Freshwater mussels enhance sediment nitrogen-removal potentials and alter bacterial communities via nutrient release and bioturbation

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Abstract: The stream benthic substrate is associated with ecosystem functioning and provides important habitat for a multitude of organisms from microbes to larger macrofauna. Microbially mediated biogeochemical transformations are critical ecological processes that occur within benthic sediments. Benthic macrofauna, such as freshwater mussels (Bivalvia: Unionoida), can influence these processes by chemically and physically altering the stream substrate, consequently altering redox conditions and the sediment microbial community. However, few studies have investigated the interactions between burrowing macrofauna, benthic nutrient fluxes, and the associated microbial communities in freshwater substrata. We used a mesocosm experiment to investigate how mussels physically (via bioturbation) and chemically (via excretion and egestion) influence sediment–water nutrient fluxes and sediment bacterial community composition. Because many microbially mediated N-transforming pathways can be stimulated by mussel-derived nutrient inputs (excreted NH_4^+ or organic matter biodeposits) or altered by bioturbation, we predicted that mussels would enhance sediment N-removal potentials and alter the sediment microbial community structure, but that these effects would be mediated by mussel species identity and functional traits. Overall, the presence of mussels enhanced N-removal and modified sediment bacteria community composition and structure. The differences across mussel treatments in sediment N-removal potentials and alpha diversity of bacterial communities suggest that mussel species identity and assemblage composition play a critical role at the benthic–water-column interface in streams. Given the current declines in mussel species richness and abundance, a better understanding of the functional effects of mussel species and assemblage composition is critical to predicting changes in the functioning of stream ecosystems.

Key words: unionid, microbiome, biogeochemical cycling, denitrification, anammox, excretion

Aquatic ecosystems harbor a disproportionately large amount of global biodiversity and provide a variety of ecosystem services, from material goods and food production to water purification and nutrient cycling (Reid et al. 2019, Lynch et al. 2023). In particular, the benthic substrate of streams provides habitat for a multitude of organisms ranging from microbes to larger macrofauna (Moore 2006, Lowell et al. 2009) and can be associated with key ecosystem functions, such as biogeochemical cycling (Krause et al. 2017). Specifically, biogeochemical cycling is often a major driver of stream ecosystem processes, such as primary and secondary production (Grimm and Fisher 1986, Cross et al. 2006). Hotspots of biogeochemical activity occur because of nutrient loading in the riparian zone (McClain et al. 2003), biotic and abiotic controls of nutrient retention time and spiraling (Newbold et al. 1981, Grimm et al. 2005), and strong redox gradients found in the benthic substratum that result in microbially active sites (Boulton et al. 1998, Groffman et al. 2009, Lau et al. 2017).

Biogeochemical cycles are closely linked to microbial communities and are controlled by interactions and feedback

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between physical and biological processes. In streams, microbially mediated transformations encompassing the N cycle are critically important because N is a limiting nutrient within downstream estuarine and marine waterbodies (Grimm and Fisher 1986, Vitousek and Howarth 1991, Rabalais et al. 2002). Control of the benthic N-cycle is dominated by redox gradients in the sediment profile where different microbes dominate based on redox potential. N-removal via denitrification and anaerobic ammonium oxidation (anammox), in particular, are important biogeochemical processes because they result in loss of N from aquatic ecosystems. In both processes, specialized groups of N-transforming microbes facilitate the movement of reactive N through the cycle. In denitrification, 2 molecules of NO_3^- are converted to N_2, whereas in anammox, 1 molecule of NO_2^- (derived from NO_3^-) combines with 1 molecule of NH_4^+ to form N_2. As a result, anammox is twice as efficient as denitrification at permanently removing N from the system (Knowles 1982, Burgin and Hamilton 2007). Critical factors that regulate heterotrophic microbial activity specifically involved in the N cycle include redox conditions and the availability of organic matter (OM) and reactive N (NO_3^-) (Knowles 1982, Burgin and Hamilton 2007). In addition to microbes, aquatic macrofauna also mediate biogeochemical processes in the N cycle by chemically and physically altering the sediment through bioturbation, sediment formation and stabilization, and release of waste products (Hoellein and Zarnoch 2014, Turek and Hoellein 2015, Nickerson et al. 2019).

Aggregations of animals at high densities can affect biogeochemical cycling through both direct and indirect effects by creating hotspots of biogeochemical activity (Vanni 2002, Atkinson et al. 2017a). Direct effects can include transformations of nutrients during ingestion and consequential excretion of bioavailable nutrients and egestion of particulate wastes, thereby affecting C, N, and P cycles (Vanni et al. 2006, Atkinson and Vaughn 2015, Hopper et al. 2021, Uno et al. 2022). Indirect effects can include affecting nutrient cycling through impacts on prey or physical habitat structure, such as size-selective predation and ecosystem engineering (Atkinson et al. 2017a), and studies have highlighted the effects of bioturbation on N-cycling pathways (Nogaro and Burgin 2014, Benelli et al. 2019, Nickerson et al. 2019). Large aggregations of animals promote biogeochemical heterogeneity by modifying their chemical and physical environment, which results in altered bioavailable nutrient fluxes, strengthened nutrient cycling pathways, and modified redox conditions (Atkinson et al. 2013, 2018, Hopper et al. 2021).

Freshwater mussels (Bivalvia: Unionoida) are a guild of burrowing bivalves that can be found in high density, patchy aggregations in many freshwater systems, where they live partially or completely burrowed in benthic sediments (Vaughn and Hakenkamp 2001, Haag 2012, Atkinson and Vaughn 2015). Mussels couple the benthic compartment and the water column in stream ecosystems by filter feeding from the water column and transferring nutrients and energy to stream sediments through biodeposition of feces and pseudofeces (Spooner and Vaughn 2008, Atkinson et al. 2011, Vaughn and Hoellein 2018). Mussels excrete highly reactive N in the form of NH_4^+ and protein-like dissolved organic matter (DOM), and they biodeposit labile OM as feces and pseudofeces within or near the stream sediments (Nickerson et al. 2019, Hopper et al. 2021, Atkinson and Forshay 2022). Mussel excretion can influence N-removal potentials by providing reactive N for microbes, and mussel feces and pseudofeces may foster denitrification by providing OM as a substrate to microbes (Trentman et al. 2018, Nickerson et al. 2019). Furthermore, OM decomposition through aerobic respiration can create anoxic environments favoring N-removal. However, the act of burrowing can increase O_2 penetration into the sediment and disrupt the redox gradient, potentially reducing N-removal potentials and providing sites for nitrification (Turek and Hoellein 2015, Trentman et al. 2018). Therefore, mussel burrowing behavior and position in the sediment could play a critical role in N-removal.

Despite being classified in the same functional group (filter-feeding bivalves), different mussel species within the same system vary in nutrient excretion and egestion rates and stoichiometries (Spooner and Vaughn 2008, Atkinson et al. 2010, 2020b), feeding and assimilation (Fogelman et al. 2022, Sánchez González et al. 2023), burrowing behavior (Schwalb and Pusch 2007, Allen and Vaughn 2009), and gut microbiomes (Weingarten et al. 2019, McCauley et al. 2021). Because species' functional characteristics strongly influence ecosystem properties through niche complementary or synergistic interactions, greater species richness may enhance ecosystem function and stability by increasing the likelihood that species will differentially utilize resources (Loreau and de Mazancourt 2013). Therefore, greater mussel functional trait diversity could influence ecosystem functions via differential nutrient release and bioturbation. However, freshwater mussels are experiencing an extinction crisis (Haag and Williams 2014), with projections of extinction rates to be as much as 50% of remaining species over the next century (Ricciardi and Rasmussen 1999). Previous declines in mussel biodiversity coupled with future reductions could have strong, but unknown, implications for stream ecosystem functioning.

