Final Report

Project Title: Influence of Algal / Bacterial Interactions on Denitrification in Stream Biofilms

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Problem and Research Objective :

Over the last century, since the development of a commercially viable process for converting atmospheric nitrogen (N_2) to ammonium (the Haber–Bosch process), the input of nitrogen to the environment has increased dramatically. In fact, the amount of nitrogen fixed annually by industry now exceeds that fixed by combined biological processes in ecosystems worldwide (Vitousek et al. 1997). Much of the N that is fixed commercially is applied to agricultural soils, and much of this N ends up in freshwater or marine systems via leaching and runoff. Compared to pre-industrial times, annual N loading rates into freshwaters in the United States have increased 6 to 50-fold (Carpenter et al., 1998), with a concurrent 20-fold increase in N loading into the oceans from the world's largest river systems (Howarth et al. 1996).

Increased nitrogen inputs to surface waters can lead to decreased water quality. For example, high levels of nitrogen can result in eutrophication (Officer et al. 1984, Nixon et al. 1996) which can lead to excessive growth of algae. Algal blooms followed by algal decomposition can lead to a decrease in the amount of dissolved oxygen in the water, a condition known as hypoxia (Nixon et al. 1996), which can cause the death of fish and other aquatic organisms (Carpenter et al. 1998, Goolsby et al. 2001). A well publicized example of this phenomenon can be seen annually in the Gulf of Mexico. Nitrogen inputs to the gulf have tripled in the last 30 years and have been linked to the development of a huge seasonal hypoxic zone (Alexander et al. 2000, Goolsby et al. 2001). Fishermen refer to this hypoxic zone as a "dead zone" due to the lack of fish, crabs, and shrimp.

Nitrogen pollution of rivers and streams is an especially significant issue for the Midwestern United States and for the State of Illinois in particular due to the high levels of agriculture in this region. The soils in this region receive high inputs of N fertilizers, and much of this N finds its way into the Mississippi River and eventually to the Gulf of Mexico. Although Illinois represents approximately 6% of the drainage area of the Mississippi River, it is responsible for about 15% of the nitrates in the river (David and

Gentry 2000), so Illinois has a disproportionately large impact on downstream water quality.

Due to the significant environmental problems linked to N pollution, there is interest in biological processes that can remove nitrogen from aquatic ecosystems. Denitrification is a microbially catalyzed dissimilatory redox process through which nitrate (NO_3^-) is reduced to gaseous nitrogen (primarily N_2). Nitrate is a soluble, biologically available form of nitrogen, but N_2 is a gas that will diffuse to the atmosphere and has limited biological availability (it is only available to N fixing bacteria). Therefore, denitrification has the capacity to reduce N pollution of surface waters by removal of nitrogen from the system.

Denitrification is catalyzed primarily by denitrifying bacteria and it tends to be favored in anoxic habitats, so high rates of denitrification are often observed in aquatic sediments just below the sediment water interface. However, a number of studies have demonstrated that significant denitrification also occurs within periphytic biofilms (Nakajima 1979; Sorensen et al. 1988; Eriksson & Weisner 1997; Eriksson 2001; Toet et al. 2003; Sladeckova et al. 1983, Baldwin et al. 2006), which are mixed communities of algae and bacteria that can be found attached to solid surfaces within aquatic ecosystems (Wetzel 1983). Within these biofilms algae grow autotrophically producing fixed carbon and heterotrophic bacteria utilize the carbon produced by algae, supplied either via extracellular release or algal decomposition (Haack & McFetters 1982; Murray et al. 1986; Paerl & Pinckney 1996). Recent studies by Co-PI Gray have demonstrated that denitrification rates per cell can actually be much higher in periphytic biofilms as compared to sediments, thus suggesting that biofilms might be hot-spots for denitrification activity within aquatic ecosystems (Sirivedhin & Gray 2006). Biofilms therefore have the potential to make significant contributions to nitrogen removal from aquatic ecosystems. Unfortunately relatively little is known about the mechanisms driving denitrification in these biofilms.

The objective of our project is to explore the ecology of periphytic biofilms with a specific focus on denitrification. We will accomplish this goal through a combination of field and laboratory-scale experiments. This project will improve our understanding of the ecology of denitrification in streams and rivers, and it will provide insight into environmental factors that influence denitrification rates. This work could have significant implications for the management and remediation of nitrogen pollution and the design of systems for nitrogen removal, including wetland restoration and the construction of artificial treatment wetlands. Specifically, our project will provide details on environmental and ecological conditions that will maximize denitrification, details that could be incorporated into the design of treatment systems in order to maximize nitrogen removal.

Prior to the IWRC award our research team conducted a short term (28 day) field experiment in two streams in DuPage County Illinois that differed in degree of human impact. The low impact stream was in a restored prairie watershed and the high impact stream was directly downstream of a wastewater treatment plant that released its

effluent to the stream. Our results demonstrated that there was a tight coupling between the species composition of the algal and bacterial communities at the low-impact site, supporting the important role that algae can play in shaping bacterial communities within biofilms. However, at the high-impact sight the algal and bacterial communities were de-coupled, suggesting a significant role of anthropogenic inputs in shaping biofilm bacterial communities. The results of this study were recently published in the journal *FEMS Microbiology Ecology* (Peterson et al., 2011). These results generated several lines of inquiry into the ecology of periphytic biofilms:

Research Element 1: Explore the influence of algae on bacterial community composition within biofilms

Element 1 Part A: The short term field project described above suggested that algae could be drivers of bacterial community development in periphytic biofilms. We wanted to explore the potential influence of specific algal species on bacterial community development in controlled lab experiments. **We explored this question as part of the IWRC funded project, as detailed below.**

Element 1 Part B: We hypothesized that algae might influence bacterial community development in biofilms through the production and release of species specific organic compounds, which might select for different bacterial communities. **We tested this hypothesis as part of the IWRC funded project, as detailed below.**

Element 1 Part C: The short term field project described above suggested a tight coupling between algal and bacterial communities in one stream during the first 28 days of biofilm development. We wanted to determine if this coupling would be observed in a wider arrays of streams over a longer period of time. We conducted a larger scale investigation of periphytic biofilms using six streams over a period of 12 weeks. **The analysis of the bacterial communities in these samples was part of the IWRC funded project, as detailed below.**

Research Element 2: Explore the mechanisms by which anthropogenic inputs might influence bacterial species composition within biofilms

Element 2 Part A: We hypothesized that the organic compounds within wastewater effluent might be disrupting the coupling between algal and bacterial communities within biofilms. To explore this we used Pyrolysis GC/MS to compare the organics within rivers with varying degrees of

wastewater inputs. We found that the dissolved organic carbon in streams that received discharge from upstream wastewater treatment plants was distinct from the organic carbon in streams that did not receive discharge, and we were able to identify specific pyrolysis fragments that were indicative of upstream wastewater treatment plant discharges. This work was completed and recently published in the journal *Water Research* (Kalscheur, Penskar, et al., 2012). This research element was not part of the IWRC funded project, but this work did contribute to our understanding of these systems and lead to further research questions that were explored in the IWRC funded project, as detailed below.

Element 2 Part B: Our next step was to explore the impacts that organics from streams with and without wastewater inputs might have on bacterial communities within biofilms. **We explored this question as part of the IWRC funded project, as detailed below.**

Methodology for IWRC Funded Project

1. **Research Element 1 Part A: Influence of algal species identity on bacterial community development in biofilms**

   We established axenic cultures of four algal species that were numerically dominant at our field sites, *Achnanthidium minutissimum* (AM), *Achnanthidium lanceolatum* (AL), *Amphora pediculus* (AP) and *Nitzschia amphibia* (NA), and we collected a mixed bacterial community from streams at both of our field sites (the sites are described in Peterson et al., 2011). We then set up replicated lab-scale microcosms each consisting of a single algal species, and amended each microcosm with the mixed bacterial community. We monitored biofilm development in the microcosms over a period of 80 days. We used quantitative real-time PCR (qPCR) to quantify denitrifying bacteria within the biofilms. We used another leading-edge DNA-based approach, tag pyrosequencing, to assess the composition of the denitrifying bacterial communities within the biofilms. **This work was conducted by a graduate student at Loyola University Chicago, Miguel Rojas, who was supported by the IWRC award.**

2. **Research Element 1 Part B and Research Element 2 Part B: Effects of organic carbon source on the development of denitrifying bacterial communities in biofilms.**

   We were interested in exploring the effects of algal-derived organic carbon (Research Element 1 Part B) and organic carbon from stream sites that varied in

anthropogenic inputs (Research Element 2 Part B). To accomplish this we isolated organic exudates from axenic cultures of seven algal species that were common in our field sites. We also isolated organic carbon from the two streams in DuPage county that we had used for our short term study (see Peterson et al., 2011). We set up small-scale microcosms in which a mixed population of bacteria that was collected from the field was incubated with organic carbon from one of these sources. We measured rates of denitrification and development of denitrifying bacterial communitties using qPCR and tag pyrosequencing. **The analysis of the bacterial communities for this project was conducted by a graduate student at Loyola University Chicago, Miguel Rojas, who was supported by the IWRC award.**

