210; Branson Ultrasonics Co., Danbury, CT) for 10 minutes to dislodge the bacterial cells. Thereafter, samples were filtered through 0.2-µm black polycarbonate filters and stained with 4,6-diamino -2- phenylindole (DAPI). Bacterial cells in 10 fields per sample were enumerated using epifluorescence microscopy (Ghosh and Leff 2013).

DNA analysis- DNA was extracted from subsamples stored at -80 degrees using the Qiagen DNAeasy Powersoil Kit (Germantown, MD, USA) according to the manufacturer's protocol. The DNA was checked for the presence of 16S rRNA gene via PCR using the universal primers 357 F and 1391 R, and subsamples were sent to the Molecular and Cellular Imaging Center of The Ohio State University for library preparation and sequencing of the hypervariable V4-V5 region (Sun et al. 2013) using Illumina Mi-Seq Next Generation Sequencing.

The abundance of the *nosZ* gene was determined via quantitative polymerase chain reaction (q-PCR) (Baxter et al. 2012; Manis et al. 2014). *Pseudomonas aeruginosa* (ATCC number BAA-47) genomic DNA was used as template for PCR-amplification of the *nosZ* gene, which was then ligated into a plasmid using the TOPO TA Cloning kit (Thermo Fisher Scientific, Waltham, MA, USA). Plasmids were then isolated and diluted to form a standard curve by serial dilutions (Ayayee et al. 2020). Primers were as in Henry et al. 2006, *nosZ1F* (WCSYTGTTCMTCGACAGCCAG) and *nosZ1R* (ATGTCGATCARCTGVKCRTTYTC). Each 20 µl qPCR reaction mixture consisted of template DNA (2 µL), PerfecTa SYBR Green SuperMix (Quanta bio, Beverly, MA, USA), water, and primers (0.2 µM each), with runs carried out with a Stratagene MX3005P Real-time PCR System (Agilent Technologies, Santa Clara, CA,

USA). Thermal conditions (according to Ayayee et al. 2020) were 96 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60s. Finally, the run was followed by a melt curve comprising the following steps:95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s.

Statistical and bioinformatics analyses- Statistical analysis was performed using R (ver. 3.3.0; R Core Team 2016), and $\alpha \leq 0.05$ was considered statistically significant. Dependent variables were either log or square-root transformed to meet homoscedasticity and normality assumptions, and then ANOVAs were performed followed by Tukey tests. For nitrate and ammonium concentrations, rate of potential denitrification, number of *nosZ* gene copies, and bacterial cell counts, a two-way or a three-way ANOVA (as appropriate) was done using treatments (control, mayfly, and worm), sampling days (second, fourth, and sixth days of the experiment), and depths (5 and 10 cm) as separate independent variables.

QIIME 2 (version 2019.7.0) microbiome bioinformatics pipeline (accessed via Docker Desktop 3.1.0. build: 51484) was used for processing sequence data and detection of amplicon sequence variants (ASVs). Use of ASVs does not involve arbitrary dissimilarity thresholds like OTU methods and maintains differences as small as one nucleotide after error-correcting Illumina sequence data. Thus, ASV methods improve microbial data resolution, and have additional benefits of being reusable across studies (Eren et al. 2015, Callahan et al. 2017, Needham et al. 2017). In order to perform ASV clustering, briefly, cutadapt (https://github.com/qiime2/q2-cutadapt, Martin 2011) was used to discard the primer sequences and then remaining sequences were further trimmed at 265 bp for forward reads and 220 bp for reverse reads, and then the paired-end reads were combined. Thereafter, the DADA2 algorithm (Callahan et al. 2016) was applied for denoising and error correction of the remaining sequences. The RDP database (Knight et al. 2018) was used to assign taxonomy to representative bacterial

sequences. Sequences were rarefied to a depth of 18308 sequences which retained 50% of the total sequences. A bacterial community data matrix was exported as an excel file for further downstream processing in R statistical environment (v3.3.1; R Core Team, 2016).

The rarefied ASV abundance data was then Hellinger transformed, followed by redundancy analysis (RDA) to assess how treatments and sediment depths influence bacterial community composition (using R packages vegan and tidyverse). Significance of experimental factors in the RDA models was determined by a random permutation test. Thereafter, the 'goodness' command was used to determine the variance explained for each ASV, with subsequent focus on those that were well-explained by the RDA model (R^2>0.3) (Mason et al. 2020). Phyla and families were then assigned to those ASVs and the changes in their relative abundances (relative to control treatments) between bioturbator types and among sediment depths were depicted as a heatmap.

RESULTS

Surface water DIN concentrations- Surface water nitrate concentration (Figure 22 A) differed significantly among treatments ($P < 0.001$) and sampling days ($P < 0.001$), but there was no significant interaction between treatment and days ($P=0.31$). Overall, the mayfly treatment had ~3 fold lower and worm treatments had ~1 fold higher nitrate concentrations than controls. However, nitrate concentration in worm treatments was not significantly different than the control. Nitrate concentration in surface water increased significantly on days 4 and 6 from day 2. In the sand microcosms (designed to assess macroinvertebrate excretion contributions), there were significant differences among treatments ($P< 0.001$), days ($P< 0.01$) and a significant interaction between treatment and day ($P < 0.001$, Figure 22 B). Overall, nitrate concentration

was ~17 fold higher in mayfly treatments relative to control and peaked on day 6 while there was no significant change in worm treatments.

For surface water ammonium concentration (Figure 23 A) only two replicates per treatment (n=2) could be analyzed due to loss of samples. There were significant differences among treatments ($P < 0.001$) and sampling days ($P < 0.05$) with no significant interaction between treatments and sampling days ($P = 0.18$). The mayfly treatment had ~5 fold and ~7 fold higher ammonium concentration compared to the control and worm treatments, respectively. Overall, ammonium concentration decreased from day 2 to day 6 in worm and control treatments. In the excretion experiment (Figure 23 B), differences among treatments were significant ($P<0.001$) while sampling days ($P = 0.51$) and interaction between treatment and sampling days ($P=0.45$) were not significant. The mayfly treatment had a ~ 4 fold higher ammonium concentration relative to controls while worm treatments were not different from control.

Interstitial DIN concentrations- For interstitial water, nitrate concentration (Figure 24 A) differed significantly among depths ($P < 0.001$) and there was a significant treatment by depth interaction effect ($P<0.05$). Treatments ($P = 0.13$), days ($P = 0.43$), and interactions were not statistically significant (treatment*day: $P=0.15$, day*depth: $P=0.39$, and treatment*day*depth: $P=0.44$). Overall, nitrate concentration was ~5 fold lower at 10 cm compared to 5 cm. In the sand microcosms (Figure 24 B), interstitial nitrate concentration differed significantly among treatments ($P<0.05$), days ($P<0.001$), and the interaction between treatment and days was significant($P<0.05$). Nitrate concentration was higher in the worm treatment on day 4 than control and mayfly treatments.

Ammonium concentration (Figure 25 A) in interstitial water differed significantly among depths ($P < 0.01$); no other significant differences were detected ($P > 0.5$). Overall, ammonium concentration was twice as high at 10 cm than at 5 cm. Interstitial concentrations in the excretion experiment (Figure 25 B) showed similar trends as surface water ammonium concentrations. Only treatments were significantly different ($P<0.001$) while day ($P=0.08$) and interaction between day and treatment ($P = 0.11$) were not significant. The mayfly treatments had a 2 fold higher interstitial ammonium concentrations compared to control and worm treatments.

Bacterial numbers - There was no significant differences in bacterial numbers among treatments ($P=0.492$), sediment depths ($P=0.348$) nor was there a significant interaction effect ($P=0.123$) between treatment and sediment depths. When abundances across depths were averaged, as depicted in Figure 26, there were no apparent differences among treatments.

Potential denitrification rates and *nosZ* gene abundance- Potential denitrification rate did not differ significantly among treatments ($P = 0.349$), sediment depths ($P = 0.208$) nor was there a significant interaction ($P = 0.942$) between treatments and depths (Figure 27 A). Abundance of the *nosZ* gene (expressed gene copies/ gram sediment dry weight), were not significantly different (Figure 27 B) among treatments ($P = 0.628$), depth ($P = 0.166$) nor was there a significant interaction between treatments and depths ($P = 0.249$).

Bacterial community composition- Analysis of the bacterial community revealed 36 phyla and 184 families. Phyla contributing most to community composition were Proteobacteria (~34%), Bacteroidetes (~21%), Chloroflexi (~15%), Firmicutes (~5%), and Acidobacteria (~5%). Bacterial sequences with an unknown phylum affiliation comprised ~7% of sequences. Phyla with relative abundance greater than 0.01% (only two phyla: Candidate_division_ZB3 and Synergistetes had < 0.01% relative abundance) are shown in Figure 28.