Here, we used a substitutive approach in a mesocosm experiment to assess how mussels physically (via bioturbation) and chemically (via excretion and egestion) influence sediment–water nutrient fluxes and sediment microbiome community composition. Because many of the microbially mediated biogeochemical processes rely on mussel-derived nutrient inputs (excreted NH_4^+ and soluble reactive P [SRP] or OM biodeposits), we predicted that 1) compared with 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) N-removal potentials will vary with different mussel assemblages as a result of species-specific functional traits of nutrient excretion/egestion rates and stoichiometry (N:P) and burrowing behavior; and 3) sediment microbial community structure will vary across treatments as a result of different mussel assemblage composition and associated N-transforming microorganisms (Fig. 1).

METHODS

To investigate the effect of mussel functional diversity on ecosystem processes, we used 32 recirculating mesocosms to mimic natural stream environments. Using these systems allowed us to control environmental variables that are often difficult to constrain in field-based studies. To disentangle multispecies interactions and isolate species-specific effects on key components of ecosystem functioning, we used 3 different mussel species in an 8-treatment design with 4 replicates each. Each mussel treatment consisted of 1 of 7 possible combinations of 1-, 2-, and 3-species communities plus a no-mussel control. At weekly intervals and at the end of the experiment we measured mussel movement and nutrient release as well as sediment nutrient fluxes and sediment bacteria composition. We used analysis of variance (ANOVA) and permutation-based analysis of variance (PERMANOVA) to assess the physical and chemical influence of each mussel assemblage on ecosystem functioning.

Study organisms

We collected mussels, riverbed sediment, and water from the Sipsey River, a 5th-order tributary of the Tombigbee River in Alabama, USA, and part of the Mobile River basin. The Sipsey River is relatively unmodified by human disturbances and harbors diverse and abundant freshwater mussel assemblages (Haag and Warren 2010). We used 3 abundant mussel species that occur in distinct evolutionary tribes: *Cyclonaias kieneriana* (Lea, 1852) (Tribe Quadrulini), *Fusconaia cerina* (Conrad, 1838) (Tribe Pleurobemini), and *Lampsilis ornata* (Conrad, 1835) (Tribe Lampsilini). These 3 species vary in shell morphology, life-history traits, and tissue and excretion stoichiometry (Williams et al. 2008, Haag 2012, Atkinson et al. 2020b).

We collected a total of 183 mussels on 20 to 22 November 2019 and transported them back to the lab where they were held in Living Stream Systems® (Frigid Units Inc., Toledo, Ohio). We measured each mussel and used standardized length–dry mass regressions to estimate mass (Atkinson et al. 2020a). We tagged each mussel with an individual identification tag by attaching 15 cm of fly line to the posterior end of each individual to track movement and burial depth (Fig. S1). We held mussels at 10°C in the Living Stream Systems until the beginning of the experiment in January 2020, and we fed them with algae cultured from Sipsey River water 3×/wk.

Mesocosm design

We used 200-L recirculating stream mesocosms (81 × 51 × 48 cm), which consisted of 2 tanks: an open-ended plastic

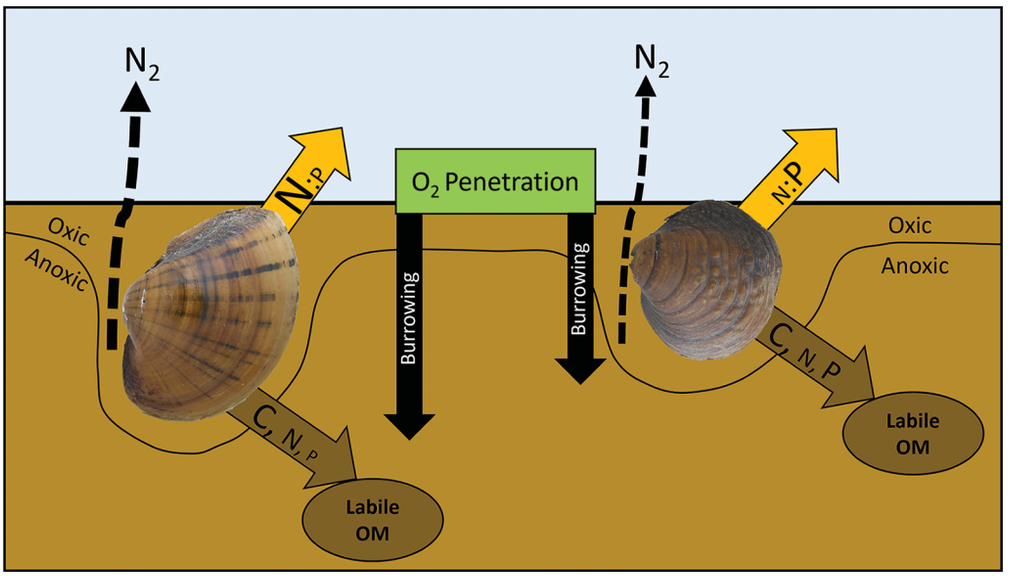

Figure 1. Conceptual figure highlighting that mussel species excrete and egest nutrients at different ratios and burrow to different depths, both which can result in differences in redox conditions, the sediment microbial community, and N-removal rates (prediction 1). Mussel species that excrete at higher N:P ratios (left) may result in higher N-removal rate potentials (prediction 2). Further, species that burrow deeper and biodeposit at higher rates have a greater potential for altering redox conditions and the microbial community at these sites (prediction 3).

liner placed inside a fiberglass outer tank. We placed the inner liner atop bricks to allow water recirculation (as in Nickerson et al. 2021) and filled the bed of the liner with a mix of pea gravel and sand from the Sipsey River and supplemented with purchased Vigoro® pea gravel pebbles (Vigoro Corporation, Chicago, Illinois). We filled each liner with water from the Sipsey River to a depth of 35 cm and used 47-W magnetic drive pumps (Danner Manufacturing, Inc., Islandia, New York) to recirculate the water at a rate of 2527 L/h. 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 2 tanks and flow directly over the pea gravel. To further facilitate a natural microbial community in the mesocosms, we assembled 32 mixed-species leaf-litter bags containing ~1.5 g of leaf tissue from tulip poplar (*Liriodendron tulipifera*), American sycamore (*Platanus occidentalis*), and black willow (*Salix nigra*), which are common riparian trees along the Sipsey River, and left them in the stream for 12 d. Five d before mussels were added to the mesocosms, we added 1 of these preconditioned mixed-species litter bags to inoculate each mesocosm.

To assess the effect of functional trait diversity on ecosystem functions, we manipulated mussel diversity through 8 treatment combinations: 3 single-species treatments (*L. ornata, C. kieneriana*, or *F. cerina*; hereafter L, C, or F), 3 three-species treatments (*L. ornata* + *C. kieneriana*, *L. ornata* + *F. cerina, C. kieneriana* + *F. cerina*; hereafter LC, LF, CF), 1 multi-species treatment (*L. ornata* + *C. kieneriana* + *F. cerina*; hereafter LCF), and 1 control treatment (no mussels; hereafter NM) (Table 1). Each treatment was replicated in 4 mesocosms ($n = 32$). We implemented a substitutive design in which each treatment assemblage contained the same mussel biomass (7.38 ± 0.15 g; ANOVA $F_{6,21} = 0.87$, $p = 0.53$), and we used a random number generator to randomly assign individuals to each of the 7 mussel treatments and to mesocosms. Mesocosms were maintained for 42 d (mussels were added on d 0) in the greenhouse on the main campus of the University of Alabama in Tuscaloosa, Alabama from January to March of 2020. We conducted weekly water changes (~10–15%) for the duration of the experiment in which we emptied 20 L of water from each tank and replaced it with 20 L of Sipsey River water, and each tank received 500 mL of a concentrated mixed algal assemblage cultured from natural river water 2×/wk. We used a Hobo® U20L temperature logger (Onset® Corporation, Bourne, Massachusetts) to monitor water temperature every 60 min in 12 mesocosms randomized across treatments and rows in the greenhouse (Fig. S2). We collected water chemistry samples bi-weekly to monitor background conditions (Fig. S3A–D).

