Microbial community composition in sediments resists perturbation by nutrient enrichment

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Abstract

Functional redundancy in bacterial communities is expected to allow microbial assemblages to survive perturbation by allowing continuity in function despite compositional changes in communities. Recent evidence suggests, however, that microbial communities change both composition and function as a result of disturbance. We present evidence for a third response: resistance. We examined microbial community response to perturbation caused by nutrient enrichment in salt marsh sediments using deep pyrosequencing of 16S rRNA and functional gene microarrays targeting the *nirS* gene. Composition of the microbial community, as demonstrated by both genes, was unaffected by significant variations in external nutrient supply, despite demonstrable and diverse nutrient–induced changes in many aspects of marsh ecology. The lack of response to external forcing demonstrates a remarkable uncoupling between microbial composition and ecosystem-level biogeochemical processes and suggests that sediment microbial communities are able to resist some forms of perturbation.

Key Words: salt marsh/eutrophication/estuaries/denitrification/resistance/nirS

Introduction

Estimates of the diversity of microbial communities have increased immensely in recent years, initially based on sequencing of clone libraries (e.g., Rappé and Giovannoni, 2003). Now next-generation sequencing has provided genomic data from habitats all over the world (Sogin *et al.*, 2006; Huber *et al.*, 2007, Galand *et al.*, 2009) and greatly increased estimates of species richness. Although there is considerable debate regarding absolute measures of diversity, it is clear that microbial communities support greater diversity than suggested by prior methodologies (Kunin *et al.*, 2009; Huse *et al.* 2010). The role this diversity plays in microbial community structure, function, and response to disturbance, however, is poorly understood. Allison and Martiny (2008) used a meta-analysis to determine the extent to which microbial communities demonstrated resistance, resilience, or functional redundancy in the face of environmental perturbations. They showed that in most cases microbial communities were sensitive to disturbance, regardless of the system or the nature of the disturbance (Allison and Martiny 2008).

Among the disturbances included in the Allison and Martiny (2008) meta-analysis, the addition of inorganic nutrients induced a perturbation response in the greatest number of studies. Enrichment of natural ecosystems by inorganic nutrients, in particular nitrogen (N), is occurring at an alarming rate and is fundamentally perturbing the global N cycle (Galloway *et al.*, 2008). Such perturbations are particularly noticeable in the coastal zone where human population growth has increased the amount of anthropogenic N entering near-shore waters. High N loading alters coastal biogeochemistry via increased primary production and associated symptoms of eutrophication, including increased severity of harmful algal blooms (Paerl, 1997), decreased areas of seagrass beds and the commercially important species that rely on them (Valiela *et al.*,

1992; Deegan *et al.*, 2002), and expanded areas of anoxic dead zones (Rabalais *et al.*, 2002; Osterman *et al.*, 2008).

Wetland biogeochemical processes are critical for removing anthropogenic N prior to its entry into the coastal ocean. Salt marshes provide this ecosystem service by sequestering some portion of C and N inputs in increasing macrophyte biomass and accreting marsh peat (Brin *et al.*, 2010), and by converting N to gaseous forms (NO, N_2O and N_2) via denitrification and, to a lesser extent, anammox (Hopkinson and Giblin, 2008). The importance of salt marsh biogeochemistry in ameliorating eutrophication is well recognized but little attempt has been made to understand how the additional supply of N to these marshes is altering the structure of the sediment microbial communities responsible for this critical ecosystem service. We used two marsh nutrient enrichment experiments to test how increased nutrient supply altered the community composition of microbes, in particular those microbes responsible for key N loss processes in the marsh. Whether the microbial response to perturbation by nutrient enrichment is classified as resistant, resilient, or functionally redundant has relevance for the ecological subsidy provided by salt marshes as well as for our understanding of the important connection between microbial community composition and biogeochemical function.