Redundancy analysis (RDA) of Hellinger transformed ASVs revealed a significant ($P < 0.001$) interaction between treatments and sediment depths at the ASV level (Figure 29). However, when analyzed at higher taxonomic levels (phylum, family, and genus) there were no significant differences. RDA indicated that bacterial community composition in the worm treatment was distinct from the control and mayfly treatments at all depths (Figure 29). Within the worm treatment, bacterial community composition at two and four cm depths were grouped separately from six and eight cm. In contrast, in the control and mayfly treatments, bacterial community composition at eight cm differed from other depths. Additionally, according to the ordination plot, sediment depth explained (8.6%) more variation than bioturbator types (2.7%).

In total, 115 ASVs had over 30% of their variation explained by the interaction effect between treatments and sediment depths in the RDA model. Changes (relative to control) in relative abundances (expressed as percentages) of these ASVs across different treatments (mayfly and worm) and depths are shown in Figure 30 (A, B, and C).

Briefly, these ASVs represented 14 phyla (Proteobacteria, Bacteroidetes, and Chloroflexi were the most common) and 19 families. For Proteobacteria (Figure 30 A), most members of families *Xanthobacteraceae* and *Rhodocyclaceae* were lower while members of *Comamonadaceae, Syntrophaceae, Geobacteraceae, Rhodospirillaceae,* and *Methylococcaceae* were higher in relative abundances in worm than mayfly treatments. For Bacteroidetes (Figure 30 B), most members of *Marinilabiliaceae* and *Cytophagaceae* had higher relative abundance in worm than mayfly treatments while the opposite trend was found for all members of the phylum Firmicutes (Figure 30 B). Members of Acidobacteria, Cyanobacteria, and *Gemmatimonadaceae* had higher relative abundance in worm than mayfly treatments according to Figure 30 C. Other bacterial taxa shown in Figure 30 either belonged to unknown taxonomic affiliations or revealed

similar shifts (higher or lower) in their relative abundances for both, *L. variegatus* and *H. bilineata* relative to control.

DISCUSSION

Prior studies have examined how bioturbators influence biogeochemical cycles and bacterial community composition in marine ecosystems (Laverock et al. 2010, Bertics and Ziebis 2009, Kristensen and Kostka 2005). The present study is one of only a few in freshwater ecosystems that relate bioturbation to nitrogen cycling and bacterial community composition (Zeng et al. 2014, Mermillod-Blondin et al. 2004). In this study, we observed that *H. bilineata* (mayfly nymph) and *L. variegatus* (annelid worm) had distinct effects on DIN concentrations and sediment bacterial community composition. The two species used in this study differed in burrowing mode (specifically, depth, stability, and configuration) suggesting that these factors may be important predictors for water nutrient (N) chemistry and sediment bacterial community composition. Compared to controls, mayfly nymphs had lower nitrate and higher ammonium concentrations in surface water while worm treatments had higher nitrate concentration in interstitial water. Despite significant differences in bacterial community composition (at finer taxonomic level) between controls and treatments, in contrast to other studies, we did not observe differences in denitrification rates (Gilbertson et al. 2012, Foshtomi et al. 2015) and bacterial abundance (Mermillod-Blondin et al. 2004, Papaspyrou et al. 2005).

In this study, *L. variegatus* altered bacterial community composition relative to the control. In contrast, Zeng et al. (2014) reported no impact of bioturbation on bacterial community composition in lacustrine ecosystem when they examined how sediments inhabited and uninhabited by bioturbators differed in bacterial community structure. However, studies in marine ecosystems have demonstrated that bioturbation affects bacterial community composition

(e.g., Papaspyrou et al. 2006, Satoh et al. 2007, Bertics and Ziebis 2009, Laverock et al. 2010). Differences among studies between marine and freshwater ecosystems may be attributable the experimental design, target organisms, environmental conditions, and methods. For example, Papaspyrou et al. (2006), Bertics and Ziebis (2009), and Laverock et al. (2010) examined differences in bacterial community composition in the burrow walls and ambient surface or subsurface sediment. Other differences include experiment duration (> 7 days), bioturbator abundance, different burrow configurations (e.g., J shaped burrows of fiddler crabs) and depths (> 10 cm). Additionally, in our study, we did not look specifically at bacterial communities within the burrows of the bioturbators but compared between control and bioturbated sediment.

Significant differences in bacterial community composition among treatments at the ASV level, with no apparent differences at the higher taxonomic levels (phylum and family), suggests there is functional redundancy. For example, an increase in specific ASVs within a higher taxonomic level (e.g., phylum, family) must have been compensated by decrease in ASVs within those same taxa. Microbiomes encompassing broadly distributed important functions (e.g., various C and N cycling processes) tend to resist changes in higher-level taxonomic community diversity due to functional redundancy (Louca et al. 2018). Additionally, several taxa performing a similar function and belonging to a specific taxonomic rank (e.g., numerous families performing denitrification are grouped under Proteobacteria) can persist in a functionally stable state with varying relative abundances (Wittebolle et al.2008). These circumstances may result in no shifts in taxonomic community at higher taxonomic levels but cause differences at the sequence level as was observed in our study. Moreover, the 16S rRNA gene sequencing method used identifies differences in sequences in hypervariable regions (V4-V5 region, Sun et al. 2013). Differences in nucleotides in those variable regions does not necessarily imply that there

will be differences in conserved regions of the gene too. Therefore, differences in sequences at a particular region of the gene might not be sufficient to alter the taxonomic affiliation of the ASVs and subsequently indicate no differences at higher taxonomic ranks (e.g., phyla, families).

In terms of bacterial community composition, Proteobacteria was the dominant phylum, as reported in other studies of freshwater ecosystems, including constructed wetlands (Ansola et al. 2014, Ma et al. 2018, Zeng et al. 2014). Additionally, certain bacterial taxa defined at a much finer level (ASVs) differed among treatments. For example, the relative abundance of the members of families *Methylococcaceae, Rhodospirillacea*, *Spirochaetaceae* and Acidobacteria were higher in worm than mayfly treatments at all depths relative to controls. This implies that certain treatments select for specific ASVs assigned to certain bacterial taxa. However, it must be noted that although the selection resulted in some shifts in the relative abundances at the ASV level it was not strong enough to completely replace or add new bacterial taxa.

We found some support of our hypothesis that *L. variegatus* burrows facilitate more oxygen-tolerant bacterial taxa than *H. bilineata* burrows. Specifically, relative abundance of the members of the aerobic family *Methylococcaceae*, which are commonly found in wetlands (Smith et al. 2018), was higher in worm than mayfly treatments. Moreover, most species of *Rhodospirillaceae* and phylum Acidobacteria are known to grow under aerobic conditions (Biebl and Pfennig 1981, Dedysh and Damsté 2018, Kalam et al. 2020) and relative abundance of the members of both these bacterial taxa was higher in worms than mayflies. Additionally, relative abundance of the members of the phylum Firmicutes (harboring mostly obligate/facultative anaerobes) was lower in worm than mayfly treatments compared to control. Prior studies have suggested that gallery network burrows are capable of increasing sediment surface area interaction with oxygen from surface water and creating aerobic microsites (Zeng et al. 2014,

Martin et al. 2005, Braker et al. 2001, Anschutz et al. 2012). Interestingly, relative abundance of the members of Nitrospirae (nitrifying taxa) (Daims et al. 2015) was lower in both bioturbator treatments relative to control; however, the difference was greater in mayfly than worm treatments. Moreover, relative to controls, we saw lower relative abundance of the members of anaerobic families *Anaerolineaceae* (phylum Chloroflexi) and *Geobacteraceae* in worm than mayfly treatments. By extension, this suggests that, in addition to N cycling processes, *L. variegatus* burrows facilitate other biogeochemical processes related to carbon cycling (organic matter mineralization by *Anaerolineaceae;* Yamada et al. 2005, Sinkko et al. 2013) and iron cycling (in *Geobacteraceae*; Yi et al. 2013). Therefore, further research regarding other predominant biogeochemical processes in the presence of bioturbation needs to be done to understand how bacterial community composition relates to ongoing nutrient cycling processes.

Denitrification rates were not significantly affected by invertebrate treatments. Similarly, *nosZ* gene abundance was not different across treatments or depths. The lack of response in the denitrifier activity and abundance may be caused by functional redundancy across several families in different phyla, including Proteobacteria which was the dominant phyla across all treatments and includes many potential denitrifiers (Shapleigh, 2013). Specifically, the ability for one dentrifier ASV to increase and compensate for the decline in another denitrifier ASV perhaps resulted in maintaining similar denitrification rates across treatments. Availability of nitrate can positively impact denitrification rates and we observed higher nitrate in interstitial water in worm than other treatments (presumably due to nitrification). Therefore, coupled nitrification-denitrification may have resulted in slightly higher (although not statistically significant) denitrification rates in worm than other treatments.