Mussel excretion and biodeposition

After 42 d, we estimated N and P excretion for each assemblage by randomly subsampling 6 mussels of each species and measuring their N (NH_4^{+}) and P (SRP) excretion rates. To control for biofilm uptake, we used a toothbrush and scour pad to gently scrub each mussel and placed each individual in excretion chambers. Depending on mussel size, we filled the container with 300 or 500 mL of filtered mesocosm water (GF/F; 0.7-μm pore size; MilliporeSigma, Burlington, Massachusetts). We used 3 control chambers without mussels. After 1 h, we gently removed each mussel, filtered the chamber water (GF/F; 0.7-μm pore size; MilliporeSigma), and stored 30 mL of the sample at −20°C until analysis. We retained filters to estimate biodeposition rates (mg nutrient mg dry mass^{-1} h^{-1}) and %C, %N, and %P content. We used a Seal AQ300 discrete analyzer (Seal Analytical, Mequon, Wisconsin) to analyze SRP with the colorimetric method (Murphy and Riley 1962) and NH_4^{+} with the phenol method for filtered excretion samples (Eaton et al. 1995). We calculated mass-specific excretion rates for each mussel (μmol NH_4^{+} g^{-1} h^{-1}) with the concentration of NH_4^{+} in the excretion chamber (μg/L), the known volume of water in the chamber, and the amount of time the mussel incubated (1 h), corrected for the controls. We calculated mussel biodeposition

Table 1. Estimated areal excretion and biodeposition nutrient fluxes of mussels scaled to the assemblage level for each of the mussel treatments (L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, F = *Fusconaia cerina*) and multi-species treatments after the 42-d experiment period. 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	117.0 (3.9)	235.0 (28.6)	13.9 (0.5)	2.0 (0.1)	1.8 (0.06)
F	4	24.3 (0.9)	23.1 (6.9)	10.4 (1.4)	1.4 (0.2)	1.2 (0.06)
L	4	11.7 (0.6)	2.0 (0.4)	6.8 (0.6)	0.9 (0.1)	0.5 (0.04)
LC	4	59.8 (2.6)	91.1 (17.2)	8.7 (0.6)	1.2 (0.1)	1.1 (0.03)
LF	4	22.6 (1.2)	19.4 (2.3)	11.1 (1.1)	1.5 (0.1)	1.1 (0.06)
CF	4	84.4 (4.6)	199.0 (16.1)	15.7 (0.5)	2.2 (0.1)	1.8 (0.07)
LCF	4	52.8 (0.9)	72.2 (6.6)	12.7 (1.0)	1.7 (0.1)	1.4 (0.02)

rates from the filters, which we dried (48 h, 50°C), weighed, then combusted (2 h, 500°C), and weighed again to calculate ash-free dry mass (AFDM) and to calculate total OM of the biodeposits. We then subsampled biodeposit samples to analyze %C, %N, and %P. To calculate %C and %N, we analyzed samples with a Carlo Erba CHNS-O EA1108-Elemental Analyzer (Isomass Scientific Inc., Calgary, Alberta, Canada). To calculate %P, samples were digested with HCl and analyzed for SRP. We used mass-specific hourly rates of excretion and biodeposition to estimate the assemblage-scale rates for each of our mesocosm treatments. Following methods similar to Atkinson and Forshay (2022), we calculated areal excretion rates of N and P and biodeposition of C, N, and P (μmol nutrient m^{-2} h^{-1}) by multiplying the species-specific population biomass (i.e., for the species combinations in each mesocosm) by the per capita excretion or biodeposition rate summed across all species for each treatment.

We tested whether there was an effect of mussel species or assemblage composition (i.e., treatment) on mass-specific and areal excretion and biodeposition rates with 1-way ANOVA. For ANOVA tests with a p-value of ≤0.05 we followed the test with post hoc Tukey's honestly significant difference (HSD) pairwise tests between treatments. We transformed excretion and biodeposition rates by the natural log to meet assumptions of normality and homoscedasticity. We performed statistical analyses, including analyses described below, in R (version 4.1.2; R Project for Statistical Computing, Vienna, Austria).

Mussel movement

To examine the impact of bioturbation on sediment nutrient fluxes and microbiome community, we tracked mussel movement and burrowing behavior. We recorded the location and depth of each individual mussel 3×/wk for the duration of the study by locating the tagged fly line on each mussel. We determined mussel location with a grid of sixty 3-cm^2 cells constructed from a PVC pipe frame. We calculated horizontal movement between grid points of consecutive days with the distance formula via the Pythagorean Theorem. We determined mussel burial depth by measuring the length of fly line exposed from the sediment. To account for differences in mussel body size, we calculated burial depth 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. We calculated vertical movement via the absolute value of the start and end burial depth for each day. Following methods similar to Allen and Vaughn (2009), we defined total mussel movement as the sum of horizontal and vertical movement measurements throughout the duration of the experiment (42 d). Finally, we tested whether total mussel movement differed among treatments with 1-way ANOVA followed by post hoc Tukey's HSD test when ANOVA tests had a p-value of ≤0.05.

Sediment N-removal potentials

To examine the influence of mussels on the biogeochemical pathways of denitrification and anaerobic NH_3 oxidation, we used isotope-pairing techniques to analyze N-removal potential in sediment slurries (Thamdrup and Dalsgaard 2002). At the end of the experiment (42 d), we extracted sediment cores (20 × 8 cm) from the top 20 cm of each mesocosm, then homogenized and wet-sieved (2 mm) them into 12-mL Exetainers® (Labco Limited, Lampeter, Wales, UK). We filled exetainers in duplicate with ~6 g of wet sediment. Each exetainer was then filled with anoxic water leaving no head space, capped, and shaken on a shaker table (7.33 rad/s) overnight to remove any residual NO_3^- and O_2 (Dalsgaard et al. 2005). The following day, isotopically-labelled $Na^{15}NO_3^-$ (98 atom %) was added to each exetainer to a final concentration of [$^{15}NO_3^-$] of 50 μM. Immediately after adding $^{15}NO_3^-$, we added a 250-μL solution of $ZnCl_2$ (50% mass/volume) to ½ of the replicate exetainers to cease all microbial activity, which represented the concentration of N_2 isotopes at time zero (T_0). The exetainers were sealed and shaken anaerobically. After 6 h, we spiked the remaining replicates with 250 μL of $ZnCl_2$, which represented the concentration of N_2 isotopes at time final (T_6). Within 24 h, we used a membrane inlet mass spectrometer to measure the production of $^{29}N_2$ and $^{30}N_2$ (Kana et al. 1994) with standard gas concentrations determined from Hamme and Emerson (2004). The mass spectrometer was equipped with an inline copper column heated to 600°C to remove residual O_2 from samples (Eyre et al. 2002). We determined denitrification rates from sediment slurries with the isotope pairing technique as described by Nielsen (1992) and determined potential anammox rates in sediment slurries as in Thamdrup and Dalsgaard (2002) and Ledford et al. (2020). To calculate N-removal potentials, we dried sediment slurries at 50°C and weighed the dried slurries to determine dry mass. We normalized concentrations of $^{29}N_2$-N and $^{30}N_2$-N (μmol/L) 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}).