The two on-going fertilization experiments used in this study were designed to investigate how increased N supply alters the ecology of salt marsh ecosystems. Unintentional enrichments that resemble these experimental manipulations are being carried out in estuaries around the world that are experiencing moderate to severe increases in N loading. The two experiments examined here differ in the duration of the experiment and in the form and mechanism of nutrient delivery but both experiments have documented wide-spread ecological responses to the nutrient perturbation (Fig. 1, Table S1). Significant responses to fertilization are reported for sediments,

food webs, and for microbially-mediated biogeochemical processes (Table S1). Some of the well documented changes to the marsh ecology include increases in % C and % N content of the sediments, increases in rates of bacterial production, and increases in microalgal and benthic invertebrate biomass (Table S1). Many of the additional effects of nutrients resulted from changes in the keystone species of marsh vegetation, *Spartina alterniflora.* Increased nutrient inputs caused a shift from the short to the tall phenotype of *S. alterniflora* (Valiela *et al.*, 1985) and accompanying this shift were well documented changes in the redox chemistry of the sediments, including increased oxidation of the rooting zone (Howes *et al.*, 1981). The extent to which fertilization led to changes in macrophyte distribution that then translated into changes in the plot-wide distribution of microbes was not addressed in this study. Rather, our interest was in explicitly testing community response to a single forcing function: nitrogen supply.

The experimental marsh enrichments and the previously documented changes in microbially-mediated biogeochemistry of the ecosystem offer a unique opportunity to examine whether chronic exposure to increased nutrient supply alters the structure and function of the marsh sediment microbial community. We used two approaches to assess this perturbation effect. First, we used pyrosequencing to catalog the taxonomic richness and relative abundance of microbes in the two replicated fertilization experiments. Second, we used microarray analysis of the same samples to investigate a key functional gene involved in salt marsh nitrogen cycling. Since denitrification is the primary N loss mechanism in these marshes (Koop-Jakobsen and Giblin 2010) we examined perturbation-induced changes to the diversity and relative abundance of *nirS*, a key gene in the denitrification pathway. This combination of methods applied to two different enrichment experiments, both of which are replicated, provides the most robust assessment to date of the microbial community response to perturbation.

Materials and Methods

Site descriptions

The on-going PIE fertilization experiment is located at the Plum Island Ecosystem Long-Term Ecological Research Station in eastern Massachusetts (42.759 N, 70.891 W). Within PIE two pairs of marshes within the Rowley River marsh complex were selected based on similarities in standing stocks of benthic microalgae, vascular plant community composition, hydrology, and nutrient dynamics (Deegan *et al.*, 2007). One marsh in each pair (Sweeney marsh in marsh pair 1 (West/Sweeney marshes), and Clubhead marsh in marsh pair 2 (Nelson/Clubhead marshes)) began receiving fertilizer additions in 2004. In the fertilized marsh NO_3^- and PO_4^{3-} were diluted into flooding marsh creek water on every high tide through the growing season to attain marsh-wide concentrations of 70 μM NO_3^- and 4 μM PO_4^{3-} (equivalent to a loading rate of 0.6 g N m^{-2} wk^{-1}). These targets were chosen because they are roughly 15-fold higher than the background nutrient concentrations in flood tide waters. Actual concentrations of N delivered to the sediments varied across the surface of the marsh but averaged 80 μM (range: 49-105 μM) in the fertilized marsh of the first marsh pair and averaged 50 μM (range: 2-78 μM) in the fertilized marsh of the second pair (Deegan *et al.*, 2007). This experimental design provides two different comparisons: 1) duplicate whole marsh enrichments that can be contrasted with specifically paired control marshes, and 2) samples taken prior to the onset of fertilization compared to samples taken in the months following the initiation of the treatment. Further details of the experimental design have been reported elsewhere (Deegan *et al.*, 2007).

The Great Sippewissett Marsh experiment (GSM) began in 1971 in Falmouth MA (41.5828 N. 70.6397 W). Four treatment levels were randomly assigned to duplicate 10 meter diameter plots (8 plots total). Each plot was selected to encompass a creek terminus to ensure that

the distribution of habitats within each plot would be similar. Since 1971 these plots have been fertilized by the biweekly addition of pelletized sewage sludge in the following concentrations: CF (control): no sewage sludge added, LF: 0.85 g N m^{-2} $week^{-1}$, HF: 2.52 g N m^{-2} $week^{-1}$ and XF: 7.46 g N m^{-2} $week^{-1}$. Thus this experiment allows for the comparison of duplicate plots that receive a range of nutrient inputs that spans an order of magnitude, and are considerably more highly loaded than the PIE experiment. Moreover, these plots have been receiving the treatment for nearly 40 years so the microbial community has had sufficient time to adjust to the perturbation. Further details of the experimental design have been reported elsewhere (Valiela *et al.*, 1973; Valiela *et al.*, 1975).