3. **Research Element 1 Part C: Biofilm development in six streams of varied anthropogenic influence**

   Six streams in DuPage County Illinois that differed in degree of human impact, with some sites located directly downstream of wastewater treatment plant effluent discharge points and some streams running through protected areas, including prairies and forests, were selected for this field study. Ceramic tiles were incubated in each stream for a total of 12 weeks to provide a uniform substrate for the development of periphytic biofilms. Tiles were collected after 3, 5, 8 and 12 weeks and returned to the lab for biofilm analysis. On each sampling date, 500 ml of stream water was also collected to assess nutrient chemistry. **The analysis of the bacterial communities within these biofilms was conducted by a graduate student at Loyola University Chicago, Miguel Rojas, who was supported by the IWRC award.**

Principal Findings and Significance

1. **Research Element 1 Part A: Influence of algal species identity on bacterial community development in biofilms**

   The results of this project indicated that different algal species supported different abundances of denitrifying bacteria (Fig. 1). In addition, the species composition of the denitrifying bacterial communities was also influenced by the identity of the algal species (Fig. 2). This work was conducted by a graduate student at Loyola University Chicago, Miguel Rojas, who was supported by the IWRC award. Miguel is currently preparing a manuscript based on this project that will be submitted to a peer reviewed scientific journal. Miguel will serve as the first author on this manuscript and the support of IWRC will be acknowledged.

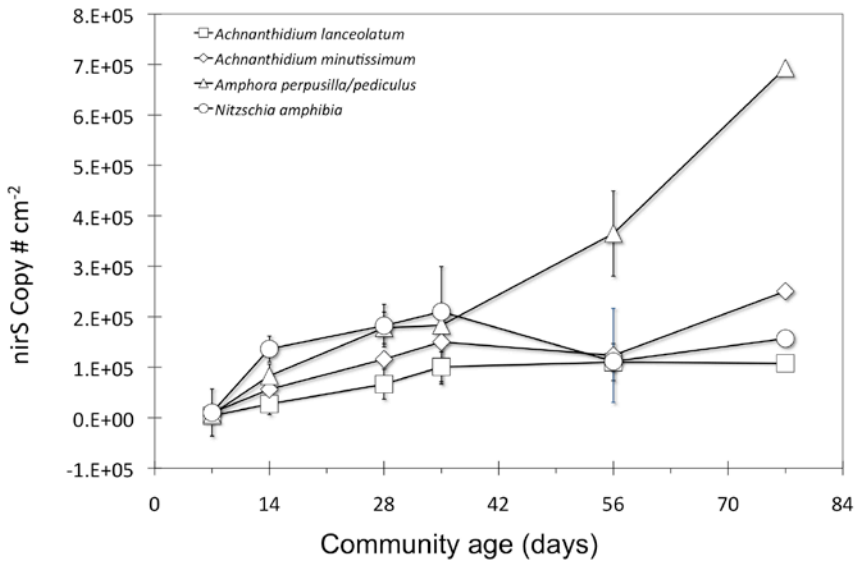

Fig 1. Effect of algal species identity on abundance of denitrifying bacteria as indicated by quantitative PCR (qPCR) quantification of *nirS* copy numbers. Each data point represents mean of four replicates and error bars represent standard error.

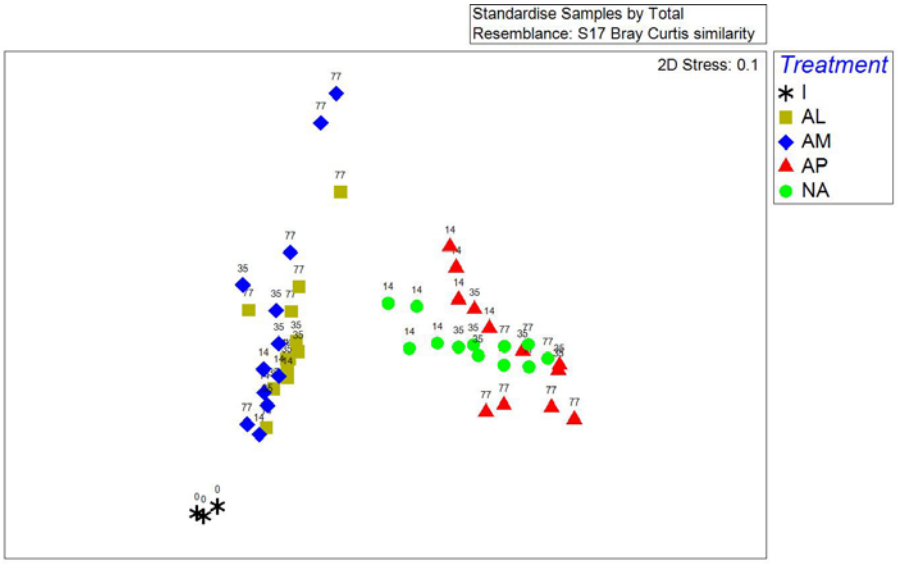

Fig 2. Effect of algal species identity on denitrifier community composition. Ordination is based on MDS analysis of *nosZ* gene sequences present within each biofilm. Gene sequences were determined via tag pyrosequencing of *nosZ* gene. Letters indicate algal species present in each microcosm (Achnanthidium lanceolatum (AL), Achnanthidium minutissimum (AM), Amphora perpusilla/pediculus (AP) and Nitzschia amphibia (AM). The initial bacterial inoculum is shown as "I" on the ordination. Numbers above each data point indicate duration of incubation (in days).

2. **Research Element 1 Part B and Research Element 2 Part B: Effects of organic carbon source on the development of denitrifying bacterial communities in biofilms.**

   Our analysis revealed differences in the organic composition of algal exudates and stream waters, which, in turn, selected for distinct communities of denitrifying bacteria. Organic carbon source also had a significant effect on denitrification rates of the communities. One especially interesting finding was that organic carbon from the stream with low human inpacts selected for a bacterial community that was similar to the community produced by incubation with algal-derived organic carbon. In contrast, organic carbon from the stream that received wastewater input selected for a distinct bacterial community that also showed the highest rates of denitrification. This result supported our earlier finding (as reported in Peterson et al., 2011) that anthropogenic inputs can alter the composition of bacterial communities within biofilms. Miguel Rojas, a graduate student at Loyola University Chicago who was supported by the IWRC award, contributed the bacterial community analysis to this project. **This work was recently published in the journal *Microbial Ecology* (Kalscheur, Rojas et al 2012) and the support of IWRC was acknowledged (see attachment).**

3. **Research Element 1 Part C: Biofilm development in six streams of varied anthropogenic influence**

   Our analysis revealed that the streams at our field sites varied significantly in a variety of physico-chemical characteristics, including depth, velocity, concentrations of N and P, and DOC concentration. Tiles from all sites developed biofilms over the course of the study, although the biofilm mass varied for the different sites. Half of the sites showed measurable rates of denitrification, and these tended to be the sites with higher levels of biofilm mass. The sites differed in algal species composition (Fig. 3). Miguel Rojas, a graduate student at Loyola University Chicago who was supported by the IWRC award, was responsible for analysis of the bacterial communities in these biofilms. Miguel used terminal restriction fragment lentgth polymorphism (T-RFLP) analysis to profile the bacterial communities within the biofilms (Fig. 4). This analysis did not show a clear separation of bacterial communities based on site. Miguel also profiled the denitrifying bacterial communities within the biofilms using tag pyrosequencing. This analysis focused on the denitrifying bacteria in particular based by targeting *nosZ*, a functional gene involved in the denitrification pathway. These results demonstrated a strong seperation of denitrifying communities based on site (Fig. 5). Our analysis of the relationships between algal and bacterial communities at these sites is ongoing, and we are in the process of preparing a manuscript based on this project that will be submitted to a peer reviewed scientific journal. Miguel will serve as a co-author on this manuscript, and the support of IWRC will be acknowledged.

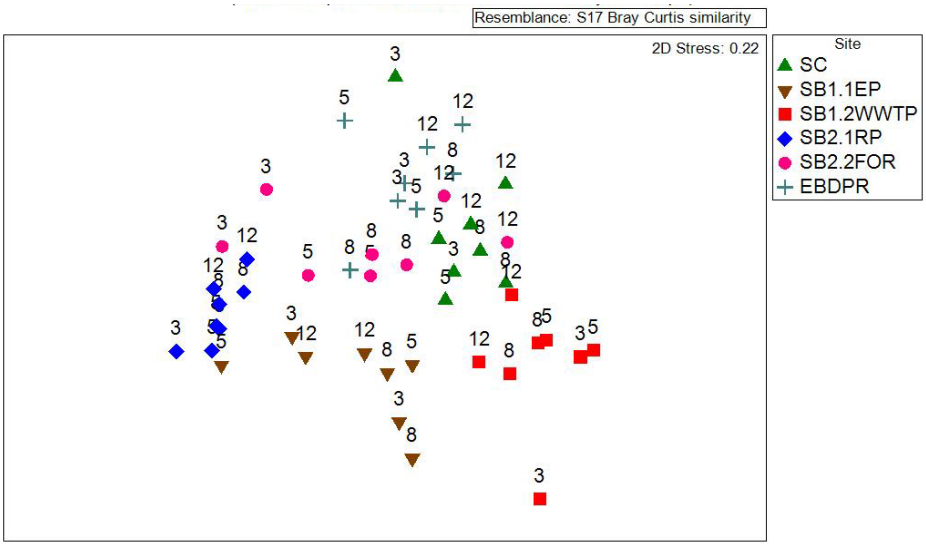

Fig 3. Algal community composition in biofilms from six field stream sites. Ordination is based on MDS analysis of algal species abundance data determined by microscopic identification of algal cells. Different colors and shapes indicate different stream sites, and numbers above data points indicate the number of weeks that tiles were incubated in the streams (i.e. time of biofilm development).

