Nitrate amendment reduces biofilm biomass and shifts microbial communities in remote, oligotrophic ponds

Carmella Vizza[1,5], Jennifer L. Pechal[2,6], M. Eric Benbow[3,7], Jennifer M. Lang[4,8], Dominic T. Chaloner[1,9], Stuart E. Jones[1,10], and Gary A. Lamberti[1,11]

[1]Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556 USA
[2]Department of Entomology, Michigan State University, East Lansing, Michigan 48824 USA
[3]Department of Entomology, Department of Osteopathic Medical Specialties, Ecology, Evolutionary Biology, and Behavior Program, Michigan State University, East Lansing, Michigan 48824 USA
[4]Department of Biology, University of Dayton, Dayton, Ohio 45469 USA

Abstract: Humans have increased the amount of reactive N available in the environment by over an order of magnitude since the industrial revolution. Most studies have been conducted in ecosystems with pervasive anthropogenic nutrient inputs, so little is understood about how naïve biofilm communities respond to elevated nutrients. Our nutrient-diffusing substrate (NDS) experiments, which were conducted in Alaskan freshwater ponds with very little anthropogenic nutrient inputs, suggest that P limits biofilm photoautotrophs. However, despite low water-column nutrient concentrations, overall biofilm biomass was not enhanced by the addition of N or P. Rather, we observed an ~60% biomass reduction with NO_3^- amendment in 15 oligotrophic ponds across 2 y. This widespread biomass reduction was accompanied by changes in microbial communities, but these trends were not observed with NH_4^+ or P amendment. Nonamended communities (i.e., no nutrient amendment other than lysogeny broth agar) were characterized by anaerobic heterotrophs and purple nonsulfur bacteria, whereas NO_3^--amended communities were characterized by aerobic heterotrophs and facultatively aerobic heterotrophs (e.g., denitrifiers). These community patterns suggest that NO_3^- can strongly affect microbial interactions during biofilm formation by altering redox conditions. The effect of NO_3^- on microbial biomass may be caused by an NO_3^- toxicity effect or competitive shifts in taxa, both of which may shape biofilm formation and community assembly. Our results reveal possible consequences for low-NO_3^-, aquatic environments after novel exposure to anthropogenic NO_3^- inputs, suggesting that a legacy of anthropogenic NO_3^- inputs may have fundamentally changed microbial community assembly and biogeochemical cycling in aquatic ecosystems.

Key words: nutrient-diffusing substrate, oligotrophic, nitrate inhibition, biofilms, microbial community composition, high-throughput sequencing, redox

From 1860 to the present, humans have increased the amount of reactive N available by more than 10× (Galloway et al. 2004). We have dramatically transformed the global N cycle via fossil fuel combustion and heavy use of N in agriculture and industry (Galloway et al. 2008). This transformation can have profound effects on terrestrial and aquatic food webs (Meunier et al. 2016). Long-term fertilization studies in terrestrial ecosystems have shown that N enrichment can strongly alter soil microbial communities and processes (Ramírez et al. 2012, Francioli et al. 2016) and lead to biodiversity loss (Isbell et al. 2013), but the long-term effects of anthropogenic N inputs on aquatic ecosystems and their microbial communities are less understood.

Primary producers in aquatic ecosystems are equally likely to be limited by N or P (Francoeur 2001, Elser et al. 2007). However, atmospheric N deposition (Elser et al. 2009) may shift nutrient limitation from primary N limita-

E-mail addresses: [5]cvizza@nd.edu; [6]pechalje@msu.edu; [7]benbow@msu.edu; [8]Present address: Department of Medicine/Division of Cardiology, David Geffen School of Medicine, University of California, Los Angeles, California 90095 USA, jenm1006@gmail.com; [9]dchalone@nd.edu; [10]sjones20@nd.edu; [11]glambert@nd.edu

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tion to secondary P limitation. In addition, researchers have demonstrated that ambient water-column chemistry and N : P stoichiometry (e.g., deviations from the Redfield ratio) can be used to predict nutrient limitation (Keck and Lepori 2012, Cooper et al. 2015). Most studies documenting relationships between water chemistry and nutrient-limitation patterns were conducted in aquatic ecosystems that have been receiving regular anthropogenic N and P inputs for close to a century. Thus, primary producers might be biologically primed to respond to enhanced nutrient inputs (Reisinger et al. 2016).

Not all freshwater ecosystems are limited by N or P. Some are limited by light availability (Karlsson et al. 2009), whereas some systems fail to show any limitation (Johnson et al. 2009). A few investigators have even found that nutrient addition can inhibit biofilm growth (reviewed by Francoeur 2001). Here, inhibition of biofilms through N and P amendment occurred in 1.7 and 3.8% of the studies, respectively (Francoeur 2001). In general, inhibition patterns are so scarce that they fall within the type 1 error range and, thus, are often ignored (Francoeur 2001). These inhibition patterns are usually documented at a single site (e.g., Biggs et al. 1998) or at particular times (e.g., Bernhardt and Likens 2004) and, therefore, are difficult to recreate. For example, Bernhardt and Likens (2004) documented N inhibition in a heterotrophic stream outside the growing season only. Their hypothesis for this pattern was that, in environments with ample organic C sources, nutrients could stimulate bacterial heterotrophs, which then inhibit periphyton growth by outcompeting them for space or other resources. If inhibition of periphyton is the result of competitive shifts in microbial taxa, then examining the effects of nutrients on microbial communities is the key to understanding inhibition patterns in aquatic ecosystems.

We must study remote ecosystems that receive very little anthropogenic N and P to understand whether anthropogenic nutrient input can fundamentally change microbial community taxonomic composition and, as a result, biogeochemical cycling in aquatic ecosystems. Our study was conducted in Alaskan ponds in the Copper River Delta (CRD). The CRD in southcentral Alaska comprises diverse wetland pond habitats, distributed along a gradient of glacial and oceanic influences (Vizza et al. 2017b). The CRD is considered to be a low-nutrient system because of its geological history and the limited anthropogenic influence in this remote area (Bryant 1991). The microorganisms in these ecosystems may be naïve to elevated nutrient supply because they have not been subjected to long-term anthropogenic nutrient loading. Our basic study objectives were to: 1) assess the nutrient-limitation status of these ponds using nutrient-diffusing substrate (NDS) experiments and 2) identify how microbial biofilm communities were affected by nutrient amendment based on targeted high-throughput gene amplicon sequencing.

