INVESTIGATING MICROBIAL COMMUNITIES IN SALT MARSH SEDIMENTS USING CARD-FISH

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Abstract

Salt marsh ecosystems are geochemically significant for its role in carbon burial and they serve as a buffer zone for coastal marine environments by actively removing nitrogen input from upstream sources. Microbial communities in salt marsh sediments that are responsible for most of the ecological roles the ecosystem play have been investigated, but very limited number of studies have compared the communities that are bound to sediment particles and the "free-floating" ones. Considering the different substrate availability and community dynamics on particles and porewater in salt marsh sediments, this study examined the microbial community structure in these two types of environments, using catalyzed reporter deposition-fluorescence *in situ* hybridization. Sediment samples were taken from the long-term fertilization plots in the Great Sippewissett Marsh, and the effect of long-term fertilization was also studied. Most (> 90%) of the microorganisms in salt marsh sediments were particle-associated. The bacterial community did not differ in relative abundance between particle-associated and free-floating communities. Long-term fertilization has decreased the relative abundance of bacteria targeted by the probe mix Eub 338 I – III, and there was an inhibitory effect of long-term fertilization on γ-proteobacteria. Further optimization of the CARD-FISH method is required for a comprehensive understanding of how the particle-associated microbial community might differ from the free-floating community in salt marsh sediments, and how long-term fertilization could alter microbial community structure.

Introduction

Salt marsh ecosystems play a variety of critical roles in the cycling of carbon, nitrogen, and sulfur. Occupying only a miniscule portion of global area, salt marshes account for a quarter of the carbon burial in marine environments due to their extremely high production and burial rate (Duarte et al. 2005). Microbial communities in salt marsh sediments account for the majority of the elemental cycling. Although they have been investigated broadly, but but very limited number of studies have compared the communities that are bound to sediment particles and the "free-floating" ones. A number of studies have shown that there is considerable difference in substrate availability between the particle and porewater in marine sediments (Fitzsimons, et al. 2006, Rysgaard et al. 1999, Wang and Lee 1994). Therefore it is reasonable to postulate that there is a difference in microbial community structure, given the different substrate availability and community dynamics on particles and porewater in salt marsh sediments. This study examined the microbial community structure in these two types of environments, using catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH). Sediment samples were taken from the long-term fertilization plots in the Great Sippewissett Marsh, and the effect of long-term fertilization was also studied.

Methods

Site description

The Great Sippewissett Marsh is located at the West Falmouth Harbor on Cape Cod, MA (41.5828 N, 70.6397 W). Circular experimental plots (10 m in radius) were fertilized biweekly during the growing season since 1971 (Figure 1). Besides the unfertilized control plots, three dosages of sewage pellets were applied: low fertilization (LF, 8.4 g/m^2/week), high fertilization (HF, 25.2 g/m^2/week), and extra-high fertilization (XF, 75.6 g/m^2/week) (Vince et al. 1981). *Spartina alternaflora* is the dominant vegetation in both high and low marsh in all plots, except for the high marsh in XF plots, which have been taken over by *Distichlis spicata*. Tall-form *S. alternaflora* dominate the low marsh in all plots, while short and intermediate-form *S. alternaflora* dominate the high marsh.

Sampling Scheme

Peaty sediments were sampled using a 60-ml syringe with the top cut off and sharpened. One C (7) and one XF (8) plots were chosen, in which a surface layer and a layer at 3 cm were sampled in both low and high marsh (Figure 1). A thin layer (~1 mm) of sediment was sliced with a scalpel into a pre-weighed 50-ml centrifuge tube. These two depths were chosen for their contrasting redox conditions, according to *in situ* oxygen measurements (Figure 2).

Separation of "free-floating" and particle-associated cells

Immediately upon arrival at the laboratory, the samples were weighed, and 20 ml of freshly prepared 4% paraformaldehyde were added to them. After vortexing to homogenize the slurry,

the samples were incubated at 4 ℃ overnight. Then they were centrifuged at 4000 rpm for 5 minutes. The supernatant was filtered through Whatman qualitative filter paper, Garde 1 (pore size = 11 μm). Cells in the filtrate were defined as "free-floating" in this study. The precipitated sediments after centrifugation were resuspended with a buffer made of 50% 1xPBS and 50% pure ethanol for storage at -20 ℃. The weight of the buffer is ten times the weight of the sediment. An aliquot of 2 ml of the resuspended sediments were transferred to a 15-ml centrifuge tube, and sonicated on ice for 120 s intermittently (1 second of sonication followed by 1 second of break) at 20% intensity. Cells in this resuspended sediment after sonication were defined as particle-associated in this study.