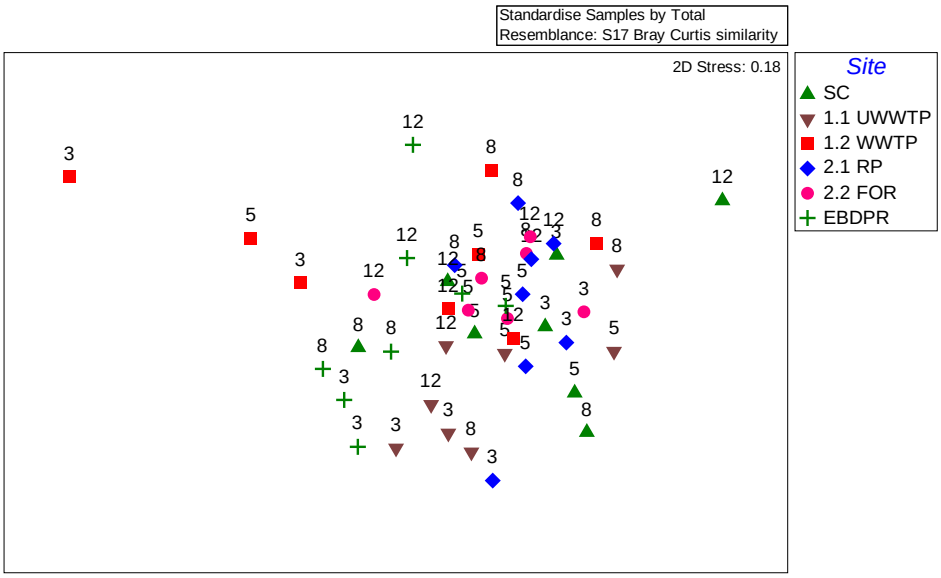

Fig 4. Total bacterial community composition in biofilms from six field stream sites. Ordination is based on MDS analysis of T-RFLP analysis of 16S rRNA genes. Different colors and shapes indicate different stream sites, and numbers above data points indicate the number of weeks that tiles were incubated in the streams (i.e. time of biofilm development).

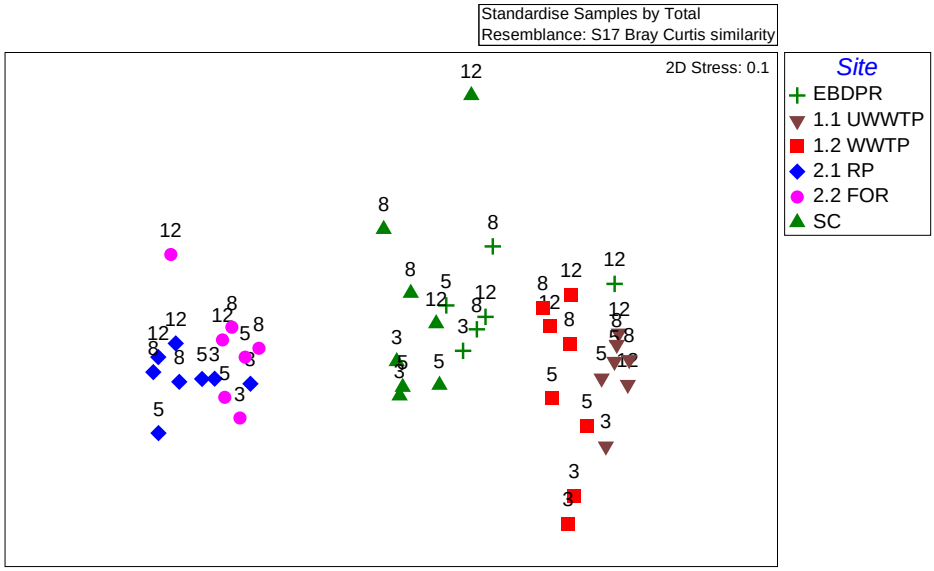

Fig 5. Denitrifying bacterial community composition in biofilms from six field stream sites. Ordination is based on MDS analysis of pyrosequencing analysis of *nosZ* genes. Different colors and shapes indicate different stream sites, and numbers above data points indicate the number of weeks that tiles were incubated in the streams (i.e. time of biofilm development).

Notable Achievements

- We demonstrated that the input of wastewater treament plant effluent to streams can disrupt the ecology of biofilms and influence an envrionmentally significant process, denitrification
- We demonstrated that pyrolysis GS/MS technology can be used to discriminate the organic carbon in streams with varying degrees of wastewater inputs, and that wastewater inputs can alter the organic composition of streams
- We have gained new insight into the realtionships between algal species and bacterial species within biofilm communities

Students Supported with Funding

Miguel Rojas, a graduate student in the MS program in Biology at Loyola University Chicago was supported by the IWRC award. Miguel entered the program in Fall of 2010 and is planning to graduate with his MS degree in Spring 2011.

Publications and Presentations

We have published one paper that included results obtained with IWRC support. This paper (which is attached) acknowledged IWRC support.

- Kalscheur, K.N., M. Rojas, C.G. Peterson, J.J. Kelly, and K.A. Gray. 2012. Algal exudates and stream organic matter influence the structure and function of denitrifying bacterial communities. Microbial Ecol. 64: 881-892.

We have two other publications in preparation that were also supported by the IWRC award. These papers will acknowledge IWRC support.

- Peterson, C.G., A.D. Daley, S.M. Pechauer, K.N. Kalscheur, M. Rojas, K.A. Gray, and J.J. Kelly. Development of algal and bacterial communities in periphytic biofilms in streams with varied anthropogenic inputs. *In preparation.*
- Rojas, M., K.N. Kalscheur, C.G. Peterson, K.A. Gray, and J.J. Kelly. Algal taxa influence the development of denitrifying bacterial communities within biofilms. *In preparation.*

Miguel Rojas, a graduate student at Loyola University Chicago who was supported by the IWRC award, presented a poster based on his work at the 2012 General Meeting of the American Society for Microbiology.

- Rojas, M., K.N. Kalscheur, K.A. Gray, C.G. Peterson, and J.J. Kelly. Algal species identity influences the development of denitrifying bacterial communities in periphytic biofilms. General Meeting of the American Society for Microbiology, June 16 - 19, 2012, San Francisco, CA.

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MICROBIOLOGY OF AQUATIC SYSTEMS

Algal Exudates and Stream Organic Matter Influence the Structure and Function of Denitrifying Bacterial Communities

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Abstract Within aquatic ecosystems, periphytic biofilms can be hot spots of denitrification, and previous work has suggested that algal taxa within periphyton can influence the species composition and activity of resident denitrifying bacteria. This study tested the hypothesis that algal species composition within biofilms influences the structure and function of associated denitrifying bacterial communities through the composition of organic exudates. A mixed population of bacteria was incubated with organic carbon isolated from one of seven algal species or from one of two streams that differed in anthropogenic inputs. Pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) revealed differences in the organic composition of algal exudates and stream waters, which, in turn, selected for distinct bacterial communities. Organic carbon source had a significant effect on potential denitrification rates (DNP) of the communities, with organics isolated from a stream with high anthropogenic inputs resulting in a bacterial community with the highest DNP. There was no correlation between DNP and numbers of denitrifiers (based on *nirS* copy numbers), but there was a strong relationship between the species composition of denitrifier communities (as indicated by tag pyrosequencing of *nosZ* genes) and DNP. Specifically, the relative abundance of *Pseudomonas stutzeri*-like *nosZ* sequences across treatments correlated significantly with DNP, and bacterial communities incubated with organic carbon from the stream with high anthropogenic inputs had the highest relative abundance of *P. stutzeri*-like *nosZ* sequences. These results demonstrate a significant relationship between bacterial community composition and function and provide evidence of the potential impacts of anthropogenic inputs on the structure and function of stream microbial communities.

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Introduction

Denitrification, the reduction of nitrate to dinitrogen, is an ecologically significant process that removes nitrogen from aquatic ecosystems and can ameliorate the effects of nitrogen pollution. However, the potential of surface waters to support denitrification has steadily diminished with the loss of habitats and aquatic buffer zones, such as forested and coastal wetlands, the losses of which continue to substantially outdistance gains [18]. With the amount of nitrogen fixed from the atmosphere for human use vastly exceeding biophysical thresholds, much of this new reactive nitrogen has accumulated in the environment, which has led to detrimental environmental changes [37] including the seasonal hypoxic zone in the Gulf of Mexico [36]. Recent results indicate that since 1980 little progress has been made in reducing nitrate concentrations in the Mississippi River and that flow-normalized nitrate inputs to the Mississippi are increasing in some areas [47]. Therefore, there is significant interest in understanding the factors that control rates of denitrification in aquatic ecosystems.

