FRESHWATER MUSSEL-MEDIATED NUTRIENT FLUXES AND BURROWING BEHAVIOR INFLUENCE ECOSYSTEM FUNCTIONING

by

MEGAN ELIZABETH KUBALA

CARLA L. ATKINSON, COMMITTEE CHAIR
ALEX HURYN
COLIN JACKSON

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ABSTRACT

In freshwater ecosystems, animals can play large roles in biogeochemical cycles by sequestering and recycling nutrients and by modifying their physical habitat. Nutrient release from consumers can support both 'green food webs' based on primary producers and 'brown food webs' based on decomposers. Microbes also play a critical role in facilitating biogeochemical processes, such as the nitrogen cycle, in which they transform organic and inorganic nitrogen compounds. Freshwater mussels (Bivalvia: Unionoida) are a guild of benthic, burrowing, filter-feeding bivalves that translocate nutrients and energy from the water column to the benthos via excretion and egestion. Despite being classified in the same functional group, mussels within the same system exhibit variation in functional traits such as nutrient stoichiometry and burrowing behavior which can strongly influence ecosystem processes. Therefore, quantifying functional trait diversity is key to improving our ability to predict the effects of mussel diversity loss on ecosystem function. Here, we used a mesocosm experiment to investigate how phylogenetically diverse mussel communities influence ecosystem functioning through direct and indirect effects. Our results suggest that mussel-mediated nutrient cycling directly influences brown and green food webs by increasing primary productivity and enhancing organic matter decomposition. Additionally, mussels physically (via bioturbation) and chemically (via excretion and egestion) influenced sediment-water nutrient fluxes and sediment microbiome community composition by increasing sediment nitrogen-removal potentials. Our study advances our understanding of how mussel species identity and community composition

regulates components of green and brown food webs as well as potentially predicting changes in ecosystem functioning as a result of a loss or shift in species composition.

DEDICATION

To my husband, Austin

My parents, George and Gloria

My brother, Michael

LIST OF ABBREVIATIONS AND SYMBOLS

AFDM	Ash-free dry mass
AL	Alabama
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Anammox	Anaerobic ammonium oxidation
BD-EF	Biodiversity-ecosystem function framework
C	Carbon
C. kieneriana	*Cyclonaias kieneriana*
cm	Centimeter
Coupled-DNF	Coupled nitrification-denitrification
d	Day
DIN	Dissolved inorganic nitrogen
DNF	Denitrification
DO	Dissolved oxygen
e.g.	exempli gratia (“for example”)
ES	Ecological stoichiometry
F. cerina	*Fusconaia cerina*
g	Gram
h^{-1}	Per hour
H_2O	Water

Hr	Hour
i.e.	id est (“that is”)
IPT	Isotope pairing method
kg^{-1}	Per kilogram
L	Liter
L^{-1}	Per liter
L. ornata	*Lampsilis ornata*
m	meter
m^2	Square meter
m^3	Cubic meter
m^{-2}	Per square meter
mg	Milligram
MIMS	Membrane inlet mass spectrometer
min^{-1}	Per minute
mL	Milliliter
mm	Millimeter
n	Sample size
N	Nitrogen
N_2	Dinitrogen gas
$^{29}N_2$	Dinitrogen gas derived from anaerobic ammonium oxidation
$^{30}N_2$	Dinitrogen gas derived from denitrification
NH_4^+	Ammonium
NO^-_2	Nitrite

NO^{-}_{3}	Nitrate
$^{15}NO^{-}_{3}$	Isotopically-labeled nitrate
N-removal	Transformation of dissolved inorganic nitrogen to dinitrogen gas
O_2	Oxygen
OM	Organic matter
P	Phosphorus
p	p-value for determining significant results in statistical analyses
PERMANOVA	Permutational multivariate analysis of variance
PCoA	Principal coordinate analysis
PCo1	Principal coordinate one
PCo2	Principal coordinate two
PVC	Polyvinyl chloride
r	Pearson correlation coefficient
Redox	Reduction-oxidation
SE	Standard error of the mean
SYC	Sycamore
t	Symbol representing the beginning of an incubation trial
T_0	Start of sediment slurry incubation
T_6	End of sediment slurry incubation
TP	Tulip poplar
Tukey HSD	Tukey's Honest Significant Difference multiple comparison test
WIL	Willow
w/v	Percent of molecule in total volume of solution

$ZnCl_2$	Zinc chloride
µL	Microliter
µm	Micrometer
µmol	Micromole
°C	Degree Centigrade
±	Plus or minus
%	Percent
#	Number of
→	Forward reaction

TREATMENT ABBREVIATIONS

NM	No mussel
L	*L. ornata*
C	*C. kieneriana*
F	*F. cerina*
LC	*L. ornata* + *C. kieneriana*
LF	*L. ornata* + *F. cerina*
CF	*C. kieneriana* + *F. cerina*
LCF	*L. ornata* + *C. kieneriana* + *F. cerina*

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CHAPTER 1:

CONCEPTUAL FRAMEWORK AND SUMMARY OF OBJECTIVES

Ecological stoichiometry (ES) is a conceptual framework based on the balance and ratios of multiple elements in ecological systems (Sterner & Elser, 2002). This framework can be used to understand and predict many important ecological processes that shape ecosystems such as nutrient cycling and trophic interactions. Elemental ratios of organisms and ecosystem fluxes have mainly focused on the key elements of Carbon (C), Nitrogen (N), and Phosphorous (P) because these core macroelements are essential to all life on Earth, contribute significantly to ecosystem functions (Sterner & Elser, 2002), and often limit productivity (Elser & Urabe, 1999). The Redfield ratio is a foundational stoichiometric relationship that describes the ratio of C:N:P in marine phytoplankton and seston (Redfield, 1958) and has been largely used to study the elemental composition of organisms in relation to ecosystem level elemental supply. Thus, ecological stoichiometry is a powerful framework that can connect multiple levels of ecological hierarchy by linking elemental composition of individual organisms to ecosystem-scale processes (Elser et al., 2000).

In freshwater ecosystems, animals can play large roles in biogeochemical cycles by sequestering and recycling nutrients (Vanni, 2002). Recently, more attention has been given to describing these roles using a more comprehensive approach, such as studying animal-mediated nutrient cycling by small animals or in dynamic environments (Hopper et al., 2018). Animal effects on ecological processes include a multitude of direct and indirect effects and thus has

been termed consumer-driven nutrient dynamics (CND) (Atkinson et al., 2017). The rules that govern the storage and release of nutrients by animals have been increasingly studied in freshwater systems (Capps et al., 2015) and ES provides an excellent framework for investigating CND on ecosystem processes (Vanni et al., 2002). One principle of ES is that animals maintain elemental homeostasis of body tissue, so they will sequester the element that is most limiting in their environment and release the element that is in excess (Boersma & Elser, 2006). Another underlying principle that influences how CND mediates ecosystem processes is ES in relation to animal growth rates, body size, and diets. The largest supply of P in invertebrates is RNA, thus an increase in body size and growth would elevate demands for P, and consequently drive variation in C, N, and P ratios within the body (Growth Rate Hypothesis; Elser et al., 2003). The effects of nutrient ingestion, assimilation, egestion, and excretion by consumers is dictated by principles of ES and can greatly influence ecosystem nutrient cycling as consumers retain and remove C, N, and P from the environment. These effects in which consumers physically transform nutrients from one form to another can be considered direct effects (Vanni, 2002). Indirect effects can be classified as when consumers affect nutrient fluxes through alterations to their physical habitat or have impacts on their prey (Vanni, 2002).

In aquatic environments, the nutrients that consumers ingest are either assimilated into body tissue or released as excretion or egestion, and directly provide nutrients to primary producers and decomposers. Nutrients that are not assimilated into body tissue are released as egestion, whereas nutrients that have been assimilated are released in dissolved form as excretion. Excreted inorganic N and P (ammonia and phosphate) from consumers is soluble and therefore readily supplies autotrophs and heterotrophs with nutrients needed for growth and thus directly supports both ‘green food webs’ based on primary producers and ‘brown food webs’

based on decomposers (Vanni, 2002). Nutrients are bound in egesta, and while heterotrophic microbes can utilize this organic nutrient source, egested nutrients are not directly available to primary producers as they require nutrients in dissolved form (Cross et al., 2005; Parr et al., 2019). However, remineralization by microbes can make egested nutrients available for primary producers (Caron, 1994; Halvorson et al., 2019).

Much research has focused on the impacts of CND on ecosystem primary production through the supply of nutrients via excretion and egestion (Allgeier et al., 2017; Elser & Urabe, 1999; Kitchell et al., 1979; McIntyre et al., 2008; Vanni, 1996), and has shown that consumers can have substantial contributions to supporting ecosystem primary producers (Atkinson et al., 2017; Sharitt et al., 2021). The degree to which consumers influence primary production depends in part on the rates and ratios at which nutrients are supplied (Tilman et al., 1982). Because consumers maintain homeostasis of body nutrient ratios, ES predicts that consumers with a high body P content (low body N:P) need to sequester more P compared to consumers with lower body P content (high body N:P) in order to maintain homeostasis (Elser & Urabe, 1999; Vanni, 2002). Thus, a consumer with low body N:P will recycle less P and release nutrients at a higher N:P ratio. Therefore, consumer-mediated nutrient availability to primary producers is greatly dependent on the identity of the consumer.

While it is clear that CND influences green food webs, considerably less attention has been given to studying CND effects on brown food webs (Atkinson et al., 2017; Evans-White & Halvorson, 2017). Like green food webs, major processes of brown food webs are also limited by N and P such as microbial respiration, growth, and production of extracellular enzymes (Jaboil et al., 2018; Moore et al., 2004). However, there are also important differences between green and brown food webs where ES assumptions that apply to green food webs do not translate

to brown systems. For example, in brown food webs, light is not a direct resource utilized by heterotrophic organisms, organic carbon in the detrital pool is consumed several times creating a "microbial loop", and detritivores tend to have a have higher body C:P than consumers (Frost et al., 2005, 2006). These factors represent areas where CND may differ between green and brown systems and therefore it is important to consider CND effects on both green and brown processes within the same system.

In aquatic systems, heterotrophic microbes (especially bacteria and fungi) play important roles in organic matter processing and nutrient cycling by providing a flow of energy to higher trophic levels and thus form the base of many freshwater food webs. Microbes play an essential role in biogeochemical processes, especially in benthic habitats where microbial biomass is the greatest (Findlay et al., 2002). Furthermore, the uptake, storage, and transformation of nutrients by microbes in the benthos provides a critical flux of energy to stream food webs (Meyer, 1994). Heterotrophic microbes utilize organic matter as a source of C for energy and mineralize it to CO_2. The conversion and assimilation of dissolved organic matter (DOM) to fine particulate organic matter (FPOM) provides an important flux of C to the rest of the aquatic food web (Findlay, 2010). Additionally, the development of biofilms and associated microorganisms, such as bacteria, fungi and algae, contribute to the decomposition of allochthonous organic material (Allan et al., 2021). Previous studies have demonstrated that the composition of the microbial community influences its ability to breakdown leaf litter due to the mixture of labile, recalcitrant, and phenolic compounds in the litter (McNamara & Leff, 2004). Microbes excrete extracellular enzymes to aid in the breakdown of organic allochthonous matter. The enzymes of primary research interest are the ones involved in the degradation of cellulose and the mineralization of

organic C, N, and P (Sinsabaugh et al., 1991). Therefore, the activities of these enzymes can serve as a proxy for microbial nutrient cycling demand.

Microbes play a critical role in mediating biogeochemical processes, such as the nitrogen cycle, in which they transform organic and inorganic nitrogen compounds. Microbial transformations of nitrogen can be summarized as a cycle where atmospheric N_2 gas is fixed to ammonium (NH_4), an inorganic bioavailable form for organisms. Ammonification transforms dissolved organic N to ammonia (NH_3) where it is subsequently oxidized to nitrate (NO_3) via the process of nitrification. Then, through denitrification or anaerobic ammonium oxidation (anammox), nitrate or nitrite is converted back to nitrogen gas (N_2) and removed from the aquatic habitat to the atmosphere. Many nitrogen-transforming microbes mediate these processes and are classified based on which processes they are involved in (Kuypers et al., 2018). In all ecosystems, the removal of fixed N to inert N_2 represents an integral part of the nitrogen cycle. The most important factors that regulate N-removal by heterotrophic microbes include: an anaerobic environment, the availability of organic C compounds, and reactive N (Knowles, 1982). In aquatic environments, benthic substrate provides an ideal habitat with suitable chemical and physical properties for N-removal by heterotrophic microbes. Previous research has demonstrated that benthic burrowing organisms can enhance conditions needed for denitrification by chemically and physically altering the sediment (Anschutz et al., 2012; Hoellein et al., 2017; Holker et al., 2015; Turek & Hoellein, 2015).

Freshwater mussels (Bivalvia: Unionoida) are a guild of benthic, burrowing, filter-feeding bivalves that dominate benthic biomass in many freshwater systems where they live partially or completely burrowed in benthic sediments (Haag, 2012; Vaughn & Hakenkamp, 2001). Mussels filter-feed across trophic levels and take in bacteria, algae, detritus, zooplankton,

and dissolved organic matter from the water column and transfer these nutrients and energy to sediments through biodeposition of feces and pseudofeces (Black et al., 2017; Vaughn et al., 2008). Direct nutrient excretion from mussels provides inorganic nutrients to autotrophic and heterotrophic organisms and thus they regulate the aquatic environment through their bottom-up effects on nutrient cycling by acting as benthic-pelagic couplers (Atkinson et al., 2018; Atkinson & Vaughn, 2015). Additionally, burrowing behavior by mussels has indirect effects on N dynamics by stimulating denitrification via enhancing sediment respiration and creating redox conditions needed for denitrification (Atkinson & Forshay, 2022; Nickerson et al., 2019; Trentman et al., 2018). Furthermore, bioturbation of sediments increases sediment water and oxygen content and releases nutrients from the sediment to the water column which can stimulate primary production and alleviate nutrient limitation (Vaughn & Hakenkamp, 2001). As a result of these processes and the spatial heterogeneity of their aggregations (Atkinson et al., 2018) mussels exert heavy control over ecosystem structure and function via biogeochemical hotspots (Atkinson & Vaughn, 2015).

Despite the fact that freshwater mussels are classified in the same functional group (filter-feeding bivalves), species within the same system exhibit variation in nutrient excretion/egestion rates and stoichiometry (Atkinson et al., 2010; Atkinson et al., 2020; Spooner & Vaughn, 2008) as well as burrowing behavior (Allen & Vaughn, 2009; Schwalb & Pusch, 2007). These traits, which affect how an organism performs (e.g., survival, growth, and reproduction) and interacts with its environment, can be classified as functional traits (Violle et al., 2007). Differences in functional traits and effects has been linked to phylogeny across organisms (Floeter et al., 2018; González et al., 2018), including mussels (Atkinson et al., 2020) and is based on the hypothesis that evolutionary processes drive trait diversification and thus enhances the functional trait space

of a community (Srivastava et al., 2012). Because species' functional characteristics strongly influence ecosystem properties, through niche complimentary or synergistic interactions, species richness may enhance ecosystem function and stability (Loreau & de Mazancourt, 2013). Therefore, better understanding functional trait diversity is key to improving our ability to understand and predict the effects of diversity loss on ecosystem stability.

Despite recent increased attention to biodiversity-ecosystem function (BEF) relationships (Srivastava & Vellend, 2005), the connection between phylogenetic and functional diversity are not well understood. Previous BEF studies have aim to isolate the effects of species richness from those of other factors known to affect ecosystem processes. It is especially critical to investigate trait-environment relationships within freshwater systems where species diversity and abundance is decreasing at a rapid rate (Dudgeon et al., 2006). Freshwater mussels are among one of the most threatened faunal groups in North America with over 70% of species being classified as imperiled (Williams et al., 2008) as they are experiencing a global decline in both species richness and abundance (Dudgeon et al., 2006; Haag & Williams, 2014; Vaughn et al., 2004). This tremendous loss of biodiversity can negatively impact stream ecosystems in a multitude of ways.

The overarching goal of my research aims to examine BEF relationships by investigating how phylogenetically diverse mussel communities influence ecosystem functioning through direct and indirect effects. Specifically, my objectives are two-fold:

Objective 1: How does mussel community composition determine how mussel-mediated nutrient cycling directly influences brown and green food webs.

Nutrient release by mussels in the form of excretion and egestion can strongly influence autotrophic and heterotrophic microbes via bottom-up nutrient alleviation. Because most

ecosystems are limited by energy in the form of carbon (C), or the nutrients nitrogen (N) and phosphorous (P), we decided to focus on C, N, and P dynamics in our mesocosm experiment. Mussels ingest particulate organic matter from the water column and excrete soluble forms of N and P as ammonium (NH_4^+) and phosphate (PO_4^{3-}) and biodeposit C in the form of organic matter (OM) (Figure 1.1). Thus, they are able to recycle and relocate essential nutrients and energy in the water column from unavailable to available forms (organic to inorganic) for autotrophs and concentrate organic matter in the benthos for heterotrophs (Hopper et al., 2021; Vaughn & Hakenkamp, 2001). The flux of soluble N and P to primary producers is one mechanism known to influence nutrient dynamics in aquatic systems and enhance primary productivity (Elser & Urabe, 1999). To assess the potential effects of mussel excretion on primary producer-based green food webs, we measured ecosystem responses of gross primary productivity (GPP) and chlorophyll-a production on inorganic (benthic substrate) and organic (leaf litter) substrates. Then, to assess the effects of mussel egestion on decomposer-based brown food webs, we measured ecosystem responses of leaf litter decomposition rates and stoichiometric content, extracellular enzyme activity, ergosterol production as a function of fungal biomass, and O_2 uptake on leaves as an indicator of microbial respiration. Additionally, to investigate differences in CND of filter-feeders that occupy different stoichiometric niches (i.e., rates and ratios of nutrients released) we used three species of mussels that occupy distinct phylogenetic tribes (Lampsilini, Pleurobemini, and Quadrulini) and manipulated mussel community composition to create a gradient of species richness (zero to three). We predicted that mussel excretion and egestion would have positive effects on enhancing green and brown food web responses via bottom-up provisioning of N and P to autotrophs and C to heterotrophs. Furthermore, we predicted that the presence of mussels will elicit a positive response in both

brown and green food webs and increasing species richness will lead to greater ecosystem functioning (e.g., algal accrual, litter decomposition) as a result of greater phylogenetic and consequential trait diversity.

Objective 2: Experimentally assess how mussels physically (via bioturbation) and chemically (via excretion and egestion) indirectly influence sediment-water nutrient fluxes and sediment microbiome community composition.

Mussel excretion, specifically ammonium (NH_4^+), and egestion can stimulate the conversion of dissolved inorganic nitrogen to dinitrogen gas (N_2) via microbial-mediated biogeochemical pathways. The conversion to N_2 and its subsequent removal from the environment (hereafter N-removal) is performed by facultative anaerobic bacteria. When mussels excrete NH_4^+, there are multiple biogeochemical pathways that may occur depending on where the mussel is buried in the sediment. Mussel excreta and pseudofeces are released through the excurrent siphon, and feces is released through the supre-anal aperture, both of which are located near the posterior end of the animal (Haag, 2012). When mussels bury into sediment, their posterior end faces up towards the water column and depending on how deep the mussel is buried, it may stick out above the surface of the sediment or be partially or completely buried (Haag, 2012; Sansom et al., 2022). Thus, if a mussel is completely exposed, NH_4^+ will be excreted directly into the water column and will either be taken up by primary producers (Atkinson et al., 2013) or converted to nitrate (NO_3^-) via the aerobic pathway nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) (Figure 1.1, mussel B). If the mussel is partially buried and closer to the oxic/anoxic boundary layer, the excreted NH_4^+ can undergo a coupled nitrification-denitrification process where NH_4^+ is first nitrified ($NH_4^+ \rightarrow NO_3^-$) in the oxic layer, then denitrified ($NO_3^- \rightarrow$

N_2) in the anoxic layer. Finally, if the mussel is completely buried and very close to the anoxic sediment layer, a third transformation can take place called anaerobic ammonium oxidation (hereafter annamox). For anammox to occur, excreted NH_4^+ combines with NO_3^- and through a series of intermediate steps, forms N_2 gas without first being nitrified (Figure 1.1, mussel A). Additionally, mussel egestion may influence N-removal potentials by providing an energy source for heterotrophic microbes, as well as a substrate for denitrification to occur. OM biodeposition in feces and pseudofeces may foster the denitrification pathway by providing an energy substrate to microbes. Furthermore, decomposition of OM is a type of aerobic respiration where oxygen is consumed, thus potentially creating anoxic environments where denitrification and anammox can occur. All of these microbial-mediated biogeochemical processes rely on mussel-derived nutrient inputs (excreted NH_4^+ or OM biodeposits), thus we predicted that the presence of mussels will enhance sediment N-removal potentials by providing microbes with reactive N and labile C. However, the act of burrowing has the potential to increase oxygen penetration in the sediment and disrupt the redox gradient, therefore reducing N-removal potentials. We predicted that different communities of mussels will vary in N-removal potentials as a result of species-specific functional traits of stoichiometric nutrient excretion/egestion and burrowing behavior.

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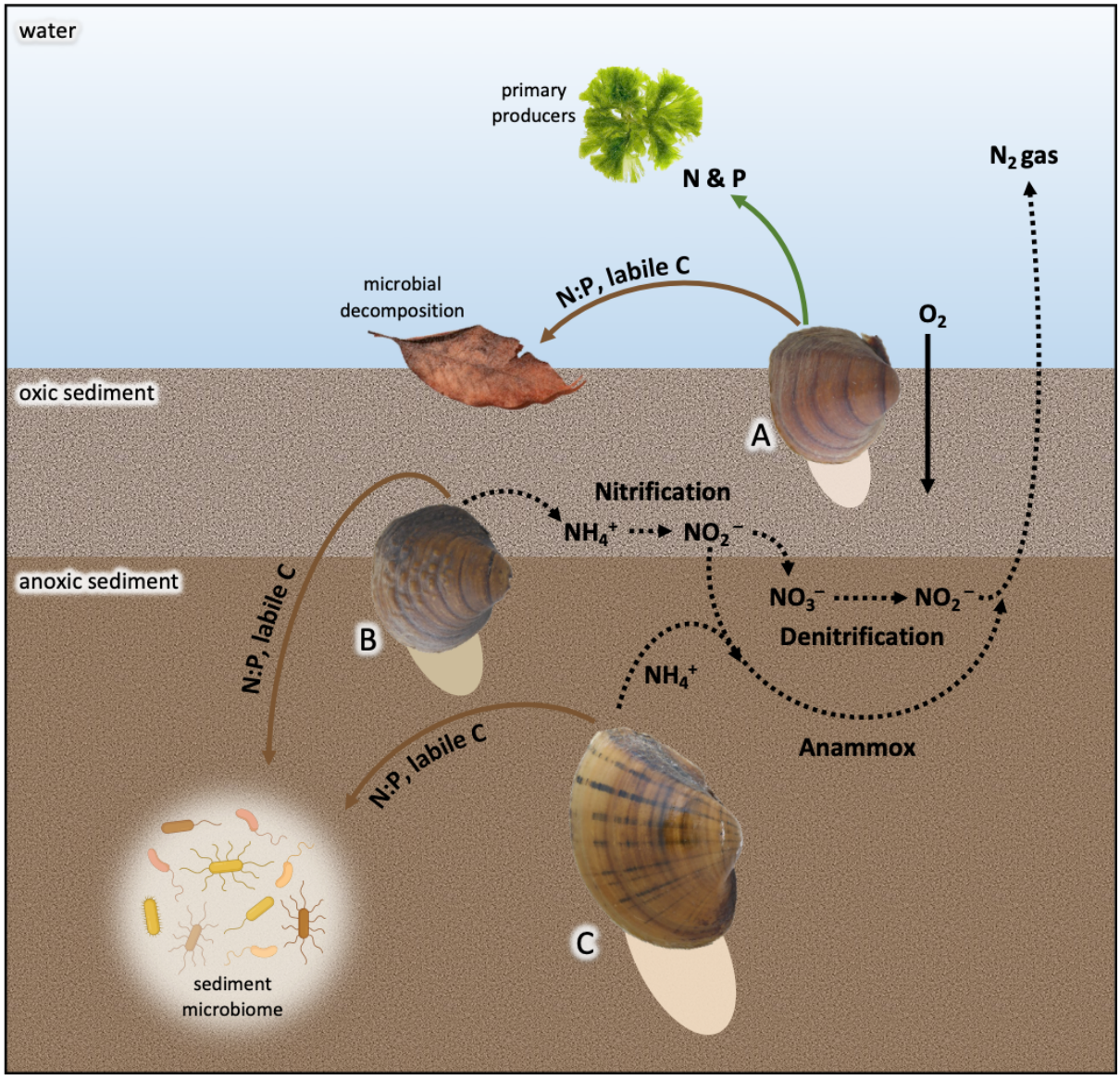
Figure 1.1. Conceptual basis for my thesis research depicting three phylogenetically distinct (i.e., occupy different tribes) species of freshwater mussels (A, B, and C), which vary in functional traits (e.g., nutrient stoichiometry and burrowing behavior), with theorized influences of how different functional traits effect pathways of green and brown food webs and sediment N-removal potentials. Solid green and brown lines represent energy flows via excreta and egesta in green and brown food webs respectively. Pathways of N-transformations (e.g., nitrification, denitrification, and anammox) are depicted with black dotted lines between oxic and anoxic zones in the sediment with O_2 penetration from burrowing behavior.