METHODS

Study area

The Copper River in southcentral Alaska is the 8th-largest river in the USA (Kammerer 1990). The Copper River drains a large region of the Chugach and Wrangell Mountains into the Gulf of Alaska, and the river and the sediments it deposits have shaped the largest contiguous wetland on the Pacific Coast of North America. The CRD encompasses about 283,000 ha of wetland pond habitat and supports extraordinary biodiversity (Bryant 1991). Within the CRD, different wetland habitats can be distinguished along a gradient of glacial and oceanic influences (Vizza et al. 2017b). Ponds were created and modified by the Great Alaska earthquake in 1964 that elevated the Delta by 1−4 m (Thilenius 1995). Our study ponds (n = 15), which we treated as independent replicates, provided a distinct gradient of habitats differing in biogeochemistry (Table 1).

Study design

We conducted 2 separate experiments. We designed the 1st experiment, conducted in 2013 (n = 9 ponds) and 2014 (n = 6 ponds), to test nutrient limitation using 4 different NDS treatments (control, N, P, and N+P). The total number of samples for this experiment was 600 (15 ponds × 4 treatments × 10 replicates). We conducted the 2nd experiment in 2014 in the same 9 ponds sampled the previous year to test for the effects of NH_4^+ compared to NO_3^- using 10 replicates of 5 different treatments (control, low NH_4^+, high NH_4^+, low NO_3^-, and high NO_3^-) for a total of 450 samples (9 ponds × 5 treatments × 10 replicates).

NDSs

We used NDSs to assess nutrient limitation in CRD ponds (Tank et al. 2017). They were constructed from 30-mL plastic cups, which were filled with a 2% lysogeny broth (LB) agar solution (Novagen; EMD Chemicals Inc., San Diego, California) and topped with glass fritted disks. We constructed different treatments for each of the 2 experiments detailed in the study design. For the nutrient limitation experiment, the treatments consisted of control (CTL; not amended except for LB agar), N (LB + 0.5 M KNO_3), P (LB + 0.5 M KH_2PO_4), and N+P (LB + 0.5 M KNO_3 + 0.5 M KH_2PO_4). The 2nd experiment, in which we specifically tested for the effects of N form (NH_4^+ or NO_3^-) and concentration (high or low), consisted of the following treatments: CTL (LB), low NO_3^- (LB + 0.05 M KNO_3), high NO_3^- (LB + 0.5 M KNO_3), low NH_4^+ (LB + 0.05 M NH_4Cl), and high NH_4^+ (LB + 0.5 M NH_4Cl). After a deployment period of 21 to 28 d, we removed substrate disks from ponds, wrapped them in foil, and froze them until they could be analyzed for chlorophyll *a* (Chl *a*) and ash-free dry mass (AFDM), or the total amount of

Table 1. Mean (±SD) values for physical and biogeochemical variables at the Copper River Delta (CRD) ponds sampled in the summers of 2013 and 2014. Water-chemistry variables were measured at the surface layer of 5 sites per pond during July and August (n = 10). All analytes were within detection limits except for NO_3^-, for which all samples were <5 μg N/L. Salinity did not vary within a pond. A map of the ponds along with detailed methods for parameter measurement was published by Vizza et al. (2017b). Temp = temperature, SpC = specific conductivity, DO = dissolved O_2, DOC = dissolved organic C, SRP = soluble reactive P, TN = total N, TP = total P.

Pond	Depth (m)	Daily temp (°C)	Light (kilolux)	pH	SpC (μS/cm)	Salinity (PSU)	DO (mg/L)	DOC (mg/L)	NH_4^+ (μg N/L)	SRP (μg P/L)	TN (μg N/L)	TP (μg P/L)
BVN	1.14 ± 0.02	16.1 ± 1.7	8.9 ± 18	5.9 ± 0.1	20 ± 1.1	0.01	8.9 ± 0.3	2.7 ± 1.2	1.1 ± 1.0	3.7 ± 1.0	130 ± 62	27 ± 12
BVS	0.86 ± 0.08	15.5 ± 1.4	1.8 ± 4.2	6.1 ± 0.2	24 ± 1.1	0.01	6.6 ± 0.3	3.6 ± 0.8	0.9 ± 1.0	3.2 ± 0.9	190 ± 33	34 ± 14
CME	0.83 ± 0.04	15.4 ± 1.8	5.4 ± 8.4	5.7 ± 0.1	52 ± 3.1	0.02	2.7 ± 0.7	4.5 ± 0.8	0.8 ± 1.7	2.9 ± 0.8	210 ± 31	18 ± 9
CMW	0.81 ± 0.04	15.9 ± 2.3	1.7 ± 3.3	7.7 ± 0.5	47 ± 1.3	0.02	10 ± 0.6	4.5 ± 0.4	0.6 ± 0.6	3.8 ± 1.0	230 ± 32	29 ± 13
EYN	0.52 ± 0.04	16.6 ± 2.3	9.3 ± 12	6.3 ± 0.1	9.5 ± 0.3	0.00	7.7 ± 0.5	7.1 ± 0.6	11 ± 2.8	4.8 ± 1.5	270 ± 40	34 ± 8
EYS	0.59 ± 0.04	17.6 ± 2.6	14 ± 18	6.6 ± 0.1	8.8 ± 0.2	0.00	8.3 ± 0.4	6.8 ± 0.9	13 ± 5.7	4.8 ± 2.5	280 ± 34	38 ± 13
LIL	0.61 ± 0.01	15.7 ± 2.5	6.1 ± 10	7.0 ± 0.1	76 ± 11	0.04	3.7 ± 0.5	4.2 ± 0.5	6.2 ± 0.8	6.9 ± 3.1	130 ± 28	24 ± 7
RHM	0.64 ± 0.12	13.6 ± 1.7	7.7 ± 11	7.2 ± 0.1	81 ± 2.4	0.04	3.6 ± 1.0	4.0 ± 0.5	15 ± 5.3	8.6 ± 2.5	120 ± 29	19 ± 8
SCS	0.89 ± 0.14	16.3 ± 2.5	11 ± 20	7.4 ± 0.1	53 ± 5.0	0.02	8.2 ± 0.4	2.7 ± 0.4	5.0 ± 1.0	8.7 ± 3.0	110 ± 25	23 ± 7
SME	0.66 ± 0.02	14.9 ± 1.2	2.4 ± 4.5	6.3 ± 0.2	44 ± 2.9	0.02	11 ± 0.4	3.8 ± 0.3	0.5 ± 0.5	5.1 ± 3.3	180 ± 32	27 ± 9
SMW	1.04 ± 0.05	15.6 ± 1.7	4.7 ± 10	6.7 ± 0.1	70 ± 0.8	0.03	11 ± 0.5	2.9 ± 0.7	0.5 ± 0.9	4.3 ± 2.0	160 ± 44	27 ± 9
STN	0.57 ± 0.01	19.4 ± 2.6	13 ± 23	7.6 ± 0.1	42 ± 0.4	0.02	8.5 ± 0.3	8.7 ± 1.6	13 ± 0.9	4.0 ± 0.7	300 ± 98	24 ± 7
STS	0.54 ± 0.09	19.1 ± 2.8	13 ± 19	7.6 ± 0.1	63 ± 1.8	0.03	8.1 ± 0.5	5.5 ± 1.7	15 ± 2.6	5.6 ± 2.1	220 ± 89	20 ± 7
TIN	0.60 ± 0.03	18.3 ± 2.6	12 ± 21	6.6 ± 0.1	11 ± 0.6	0.00	6.2 ± 1.1	8.0 ± 0.4	14 ± 2.4	5.2 ± 1.0	250 ± 10	31 ± 6
TIS	0.73 ± 0.04	18.1 ± 2.7	14 ± 23	6.6 ± 0.1	8.2 ± 0.9	0.00	7.8 ± 0.5	6.4 ± 0.5	11 ± 1.1	4.8 ± 0.9	200 ± 22	27 ± 5