Within aquatic ecosystems, periphytic biofilms can serve as hot spots for a myriad of biogeochemical processes including denitrification [8, 46, 48, 50]. The metabolic processes of bacteria within biofilms can be influenced significantly by interactions with resident microalgae, as bacteria often rely on algal-derived organic carbon to fuel their metabolism [6, 14, 24]. The strength of algal/bacterial interactions and, by inference, their importance in mediating biogeochemical processes within biofilms can be influenced by exogenous factors such as nutrient concentrations and supply rates [33, 35, 42, 54]. Less is known about the degree to which variation in algal species composition influences the structure and metabolic performance of bacterial consortia in freshwater biofilms. Results of Ishida et al. [27] suggested that rates of denitrification in biofilms varied depending on the degree of dominance of certain algal genera within those biofilms. This led us to investigate more closely the relative influence of algal species assemblage on the composition and performance of consortia of denitrifying bacteria within benthic biofilms.

We hypothesize that algal communities influence the composition and function of denitrifying bacterial communities within periphyton through interspecific variation in the composition of organic exudates produced by the algae. The rationale for this hypothesis is based on previous data illustrating that bacteria within periphyton use algal-derived exudates as a carbon source to fuel their metabolism [6, 24], that different algal taxa can produce chemically distinct exudates [3, 25, 52], and that different algal species can support taxonomically distinct communities of satellite bacteria [22, 30, 40]. In addition, the ability to denitrify is widely dispersed among the prokaryotes, being found in about 50 bacterial genera [43]. There are differences among these genera in their oxygen thresholds, carbon requirements, and kinetic parameters [49], suggesting that the species composition of denitrifying bacterial communities may have significant functional implications. Several studies have demonstrated a relationship between the composition of denitrifying communities and the process of denitrification in terrestrial ecosystems [16, 26] and stream sediments [5].

To test our hypothesis, we designed an experiment that used a mixed population of bacteria obtained from local streams and incubated this mixed community with organic carbon isolated either from one of seven different algal species or from the waters of two streams that varied in degree of anthropogenic inputs. To determine if the organic carbon derived from these different sources influenced the composition and function of denitrifying bacteria, we quantified denitrifying bacteria based on quantitative real-time polymerase chain reaction (qPCR), assessed the composition of denitrifying bacterial communities using tag pyrosequencing, and measured potential denitrification rates using the acetylene inhibition method. Denitrifying bacteria were profiled based on functional genes involved in the denitrification pathway (*nirS* and *nosZ*), because the phylogenetic diversity of denitrifiers makes 16S rRNA gene-based approaches inadequate to target this functional guild [12]. In this experiment, we also used Py-GC/MS to fingerprint the salient chemical characteristics of each organic source and then used these data to determine if we can identify distinct chemical structural features that are associated with the selection of particular bacterial community composition and function [28, 35, 46].

Methods

Algal Exudates and Stream Water Organic Carbon

Triplicate axenic algal batch cultures were established for five diatoms [*Achnanthidium minutissimum* (Kütz.) Czarnecki (AM), *Achnanthidium lanceolatum* Bréb. ex Kütz. (AL), *Amphora pediculus* (Kütz.) Grun. (AP), *Gomphonema parvulum* (Kütz.) Kütz. (GP), *Nitzschia amphibian* Grun. (NA)] and two green algae [*Scenedesmus armatus* (R. Chodat) R. Chodat (SA) and *Stigeoclonium tenue* (C. Agardh) Kütz. (ST)] from algal inocula obtained from The Culture Collection of Algae at the University of Texas at Austin. These algal species were selected based upon previous studies and on our field and artificial stream observations of their potential influence on denitrification [27]. Cultures were grown in 250 ml Erlenmeyer flasks filled with 150 ml of Chu's Medium No. 10 and capped with a sponge and stainless steel cap. Growth was monitored by cell counts, and when stationary phase growth was observed, the extracellular organics were separated from the cells by filtering (pre-rinsed 0.45 mm Tuffryn® membrane filters).

The effects of organic carbon from the axenic algal cultures were also compared to the dissolved organic carbon isolated from two stream waters with contrasting anthropogenic influences. Water samples were collected on August 4, 2009 from two streams in DuPage County, IL, one within a Restored Prairie (RP) site that did not receive significant anthropogenic inputs and one located downstream of a wastewater treatment plant effluent release (DER). These sites are described in detail in [28, 35]. Nearly 100 % of the stream flow at the DER site on this date can be attributed to the Wheaton Sanitary District Plant's effluent discharge as calculated from field measurements and plant operating data. Stream waters were collected in acid-washed amber glass jugs, filtered (pre-rinsed 0.45 μm Tuffryn® membrane filters) and stored at 4 °C.

The dissolved organic carbon concentration in the filtered cultures and stream waters was measured by high

temperature catalytic oxidation (APHA Standard Method 5310 B; Dohrmann Apollo 9000). Each organic carbon sample was then split, with a portion reserved for organics analysis via Py-GC/MS and a portion used as the carbon source in the bacterial incubations.

Bacterial Inoculum

A mixed bacterial consortia was collected from periphyton attached to rocks in three streams with varying anthropogenic influences, including the RP and DER sites mentioned above and a site on the E. Branch DuPage River that had upstream input of wastewater treatment plant effluent. Periphyton was removed from rocks by scraping with a stiff-bristle toothbrush into stream water. The collected periphyton from all three sites was homogenized and filtered through a 5 μm Teflon filter (Micron Separations Inc.) with the filtrate containing the bacterial consortia preserved at -85 °C in a 15 % (v/v) glycerol solution. Initial experiments indicated insufficient bacterial mass for the measurement of denitrification. Therefore, prior to the start of the incubations with different carbon substrates, the bacteria were pre-fed M9 Minimal Media [39] with glucose (G) as the carbon source until exponential growth was observed. The bacteria were then washed ten times in M9 Minimal Media without a carbon source.

Incubations

Subsamples of the bacterial inoculum (IN) were incubated for 14 days with equal amounts (1 mg) of the different carbon substrates from seven algae and two stream waters. A G incubation was also included as a control. Each incubation was done in duplicate. Carbon substrates included G, carbon isolated from one of the monoalgal cultures (AL, AM, AP, GP, NA, SA, or ST) or carbon isolated from one of the two field sites (DER or RP). Incubations were conducted in 250 ml gas-tight jars (I-CHEM septa jars) containing 190 ml M9 minimal media (without glucose) and 40 mg l^{-1} NO_3-N as KNO_3 to provide enriched conditions for denitrification. At the start of the incubation, the jars were sealed and flushed with N_2 for 3 min. During the incubation, jars were agitated gently at 150 rpm, under dark conditions at room temperature (23± 1 °C). The dissolved organic carbon concentration in each of the jars was measured four times during the 14-day incubation period by removing 3 ml of solution by syringe through the jar's septa and measuring dissolved organic carbon (DOC) by high-temperature catalytic oxidation (APHA Standard Method 5310 B; Dohrmann Apollo 9000).

Measurement of Denitrification Potential

Denitrification potential (DNP) of incubated bacterial communities was determined by a modified version of the standard acetylene inhibition method for ecological research [2, 21, 46]. To ensure enriched conditions for the DNP analyses, 15 ml containing 40 mg l^{-1} NO_3-N as KNO_3 and 100 mg l^{-1} carbon as G were added to the jars by syringe along with 225 mg l^{-1} chloramphenicol to inhibit microbial growth. The DNP incubation started immediately after the jars were flushed with N_2 for 3 min and acetylene was added (10 % v/v) to inhibit transformation of N_2O to N_2. During the DNP incubation, the jars were agitated gently at 150 rpm under dark conditions at room temperature (23±1 °C). Headspace samples were measured using a gas chromatograph (Hewlett Packard 5890) equipped with ^{63}Ni electron capture detector at an operating temperature of 320 °C. A stainless steel Porapak Q (80/100 mesh) column was used to separate the gases at 60 °C with high purity N_2 as a carrier gas (18–20 ml min^{-1}). Denitrification rates were calculated from linear regression of N_2O accumulation in the headspace, after the concentrations were corrected for solubility using the Bunsen coefficient [51]. Effect of carbon source on DNP rates was assessed by analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test using Systat version 13 (Systat Software, San Jose, CA). DNP rates were square root transformed prior to ANOVA to account for non-homogeneity of variances.

Sample Collection and Processing

At the end of the incubation period jars were stored overnight at 4 °C. From each jar 200 ml of microbial suspension was removed and centrifuged at 12,000×*g* for 10 min. Supernatant was removed by decanting and the pellet was resuspended in 1.5 ml of supernatant and transferred to a 2 ml screw cap microcentrifuge tube. Each suspension was centrifuged at 12,000×*g* for 5 min, supernatant was removed by pipeting and the remaining pellet was stored at -20 °C. DNA was isolated from each pellet using the UltraClean microbial DNA kit (MoBio Laboratories, Carlsbad, CA). Successful DNA isolation was confirmed by agarose gel electrophoresis.