Sample collection and DNA extraction

Sediments from both experiments were collected using a sterile 15 mm diameter syringe corer and were taken from surface sediments (less than 1 cm depth) within the rooting zone of the tall ecotype of *Spartina alterniflora*. In the PIE experiment sediments were collected in May (prior to the onset of that season's fertilization), June, August, and September of 2005 and at GSM sediments were collected in September 2007. Ten cores were taken from sediments at each site, combined and mixed in sterile 20 mL scintillation vials, and stored on ice until arrival at the laboratory. We initially hypothesized that homogenizing multiple samples from a single site would minimize local-scale variability although tests of this hypothesis indicate that homogenizing samples and single samples capture the same snapshot of the community (Bowen et al. in prep). Aliquots of homogenized sediments were removed with a sterile spatula and were stored frozen at -80°C until extraction. The remaining sediments were used to measure bacterial production (Bowen *et al.*, 2009), nitrogen fixation, following the methods of Tyler *et al.* (2003), and the carbon and nitrogen content of the sediments using a PerkinElmer model 2400 CHN

analyzer. DNA was extracted from triplicate aliquots of sediments using the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) following manufacturers instructions. Presence of DNA was confirmed by gel electrophoresis and DNA from the triplicate extractions were pooled and stored at -80°C for further use.

Pyrosequencing analysis

Pyrosequencing methodology has been described in detail (Sogin *et al.*, 2006; Huber *et al.*, 2007; Huse *et al.*, 2007; Huse *et al.*, 2008; Galand *et al.*, 2009; Huse *et al.*, 2010). Briefly, the hypervariable V6 region of 16S rRNA was amplified using a combination of nine forward and reverse primers that are thought to amplify most known bacteria. Sequence adaptors of five base oligonucleotides were attached to the primer set used with each sample, thereby allowing for post-sequencing bioinformatic separation of the samples (Huber *et al.*, 2007). Amplified DNA was spin column purified using the MinElute PCR purification kit (Qiagen, Valencia, CA) and was quantified on a Bioanalyzer 2100 (Agilent, Palo Alto, CA). Purified DNA was then sequenced on a Roche GSFLX pyrosequencer following the methods outlined in Sogin *et al.* (2006) and Huber (2007). Sequence data were then subject to rigorous quality control checks as described in Huse *et al.* (2007, 2008). OTUs of sequence tags were assigned based on a 2% single linkage pre-clustering using a pairwise alignment followed by a 3% average linkage clustering (Huse *et al.*, 2010). All V6 sequence data are available at the VAMPS database (http://vamps.mbl.edu/) and in the NCBI short read archive (accession # SRP004028).

Microarray analysis

The *nirS* microarray consists of 39 90-mer oligonucleotide probes that contain a 70-mer region complementary to a portion of the *nirS* gene and a 20-mer control region that is bonded to a CMT-GAPS aminosilane coated glass slide (Corning Inc, Massachusetts, USA). Probe selection

was based on an algorithm to select archetypes that hybridize with sequences that are ≥85% identical to the probe sequence and was generated from a combination of known and environmental *nirS* sequences present in the public data bases when the array was designed (Bulow *et al.*, 2008). DNA hybridization targets (256 bp) were generated from triplicate PCR products using primers nirS1F and nirS3R (Braker *et al.*, 1998). After amplification PCR products were pooled and purified with the QIAquick PCR purification kit (Qiagen, Valencia CA). PCR products were then labeled using the Klenow enzyme and hybridized to the array following standard methods (Ward *et al.*, 2007; Bulow *et al.*, 2008). After hybridization the slides were washed (Ward *et al.*, 2007) and scanned on an Agilent Laser Scanner (Agilent Technologies, Palo Alto, CA).

Scanned images of the slide were analyzed with Gene Pix Pro (version 6.0, Molecular Devices, Sunnyvale, CA) and analyzed in Microsoft Excel. Each slide contains eight arrays and each array consists of four replicate blocks. Data were quality checked (Ward *et al.*, 2007; Bulow *et al.*, 2008) and then the fluorescence from the Cy3 bound *nirS* sequences were compared to the Cy5 fluorescence to determine the fluorescence ratio for each probe after background fluorescence was subtracted. The Cy3/Cy5 ratios were first normalized for variations in fluorescence intensities across the slide by comparing Cy3/Cy5 fluorescence from internal standards within each array. Next we calculated the relative fluorescence ratio (RFR) by comparing the fluorescence ratio of any one probe to the total fluorescence across all probes within a given block. The four RFR for each probe within an array (one from each block) were then averaged to derive a mean RFR for each probe that could be compared among samples on different slides. Microarray data are archived at the NCBI Gene Expression Omnibus (Accession #:GSE25227).

