

Visualizing changes in the community structure of sulfur cycling bacteria in nitrogen
enriched salt marsh sediments

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Abstract

Salt marshes are some of the world's most productive ecosystem, and in these ecosystems over 50% of carbon mineralized is due to sulfate reduction. Anthropogenic inputs of nitrogen to these ecosystems might be responsible a perturbation of the sulfur cycle. Pyrite concentrations and total sulfur in marsh sediments suggests variation the very highly fertilized plots relative to the control. This variation may be able to be tied to the quantitative labeling of microbes by FISH probes and DAPI stain. DAPI counts suggest consistency in dislodgment protocol, and the hybridization of some microbes by targeted probes provides a proof of concept. Work remains to determine whether the counts are quantitative or qualitative, and hybridization techniques must be refined to more accurately label all targeted microbes.

Introduction

Salt marshes are considered some of the most productive ecosystems in the world, with estimates as high as $1500 \text{ g C m}^{-2} \text{ yr}^{-1}$ (Giblin 1992). These ecosystems have large stores of carbon in peat that is periodically, or continuously inundated with water. This provides plenty of sulfate to fuel the mineralization of organic matter. In new England salt marshes, sulfate reduction is responsible for as much as 50% or more of carbon mineralization in salt marsh ecosystems (Howes 1984,1985).

The frequent introduction of sulfate in seawater, and the contrast of oxic conditions on the surface, and anoxic conditions just below the surface fuels a tight cycling of sulfur by sulfate reduction, and sulfide oxidation. An alternation of sulfur cycling might result in the mineralization of carbon more rapidly, or more slowly than current rates. Nitrogen loading could perturb the sulfur cycle through the introduction of more below ground biomass and root exudates (Giblin 1992). And if such changes are occurring, FISH probes might be used to view changes in bacterial community composition associated with this important salt marsh process.

Nitrogen loading may perturb this cycle in many ways: by increasing plant growth and dissolved inorganic carbon inputs to the soil, by making the soils more oxidizing (Howes 1981), or by altering the below-ground root biomass and structure. Oxidation Reduction potentials were found to have a positive correlation with primary production

(Howes 1981) and the fertilized plots studied had higher rates of primary production and subsequently, more oxidizing soils.

All of these variables might be driving changes in microbial community structure between plant species, and between fertilization treatments. Pyrite can be used as an indicator of relatively more oxidizing, or reducing conditions, and the differences in bacterial abundance between plant species and fertilizer treatments. FISH probes, which have been used as diagnostic tools in medicine, and fingerprinting methods in environmental samples, might be used as indicators of this change.

Methods

Study Area – Experiment was conducted in Great Sippewissett Salt Marsh, Cape Cod, Massachusetts (*see* Valiela et al. 1976). Control plots receive no fertilizer, while LF plots receive 16.8g fertilizer per square meter, and XF plots receive 151 g fertilizer per square meter. The fertilizer is in the form of sewage sludge, and is applied twice a month from April through November.

Samples were taken in the control, low fertilized (LF), and very high fertilized plots (XF). Of interest were the patches of tall *Spartina alterniflora* nearest the tidal creek, and the patches of mostly *Spartina patens* and *Distichlis spicata* in plots 5, 6 and 7.

Coring – Two cores were taken in each of the plots, by first brushing away some of the plant matter which had just recently fallen, and placing the PVC pipe in an area devoid of visibly alive plants. A PVC pipe, with a diameter of 5.2 centimeters and a sharpened edge, was hammered into the marsh platform with a rubber mallet. The tubing was twisted, and a rubber stopper placed in the top of pipe. The core was lifted out of the marsh, wrapped in aluminum foil and stored on ice.

Pyrite – Core material was dried in a drying oven at 50° C, and homogenized. 0.1 g of sediment was taken from dried and homogenized core material and placed in 50 mL Falcon centrifuge tubes. The material was digested in 5 mL 5.5 N hydrochloric acid, filtered, and digested again in 5 mL concentrated nitric acid for 2-3 hours at 70° C, 5 mL trace metal grade hydrochloric acid was then added and samples were returned to 70° C

for 2-3 hours. The extracts were filtered Fisherbrand Q8 filter paper and diluted to 100 mL and run on a Perkin Elmer 2380 Atomic Absorption Spectrophotometer to measure the iron remaining as pyrite (FeS₂).

Sampling bacteria – Sediment cores were cored horizontally at the depths 3, 10 and 20 cm by driving a piece of 2.25 cm inner diameter pipe through the core. 0.25 cm of material was extruded from the pipe and discarded, and 1.5 cm of material extruded and placed in scintillation vials. The total volume of sampled material was 10.6 cm³. Samples were preserved in 9 mL of 10% formalin for a period of 2 weeks at 4° C.