We used ANOVA and correlation tests to address our questions about the relationships between N-removal potentials and the effects of mussels. To assess our prediction that the presence of mussels would enhance N-removal potentials and that N-removal potentials would vary with differing mussel assemblage composition, we tested the effect of mussel species assemblage composition (i.e., treatment) on sediment N-removal potentials with 1-way ANOVA followed by post hoc Tukey's HSD tests between treatments if ANOVA had a p-value ≤0.05. We also assessed relationships between the N-removal pathways of denitrification and anammox and mussel movement, as well as excretion and biodeposition, with Pearson's correlation coefficients. We transformed all N-removal potentials by the natural log to meet assumptions of normality and homoscedasticity.

Sediment microbiome

To characterize the sediment microbiome, we collected sediment samples from each mesocosm at the beginning of the experiment on d 0 (1 replicate was chosen randomly from the 4 replicate mesocosms for each treatment), on d 14 (4 replicates/treatment), and at the end of the experiment on d 42 (4 replicates/treatment). To collect samples, we used a sterile spatula to gently scoop the top 3 cm of sediment into a 15-mL conical tube, which we immediately froze at −80°C until subsequent DNA extraction.

Characterizing the sediment bacterial community Prior to extracting DNA with a DNeasy® PowerSoil® Pro kit (Qiagen®, Hilden, Germany), we pretreated sediment samples for optimization. Briefly, we thawed samples, added ~8 to 10 mL of sample to sterile 50-mL centrifuge tubes, and weighed them. We added a 5-mL solution of Tris-EDTA and 0.1% Tween™ 20 buffer (Croda International PLC, East Yorkshire, UK) to each sample before vortexing (mid-speed for 10 min). After the samples settled for 10 min at ambient temperature, we transferred the supernatant to 15-mL Falcon™ tubes (Thermo Fisher Scientific, Waltham, Massachusetts). We then centrifuged the samples (4000 × g, 4°C, 15 min) and poured off the supernatant. We resuspended the pellet in 500 μL of bead-beating solution from the PowerSoil kit and transferred it to the bead-beating tubes. We then extracted the samples 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). Briefly, 1 μL of genomic DNA was combined with 1 μL of each primer (10 μM) and 17 μL of Invitrogen™ AccuPrime™ Pfx SuperMix (Thermo Fisher Scientific) and amplified with an initial denaturation at 95°C for 2 min; 30 cycles of denaturing at 95°C for 20 s, annealing at 55°C for 15 s, extension at 72°C for 2 min; and final elongation at 72°C for 10 min. Amplification products were standardized with SequalPrep™ Normalization Plates (Thermo Fisher Scientific) and pooled prior to sequencing on a MiSeq system (Illlumina®, San Diego, California) at the University of Mississippi Medical Center Molecular and Genomics Core facility.

Raw sequencing files from 72 samples were processed in mothur v.1.41.1 (Schloss et al. 2009) 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 (v18; http://rdp.cme.msu.edu/). After nonbacterial sequences were removed, 2 samples (14_CF2, 14_LCF1) were removed because 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% sequence similarity.

Effect of mussel assemblage on sediment microbiome To test for differences in the relative abundances of different bacterial phyla in the sediment community across sampling date and treatments, we used the *car* package in R (version 3.0.12; Fox and Weisberg 2019) to run 2-way ANOVAs (Type II sums of squares) with sampling day and treatment as factors, followed by post hoc Tukey's HSD tests. To assess alpha diversity of the bacterial community, we calculated Shannon's Index for community evenness, $Chao_1$ for species richness, and the Inverse Simpson index for overall diversity. All alpha diversity indices were calculated with the R package *phyloseq* (version 1.36.0; McMurdie and Holmes 2013). To address our prediction that mussel species assemblage would affect the composition and alpha diversity of sediment bacterial phyla, we used 2-way ANOVAs (Type II sums of squares) followed by post hoc Tukey's HSD tests to determine if evenness, richness, and diversity differed between treatments and sampling days. We examined beta diversity through principal coordinate analysis based on Bray–Curtis dissimilarities and PERMANOVA (permutations = 999; R package *vegan* version 2.6-2; Oksanen et al. 2013) to assess differences in sediment microbiome structure between treatments and sampling days. Finally, we quantified the relative abundance of N-cycling taxa across treatments and sampling date. However, in all cases only 1 associated taxon was detected in a sample, so statistical analyses were not done.

RESULTS

Mussel nutrient inputs

Mussel excretion In line with our prediction, mussel nutrient excretion rates and stoichiometry varied across species. There were differences in mass-specific N excretion rates among species (ANOVA $F_{2,15} = 4.7$, $p = 0.03$; Table S1, Fig. S4A, and see Appendix S1 for all ANOVA and Tukey's HSD results), with *L. ornata* having >1.5× higher mass-specific N excretion rates than *F. cerina* (Tukey HSD $p = 0.03$). When scaled to the assemblage level, there were differences in N areal excretion rates among treatments (ANOVA $F_{6,21} = 215.4$, $p < 0.0001$; Table 1). Specifically, the *C. kieneriana* treatment had higher N areal excretion rates compared with all other treatments (Tukey HSD $p < 0.001$), followed by the CF treatment (Tukey HSD $p < 0.001$; Table 1). Differences in N areal excretion between LC and LCF treatments were not clear (Tukey HSD $p = 0.5$), but both LC and LCF N areal excretion rates 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 N areal excretion than the L treatment (Tukey HSD $p = 0.03$).

Differences in mass-specific P excretion rates among species were not clear (ANOVA $F_{2,15} = 0.02$, $p > 0.9$; Table S1, Fig. S4B). However, when scaled to the assemblage level, there were differences in P areal excretion rates among treatments (ANOVA $F_{6,21} = 40.0$, $p < 0.0001$; Table 1, Fig. 2A). The C and CF treatments did not differ in areal P excretion

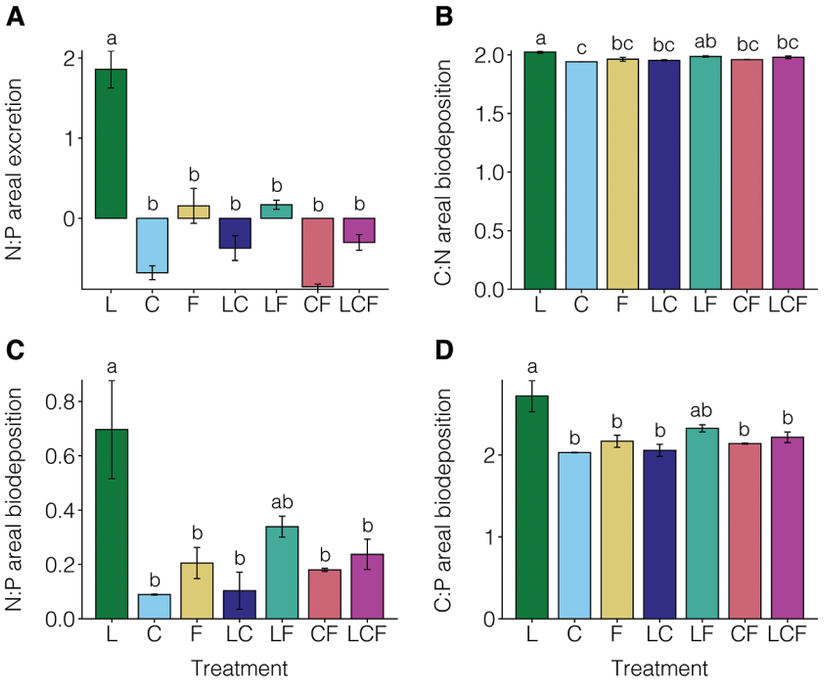

Figure 2. Mean (±SE) freshwater mussel N:P areal excretion (A) and C:N (B), N:P (C), and C:P (D) areal biodeposition stoichiometric molar ratios measured at the end of the 42-d experiment period and scaled to the assemblage level. All data are transformed by the natural log. Treatments are combinations of the following species: L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, and F = *Fusconaia cerina*. Different lowercase letters indicate differences between treatments based on post hoc Tukey's honestly significant difference tests.