CHAPTER 2:

PHYLOGENETIC AND TAXONOMIC DIVERSITY OF FILTER-FEEDING BIVALVES IMPACTS GREEN AND BROWN FOOD WEBS

Key words:

freshwater mussels, nutrient excretion, decomposition, ergosterol

ABSTRACT

Freshwater mussels (Bivalvia: Unionidae) can dominate benthic biomass in aquatic systems as they often occur in dense aggregations that create biogeochemical hotspots that can control ecosystem structure and function through direct and indirect pathways. Mussel nutrient excretion can enhance benthic algal production (i.e., primary producer based 'green food webs') as well as stimulating microbial decomposers (i.e. detrital based 'brown food webs') via priming. Additionally, bioturbation of benthic sediment at the oxic-anoxic boundary layer influences nutrient dynamics and alters redox conditions by indirectly stimulating denitrification and microbial growth. However, despite functional similarities as infaunal filter-feeders, mussels exhibit variation in nutrient excretion and tissue stoichiometry due in part to their phylogenetic origin. Here, we conducted a six-week mesocosm experiment to evaluate how monoculture and polyculture treatments of three phylogenetically distant species of mussels influence components of green and brown food webs. Compared to controls, mussel treatments substantially increased algal production on benthic substrates and leaves. Further, species richness was positively correlated with benthic extracellular enzyme activity and negatively correlated with litter C:N composition. Our results suggest that mussels affect the functioning of green and brown food

webs through altering nutrient availability for both autotrophic and heterotrophic microbes. These effects are likely driven by phylogenetic constraints on tissue nutrient stoichiometry and consequential excretion stoichiometry which can have large scale functional effects on ecosystem processes.

INTRODUCTION

Across ecosystems, microbes (i.e. bacteria and fungi) are important agents of nutrient cycling (Gessner et al., 2010). In stream ecosystems, spatial hotspots of nutrient transformations are generally attributed to physical and microbial processes (Bernhardt et al., 2017; McClain et al., 2003; McIntyre et al., 2008). Microbes mediate nutrient processing and drive organic matter decomposition and nutrient flows to higher trophic levels (Meyer, 1994; Zeglin, 2015). However, within the past few decades, research has increasingly shown that animal consumers play a large role in driving nutrient dynamics, especially in freshwater systems (Atkinson et al., 2017; Hopper et al., 2018; Vanni, 1996). These consumer-driven nutrient dynamics (CND) are extensive and include direct mechanisms such as the physical transformation of nutrients from one form to another via nutrient excretion and egestion, as well as indirect effects that regulate ecosystem processes such as enhancing primary productivity and decomposition (Vanni, 2002).

Biogeochemical hotspots created by animals can fuel both ‘green food webs’ (i.e., primary producers) and ‘brown food webs’ (i.e., decomposers) from their excretion of nitrogen (N) and phosphorus (P). Previous work demonstrates that these green and brown food web effects are important pathways that maintain ecosystem stability and function (Mougi, 2020; Zou et al., 2016). Attention has focused on the cycling of N and P because they are the nutrients most likely to limit primary producers and perhaps heterotrophic microbes (Vanni, 2002). Direct excretion of N and P by animals affects the supply of bioavailable nutrients to producers and

may result in differences in nutrient limitation patterns (Allgeier et al., 2013; Atkinson et al., 2013).

Freshwater mussels (Bivalvia: Unionoida) are a guild of benthic, burrowing, filter-feeding bivalves that dominate benthic biomass in many freshwater systems (Haag, 2012; Vaughn & Hakenkamp, 2001) and regulate the aquatic environment through their bottom-up effects on nutrient cycling by acting as benthic-pelagic couplers (Atkinson et al., 2018; Atkinson & Vaughn, 2015). Mussels filter-feed across trophic levels and take in bacteria, algae, detritus, zooplankton, and dissolved organic matter (DOM) from the water column and transfer these nutrients, DOM, and energy to sediments through biodeposition of feces and pseudofeces (Black et al., 2017; Vaughn et al., 2008). Additionally, bioturbation of sediments through bivalve movements increases sediment water and oxygen content and releases nutrients from the sediment to the water column which can stimulate primary production and alleviate nutrient limitation (Vaughn & Hakenkamp, 2001). As a result of these processes and the spatial heterogeneity of their aggregations (Atkinson et al., 2018) mussels exert heavy control over ecosystem structure and function via biogeochemical hotspots (Atkinson & Vaughn, 2015).

Despite the fact that freshwater mussels are classified in a single functional group, different species within the same system exhibit variation in nutrient excretion rates and stoichiometry (Atkinson et al., 2010; Atkinson, van Ee, et al., 2020; Spooner & Vaughn, 2008). Differences in functional traits and effects has been linked to phylogeny across organisms (Floeter et al. 2018; Gonzalez et al. 2018), including mussels (Atkinson, van Ee, et al., 2020) and is based on the hypothesis that evolutionary processes drive trait diversification and thus enhances the functional trait space of a community (Srivastava et al., 2012). Through niche complimentary or synergistic interactions, species richness may enhance ecosystem function and

stability (Loreau & de Mazancourt, 2013). Therefore, better understanding functional trait diversity is key to improving our ability to understand and predict the effects of diversity loss on ecosystem stability. It is especially critical to investigate trait-environment relationships within freshwater systems where species diversity and abundance is decreasing at a rapid rate (Dudgeon et al., 2006) with freshwater mussels being one of the most threatened faunal groups. Over 70% of North American species are classified as imperiled (Williams et al., 2008) and they are experiencing a global decline in both species richness and abundance (Dudgeon et al., 2006; Haag & Williams, 2014; Vaughn et al., 2004). This tremendous loss of biodiversity can negatively impact stream ecosystems in a multitude of ways.

Despite recent increased attention to biodiversity-ecosystem function (BEF) relationships (Srivastava & Vellend, 2005), the congruence between phylogenetic and functional diversity are not well understood. Here, we use a mesocosm approach to examine BEF relationships to investigate how phylogenetically diverse mussel communities influence ecosystem functioning including 'green' and brown' food web responses. These ecosystem functions have a large impact on the flow of energy and nutrients in stream ecosystems, thus providing a framework to investigate the influence of mussel aggregations on large-scale ecosystem responses. We hypothesized that: 1) Based on previous findings, bottom-up provisioning of nitrogen and phosphorus via excretion and egestion will vary among mussel treatments; 2) compared to no mussel controls, the presence of mussels will elicit a positive response in both brown and green food webs, and 3) increased species richness will lead to greater ecosystem functioning (e.g., increased algal accrual, faster litter decomposition) as a result of greater phylogenetic and consequential trait diversity.

MATERIALS AND METHODS

Study Design

Study Organisms

We manipulated mussel diversity by creating treatments of species that varied in phylogenetic tribe. We collected mussels, sediment, and water from the Sipsey River, a fifth-order tributary of the Tombigbee River in Alabama. The Sipsey River is relatively unmodified by human disturbances and harbors diverse and abundant communities of freshwater mussels—making it an ideal study system (Haag & Warren, 2010). For these reasons, we utilized three abundant species in the Sipsey River: *Cyclonaias kieneriana* (Tribe Quadrulini)*, Fusconaia cerina* (Tribe Pleurobemini)*,* and *Lampsilis ornata* (Tribe Lampsilini). These three species vary in shell morphology, life-history traits, and tissue stoichiometry (Atkinson, van Ee, et al., 2020; Williams et al., 2008).

We manipulated mussel diversity by creating four replicates of eight treatments: (i) three single-species treatments (*L. ornata, C. kieneriana, F. cerina; hereafter* L, C, F), (ii) three two-species treatments (*L. ornata + C. kieneriana*, *L. ornata + F. cerina, C. kieneriana + F. cerina; hereafter* LC, LF, CF), (iii) one three-species treatment (*L. ornata + C. kieneriana + F. cerina; hereafter* LCF), and (iv) one control treatment (no mussels; hereafter NM) (Table 1). We implemented a substitutive design in which each treatment contained the same biomass of mussels (ANOVA $F_{6,21}$=0.87 p=0.53). At the start of the study, we assigned treatments to a mesocosm using a random number generator.

We collected 183 mussels November 20-22, 2019 from the Sipsey River and transported them back to the lab where they were held in Living Stream Systems® (Frigid Units Inc., Toledo, Ohio). Each mussel was measured, and standardized length dry mass regressions were

used to obtain weight (Atkinson, Parr, et al., 2020). We tagged each mussel with fly line each 15cm in length and attached an individual identification tag to the end. We randomly assigned individuals to each of the eight treatment groups (n=32). Mussels were held at 10° C in Living Stream Systems® until the beginning of the experiment and fed with cultured algae 3x weekly.

Mesocosm Design

We conducted a 42-day mesocosm study in the greenhouse on the main campus of the University of Alabama in Tuscaloosa, AL from January to March of 2020. We used 200 L recirculating stream mesocosms (81x51x48 cm) which consisted of two tanks, an open-ended plastic liner placed inside a fiberglass outer tank. The inner liner was placed atop bricks to allow water recirculation as in (Nickerson et al., 2019) and the bed of the liner was filled with a mix of pea gravel and sand from the Sipsey River and supplemented with purchased pea gravel (Vigoro® Pea Gravel Pebbles). We filled each liner to a depth of 35cm with water from the Sipsey River and used 47 w magnetic drive pumps (Dammer Mfg, Islandia NY) to recirculate the water at a rate of 2527 L h^{-1}. We placed each pump atop the pea gravel at one end of the mesocosm so that the orientation of the pump caused water to be drawn up from the space between the two tanks and flow directly over the pea gravel. We conducted weekly water changes (15%) in which we emptied 20L of water from each tank and replaced it with 20L of Sipsey water weekly for the duration of the experiment. In addition, each tank received 500mL of a concentrated mixed algal assemblage cultured from natural river water every week. We monitored water temperature in 12 mesocosms using temperature loggers that collected records every 60 minutes (Hobo U20L, Onset Corp, Bourne, MA).

Leaf Pack Assembly

To assess the effect of mussel communities on macrophyte litter decomposition, we assembled leaf litter bags with dried leaves of *Salix nigra* (Black willow), *Platanus occidentalis* (American sycamore), and *Liriodendron tulipifera* (Tulip poplar). These species are common riparian trees along the Sipsey River. Leaves that were freshly fallen and had no signs of herbivory were collected October-November 2019 and oven-dried at 40ºC for 24 hours. Dried leaves were weighed and placed in 20 x 15cm fiberglass mesh litter bags with 1 x 1.5mm size mesh. To facilitate a natural microbial community in the mesocosms, we assembled 32 mixed leaf litter bags containing approximately 1.5g of leaf tissue of each species and left them in the Sipsey River to incubate for 12 days. Five days before mussels were added to the mesocosms, we added one of these pre-conditioned mixed-species litter bags to each mesocosm. To assess litter decomposition rates, we assembled 288 single-species litter bags (96 bags of each species) each containing 3.0-3.2g of leaf tissue. Exact tissue weights and species were labeled on aluminum tags and attached to bags via zip-ties. Three bags of each leaf species were placed in each mesocosm and secured together and sampled at weeks one, three, and six. We allowed the mesocosms to acclimate for 5 days with leaf pack inoculators prior to the addition of the single-species leaf litter bags and mussels.

Study Organism Measurements

Excretion Trials: At the end of six weeks, we estimated nitrogen and phosphorus excretion for each community by randomly subsampling six mussels of each species and measuring their NH_4^+ (N) and Soluble Reactive Phosphorus (P) excretion rates. Using a toothbrush and scour pad, we gently scrubbed each mussel and placed the individuals in separate excretion chambers. Depending on mussel size, we filled each container with 300 or 500mL of

filtered mesocosm water (GF/F; 0.7 μm pore size; Millipore). We used three control containers which were incubated simultaneously without bivalves to control for biofilm uptake. After 1 hour, we gently removed each mussel, filtered the chamber water (GF/F; 0.7 μm pore size; Millipore), and stored 30mL of the sample at -20°C until analysis. Filters were retained to estimate biodeposition rates (mg DM^{-1} h^{-1}). We used a Seal AQ300 discrete analyzer (Seal Analytical, Mequon, Wisconsin, USA) to analyze Soluble Reactive Phosphorus using the colorimetric method (Murphy & Riley, 1962) and NH_4^+ using the phenol method for filtered excretion samples. Mass-specific excretion rates for each mussel (μmol NH_4^+ hr^{-1} g^{-1}) were calculated using the concentration of NH_4^+ in the excretion chamber (μg L^{-1}), the known volume of water in the chamber, and the amount of time the mussel incubated (1 hr) after correcting for the controls. Solid material collected on the filters was used to calculate mussel biodeposition rates. The filters were dried for 48h at 50°C, weighed on an analytical balance, then combusted at 500°C for 2h and weighed again to calculate ash-free dry mass AFDM and calculate total organic matter (OM) of the biodeposits.

We used mass-specific hourly rates of excretion and biodeposition to estimate the community-scale rates for each of our mesocosm treatments. Following the methods similar to Atkinson & Forshay (2022), we calculated areal excretion rates of N and P (μmol nutrient m^{-2} hr^{-1}) and biodeposition of C, N, and P (μmol nutrient m^{-2} hr^{-1}) by multiplying the species-specific population biomass (i.e. mesocosm) by the per capita excretion or biodeposition rate summed for across all species for each treatment.

Green Food Web

Benthic and Leaf Litter Algal Accrual

At the onset of the experiment, we placed four ceramic tiles (25.81 cm^2) in each mesocosm, three of which had silica discs (0.424 cm^2, Leco Corp., St. Joseph, MI) attached with waterproof epoxy. The discs served as a proxy for benthic algal accrual in a mesocosm as the surface area of the discs allowed algae to colonize in a similar fashion as on the pea gravel substrate. In order to simulate up-stream and down-stream effects, the tiles were placed equal distances apart lengthwise down the center of the mesocosm. A silica disc was removed at the end of two, four, and six weeks for determination of benthic chlorophyll-a (chl-a) concentration. We wrapped the discs individually in foil and immediately froze them in the dark at -20° C until analysis using methods similar to (Atkinson et al., 2021). We extracted the chl-a from each disc in a black-plastic film canister with 15mL acetone-bicarbonate extraction reagent (90 vol% acetone + 1 g $MgCO_3$) and incubated the discs for 18-hrs at -20 °C. After 24-hrs, we returned the samples to room temperature and analyzed the extraction solution for [chl-a] on a Genesys 10S UV/Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Benthic algal accrual (µg cm^{-2}) was estimated by dividing the concentration of chl-a (µg mL^{-1}) by the known surface area of the silica disc (0.424 cm^2).

After 8, 21, and 42 days we collected one leaf pack of each leaf species to assess algal biomass on leaf litter which was estimated using chlorophyll-a. We used a corer (2.01 cm^2) to cut two leaf discs (one from each leaf) which were stored in 15mL polypropylene conical tubes (in the dark) and frozen at -20°C until analysis. Following similar methods to Kuehn et al., (2014), chlorophyll-a was extracted using the hot ethanol technique, where leaf discs were submerged in

90% ethanol (80°C for 5 min), steeped overnight in the dark (4°C), then quantified using high-performance liquid chromatography (HPLC).

Gross Ecosystem Production

At the end of 2, 4, and 6 weeks we also collected silica discs to estimate mesocosm metabolism. Following the methods of Tank et al. (2006), we gently removed the silica disc from the ceramic tile and placed it into a 50-ml centrifuge tube filled completely with deionized water and measured the initial temperature and dissolved oxygen (DO) concentrations. We then placed the tubes in a light incubator for two hours. Following incubation, temperature and DO were measured again and tubes were incubated for another two hours in the dark. We used the change in DO between the light and dark incubations to estimate gross primary production (GPP), ecosystem respiration (ER), and net ecosystem production (NEP) on the benthic substrate.

Brown Food Web

Leaf Decomposition and C:N

We removed leaf packs at the end of one, three, and six weeks and subsampled each pack for O_2 uptake, chl-a accrual, enzyme activity, and ergosterol production. We then dried all bags at 50°C for 72 hours. Once dry, we weighed each bag to determine the percent mass loss. As litter bags started with a slightly different initial mass, all initial masses were standardized to 100%, and values for decomposition were reported as the percent of mass loss over time. In order to account for mass-loss due to handling, we assembled 15 additional leaf litter bags. We submerged these control bags in stream water, dried at 50°C for 72 hr, and weighed to calculate tissue loss due to handling.

We analyzed the leaf-litter for C:N by homogenizing litter using a Wiley® Mini-Mill Grinder (Thomas Scientific, Swedesboro, NJ). We then took a subsample of ground tissue (2.5-

3.0 mg), and determined C:N using a Carlo Erba CHNS-O EA1108-Elemental Analyzer (Isomass Scientific Inc., Calgary, Alberta, Canada).

Enzyme Activity

Heterotrophic microbes degrade complex organic compounds by secreting extracellular enzymes into the environment. Therefore, the activities of these enzymes serve as a proxy for microbial nutrient cycling demand (Jackson et al., 2013). Here, we assayed three extracellular enzymes, β-glucosidase, β-N-acetylglucoaminidase (hereafter NAGase), and phosphatase as indicators of C, N, and P acquisition efforts by decomposer microorganisms. At the end of six weeks, tulip poplar leaves were cut using a corer (2.01 cm^2) and four leaf discs were subsampled (one for each of the three enzymes, and one for a control). Enzyme assays were performed colorimetrically using p-nitrophenyl linked substrates following the procedures of Jackson et al., (2013). Briefly, each leaf disc was patted dry and added to 500 µl of the appropriate substrate for 0.5-2 hr. Following incubation, 150 µl of the reaction mixture was transferred to a 96-well microplate, where it received 10 µl of 1M NaOH and 140 µl water. Absorbance of the resulting solution at 410 nm was measured on a BioTek Synergy 2 plate reader.

Leaf Disc Respiration

We used respiration as an indicator of microbial respiration on leaves and measured as oxygen uptake on leaf discs pre- and post-incubation following the methods of Rugenski et al., (2012). Respiration measurements were taken at the end of one, three, and six weeks from one leaf litter bag of Tulip poplar leaves from each mesocosm. Leaves were cut using a corer (2.01 cm^2) and five leaf discs (1-3 from each leaf) were placed in a 60mL centrifuge tube containing filtered mesocosm water (GF/F; 0.7 µm pore size; Millipore) and incubated for 90 min in the dark. There were three controls for each sampling event containing only stream water. Dissolved

oxygen readings were recorded at 0 min and 90 min using a YSI ProODO dissolved oxygen meter (Yellow Springs Instrument Co., Inc., Yellow Springs, OH, U.S.A.). We calculated respiration rates from change in dissolved oxygen concentrations between samples and controls, and final rates were calculated based on leaf disc dry mass (DM) (mg O_2 DM^{-1} h^{-1}). Following respiration measurements, leaves were frozen and preserved for ergosterol measurements.

Fungal Biomass

In order to assess the quantitative importance of fungi in leaf litter decomposition, we used ergosterol as a measure of fungal biomass. Determination of ergosterol was achieved by following the methods of Atkinson et al., (2021) and Gessner, (2005). Weighed leaf discs were ground into powder using a Wiley Mill (Thomas Scientific, Swedesboro, NJ) and 100mg of leaf powder from each leaf pack was taken as a subsample for ergosterol determination. We extracted ergosterol following lipid extraction in alkaline methanol and purification of the extract by means of solid-phase extraction (SPE). We then used high-performance liquid chromatography to quantify ergosterol levels (Gessner, 2005). We converted ergosterol concentrations to fungal C assuming 5 μg ergosterol/mg fungal dry mass and 43% fungal C (Findlay et al., 2002; Kuehn et al., 2014).

Data Analyses

To test the effect of mussel species community composition on ecosystem responses, we used two-way and three-way analysis of variance (ANOVA) tests followed by a post hoc Tukey test, with treatments, leaf litter species, and sampling day as factors. All statistical analyses were performed using the 'stats' package in R (R Development Core Team 2015) and data were log transformed as necessary to meet assumptions of normality and homoscedasticity.

We used Hedge's *g* as a measure of effect size to directly compare the effects of mussel treatments on ecosystem functions of differing units. To account for time, we calculated all effect sizes using measurements taken at the end of the experiment (6 weeks) and used tulip poplar leaves to compare all leaf-related responses. We calculated Hedge's *g* values using the R package *effsize* (Torchiano, 2020), where the difference between the mean treatment effect and mean control is divided by the pooled weighted standard deviation of the two treatments (Hedges, 1981). A large effect size is considered to be $-0.8 < g > 0.8$, a medium effect size is $-0.5 < g > 0.5$, and a small effect size is $-0.2 < g > 0.2$. Hedge's g in particular has been found to outperform other effect size analyses (such as Cohen's d) when dealing with small sample sizes (Grissom & Kim, 2005). In addition, Hedge's g uses pooled weighted standard deviations—compared to pooled standard deviations–which allowed us to compare treatment effects with different sample sizes (Durlak, 2009).

RESULTS

Mussel Nutrient Inputs

Mussel Excretion

There were significant differences in mass-specific NH_4^+ excretion rates among species (ANOVA $F_{2,15}=4.69$, $p< 0.05$; Table 2.2, Figure 2.1A), with *L. ornata* having higher mass-specific NH_4^+ excretion rates than *F. cerina* (Tukey HSD, $p<0.05$). When scaled to the community level, there were significant differences in NH_4^+ areal excretion rates among treatments (ANOVA $F_{6,21}=215.36$, $p< 0.0001$; Table 2.3). The C treatment had higher NH_4^+ areal excretion rates compared to all other treatments (Tukey HSD, $p<0.001$) followed by the CF treatment (Tukey HSD, $p<0.001$). The LC and LCF treatments did not differ in NH_4^+ areal excretion (Tukey HSD, $p=0.50$), but were greater than the F, LF, and L treatments (Tukey HSD,

$p<0.0001$). The LF treatment did not differ from the F or L treatment (Tukey HSD, $p=0.09$), but the F treatment had greater areal excretion than the L treatment (Tukey HSD, $p<0.05$).