Nitrate utilization via dissimilatory processes (denitrification, dissimilatory nitrogen reduction to ammonium) combined with low nitrifying taxa abundance might have resulted in lower nitrate concentration in mayfly treatments while higher levels of nitrate in interstitial water for worm treatments may indicate nitrification due to higher oxygen penetration. Along with ammonia oxidizing bacteria, archaea can perform N transformations, including ammonia oxidation and bioturbation can affect archaeal abundance and *amoA* expression (Huang et al. 2016). Therefore, it is possible that the archaeal community was also driving the observed changes in DIN. The unexpected high concentration of nitrate in the sand microcosms (having autoclaved sand and site water) with mayflies maybe due to N- transformation (specifically, nitrification) mediated by the gut microbes (released via excretion) of the mayfly nymphs.

In addition to the role of certain bacterial taxa, factors like burrowing mode and animal excretion can also explain the shifts in the water DIN concentration in different treatments to some extent. For example, *L. variegatus*, burrow head-down, feed in anoxic layers, and excrete at the sediment water interface (Anschutz et al. 2012) resulting in translocation of ions from bottom anoxic to top oxic layers. Such movement of ions can facilitate oxidation of ions, including ammonium, that may have resulted in lower ammonium in interstitial water in *L. variegatus* than *H. bilineata* treatments. High ammonium concentration in mayfly microcosms that increased over time was likely due to excretion based on our results and as reported for other U-shaped burrowers (Kuntz and Tyler 2018). Overall, lower nitrate and higher ammonium concentrations at increasing sediment depths was possibly due to lower oxygen penetration resulting from lack of burrowing activity.

CONCLUSIONS

This study looked at how distinct bioturbator species with unique burrow configuration (U shaped burrows: *H. bilineata* and gallery network burrows: *L. variegatus*) and burrowing depths impact N- cycling processes (specifically, denitrification) and bacterial community composition in freshwater ecosystems. Higher relative abundances of some ASVs assigned to aerobic bacterial taxa in *L.variegatus* treatments in comparison to *H. bilineata* (mayfly nymphs) provided was consistent with the prediction that *L. variegatus* enhances oxygen penetration. Our results also indicated that burrowing mode and excretion may impact water DIN concentration. However, we did not see expected differences in denitrification rates, perhaps due to functional redundancy (which may be one of the plausible causes). Future studies can be designed to test these results in actual ecosystems to obtain more insights regarding bioturbation-mediated shifts in N dynamics and bacterial community composition.

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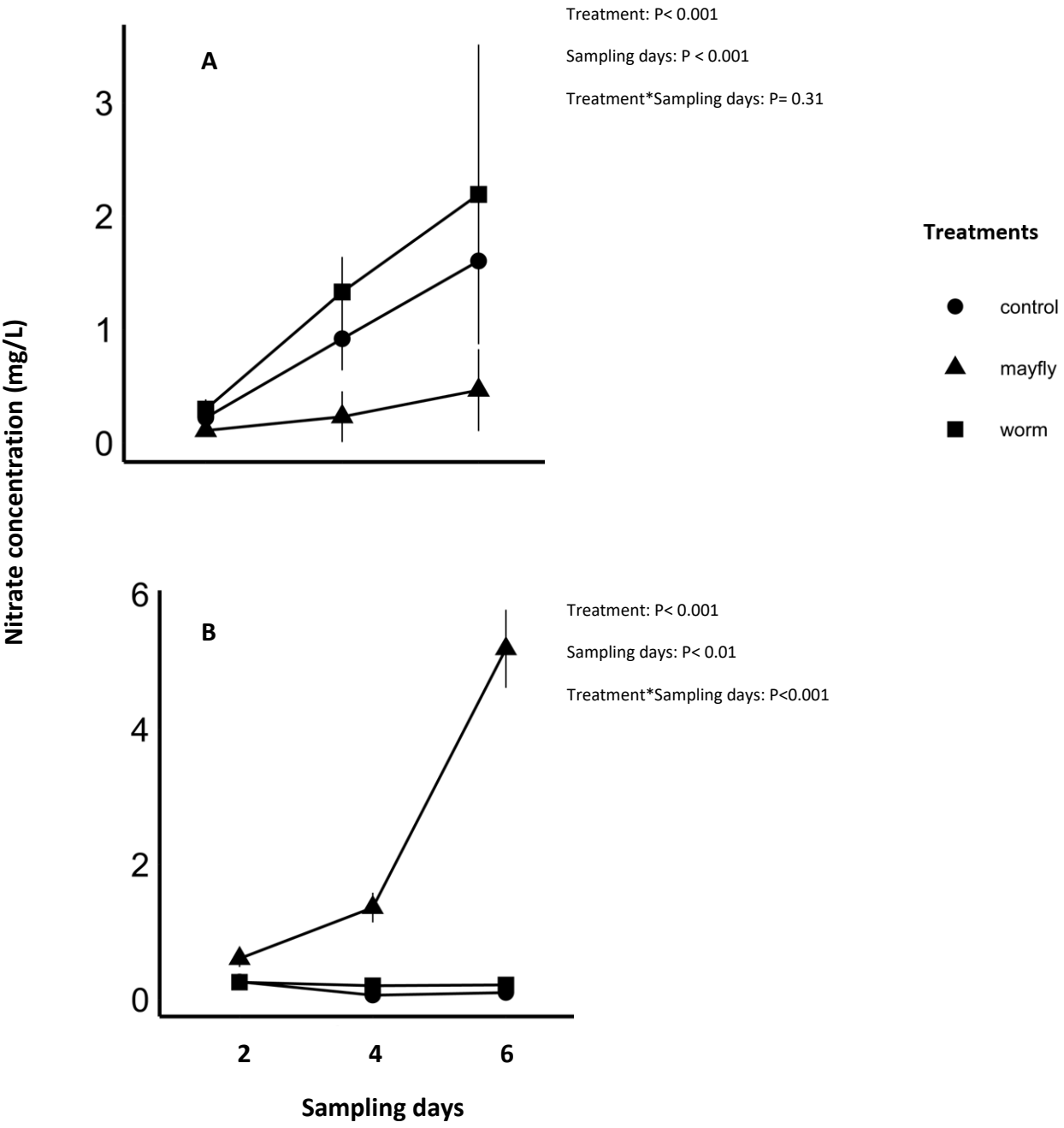


Figure 22. Differences in surface water nitrate concentration (expressed as mg/L; untransformed data) for bioturbation experiment (A), and excretion experiment (B) among treatments at various sampling days. Symbols represent mean ± standard deviation (n=3).