Optimization of cell density on filters

In order to determine the optimal volume of samples to be used for epifluorescence microscopy, varying amounts of samples were diluted into 20 ml of 1xPBS and gently vacuum filtered onto a 0.2 μm polycarbonate filter (47 mm, GTTP, Millipore). A glass filter holder assembly with funnel, fritted base, stopper, and clamp were used and the filtration area is 9.6 cm^2. After embedding the cells on the filter with 0.2% agarose gel with a sprayer, a subsection of the filter was stained with 4',6'-diamidino-2'-phenylindole (DAPI, 1 μg/ml) for 10 minutes. The filters were then rinsed with DI water and 80% ethanol, and dried on blotting paper. Finally, the filter sections were mounted onto a glass slide with a drop of Citifluo/Vectashield (4:1) mounting medium. The number of cells were enumerated with an Axio Imager M2 epifluorescence

microscope (Carl Zeiss, Jena, Germany), and the filters with about 20 to 100 cells per 100 μm by 100 μm grid were chosen for downstream hybridization. For all 16 samples, 0.25 to 1 ml of the filtrate and 5 to 10 μl of resuspended sediments were the optimal range of volume to be used for cell counting (Table 1). Due to limited time, only four of the 16 samples were chosen for CARD-FISH (sediments from 3 cm depth in the high marsh in both C and XF plots, see Table 1).

CARD-FISH

Seventeen horseradish peroxidase-labeled oligonucleotide probes (50 ng/μl) targeting a broad range of organisms were chosen, including a general bacterial, three archaeal, five proteobacterial probes, as well as two probes targeting sulfate-reducing bacteria, two probes targeting sulfur-oxidizing bacteria, one probes targeting β-proteobacterial ammonia-oxidizing bacteria (β-AOB), one probe targeting planctomycetes, and one probe targeting *Methylomirabilis sp.* (Table 2).

Following the embedment of cells onto filters with 0.2% agarose gel, the cells were permeabilized using freshly prepared lysozyme (Sigma, 10000 U/ml in 0.05 M EDTA, pH 8.0, and 0.1 M Tris-HCl, pH 8.0) for 60 minutes in a 37 ℃ oven for bacteria, and proteinase K (Roche, PCR grade, 20 mg/ml, final concentration 15 μg/ml in 0.05 M EDTA, pH 8.0, 0.1 M Tris-HCl, pH 8.0, and 0.5 M NaCl) for 5 minutes at room temperature for archaea. All incubations were performed by soaking the filters in a 50-ml centrifuge tube. The filters were washed with excess Milli-Q water.

Endogenous peroxidases were inactivated using 3% H_2O_2 in a 50-ml centrifuge tube at room

temperature for 10 minutes. After the incubation, excess Milli-Q water and 96% ethanol were sequentially used to wash the filters in the tube (by inverting the tube a few times). Then the filters were dried on a blotting paper until hybridization.

The filters were hybridized with 300 μl of hybridization buffer and 1 μl of probe working solution (50 ng DNA/μl), in a 1.5 ml microcentrifuge tube, in a 46 ℃ oven for 3 hours. The concentration of formamide in each hybridization buffer were adjusted according to the stringency of each probe. Post-hybridization washing was performed for 10 minutes in a 48 ℃ water bath in one pre-warmed 50-ml centrifuge tube for filters hybridized under the same formamide concentration. Another wash with 1xPBS for 15 minutes at room temperature was performed after the washing buffer was poured out.