organic matter. Chl *a* represents the photoautotrophs of the biofilm including both algae and cyanobacteria, whereas AFDM represents both autotrophs and heterotrophs in the biofilm.

LB agar promotes colonization of heterotrophic biofilms because it is supplemented with yeast extract (5 g/L), peptone (10 g/L), and NaCl (10 g/L), whereas the fritted disk promotes colonization of autotrophic biofilms (Johnson et al. 2009). Different substrates, such as a cellulose sponge, have been used to promote the heterotrophic community, but our design allowed us to mimic natural organic substrates in these ponds, such as macrophyte stems, which can structurally and chemically support biofilms composed of microbial autotrophs and heterotrophs (Cattaneo et al. 1998, He et al. 2014). The NDS method used can affect the nutrient limitation patterns detected (Capps et al. 2011). For example, Capps et al. (2011) found slight variations in N and P colimitation patterns of a single stream depending on substrate type and diffusion rates. Therefore, diffusion rates and ambient chemistry should be reported when using standardized nutrient limitation methods.

To better understand nutrient release from a control substrate with LB agar relative to agar-agar, we assessed laboratory diffusion rates of dissolved organic C (DOC; Fig. S1A), total N (TN; Fig. S1B), and total P (TP; Fig. S1C) from these 2 agar types. Our diffusion rates for LB agar (Appendix S1, Fig. S1A–C) were orders of magnitude lower than rates in other studies (Bernhardt and Likens 2004, Rugenski et al. 2008), but our C, N, and P release rates tended to be about an order of magnitude higher for LB agar relative to agar-agar. Stoichiometry was similar between the agar types, suggesting that biofilms would experience primarily C limitation followed by N limitation based on the amendments alone (C : N : P after 24 h of LB agar diffusion was 35 : 11 : 1 and that of agar-agar was 36 : 6 : 1; Appendix S1, Fig. S1A–C). We also assessed diffusion rates in high and low NO_3^--amended LB agar (Appendix S1, Fig. S1A–C) because of the strong inhibition response exhibited by biofilms on NO_3^--amended substrates.

Chl *a* and AFDM analyses

Within 60 d of collection, we extracted Chl *a* from disks overnight in 20 mL of 90% buffered acetone. The next day, we used a fluorometer (TD-700; Turner Designs, San Jose, California; after Steinman et al. 2017) to measure Chl *a* in a subsample of the extract. We estimated total biofilm biomass by measuring AFDM (after Steinman et al. 2017). We air-dried disks and their respective acetone extracts (including the subsample used for Chl *a* analysis) for a week, and then oven-dried them for at ≥48 h at 60°C, weighed them, and combusted them at 500°C for 4 h. Last, we rewetted the disks and dried them at 60°C for ≥48 h before the final weighing. We used the difference in mass before and after combustion to estimate AFDM. We report Chl *a* and AFDM in areal units based on the top surface area of a fritted glass disk (3.9 cm^2).

Microbial community study design

We used automated ribosomal intergenic spacing analysis (ARISA) to generate initial microbial community fingerprints from 1 replicate of each treatment per pond in 2013 (Lang et al. 2015). ARISA results demonstrated that microbial communities on disks varied among nutrient treatments (Appendix S2, Fig. S2). Therefore, in 2014, we used a more advanced sequencing platform (MiSeq; Illumina, San Diego, California) to obtain more detailed information on microbial taxa by analyzing 1 replicate of each treatment per pond. In total we used samples from 4 treatments (CTL, N, P, and N+P) in the 6 ponds sampled in 2013/2014 and 5 treatments from 9 other ponds (CTL, low NO_3^-, high NO_3^-, low NH_4^+, high NH_4^+) sampled in 2014 to generate a total of 69 samples. In both years, we sterilized 2-mL centrifuge tubes in a boiling water bath and rinsed them with 95% ethanol because no autoclave was available near these remote field sites. We removed biofilm samples from disks with a flame-ethanol-sterilized razor blade and placed them in the 2-mL centrifuge tubes. We had no access to a −80°C freezer, so we covered the tubes with sterile glass-fiber filters but left them uncapped to air-dry for ~48 h to prevent mold growth and to preserve samples. Two weeks later, they were transported to Michigan State University and frozen at −80°C upon arrival. Studies comparing preservation methods indicate this approach was sufficient to enable us to identify community differences based on environmental factors (Piggott and Taylor 2003, Lauber et al. 2010).