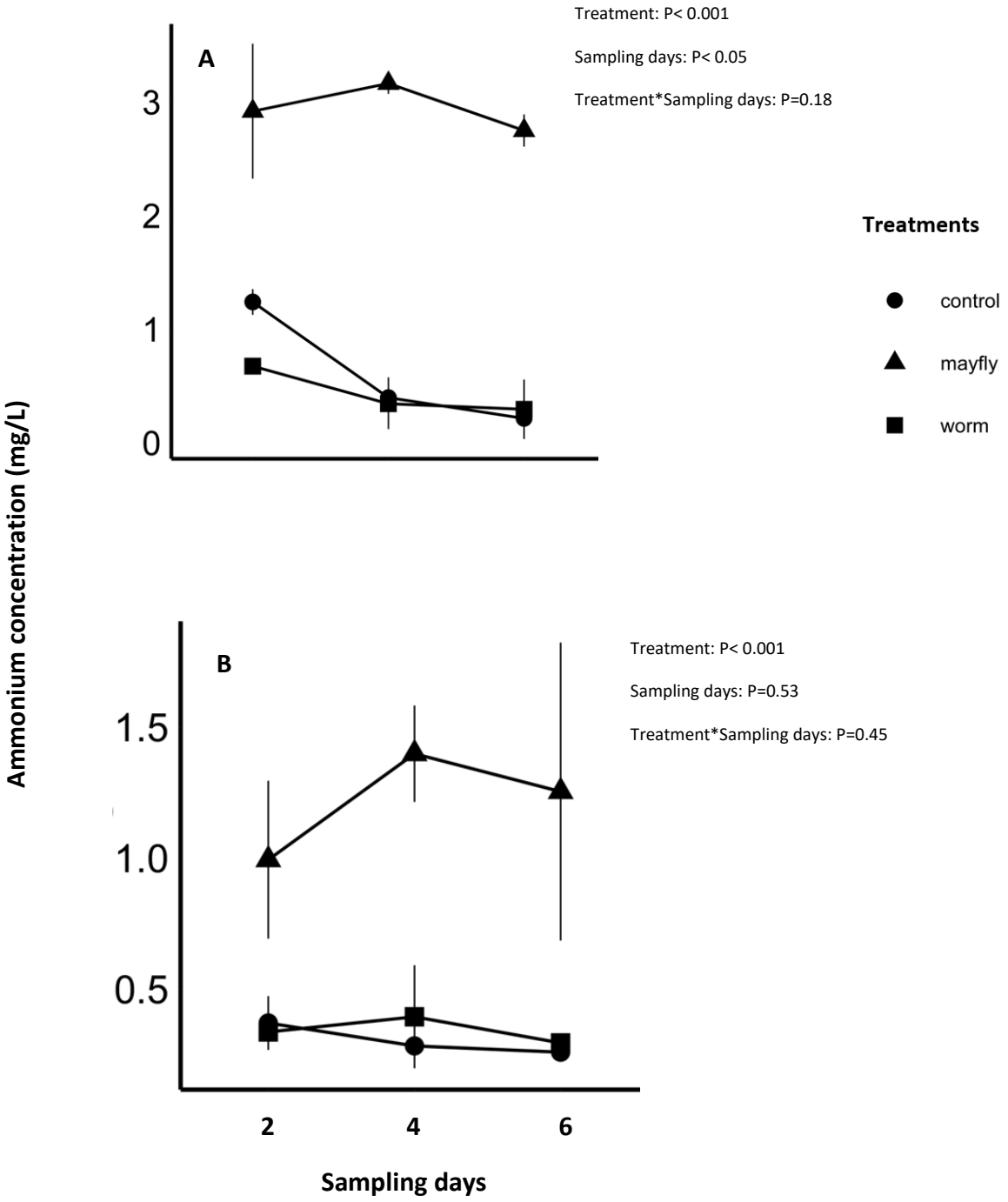


Figure 23. Differences in surface water ammonium concentration (expressed as mg/L; untransformed data) for bioturbation experiment (A), and excretion experiment (B) among treatments at various sampling days. Symbols represent mean ± standard deviation (n=2 for A and n=3 for B).

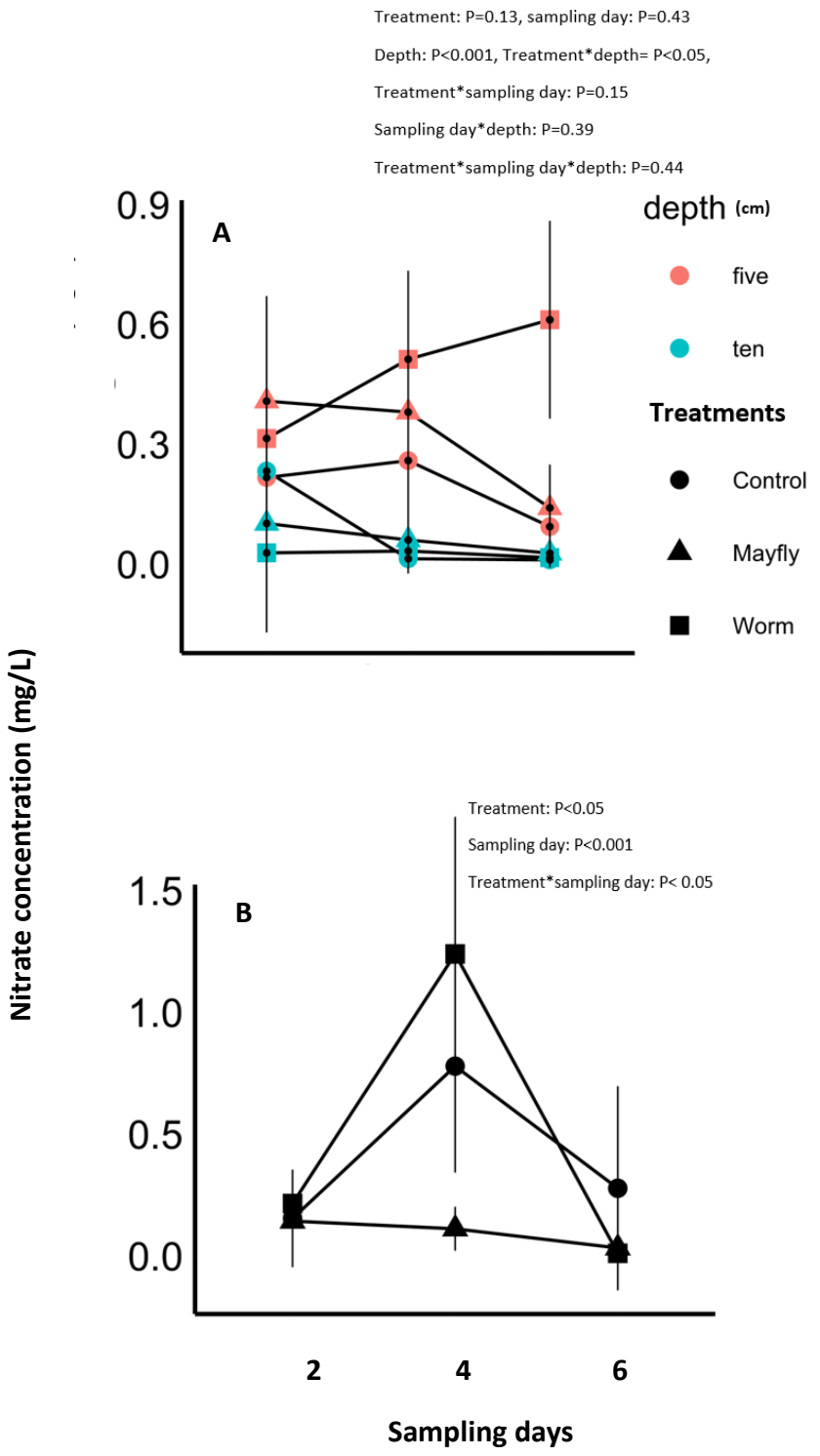


Figure 24. Differences in interstitial water nitrate concentration (expressed as mg/L; untransformed data) for bioturbation experiment (A), and excretion experiment (B) among treatments at various sampling depths over sampling days. Symbols represent mean ± standard deviation (n=3). For excretion experiment only one depth (5cm) was considered.

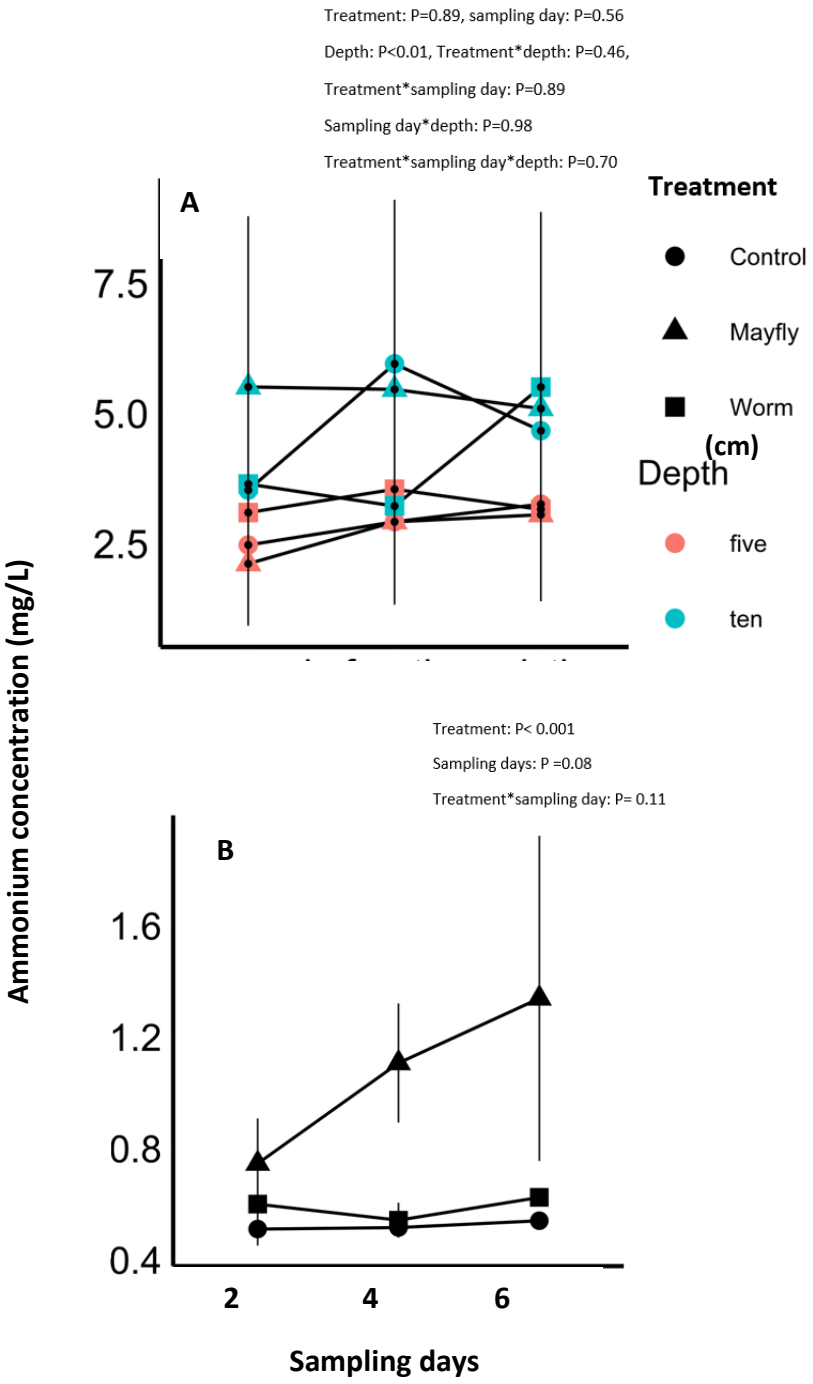


Figure 25. Differences in interstitial water ammonium concentration (expressed as mg/L; untransformed data) for bioturbation experiment (A), and excretion experiment (B) among treatments at various sampling depths over sampling days. Symbols represent mean ± standard deviation (n=3). For excretion experiment only one depth (5cm) was considered.