(Tukey HSD, $p = 0.6$) 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 $p > 0.9$), nor was there a difference between the LCF, F, and LF treatments (Tukey HSD $p = 0.2$–1.0). The LC treatment had greater areal P excretion than F (Tukey HSD $p = 0.04$), LF (Tukey HSD $p = 0.03$), and L (Tukey HSD $p = 0.004$) treatments.

As a result of the variation in elemental excretion across species (Tables 1, S1), there were differences in areal N:P molar excretion among treatments (ANOVA $F_{6,21} = 11.1$, $p < 0.0001$; Fig. 2A) with the single-species *L. ornata* treatment having higher N:P than all other treatments (Tukey HSD $p < 0.0001$). Generally, treatments containing *C. kieneriana* had lower areal N:P excretion, and treatments containing *L. ornata* had higher areal N:P excretion (Fig. 2A).

Mussel egestion In agreement with our prediction, mussel nutrient egestion rates and stoichiometry varied across species (Table S1). Mass-specific egestion rates differed among mussel species for N (ANOVA $F_{2,15} = 4.3$, $p = 0.03$) and P (ANOVA $F_{2,15} = 7.7$, $p = 0.005$), but not for C (ANOVA $F_{2,15} = 3.3$, $p = 0.06$). *Fusconaia cerina* deposited higher quantities of N compared with *L. ornata* (Tukey HSD $p = 0.03$), and both *F. cerina* and *C. kieneriana* deposited higher quantities of P compared with *L. ornata* (Tukey HSD $p =$ 0.008–0.01). Total OM biodeposition rates across species varied (ANOVA $F_{2,15} = 3.6$, $p = 0.05$), with *F. cerina* having higher mass-specific biodeposition rates compared with *L. ornata* (Tukey HSD $p = 0.05$).

As a result of species-specific variation in biodeposition content (Table S1), when scaled to the assemblage level, there were differences in stoichiometric ratios of C:N (ANOVA $F_{6,21} = 9.3$, $p < 0.0001$; Fig. 2B), N:P (ANOVA $F_{6,21} = 4.2$, $p = 0.006$; Fig. 2C), and C:P (ANOVA $F_{6,21} = 4.4$, $p = 0.005$; Fig. 2D) of biodeposits among mussel treatments. Generally, treatments containing *L. ornata* or *F. cerina* had greater areal C:N, N:P, and C:P stoichiometric biodeposition compared with other treatments (Fig. 2B–D).

Burrowing movement

Mussel species varied in their burrowing behavior. Mussel movement varied among mussel species vertically (ANOVA $F_{2,286} = 178.1$, $p < 0.0001$; Fig. S5B) and in total movement (ANOVA $F_{2,286} = 41.5$, $p < 0.0001$; Fig. S5C), with *L. ornata* moving more vertically and overall compared with *C. kieneriana* or *F. cerina* (Tukey HSD $p <$ 0.0001). Although horizontal movement did not vary among species (Fig. S5A), *L. ornata*'s horizontal movement was, on average, greater and more variable than the other species.

Sediment N-removal potentials

As we predicted, the presence of mussels enhanced sediment N-removal potentials, and the species composition of the treatments resulted in varying N-removal potentials. There was a treatment effect on rates of the N-removal pathways of denitrification (ANOVA $F_{7,24} = 5.5$, $p < 0.001$; Fig. 3A) and anammox (ANOVA $F_{7,24} = 6.5$, $p < 0.001$; Fig. 3B), as well as when both pathways were combined to estimate total N-removal potential (ANOVA $F_{7,24} = 6.9$, $p < 0.001$; Fig. 3C). In general, the single species treatments of *L. ornata* and *C. kieneriana* had greater rates of denitrification and total N-removal than most other treatments (Fig. 3A, C) and were the only 2 treatments that were greater than the no-mussel control when we combined denitrification and anammox (Tukey HSD $p = 0.002–0.01$).

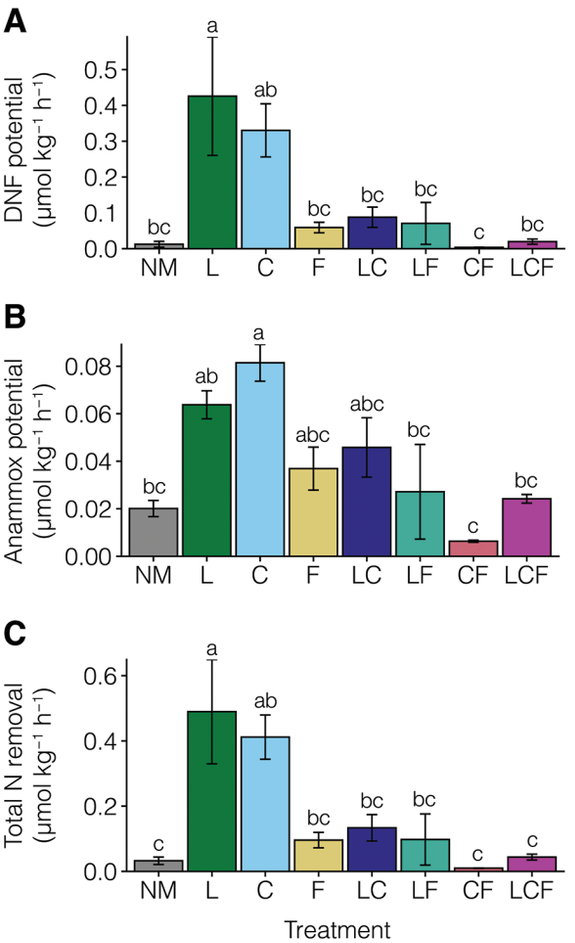

Figure 3. Mean (±SE) rates of denitrification (DNF) (A), anammox (B), and total combined N removal (C) across all treatments ($n = 4$), measured at the end of the 42-d experiment period. Treatments are combinations of the following species: L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, and F = *Fusconaia cerina*. NM is the no-mussel control. Different lowercase letters indicate differences between treatments based on post hoc Tukey's honestly significant difference tests.

Mussel vertical movement and N:P areal excretion were correlated to N-removal pathways (Table S2, Fig. 4A). There was a positive correlation between N:P areal excretion and total N-removal potentials (Pearson's $r_{26} = 0.44$, $p = 0.02$; Table S2, Fig. 4B) and negative correlation between mussel vertical movement and total N-removal potentials (Pearson's $r_{26} = –0.39$, $p = 0.04$; Table S2, Fig. 4C).