DNA extraction

DNA was extracted from biofilm samples according to the manufacturer's protocol using a PowerBiofilm® DNA isolation kit (Mo Bio, Carlsbad, California). Sufficient reads were obtained for only 27 of the 69 samples in the first sequencing run because of a combination of low quality (probable inhibition) and low quantity (0.206–67.0 ng/μL) of DNA products. Therefore, we used a PowerClean Pro DNA clean-up kit (Mo Bio) for the remaining 42 samples and then sequenced these for a 2nd run. These 2 runs resulted in sequencing all 24 samples from the nutrient limitation experiment (CTL, N, P, and N+P), and 35 samples from the N-form experiment. However, only 5 of the 9 ponds (EYN, EYS, SCS, STN, and TIN) from this experiment had all treatments (CTL, low NO_3^-, high NO_3^-, low NH_4^+, high NH_4^+) represented; therefore, we included only those 25 samples (5 treatments × 5 ponds) in analyses. Even though we conducted 2 different sequencing runs with Illumina MiSeq, <10% of the variation was explained by sequencing run (see Appendix S3, Fig. S3), and microbial community separation by treatment was similar to ARISA where a clean-up kit was used for all samples (see Appendix S2, Fig. S2).

16S ribosomal RNA (rRNA) gene amplicon high-throughput sequencing

Targeting the 16S rRNA gene allowed us to gather phylogenetic information about Bacteria and Archaea. After DNA extraction, we quantified the DNA using a Quant-iT dsDNA HS Assay kit and a Qubit 2.0 (Thermo Fisher, Grand Island, New York) and then stored all samples at −80°C. Illumina MiSeq 16S library construction (2 × 250 base pair [bp] paired-end reads) and sequencing was performed in the Michigan State University Genomics Core Facility with a modified version of the protocol adapted for the Illumina MiSeq described by Pechal and Benbow (2016). Briefly, V4 regions of the 16S rRNA gene amplicon region were amplified with region-specific primers that include Illumina flowcell adapter sequences (515f [5′ GT GCCAGCMGCCGCGGTAA] and 806r [5′ GGACTACH VGGGTWTCTAAT]) (Caporaso et al. 2010). All sequencing data were curated using the mothur software package (version 1.37; https://www.mothur.org/) and the procedure detailed at https://www.mothur.org/wiki/MiSeq_SOP (Kozich et al. 2013). Sequences were classified against the SILVA (version 123) reference taxonomy (Pruesse et al. 2007). We assessed the error rate of our sequences (7.25×10^{-5}) using the mock community described by Kozich et al. (2013). We then performed rarefaction to ensure an even sequence depth of 1000 sequences/sample subsampled 1000×; the range in coverage of these rarefied sequences was 0.879 to 0.996, which indicates sufficient sampling of the microbial communities. Sequence files for all samples used in this study are deposited in the Sequence Read Archive at the EMBL European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena): PRJEB19927.

Statistical analyses

We converted the raw Chl *a* and AFDM data to average response ratios (Francoeur 2001) at the site level per pond, which resulted in 5 response ratios per treatment per pond. Response ratios were calculated by averaging the 2 replicates per treatment at each deployment site within a pond (if applicable) and then dividing the average nutrient treatments by the average CTL treatments. For the nutrient-limitation experiment, we used a blocked analysis of variance (ANOVA) design where either Chl *a* or AFDM response ratio was the response variable, pond was a blocking variable, and treatment (N, N+P, P) was the factor of interest. Within the ANOVA design, we tested 3 potential data distributions (normal, log-normal, and gamma) to determine which distribution had the lowest Akaike's Information Criterion value and, therefore, was the best distribution to model each response variable (Burnham and Anderson 2002). We used a log-normal distribution for the Chl *a* response ratios of the nutrient-limitation experiment and a gamma distribution for the AFDM response ratios. For the N-form experiment, we also used a blocked ANOVA design where either Chl *a* or AFDM response ratio was the response variable, pond was a blocking variable, and form of N (NH_4^+ or NO_3^-) and concentration (0.05 or 0.5 M) were the factors of interest. We included an interaction between N form and concentration. We used a gamma distribution for the Chl *a* response ratios for the N-form experiment, and a normal distribution for the AFDM response ratios. We performed post hoc pairwise comparisons on all 4 ANOVAs (i.e., Tukey's Honestly Significant Difference [HSD]; Zar 2010). For all ANOVAs and Tukey's HSD tests, we set $\alpha = 0.05$.

To examine the effects of nutrient amendment on microbial community diversity, we calculated operational taxonomic unit (OTU) richness and Shannon entropy, an index that accounts for both species richness and evenness (Jost 2007). We then performed 2 blocked ANOVAs (1 per experiment) on OTU richness, which was $\log_{10}(x)$-transformed to ensure normality, with treatment as the factor of interest and pond as a blocking variable. We used 2 similar blocked ANOVAs to test Shannon entropy.

To analyze microbial community data, we calculated pairwise Bray–Curtis dissimilarity indices for the relative abundance data from the 2 experiments separately. To visualize patterns in microbial community composition in our experiments, we used principal coordinates analyses (PCoAs) to ordinate microbial communities. We then evaluated whether our treatments had statistically significant effects on microbial community composition of biofilms using permutational multivariate analyses of variance (PERMANOVA; Anderson and Walsh 2013). All PERMANOVA models included pond and sequencing run as blocking variables, and we set $\alpha = 0.05$.

Last, we used indicator species analysis as a heuristic tool to identify which OTUs were representative of NO_3^- and CTL treatments because these 2 treatments exhibited the greatest difference in biomass and microbial community composition. Indicator species are those that are strongly associated with a particular habitat (Carignan and Villard 2002) or that can be used to reveal evidence for the effect of environmental changes (McGeoch 1998). To identify which OTUs were the best indicator "species" for each treatment, we calculated indicator value indices (Cáceres and Legendre 2009) for the 1090 OTUs found on the CTL and high-NO_3^- treatments from both experiments. We present only the subset for which the unadjusted *p*-value was ≤ 0.01. All statistical analyses were conducted in the R software environment using the *base*, *vegan*, and *indicspecies* packages (version 3.3.0; R Project for Statistical Computing, Vienna, Austria).