There were no differences in mass-specific PO_4^{3-} excretion rates among species (ANOVA $F_{2,15}=0.02$, $p=0.98$; Table 2.2, Figure 2.1B). However, when scaled to the community level, there were significant differences in areal excretion rates among treatments (ANOVA $F_{6,21}=40.04$, $p< 0.0001$; Table 2.3). Generally, treatments containing *Cyclonaias* had higher areal PO_4^{3-} excretion and treatments containing *Lampsilis* had lower areal PO_4^{3-} areal excretion. The C and CF treatments did not differ between each other (Tukey HSD, $p=0.57$), but were higher than all other treatments (Tukey HSD, $p<0.0001$). There was no difference in areal excretion rates between the LC and LCF treatments (Tukey HSD, $p=0.96$), nor was there a difference between the LCF, F, and LF treatments (Tukey HSD, $p=0.20$). The LC treatment had greater areal excretion than F, LF, and L treatments (Tukey HSD, $p<0.05$).

As a result of the variation in elemental excretion across species (Table 2.2, Figure 2.1A-B), there were differences in areal N:P molar excretion among treatments (ANOVA $F_{6,21}=11.09$, $p<0.0001$; Table 2.3, Figure 2.3A) with the L treatment having higher N:P than all other treatments (Tukey HSD, $p<0.0001$).

Mussel Egestion

Mass-specific elemental biodeposition rates differed significantly among mussel species for nitrogen (ANOVA $F_{2,15}=4.28$, $p<0.05$; Table 2.2, Figure 2.2B) and phosphorus (ANOVA $F_{2,15}=7.72$, $p<0.01$; Table 2.2, Figure 2.2C), but not for carbon (ANOVA $F_{2,15}=3.32$, $p=0.06$; Table 2.2, Figure 2.2A). *F. cerina* deposited higher quantities of nitrogen compared to *L. ornata* (Tukey HSD, $p<0.05$) and both *F. cerina* and *C. kieneriana* deposited higher quantities of phosphorus compared to *L. ornata* (Tukey HSD, $p<0.05$). There were also differences in total

biodeposition rates of nutrients and carbon across species (ANOVA $F_{2,15}$=3.61, p=0.05; Figure 2.2D) with *F. cerina* having higher biodeposition rates compared to *L. ornata* (Tukey HSD, p<0.05).

As a result of these species-specific variation in biodeposition content, when scaled to the community level, there were significant differences in stoichiometric ratios of C:N (ANOVA $F_{6,21}$=9.32, p<0.001; Table 2.3, Figure 2.3B), N:P (ANOVA $F_{6,21}$=4.18, p<0.01; Table 2.3, Figure 2.3C), and C:P (ANOVA $F_{6,21}$=4.35, p<0.01; Table 2.3, Figure 2.3D) among treatments. Generally, treatments containing *Lampsilis* and or *Fusconaia* species had greater C:N, N:P, and C:P stoichiometric biodeposition compared to other treatments.

Green Food web

Benthic algal biomass accrual

Chlorophyll biomass production on the silica discs increased over the course of the experiment, but did not vary across treatments (ANOVA $F_{7,88}$=1.57 p=0.15; Figure 2.4B). Sampling week also had a marginal effect on chl-a biomass accrual (ANOVA $F_{2,93}$=2.79, p=0.07).

Ecosystem Productivity (GPP)

There were no significant mussel treatment effects, however there was significantly different gross primary productivity between sampling weeks (ANOVA $F_{2,93}$=28.98 p<0.0001; Figure 2.4A). GPP was greatest at the end of the study (Tukey HSD, p<0.0001), and increased significantly from weeks two to four (Tukey HSD, p<0.0001), and from weeks four to six (Tukey HSD, p<0.05).

Leaf litter algal accrual

Chl-a production on leaf litter was mainly driven by the identity of leaf species (ANOVA $F_{2,279}$=2.77 p=0.06; Figure 2.4C) and sampling week (ANOVA $F_{2,279}$=10.29 p<0.0001), as well as a significant interaction between the two factors (ANOVA $F_{4,279}$=5.53 p<0.0001). After one week, willow leaves generally had greater chl-a production than sycamore leaves (Tukey HSD, p=0.06). After three weeks, both tulip poplar and willow leaves had significantly greater chl-a production than sycamore leaves (Tukey HSD, p<0.05). Finally, after six weeks tulip poplar leaves had the greatest chl-a production, followed by willow leaves, then sycamore leaves (Tukey HSD, p<0.05).

On tulip poplar leaves specifically, sampling week had a significant effect on chl-a production (ANOVA $F_{2,72}$=6.46 p<0.01) with week six accruing the greatest algal biomass compared to weeks one and three (Tukey HSD p<0.0001). We did not observe a significant effect of mussel community composition on chl-a production (ANOVA $F_{7,72}$=0.02 p=1.00), however post hoc pairwise comparisons revealed that compared to the no mussel control, the L and LC treatments had greater chl-a production after six weeks (p<0.05; Figure 2.4C).

On sycamore leaves, sampling week had a significant effect on chl-a production (ANOVA $F_{2,72}$=7.56 p<0.001) with week six accruing the greatest algal biomass compared to weeks one and three (Tukey HSD p<0.0001). We did not observe a significant effect of mussel community composition on chl-a production (ANOVA $F_{7,72}$=0.02 p=1.00), however post hoc pairwise comparisons revealed that the most species-rich treatment (LCF) had greater chl-a production than the F and LC treatments after six weeks (p<0.05; Figure 2.4C).

On willow leaves, we observed a significant interaction between mussel treatment and week (ANOVA $F_{14,72}$=3.02 p<0.01) as well as a significant effect of sampling week (ANOVA

$F_{2,72}$=29.58 p<0.0001) with week six accruing the greatest algal biomass compared to weeks one and three (Tukey HSD p<0.0001). After six weeks, the single-species C treatment had greater algal accrual compared to all other treatments (Tukey HSD p<0.001; Figure 2.4C).

Brown Food web

Leaf decomposition

Mussel treatments did not have a significant effect on mass loss of leaf litter (ANOVA $F_{7,288}$=1.06 p=0.34), but we observed a significant effect of leaf species identity (ANOVA $F_{2,288}$=30.69 p<0.0001; Figure 2.5), sampling week (ANOVA $F_{3,288}$=31.12 p<0.0001), as well as a significant interaction between leaf species and sampling week (ANOVA $F_{6,288}$=17.36 p<0.0001). Overall, leaf species identity drove mass loss with tulip poplar leaves having the greatest mass loss (Tukey HSD, p<0.0001), followed by sycamore leaves (Tukey HSD, p<0.0001), and willow leaves (Tukey HSD, p<0.0001). After week one, tulip poplar leaves had greater mass loss compared to both sycamore (Tukey HSD, p<0.0001) and willow leaves (Tukey HSD, p<0.0001). In addition, sycamore leaves lost more mass than willow leaves (Tukey HSD, p=0.01) after one week. After three weeks, tulip poplar leaves had the greatest mass loss compared to both sycamore (Tukey HSD, p<0.0001) and willow leaves (Tukey HSD, p<0.0001), and sycamore leaves lost more mass than willow leaves (Tukey HSD, p<0.0001). At the end of six weeks, we observed the same trend of tulip poplar leaves losing the greatest percent mass compared to the other two species (Tukey HSD, p<0.0001) as well as sycamore losing more than willow (Tukey HSD, p<0.0001), and willow losing the least compared to the other two species (Tukey HSD, p<0.0001).

On tulip poplar leaves specifically, we observed a significant effect of sampling week (ANOVA $F_{3,96}$=70.36 p<0.0001) on mass loss, but no mussel effect (ANOVA $F_{7,96}$=0.85

p=0.55). However, post hoc pairwise comparisons revealed that compared to the no mussel control, the C, L, and LF treatments had greater mass loss after six weeks ($p<0.05$; Figure 5). On sycamore leaves, we observed a significant effect of sampling week (ANOVA $F_{3,96}=50.89$ $p<0.0001$) as well as a significant interaction between treatment and week (ANOVA $F_{21,96}=1.95$ $p<0.05$; Figure 2.5). After three weeks, the LC treatment had lost more mass compared to all other treatments except the C treatment ($p<0.01$) and after six weeks, the L treatment had lost more mass than the F treatment ($p<0.05$). On willow leaves, we observed a significant effect of sampling week (ANOVA $F_{3,96}=33.83$ $p<0.0001$) on mass loss, but no treatment effect (ANOVA $F_{7,96}=0.20$ $p=0.99$). However, post hoc pairwise comparisons revealed that after six weeks, the C and L treatments had greater mass loss compared to the no mussel control ($p<0.05$; Figure 2.5) and the L treatment had lost more mass than LC treatment ($p<0.05$).

Ergosterol production

Ergosterol production varied across treatments (ANOVA $F_{7,216}=2.48$, $p<0.05$; Figure 2.6) and leaf species (ANOVA $F_{2,216}=3.71$ $p<0.05$; Figure 2.6), but not over time (ANOVA $F_{2,216}=1.99$ $p=0.14$). Overall, tulip poplar leaves had greater ergosterol biomass compared to both willow and sycamore leaves over the course of the entire experiment (Tukey HSD $p<0.0001$). Differences between mussel treatments were not detected with a post hoc Tukey test, but we noted a trend across tulip poplar and willow leaves that the no mussel control had lower mean ergosterol production compared to all other treatments.

Leaf C:N

We observed differences in stoichiometric C:N composition of leaves among treatments (ANOVA $F_{7,216}=3.21$ $p<0.01$; Figure 2.7) as well as a significant interaction between leaf species and sampling week (ANOVA $F_{4,216}=2.93$ $p<0.05$; Figure 2.8). On sycamore leaves, the LC

treatment had significantly lower C:N content than the no mussel control as well as the F and CF treatments (Tukey HSD, $p<0.05$). On tulip poplar leaves, the L, C, LC, and LCF treatments all had significantly lower C:N content compared to the no mussel control (Tukey HSD, $p<0.05$). After one, three, and six weeks, tulip poplar leaves had consistently lower C:N content compared to both sycamore and willow leaves (Tukey HSD, $p<0.001$; Figure 2.8). Only after three and six weeks did willow leaves have lower C:N content than sycamore leaves (Tukey HSD, $p<0.01$).

Leaf Respiration and Enzyme Activity

Respiration rates did not vary among treatments (ANOVA $F_{7,72}=0.45$ $p=0.86$; Figure 2.9) or sampling week (ANOVA $F_{2,72}=0.93$ $p=0.40$; Figure 2.9). Enzyme activities did not differ among treatments for β-glucosidase (ANOVA $F_{7,24}=0.56$ $p=0.78$; Figure 2.10), NAGase (ANOVA $F_{7,24}=0.50$ $p=0.83$; Figure 2.10), or Phosphatase (ANOVA $F_{7,24}=1.06$ $p=0.42$; Figure 2.10).

Effect Size Analysis

Overall, we observed that the presence of mussels had a strong positive effect on two components of the green food web by enhancing algal accrual on both organic and inorganic substrate (Figure 2.11). However, contrary to our predictions, we observed a mixture of positive and negative mussel effects on ecosystem productivity (GPP). We also saw mixed effects on brown food web components of litter decomposition, ergosterol production, and leaf respiration compared to the no mussel control. Mussels did however, have a large effect on decreasing leaf litter C:N content and generally decreasing NAGase production. Furthermore, for the most part the presence of mussels had a small to medium effect on increasing β-glucosidase and Phosphatase activity. Even though we observed large variation in and among treatments, when

taken as a whole, we show that the presence and species composition of mussels alters the magnitude and direction of ecosystem functions of green and brown food webs.

DISCUSSION

Our results show that the presence of freshwater mussels and community composition have effects on multiple components of brown and green food webs, which corroborates findings from previous research that filter-feeding consumers have differential effects on ecosystem functions (Atkinson et al., 2021). Despite weak or no treatment effects on many of the functional responses, in general, the presence of mussels enhanced green and brown food webs. In particular, mussels had effects on green food web components such as increasing algal accrual on organic and inorganic substrate possibly through bottom-up nutrient alleviation in the form of mussel waste products (excretion and egestion) which stimulate productivity of autotrophic microbes (Atkinson et al., 2013). Additionally, we saw trends that mussels augmented components of the brown food web. For example, on tulip poplar leaves, mussel treatments that had greater species richness also tended to have higher enzyme production and higher ergosterol production. We also observed significant differences among treatments in stoichiometric C:N composition of leaves, suggesting that mussels play a key role in brown food webs based on heterotrophic microbes via microbial priming (Halvorson, Francoeur, et al., 2019).

Mussel bottom-up provisioning

Previous studies have shown that mussel-mediated nutrient release (via excretion and biodeposition) can stimulate benthic productivity, alleviate nutrient limitation, enhance decomposition, and drive biogeochemical nutrient cycles (Atkinson et al., 2018, 2021; Spooner & Vaughn, 2012; Vaughn et al., 2007). This bottom-up provisioning of nutrients provides a flow of energy to autotrophic and heterotrophic microbes that cascades up trophic food webs. In our

study, we demonstrated that mussels augment ecosystem functioning via bottom-up nutrient release, and observed significant differences in mass-specific excretion and biodeposition rates and ratios among species. Moreover, we show that when scaled to the community level, variation in species-specific traits results in differences in areal nutrient fluxes across treatments. Previous studies have shown mussels' ability to regulate energy flow becomes more pronounced in systems where they dominate benthic biomass (Vaughn et al., 2008) and are thus able to alter nutrient dynamics and ecosystem functioning at a large scale (Atkinson & Forshay, 2022). Taken together, our results highlight the importance of how mussel species diversity and community composition affect the flow of energy through bottom-up nutrient provisioning.

Green food web impacts

As anticipated, ecosystem productivity and algal accrual on benthic substrate and leaf litter increased over the course of our six-week experiment. Despite no significant treatment effects on ecosystem productivity and benthic algal accrual, we observed trends that the presence of mussels enhanced these components of the green food web, likely by the addition of excretion and biodeposits which are nutrient rich in N and P and a labile source of C (Christian et al., 2008; Hopper et al., 2021; Howard & Cuffey, 2006). In particular, the no mussel control consistently produced less chl-a over the course of the experiment compared to the mussel treatments, suggesting mussels have an influence on stimulating primary productivity via bottom-up nutrient excretion. We observed similar trends with chl-a production on tulip poplar leaf litter, with the no mussel control producing significantly less algal biomass compared to two of our mussel treatments (Figure 4C). Our results are corroborated by previous research that also found higher benthic algal biomass and increased primary production with the presence of mussels (Atkinson et al., 2018; Howard & Cuffey, 2006; Spooner & Vaughn, 2006). Overall, our

results support our predictions that mussels can enhance the growth of primary producers by supplying nutrients necessary for growth via excretion and biodeposition.

Brown food web impacts

Our experimental results support an effect of mussel CND on brown food web responses and corroborates previous research findings that suggest mussels mediated priming effects on decomposition (Atkinson et al., 2021). Compared to the no mussel control, we observed a decrease in litter C:N and an increase in decomposition rates, ergosterol production, and leaf litter enzyme activity in the mussel treatments, which suggests a mussel effect on brown food web dynamics. Heterotrophic microbes synthesize and excrete degrative enzymes to acquire C, N, and P from leaf litter (Sinsabaugh et al., 1991) and thus drive decomposition and alter the nutrient ratios of the litter. These microbes play key roles in aquatic systems but rely on labile C for growth and enzyme production. Previous studies (Nickerson et al., 2021) have shown that freshwater mussels may contribute to a priming effect by providing labile C to heterotrophic microbes in the form of biodeposition. In our study we observed similar results of increased microbial growth on tulip poplar leaves measured by increased levels of ergosterol in mussel treatments compared to the no mussel control. Additionally, we saw a trend that mussel treatments generally had elevated enzyme activities compared to the controls, suggesting that heterotrophic microbes may have utilized labile C from mussel biodeposits for growth and enzyme production. Furthermore, we observed a mussel effect on tulip poplar decomposition rates. Over the course of the experiment, the control litter packs consistently lost less mass than the mussel treatments (Figure 5), and at the end of six weeks the C, L, and LF treatments lost more mass compared to the controls. We also observed significant differences in stoichiometric C:N composition of tulip poplar leaves across treatments, with the L, C, LC, and LCF treatments

having significantly lower C:N content compared to the no mussel control. Taken together, our results suggest that mussels affected leaf litter decomposition and subsequent stoichiometric content by enhancing fungal growth and enzyme activity.

Interaction Effects

Even though mussels are classified in the same functional guild as benthic filter-feeders, they typically occur in diverse, multispecies aggregations (Haag, 2012; Vaughn, 1997) and have evolved to vary in species-level functional traits (Atkinson, van Ee, et al., 2020). Therefore, mussel community structure and underlying species interactions are important to consider when measuring ecosystem functions. In our study, we observed significant differences in mass-specific excretion and biodeposition rates among species which resulted in significant differences in nutrient fluxes across treatments when scaled to the community level. For example, at the mass-specific level, *Lampsilis* excreted significantly greater N and biodeposited significantly less N compared to *Fusconaia.* When we scaled these nutrient flux rates to the community level, we saw that the single *Lampsilis* treatment had significantly greater N:P areal biodeposition compared to the single *Fusconaia* treatment, however when these two species are in the same treatment (LF), the N:P areal biodeposition did not differ significantly from either of the single L or F treatments. This potential 'counterbalancing effect' may have contributed to the weak or no treatment effects that we observed on various ecosystem responses. Beyond the results of our experiment, this functional trait complementarity effect has implications for natural systems where many species of freshwater mussels co-occur. Thus, through niche complementarity or synergistic interactions, the overall contribution to ecosystem functions may be enhanced as a result of species-specific functional traits. Results from our study and many others (Allen et al., 2012; Atkinson, van Ee, et al., 2020; Nickerson et al., 2021; Spooner &

Vaughn, 2008; Vaughn et al., 2007) show that mussel nutrient excretion and egestion is species-specific and that mussel community composition plays a role on the direction and magnitude of ecosystem functions. Therefore, the loss of certain species, especially native species, within the mussel community may affect ecosystem functioning via nutrient fluxes and stoichiometry (Benelli et al., 2019).

Conclusions

Within our controlled mesocosm design, we attempted to mimic the natural conditions in which mussels live. Though, it is important to recognize that our mesocosm design differs greatly in a number of ways compared to the natural environment. Excretion and biodeposition rates of mussels vary widely and previous studies have documented rates that are similar to, less than, and greater than rates that we measured in our study (Atkinson et al., 2021; Atkinson, van Ee, et al., 2020; Christian et al., 2008; Nickerson et al., 2019). Some of this variation can be attributed to temperature, seston quantity and quality, and species identity. Freshwater mussels are powerful filter feeders, with adult mussels capable of turning over water at a rate of approximately 0.5 to 1 L/h (Vaughn et al., 2008). Given that mussels are capable of filtering large volumes of water, the food quantity and quality they are filtering from the water column has a direct impact on their metabolism and thus nutrient release (both excretion and egestion). Previous studies using freshwater mussels in artificial mesocosms found that captive animals are likely to experience dysbiosis and a reduction in metabolic and physiological condition (Allison Aceves et al., 2020). In addition to potentially low quality and quantity of added seston, the growth of algal biofilms in the mesocosms may have affected the brown food web responses by accumulating on decomposing material and suppressing the accumulation of fungal biomass (Halvorson, Barry, et al., 2019). While we show that excreted and egested nutrients enhance both

green and brown food webs, this leads to opposing effects on litter resource quality; nutrient additions stimulate primary producers but can also stimulate decomposition rates which reduce litter quality (Rosemond et al., 2015). However, the ability of increased nutrients (N and P) to fuel an increase in algal carbon was observed over long periods of time (2-5yrs). Thus, this may only be weakly contributing to the contrasting effects we observed in our study.

Our study is among the first to examine CND of multiple species composition using multiple species of natural leaf litter that vary in recalcitrance, while linking both brown and green food webs in the same study system. Our results, particularly the effect size analysis, highlights the importance of measuring multiple functional metrics across a gradient of diversity in ecologically similar consumers. While we did not observe many treatment effects in our mesocosm study, we did observe many positive trends that the presence of mussels augmented components of both green and brown food webs. Additionally, our effect size analysis emphasizes that the mussel community composition has variable effects on the direction and magnitude of different ecosystem functions. Mussels occur in dense, species-rich aggregations in natural systems, with species having variable functional roles. We captured and quantified this variability by implementing an additive experimental design (Wright et al., 2021) in order to elucidate phylogenetic contributions of freshwater mussels to large scale ecosystem functioning. As mussels are experiencing a global decline in both species richness and abundance (Dudgeon et al., 2006; Haag & Williams, 2014; Vaughn et al., 2004), future studies should address how these declines as well as shifts in community dominance (specifically a shift from native to invasive species) alter CND on ecosystem function.

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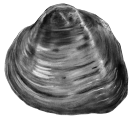
Table 2.1 Treatment code abbreviations.

Treatment Code	Species Composition
L	*Lampsilis ornata*
C	*Cyclonaias kieneriana*
F	*Fusconaia cerina*
LC	*Lampsilis ornata* + *Cyclonaias kieneriana*
LF	*Lampsilis ornata* + *Fusconaia cerina*
CF	*Cyclonaias kieneriana* + *Fusconaia cerina*
LCF	*Lampsilis ornata* + *Cyclonaias kieneriana* + *Fusconaia cerina*
NM	No mussel control

Lampsilis ornata
Tribe: Lampsilini

Cyclonaias kieneriana
Tribe: Quadrulini



Fusconaia cerina
Tribe: Pleurobemini

Table 2.2 Mass-specific excretion and biodeposition rates. Values are given as means (±SE).

		Nutrient Flux (μmol g^{-1} h^{-1})				
Species	**N**	**N Excretion**	**P Excretion**	**C Biodeposition**	**N Biodeposition**	**P Biodeposition**
Cyclonaias kieneriana	6	1.58 (0.15)	0.08 (0.01)	0.97 (0.27)	0.14 (0.03)	0.12 (0.03)
Fusconaia cerina	6	1.35 (0.17)	0.08 (0.01)	1.30 (0.37)	0.17 (0.04)	0.11 (0.02)
Lampsilis ornata	6	2.24 (0.29)	0.07 (0.01)	0.34 (0.05)	0.05 (0.01)	0.03 (0.00)

Table 2.3 Areal excretion and biodeposit nutrient fluxes scaled to the community level. Values are given as means (±SE).

		Nutrient Flux (μmol m^{-2} h^{-1})				
Treatment	**N**	**N Excretion**	**P Excretion**	**C Biodeposition**	**N Biodeposition**	**P Biodeposition**
C	4	116.97 (3.87)	235.40 (28.63)	13.92 (0.51)	2.00 (0.07)	1.83 (0.06)
CF	4	84.37 (4.59)	198.91 (16.10)	15.65 (0.53)	2.21 (0.08)	1.84 (0.07)
F	4	24.26 (0.93)	23.08 (6.86)	10.35 (1.43)	1.44 (0.17)	1.16 (0.06)
L	4	11.74 (0.63)	2.00 (0.41)	6.76 (0.63)	0.89 (0.07)	0.45 (0.04)
LC	4	59.82 (2.60)	91.10 (17.22)	8.73 (0.58)	1.24 (0.08)	1.11 (0.03)
LCF	4	52.82 (0.95)	72.17 (6.59)	12.71 (1.00)	1.75 (0.12)	1.37 (0.02)
LF	4	22.63 (1.23)	19.38 (2.29)	11.08 (1.09)	1.52 (0.14)	1.07 (0.06)

Figure 2.1. Mass -specific A) nitrogen and B) phosphorous excretion rates for our three study taxa (n=6). Different letters indicate significant differences among species based on Tukey's HSD.