Catalyzed reporter deposition reaction was performed using 1 ml of amplification buffer in a 1.5 ml microcentrifuge tube for 30 minutes in a 46 ℃ oven. There was 10 μl of 0.15% H_2O_2 and 1 μl of Alexa Fluor 594 tyramide (1 mg/ml) in 1 ml of amplification buffer. Ten filter subsections were incubated together in each ml of amplification mix. After amplification, all 60 filters were transferred into 50 ml of 1xPBS and incubated for 10 minutes at room temperature in the dark. Then the filters were washed with excess Milli-Q water and 96% ethanol sequenctially for about 1 minute each. The filters were dried on a blotting paper. Dual CARD-FISH were performed on two sets of probes (Table 2). Horseradish peroxidase-labeled probes were inactivated with 3% H_2O_2 as described above, prior to the second hybridization and amplification (using Alexa Fluor 488 tyramide). Finally, cells were stained with DAPI as described above. The filters were

mounted onto glass slides as described above, for enumeration of cells (Ruff et al. 2014). Image acquisition and processing was performed using Zen 2012 SP2.

Results

The vast majority of the cells (>90%) in the Great Sippewissett Marsh sediments at 3 cm depth were particle-associated (Figure 3). Cell numbers in fertilized plots (3.8×10^9 cells/g wet weight) were three times as high as in control plots (1.4×10^9 cells/g wet weight).

Among the 17 probes used in this study, only three (Eub338, Gam42a, and NSO1225) resulted in significant cell counts in all four samples for comparison across the board. The other probes were detected in some but not all samples. For example, planctomycetes, β-proteobacteria, γ-proteobacteria, sulfur-oxidizing bacteria, and euryarchaeota were found among the free-floating cells (Figure 4).

The relative abundance of bacteria did not differ much between particle-associated and free-floating communities (Figure 5). The proportion of bacteria relative to total cell counts was significantly higher in the control plots than in the fertilized plots ($p = 0.013$). Gamma-proteobacteria accounted for 5 – 7% of the total number of cells in unfertilized plots, and <1% in fertilized plots. Beta-proteobacterial ammonia-oxidizing archaea were only found in the free-floating community and accounted for about 1% of the total population.

Discussion

Abundance of microorganisms

Bacterial abundance in the Great Sippewissett Marsh sediments is comparable to that in other salt marsh ecosystems in Rhode Island, South Carolina, Georgia, and South Africa (Caffrey et al 2007, Frischer et al. 2000, Sundareshwar et al. 2003, Tibbles et al. 1992), confirming the high abundance of microorganisms in salt marsh sediments. A couple caveats include the unspecific staining of DAPI which could lead to an overestimation, and the loss of cells from the filters which could lead to an underestimation. Moreover, whole-cell extraction efficiency from sediment particles has been a long-standing challenge for microbial ecologists, and the cell extraction efficiency using sonication, which could possibly lyse cells, was not tested in this mini-project (Frischer et al. 2000).

Comparison between "free-floating" and particle-associated communities

Most (> 90%) of the microorganisms were particle-associated, probably because attachment provide them with greater stability, either through avoiding or minimizing physical disturbance, or through the steady supply of substrates for metabolism. Furthermore, it's likely to be "convenient" for microorganisms to form syntrophic relationship on particles. For instance, microenvironments ranging from oxic to anoxic conditions could develop on a sediment particle, where heterotrophic bacteria could remineralize organic nitrogen to ammonium, which could be subsequently oxidized to nitrate by nitrifiers, and finally converted to nitrogen gas by denitrifiers. Nitrification and denitrification are usually spatially decoupled since one requires an

oxic and the other an anoxic environment. However, such a gradient could potentially be found on a fine particle that harbors both nitrifiers and denitrifiers.

On the other hand β-proteobacterial ammonia-oxidizing bacteria were present only in the free-floating community, suggesting that they were only capable of using the ammonium in porewater, or, if ammonium was available in both porewater and particles, it is more favorable for them to utilize the dissolved ammonium.

Effect of long-term fertilization

Long-term fertilization has increased the total number of cells three-fold in the Great Sippewissett Marsh at 3 cm, contrary to what was reported in a fertilization experimental plot in Rhode Island (Caffrey et al. 2007). This could be because the experimental plots in Rhode Island were fertilized with a lower dosage (0.7 g N /m^2/week) and for a shorter term (28 months), compared to the fertilization plots at the Great Sippewissett Marsh (75.6 g N/m^2/week for 40 years). On the other hand, biological replicates are necessary to test if the increase in microbial abundance was significant in Great Sippewissett Marsh.