Quantitative PCR

Quantitative PCR (qPCR) was performed on DNA extracts from the GSM fertilized plots. *nirS* and 16S rRNA standards were quantified using Quant-iT Picogreen (Molecular Probes Inc., Eugene OR) and serially diluted to generate known standards. Environmental DNA from each plot was amplified in triplicate, along with triplicate standards and internal controls, on a Strategene MX-3000 (Stratagene, LaJolla, CA) under the following conditions: 16S rRNA was amplified following the protocol outlined in Biddle *et al.* (2008). For each sample of 16S rRNA, 1.125 μL of 20 mM primers 357F and 519R were added along with1 μL DNA, 12.5 μL SybrGreen master mix (Applied Biosystems, Carlsbad, CA) and 10.5 μL DI water. They were amplified with a 10 minute initial denaturing step at 95°C followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for one minute. All amplification products were checked for quality by examination of melt curves and by gel electrophoresis. The *nirS* gene was amplified following the method of Jayakumar *et al.* (2009). Briefly, 0.5 μL of 100 mM primers (nirS1F and nirS3R (29)), 1 μL of DNA, 12.5 μL of SybrGreen master mix (Applied Biosystems, Carlsbad, CA) and 10.5 μL DI water were amplified using the following conditions: 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 second, 62°C for 30 seconds, and 72°C for 30 seconds. Again, amplification products were checked for quality by examining melt curves and gel electrophoresis of the amplification products.

Statistical analyses

Pyrosequencing analysis provides data on the both the composition and relative abundance of OTUs in each sample thereby making it possible to analyze whether particular members of the microbial community are either stimulated or inhibited by nutrient enrichment. If either stimulation or inhibition were to occur as a result of the treatment then the abundance of a given

OTU in fertilized marshes would deviate from the abundance of that same OTU in control marshes to a greater extent than would be expected by chance. To analyze the pyrosequencing data we used a Wilcoxon Mann Whitney test (aka Mann Whitney U test) to determine whether the distribution of OTUs differed as a result of the treatment in both the PIE and GSM experiments. Rejection of the null hypothesis in these tests would indicate that chance alone cannot explain the distribution in the OTUs. The Wilcoxon Mann Whitney tests were performed using the freeware statistical program R (http://cran.r-project.org/).

To analyze the *nirS* microarray data, the relative fluorescence ratios (RFR) from each sample were arcsine transformed and analysis of variance was performed to test for a significant treatment effect in each experiment. In the PIE experiment this was done by comparing the RFR from the two unfertilized marshes with the RFR from the two fertilized marshes in each month of the fertilization. For GSM this was done by comparing the RFR of the control plots compared with each of the treatment plots. Furthermore, for GSM we used analysis of variance to test the treatment effect on the number of *nirS* genes present in the control vs. treatment pots as determined by qPCR. All analysis of variance tests were performed using R.

Results and Discussion

We compared between 20,000 and 25,000 pyrosequences per site from bacterial DNA extracted from sediments of multiple fertilized and control marsh plots at both PIE and GSM. This degree of sequencing is 20-25x deeper than is typical of clone library studies and provides the most robust examination of microbial response to perturbation to date. Furthermore, cumulative frequency distribution curves suggest that sampling to this depth captures all the dominant taxa present in the sample (Bowen et al. in prep). In both marsh experiments we controlled for variations in microbial communities induced by the dominant vegetation or by