Sediment microbiome

Bacterial community composition The final dataset consisted of 1,608,834 sequence reads corresponding to 16,381 unique OTUs. After normalizing the number of reads to the sample with the lowest number of sequence reads ($n = 2112$), we retained 1,548,434 sequences for analysis (Table S3). Overall, the relative abundances of bacterial phyla were similar across sampling weeks with Proteobacteria accounting for the most sequences on all dates (45.1, 39.6, and 21.7% of the total sequences recovered on d 0, 14, and 42, respectively). The most prevalent subdivisions of Proteobacteria were Betaproteobacteria (20.9, 16.7, and 7.0 of all sequences for d 0, 14, and 42, respectively), and Alphaproteobacteria (15.4, 14.5, and 10.0% of sequences for d 0, 14, and 42). Proteobacteria relative abundance did not differ among treatments but did decrease across sampling days (ANOVA $F_{2,12120} = 3.7$, $p = 0.02$; Fig. 5), with sediment samples from d 42 having lower percentages of Proteobacteria in the bacterial community than either d 0 or 14 (Tukey HSD $p < 0.0001$). The abundance of the 11 other most-abundant bacterial phyla were not different across sample dates or mussel treatments.

Bacterial community alpha and beta diversity Alpha diversity of the sediment bacterial community declined over the course of the experiment. At d 0, mussel treatments generally had higher alpha diversity indices compared with the no-mussel controls. However, at the end of the experiment, bacterial diversity indices were lower in the mussel treatments compared with the no-mussel controls. Bacterial communities sampled on d 42 were less even (Shannon; ANOVA $F_{1,46} = 3.8$ $p = 0.06$; Fig. 6A) and had lower richness ($Chao_1$; ANOVA $F_{1,46} = 6.3$, $p = 0.02$; Fig. 6B) compared with communities sampled after d 14. Specifically, after 42 d, sediment bacterial communities in the no-mussel controls had greater species richness as measured by $Chao_1$ compared with the C, F, and 3-species LCF treatments (ANOVA $F_{7,46} = 3.1$, $p = 0.01$; Tukey HSD $p = 0.005–0.04$; Fig. 6B). Neither treatment nor sample date influenced bacterial community diversity as measured by the Inverse Simpson index ($F = 0.50–1.00$, $p = 0.45–0.49$; Fig. 6C).

Overall, the sediment microbiome varied temporally across the sample dates (PERMANOVA pseudo-$R^2 = 0.33$, permutational-$p < 0.001$; Fig. 7A). Principal coordinate analysis ordination ($k = 2$; Figs 7A, S6) showed separation in the bacterial community based on sample date, with samples from each sampling time point clustering together. In further

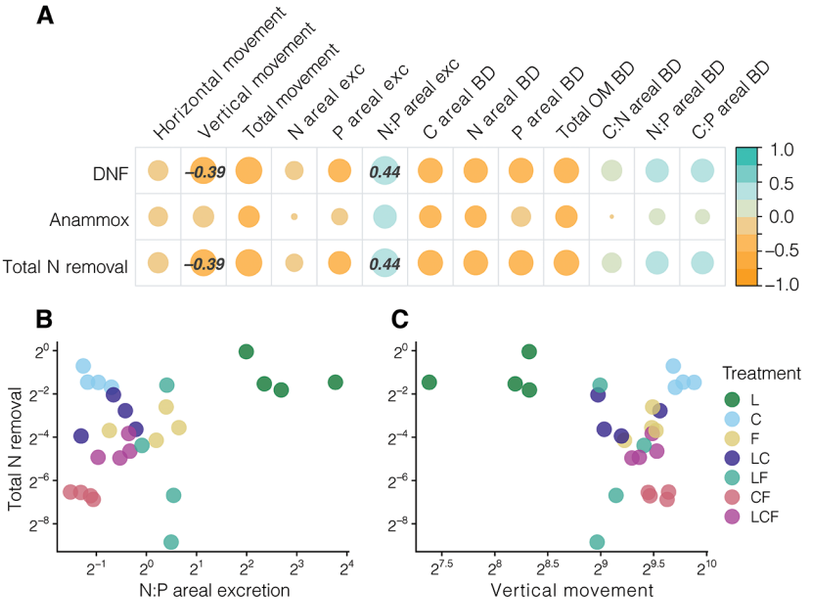

Figure 4. Pearson correlation plot showing the relation of mussel functional traits to N-removal pathways (anammox and total N removal) at the end of the 42-d experiment period, with size of the circle representing strength of correlation (A). Correlation coefficients (r) are shown for relationships with $p < 0.05$. Exc is areal excretion, BN is areal biodeposition, OM is organic matter, and DNF is denitrification. N:P areal excretion (B) and vertical movement (C) correlated to total combined N-removal potentials across all mussel treatments ($n = 4$). Treatments are combinations of the following species: L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, and F = *Fusconaia cerina*.

exploring treatment effects on each sample date, we observed a difference among treatments in microbiome communities (PERMANOVA pseudo-$R^2 = 0.25$, permutational-$p = 0.07$; Fig. 7B). Specifically, principal coordinate analysis ordination ($k = 2$; Figs 7B, S7 illustrated that after 42 d, bacterial communities were different between the NM and C treatments (pairwise ADONIS $p = 0.05$) and between the NM and LCF treatments (pairwise ADONIS $p = 0.05$), and there was a weaker effect of the LC treatment on bacterial communities (pairwise ADONIS $p = 0.05$; Fig. 7B).

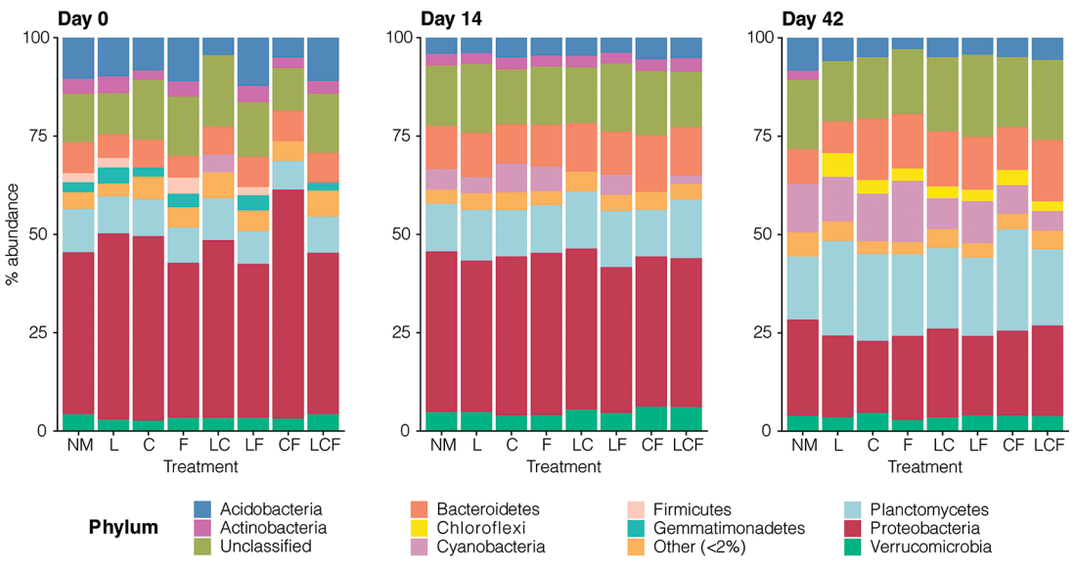

Figure 5. Bacterial phyla in the sediment among treatments, as determined by 16S rRNA gene sequencing, at the start of the experiment (d 0; $n = 1$ replicate/treatment) and after 14 and 42 d ($n = 4$ replicates/treatment). Stacked bar plots represent the 11 most abundant bacterial phyla based on sequence data, with taxa represented by <2% of sequences grouped as Other. Treatments are combinations of the following species: L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, and F = *Fusconaia cerina*. NM is the no-mussel control.