RESULTS

For the nutrient-limitation experiment, biofilm photoautotrophs (Chl *a*) and total biomass (AFDM) responded differently to N and P (Fig. 1A, B, Table 2). PO_4^{3-} (P, N+P) had a positive effect, and approximately doubled the amount

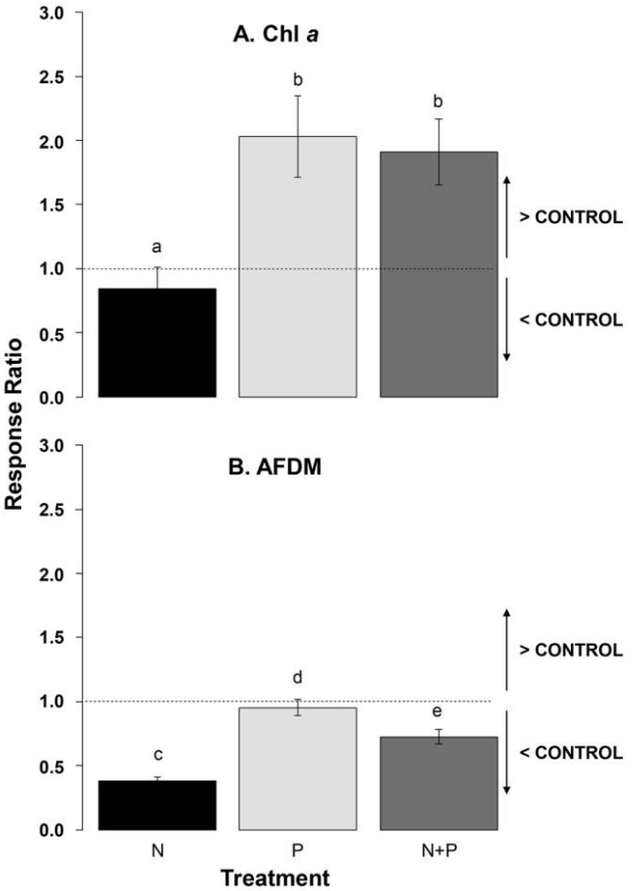

Figure 1. Mean (±95% CI) response ratios of chlorophyll *a* (Chl *a*) (A) and ash-free dry mass (AFDM) (B) on nutrient-amended substrates (N, P, and N+P) relative to the control in the 15 study ponds in 2013 ($n = 9$) and 2014 ($n = 6$). Response ratios of 1.0 indicate equal growth relative to the lysogeny broth agar control. Bars with the same lowercase letters are not significantly different ($p > 0.05$).

of Chl *a* relative to the CTL substrates, whereas NO_3^- alone had relatively little effect (Fig. 1A). In contrast, NO_3^- reduced biofilm biomass by 60%, but P had relatively little effect (Fig. 1B). N+P substrates had an intermediate amount of biomass relative to the N and P treatments (Fig. 1B).

For the N-form experiment, biofilm photoautotrophs were slightly reduced in the presence of high N concentrations, whereas total biofilm biomass was drastically lower for NO_3^- amendments at both concentrations (Fig. 2A, B, Table 2). Both treatments with lower concentrations of NH_4^+ and NO_3^- had approximately the same amount of Chl *a* as CTL substrates, whereas the higher concentrations of NH_4^+ and NO_3^- had ~80 and 60% of CTL Chl *a*, respectively (Fig. 2A). Biofilm biomass exhibited a 40 and 60% reduction in the presence of low- and high-NO_3^- treatments, respectively. In contrast, NH_4^+ treatments had approximately the same AFDM as the CTL substrates (Fig. 2B).

Biofilm α diversity did not respond to N or P. The number of observed microbial OTUs ranged from 19 to 157 (mean ± SD, 60 ± 37) in the nutrient-limitation experiment and from 16 to 80 in the N-form experiment (38 ± 17). However, nutrient amendments and pond did not significantly affect the number of OTUs present for either experiment ($p \geq 0.17$). In addition, species richness/evenness was similar between experiments (Shannon entropy for nutrient limitation: 2.1 ± 0.6; N form: 1.9 ± 0.6). Treatment had no effect on species richness/evenness for either experiment ($p \geq 0.16$), but pond was a significant factor in the nutrient-limitation experiment ($p = 0.042$).

In contrast to α diversity, biofilm microbial community composition responded differently to N and P. In the nutrient-limitation experiment, N-amended communities were the most different from CTL communities, whereas P-amended communities grouped closer to CTL treatments (Fig. 3A). N+P-amended communities were clustered between the communities associated with N and P treatments (Fig. 3A). Only treatment had a significant effect on biofilm microbial communities (PERMANOVA, $R^2 = 0.57$, $p = 0.001$; Fig. 3A). Pond only weakly influenced microbial communities ($R^2 = 0.14$, $p = 0.08$), and sequencing run was not significant ($R^2 = 0.03$, $p = 0.18$).

Biofilm microbial community composition also responded differently to NH_4^+ and NO_3^-. The CTL communities grouped with the low- and high-NH_4^+-treated communities, and these communities differed from those grown at both concentrations of NO_3^- (Fig. 3B). Treatment and pond had significant effects on the biofilm microbial communities (treatment: $R^2 = 0.38$, $p = 0.001$; pond: $R^2 = 0.24$, $p = 0.002$), whereas sequencing run had a small and nonsignificant effect ($R^2 = 0.04$, $p = 0.09$).

The CTL and high NO_3^- treatments differed substantially in biomass and microbial community composition, so we identified OTUs that were associated with these differences. A total of 8 and 6 indicator OTUs for the CTL and high NO_3^- treatments, respectively, were diagnostic (Table 3). In general, CTL indicators tended to be anaerobic chemoorganotrophs or phototrophs, whereas NO_3^- indicators were aerobic or facultatively aerobic chemoorganotrophs with an ability to reduce NO_3^- (Table 4).

DISCUSSION

Our study suggests that nutrients can have strong positive and negative effects on microbial biofilms in low-nutrient aquatic ecosystems. Pond photoautotrophs (measured as Chl *a*) probably were limited by P, but total biofilm biomass (measured as AFDM) did not increase in the presence of N or P, but rather experienced a 60% reduction with the addition of NO_3^-. This reduction in biomass with NO_3^- addition was observed across 15 remote, oligotrophic ponds differing in biogeochemistry across 2 y, a result not observed with NH_4^+ addition. NO_3^- amendment shifted het-

Table 2. Results of the analyses of variance (ANOVA) conducted for the nutrient-limitation and N-form experiments. Chlorophyll *a* (Chl *a*) and ash-free dry mass (AFDM) were the response variables.