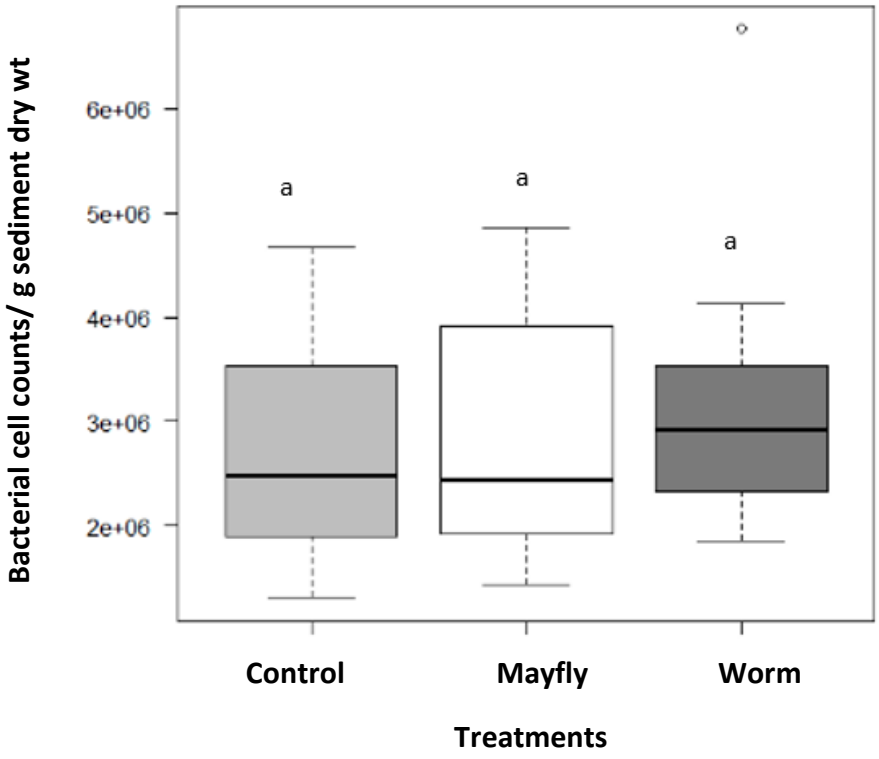
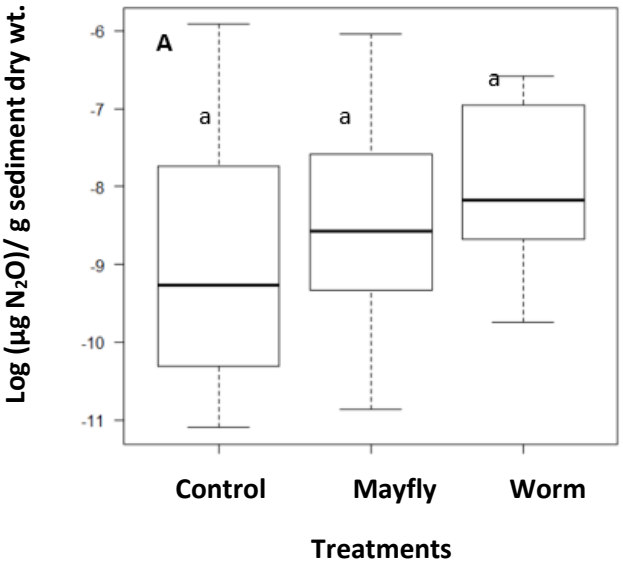


Figure 26. Difference in bacterial cell numbers (expressed as cells/g sediment dry weight) among treatments (averaged across all depths). Horizontal bar represents median value of sample, box represents the interquartile range (IQR), and whiskers represent 1.5 × IQR. Lower case letters directly above boxplots indicate significant (letters differ) or no significant (letters are the same) differences.



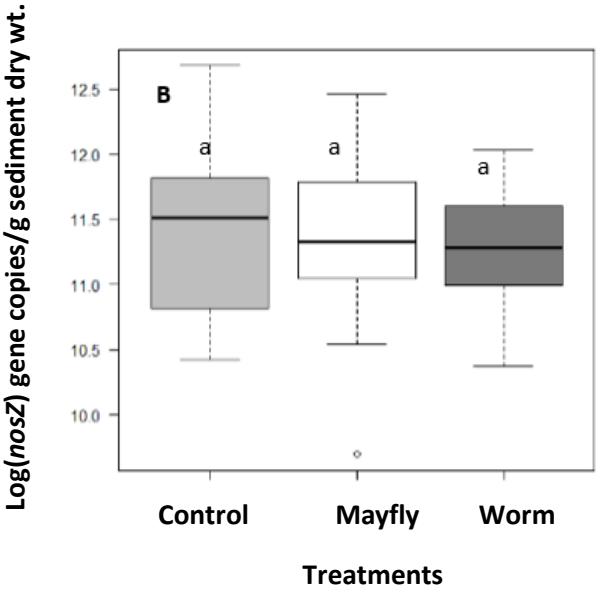


Figure 27. The effect of treatment (averaged across depths) in respect to log transformed potential denitrification rate expressed as µg N_2O/gram sediment dry weight/hour (A) and log transformed *nosZ* gene abundance expressed as gene copies/ gram sediment dry weight (B). Horizontal bar represents median value of sample, box represents the interquartile range (IQR), and whiskers represent 1.5 × IQR. Lower case letters directly above boxplots indicate significant (letters differ) or no significant (letters are the same) differences.

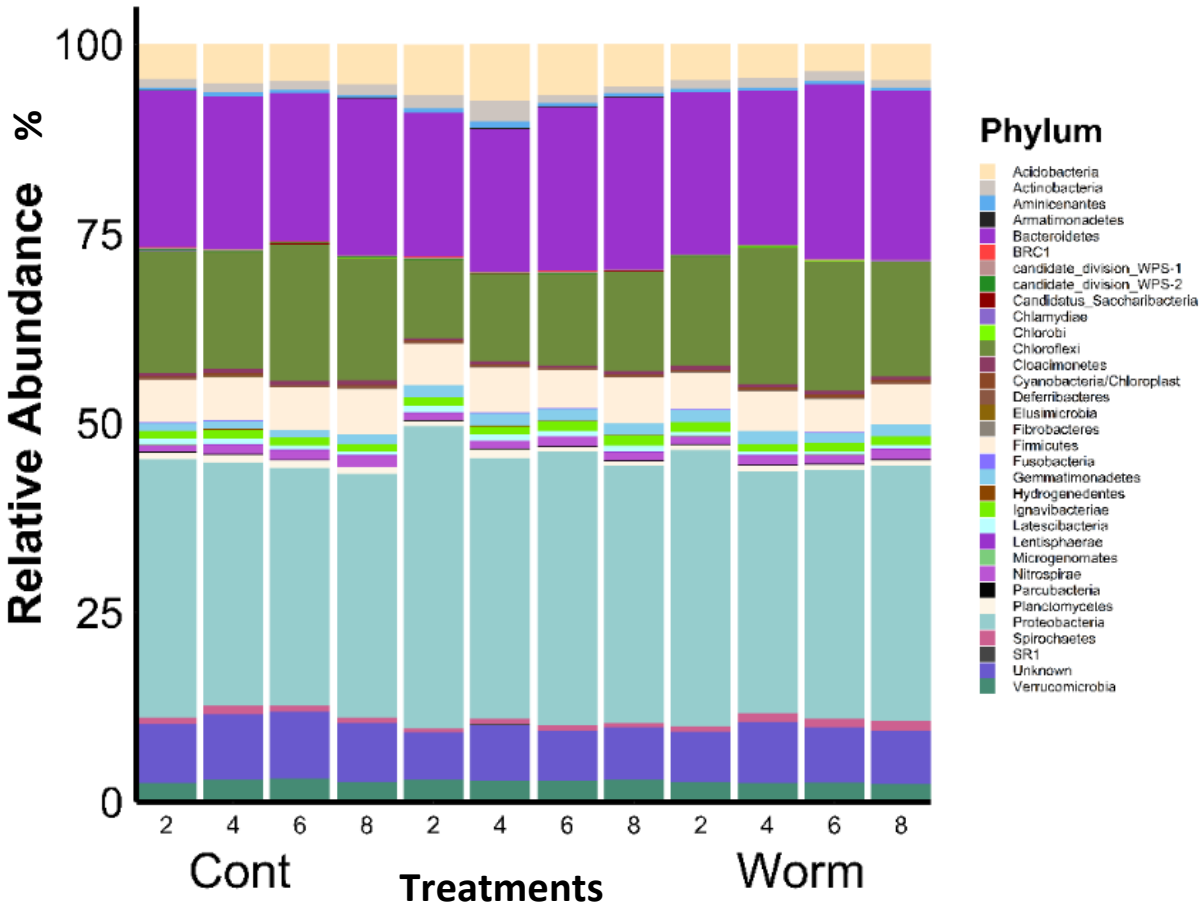


Figure 28. Relative abundance (% total) of all phyla (>0.01%) across different treatments (bioturbator types) along with respective sediment depths (in cm).