Dislodging Bacteria – Bacterial samples were prepared with one of three methods. First, a gentle sonication method adapted from Kallmeyer et al. 2008 was used. Samples were sonicated (setting 1, 200 watt centrifuge) for 30 seconds in treatments of 10 seconds with 10 seconds in between to prevent overheating. Samples were spun at low speed for 1 minute and the supernatant was removed to a new scintillation vial. The remaining material was washed twice with a 50% solution of PBS and 100% ethanol, hand agitated, and spun. The supernatant was removed to the same scintillation vial.

Secondly, the same procedure was repeated, but with 30 seconds of uninterrupted sonication, at the same setting as before. Both techniques were repeated with bacterial samples diluted to 25 ml in sterile PBS. Finally, a separate technique of suspending sample in 25 mL of PBS and vortexing at highest setting for two one minute periods was used.

Hybridization –The probes used are detailed in figure 1. Probes DSB985, targeting *Desulfobacter*, *Desulfobacula*, and *Desulfospira* genera, and G123T targeting sulfur-oxidizing *Thiotrix spp.*, as well as a Eubacterial probe (Eub338) were chosen to be used in the samples. Probe specificity was accounted by introducing 3 irrelevant probes to other samples. Hypho143 for *Hyphomonas neptunium*, Kpm1707 for *Klebsiella pneumoniae*, and Erec482 which targets *Eubacterium rectale*.

1.5 mL of sample was pipetted into microcentrifuge tube and centrifuged for 5 minutes at 13,200 rpm to pellet the bacteria. The supernatant was removed and discarded

to remove the formalin in the sample. The pellet was resuspended in sterile PBS, pelleted, the pbs removed, and the bacteria resuspended in a 50% solution of sterile PBS and 100% ethanol and allowed to incubate for at least 15 minutes.

A hybridization mix of 18 μL 5 M NaCl, 2 μL 1 M Tris 7.5, 1 μL 1% SDS, 20 μL Hi-Di formamide, 56 μL sterile H₂O, and 1 μL of each probe at .2 nmol/ μL concentration was prepared for each sample. The final formamide concentration was based on literature values available at probeBase. A wash solution of 2.15 mL 5 M NaCl, 1 mL 1 M Tris 7.5, 0.5 mL 0.5 M EDTA, and 46.35 mL sterile H₂O was prepared and warmed in a water bath to a temperature of 48° C. Excess wash solution was prepared and stored in a 50 mL centrifuge tube. Samples were once again centrifuged, and the supernatant discarded. Cells were resuspended in 194 μL of the hybridization mix and the samples were split into two tubes of 97 μL . 3 μL of the relevant probe mix was added to one set of tubes and 3 μL of the irrelevant probe mix was added to the other set of tubes. The tubes were allowed to incubate for 3 hours at 46° C (2.5-4 hours required).

After incubation, samples were spun for 5 minutes at 13,200 rpm, and the supernatant was discarded. The pellet was resuspended in 200 μL of wash solution and incubated for 15 minutes at 48° C. This was repeated for a second 15 minute incubation. The samples were spun again, the supernatant was removed, and resuspended in 100 μL of 1 $\mu\text{g}/\text{mL}$ DAPI stain in wash solution. After waiting 5 minutes, the samples were pelleted, the supernatant removed, and resuspended in wash buffer to the appropriate dilution. 100 μL of wash buffer was used for samples. 20 μL of liquid was spotted onto an UltraStick slide and allowed to air dry. To remove the salt crystals from the sample, the slides were then dipped into ice-cold sterile H₂O for 1 second, then 95% ethanol for 1 second, and allowed to air dry. An antifade medium (VectaShield) was added to the slide, and the coverslip was placed on the slide. Samples were viewed on an AxioVision Spectral imaging microscope using the Nuance interface. DAPI counts were also performed by randomly counting the outside rim of the mounted sample and counting all cells in the 100x100 μm grid at 6 locations.

Results

In the pyrite digestions, it was found that pyrite tended to increase with depth. In the core taken near *Spartina alterniflora*, at 3cm in the control plot pyrite was present in an average concentration of 2.59 mg/g dry weight, which increased to 4.91 mg/g at 10cm and 7.81 mg/g at 20 centimeters. In the *Spartina alterniflora* of the XF plot, at 3 cm pyrite concentrations were low, with only 1.48 mg/g present. Pyrite increased rapidly to 13.49 mg/g at 10 cm, and ultimately 14.13 mg/g at 20 centimeters. The magnitude of these . (Figure 3) In the cores taken nearest to *Spartina patens* these trends were quite similar but the magnitude of change was much lower. Pyrite increased from 1.75 mg/g at 3 cm to 2.29 at 10cm, and 2.56 at 20 cm in the control plot. In the XF plot pyrite concentrations were an average of 1.17 mg/g at 3 cm, 0.98 mg/g at 10 cm, and 4.62 mg/g at 20 cm.