Experiment	Response variable	Categorical variable	F_{df}	p
Nutrient limitation	Chl *a*	Pond	$F_{14,208} = 6.1$	0.001
		Treatment	$F_{2,208} = 80$	0.001
	AFDM	Pond	$F_{14,208} = 4.8$	0.001
		Treatment	$F_{2,208} = 150$	0.001
N form	Chl *a*	Pond	$F_{8,164} = 5.1$	0.001
		N form	$F_{1,164} = 1.9$	0.170
		Concentration	$F_{1,164} = 17$	0.001
		Interaction	$F_{1,164} = 4.5$	0.035
	AFDM	Pond	$F_{8,164} = 7.5$	0.001
		N form	$F_{1,164} = 440$	0.001
		Concentration	$F_{1,164} = 25$	0.001
		Interaction	$F_{1,164} = 15$	0.001

erotrophic microbial composition from predominantly anaerobic to aerobic with many of the aerobic taxa capable of using both O_2 and NO_3^- as electron acceptors. These community patterns suggest that NO_3^- probably has a significant effect on microbial interactions during biofilm formation, at least in ecosystems that receive little geologic or anthropogenic inputs of NO_3^-.

P was the primary nutrient limiting photoautotroph growth in CRD biofilms as shown by a doubling of Chl *a*, a widely used surrogate for algal and cyanobacterial biomass (Wetzel and Likens 2000). P limitation of photoautotroph growth is common in both lakes and streams (Elser et al. 1990, Francoeur 2001), and water nutrient concentrations and N : P stoichiometry are generally considered good predictors of nutrient limitation (Keck and Lepori 2012). In our study, water-column TN : TP molar ratios were ~17, which suggests that the ponds could be on the verge of P limitation because the ratio is higher than the Redfield N : P ratio of 16 : 1, which is considered an optimal nutrient ratio for oceanic seston (Redfield 1958). In contrast, Kahlert (1998) found that periphyton N : P > 32 indicates P limitation, which suggests that periphyton assimilating nutrients from the water column in the CRD could be limited by N instead of P. Nonetheless, we observed widespread P limitation of primary producers across ponds with P amendments significantly increasing Chl *a*.

In contrast to the photoautotrophs, total biofilm biomass was not enhanced by nutrient addition. Instead, AFDM was strongly reduced in the presence of NO_3^-. Results of some algal studies tend to show similar trends for Chl *a* and AFDM (e.g., Wyatt et al. 2010), but Lang et al. (2012) found that N appears to be more limiting for photoautotrophs than total biofilm biomass. In addition, different nutrient-limitation patterns for fungi and algae have been identified when using wood substrates (Tank and Dodds 2003). We also found different response patterns between total biofilm biomass and the photoautotrophic components of biofilms on glass fritted disks amended with LB agar. The strong decrease in biomass with NO_3^- amendment was unexpected given that inhibition patterns (i.e., reduction in Chl *a* or AFDM relative to the CTL) are rare and often difficult to reproduce in time and space (Francoeur 2001, Tank and Dodds 2003, Bernhardt and Likens 2004, Sanderson et al. 2009). We are the first investigators to document strong NO_3^- inhibition of total biofilm biomass across multiple sites and years.

NH_4^+ did not limit or reduce biofilm biomass. Differential response to N form usually is expected because NH_4^+ is energetically less expensive to assimilate than NO_3^- (Von Schiller et al. 2007). Nevertheless, some investigators have shown that biofilm response does not differ in response to NO_3^- or NH_4^+ (Hoellein et al. 2010), whereas biofilms of midwestern rivers (USA) responded more to NO_3^- than to NH_4^+ (Reisinger et al. 2016). An explanation is that organisms adapt to the most common N form. For example, Reisinger et al. (2016) hypothesized that positive biofilm responses to NO_3^- amendment in agriculturally influenced streams are related to microbial acclimation to elevated NO_3^- levels from fertilizer runoff. In ecosystems where nutrient concentrations are very low, differences in assimilation are unlikely to cause strong reductions in biofilm caused by NO_3^- amendment. However, in the CRD, water-column NO_3^- levels were very low (<5 μg/L) as were sediment porewater concentrations (<5 nmol/g dry sediment; Vizza et al. 2017a). Therefore, NO_3^- amendment could represent a relatively novel source of N in these oligotrophic ponds to which biofilm communities are not well adapted.

NO_3^- had a larger effect on community composition than did P or NH_4^+, even at log-lower concentrations. Indicator species analyses revealed that the bacterial OTUs on the CTL substrates were characterized by anaerobic

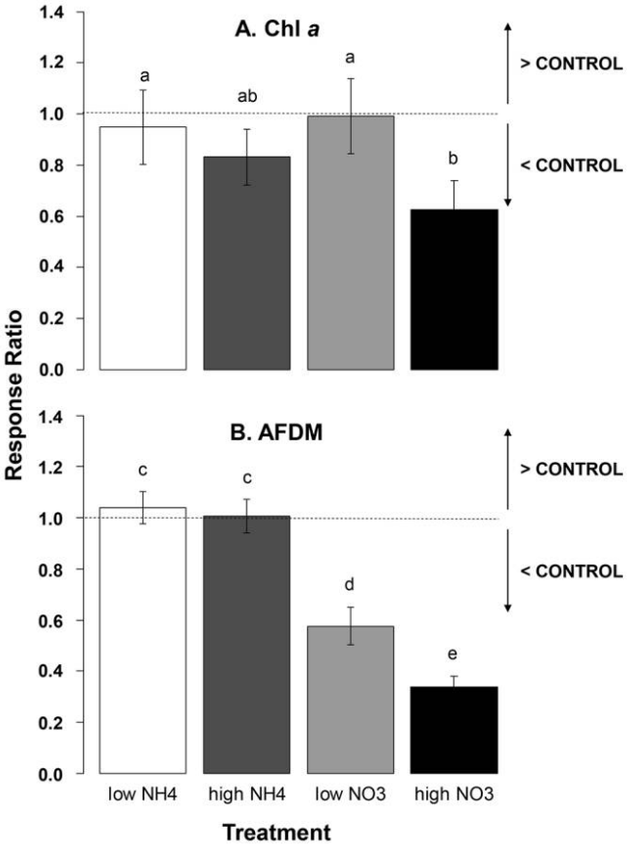

Figure 2. Mean (±95% CI) response ratios of chlorophyll *a* (Chl *a*) (A) and ash-free dry mass (AFDM) (B) on nutrient-amended substrates (low NH_4^+, high NH_4^+, low NO_3^-, high NO_3^-) relative to the control in 9 ponds in 2014. Response ratios of 1.0 indicate equal growth relative to the lysogeny broth agar control. Bars with the same lowercase letters are not significantly different ($p > 0.05$).