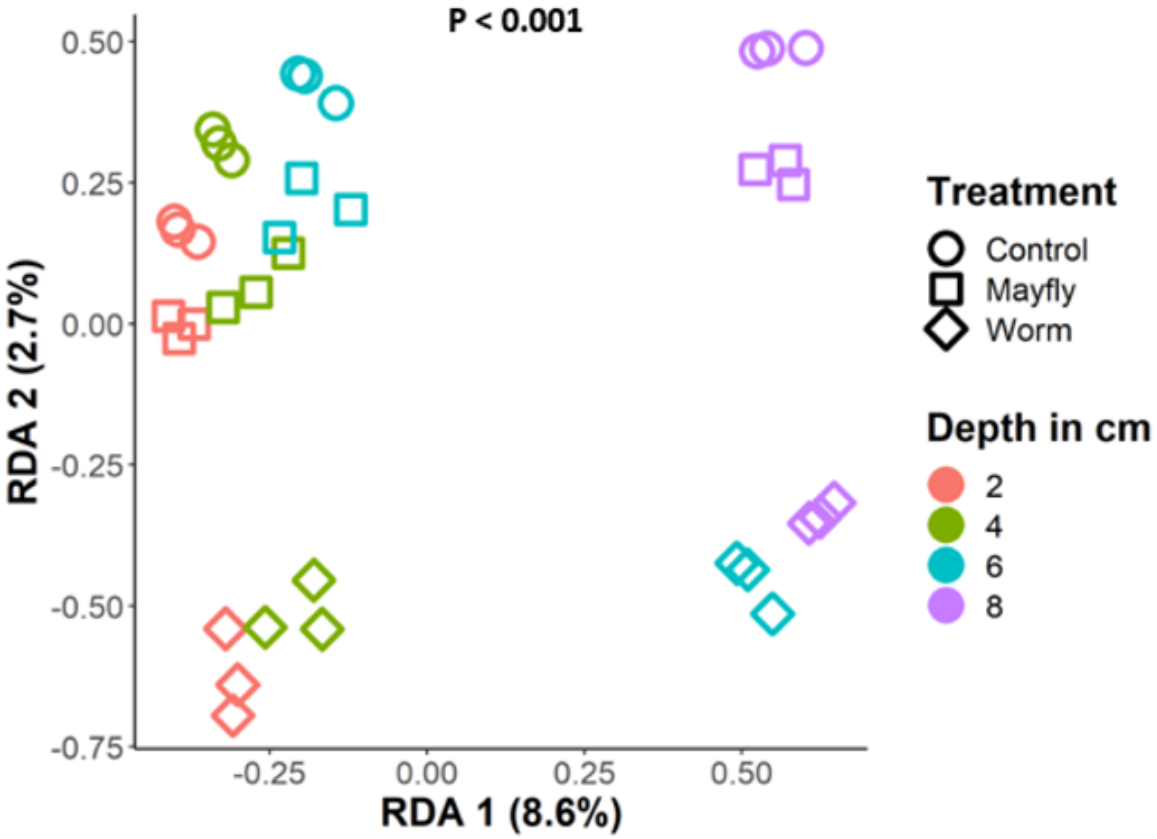


Figure 29. RDA plot of the interaction effect between different treatments (bioturbator types) and sediment depths (in cm) on Hellinger transformed 16S ASV profiles.

A

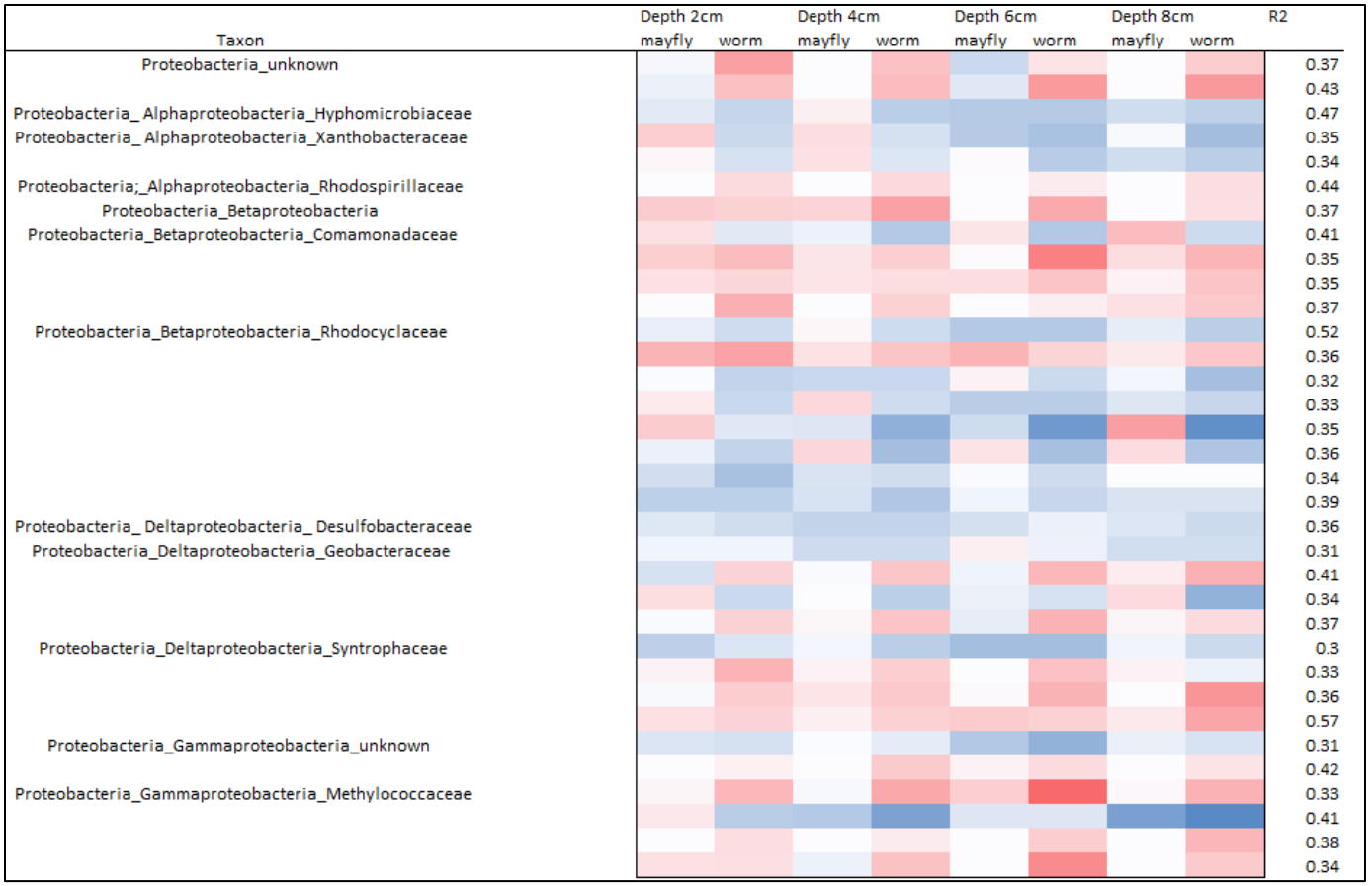


Figure 30 (A). Heatmap depicting changes in relative abundances (expressed as percentages) across different treatments and depths relative to control for the ASVs (taxonomic affiliations assigned to family level to avoid complexity) that were explained most (R^2>0.03) by the treatment*depth RDA model for the phylum Proteobacteria. Lower abundance compared to the control are shaded blue, while a higher abundance compared to the control are shaded red. White indicates zero change in relative abundance.