The significantly higher relative abundance of microorganisms in the control plot than in the XF plot suggests that long-term fertilization has disproportionally promoted the microbial abundance of archaea and eukaryotes, assuming that the probe mix Eub I – III captured all bacteria in salt marsh sediments (Loy et al. 2007), and there was no interference with the CARD-FISH by any sediment material. It is conceivable that a group of rare bacteria in control plot not targeted by Eub I – III was enriched during the long-term fertilization, and accounted for a

significant portion of the bacteria in the XF plot.

It is unclear why γ-proteobacteria was not present in the XF plot, while it accounted for up to 7% of the total community in the control plot, especially considering that some of the key players in nitrogen and sulfur cycling in salt marsh sediments, such as denitrifiers and sulfur bacteria, belong to γ-proteobacteria. Regardless, the disappearance of the γ-proteobacteria, along with the evidence presented above, demonstrated that long-term fertilization has at least partially altered the microbial community in salt marsh sediments. This is inconsistent with what was reported previously, where microbial community was investigated with both deep pyrosequencing of 16S rRNA and functional gene microarray (Bowen et al. 2011). This is likely a result of different marsh habitat and redox environment: this mini-project examined sediments at 3 cm depth (anoxic) from the high marsh, whereas Bowen and colleagues (2011) sampled the surface sediments from the low marsh, dominated by tall-form *S. alternaflora*. *Distichlis spicata* was the dominant vegetation in the high marsh of XF plot, and short-form *S. alternaflora* in the high marsh of control plot.

Conclusion

Most microorganisms (> 90%) were bound to particles in salt marsh sediments. Certain groups of microorganisms, such as β-proteobacterial ammonia-oxidizing bacteria, were only found in the free-floating community. Long-term fertilization has elevated the microbial abundance by three-fold and possibly inhibited the γ-proteobacterial community at 3 cm in the Great Sippewissett Marsh. Further optimization of the CARD-FISH method is required for a

comprehensive understanding of how the particle-associated microbial community might differ from the free-floating community in salt marsh sediments.

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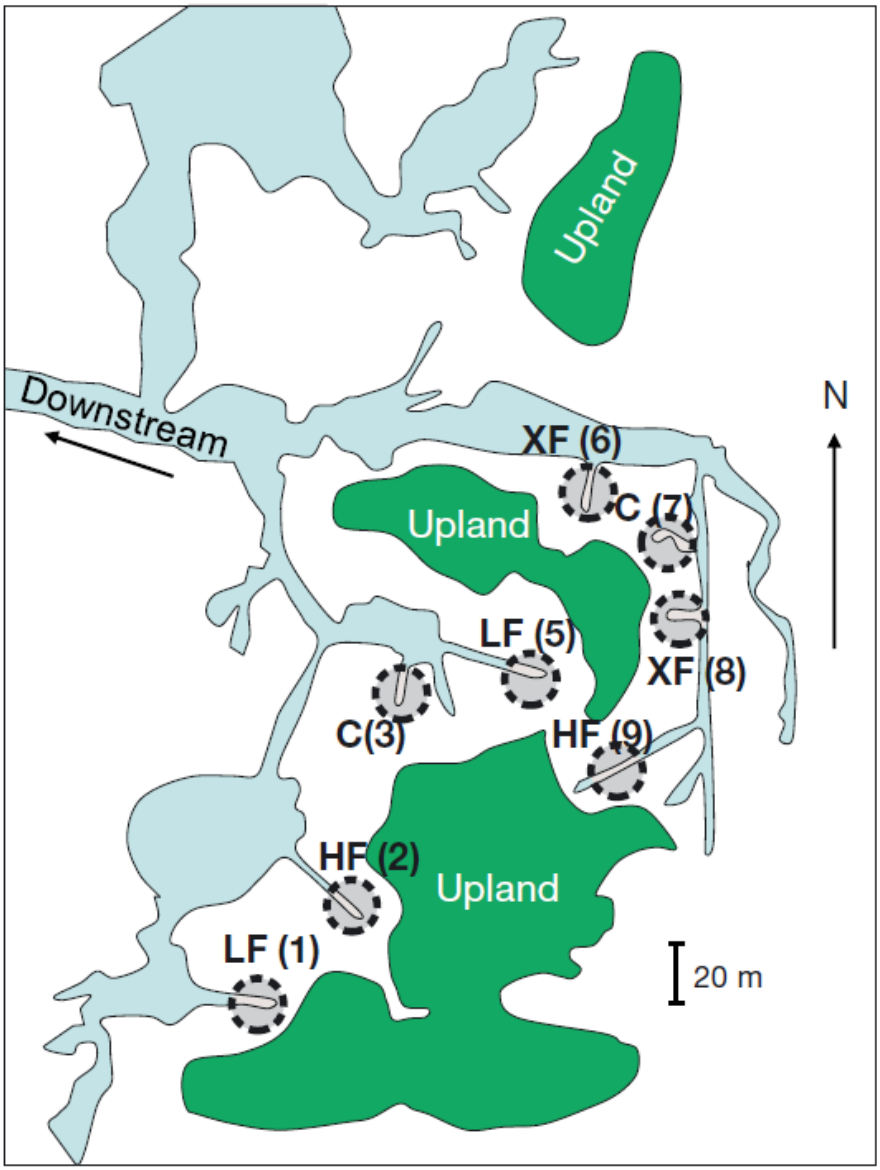