Organic Characterization by Py-GC/MS

For the stream waters, sample preparation involved first distilling approximately 4 L of the filtered water sample in a series of evaporations carried out using rotary vacuum evaporation (Buchi rotavapor Model R114 with B480 Waterbath) operating at 25 °C and under a vacuum pressure of approximately 27.5 in. Hg. To reduce salt interferences, which can exert matrix effects during pyrolysis, the concentrated liquid samples were dialyzed with a 2000 molecular weight cutoff membrane (Thermo Scientific Slide-A-Lyzer G2 Dialysis Cassette).

The concentrated liquid samples and filtered algal exudates were lyophilized, homogenized, and loaded into

pyrolysis tubes (unsealed quartz tube, Scientific Instrument Services, Inc.) to approximately 0.8–1.0 mg of carbon and plugged with deactivated glass wool. External standards of polystyrene and poly-L-tyrosine were run before each analysis series and within a series to verify proper instrument functioning. After preparation, the sample or standard was placed inside the platinum filament coil of the pyrolysis probe (Chemical Data Systems Pyroprobe, 2000) which was then inserted into the interface (Chemical Data Systems 1500 valved GC interface). Conditions of the pyrolysis unit used to ensure reproducibility of the analysis include: (1) pyrolysis interface temperature of 250 °C, (2) final pyrolysis temperature of 625±5 °C, (3) total pyrolysis time of 1 min, and (4) ramping rate of 20 °C ms^{-1}. After flash pyrolysis, the volatile pyrolyzates were directly swept onto a GC column to be separated (60-m, 0.25-mm internal diameter, cross-bond, carbowax column; Restek: Stabilwax®). The gas chromatograph (Fisons 8030) was operated in a splitless injection mode with a column head pressure of 25 psi. Oven temperature was held at 45 °C for 15 min, then ramped up to 240 °C at 2 °C min^{-1}, and finally, held at 240 °C for 10 min. The separated fragments were identified by MS (Fisons MD 800) that operated at 70 eV and scanned from 20 to 400 amu at 1 scan s^{-1}. Positive electron ionization (EI+) mode was used as an ion source. The source temperature was set at 200 °C and the GC/MS interface temperature was 250 °C. A GC/MS data acquisition software, Xcalibur version 1.2, was used to collect the mass to charge (*m*/*z*) scan and produce a pyrochromatogram. The National Institute of Standard Technology (NIST) Library (match at >800/1000) was then used to identify the fragments in the pyrochromatogram. Principal component analysis (PCA) was employed to assess between species differences in organic signatures. PCA of the organic profiles was performed using prcomp function in R (R Development Core Team, 2008).

Quantification of Denitrifying Bacteria

qPCR was used to determine copy number of *nirS* genes as an indicator of the number of bacteria within the communities with the potential for denitrification. qPCR reactions followed the protocol described by Mincer et al. [31]. Briefly, primers nirS1F and nirS6R [11] were used to amplify an 890 base pairs (bp) fragment of the *nirS* gene. The standard used for quantification was genomic DNA isolated from *Pseudomonas stutzeri* ATCC 11607, which was assumed to have a genome size of 4 Mbp and 1 *nirS* copy per genome [23]. The *P. stutzeri* dilution series included tenfold dilutions ranging from 1.2×10^5 to 12 copies of *nirS*. qPCR reactions were run using an MJ Research DNA Engine Opticon 1 thermal cycler equipped with Opticon software version 3.1 (Bio-Rad, Hercules, CA). The conditions for all qPCR reactions were as follows: 12.5 μl QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 0.5 μM final concentration of each primer, 1 μL template, and water were added to a final 25 μL volume. All primers were synthesized by Eurofins MWG Operon (Huntsville, AL). All reactions were performed in low-profile 0.2 ml white strip tubes with optical ultraclear strip caps (Bio-Rad). Three analytical replicates were run for each sample. The specificity of qPCR reactions was confirmed by melting curve analysis and agarose gel electrophoresis. Thermal cycling was conducted as follows: initial denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 1 min, primer annealing at 57 °C for 1 min, extension at 72 °C for 1 min, hold at 78 °C for 1 sec, and plate read. Finally, a melting curve was run from 50 to 95 °C with a read every 1 °C and a hold of 1 s between reads. Effect of carbon source on *nirS* copy numbers was assessed by ANOVA and Tukey's honestly significant difference (HSD) test using Systat version 13 (Systat Software). Copy numbers were log transformed prior to ANOVA to account for nonhomogeneity of variances. Relationship between *nirS* copy numbers and DNP was assessed by determining Pearson product–moment correlation coefficients and probabilities using Systat version 13 (Systat Software).

Composition of Denitrifying Consortia

Tag pyrosequencing of *nosZ* genes was used to profile the bacteria within these communities with the potential for denitrification. DNA from each sample was sent to the Research and Testing Laboratory (Lubbock, TX). PCR amplification was performed using primers nosZF and nosZR [38]. Sequencing reactions utilized a Roche 454 FLX instrument (Roche, Indianapolis, IN) with titanium reagents. Sequences were processed using MOTHUR v.1.20.1 [41]. Briefly, any sequences containing ambiguities or homopolymers longer than eight bases were removed. Remaining sequences were individually trimmed to retain only high-quality sequence reads, and sequences were aligned using MAFFT (http://mafft.cbrc.jp/alignment/software/). Aligned sequences were trimmed to a uniform length of 136 bp, and chimeric sequences were removed using UCHIME [19] run within MOTHUR. After these pretreatment steps were completed, the data set included a total of 160,811 sequences for an average of 7657 sequences $sample^{-1}$. Sequences were clustered into operational taxonomic units (OTUs) based on 97 % sequence identity using the average neighbor algorithm. This clustering identified a total of 3963 OTUs. However, the communities were dominated by a relatively small number of OTUs, with the 20 most numerically dominant OTUs accounting for 83 % of the sequences. Therefore, subsequent analyses were based on this set of the 20 most numerically dominant OTUs. The community compositions of the individual samples were compared by

nonmetric multidimensional scaling (MDS) using the Primer V.5 software package (Primer-E, Plymouth, UK). For a full description of the MDS procedure, see Clarke and Warwick [17]. Briefly, the relative abundance of each of the top 20 OTUs within each of the samples was imported into Primer, and a similarity matrix was calculated using the Bray–Curtis coefficient [13]. The MDS procedure was then used to ordinate the similarity data following 100 random restarts. SIMPER analysis in Primer was used to compare the community composition of the samples that were DNP-positive, which showed production of N_2O in the DNP assay, and the samples that were DNP-negative, which did not show production of N_2O in the DNP assay. Selected OTUs were identified by comparing representative sequences to *nosZ* sequences available within Genbank via BLAST [1]. Primer was also used to quantify the diversity of the DNP-positive and DNP-negative samples based on the inverse Simpson index [44]. Finally, the relationship between the relative abundance of OTU1 and DNP rates was assessed by determining Pearson product–moment correlation coefficients and probabilities using Systat version 13 (Systat Software).

Results

Characterization of Organic Carbon

Initial comparisons of the Py-GC/MS fingerprints revealed that the organic mixtures of the algal exudates (*Achnanthidium lanceolatum* [AL] is provided as an example; Fig. 1) were very simple in contrast to the more complex organic fingerprint found in the stream waters (Fig. 2). For the algal exudates, the total number of peaks in the pyrochromatograms ranged from 35 to 53, while there were over 200 peaks in each of the stream water pyrochromatograms. Only seven peaks were identified in the exudates from *Amphora pediculus* [AP], since its low organic carbon concentration did not allow for detection of moderate to small peaks. Acetonitrile was the dominant peak (by area and height) in all of the algal exudate organic signatures, indicating a strong nitrogen containing aliphatic nature, which others have identified as a pyrolysis product of proteins [9, 32]. Other much less dominant common fragments include dipropylene glycol, phenol, styrene, propanols, and propenenitriles (Fig. 1). Differences among the algal exudates are found among the less dominant peaks, and we were unable to correlate these features with algal type or the denitrification potentials of the incubations.