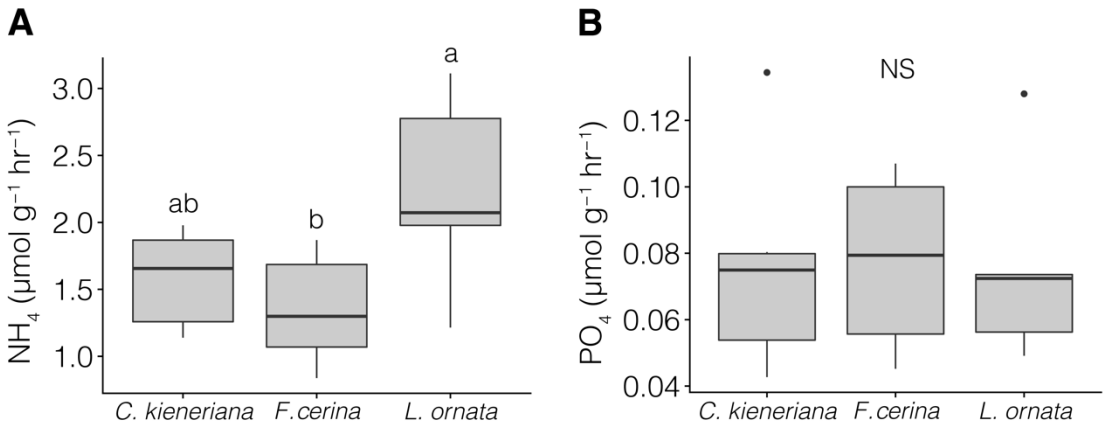


Figure 2.2. Mass -specific A) carbon, B) nitrogen, C) phosphorous, and D) total biodeposition rates for our three study taxa (n=6). Different letters indicate significant differences among species based on Tukey's HSD.

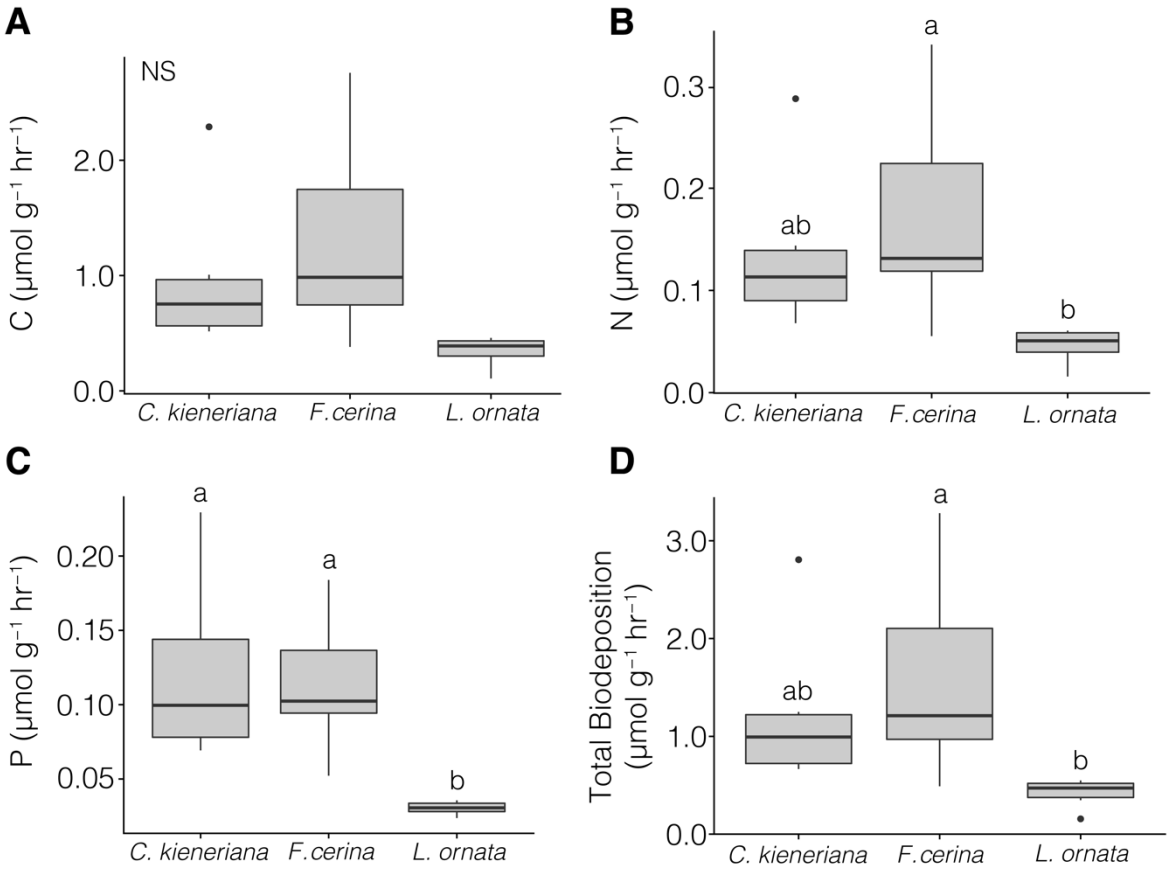


Figure 2.3. Mean (±SE) freshwater mussel A) N:P areal excretion and B) C:N, C) N:P, and D) C:P areal biodeposition stoichiometric ratios scaled to the community level. Different letters indicate significant differences among treatments based on Tukey's HSD.

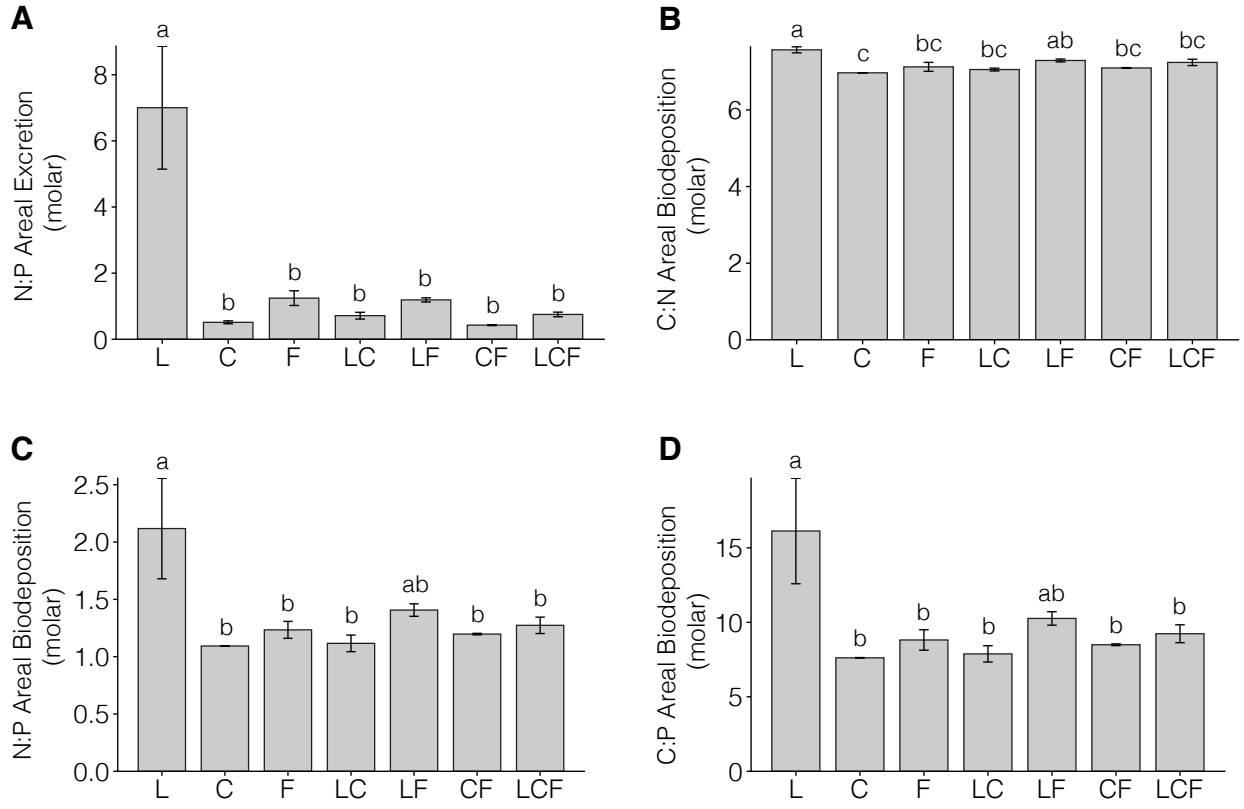


Figure 2.4. Mean (±SE) A) gross primary production and algal biomass accrual on B) inorganic benthic tiles and C) organic leaf litter over six weeks across the mesocosm treatments (n=4 per treatment). NS indicates no significant differences.

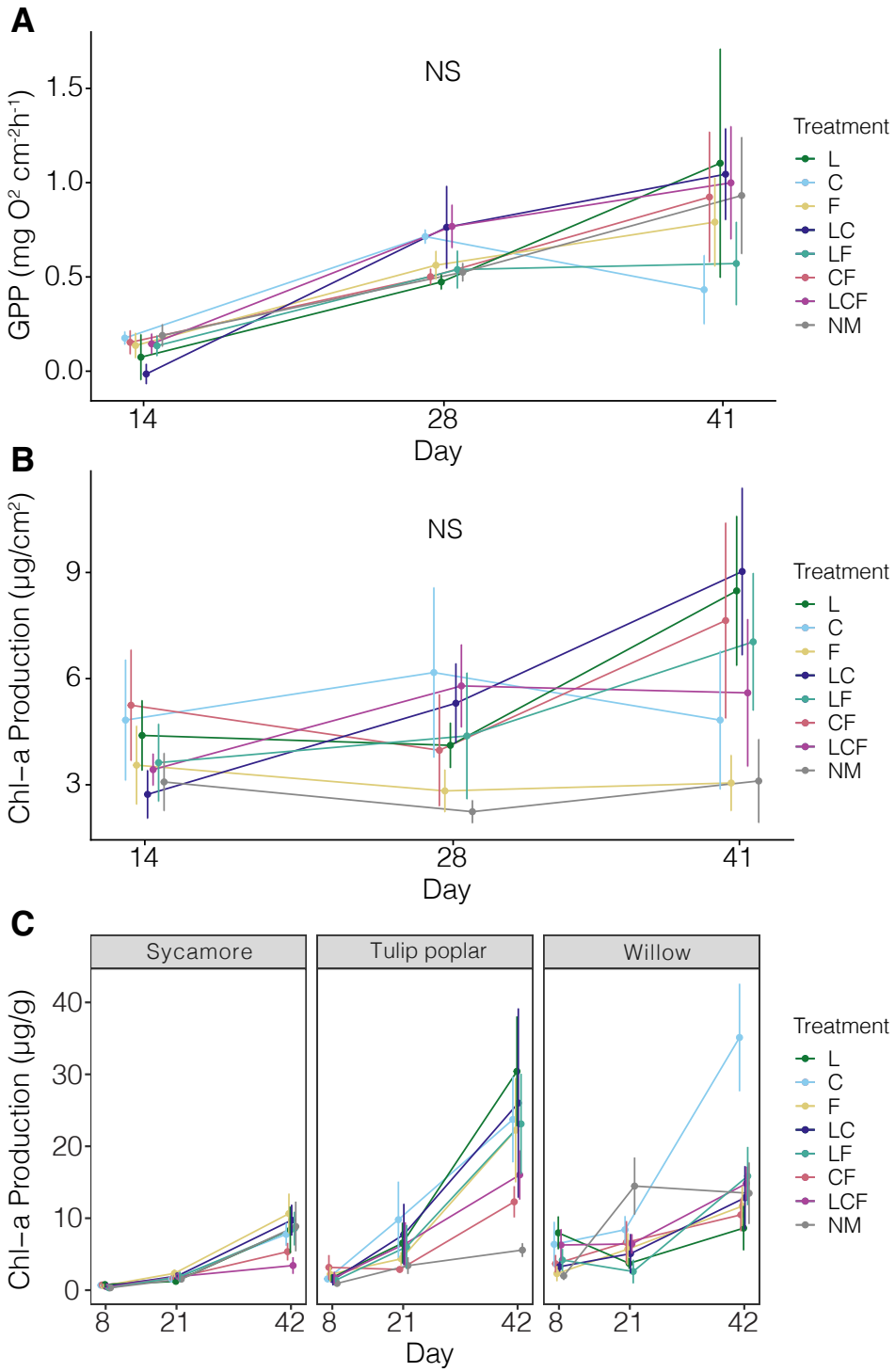


Figure 2.5. Percent litter remaining of each leaf species across treatments over six weeks.

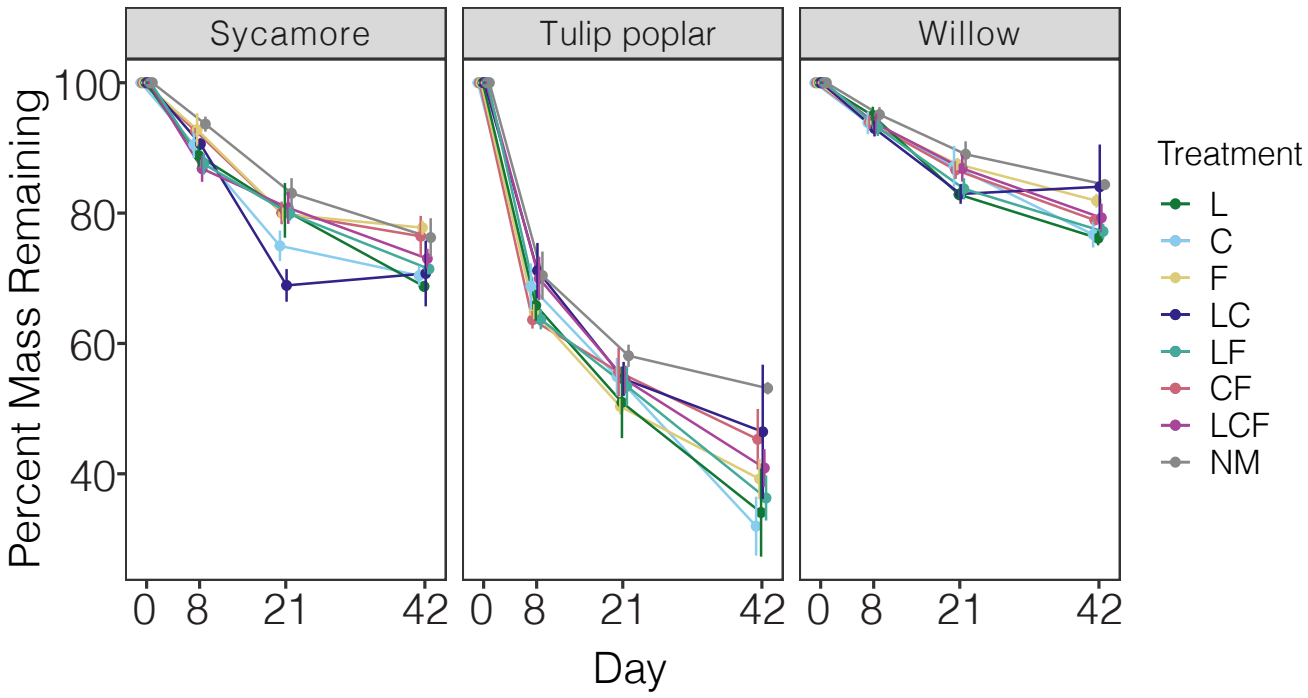


Figure 2.6. Mean (±SE) ergosterol activity measured on leaf litter. Sampling week did not have a significant effect on ergosterol production so here we show average production over six weeks. Ergosterol production was greatest on tulip poplar leaves (Tukey HSD $p<0.0001$) and varied across mussel treatments (ANOVA $F_{7,216}=2.48$ $p<0.05$) but our post hoc test failed to detect where those differences occurred.

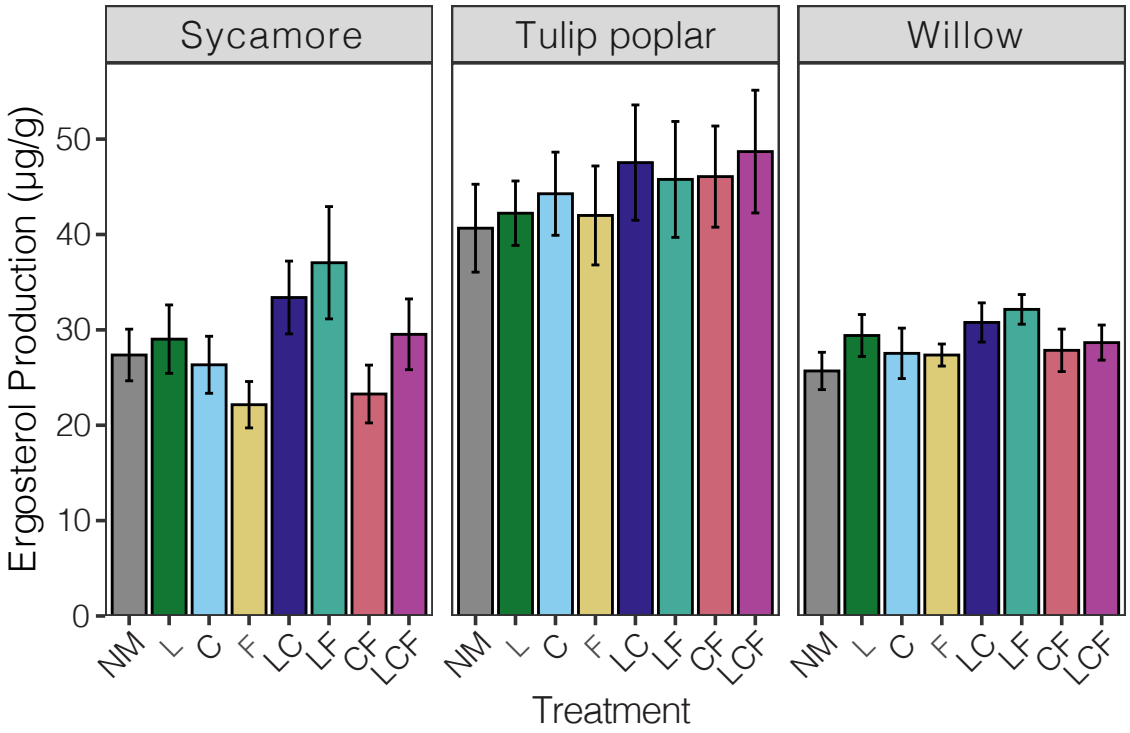


Figure 2.7. Mean (±SE) C:N molar content of leaf species among treatments averaged across six weeks. Different letters indicate significant differences among treatments based on Tukey's HSD and NS indicates non significance.

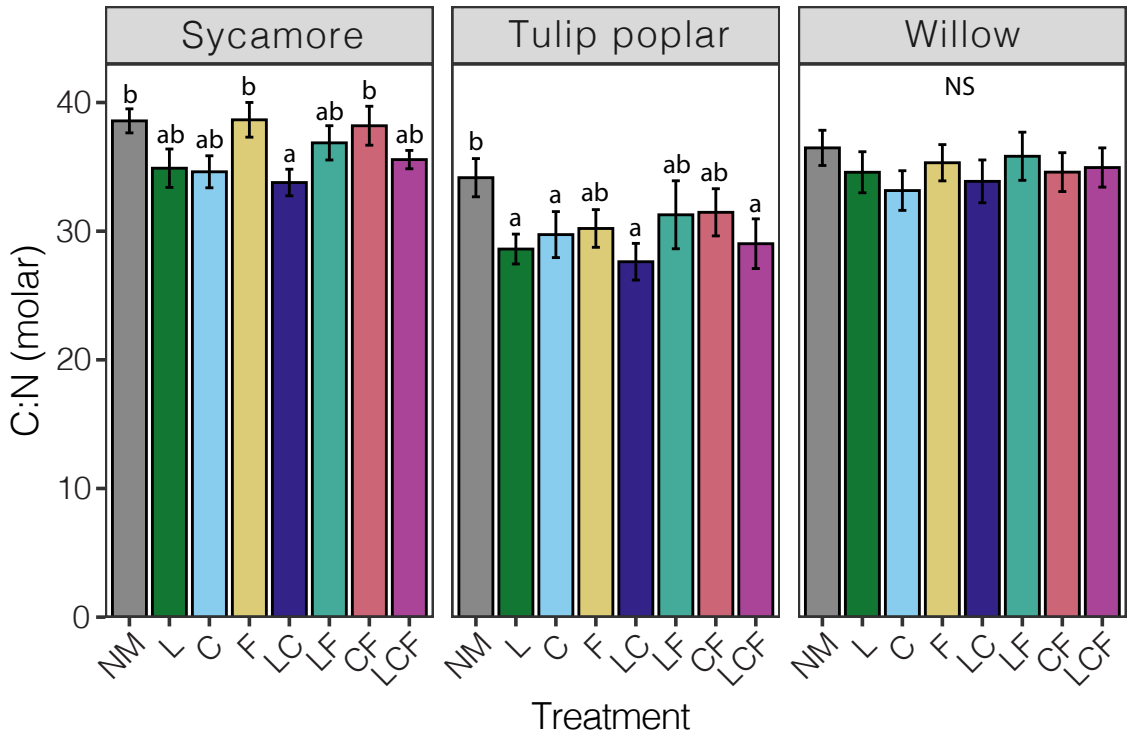


Figure 2.8. Mean (±SE) C:N molar content of all treatments of each leaf species over the 6-week experiment.

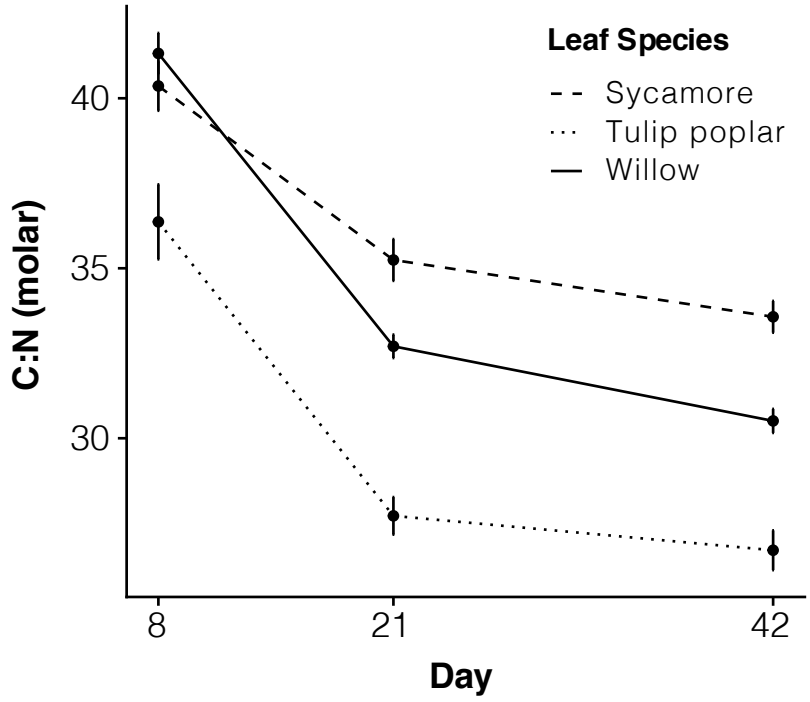


Figure 2.9. Mean (±SE) microbial activity measured as oxygen uptake on tulip poplar leaves. NS indicates no significant differences.