heterotrophs (e.g., *Desulfovibrio* and *Paludibacter*), which suggests that biofilms were thick enough to reduce O_2. For example, *Desulfovibrio* is a genus known to use SO_4^{2-} as an alternative electron acceptor (Heidelberg et al. 2004). In addition, purple nonsulfur bacteria (e.g., *Rhodoblastus* and *Rhodocyclus*) were also abundant in the CTL communities and have extremely flexible metabolisms; they can be photoorganotrophic (i.e., use light for energy and organic compounds as a source for C and electrons), photolithotrophic (i.e., use light for energy, CO_2 as a C source, and H_2 or other inorganic compounds as electron donors), or chemoorganotrophic (i.e., use organic compounds for sources of energy, C, and electrons) in dark, oxic conditions (Madigan et al. 2014). In contrast, NO_3^--amended communities were characterized by aerobic heterotrophs (e.g., *Janthinobacterium*), probably because the biofilms were not as thick, but also by facultative aerobic heterotrophs (e.g., *Microvirgula* and *Pseudomonas*) that generate energy via fermentation processes or by using NO_3^- as an alternative electron acceptor when O_2 is not present (i.e., denitrification). For example, *Microvirgula aerodenitrificans* has the unique ability to simultaneously use both O_2 and NO_3^- as electron acceptors during respiration (Patureau et al. 1998). Organisms found on CTL substrates had different metabolisms than those on NO_3^--amended substrates, so differences in redox conditions probably contributed to these divergent microbial communities. These drastic shifts in microbial communities occurred on an artificial medium supplemented with organic C and other nutrients. Therefore, direct extrapolation of these results or those of any NDS experiment to how

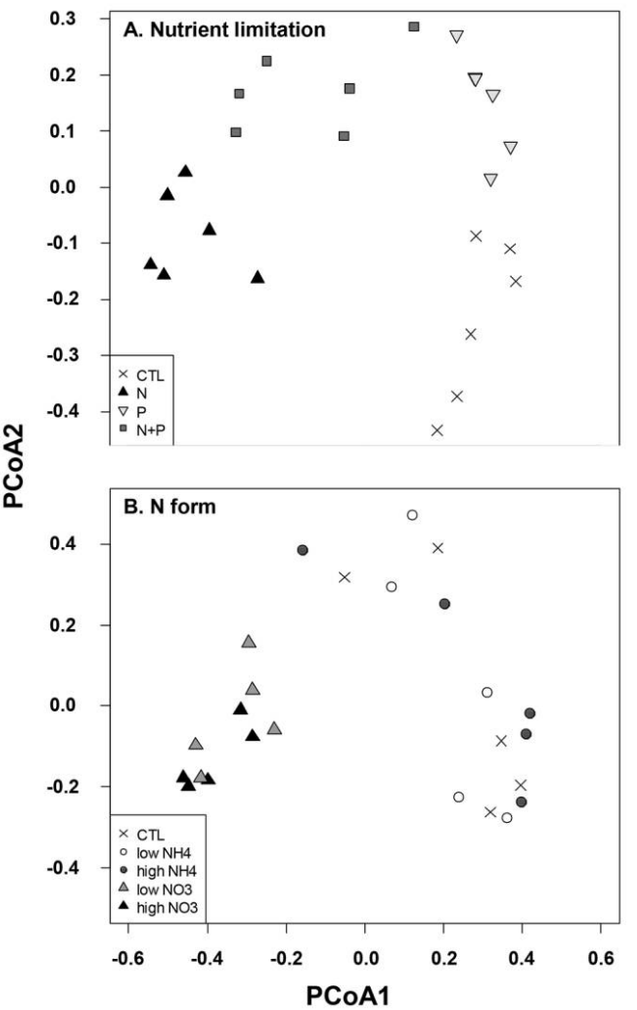

Figure 3. Principal coordinates analysis (PCoA) plots based on a Bray–Curtis relative abundance distance measure on 16S ribosomal RNA gene amplicon sequencing data from the nutrient-limitation experiment where treatments consisted of control (CTL), N, P, and N+P (A) and the N-form experiment where treatments consisted of CTL, low NH_4^+, high NH_4^+, low NO_3^-, and high NO_3^- (B). Each dot represents a nutrient-diffusing substrate disk.

Table 3. Indicator species analysis on the control (CTL) and high NO_3^- nutrient-diffusing substrate treatments from 11 ponds ($p \leq 0.01$ for all indicator taxa). Each taxon is identified to the lowest level possible. UC (unclassified) is used to mark different operational taxonomic units (OTUs) sharing the same taxonomic group. An indicator value is the product of components A and B. Component A is the probability that a sample belongs to the treatment group given that the OTU has been found, whereas component B is the probability of finding the OTU in samples belonging to the treatment group. Mean (±SD) sequence reads are given for each indicator OTU by treatment.

Treatment	Taxon	A	B	Indicator value	Reads (CTL)	Reads (NO_3^-)
CTL	Clostridiaceae	0.91	1.00	0.96	16 ± 18	1 ± 5
	Rhodocyclus	0.99	0.91	0.95	270 ± 220	2 ± 4
	Paludibacter	1.00	0.73	0.85	4 ± 6	0 ± 0
	Desulfovibrio	1.00	0.73	0.85	54 ± 86	0 ± 0
	Phaeospirillum	0.99	0.73	0.85	25 ± 64	0 ± 0
	Bacteroidales	0.99	0.73	0.85	37 ± 53	1 ± 1
	Rhodoblastus	0.98	0.64	0.79	4 ± 6	0 ± 0
	Veillonellaceae	1.00	0.55	0.74	1 ± 2	0 ± 0
High NO_3^-	Enterobacteriaceae UC1	0.98	1.00	0.99	3 ± 5	170 ± 100
	Enterobacteriaceae UC2	0.96	1.00	0.98	5 ± 9	120 ± 180
	Pseudomonas	0.95	1.00	0.98	8 ± 14	170 ± 160
	Microvirgula	0.98	0.82	0.90	4 ± 5	170 ± 130
	Paenibacillus	0.98	0.73	0.84	0 ± 1	9 ± 9
	Janthinobacterium	0.83	0.82	0.83	2 ± 3	9 ± 8

a natural system would respond would be difficult without conducting an ecosystem-scale manipulation.