Figure 1. Fertilization experimental plots in Great Sippewissett Marsh, Falmouth, MA (41.5828 N, 70.6397 W). C, unfertilized control; LF, low fertilization; HF, high fertilization; XF, extra-high fertilization. Samples for this study were taken from C (7) and XF (8). This figure is from Kinney and Valiela 2013.

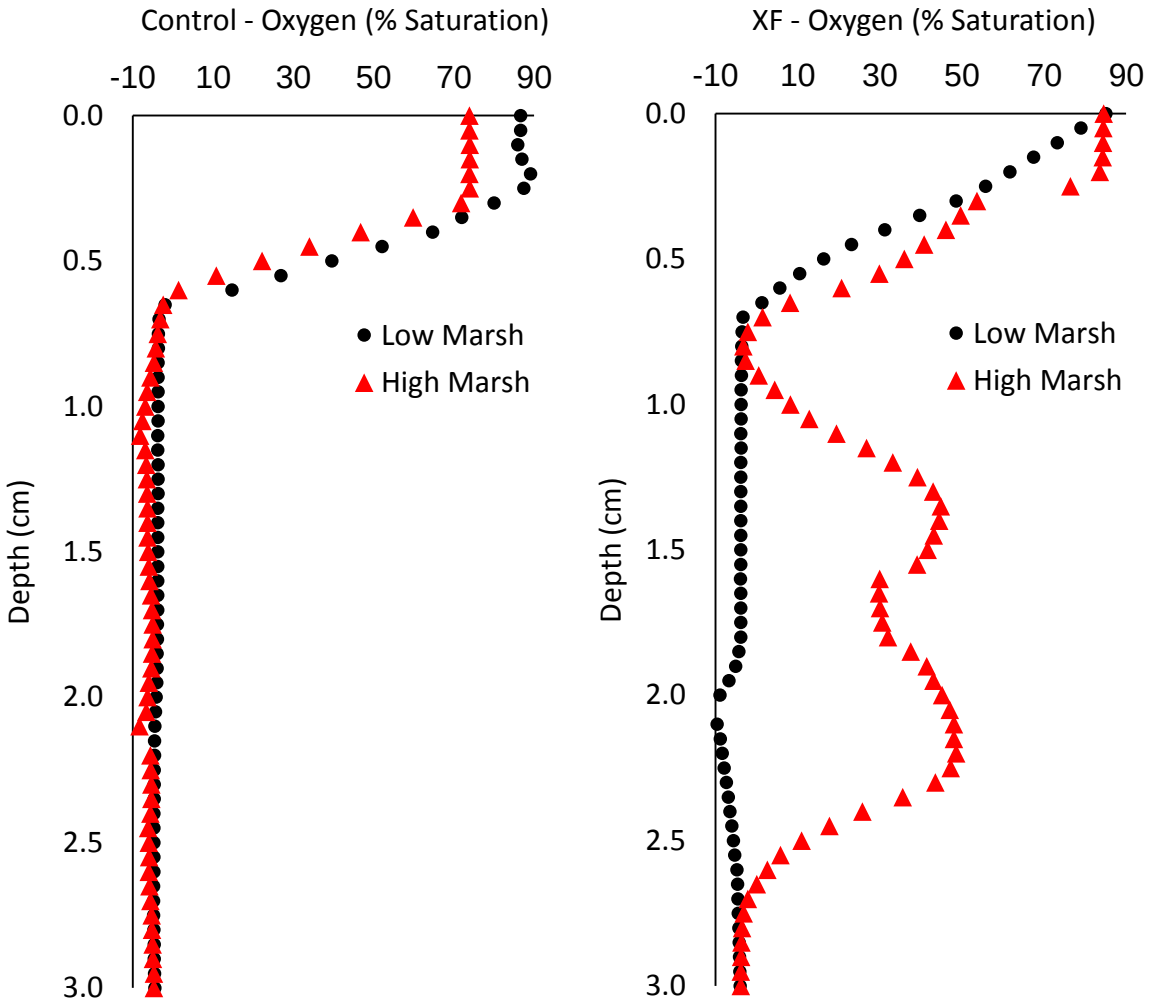