In contrast, the stream waters showed a strong aromatic chemical signature with dominant pyrolysis fragments such as 1,2-dimethyl benzene, phenol and 4-methyl phenol (Fig. 2). Some clear differences between the organic signatures of the two stream waters are also evident (Fig. 2). The organic signature of RP water exhibited stronger peaks of 2-cyclopenten-1-one and 2-cyclopenten-1-one, 2-methyl-, which have been shown to be derived from soil polycarboxylic acids [10, 53], while the organic signature of DER water displayed the characteristics of wastewater effluent with dominant pyrolysis fragments such pyridine, pyrrole, and acetamide, products of protein, and aminosugar parent structures [7, 20, 28, 32, 34, 53]. To further compare the Py-GC/MS results, a semiquantitative technique [20] was used to assess differences in organic carbon signatures among all the samples analyzed. After the chemical peaks were identified in each sample, they were classified among four categories (aromatic, aliphatic, nitrogen-containing aromatic, and nitrogen-

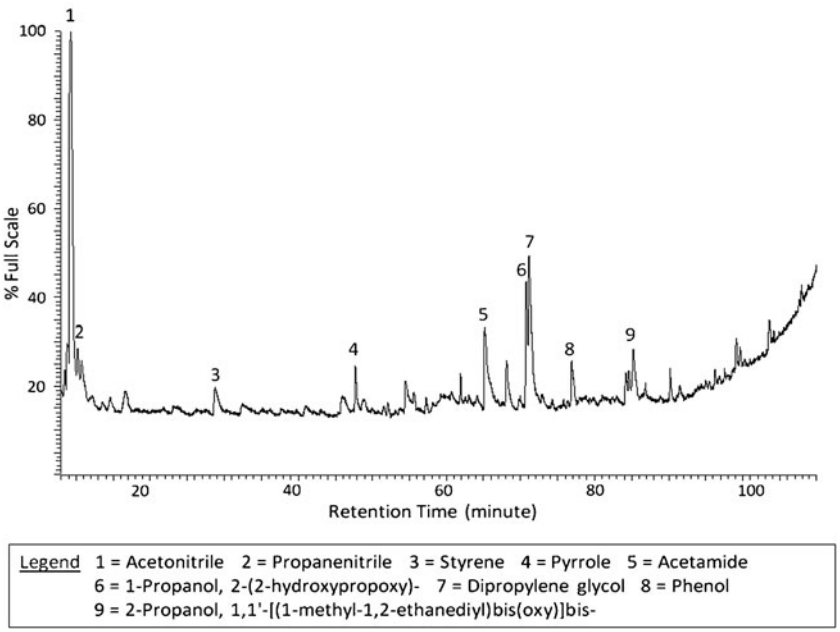

Fig. 1 Pyrochromatogram of *Achnanthidium lanceolatum* (AL) exudates

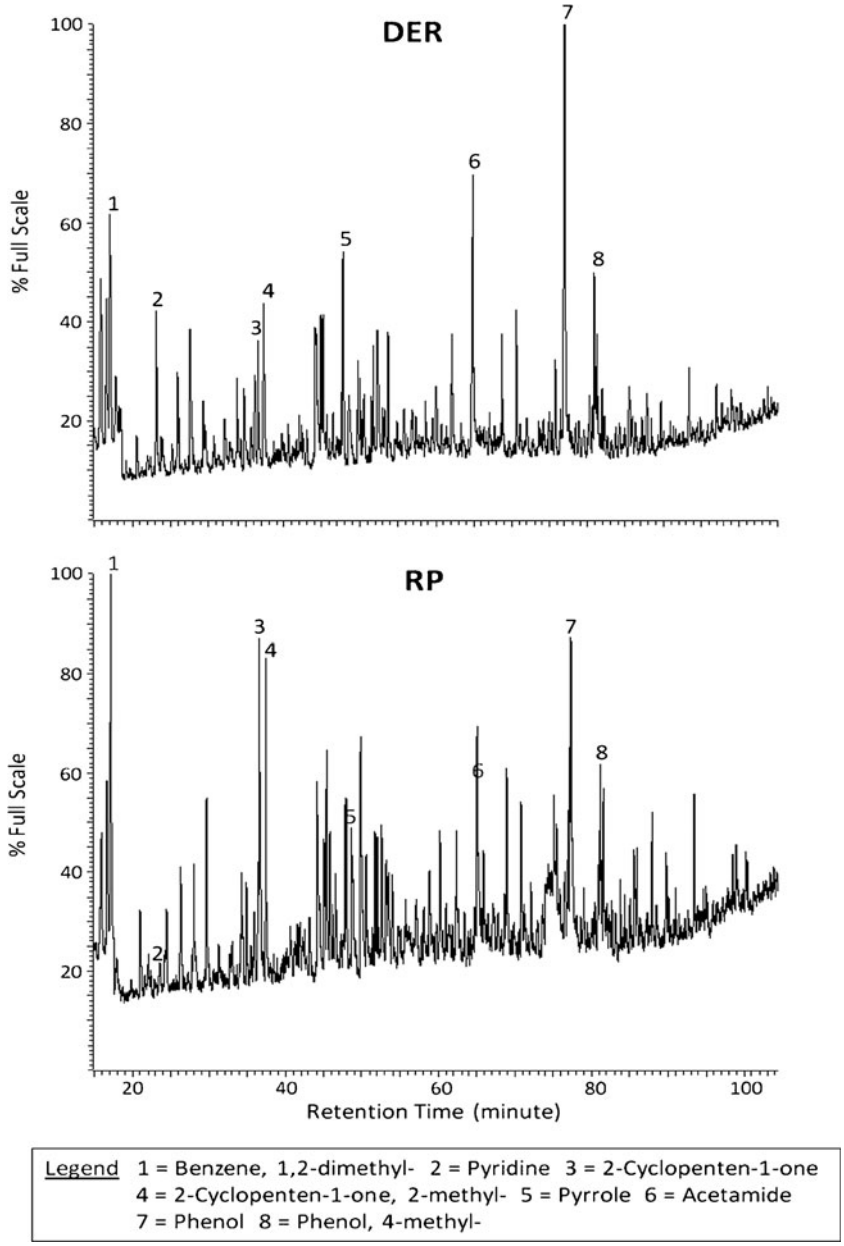

Fig. 2 Pyrochromatograms of organics from two field streams, DER and RP

containing aliphatic) with the percentage in each category based on the percentage of the total identified peak area. This comparison shows that the two stream waters had a much stronger aromatic signature than the algal exudates (Fig. 3, stream waters 60–67 % aromatic, algal exudates 13–29 % aromatic, Student's *t*-test, $t=10.2$, $p<0.007$). The AP sample was only 5 % aromatic, since its low organic carbon concentration did not allow for detection of moderate to small peaks and was therefore not included in the above statistical comparison of the stream waters and algal exudate aromatic signatures.

To further reduce the large amount of highly correlated data to a small number of independent variables while preserving most of the variance in the data, PCA was employed to assess among-species differences in organic signatures, which were comprised of the 57 unique pyrolysis fragments identified in the algal exudate pyrochromatograms. The peak areas were normalized by the maximum peak area of the identified fragments, which for all algal exudate samples was acetonitrile. PC1 accounted for 33.9 % of the total variance of the data, while PC2 accounted for 21.1 % (Fig. 4). The distance between all the samples on this score plot reveals that, while there are common dominant chemical fragments in the organic signatures, the underlying organic structure of all the algal exudates differs. The AP sample is located at the center of the score plot since its low organic carbon concentration did not allow for detection of moderate to small peaks, which would distinguish its

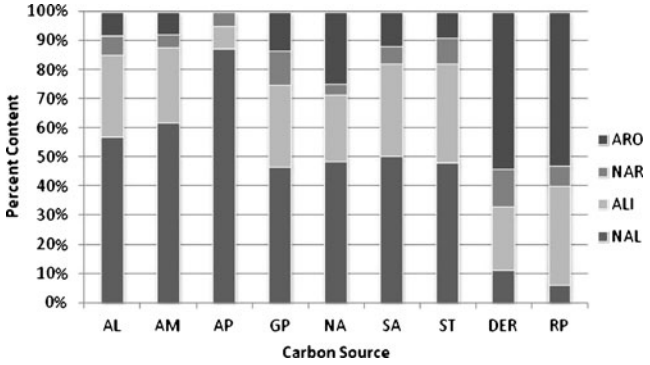

Fig. 3 Semi-quantitative analysis of organic signatures (ARO = aromatic , NAR = nitrogen-containing aromatic, ALI = aliphatic and NAL = nitrogen-containing aliphatic)

organic signature. However, the two green algae species (*S. armatus* [SA], *S. tenue* [ST]) did group near each other (PC1>3.7 and PC2>-0.2) and the two *Achnanthidium* species (AM, AL) also grouped near each other (PC1<-4.9 and PC2>0.9). Due to the highly differing nature of the algal exudate and stream water organic signatures, multivariate analysis comparing all these samples (not shown) was only able to highlight the dissimilarity of these two data sets, but was not useful for further comparison.