NO_3^- could alter redox conditions in ecosystems with anaerobic conditions (D'Angelo and Reddy 1999) and relatively low NO_3^- inputs from surrounding geology, agricultural runoff, and urban wastewater. Most NDS studies have been conducted in well-aerated streams rather than in lakes and wetlands, which are more likely to experience anaerobic conditions because of stagnant water. A biofilm nutrient-limitation survey conducted across Great Lakes

Table 4. Taxonomic and metabolic information for the indicator operational taxonomic units (OTUs) from indicator species analysis on the control (CTL) and high NO_3^- nutrient-diffusing substrate treatments listed in Table 3 including class and the ability to reduce NO_3^-. Each taxon is identified to the lowest level possible. UC stands for unclassified and is used to mark different OTUs sharing the same taxonomic group. Taxa that are nonsulfur purple bacteria, which have flexible metabolisms, are marked with an asterisk (*). References used to compile this table are listed in Appendix S4.

Treatment	Taxon	Class	Metabolism	Reduces NO_3^-?
CTL	*Desulfovibrio*	Deltaproteobacteria	Obligate anaerobic chemoorganotrophs	No
	Paludibacter	Bacteroidia	Obligate anaerobic chemoorganotrophs	No
	*Phaeospirillum**	Alphaproteobacteria	Anoxygenic photoorganotrophs	No
	*Rhodoblastus**	Alphaproteobacteria	Anoxygenic photoorganotrophs	No
	*Rhodocyclus**	Betaproteobacteria	Anoxygenic photoorganotrophs	No
	Clostridiaceae	Firmicutes	Obligate anaerobic chemoorganotrophs or chemolithotrophs	Some
	Veillonellaceae	Firmicutes	Obligate anaerobic chemoorganotrophs	Some
	Bacteroidales	Bacteroidia	Obligate anaerobic chemoorganotrophs	Some
High NO_3^-	*Janthinobacterium*	Betaproteobacteria	Obligate aerobic chemoorganotrophs	Yes
	Microvirgula	Betaproteobacteria	Facultative aerobic chemoorganotrophs	Yes
	Paenibacillus	Firmicutes	Aerotolerant anaerobic chemoorganotrophs	Yes
	Pseudomonas	Gammaproteobacteria	Obligate or facultative aerobic chemoorganotrophs	Some
	Enterobacteriaceae UC1 and 2	Gammaproteobacteria	Facultative aerobic chemoorganotrophs	Most

coastal wetlands demonstrated increased biomass in response to NO_3^- (Cooper et al. 2015), but the communities of these wetlands with their high NO_3^- (97 ± 220 μg/L) and TN (920 ± 710 μg/L) concentrations may have been adapted to NO_3^- inputs. In ecosystems with relatively little anthropogenic NO_3^- input like the CRD, NO_3^- amendment could provide a novel substrate that alters redox conditions in anaerobic biofilms. If thick biofilms experience O_2 depletion and are given a novel electron acceptor, such as NO_3^-, denitrifiers could outcompete organisms using other alternative electron acceptors because Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2 are less energetically profitable than NO_3^- (Stumm and Morgan 1996). This advantage may explain why we observed a shift in microbial communities on NO_3^--amended substrates toward organisms with the ability to reduce NO_3^-.

The shift in redox conditions can explain the observed patterns in microbial community composition, but the reduction in biofilm biomass caused by NO_3^- is more difficult to interpret. Likens et al. (1970) suggested that high concentrations of NO_3^- may be toxic to certain bacterial species, but did not offer a mechanism or toxicity threshold. NO_3^- is used as a preservative to reduce the growth of *Clostridium botulinum* and other microorganisms (Roberts 1975), and NO_2^- can retard lipid oxidation (Gray et al. 1981). Reduction of NO_3^- to NO_2^- in these pond biofilms could inhibit microbial growth. We did see a larger reduction in biomass at higher concentrations of NO_3^- (>60% at 0.5 M), but still observed a 40% reduction in biofilm biomass at the log-lower concentration (0.05 M). The N diffusion rate of the high NO_3^- (0.1 mg/h) and low NO_3^- (0.01 mg/h) substrates early in deployment was much lower than for N substrates used in other studies with comparable concentrations (Bernhardt and Likens 2004: 10 mg/h, Rugenski et al. 2008: 0.1–1 mg/h). However, NO_2^- still could have accumulated in the thick CRD biofilms at high enough concentrations to approach toxicity.

An alternative way to explain the reduction in total biofilm biomass is that NO_3^- alters microbial interactions by selecting for taxa that produce natural antimicrobial compounds. Thus, the reduction in biomass could be caused by certain bacterial taxa spending their energy on the production of toxins instead of growth. The toxins, in turn, could reduce the growth of other microbial competitors. Many of the indicator OTUs for the NO_3^--amended substrates have been documented to produce antibiotics. For example, *Janthinobacterium* spp., an indicator of our NO_3^--amended communities, produces a violet pigment with antimicrobial properties called violacein (Pantanella et al. 2007, Kim et al. 2012), whereas *Janthinobacterium lividum* produces the antibiotic prodigiosin (Schloss et al. 2010). A strain of *Paenibacillus*, another indicator of our NO_3^--amended communities can produce polymyxin E1, an antibiotic active against Gram-negative bacteria, and 2983-Da, an unknown antibiotic active against Gram-positive bacteria (He et al. 2007). We did not find similar evidence of CTL OTUs producing antimicrobial compounds in the literature. We cannot verify whether our related OTUs produce antibiotics without culturing these strains in the laboratory and testing them under similar conditions to those in the experiment, but their potential antimicrobial properties could help explain the reduction in total biofilm biomass.

Conclusions

We demonstrated that NO_3^- can greatly reduce biofilm biomass and strongly alter microbial community composition in low-nutrient environments. The reduced biomass observed on NO_3^--amended substrates may be caused by NO_3^- toxicity or shifts in competitive advantages among taxa, which affect biofilm formation and community assembly. We suggest that other aquatic ecosystems that receive little geologic or anthropogenic NO_3^- input may exhibit strong changes in microbial community structure and potentially function. Future researchers should test whether a legacy of anthropogenic NO_3^- inputs fundamentally changes microbial community assembly and biogeochemical cycling in aquatic ecosystems.

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