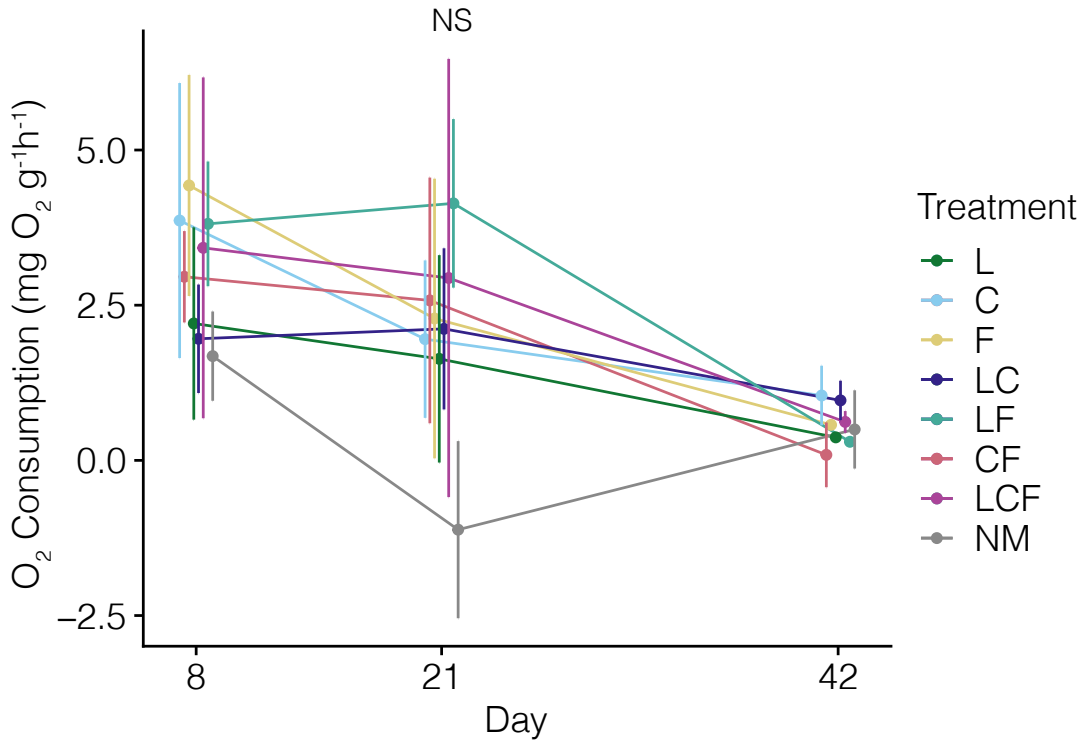


Figure 2.10. Mean (±SE) enzyme activity measured on tulip poplar leaves. NS indicates no significant differences.

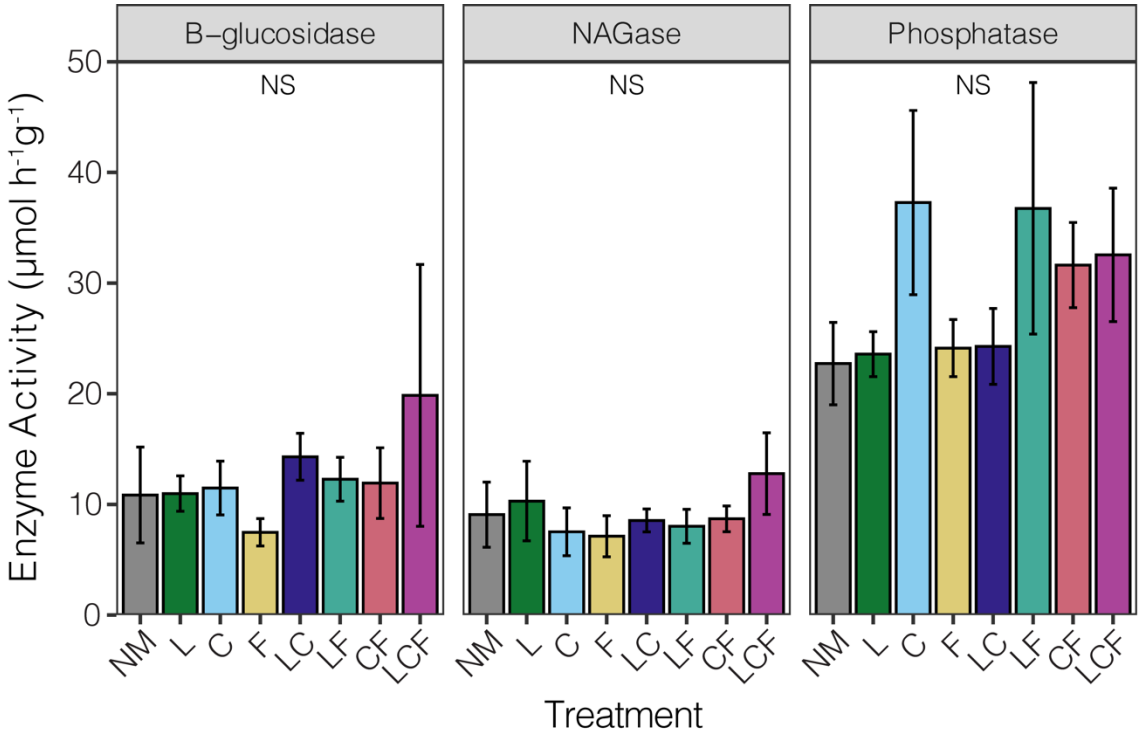
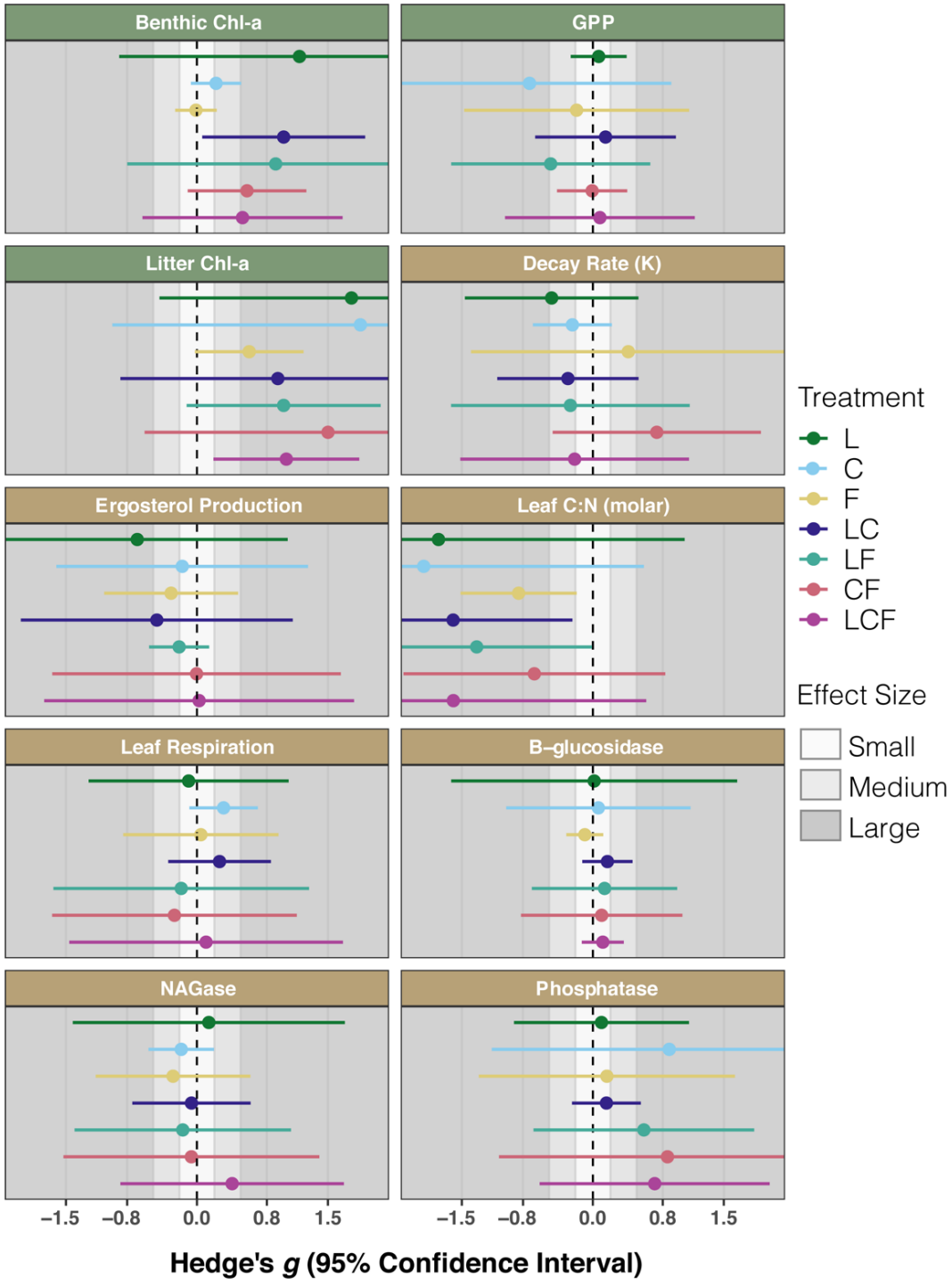


Figure 2.11. Scatterplots visualizing the results of Hedge's g effect size analysis. Each plot represents one ecosystem function of interest and each treatment group within. Green and brown food web responses are represented in green and brown boxes, respectively. All leaf related responses were calculated using tulip poplar leaves. The horizontal axis is the effect size index (Hedge's g). The vertical dashed line represents our no mussel control and shaded regions represent thresholds for effect sizes: small (|0.2| < g > |0.5|); medium (|0.5| < g > |0.8|); large (g > |0.8|).



CHAPTER 3:

FRESHWATER MUSSELS ENHANCE SEDIMENT N-REMOVAL POTENTIALS AND ALTER SEDIMENT BACTERIAL COMMUNITIES THROUGH BOTTOM-UP EFFECTS ON NUTRIENT RELEASE

Key words:

Unionid, sediment microbiome, biogeochemical cycling, nitrogen removal, denitrification, anammox

ABSTRACT

The benthic substratum in lotic systems is associated with several ecosystem functions and provides important habitat for a multitude of organisms from microbes to larger macrofauna including macroinvertebrates and fishes. One of the most critical ecological processes that occur within benthic sediments are microbial-mediated biogeochemical transformations. Microbes play a critical role in processes such as the nitrogen cycle. Benthic macrofauna, such as freshwater mussels, have the potential to influence these processes through chemically and physically altering the stream substrate consequentially altering redox conditions and the sediment microbial community. However, there is a lack of studies that investigate the interactions between burrowing macrofauna, benthic nutrient fluxes, and the associated bacteria communities in freshwater substrata. Here, we use a mesocosm experiment to investigate how mussels physically (via bioturbation) and chemically (via excretion and egestion) indirectly influence sediment-water nutrient fluxes and sediment microbiome community composition. Because many microbial-mediated N-transforming pathways can be stimulated by mussel-derived

nutrient inputs (excreted NH_4^+ or OM biodeposits), we predicted that mussels would enhance sediment N-removal potentials and alter the sediment microbial community structure via nutrient inputs and burrowing behavior. We found that the presence of mussels had a significant effect on enhancing cycling-removal and modifying sediment bacteria community composition and structure. We observed differences across treatments in sediment N-removal potentials and alpha diversity metrics of bacteria communities suggesting that mussel species identity and community composition play a critical role at the benthic-pelagic interface in stream ecosystems. Thus, a decline in mussel species richness and diversity in natural systems may result in altered nutrient fluxes and shifts in microbial community compositions. Therefore, better understanding the functional effects of mussel species and community composition is critical to predicting changes in stream ecosystem functioning.

INTRODUCTION

The benthic substratum in lotic systems provides habitat for many organisms from microbes to macroinvertebrates (Lowell et al., 2009) and is an especially important habitat for microbial communities (Findlay, 2010). Because of the heterogeneity of physical and chemical properties, stream benthic substrata are associated with many ecosystem functions such as biogeochemical cycling, primary productivity, microbial decomposition, and trophic interactions (Lewandowski et al., 2019). Decomposition of organic matter and recycling of nutrients are two of the most critical ecological processes that occur in freshwater sediments (Palmer et al., 1997). These biogeochemical transformations are carried out by various microbial metabolic pathways: photoautotrophs such as certain bacteria and cyanobacteria use light as a source of energy to produce biofilms, chemolithotrophic bacteria oxidize inorganic compounds and utilize carbon as a source of energy for carbon mineralization, and chemoorganotrophic bacteria and fungi

breakdown organic matter and release nutrients. Thus, microbial transformations of nutrients have great significance in sediments, especially the nitrogen cycle, as it is one of the most important nutrient cycles found in both terrestrial and aquatic ecosystems (Rabalais, 2002).

Many nitrogen-transforming microbes mediate N-removal processes and are classified based on the processes they are involved in (e.g., nitrifiers carry out nitrification, denitrifiers carry out denitrification, etc.) (Kuypers et al., 2018). The first step of nitrification (aerobic oxidation of ammonia to nitrite, $NH_4^+ \rightarrow NO^-_2$) is mainly carried out by chemoautotrophic ammonium-oxidizing bacteria (AOB), and members of the *Nitrosomonas* genera (Phylum Pseudomonadota, synonym = Proteobacteria) are typically the dominant AOB in freshwater systems (Whitby et al., 2001). Microbial ammonia oxidation is the first and rate-limiting step of autotrophic nitrification, thus the microbes that mediate this step serve a functionally important role. The second step of nitrification (aerobic oxidation of nitrite to nitrate, $NO^-_2 \rightarrow NO^-_3$) is mainly carried out by nitrite-oxidizing bacteria (NOB), and genus *Nitrospira* (Phylum Nitrospirota) is the most diverse and widespread known lineage of NOB and are found in a range of habitats from freshwater systems to wastewater treatment plants (Daims, 2014). Following nitrification, denitrifiers reduce NO_3^- to nitric oxide (NO), nitrous oxide (N_2O), and N_2 gas in anoxic and high C environments (Hayatsu et al., 2008). However, a specialized group of anammox bacteria in the phylum Planctomycetes can utilize NO^-_2 (produced from AOB) as an electron acceptor and oxidize NH_4^+ and produce N_2 gas (Kuenen, 2008) as a direct removal of N such as classical denitrification. Due to dependence on NO^-_2 produced by AOB, anammox bacteria thrive at the interface of oxic–anoxic conditions (Thamdrup, 2012) and have been detected in various freshwater systems including river sediment in the Southeastern United States (Hirsch et al., 2011).

In addition to microbes, aquatic macrofauna also play a critical role in mediating biogeochemical processes in the nitrogen cycle. The most important factors that regulate heterotrophic microbial activity include redox conditions, the availability of organic matter (OM), and reactive N (NO^{-}_{3}) (Knowles, 1982). In aquatic environments, consumers can enhance these conditions for nitrification an denitrification by chemically and physically altering the sediment through bioturbation, sediment formation and stabilization, and release of waste products (Anschutz et al., 2012; Hoellein et al., 2017; Holker et al., 2015; Turek & Hoellein, 2015). Previous studies have shown that benthic invertebrates modify aerobic and anaerobic microbial processes in benthic sediments and as a result have the potential to affect microbial activities (Boeker et al., 2016; Boeker & Geist, 2015; Lavrentyev et al., 2000; Mermillod-Blondin et al., 2001). However, over the last century, anthropogenic impacts such as changes in land use and land cover has altered flow and degraded stream substratum quality (Hancock, 2002). For example, increased loading of fine sediments has been shown to have adverse effects on benthic organisms such as fish (Chapman et al., 2014) and freshwater mussels (Lummer et al., 2016).

Freshwater mussels (Bivalvia: Unionoida) are a guild of burrowing, filter-feeding bivalves that dominate biomass in many freshwater systems where they live partially or completely burrowed in benthic sediments (Haag 2012; Vaughn & Hakenkamp 2001). Mussels act as benthic-pelagic couplers by filter-feeding across trophic levels feeding on bacteria, algae, detritus, and zooplankton from the water column and transferring these nutrients and energy to sediments through biodeposition of feces and pseudofeces (Atkinson et al., 2011; Black et al., 2017; Spooner & Vaughn, 2008; Vaughn et al., 2008). Mussels excrete highly reactive N in the form of NH_4^+ and biodeposit labile OM in feces and pseudofeces (Atkinson & Forshay, 2022;

Nickerson et al., 2019). Mussel egestion may influence N-removal potentials by providing reactive N for microbes and mussel feces and pseudofeces may foster denitrification by providing OM as an energy substrate to microbes (Trentman et al., 2018). Furthermore, decomposition of OM is a type of aerobic respiration where oxygen is consumed, thus potentially creating anoxic environments where denitrification and anammox can occur. However, the act of burrowing has the potential to increase oxygen penetration in the sediment and disrupt the redox gradient, therefore reducing N-removal potentials and providing sites for nitrification (Nickerson et al., 2019; Trentman et al., 2018). Therefore, the burrowing behavior and position of the mussel in the sediment plays a critical role in N-removal fluxes.

Despite the fact that all freshwater mussels are classified in the same functional group (filter-feeding bivalves), species within the same system exhibit variation in nutrient excretion/egestion rates and stoichiometry (Atkinson et al., 2010; Atkinson, van Ee, et al., 2020; Spooner & Vaughn, 2008) as well as burrowing behavior (Allen & Vaughn, 2009; Schwalb & Pusch, 2007). These traits, which affect how an organism performs (e.g., survival, growth, and reproduction) and interacts with its environment, can be classified as functional traits (Violle et al., 2007). Differences in functional traits and effects has been linked to phylogeny across organisms (Floeter et al. 2018; Gonzalez et al. 2018), including mussels (Atkinson, van Ee, et al., 2020) and is based on the hypothesis that evolutionary processes drive trait diversification and thus enhances the functional trait space of a community (Srivastava et al., 2012). Because species' functional characteristics strongly influence ecosystem properties, through niche complimentary or synergistic interactions, species richness may enhance ecosystem function and stability (Loreau & de Mazancourt, 2013). However, mussels are experiencing an extinction

crisis (Haag & Williams, 2014), with projections of extinction rates to be as much as 50% of species remaining in the next century (Ricciardi & Rasmussen, 1999).

Here, we use an additive partitioning approach with a mesocosm experiment to assess how mussels physically (via bioturbation) and chemically (via excretion and egestion) indirectly influence sediment-water nutrient fluxes and sediment microbiome community composition. Because many of the microbial-mediated biogeochemical processes rely on mussel-derived nutrient inputs (excreted NH_4^+ or OM biodeposits), we predicted that: 1) compared to no mussel controls, the presence of mussels will enhance sediment N-removal potentials by providing microbes with reactive N through excreta and labile C from biodeposits; 2) different communities of mussels will vary in N-removal potentials as a result of species-specific functional traits of stoichiometric nutrient excretion/egestion rates and ratios and burrowing behavior; and 3) sediment microbial community structure will vary across treatments as a result of different mussel community composition and associated nitrogen-transforming microorganisms.

MATERIALS AND METHODS

Study Organisms

We collected mussels, sediment, and water from the Sipsey River, a fifth-order tributary of the Tombigbee River in Alabama. The Sipsey River is relatively unmodified by human disturbances and harbors diverse and abundant communities of freshwater mussels—making it an ideal study system (Haag & Warren, 2010). For these reasons, we utilized three abundant species that occur in three distinct evolutionary tribes collected from the Sipsey River: *Cyclonaias kieneriana* (Tribe Quadrulini), *Fusconaia cerina* (Tribe Pleurobemini), and *Lampsilis ornata* (Tribe Lampsilini). These three species vary in shell morphology, life-history

traits, and tissue stoichiometry (Atkinson, van Ee, et al., 2020; Haag, 2012; Williams et al., 2008).

We collected 183 mussels November 20-22, 2019 from the Sipsey River and transported them back to the lab where they were held in Living Stream Systems® (Frigid Units Inc., Toledo, Ohio). Each mussel was measured, and standardized length dry mass regressions were used to estimate weight (Atkinson, Parr, et al., 2020). We tagged each mussel with fly line each 15cm in length and attached an individual identification tag to the end. Mussels were held at 10° C in Living Stream Systems® until the beginning of the experiment and fed with cultured algae 3x weekly.

We manipulated mussel diversity by creating four replicates of eight treatments: (i) three single-species treatments (*L. ornata, C. kieneriana, F. cerina; hereafter* L, C, F), (ii) three two-species treatments (*L. ornata + C. kieneriana*, *L. ornata + F. cerina, C. kieneriana + F. cerina; hereafter* LC, LF, CF), (iii) one multi-species treatment (*L. ornata + C. kieneriana + F. cerina; hereafter* LCF), and (iv) one control treatment (no mussels; hereafter NM) (Table 1). We implemented a substitutive design in which each community contained the same biomass of each species (ANOVA $F_{6,21}$=0.87 p=0.53). We randomly assigned individuals to each of the eight treatment groups (n=32) and treatments to mesocosms using a random number generator.

Mesocosm Design

We conducted a 42-day mesocosm study in the greenhouse on the main campus of the University of Alabama in Tuscaloosa, AL from January to March of 2020. We used 200 L recirculating stream mesocosms (81x51x48 cm) which consisted of two tanks, an open-ended plastic liner placed inside a fiberglass outer tank. The inner liner was placed atop bricks to allow water recirculation as in (Nickerson et al., 2021) and the bed of the liner was filled with a mix of

pea gravel and sand from the Sipsey River and supplemented with purchased pea gravel (Vigoro® Pea Gravel Pebbles). We filled each liner to a depth of 35cm with water from the Sipsey River and used 47 w magnetic drive pumps (Dammer Mfg, Islandia NY) to recirculate the water at a rate of 2527 L h^{-1}. We placed each pump atop the pea gravel at one end of the mesocosm so that the orientation of the pump caused water to be drawn up from the space between the two tanks and flow directly over the pea gravel. We conducted weekly water changes (15%) in which we emptied 20L of water from each tank and replaced it with 20L of Sipsey water weekly for the duration of the experiment. In addition, each tank received 500mL of a concentrated mixed algal assemblage cultured from natural river water every week. We monitored water temperature every 60 minutes in 12 mesocosms using Hobo U20L temperature (Onset Corp, Bourne, MA).

Study Organism Measurements

Excretion Trials: At the end of six weeks, we estimated nitrogen and phosphorus excretion for each community by randomly subsampling six mussels of each species and measuring their NH_4^+ (N) and Soluble Reactive Phosphorus (P) excretion rates. Using a toothbrush and scour pad, we gently scrubbed each mussel and placed the individuals in individual excretion chambers. Depending on mussel size, we filled each container with 300 or 500mL of filtered mesocosm water (GF/F; 0.7 μm pore size; Millipore). We used three control containers which were incubated simultaneously without bivalves to control for biofilm uptake. After 1 hour, we gently removed each mussel, filtered the chamber water (GF/F; 0.7 μm pore size; Millipore), and stored 30mL of the sample at -20°C until analysis. Filters were retained to estimate biodeposition rates (mg DM^{-1} h^{-1}) and %C, %N, and %P content. We used a Seal AQ300 discrete analyzer (Seal Analytical, Mequon, Wisconsin, USA) to analyze Soluble

Reactive Phosphorus using the colorimetric method (Murphy & Riley, 1962) and NH_4^+ using the phenol method for filtered excretion samples. Mass-specific excretion rates for each mussel (μmol NH_4^+ hr^{-1} g^{-1}) were calculated using the concentration of NH_4^+ in the excretion chamber (μg L^{-1}), the known volume of water in the chamber, and the amount of time the mussel incubated (1 hr) after correcting for the controls. Solid material collected on the filters was used to calculate mussel biodeposition rates. The filters were dried for 48h at 50°C, weighed on an analytical balance, then combusted at 500°C for 2h and weighed again to calculate ash-free dry mass (AFDM) and calculate total organic matter (OM) of the biodeposits. Biodeposit samples were then subsampled to calculate %C, %N, and %P. To calculate %C and %N, samples were analyzed with a Carlo Erba CHNS-O EA1108-Elemental Analyzer (Isomass Scientific Inc.). To calculate %P, samples were digested with HCl and analyzed for soluble reactive P.

We used mass-specific hourly rates of excretion and biodeposition to estimate the community-scale rates for each of our mesocosm treatments. Following the methods similar to Atkinson & Forshay (2022), we calculated areal excretion rates of N and P (μmol nutrient m^{-2} hr^{-1}) and biodeposition of C, N, and P (μmol nutrient m^{-2} hr^{-1}) by multiplying the species-specific population biomass (i.e. mesocosm) by the per capita excretion or biodeposition rate summed for across all species for each treatment.