Bacterial Community Activity and Composition

Carbon source had a significant effect on denitrification potential rates ($p<0.001$), with measurable DNP in both replicates when bacteria were fed carbon isolated from the two stream waters (DER and RP) and in one of the two replicates when bacteria were fed G or organic carbon isolated from the diatoms AL and NA and the green alga ST (Fig. 5a). Bacterial communities fed carbon isolated from the DER site showed the highest DNP rate (Fig. 5a). There was also a significant effect ($p<0.001$) of carbon source on denitrifier abundance

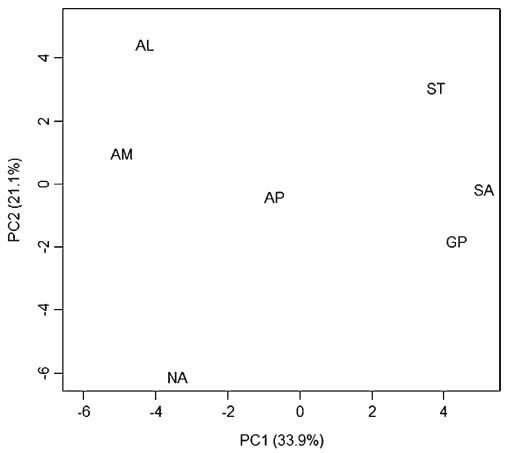

Fig. 4 Principal components analysis of algal exudate organic signatures

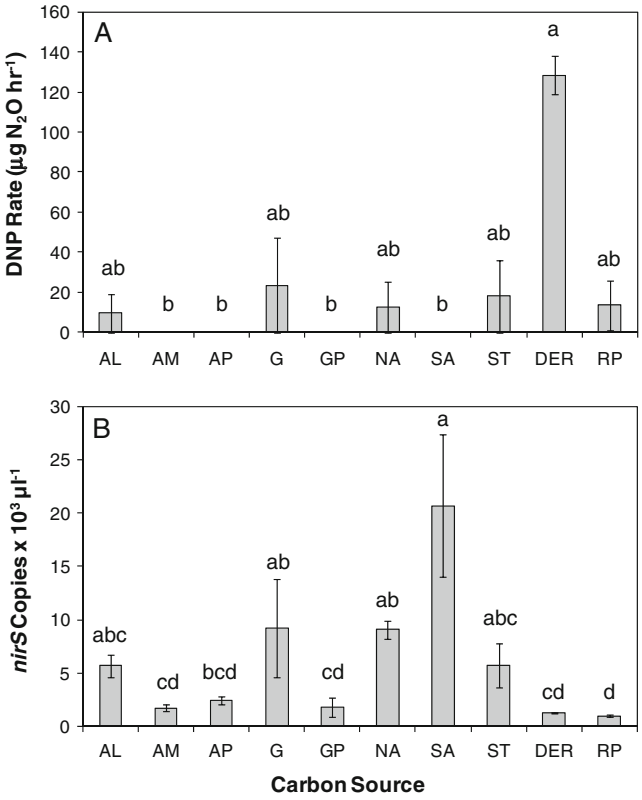

Fig. 5 Effect of carbon source on denitrification potential (**a**) and copy numbers of *nirS* genes (**b**). Each data point represents mean value with standard error bars ($n=2$). ANOVA indicated significant treatment effects on both DNP rate and *nirS* copy numbers ($p<0.001$). Different letters above bars indicate significant differences between treatments ($p<0.05$) based on Tukey's HSD test

based on copy numbers of *nirS* genes, with bacterial communities fed organics isolated from *S. armatus* showing the highest copy numbers (Fig. 5b). However, there was no significant correlation ($p=0.504$) between *nirS* copy numbers and denitrification potential (Fig. 6a).

MDS analysis of denitrifier community composition based on tag pyrosequencing of *nosZ* genes demonstrated that incubation of the bacterial consortia with the various carbon sources resulted in significant shifts in denitrifier community composition as compared to the initial inoculum (IN; Fig. 7). MDS indicated that all of the DNP-positive samples (those that showed production of N_2O in the DNP assay) were similar in composition and were generally distinct from the samples that were DNP-negative (those that did not show production of N_2O in the DNP assay; Fig. 7). For example, all of the incubations with organics from the natural streams (DER and RP) were DNP-positive and were located above and to the left of the inoculum on the MDS ordination. In contrast, all of the incubations with exudates from GP, AM, and SA were DNP-negative and were located to the right of the inoculum on the MDS ordination (Fig. 7). Results from some of the other treatments were less

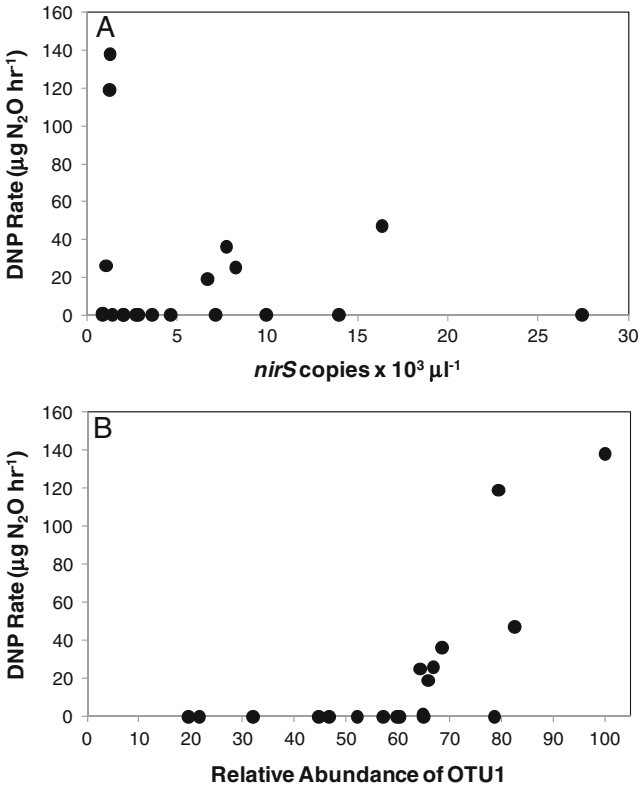

Fig. 6 Relationship between DNP rate and copy numbers of *nirS* genes (**a**) and relative abundance of OTU1 (**b**). Correlation analysis showed no significant correlation between DNP and *nirS* copy number (p=0.504) but a significant correlation between DNP and relative abundance of OTU1 (p=0.001)

consistent. For example, the replicate incubations with G were similar to each other in denitrifier species composition and were similar to the other DNP-positive samples in composition (based on their position on the MDS ordination), but only one of the replicate incubations was DNP-positive. Interestingly, the two G replicates had a large difference in denitrifier

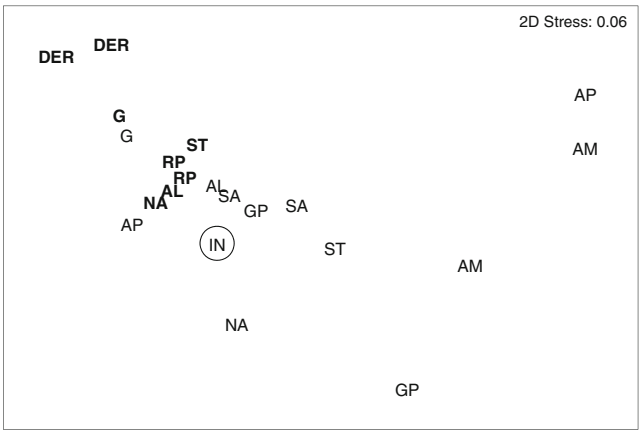

Fig. 7 MDS analysis of denitrifier community composition based on tag pyrosequencing of *nosZ* gene. Letters indicate carbon sources. Bacterial inoculum (IN) is circled. Samples in bold resulted in denitrification (i.e. production of N_2O) during DNP assay

abundance (based on *nirS* copy numbers) with the DNP-positive replicate having 1637 copies μl^{-1} and the DNP-negative replicate having 713 copies μl^{-1}. The AL and NA incubations also showed inconsistent results, with only one of the two replicates for each being DNP-positive. However, for both AL and NA exudates, there was a difference in denitrifier community composition between the replicates indicated by the MDS ordination, with the DNP-positive replicates being most similar to the other DNP-positive communities and the DNP-negative replicates being more similar to the other DNP-negative communities (Fig. 7). Finally, denitrifier communities incubated with organics from the DER site, which showed the highest rates of DNP (Fig. 5a), were clearly distinct in composition from communities from the other incubations (Fig. 7). In contrast, denitrifier communities incubated with organics from the RP site were similar in composition (Fig. 7) and DNP rate (Fig. 5a) to communities incubated with organics isolated from the algal species.

A comparison of the diversity of the denitrifier communities in the DNP-positive and DNP-negative samples based on the inverse Simpson index calculated from the tag pyrosequencing data indicated a significant difference in diversity (p<0.01), with the DNP-positive samples having significantly lower denitrifier diversity than the DNP-negative samples (Fig. 8a). SIMPER analysis of the tag pyrosequencing data indicated that the two most numerically dominant denitrifier OTUs, OTU1 and OTU2, accounted for 63 % of the variation between the DNP-positive and DNP-negative samples. ANOVA revealed that the relative abundance of OTU1 and the relative abundance of OTU2 were significantly different (p<0.05) between the DNP-positive and DNP-negative samples (Fig. 8b). Specifically, the relative abundance of OTU1 was significantly higher and the relative abundance of OTU2 was significantly lower in the DNP-positive samples as compared to the DNP-negative samples (Fig. 8b). Bacterial communities incubated with organics from the DER site, which showed the highest rates of DNP (Fig. 5a), also had the highest concentration of sequences from OTU1 (89 %) as compared to those incubated with G (80 %) or organics from the RP site (66 %) or the inoculum (57 %). These results suggested a possible relationship between the abundance of OTU1 and DNP rates, which was supported by a significant correlation (p=0.001) between these two variables (Fig. 6b). BLAST analysis indicated that sequences within OTU1 were most similar to *nosZ* sequences from *P. stutzeri* (100 % identity) and sequences within OTU2 were most similar to *nosZ* sequences from *Paracoccus denitrificans* (85 % identity).