elevational gradients in the marsh by sampling from sediments found only in the zone dominated by the tall phenotype of *S. alterniflora.* The extent to which fertilization altered the microbial community associated with other marsh habitats was not addressed in this study, however prior DNA fingerprinting work indicates that the fertilization effect in other habitats was also very small (Bowen et al. 2009). To test the effect of increasing nutrient supply on the structure of the microbial community in the PIE experiment, we compared the number of times an OTU was identified within each marsh pair (Fig. 2). If each OTU was present in equal numbers in the control and fertilized marsh of marsh pair one then each point, representing an individual OTU, would fall exactly on a 1:1 line (Fig. 2, top). Due to incomplete sequencing of the microbial community, exact adherence to the 1:1 line would not be expected, rather there would be some variance around the line that would be expected to occur solely by chance. Any effect of treatment would be detected by a deviation from the 1:1 line that is greater than could be predicted by chance alone. However no such treatment effect was detected in either marsh pair one (Fig. 2, top, U=0.107, p=0.915) or in marsh pair 2 (Fig. 2, bottom : U= -0.668, p=0.504). Further evidence that the distribution of points around the 1:1 line can be attributed entirely to chance can be found by comparing the OTU distribution prior to the onset of fertilization (blue diamonds), where no treatment effect should be detected, with the distribution of OTUs in the months following fertilization (red squares). The similar variation in the two groups of samples provides another indication that the microbial community structure in these two PIE marsh pairs are largely unaffected by nutrient enrichment.

The GSM plots, fertilized at rates up to 10-fold higher and for decades longer than the PIE experiment, provide further evidence of microbial resistance to nutrient enrichment. As with the PIE fertilization (Fig. 2), OTU abundance in the GSM sediment microbial community was neither

stimulated nor inhibited by fertilization, regardless of the dose of fertilizer added (Fig. 3). The distribution of OTUs when comparing the duplicate control plots to the lowest dose fertilized plots (fertilized at roughly similar rates to the marshes in the PIE experiment) do not deviate from one another more than can be explained by chance (Fig. 3, top: U=1.065, p=0.287). Similarly, comparing OTU data from the two control plots with the two HF plots indicate no significant effect of fertilization (Fig. 3, middle: U=0.771, p=0.441) and the same is true for the most highly fertilized plots (Fig. 3, bottom: U=0.745, p=0.456). The lack of response by the microbial community is all the more surprising in light of the dramatic treatment-induced changes that have occurred to both the biogeochemistry and ecology of these highly fertilized plots (Table S1, Fig. 1). Both the two marsh pairs in the PIE experiment and the more intensively fertilized GSM plots provide evidence that marsh sediment microbial communities, at least within the dominant S*partina alterniflora* habitat, are resistant to nutrient induced perturbation, in spite of measurable differences to other ecosystem components across a broad range of N loads.

The possibility remains that microorganisms may respond to perturbation through physiological adaptation within some functional genes that may not be detectable by examination of 16S rRNA alone. One functional gene likely to reflect changes in N transformations as a result of N fertilization is *nirS*, the gene that encodes the denitrification enzyme that catalyses the reduction of NO_2^- to NO (Zumft, 1997). Denitrification is a critical biogeochemical pathway in salt marshes because it removes land-derived N before it enters coastal waters and stimulates eutrophication. In both marshes rates of denitrification have increased as a result of nutrient enrichment. At PIE, fertilized creek bottom sediments had denitrification rates that were 47% higher (p=0.013) than control marshes (Koop-Jakobsen and Giblin 2009). At GSM there was a net stimulation of coupled nitrification-denitrification as a result of fertilizer inputs, with late

summer rates ranging from 0 to 12 mmol N m^{-2} d^{-1} in control plots and 48 to 69 mmol N m^{-2} d^{-1} in fertilized plots (Hamersley and Howes 2005).

We used the relative fluorescence ratio (RFR) from the functional gene microarray (Ward *et al.*, 2007; Bulow *et al.*, 2008) to quantify the relative contribution of each archetype to the total *nirS* signal and determine the distribution of *nirS*-containing phylotypes in both the PIE and GSM marshes. The RFR for the 39 archetypes found in both marshes were remarkably consistent, with only minor differences as a result of fertilization in the GSM experiment, and no differences as a result of fertilization in the PIE experiment (Fig. 4). The archetype CB2-S-138 dominated the *nirS* community in all marshes. In the GSM plots only two of the 39 archetypes (~5%) demonstrated a significant change in signal intensity as fertilizer dose increased (Fig. S1). The second strongest hybridization signal after archetype CB2-S-138, identified by archetype probe CB3-S-15, was negatively correlated with N supply and one minor archetype, CB3-S-102, appeared to increase with N supply in the GSM plots (Fig. S1). The remaining 37 archetypes, however, showed no difference in RFR as a result of fertilization in either set of experiments. The remarkable consistency in *nirS* community composition among the marshes and the stability of those communities in the face of considerable perturbation stands in contrast to the spatially variable community of denitrifiers found in the Chesapeake Bay using the same microarray (Bulow *et al.*, 2008).