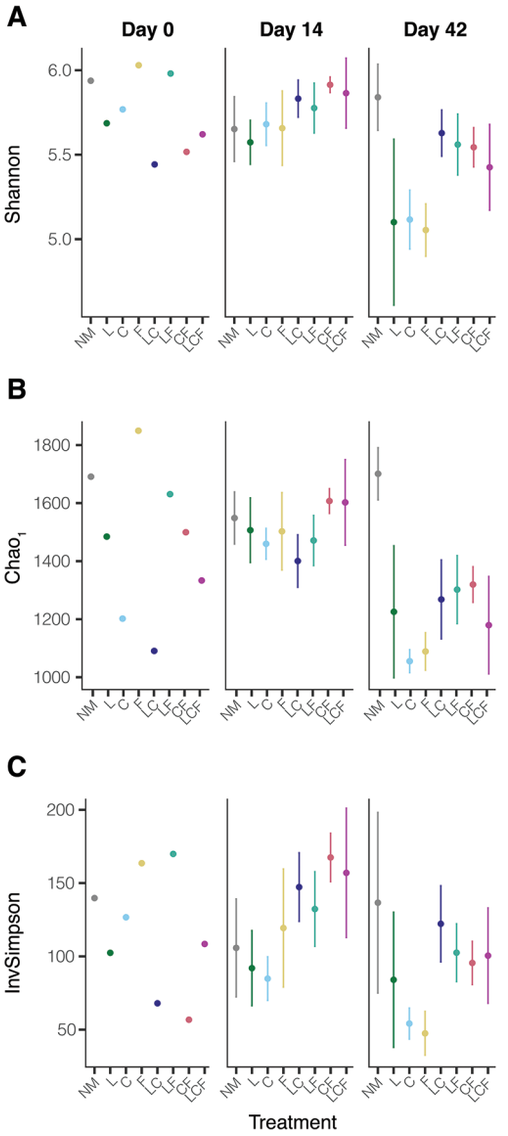

Figure 6. Scatter plots representing alpha diversity indices for sediment bacterial communities in mesocosms where the freshwater mussel assemblage was manipulated. Over the course of the experiment, the presence of mussels decreased bacterial community composition richness. Diversity was derived from 16S rRNA sequence data and calculated as Shannon's diversity (A), $Chao_1$ (B), and Inverse Simpson (InvSimpson) (C) across all treatments at each sampling time point. Treatments are combinations of the following species: L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, and F = *Fusconaia cerina*. NM is the no-mussel control. Values are given as means (±SE). For d 0, n = 1 replicate/treatment, and for d 14 and 42, n = 4 replicates/treatment.

DISCUSSION

We used a mesocosm experiment to investigate how mussels physically (via bioturbation) and chemically (via excretion and egestion) influence sediment–water nutrient fluxes and sediment bacterial community composition. Our mesocosm study showed that mussel species identity and assemblage composition altered N-removal potentials and sediment microbial communities. Species-specific functional traits of nutrient excretion and egestion rates, stoichiometry, and mussel movement in the sediment resulted in varying N-removal potentials across mussel treatments. We observed interactive effects between treatments when excretion and egestion nutrient fluxes were scaled to the assemblage level, in that a strong effect of 1 species was dampened by other species with a similar biomass. Furthermore, there was a positive correlation between mussel stoichiometric traits (areal N:P excretion) and sediment N-removal potentials, suggesting that mussels can stimulate coupled nitrification–denitrification (Fig. 1). There was a negative correlation between total vertical movement of mussels and N-removal potentials, which suggests that mussel burrowing behavior disrupts sediment redox conditions and decreases N-removal. Finally, our findings suggest that the presence of mussels can influence the composition and diversity of the sediment bacterial community, with more species-poor bacterial communities in some mussel treatments compared with the no-mussel controls. Our work highlights that functional diversity resulting from differences in mussel assemblage composition can have differential effects on nutrient cycling and sediment microbiome composition.

Excretion stoichiometry influences N-removal

Our results support previous research demonstrating species-specific differences in mussel excretion N:P that are partially attributable to phylogenetic differences (Atkinson et al. 2020b). Similar to Atkinson et al. (2020a), we observed that *L. ornata* had higher N:P excretion than *C. kieneriana* and *F. cerina*. When scaled to the assemblage level, these mass-specific differences become more evident, especially when mussels are in dense aggregations (Atkinson and Vaughn 2015, Atkinson et al. 2017b). Our findings from this mesocosm study support our prediction that mussels would enhance sediment N-removal potentials because of assemblage-level differences in excretion stoichiometry. We observed greater denitrification and anammox potentials in treatments containing *L. ornata*, which had greater total N-removal potentials than the no-mussel control and all other mussel treatments except the single *C. kieneriana* treatment. This result is corroborated by a previous study in small chamber incubations that found single-species treatments of *L. ornata* to have a stronger influence on sediment N-removal potentials compared with the same species in this study, likely because of their species traits (e.g., excretion stoichiometry and biodeposition rates; Nickerson

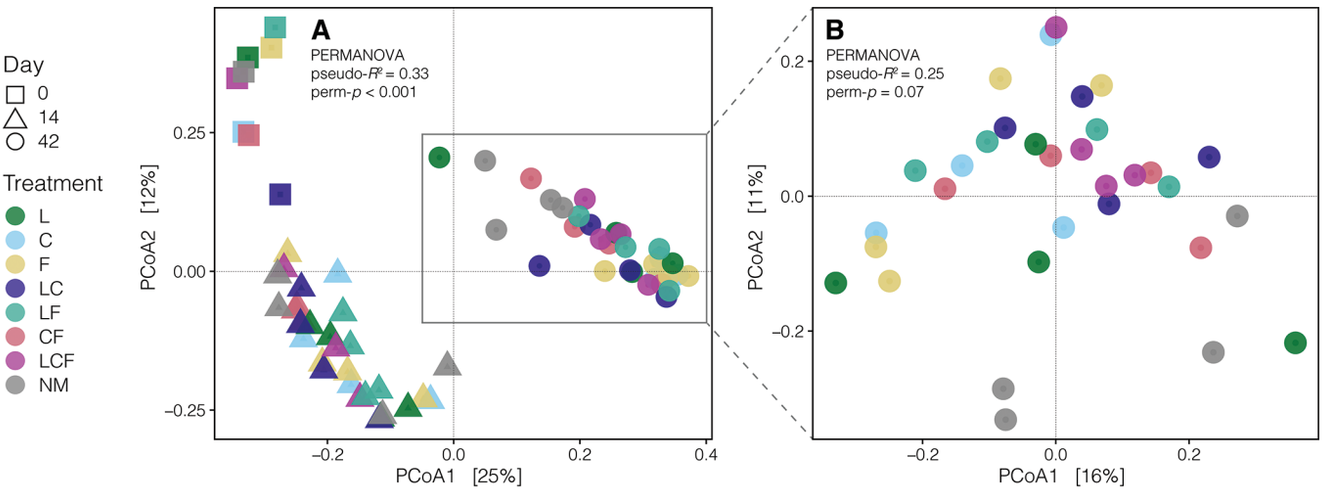

Figure 7. Both time and mussel presence affected the sediment bacterial community composition. Principal coordinate analyses of sediment bacterial communities based on Bray–Curtis dissimilarities between mesocosm treatments over all sampling timepoints (k = 2) (A), and ordinated separately for the last sample date, d 42 (k = 2) (B). For d 0, n = 1 replicate/treatment, and for d 14 and 42, n = 4 replicates/treatment. Treatments are combinations of the following species: L = *Lampsilis ornata*, C = *Cyclonaias kieneriana*, and F = *Fusconaia cerina*. NM is the no-mussel control. Permutational-p abbreviated as perm-p.