B

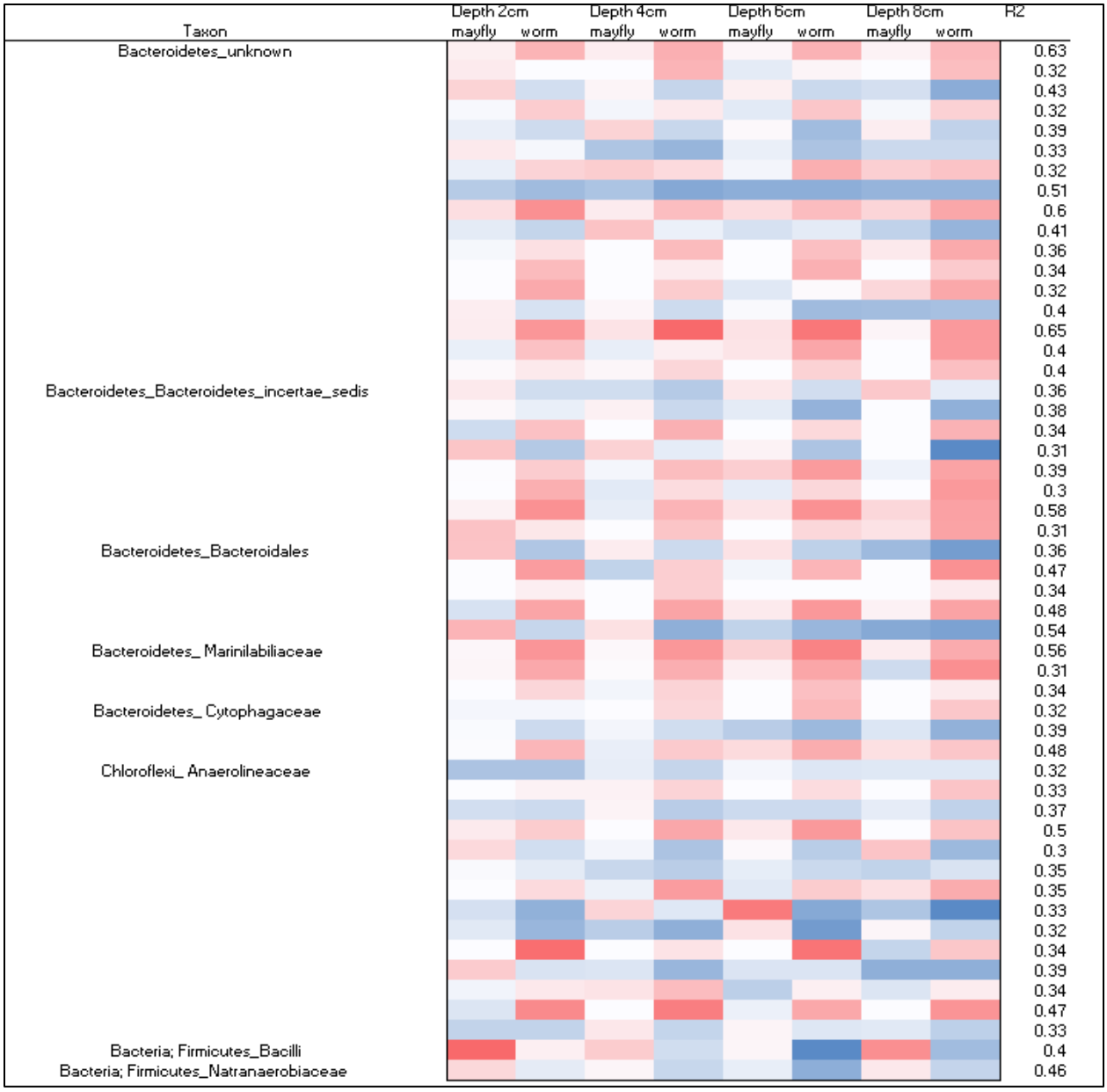


Figure 30 (B). Heatmap depicting changes in relative abundances (expressed as percentages) across different treatments and depths relative to control for the ASVs (taxonomic affiliations assigned to family level to avoid complexity) that were explained most (R^2>0.03) by the treatment*depth RDA model for phyla Bacteroidetes, Chloroflexi, and Firmicutes. Lower abundance compared to the control are shaded blue, while a higher abundance compared to the control are shaded red. White indicates zero change in relative abundance.

C

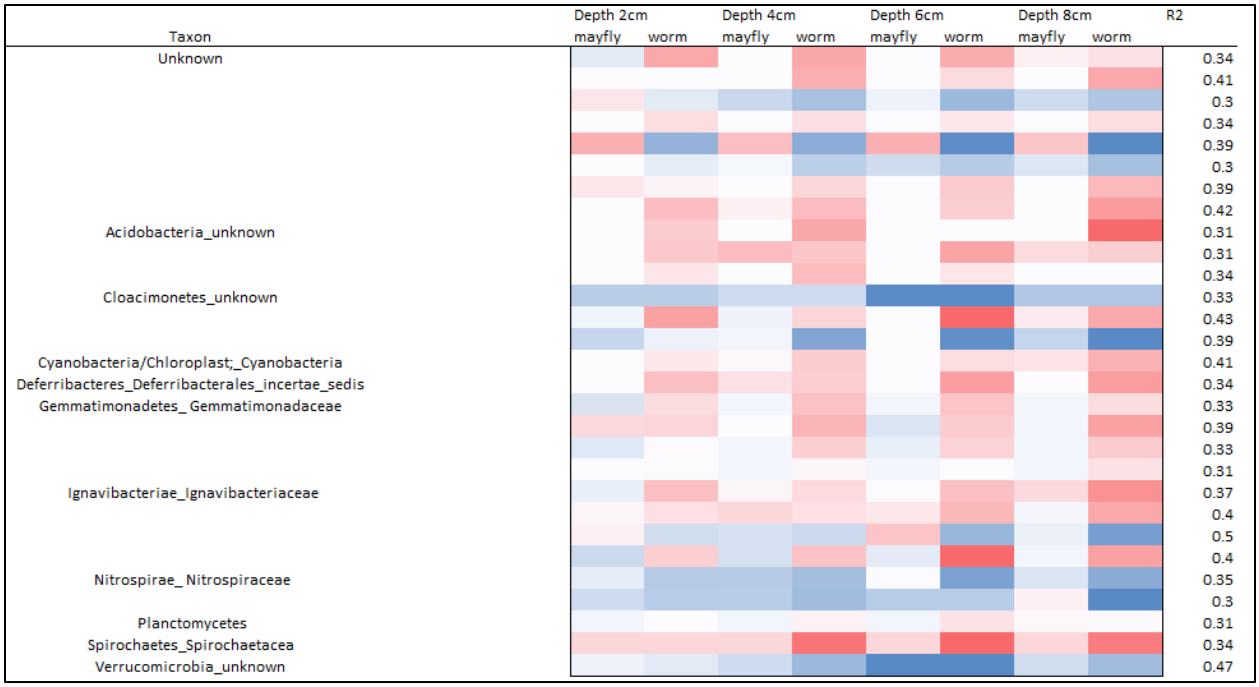


Figure 30 (C). Heatmap depicting changes in relative abundances (expressed as percentages) across different treatments and depths relative to control for the ASVs (taxonomic affiliations assigned to family level to avoid complexity) that were explained most (R^2>0.03) by the treatment*depth RDA model for all other phyla. Lower abundance compared to the control are shaded blue, while a higher abundance compared to the control are shaded red. White indicates zero change in relative abundance.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

Increased anthropogenic activities have disrupted freshwater ecosystems, including streams (Zhang et al. 2020, Camargo and Alonso 2006, Hai et al. 2009). Pollutants ranging from nutrients to antibiotics and other pharmaceuticals can adversely impact freshwater ecosystems by altering biogeochemical processes and bacterial community structure and function (e.g., Roberto et al. 2019, Wymore et al. 2021, Van Gray et al. 2020, Yan et al. 2016). In addition to bacterial assemblages in free-living microbiomes, human mediated disturbances also negatively impact freshwater macroinvertebrate communities (Camargo and Alonso 2006, Arimoro and Keke 2017, Gezie et al. 2017).

Macroinvertebrate guts harbor bacteria capable of benefiting their hosts in multiple ways, including as an aide in nutrition (breakdown of organic compounds, providing growth factors, access to N), providing protection against plant secondary metabolites, and resisting colonization by harmful transient bacteria (Dillon and Dillon 2004, Cummins and Klug 1979, Mason et al. 2014). Furthermore, freshwater macroinvertebrate gut microbiomes perform ecologically significant functions, including N-transformations and dispersal of ARGs (Stief et al. 2009, Leff et al. 1994). Moreover, macroinvertebrate taxa have unique gut morpho- physiological features

and vary in feeding mode, habitat preferences, and physical alteration of the benthos (Cummins and Klug 1979, Mermillod-Blondin 2011) which can influence microbiome structure and function (Ayayee et al. 2018, Cariou et al. 2021). However, the connections between macroinvertebrates, their gut microbiomes, and their surrounding benthic and plankton microbiomes are not well documented.