Mussel Movement

We tracked mussel movement and burrowing behavior by recording the location and depth of each individual mussel three times per week for the duration of the study. As previously stated, prior to the start of the experiment each mussel was tagged with a piece of fishing line that was glued to the shell near the incurrent and excurrent siphons. Mussel location was determined using a grid of 60, $3cm^2$ cells constructed from a PVC pipe frame. Mussel burial

depth was determined by measuring the length of fly line exposed from the sediment. To account for differences in mussel body size, burial depth was calculated by adding the recorded depth of fly line and the individual's total body length to accurately express the total distance the mussel was buried in the sediment. Horizontal movement was calculated between grid points of consecutive days using the distance formula derived from the Pythagorean Theorem such that:

$$d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$$

where $d =$ distance (cm)

$(x_2 - x_1)$ = coordinates of the first point

$(y_2 - y_1)$ = coordinates of the second point

Vertical movement was calculated using the absolute value of the start and end burial depth for each day. Following methods similar to Allen & Vaughn (2009), we define total mussel movement as the sum of horizontal and vertical movement measurements throughout the duration of the experiment (42d).

Sediment N-Removal Potentials

To examine the influence of mussels on the biogeochemical pathways of denitrification (hereafter DNF) and anaerobic ammonia oxidation (hereafter anammox), we analyzed Nitrogen-removal potential in sediment slurries using isotope-pairing techniques (IPT) (Thamdrup & Dalsgaard, 2002). At the end of six weeks, sediment cores (20cm x 8cm) were extracted from the top 20cm of each mesocosm, homogenized, and wet-sieved (2mm) into 12mL exetainers. Exetainers were filled in duplicate with approximately 6g of wet sediment. Each exetainer was then filled with anoxic water, capped, and placed on a shaker table overnight to remove any residual NO_3^- and O_2. The following day, isotopically-labelled $^{15}NO_3^-$ was added to each exetainer to a final concentration of [$^{15}NO_3^-$] = 50 µM0. Immediately after adding $^{15}NO_3^-$, a 250

μL solution of $ZnCl_2$ (50% w/v) was added to half of the replicate exetainers, which ceased all microbial activity and represented the concentration of N_2 isotopes at time zero (T_0). The exetainers were sealed and shook anaerobically for an additional 6 hours. After 6 hours, the remaining replicates were spiked with 250 μL of $ZnCl_2$ and thus represented the concentration of N_2 isotopes at time final (T_6). The concentrations of $^{29}N_2$ and $^{30}N_2$ were measured within 24 hours using a membrane inlet mass spectrometer. To calculate N-removal potentials, sediment slurries were dried at 50°C and weighed to determine dry mass. We normalized concentrations of $^{29}N_2$-N and $^{30}N_2$-N (μmol L^{-1}) by slurry incubation time (6 h) and sediment dry mass (kg) to report maximum N-removal potential as an hourly rate per kg of sediment (μmol N_2-N kg^{-1} h^{-1}).

Sediment Microbiome

Sample Collection:

To characterize the sediment microbial community, we collected sediment samples from each mesocosm at the beginning of the experiment (hereafter week zero), and after two and six weeks. Samples were collected by gently scooping the top layer of sediment into a 15mL conical tube and immediately freezing at -80°C until analysis. Frozen samples were shipped overnight on dry ice to the University of Mississippi for subsequent DNA extraction.

DNA Extraction & Sequencing:

Prior to extracting DNA with a commercially available DNA kit (Qiagen DNeasy PowerSoil Pro kit; Qiagen, Germantown, MD, U.S.A.), sediment samples were first pre-treated for optimization. Briefly, samples were thawed and approximately 8-10mL of sample was added to sterile 50mL Falcon tubes and weighed. A 5mL solution of Tris-EDTA and 0.1% Tween 20 buffer was added to each sample and vortexed (mid-speed for 10min). After the samples settled for 10min at ambient temperature, the supernatant was transferred to 15mL Falcon tubes.

Samples were then centrifuged at 4,000 xg at 4°C for 15min and the supernatant was carefully poured off. Next, the pellet was re-suspended using 500µL of bead beating solution from the PowerSoil kit and transferred to the bead-beating tubes. Samples were then extracted following standard procedures. Recovered DNA was amplified, targeting the V4 region of the 16S rRNA gene, using dual-indexed barcoding and primers following the methods of Kozich et al., (2013). Next, 1µl of genomic DNA was combined with 1µl of each primer (10µM) and 17µl of AccuPrime Pfx SuperMix (Life Technologies Corporation, Carlsbad, CA, U.S.A.). Polymerase chain reaction (PCR) amplification was performed as follows: an initial denaturation at 95 °C for 2min; 30 cycles of denaturing at 95°C for 20s, annealing at 55°C for 15s, and extension at 72°C for 2min; and finally elongation at 72 °C for 10min. Amplification products were standardized with SequalPrep Normalization Plates (Invitrogen Corporation, Carlsbad, CA, U.S.A.) and pooled prior to sequencing on an Illumina MiSeq at the University of Mississippi Medical Center Molecular and Genomics Core facility.

Sequence Processing:

Raw sequencing files from 72 samples were processed in mothur v.1.41.1 using the pipeline and mothur SOP (https://www.mothur.org/wiki/MiSeq_SOP). Sequences were aligned to the Silva database (v138) and classified based on the Ribosomal Database Project (RDP; v18). After nonbacterial sequences were removed, two samples (14_CF2, 14_LCF1) were removed as there were too few sequences remaining, leaving a total of 70 samples in the final dataset. Sequences were grouped into operational taxonomic units (OTUs) based on 97% sequences similarity.

Data Analyses

Nutrient inputs, movement, and N-removal potentials

We used one-way analysis of variance (ANOVA) tests followed by post hoc Tukey tests to test for differences in mass-specific and areal excretion and biodeposition rates and movement between species and treatments. To test the effect of mussel species community composition on sediment N-removal potentials, we used one-way ANOVAs followed by post hoc Tukey tests with treatments as a factor. We used Pearson's correlations to assess the relationships between mussel movement, excretion, and egestion on N-removal pathways of DNF and anammox. All statistical analyses were performed in R (R Development Core Team 2015) and data were log(+1) transformed as necessary to meet assumptions of normality and homoscedasticity.

Sediment microbiome

To test for differences in relative phyla abundances across sampling week and treatments, we used two-way ANOVAs followed by post hoc Tukey tests with sampling week and treatments as factors. To assess alpha diversity, Shannon's Index was used to calculate community evenness, Chao1 was used for species richness, and the Inverse Simpson index was used for calculating overall diversity. All alpha diversity indices were calculated using the R package *phyloseq* and two-way ANOVAs were used to determine if evenness, richness, and diversity differed between treatments and sampling week. To assess beta diversity, we conducted principal coordinate analysis based on Bray-Curtis dissimilarities and conducted Permutation Based Analysis of Variance (PERMANOVAs) to assess differences in microbiome structure between treatments and sampling week**.** To test the effect of mussel species community composition on the eleven most abundant phyla, we used two-way ANOVA tests followed by a post hoc Tukey test, with treatments and week as factors. We also used one-way ANOVAs to test the effect of mussel treatments on measures of alpha diversity between weeks two and six, with treatment as a factor. To test the effect of mussel community composition on beta diversity,

we used PERMANOVAs with treatment and sampling week as factors. We quantified relative abundance of N-cycling taxa across treatments and sampling week (Figure 11, Table 4). However, in all cases only one associated taxon was detected in a sample so statistical analyses were limited.

RESULTS

Mussel Nutrient Inputs

Mussel Excretion

There were significant differences in mass-specific NH_4^+ excretion rates among species (ANOVA $F_{2,15}$=4.69, p< 0.05; Table 3.2, Figure 3.2A), with *L. ornata* having higher mass-specific NH_4^+ excretion rates than *F. cerina* (Tukey HSD, p<0.05). When scaled to the community level, there were significant differences in NH_4^+ areal excretion rates among treatments (ANOVA $F_{6,21}$=215.36, p< 0.0001; Table 3.3). The C treatment had higher NH_4^+ areal excretion rates compared to all other treatments (Tukey HSD, p<0.001) followed by the CF treatment (Tukey HSD, p<0.001). The LC and LCF treatments did not differ in NH_4^+ areal excretion (Tukey HSD, p=0.50), but were greater than the F, LF, and L treatments (Tukey HSD, p<0.0001). The LF treatment did not differ from the F or L treatment (Tukey HSD, p=0.09), but the F treatment had greater areal excretion than the L treatment (Tukey HSD, p<0.05).

There were no differences in mass-specific PO_4^{3-} excretion rates among species (ANOVA $F_{2,15}$=0.02, p=0.98; Table 3.2, Figure 3.2B). However, when scaled to the community level, there were significant differences in areal excretion rates among treatments (ANOVA $F_{6,21}$=40.04, p< 0.0001; Table 3.3). Generally, treatments containing *Cyclonaias* had higher areal PO_4^{3-} excretion and treatments containing *Lampsilis* had lower areal PO_4^{3-} areal excretion. The C and CF treatments did not differ between each other (Tukey HSD, p=0.57), but were higher than

all other treatments (Tukey HSD, $p<0.0001$). There was no difference in areal excretion rates between the LC and LCF treatments (Tukey HSD, $p=0.96$), nor was there a difference between the LCF, F, and LF treatments (Tukey HSD, $p=0.20$). The LC treatment had greater areal excretion than F, LF, and L treatments (Tukey HSD, $p<0.05$).

As a result of the variation in elemental excretion across species (Table 3.2, Figure 3.2A-B), there were differences in areal N:P molar excretion among treatments (ANOVA $F_{6,21}=11.09$, $p<0.0001$; Table 3.3, Figure 3.3A) with the L treatment having higher N:P than all other treatments (Tukey HSD, $p<0.0001$).

Mussel Egestion

Mass-specific elemental biodeposition rates differed significantly among mussel species for nitrogen (ANOVA $F_{2,15}=4.28$, $p<0.05$; Table 3.2, Figure 3.1B) and phosphorus (ANOVA $F_{2,15}=7.72$, $p<0.01$; Table 3.2, Figure 3.1C), but not for carbon (ANOVA $F_{2,15}=3.32$, $p=0.06$; Table 3.2, Figure 3.1A). *Fusconaia* deposited higher quantities of nitrogen compared to *Lampsilis* (Tukey HSD, $p<0.05$) and both *Fusconaia* and *Cyclonaias* deposited higher quantities of phosphorus compared to *Lampsilis* (Tukey HSD, $p<0.05$). There were also differences in total OM biodeposition rates across species (ANOVA $F_{2,15}=3.61$, $p=0.05$) with *Fusconaia* having higher biodeposition rates compared to *Lampsilis* (Tukey HSD, $p<0.05$).

As a result of these species-specific variation in biodeposition content, when scaled to the community level, there were significant differences in stoichiometric ratios of C:N (ANOVA $F_{6,21}=9.32$, $p<0.001$; Table 3.3, Figure 3.3B), N:P (ANOVA $F_{6,21}=4.18$, $p<0.01$; Table 3.3, Figure 3.3C), and C:P (ANOVA $F_{6,21}=4.35$, $p<0.01$; Table 3.3, Figure 3.3D) of biodeposits among mussel treatments. Generally, treatments containing *Lampsilis* and or *Fusconaia* species had greater C:N, N:P, and C:P stoichiometric biodeposition compared to other treatments.

Burrowing Movement

Mussel movement varied among mussel species vertically (ANOVA $F_{2,286}$=178.12, p<0.0001; Figure 3.4B) and in total movement (ANOVA $F_{2,286}$=41.52, p<0.0001; Figure 3.4C) with *Lampsilis* moving significantly more vertically and overall compared to both *Cyclonaias* and *Fusconaia* (Tukey HSD, p<0.0001).

Sediment N-Removal Potentials

We saw significant treatment effects on both N-removal pathways of DNF (ANOVA $F_{7,24}$=5.49, p<0.001; Figure 3.5A) and anammox (ANOVA $F_{7,24}$=6.51, p<0.001; Figure 3.5B), as well as when we combined both pathways to estimate total N-removal potential (ANOVA $F_{7,24}$=6.87, p<0.001; Figure 3.5C). In general, the single species treatments of *Lampsilis* and *Cyclonaias* had greater DNF than most other treatments (Figure 3.5C) and were the only two treatments that were significantly greater than the no mussel control when we combined DNF and anammox (Tukey HSD, p<0.05).

We found significant relationships between mussel vertical movement and N:P areal excretion with N-removal pathways (Table 3.3). We found there was a significant negative correlation between mussel vertical movement and total N-removal potentials (Pearson's r(26) = -0.391, p=0.040; Table 3.3, Figure 3.6C), and a significant positive correlation between N:P areal excretion (Pearson's r(26) =0.444, p=0.018; Table 3.3, Figure 3.6B) and total N-removal potentials. We also observed that C:P areal biodeposition was more correlated with the N-removal pathway of DNF (Pearson's r(26) =0.297, p=0.124; Figure 6A) compared to anammox (Pearson's r(26) =0.116, p=0.558).

Sediment Microbiome

Composition

The final dataset consisted of 1,608,834 sequence reads corresponding to 16,381 unique OTUs. After standardizing to the lowest sequencing read (n=2,112), 1,548,43412 sequences remained. Overall, the relative abundances of bacterial phyla sampled in our study were similar across sampling weeks with Protobacteria accounting for the most abundant taxa across all weeks (45.1%, 39.1%, and 21.7% for weeks zero, two, and six respectively). Protobacteria consisted mostly of Betaproteobacteria (20.9%, 16.7%, and 6.9% for weeks zero, two, and six, respectively) and Alphaproteobacteria (15.8%, 14.3%, and 10.2% for weeks zero, two, and six, respectively). We observed a significant decrease in the mean percent relative abundance of Proteobacteria across sampling weeks (ANOVA $F_{2,12120}$=3.73, $p<0.05$; Figure 3.7), with week six having lower abundance than both weeks zero and two (Tukey HSD, $p<0.0001$). We did not observe any significant effects of treatment or sampling week on any of the other top eleven phyla.

Alpha and Beta Diversity

Overall, we saw a decrease in alpha diversity metrics over the course of the experiment. At day zero, mussel treatments generally had higher alpha diversity indices compared to the no mussel controls. However, at the end of the experiment, mussel treatments had lower diversity indices compared to the no mussel controls. Bacterial communities sampled at the end of six weeks presented a significantly lower richness (Chao1; ANOVA $F_{1,46}$=6.33 $p<0.05$; Figure 3.8B) and were less even (Shannon; ANOVA $F_{1,46}$=3.83 $p=0.06$; Figure 3.8A) compared to communities sampled after two weeks. Specifically at the end of six weeks, the no mussel controls had significantly greater species richness compared to the *Cyclonaias* treatment,

Fusconaia treatment, and the three species treatment of *Lampsilis* + *Cyclonaias* + *Fusconaia* (Chao1; ANOVA $F_{7,46}$=3.08 p<0.01; Tukey p<0.05; Figure 3.8B). Treatment and sampling week did not have a significant impact on bacterial community diversity (Inverse Simpson; Figure 3.8C; p =0.45-0.49, F =0.50-1.00), but a moderate affect on evenness (Shannon; p = 0.06-0.08, F =2.00-3.83).

Overall, there was a significant temporal difference in microbiome communities sampled across weeks (PERMANOVAs on Bray-Curtis, R^2=0.33 p<0.001; W-Unifrac, R^2=0.33 p<0.001; Figure S3). The PCoA plot showed that bacterial community composition is strongly influenced by sampling week, with OTUs from each sampling time point forming a tight cluster. In further exploring treatment effects at each week, we observed a significant effect of treatment in microbiome communities sampled at week six (PERMANOVA on Bray-Curtis, R^2=0.26 p=0.05; Figure 3.9). Post-hoc pairwise comparisons revealed that the bacteria communities were significantly different among the NM and C (p<0.05), and NM and LCF treatments (p<0.05), with a marginally significant difference between the NM and LC treatments (p=0.051).

DISCUSSION

Our results suggest that mussel species identity and community composition play a critical role at the benthic-pelagic interface by stimulating N-removal in sediments, with the single species, *Lampsilis ornata*, having the largest influence. We also observed interaction effects between treatments when excretion and egestion nutrient fluxes were scaled to the community level (i.e., strong effects of one species was dampened by other species in the community). Furthermore, we found a positive correlation between mussel nutrient release (areal N:P excretion) and sediment N-removal potentials which suggests mussels have an important influence on stimulating coupled nitrification-denitrification. Differences in mussel nutrient

excretion/egestion rates and ratios directly impacts autotrophic and heterotrophic microbes by stimulating primary producers and decomposers (Atkinson et al., 2017, 2021; Sharitt et al., 2021). We observed a negative correlation between total vertical movement of mussels on N-removal potentials, which may be indicative that mussel burrowing behavior is disrupting sediment redox conditions and decreasing N-removal. Finally, our findings suggest that the presence of mussels may have an effect on sediment bacterial community, with some mussel treatments having different communities compared to the treatment without mussels.

Excretion stoichiometry impacts N-removal

Our results support previous research that demonstrates species-specific differences in mussel excretion N:P that is partially attributable to phylogenetic differences (Atkinson, van Ee, et al., 2020). Similar to Atkinson, van Ee, et al., (2020), we observed that *L. ornata* had significantly higher N:P excretion than *C. kieneriana* and *F. cerina*. When scaled to the community level, these mass-specific differences become more evident, especially when mussels are in dense aggregations (Atkinson et al., 2018; Atkinson & Vaughn, 2015). Our findings from this mesocosm study supports our prediction that mussels would enhance sediment N-removal potentials with community-level differences resulting from differences in excretion stoichiometry. We observed greater DNF and anammox potentials in treatments containing *Lampsilis*, with the single *Lampsilis* treatment having significantly greater total N-removal potentials than the no mussel control and all other mussel treatments except the single *Cyclonaias* treatment. This result is corroborated by a previous study which found single-species treatments of *Lampsilis ornata* to have a stronger influence on sediment N-removal potentials compared to other species in small chamber incubations (Nickerson et al., 2019). Furthermore, Atkinson and Forshay (2022) found that denitrification rates in river sediments increased with

increasing mussel biomass. They also found a positive correlation between excretion N:P and denitrification which suggests mussels can enhance denitrification rates likely through providing nutrient subsidies. Additionally, Trentman et al., (2018) found that in a river system, sediment denitrification rates decreased with increasing mussel density. However, in sediments amended with N, denitrification rates increased with increasing mussel density, which suggests an interaction effect of mussel biomass and nutrient limitation on denitrification rates. Our results corroborate this study and provide evidence that mussels can play an important role in mediating biogeochemical cycling and that mussel-provisioned NH_4^+ in the form of excreta can be an important reactant in N-removal potentials.

The role of biodeposition in N-removal

We observed species-specific differences in biodeposition rates and ratios among our three study species. Similar to excreta, *Lampsilis* had greater biodeposit N:P and when scaled to the community level, the single *Lampsilis* treatment also had significantly greater biodeposit N:P than all other treatments except LC. We observed this same trend with areal C:P biodeposition, with the L treatment being significantly greater than all other treatments except LC. Given the large role that bacteria play in biogeochemical transformations, mussel biodeposition in the form of labile OM may alleviate C limitation for heterotrophic bacteria as well as influence sediment chemical properties which can influence bacterial community composition and vice versa (Black et al., 2017). Though not significant, it is worth noting that C:P areal biodeposition was more strongly correlated with the N-removal pathway of DNF compared to anammox. The pathway of DNF requires C as a source of energy, whereas anammox does not (Kuenen, 2008), which may explain this observation and supports our prediction that OM in mussel biodeposits can stimulate DNF. Additionally, mussel biodeposits may indirectly enhance N-removal by creating suitable

anoxic environments in the sediment through microbial decomposition. Previous studies have found that decomposition of marine mussel biodeposits significantly decreased sediment dissolved oxygen levels (Carlsson et al., 2010; Giles & Pilditch, 2006). Thus, the addition of mussel biodeposits may enhance DNF directly by providing microbes labile C as a source of energy and indirectly by stimulating microbial decomposition and creating micro anoxic habitats where these biogeochemical processes can occur.

Mussel movement varies among species

We observed a significant negative correlation between mussel vertical movement and sediment N-removal potentials, as well as species-specific patterns in overall movement. Mussel burrowing behavior can vary with species (Allen & Vaughn, 2009), season and reproductive cycle (Watters et al., 2011), and substrate (Lewis & Reibel, 1984; Sansom et al., 2022). Thus, there are multiple factors governing the activity of mussel burrowing behavior which may vary spatially and temporally in field settings. High temperatures have been shown to cause mussels physiological stress (Spooner & Vaughn, 2008), which can also influence their activity (Beggel et al., 2017). During our experiment, water temperatures peaked to nearly 28°C (Figure A1), a level shown to influence the rate at which mussels activate stored energy reserves (Said & Nassar, 2022; Spooner & Vaughn, 2008). Additionally, previous studies have found that reduced energy stores and increased vertical movement towards the sediment surface are associated during the reproductive period (Erk et al., 2011; Watters et al., 2011). Further study of how environmental conditions alter mussel activity and impacts to ecosystem function are warranted.

Sediment Microbiome

We observed a strong temporal effect on sediment bacterial community as well as a mussel effect with some mussel treatments having a significantly different community

composition compared to the no mussel controls. Proteobacteria had the highest relative abundance across all sampling timepoints, which corroborates previous studies that have found Proteobacteria to be the most abundant phyla in freshwater sediments (Zhang et al., 2015) and dominant in mussel microbiomes (Aceves et al., 2018; Aceves et al., 2020; McCauley et al., 2021; Weingarten et al., 2019). Previous studies have found that burrowing movements by aquatic benthic organisms influences the physiochemical and microbial properties in freshwater sediments (Anschutz et al., 2012; Boeker et al., 2016; Boeker & Geist, 2015; Mermillod-Blondin et al., 2003). Thus, changes in sediment microbial communities in our study may have been influenced by mussel burrowing activities as this causes particle reworking and biomixing of the substrate which displaces microorganisms within the sediment (Kristensen et al., 2012). Additionally, the chemical composition of biodeposits as well as the microbes living in and on the mussels may have attributed to changes in the bacterial community. Indeed, we observed considerable algal growth on both benthic substrates and on the shells of the mussels (Figure A2) as our experiment progressed. These surfaces may have also provided favorable microhabitat for photosynthetic bacteria which increased in relative abundance towards the end of our experiment. Previous studies have found that the shells of marine mussels provide a nutrient-rich microenvironment for biofilm growth that is ideal for nitrifying bacteria, further contributing to N-cycling (Heisterkamp et al., 2013; Svenningsen et al., 2012). We also observed an overall decrease in alpha diversity metrics in treatments with mussels compared to the control treatment without mussels. These results are supported by Black et al., (2017) which found that sediment bacterial communities associated with mussels had lower evenness and richness compared to sediments without mussels. This may be explained by mussels creating a niche for specific microorganisms via physical and chemical changes to the sediment. Previous studies have shown

that nitrogen enrichments decrease microbial diversity in marsh sediments (Kearns et al., 2016), and similarly in our study, mussel nutrient inputs may be reducing community diversity.

Caveats

Within our controlled mesocosm setting, we attempted to recreate natural river conditions in which mussels live. While it is important to recognize that our mesocosm design differs greatly in a number of ways compared to a natural system, our controlled study provides important insights into how native freshwater mussels alter sediment microbial communities and biogeochemical pathways. Similar to what was found in Nickerson et al., (2019), we found that NH_4^+ excretion was a significant predictor of N-removal potentials in the mussel treatments. In another study, Atkinson & Forshay (2022) found that higher mussel biomass enhanced denitrification rates. Our study controlled biomass in order to elucidate how species identity and community composition contributed to denitrification fluxes, and found that *Lampsilis ornata* individuals had the greatest contribution. In a field study, Trentman et al., (2018) also showed that species identity can play a role in mediating N-cycling pathways; they showed that areas with higher abundances of more mobile species, *A. ligamentina* (*Ortmania ligamentina*) (Allen & Vaughn, 2009), resulted in higher nitrification rates. Similarly, we found that vertical movement was negatively correlated with N-removal potentials, suggesting that bioturbation by mussels has the potential to decrease anaerobic processes like denitrification. Finally, our study corroborates findings from Black et al., (2017), the microbiome of sediments associated with mussels was distinctly different than sediments without mussels.