et al. 2019). Single-species mussel communities rarely occur naturally, so these results suggest species composition may play a role in generating hotspots of nutrient availability (e.g., Atkinson et al. 2013, Atkinson and Vaughn 2015) and heterogeneity in biogeochemical transformations. In a field study, Atkinson and Forshay (2022) found that denitrification rates in stream sediments increased with increasing mussel biomass and species richness. They also found a positive correlation between excretion N:P and denitrification, which suggests mussels and their trait expression may enhance denitrification rates, likely through providing nutrient subsidies. Additionally, Trentman et al. (2018) found that in an N-limited stream system, sediment denitrification rates decreased with increasing mussel density, but in sediments amended with N, denitrification rates increased with increasing mussel density, which suggests an interactive effect between mussel biomass and nutrient limitation status on denitrification rates. Our results corroborate these studies and provide evidence that mussels can play an important role in mediating biogeochemical cycling and that mussel-provisioned N in the form of excreta can be an important reactant in N-removal potentials in stream sediments.

The role of biodeposition in N-removal

We observed species-specific differences in biodeposition rates and ratios among the 3 study species. As with excreta, *L. ornata* had greater biodeposit N:P, and when scaled to the assemblage level, the single *L. ornata* treatment also had greater biodeposit N:P, as well as greater areal C:P, than all other treatments except LC. Mussel biodeposition of labile OM may alleviate C limitation for heterotrophic bacteria. Given the large role that bacteria play in biogeochemical transformation, these processes can also influence sediment chemistry, which could, in turn, influence bacterial community composition and vice versa (Black et al. 2017). The denitrification pathway requires organic C as a source of energy, whereas anammox does not (Kuenen 2008), which informed our prediction that OM in mussel biodeposits can stimulate denitrification. 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 decreased sediment dissolved O_2 levels (Giles and Pilditch 2006, Carlsson et al. 2010). Thus, the addition of mussel biodeposits may enhance denitrification directly, by providing microbes with a source of labile C, and indirectly, by creating anoxic microhabitats where these biogeochemical processes can occur.

Mussel movement varies by species

Mussels exhibited species-specific movement patterns, and mussel vertical movement was negatively correlated with sediment N-removal potentials. We found that *L. ornata* burrowed the deepest into the sediment, potentially because this species is larger and more active than the other species. Similar results were found in a field study (Trentman et al. 2018), where species identity also played a role in mediating N-cycling. They showed that areas with higher abundances of *Actinonaias ligamentina* (reassigned as *Ortmanniana ligamentina* [Lamark, 1819]), which is a more mobile species (Allen and Vaughn 2009), resulted in higher nitrification rates than less mobile species. Similarly, this intraspecies variability, along with the negative correlation between vertical movement and N-removal potentials,

suggests that bioturbation by mussels has the potential to decrease anaerobic processes like denitrification by increasing O_2 penetration and disrupting the oxic/anoxic sediment boundary layer (Fig. 1). Mussel burrowing behavior varies with species (Allen and Vaughn 2009), season, reproductive cycle (Watters et al. 2001), and substrate (Lewis and Riebel 1984, Sansom et al. 2022). Thus, there are multiple factors governing the activity of mussel burrowing behavior that may vary spatially and temporally in field settings and influence behavior in a mesocosm study. High temperatures cause physiological stress in mussels (Spooner and Vaughn 2008, van Ee et al. 2022), which can also influence their activity (Beggel et al. 2017). During our experiment, water temperatures peaked at nearly 28°C (Fig. S2), a level shown to influence the rate at which mussels activate stored energy reserves (Spooner and Vaughn 2008, Said and Nassar 2022). Further studies of how environmental conditions alter mussel activity and its impact on ecosystem function are warranted.

Sediment microbiome

There was a strong temporal effect on sediment bacterial community structure in the mesocosms. In addition, some mussel treatments (i.e., C and LCF) had different microbial community composition compared with the no-mussel controls, supporting our prediction that the presence of mussels would affect the community structure of the sediment microbiome. Our finding that Proteobacteria was the most abundant bacterial phyla on all 3 sample dates is not surprising given that Proteobacteria are typically the most abundant phyla in freshwater sediments (Zhang et al. 2015) and are often dominant in mussel gut microbiomes (Aceves et al. 2018, 2020, Weingarten et al. 2019, McCauley et al. 2021).

Previous studies have found that burrowing by benthic organisms influences the physicochemical and microbial properties of freshwater sediments (Mermillod-Blondin et al. 2003, Mermillod-Blondin and Rosenberg 2006, Boeker and Geist 2015, Boeker et al. 2016). Thus, differences in sediment microbial communities in our study may have been influenced by mussel burrowing activity because burrowing causes particle reworking and biomixing of the substrate and 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 contributed to changes in the bacterial community (Higgins et al. 2022). Indeed, there was considerable algal growth both on benthic substrates and on the shells of the mussels (Fig. S1) 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 (Svenningsen et al. 2012, Heisterkamp et al. 2013). We also observed a decrease in alpha diversity metrics in treatments with mussels compared with the control treatment without mussels. These results support those by Black et al. (2017), who found that sediment bacterial communities associated with mussels had lower evenness and richness compared with sediments without mussels. It could be that mussels create ecological niches for specific microorganisms by physically and chemically engineering the sediment. N enrichments to marsh sediments decrease microbial diversity (Kearns et al. 2016), and in our study, nutrient inputs from mussels could be having a similar effect. Overall, our study corroborates findings from previous work that indicates that the presence of mussels can result in changes in the sediment bacterial community.

Broader implications

In an experimental system, the presence of mussels enhanced N-removal and decreased sediment bacterial community diversity, and the response varied with mussel assemblage composition and their associated functional traits. Taken as a whole, we provide evidence that mussels are ecosystem engineers by supplying bacteria with 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, Atkinson et al. 2023, Hopper et al. 2023), but mussels are one of the most threatened faunal groups in the world (Strayer et al. 2004, Lopes-Lima et al. 2018). Bivalves in both freshwater and marine systems have been shown to provide important ecological functions and services, especially through their roles in nutrient translocation, storage, and transformation (Vaughn and Hoellein 2018). Furthermore, a previous study demonstrated that large aggregations of marine bivalves—the Eastern Oyster *Crassostrea virginica* (Gmelin, 1791)—were associated with higher rates of sediment denitrification, likely as a result of increased OM provided to sediment microbes in biodeposits (Hoellein and Zarnoch 2014). Results from our study support the idea that freshwater mussels can influence sediment N-removal and that mussels are critical for maintaining this important ecosystem service. In our controlled mesocosm experiment, we attempted to mimic the natural conditions in which mussels live, but it is important to recognize that mesocosms differ from the natural river environment. Elevated water temperatures in our mesocosms may have influenced mussel movement by mimicking spawning season and increasing vertical movement towards the surface. In addition to raised water temperature, because mussels are filter feeders, seston quality and quantity can have a direct impact on metabolism and, thus, nutrient release. These factors have the potential to alter mussel excretion, egestion, and bioturbation, which can thus influence sediment nutrient fluxes and microbiome community. Better understanding

of how species richness and assemblage structure influence nutrient cycling is critical for understanding ecosystem functioning. Therefore, assessing both the direct and indirect effects of mussels can aid in conservation efforts by improving the overall understanding of mussel provisioning of ecosystem functioning. Future studies are still needed to better understand how freshwater mussels can influence the sediment microbial function and nutrient transformations.

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