Functional gene microarrays can only detect relative changes in archetype abundance, not absolute differences (Ward *et al.*, 2007). Quantitative PCR amplification using primers that quantify the total *nirS* abundance that is represented on the array, however, indicated that there was no increase in the absolute abundance of the *nirS* genes among the plots (Fig. 5), implying that an increase in cell number cannot explain the apparent disconnect between the reported

increase in rates of denitrification and the lack of response among denitrifiers represented by the microarray. Microorganisms must thus be responding to nutrient enrichment by altering their gene expression without concomitant changes in their community composition. The possibility remains that there were unknown denitrifiers present that are not represented on the microarray. *In silico* testing of the current array against *nirS* sequences published since its design shows that by far most, if not all, of the newly published estuarine sequences would be covered by the probe set used in this study. Interestingly, the major groups identified in clone libraries from estuaries in China, for example, are well represented in the estuarine probes on the current array. It seems unlikely, but difficult to test, that denitrifiers not represented on the array had varying and contradictory responses to the nutrient perturbation, compared to the major estuarine clades whose relative contributions to the assemblage were measured here.

The data presented here, therefore, robustly demonstrate that microbial community composition and relative abundance are both largely unaffected by additions of N and by indirect N induced ecological changes in the marsh habitat that we sampled. We showed this both by examining the distribution of OTU abundance in replicated experiments in Plum Island Sound and in replicated marsh plots at Sippewissett Marsh as well as by microarray analysis of the *nirS* functional gene that is a key component of the nitrogen cycle. A similar resistance by the microbial community to increased nutrient supply has been reported in salt marsh fertilization experiments in which researchers examined the phospholipid fatty acid structure of the bulk microbial community (Lovell et al. 2001) and the structure of the diazatrophs in the marsh sediments (Bagwell and Lovell 2000). It is difficult to reconcile the remarkable consistency within the microbial community with the multitude of documented changes observed in other aspects of the marsh ecology in both systems (Fig. 1, Table S1), particularly with regard to the

consistency within the denitrifying community. Studies that simultaneously assess rates of denitrification and expression of key N cycling genes such as *nirS* are needed to shed light on the apparent disconnect between the microbial community composition and biogeochemical function, as well as how both respond to environmental perturbation. The evidence presented here, however, suggests that microbial communities can show a greater degree of resistance to perturbation than has previously been demonstrated. The surprising resistance of these microbial communities suggests a difference between sediment and aquatic systems, the latter showing regular patterns of change with changing environmental conditions (Fuhrman *et al.*, 2006; Fuhrman *et al.*, 2008; Crump *et al.*, 2009), and indicates that there is much to be learned about the relationship between microbial community composition and ecological function.

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Figure legends

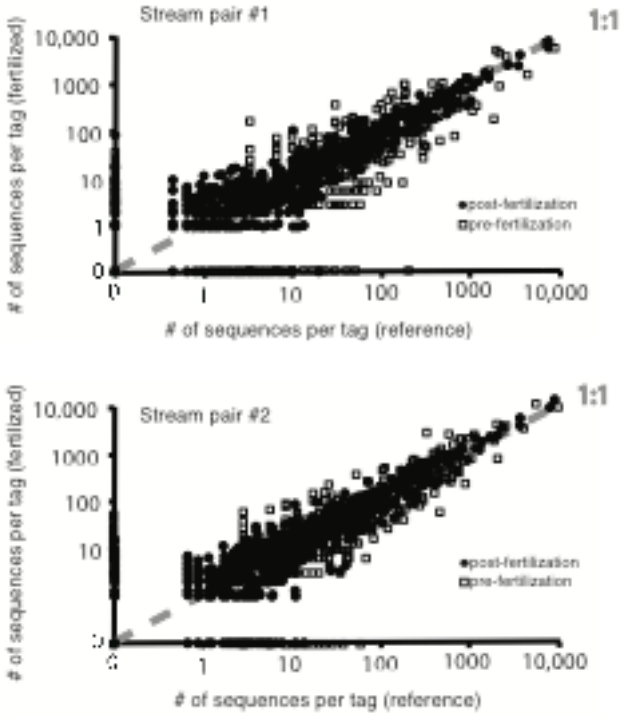
Figure 1 Photograph of very highly fertilized plot at Great Sippewissett Marsh taken Autumn 2007. The fertilized plot is clearly visible in the center of the photograph and is surrounded by unfertilized marsh that has begun fall senescence. Note the difference in macrophyte community composition, above ground biomass, and elevation between the fertilized and unfertilized portions of the marsh. Photo by I. Valiela.