Figure 2. Oxygen profile in situ measured using an oxygen microsensor (Unisense, Aarhus, Denmark). Courtesy of Jennifer Bowen, John Angell and Patrick Kearns, University of Massachusetts Boston.

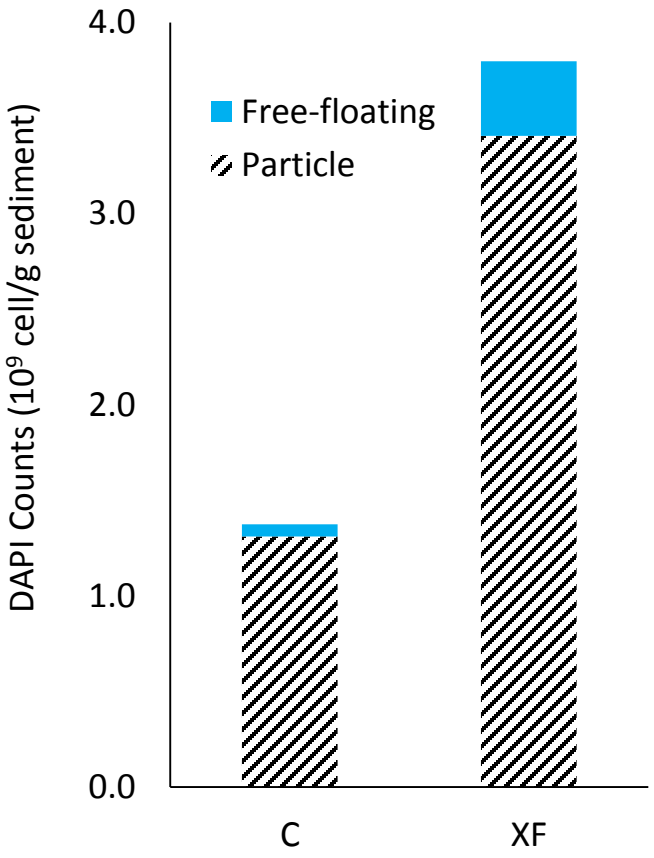

Figure 3. Cell number enumerated with DAPI staining in control (C) and extra-high fertilized (XF) plots.