The overarching goal of this dissertation was to add to examine how the host associated microbiome (freshwater macroinvertebrate guts) perform certain ecosystem processes (specifically, denitrification, harboring of ARGs and their dissemination). Furthermore, I also attempted understanding the interconnections between host-associated and free-living microbiomes with the help of studies involving N dynamics (specifically, denitrification), and antibiotic resistance in freshwater ecosystems. The questions posed in each chapter were investigated using a combination of traditional and advanced molecular methods using both, field oriented and laboratory-based microcosm approaches.

Stream macroinvertebrate gut microbiomes can be favorable habitats of ARGs, subsequently facilitating ARG abundance presumable due to conducive gut conditions for horizontal gene transfer (Lerner et al. 2017) and the potential of certain bacterial taxa to harbor specific ARGs (Roberto et al. 2019). Therefore, in Chapter II, I examined the role of stream macroinvertebrate gut microbiomes in harboring and disseminating ARGs. The abundance of three ARGs, *sulI, sulII, and tetW*, and bacterial community composition were compared among macroinvertebrate gut, sediment, and water microbiomes of an urban Northeast Ohio stream and two reference sites. The results showed that, in most instances, macroinvertebrate guts had higher ARG abundances than free living microbiomes implying that macroinvertebrate guts can be potential reservoirs of ARGs. ARG abundances and bacterial community composition also

differed among macroinvertebrate functional feeding groups. Additionally, our study also showed that certain bacterial taxa was correlated to ARGs implying that they may harbor the respective ARGs; however, further research including other potential stressors is required to fully understand the relationship between bacterial community composition and ARG abundance. Although macroinvertebrates were shown to be potential reservoirs of ARGs, we did not find evidence to support the hypothesis that macroinvertebrates are vectors of ARGs in streams. This was perhaps due to the physical movement of the macroinvertebrate taxa (*Corbicula*) used in the study that influenced the abundance of bacterial cells in the sediment.

Along with antibiotics, excess nutrients (nitrogen in this work) produced via anthropogenic activities also pollute freshwater ecosystems and adversely impact various N cycling processes, including denitrification (Mulholland et al. 2008, Wymore et al. 2021). Denitrification results in production of nitrogenous gases whereby incomplete denitrification results in production of nitrous oxide, a greenhouse gas (Phillipot et al. 2013). Freshwater macroinvertebrate guts are microsites for incomplete denitrification in comparison to their relatively oxic surrounding free-living microbiome (Stief et al. 2009). Therefore, freshwater macroinvertebrate gut-associated incomplete denitrification may contribute significantly towards the global nitrous oxide budget. However, the dominance of incomplete and complete denitrification varies among macroinvertebrate taxa (Stief et al. 2009, Peter et al. 2013), and our knowledge of what controls the relative nitrous oxide production in the macroinvertebrate guts is limited. Therefore, for chapter III, I assessed whether incomplete denitrification exceeds complete denitrification in the gut microbiome of a readily available model organism: freshwater crayfish (*Orconectes obscurus*). The gut microbiome was modified (after reducing bacterial load for facilitating bacterial colonization) by enriching it with denitrifiers possessing (*S. marcescens*)

or lacking (*P. chlororaphis*) *nosZ* (the nitrous oxide reductase gene that mediates conversion of nitrous oxide to di-nitrogen gas) and thereafter, denitrification rates and functional gene expression were measured. The results showed that altering the gut denitrifier community composition by enriching it with *S. marcescens* reduced incomplete denitrification by lowering the relative N_2O production rate and increased di-nitrogen emission. Additionally, *nosZ* expression was high in *S. marcescens* enriched than control and *P. chlororaphis* treatments. This suggested that complete denitrification can be dominant in freshwater macroinvertebrate guts and the denitrifying taxa may play an important role in determining the end product of denitrification. Moreover, detectable denitrification rates and functional gene expression in our controls suggested that extant gut bacteria may be contributing towards macroinvertebrate gut denitrification. However, like previous studies (Chen et al. 2015, Dandie et al. 2011) we did not detect any significant relationship between functional gene expression and denitrification rates possibly due to other factors (e.g., nitrate concentration) that may be affecting rates of nitrogenous gas production and gene expression that were not accounted for in the current study.

Aquatic macroinvertebrates can also impact N dynamics and bacterial community composition in their surrounding free-living microbiome via bioturbation (Foshtomi et al. 2015, Anschutz et al. 2012, Bertics and Ziebics 2009). Different burrow configurations of macroinvertebrate taxa can cause variation in oxygen penetration and nutrient translocation at different sediment depths; thus, affecting N cycling processes, including denitrification, and relative abundances of aerobic bacterial taxa. Therefore, for chapter IV, I focused on examining how two freshwater bioturbators (*Hexagenia bilineata* and *Lumbriculus variegatus*) with distinct modes of burrowing, impact dissolved inorganic nitrogen concentrations (nitrate and ammonium), potential denitrification rates, and bacterial attributes (abundance and overall

community composition). Results indicated that possibly due to increased oxygen penetration, gallery network burrows formed by *L. variegatus* increased nitrate concentration of interstitial water and abundance of aerobic bacterial taxa in comparison to U-shaped burrows formed by *H. bilineata*. Increase in ammonium concentration in *H. bilineata* treatments was attributed to macroinvertebrate excretion. Although significant differences in the sediment bacterial community composition (at the ASV level) was observed between the two bioturbator treatments, denitrification rates and *nosZ* abundance were not significantly different among treatments, perhaps, due to functional redundancy in the total bacterial community.

We know that anthropogenic activities have widely altered freshwater ecosystems, including streams. Yet, the full impact of human-mediated activities on bacterial community composition and function is still not well understood. The three projects described in this dissertation addressed the role of freshwater macroinvertebrates and their gut microbiomes in two ecologically significant processes that are strongly impacted by anthropogenic activities: denitrification and antibiotic resistance. In general, this work provided insights about how freshwater macroinvertebrates and their gut microbiomes influence N dynamics and antibiotic resistance and how host-associated and free-living microbiomes vary in performing specific ecosystem processes (e.g., serving as reservoirs of ARGs).

Overall, the results obtained from this dissertation emphasize the ecological significance of freshwater macroinvertebrates and their gut microbiomes. Moreover, as was reported previously (Van Gray et al. 2020, Baxter et al. 2012, Chen et al. 2015, Roberto et al. 2019), this work also suggests that bacterial community structural and functional attributes (e.g., taxonomic composition, gene abundances, and expression of relevant functional genes) may be important predictors of variations in biogeochemical processes (e.g., denitrification) or spread of pollutants.

Furthermore, this dissertation documented for the first time that freshwater macroinvertebrate gut microbiomes can serve as "reservoirs" of ARGs in comparison to their free-living counterparts. In addition to the above-mentioned findings, the work also supported well-established ideas like functional redundancy within microbiomes (Louca et al. 2018) and bacterial community composition being unique for different microbiomes (Hosen et al. 2017). Overall, the major conclusion is that freshwater macroinvertebrates along with their gut microbiomes play ecologically important roles in mediating certain ecosystem functions like their free-living counterparts. However, it is important to note that based on our observations further research is necessary for drawing generalities about the role of macroinvertebrates and their gut microbiomes in freshwater ecosystems as until date the ecological significance of macroinvertebrate gut microbiomes has mostly remained as a black box.

The results obtained in this work can be utilized to design future experiments which can help us answer questions in the following topics:

- Management decisions and restoration efforts: From our study we know that distinct burrowing bioturbators impact dissolved inorganic nitrogen and bacterial community composition differently based on their burrow configuration and depths. Therefore, ecosystem scale bioturbation studies can help researchers figure out which bioturbator species can facilitate or restrain excess nutrient removal and increase abundance of bacterial taxa positively or negatively related with removal of certain pollutants (e.g., organic compounds) in impaired aquatic environments.
- Anthropogenic impacts and ARG abundance in macroinvertebrates guts: Our work has shown that freshwater macroinvertebrate gut microbiomes are potential reservoirs of ARGs. However, the current work addressed local variations in host-associated and free-

living microbiomes. Therefore, future studies can focus on assessing how anthropogenic activities (e.g., watershed land use patterns, cooccurrence of other stressors like metal resistance genes), seasonal variations, or dominance of specific macroinvertebrate taxa might be driving differences in ARG abundances between host-associated and free-living microbiomes. This will facilitate drawing generalities about the role of macroinvertebrates in harboring and disseminating ARGs in freshwater ecosystems.

- Anthropogenic impacts and freshwater macroinvertebrate gut denitrification: Given that denitrifying taxa can be important predictors of relative N_2O production in freshwater macroinvertebrate guts, it is important to gain better insights about the shifts in physiology of denitrifiers when they move from relatively oxic to anoxic environments. Moreover, it will be worthwhile to assess how nitrous oxide production rates differ among macroinvertebrates inhabiting variedly impaired (e.g., receiving high nitrate inputs) freshwater ecosystems and identify the macroinvertebrate taxa that contribute relatively high amounts of nitrous oxide (specifically, the ones in which incomplete denitrification outcompetes the complete process in the guts). This will help us to better comprehend the contribution of macroinvertebrates towards the global N_2O budget.

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