Conclusions

Within our experimental system, we observed that the presence of mussels enhanced N-removal and decreased the sediment bacteria community diversity. Taken as a whole, we provide

evidence that mussels are important ecosystem engineers by supplying heterotrophic bacteria nutrients and energy needed to perform critical biogeochemical processes. There is growing recognition of the importance of freshwater mussels to ecosystem health and function (Vaughn, 2018), but mussels are one of the most threated faunal groups in the world (Strayer et al., 2004). Bivalve molluscs in both freshwater and marine systems have been shown to provide important ecological functions and services, especially their role in nutrient translocation, storage, and transformation (Vaughn & Hoellein, 2018). Furthermore, previous studies have demonstrated that large aggregations of marine bivalves (the eastern oyster, *Crassostrea virginica*) have been associated with higher rates of sediment denitrification likely as a result of increased OM provided to sediment microbes in biodeposits (Hoellein et al., 2015). Results from our study support that freshwater mussels can influence sediment N-removal and are critical for maintaining this important ecosystem service. Better understanding how species richness and community structure influence nutrient cycling is critical for understanding ecosystem functioning. Therefore, assessing both direct and indirect effects of mussels can aid in conservation efforts by improving the overall understanding of mussel provisioning of ecosystem functioning.

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Table 3.1. Mass-specific excretion and biodeposition rates. Values are given as means (±SE).

		Nutrient Flux (μmol g^{-1} h^{-1})				
Species	**N**	**N Excretion**	**P Excretion**	**C Biodeposition**	**N Biodeposition**	**P Biodeposition**
Cyclonaias kieneriana	6	1.58 (0.15)	0.08 (0.01)	0.97 (0.27)	0.14 (0.03)	0.12 (0.03)
Fusconaia cerina	6	1.35 (0.17)	0.08 (0.01)	1.30 (0.37)	0.17 (0.04)	0.11 (0.02)
Lampsilis ornata	6	2.24 (0.29)	0.07 (0.01)	0.34 (0.05)	0.05 (0.01)	0.03 (0.00)

Table 3.2 Areal excretion and biodeposit nutrient fluxes scaled to the community level. Values are given as means (±SE).

		Nutrient Flux (μmol m^{-2} h^{-1})				
Treatment	**N**	**N Excretion**	**P Excretion**	**C Biodeposition**	**N Biodeposition**	**P Biodeposition**
C	4	116.97 (3.87)	235.40 (28.63)	13.92 (0.51)	2.00 (0.07)	1.83 (0.06)
CF	4	84.37 (4.59)	198.91 (16.10)	15.65 (0.53)	2.21 (0.08)	1.84 (0.07)
F	4	24.26 (0.93)	23.08 (6.86)	10.35 (1.43)	1.44 (0.17)	1.16 (0.06)
L	4	11.74 (0.63)	2.00 (0.41)	6.76 (0.63)	0.89 (0.07)	0.45 (0.04)
LC	4	59.82 (2.60)	91.10 (17.22)	8.73 (0.58)	1.24 (0.08)	1.11 (0.03)
LCF	4	52.82 (0.95)	72.17 (6.59)	12.71 (1.00)	1.75 (0.12)	1.37 (0.02)
LF	4	22.63 (1.23)	19.38 (2.29)	11.08 (1.09)	1.52 (0.14)	1.07 (0.06)

Table 3.3. Pearson correlation coefficients (r^2) for the relation of mussel functional traits to N-removal pathways (DNF, Anammox, and Total N-removal). Values in bold are considered to be significant.

	N-Removal Pathway (μmol N kg^{-1} h^{-1})					
	DNF		Anammox		Total N-removal	
Functional Trait	**r^2**	***p***	**r^2**	***p***	**r^2**	***p***
Vertical Movement	**-0.392**	**0.039**	-0.248	0.203	**-0.391**	**0.040**
Horizontal Movement	-0.226	0.248	-0.209	0.287	-0.236	0.227
Total Movement	-0.332	0.085	-0.228	0.244	-0.334	0.082
N areal excretion	-0.187	0.341	-0.019	0.922	-0.173	0.378
P areal excretion	-0.296	0.126	-0.153	0.438	-0.291	0.132
N:P areal excretion	**0.442**	**0.019**	0.306	0.113	**0.444**	**0.018**
C areal biodeposition	-0.339	0.077	-0.275	0.156	-0.350	0.068
N areal biodeposition	-0.329	0.088	-0.263	0.176	-0.339	0.078
P areal biodeposition	-0.342	0.075	-0.217	0.269	-0.343	0.074
C:N areal biodeposition	0.242	0.215	-0.006	0.975	0.217	0.268
N:P areal biodeposition	0.302	0.118	0.145	0.463	0.293	0.131
C:P areal biodeposition	0.297	0.124	0.116	0.558	0.284	0.143
Total OM biodeposition	-0.350	0.068	-0.272	0.162	-0.359	0.061

Table 3.4. Percent relative abundances of bacterial phyla found in mesocosm sediment (only top five phyla from each treatment are listed). Abundances are based on sequence data.

	Day 0		Day 14		Day 42	
Treatment	**Bacterial Phylum**	**Percent Abundance**	**Bacterial Phylum**	**Percent Abundance**	**Bacterial Phylum**	**Percent Abundance**
NM	Proteobacteria	41.18	Proteobacteria	40.84	Proteobacteria	24.41
	Unclassified	12.26	Unclassified	15.37	Unclassified	17.61
	Planctomycetes	11.02	Planctomycetes	12.08	Planctomycetes	15.99
	Acidobacteria	10.42	Bacteroidetes	10.95	Cyanobacteria	12.32
	Bacteroidetes	7.94	Cyanobacteria	5.19	Bacteroidetes	8.88
L	Proteobacteria	47.4	Proteobacteria	38.47	Planctomycetes	24.03
	Unclassified	10.44	Unclassified	17.49	Proteobacteria	20.76
	Acidobacteria	9.86	Planctomycetes	12.91	Unclassified	15.31
	Planctomycetes	9.34	Bacteroidetes	11.27	Cyanobacteria	11.38
	Bacteroidetes	6.04	Verrucomicrobia	4.87	Bacteroidetes	8.10
C	Proteobacteria	46.92	Proteobacteria	40.48	Planctomycetes	22.11
	Unclassified	15.28	Unclassified	13.89	Proteobacteria	18.35
	Planctomycetes	9.32	Planctomycetes	11.79	Bacteroidetes	15.72
	Acidobacteria	8.3	Bacteroidetes	10.10	Unclassified	15.51
	Bacteroidetes	7.16	Cyanobacteria	7.26	Cyanobacteria	12.10
F	Proteobacteria	39.42	Proteobacteria	41.28	Proteobacteria	21.43
	Unclassified	15.06	Unclassified	14.80	Planctomycetes	20.69
	Acidobacteria	11.1	Planctomycetes	12.07	Unclassified	16.46
	Planctomycetes	8.96	Bacteroidetes	10.62	Cyanobacteria	15.59
	Bacteroidetes	5.52	Cyanobacteria	6.29	Bacteroidetes	13.93
LC	Proteobacteria	45.24	Proteobacteria	40.93	Proteobacteria	22.62
	Unclassified	18.22	Planctomycetes	14.46	Planctomycetes	20.39
	Planctomycetes	10.62	Unclassified	14.17	Unclassified	18.82
	Bacteroidetes	7.08	Bacteroidetes	12.33	Bacteroidetes	14.08
	Cyanobacteria	4.56	Verrucomicrobia	5.48	Cyanobacteria	7.88
LF	Proteobacteria	39.16	Proteobacteria	37.13	Unclassified	20.88
	Unclassified	13.88	Unclassified	17.33	Proteobacteria	20.22

	Acidobacteria	12.3	Planctomycetes	14.27	Planctomycetes	19.90
	Planctomycetes	8.24	Bacteroidetes	10.95	Bacteroidetes	13.45
	Bacteroidetes	7.72	Cyanobacteria	5.09	Cyanobacteria	10.79
CF	Proteobacteria	58.28	Proteobacteria	38.19	Planctomycetes	25.79
	Unclassified	10.72	Unclassified	16.42	Proteobacteria	21.61
	Bacteroidetes	7.84	Bacteroidetes	14.35	Unclassified	17.79
	Planctomycetes	7.26	Planctomycetes	11.87	Bacteroidetes	10.93
	Acidobacteria	5.08	Verrucomicrobia	6.20	Cyanobacteria	7.29
LCF	Proteobacteria	41.04	Proteobacteria	37.89	Proteobacteria	23.02
	Unclassified	15.06	Planctomycetes	14.83	Unclassified	20.35
	Acidobacteria	11.02	Unclassified	14.13	Planctomycetes	19.36
	Planctomycetes	9.14	Bacteroidetes	12.32	Bacteroidetes	15.66
	Bacteroidetes	7.58	Verrucomicrobia	6.08	Acidobacteria	5.58

Table 3.5. Percent relative abundances of bacteria genera that are associated with major biogeochemical transformations in the N-cycle across sampling weeks and treatments. Biogeochemical cycles represented are: AOB; ammonium-oxidizing bacteria, NOB; nitrite-oxidizing bacteria, and Anammox bacteria. Abundances are based on sequence data.

			Percent Relative Abundance							
Week	**Taxon**	**N-cycle role**	**NM**	**L**	**C**	**F**	**LC**	**LF**	**CF**	**LCF**
0	Nitrosomonas *spp.*	AOB	0.000	0.000	0.000	0.020	0.000	0.000	0.000	0.020
	Nitrospira *spp.*	NOB	0.240	0.160	0.620	0.700	0.600	0.620	0.060	0.280
	Candidatus Brocadia *spp.*	Anammox	0.000	0.000	0.000	0.000	0.000	0.020	0.000	0.000
2	Nitrosomonas *spp.*	AOB	0.005	0.000	0.005	0.000	0.000	0.005	0.000	0.007
	Nitrospira *spp.*	NOB	0.105	0.075	0.190	0.115	0.130	0.125	0.140	0.253
	Candidatus Brocadia *spp.*	Anammox	0.095	0.020	0.020	0.010	0.040	0.025	0.040	0.087
6	Nitrosomonas *spp.*	AOB	0.000	0.010	0.000	0.000	0.005	0.005	0.007	0.000
	Nitrospira *spp.*	NOB	0.485	0.630	0.240	0.080	0.535	0.265	0.260	0.425
	Candidatus Brocadia *spp.*	Anammox	0.005	0.005	0.000	0.000	0.010	0.000	0.020	0.000

Figure 3.1. Mass -specific A) carbon, B) nitrogen, C) phosphorous, and D) total biodeposition rates for our three study taxa (n=6). Different letters indicate significant differences among species based on Tukey's HSD.

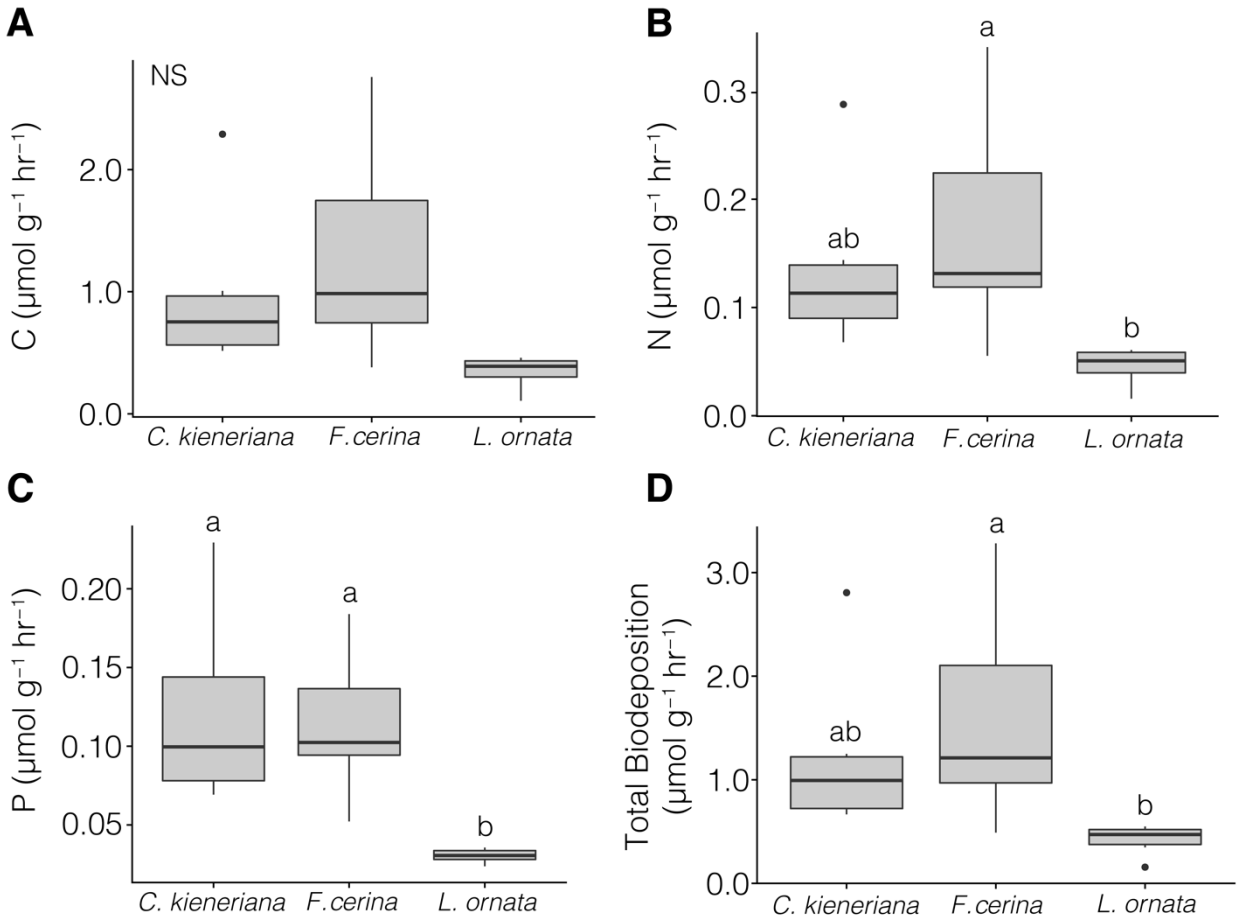


Figure 3.2. Mass -specific A) nitrogen and B) phosphorous excretion rates for our three study taxa (n=6). Different letters indicate significant differences among species based on Tukey's HSD.

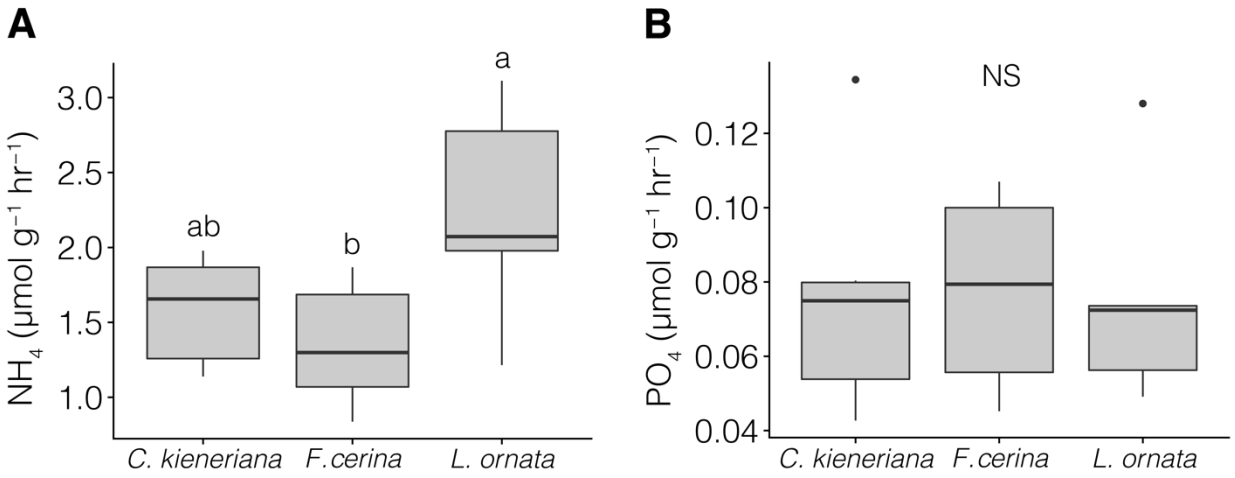


Figure 3.3. Mean (±SE) freshwater mussel A) N:P areal excretion and B) C:N, C) N:P, and D) C:P areal biodeposition stoichiometric ratios scaled to the community level. Different letters indicate significant differences among treatments based on Tukey's HSD.

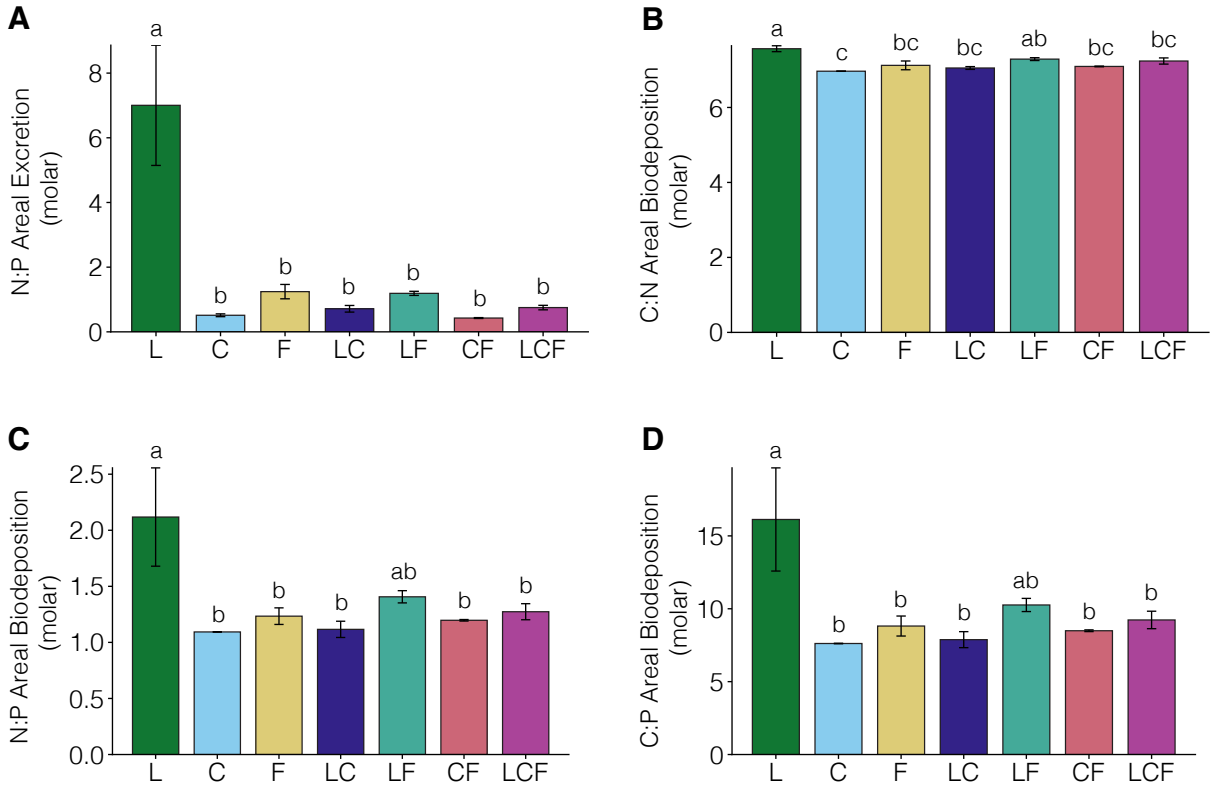


Figure 3.4. Mean (±SE) A) horizontal, B) vertical, and C) total movement of each individual mussel used in our experiment. NS denotes no significance.

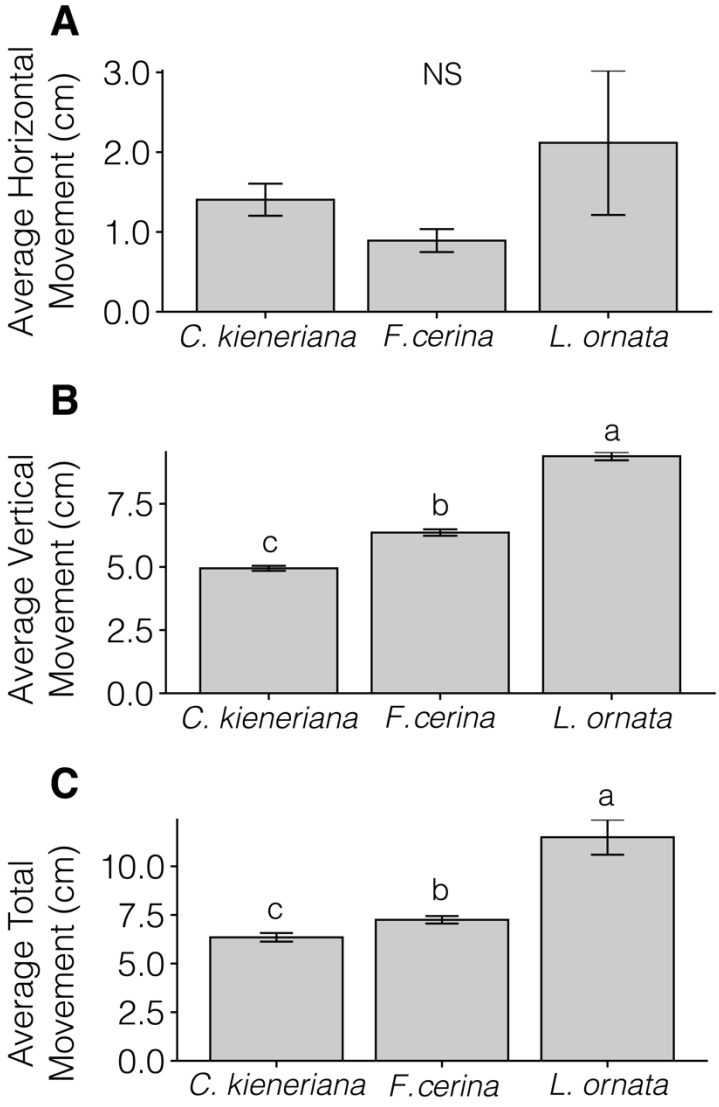


Figure 3.5. Mean (±SE) N-removal potentials of A) DNF, B) Anammox, and C) total combined N- removal across all treatments (n=4). Different letters indicate significant differences among treatments based on Tukey's HSD.