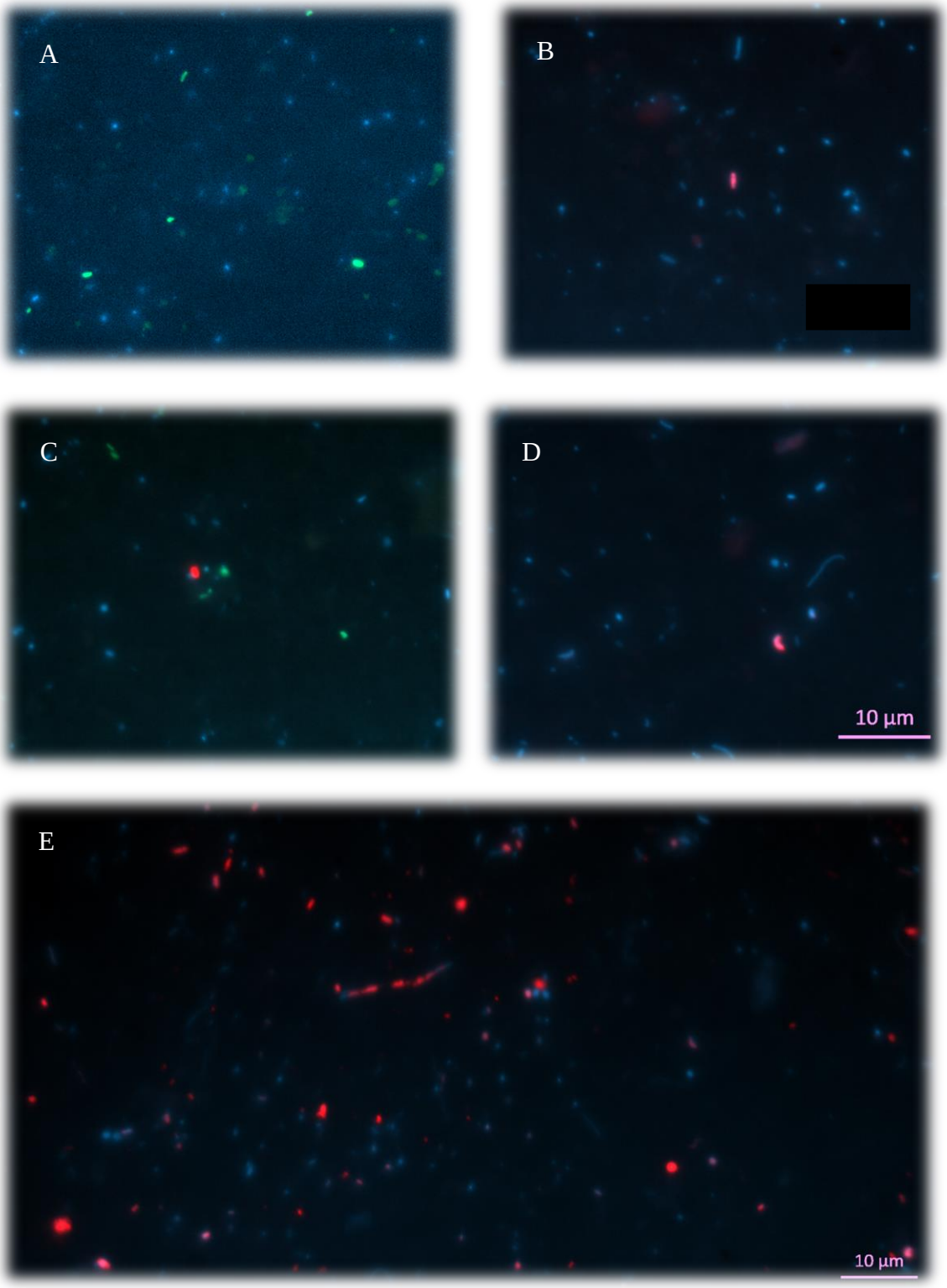

Figure 4. Micrographs of A) planctomycetes, B) β-proteobacteria, C) γ-proteobacteria and sulfur-oxidizing bacteria, D) euryarhaea, and E) Bacteria.