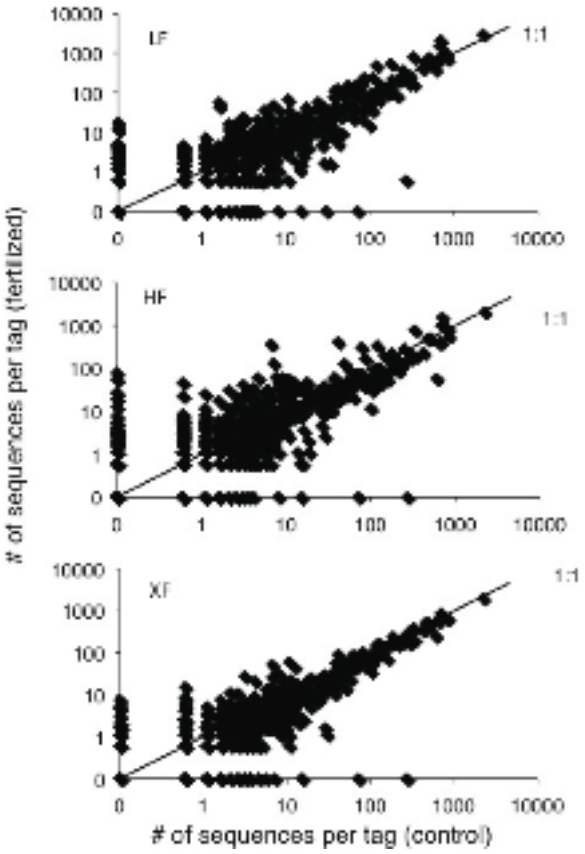
Figure 2 Number of sequences from different microbial taxa in control and fertilized marshes at PIE. Top: Marsh pair one compares Sweeney (fertilized) with West (control). Bottom: Marsh pair two compares Clubhead (fertilized) and Nelson (control).

Figure 3 Number of sequences from different microbial taxa in CF compared to LF (top), HF (middle) and XF (bottom) from GSM.

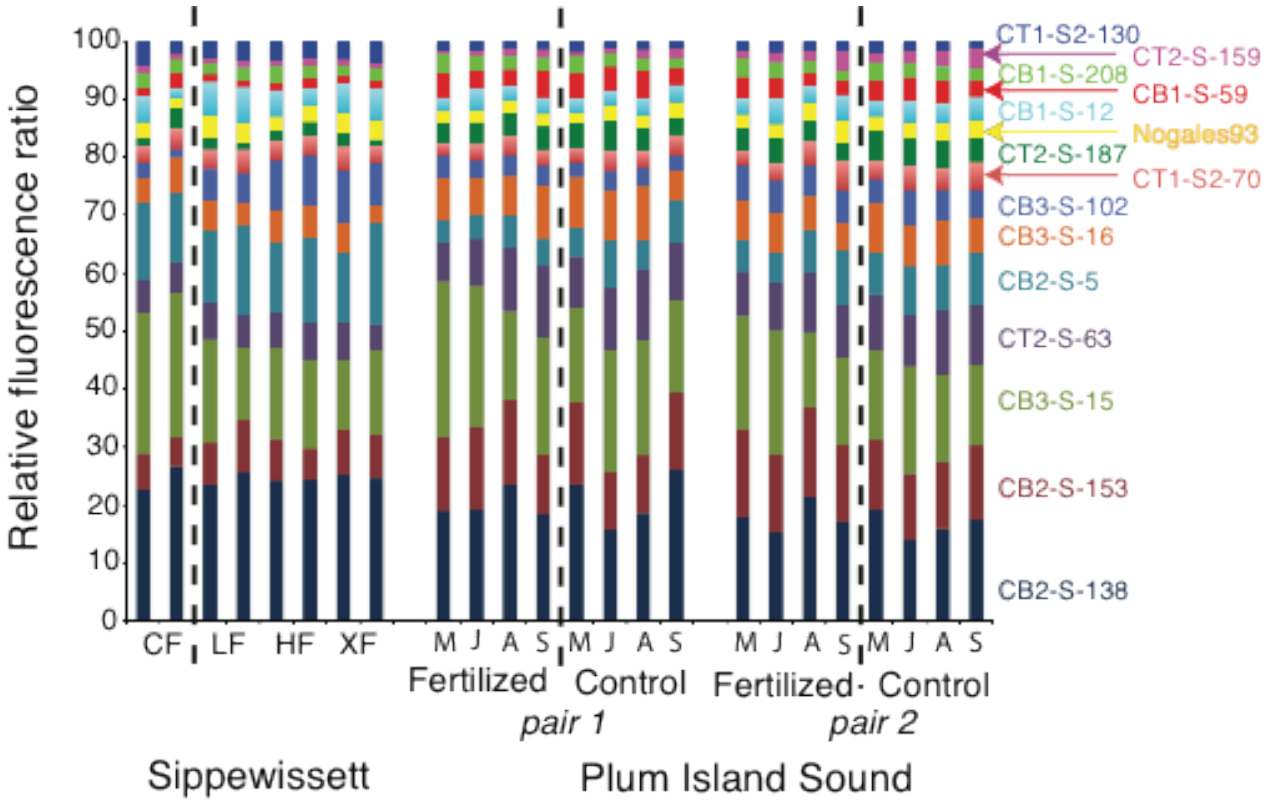
Figure 4 Stacked bar plot of the magnitude of the hybridization signal of the 15 most abundant *nirS* targets from GSM and PIE. The relative fluorescence ratio (RFR) indicates the relative signal strength of that archetype in each sample. ANOVA results indicate no significant differences between control and fertilized marshes in either experiment (GSM: F=0.846, p=0.471; PIE: F=0.115, p =0.734).