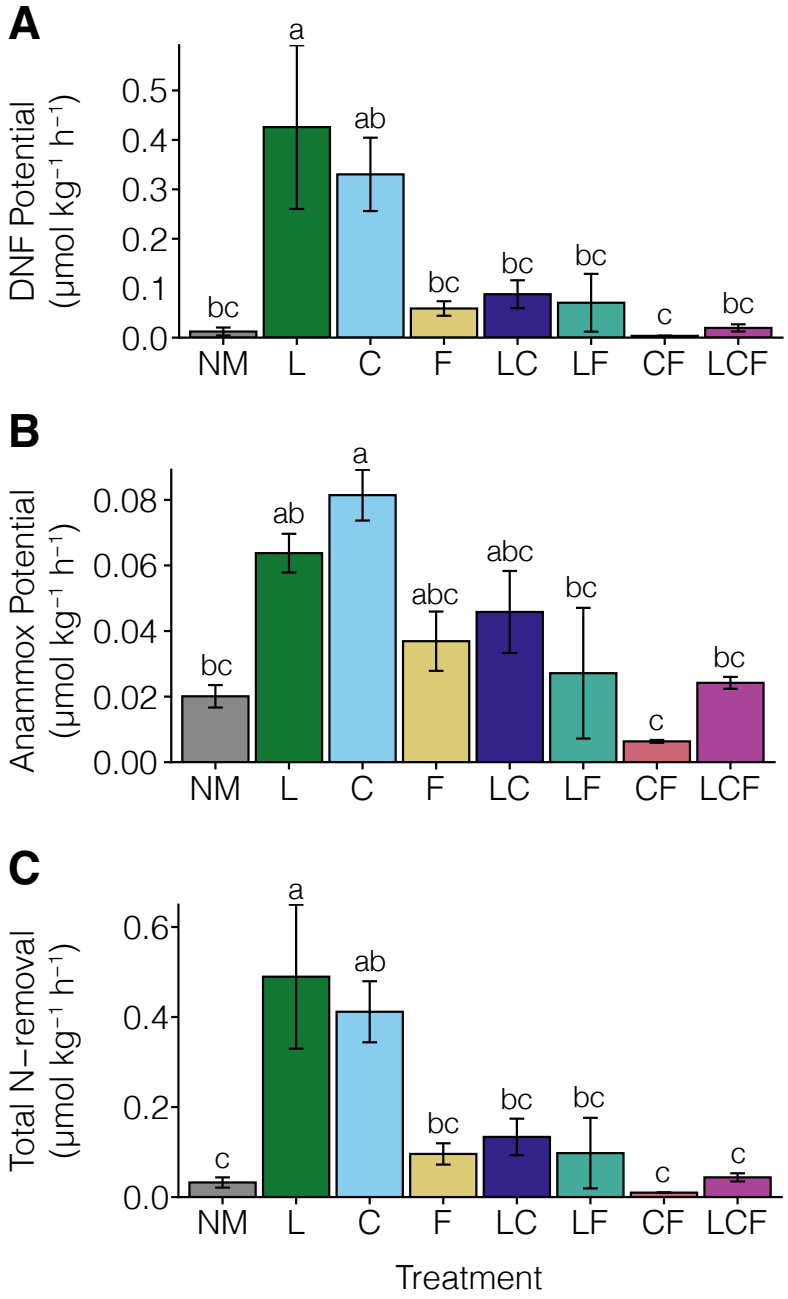


Figure 3.6. A) Pearson correlation plot showing the relation of mussel functional traits to N-removal pathways (DNF, Anammox, and Total N-removal) with size of the circle representing proximity to p=0.05. Correlation coefficients (r^2) are shown for significant relationships ($p<0.05$) with B) N:P areal excretion, and C) Vertical Movement to total combined N-removal potentials across all mussel treatments (n=4). Areal excretion is abbreviated as Exc and areal biodeposition is abbreviated as BD.

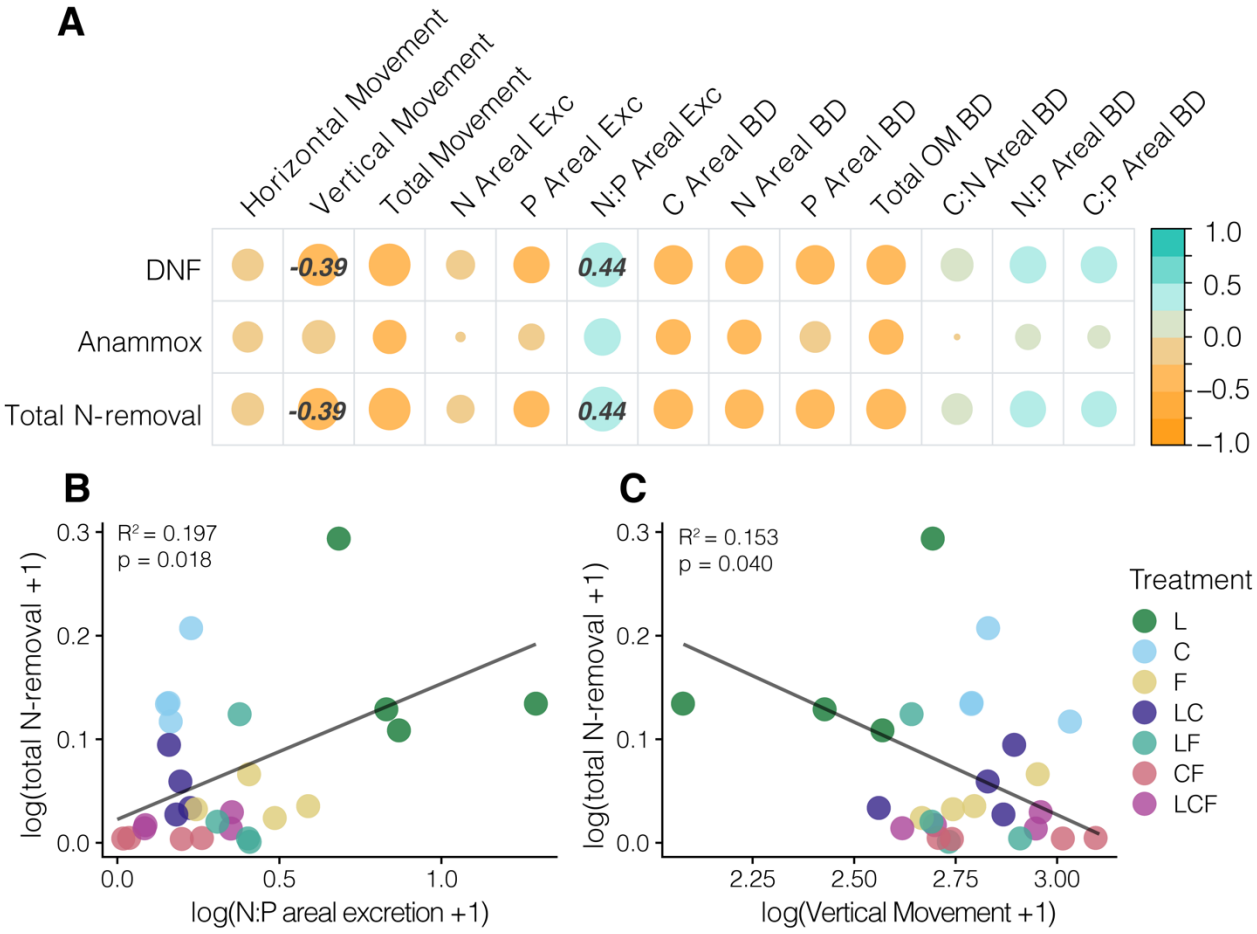


Figure 3.7. Bacterial phyla in the sediment among treatments over three sampling time points as determined by 16S rRNA gene sequencing. Stacked bar plots represent eleven most abundant phyla with taxa represented by <2% of sequences grouped as Other.

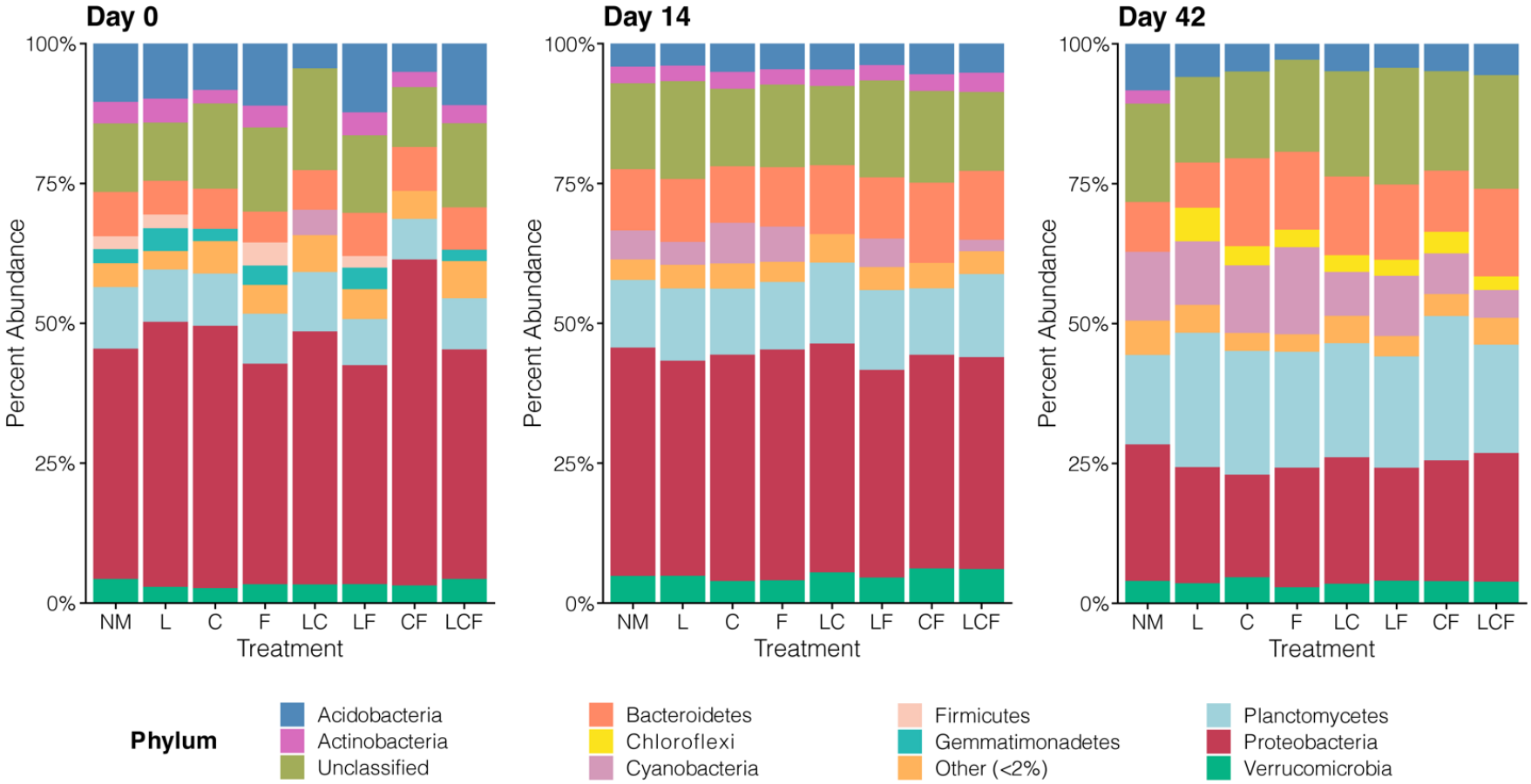


Figure 3.8. Scatter plots representing alpha diversity indices of A) Shannon's diversity, B) Chao1, and C) Inverse Simpson across all treatments at each sampling time point.

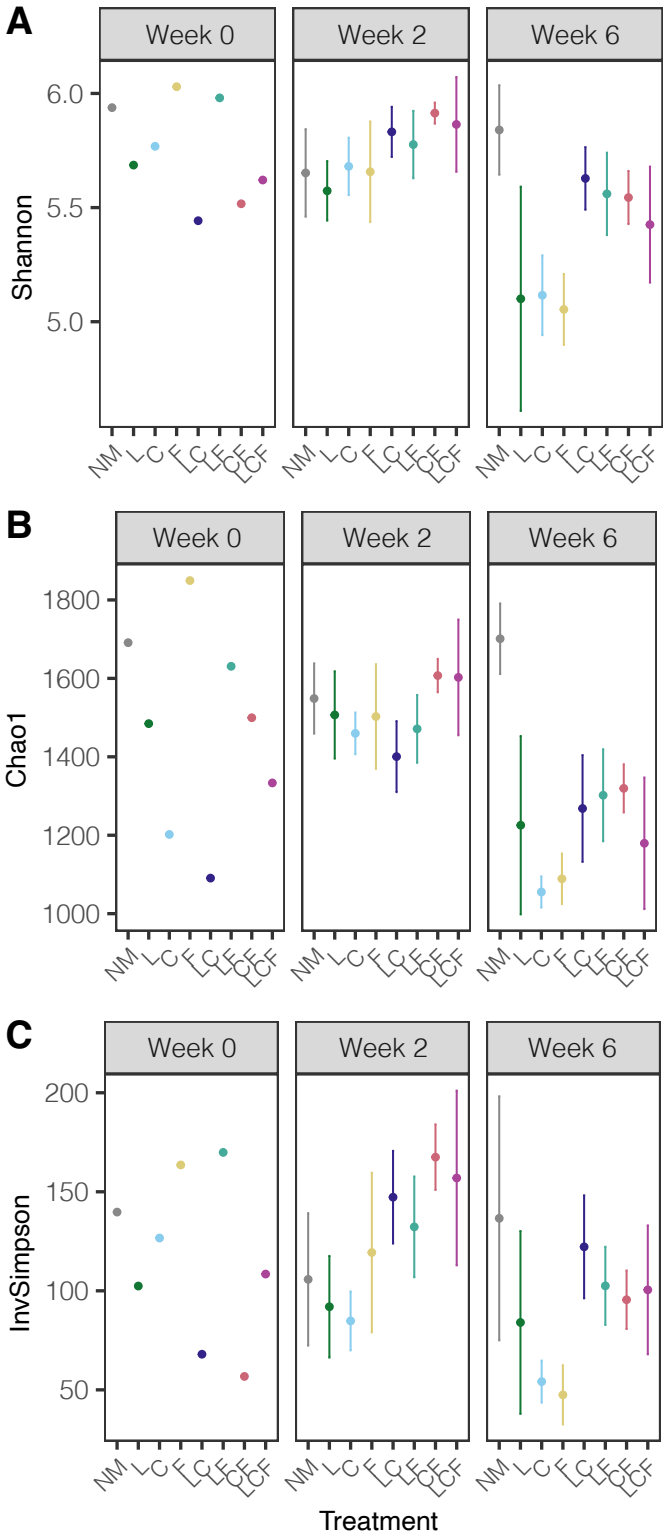


Figure 3.9. Principle coordinates analyses of sediment microbial communities based on Bray-Curtis dissimilarities between mesocosm treatments over all sampling timepoints (A) and at the end of six weeks (B).

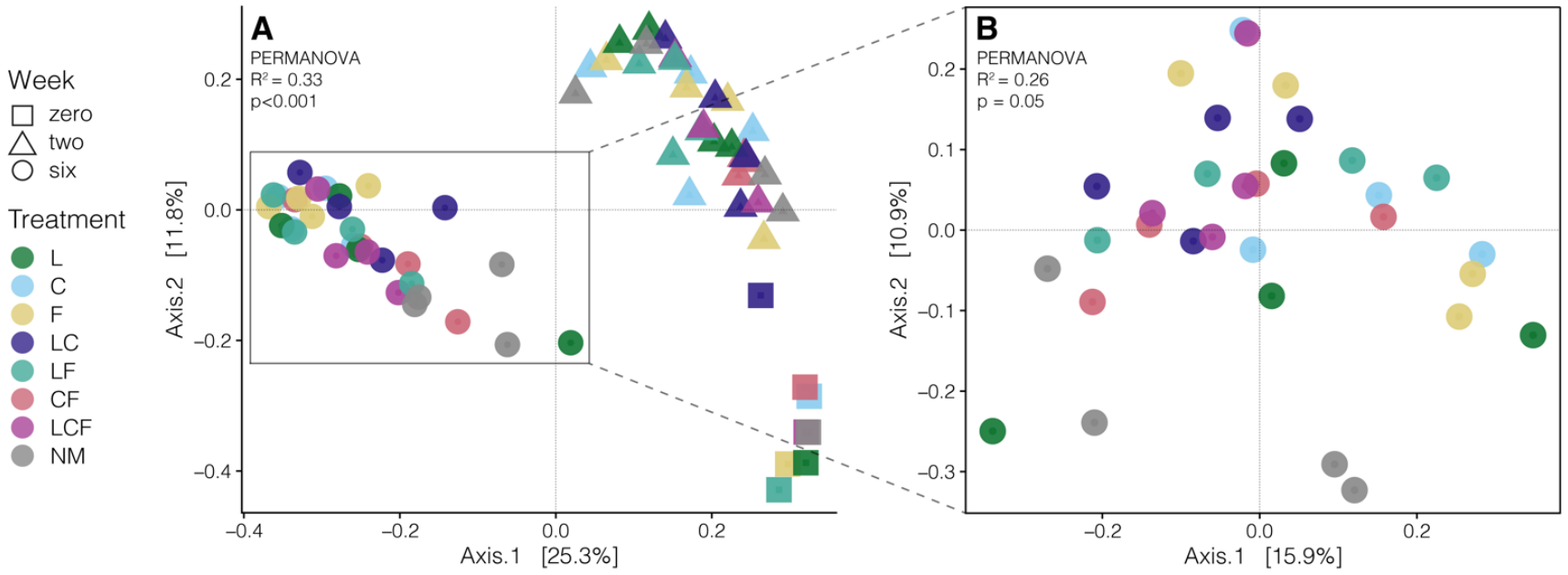
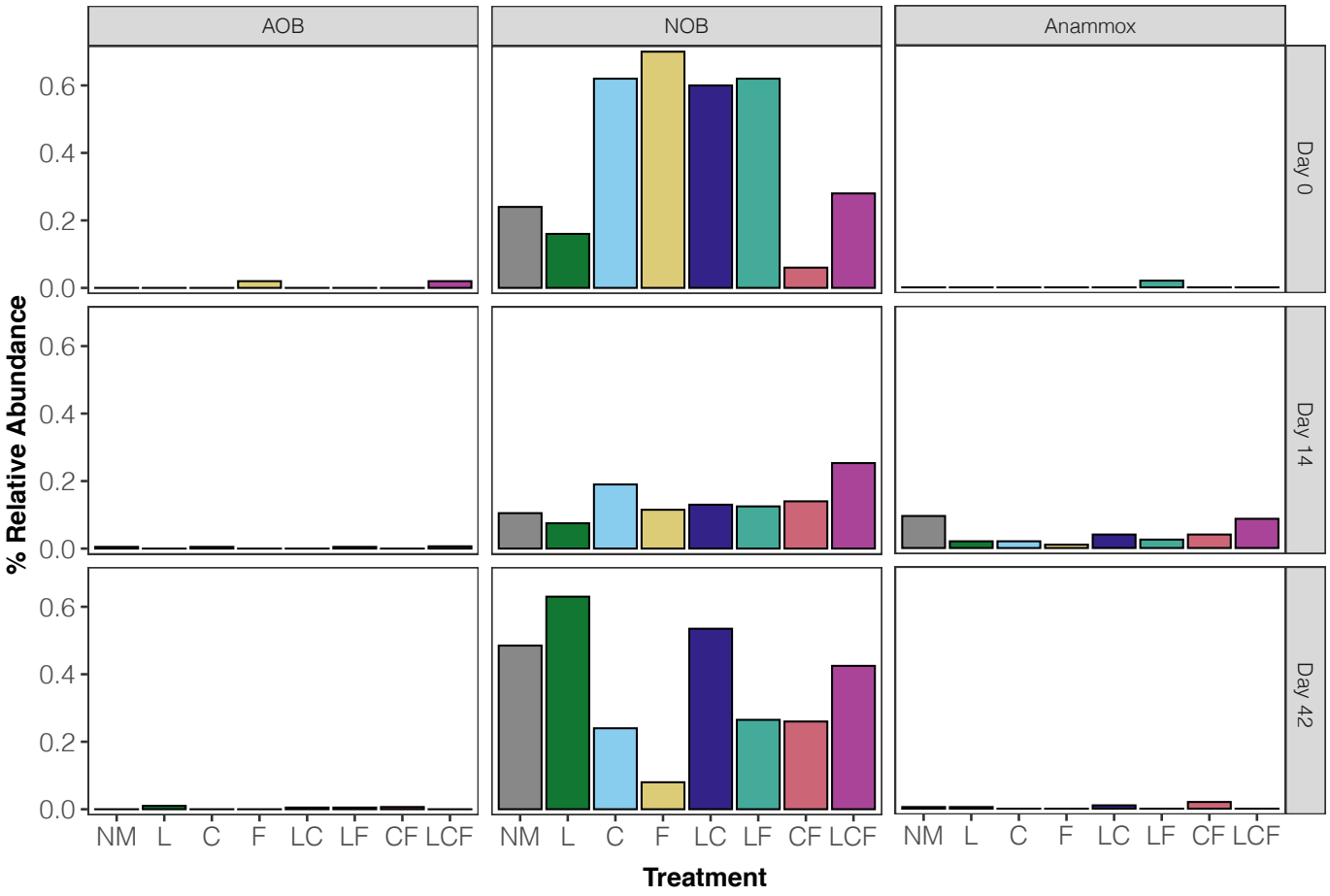


Figure 3.10. Percent relative abundances of bacteria genera that are associated with major biogeochemical transformations in the N-cycle across sampling weeks and treatments. Biogeochemical cycles represented are: AOB; ammonium-oxidizing bacteria, NOB; nitrite-oxidizing bacteria, and Anammox bacteria.



APPENDIX

Figure A1. Water temperature (°C) in the mesocosms throughout the length of the study. Prior to beginning the study, we randomly selected 12 mesocosms into which we placed a HOBO temperature logger (Hobo U20L, Onset Corp, Bourne, MA) that collected water temperature measurements at the rate of one record h-1.

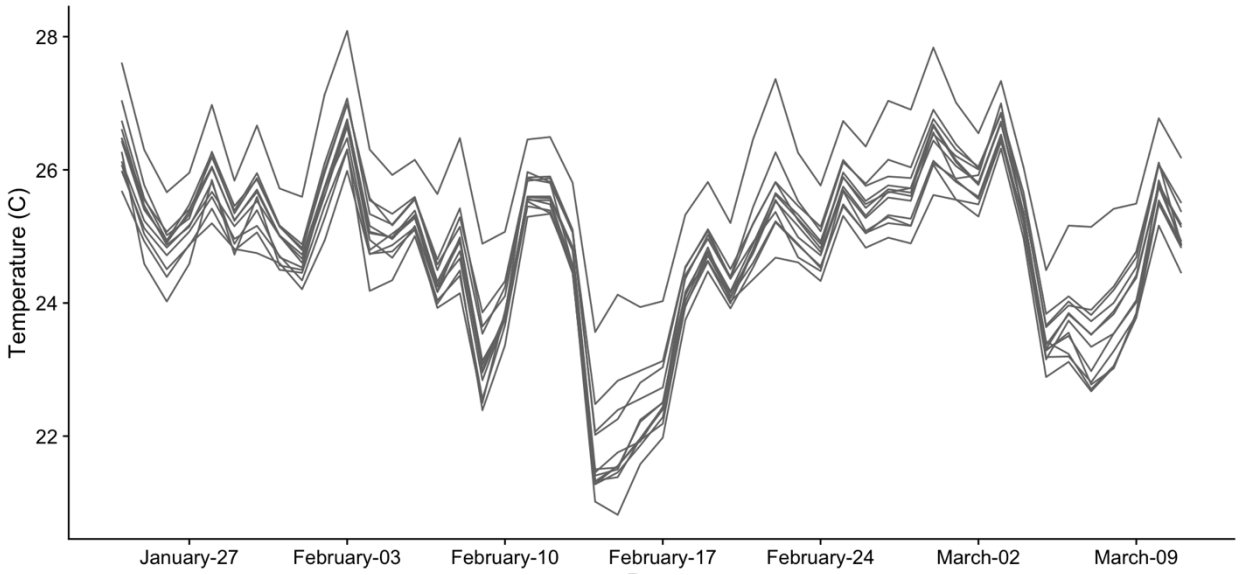


Figure A2. Considerable algal biofilm accrual on benthic substrate and on the shell of a mussel (*L. ornata*) partially buried in the sediment.