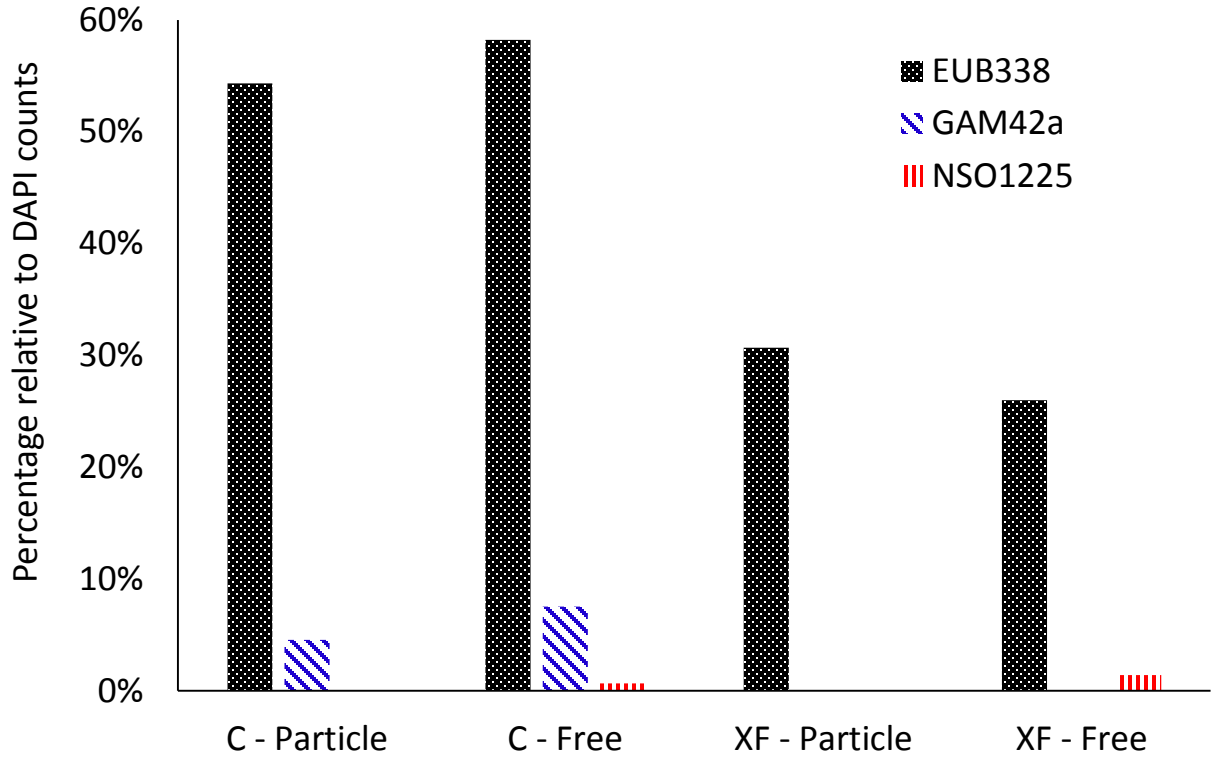

Figure 5. Relative abundance of cells that hybridized with Eub338 (most bacteria), Gam42a (γ-proteobacteria), and Nso1225 (β-proteobacterial ammonia-oxidizing bacteria). C, control plots; XF, extra-high fertilized plots; particle, particle-associated cells; free, "free-floating" cells.

Table 1. Optimized volume of samples filtered onto a 47 mm polycarbonate filter.

Sample form	Plot	Habitat	Depth	Volume of sample used
Resuspended Sediment	Control	High Marsh	Surface	5 µl
			3 cm	10 µl
		Low Marsh	Surface	5 µl
			3 cm	5 µl
	XF	High Marsh	Surface	5 µl
			3 cm	5 µl
		Low Marsh	Surface	5 µl
			3 cm	5 µl
Filtrate through 11 µm	Control	High Marsh	Surface	0.25 ml
			3 cm	1.0 ml
		Low Marsh	Surface	0.25 ml
			3 cm	0.25 ml
	XF	High Marsh	Surface	0.25 ml
			3 cm	0.25 ml
		Low Marsh	Surface	0.25 ml
			3 cm	0.25 ml

Table 2. Horseradish peroxidase probes used in this study (Loy et al. 2007).

First Probe	Target	Second Probe	Target
Arch915	Archaea		
Cren554	Most Crenarchaeota		
Eury806	Euryarchaea		
Eub338 I – III	Most Bacteria		
Non338	Nonsense		
Bet42a	β-proteobacteria		
Delta495a	δ-proteobacteria		
Epsy549	ε-proteobacteria		
PSB PiBC 467	Purple sulfur bacteria	Alph968	α-proteobacteria
JTB255	Marine benthic γ-proteobacteria	Gam42a	γ-proteobacteria
Dss658	Desulfobacteraceae and other Bacteria		
Dbb660	some Desulfobulbus		
Nso1225	β-proteobacterial AOB		
Pla46	Planctomycetes		
Dbact0193	Methylomirabilis sp.		