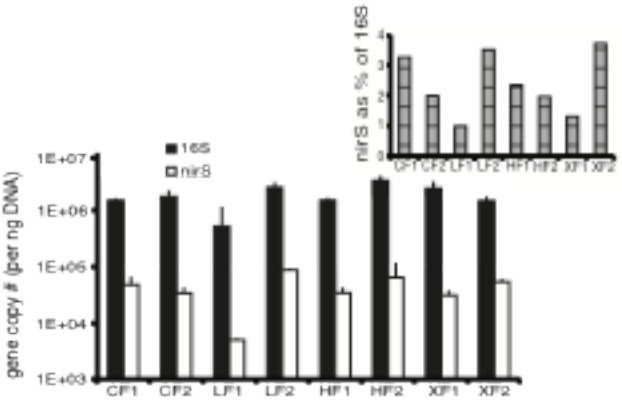
Figure 5 Quantitative -PCR results comparing the absolute abundance of *nirS* genes in DNA from each of the GSM plots with the absolute abundance of the 16S rRNA genes in the same plots. Inset: the % of 16S rRNA that is *nirS.* There were no significant differences among treatments with respect to gene copy number for either 16S (F=1.11, p=0.37) or nirS (F=0.08, p=0.96), although the difference between the abundance of nirS and 16S were clearly different from each other (F=91.07, p<0.01).


Stream pair #1
1:1
10,000
1000
100
10
1
0
0
1
10
100
1000
10,000
# of sequences per tag (fertilized)
# of sequences per tag (reference)
post-fertilization
pre-fertilization
Stream pair #2
1:1
10.000
1000
100
10
1
0
1
10
100
1000
10,000
# of sequences per tag (fertilized)
# of sequences per tag (reference)
post-fertilization
pre-fertilization


LF
HF
XF
1:1
# of sequences per tag (fertilized)
# of sequences per tag (control)
0
1
10
100
1000
10000

500


Relative fluorescence ratio
100
90
80
70
60
50
40
30
20
10
0
CF
LF
HF
XF
M J A S
M J A S
M J A S
M J A S
Fertilized
Control
Fertilized
Control
pair 1
pair 2
Sippewissett
Plum Island Sound
CT1-S2-130
CT2-S-159
CB1-S-208
CB1-S-59
CB1-S-12
Nogales93
CT2-S-187
CT1-S2-70
CB3-S-102
CB3-S-16
CB2-S-5
CT2-S-63
CB3-S-15
CB2-S-153
CB2-S-138


16S
nirS
gene copy # (per ng DNA)
1E+07
1E+06
1E+06
1E+04
1E+03
CF1
CF2
LF1
LF2
HF1
HF2
XF1
XF2
nirS as % of 16S