Discussion

The results of this research demonstrate that DOC extracted from different algal species and stream waters varied in

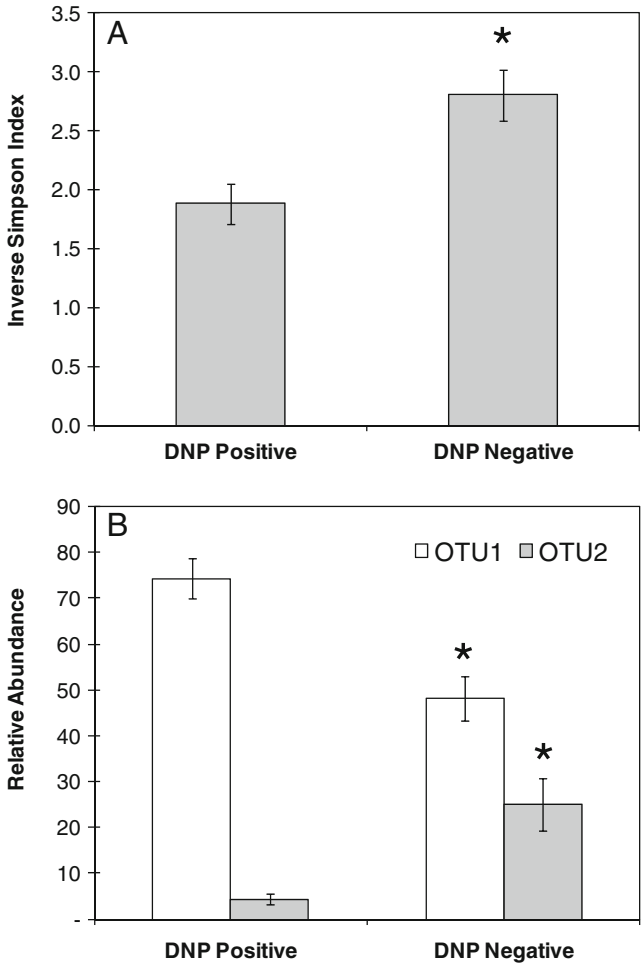

Fig. 8 (**a**) Diversity of denitrifier communities in DNP positive and DNP negative samples. ANOVA indicated significant difference between DNP positive and DNP negative samples ($p<0.01$). (**b**) Relative abundance of the two most numerically dominant OTUs in DNP positive and DNP negative samples. Asterisks indicate significant differences between DNP positive and DNP negative samples ($p<0.05$)

chemical quality and directly influenced the numbers, species composition and activity of denitrifying bacteria. Previous work by our group and others demonstrated that different algal taxa select for taxonomically distinct communities of associated bacteria [22, 30, 40] and that the chemical quality of organic carbon has a measurable effect on the denitrification potential of bacterial communities [35, 46]. However, this research is the first to directly connect DOC quality to both species composition and the activity of denitrifying bacteria, a group of microorganisms that are highly relevant to the nitrogen removal capacity of aquatic ecosystems. Specifically, the present study showed that incubation of bacteria with organic carbon from a site (DER) highly influenced by anthropogenic sources, specifically wastewater treatment effluent, showed the highest rates of DNP. In contrast, incubation of bacteria with organic carbon from a stream with relatively low anthropogenic influence (RP) resulted in DNP rates that were an order of magnitude lower than for DER and were similar to rates for incubations with algal-derived organics. This finding is consistent with our previous results [46]. However, the current study also demonstrated that DNP rates across all incubations were not correlated with the numbers of denitrifying bacteria but rather with the species composition of the denitrifying consortia. Specifically, denitrifier communities that showed measureable denitrification were less diverse and showed significantly higher relative abundances of *P. stutzeri*-like sequences and significantly lower relative abundances of *P. denitrificans*-like sequences. Furthermore, there was a significant correlation between DNP rate and the relative abundance of *P. stutzeri*-like sequences. The relationship between relative abundance of *P. stutzeri* sequences (OTU1) and DNP rate (Fig. 6b) suggested a threshold effect, as communities with less than 60 % relative abundance of *P. stutzeri* sequences showed no denitrification. This apparent threshold effect may reflect the sensitivity of the assay; that is, some communities with less than 60 % relative abundance of *P. stutzeri* sequences may have denitrified at a rate that was below the limit of detection of the DNP assay.

The lack of correlation between denirifier abundance and DNP rates was rather surprising, as we expected that overall denitrifier density would influence denitrification rates directly as others have reported [5]. However, these results highlight the potentially significant influence that microbial community composition can have on community activity. The distinct responses of *P. stutzeri* and *P. denitrificans* in the experimental incubations may be related to difference in the physiological properties of these two organisms. Both *P. stutzeri* and *P. denitrificans* are Gram-negative bacteria from the proteobaterial phylum that are common in terrestrial and aquatic environments. Both are facultative anaerobes capable of both aerobic respiration and denitrification [55]. Each has been shown to require both low oxygen conditions and the presence of nitrogenous oxides to induce the genes involved in the denitrification pathway [4, 29]. These two species also contain the cytochrome cd1 version of nitrite reductase (encoded by the gene *nirS*), the enzyme that catalyzes the second step of the denitrification pathway (the reduction of nitrite to nitric oxide), and nitrous oxide reductase (encoded by the gene *nosZ*), the enzyme that catalyzes the final step in the denitrification pathway (conversion of N_2O to N_2) [55]. However, in a previous study that compared the denitrification properties of these two organisms under conditions similar to our DNP assay (i.e. high organic carbon, high nitrate, oxygen purged), *P. stutzeri* showed significantly higher rates of N_2 production than *P. denitrificans* [15], which may explain the results of our study in which only communities dominated by *P. stutzeri* showed denitrification in the DNP assay. Interestingly, *P.*

denitrificans has a K_m for nitrate that is ten times lower than that of *P. stutzeri* [55], which could give *P. denitrificans* an advantage under low nitrate conditions, but under the high nitrate conditions in our DNP assay this difference in substrate affinity was likely not a factor.

The results of our study did indicate differences in the chemical composition of algal organics and organics from the two stream sites. These results confirm earlier work in which we used Py-GC/MS to distinguish natural versus anthropogenic organic signatures in aquatic systems [28, 45, 46]. We were unable, however, to identify which chemical features as characterized by Py/GC/MS are associated with DNP. The organic fingerprints of the algal exudates that produced detectable DNP do not display common chemical patterns and show little similarity to DER and RP. This is likely due to methodological limitations associated with isolating algal exudates under laboratory conditions, where we were unable to produce sample volumes sufficient to cultivate bacterial communities and resolve the fine details of organic quality by Py-GC/MS.

One of the most interesting findings of our study is the marked difference in the response of bacterial communities fed organics from stream water dominated by wastewater inputs (DER) compared to that of communities provided organics from a stream with relatively small anthropogenic influence (RP). The organics in water from both of these streams resulted in relatively low numbers of denitrifying bacteria, yet the organics from the DER site produced DNP rates an order of magnitude higher than any of the other carbon sources. Our data indicate that this high rate of denitrification was likely based on the fact that organics from DER selected for a denitrifier community that was distinct in composition, was less diverse and was dominated by *P. stutzeri* as compared with communities developed under any other carbon source. In contrast, organics from the RP site supported DNP rates and denitrifier community composition that were similar to those supported by organics from several of the algal species. These results are consistent with the conclusions of Peterson et al. [35] that bacterial denitrifiers and algae in biofilms developed in RP were coupled and in-biofilm processes were likely strongly influenced by autogenic factors, while, in DER, algal and bacterial assemblages were decoupled, and biofilm processes were more influenced by the organic carbon and nutrient pools in stream water. This highlights the potentially significant impacts of anthropogenic inputs on stream microbial communities. The role of anthopogenic impacts on internal structure and processes with periphytic biofilms warrants further research, as these communities serve as critically important sites of ecosystem services.

Care should be taken not to broadly generalize when interpreting our results for several reasons. First, while denitrifier communities dominated by *P. stutzeri* showed the highest denitrification rates in the DNP assay, it would be inappropriate to conclude from these results that bacterial communities dominated by *P. stutzeri* will be the most effective denitrifying communities under all circumstances. For example, the lower nitrate K_m for *P. denitrificans* suggests that it may perform better under nitrate limiting conditions. We can conclude from our results that bacterial communities dominated by *P. stutzeri* were the most effective denitrifying communities under the conditions of our DNP assay, i.e., high carbon, high NO_3^-, and anoxic. Secondly, while this study focused on the influence of organic carbon, it is possible that other components of the DER and RP stream waters, such as inorganic nutrients or the presence of other unidentified organic or inorganic wastewater contaminants, may also have influenced the development of the bacterial communities. While further work is needed to determine the specific components of the DER stream water that induced the denitrifying bacterial response we observed, our results suggest a potentially important role of species composition, both algal and bacterial, in the variation in rates of biogeochemical processes within periphytic biofilms.

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