The trends in the LF plot do not appear to be as reliable, the average of the *Spartina alterniflora* core increases from 4.43 mg/g at 3 cm to 11.28 mg/g at 10 cm and 12.59 mg/g at 20 cm. In the cores taken from an area of *Spartina patens* there is an average of 9.12 mg/g at 3 cm, 11.52 mg/g at 10 cm, and 8.375 mg/g at 20 cm. (Figure 4)

DAPI counts indicate no statistical significance in the number of microbes dislodged or visible between samples. Six counts of each slide preparation were performed and averaged. The counts in Figure 5 seem to reflect consistency between sediment compositions in bacterial yield. All counts are within one standard deviation of each other.

Very few cells were successfully hybridized by the above protocol. Cells in all samples were successfully stained with DAPI (Figure 6), however all of the bacterial samples taken from plots of *Spartina alterniflora* were not hybridized with the probe. In the control plot in a bacteria sample at 3 cm, there was found to be probe conferred florescence for eubacteria (Figure 7) in the 470nm image, in which the eubacteria probe labeled with Alexa 488 is visible. In the image taken at 570nm there is visible probe conferred florescence, as two bacteria at the upper middle of the field remain are labeled. Because Alexa 488 has an excitation maxima of 495 nm, it is likely this is the G123T

probe targeting *Thiotrix sp.* that is labeled with Alexa 647. In the XF plot at 3 centimeters (Figure 8) the eubacterial probe is excited, and able to be isolated at 470 nm.

Discussion

The pyrite data suggests that there is some alteration of the sulfur cycle in the more fertilized plots. While no significant change was observed in the *S. patens* cores, pyrite concentrations in the *S. alterniflora* XF cores were two times that in the control core. Consistent with this, Katie Harrold found that the percent of total sulfur in XF plots was elevated more than one percent over the control plots. This total sulfur might be due to the accumulation of pyrite and iron sulfide, which can be effective in the retention of hydrogen sulfide which might otherwise be lost to the atmosphere. In marshes, the majority of sulfur is carbon bonded, and only small amounts are found as reduced inorganic compounds (H_2S , FeS , FeS_2) (Schlesinger 236). This phenomenon of increasing percent sulfur in fertilized plots may indicate a perturbation of the sulfur cycling, and at the very least warrants further study. Pyrite concentrations may potentially be skewed by the time of sampling, as most plants on the marsh are senescing. However, the accumulation of pyrite operates on a much longer timescale than the accumulation of more transient sulfur compounds such as H_2S .

Of the techniques employed to dislodge bacteria, the gentle sonication technique (10 seconds on, 10 seconds off) appeared to be the most effective, and only in minimal liquid. Samples sonicated had visibly fewer detached bacteria, and the samples that were vortexed had much larger particles. These large particles pushed the coverslip away from the slide when mounted, and resulted in a much thicker mount, and poorer resolution and movement of light through the sample.

Most important to the success of the hybridization technique was the potential overfixing of the bacteria. The bacteria were left in 10% formalin for a period of two weeks at 4° C when 16 hours in 4% formaldehyde is more than adequate for natural samples. Given more time for experimental trials manipulation of formamide concentration might provide

Additionally, it is difficult to tell if the bacteria were dislodged from the detritus preferentially, or without bias. Counts could be compared to ^{14}C -Leucine productivity estimates to get an idea of how many bacteria are present (Epstein, et al. 1997) and more DAPI counts could be performed to check for variation. The ability of the probes to bind to targeted organisms might be assessed by performing hybridizations on either pure cultures, or samples that are known to contain the targeted microbe.

Further experiments might target bacteria closer to the roots of *Spartina alterniflora*, and *Spartina patens*, and might involve the mounting and hybridization of small root cross sections to see if there are bacteria more closely related to the rhizosphere of the plant species. To tease apart the role of fertilization in on sulfur cycling sulfides and sulfates, and dissolved organic carbon and reduction oxidation potential would need to be measured. By comparing these values between the control and XF plots, it might be more clear as to whether DOC from root exudates might be driving a change in sulfur cycling.

QuickTime™ and a
decompressor
are needed to see this picture.

Figure 1. Map of field sites at Great Sippewissett Marsh. Sites sampled were plots 5, 6, and 7.

Name	Target	Fluor	Excitation Maxima
Experimental Probes			
DSB985	Desulfobacter, Desulfobacula,	Alexa 488	495nm
G123T	Desulfospira, et al.	Alexa 647	655nm
Eub338	Eubacteria	RhodRedX	570nm
Irrelevant Probes			
Hypho143	Hyphomonas neptunium	Alexa 647	655nm
Kpn1707	Klebsiella pneumoniae	RhodRedX	570nm
Erec482	Eubacterium rectale	Elexa 488	495nm

Figure 2. Probes used, with attached fluor and excitation maxima. Probe data was found using probeBase, and probes were supplied by Invitrogen.

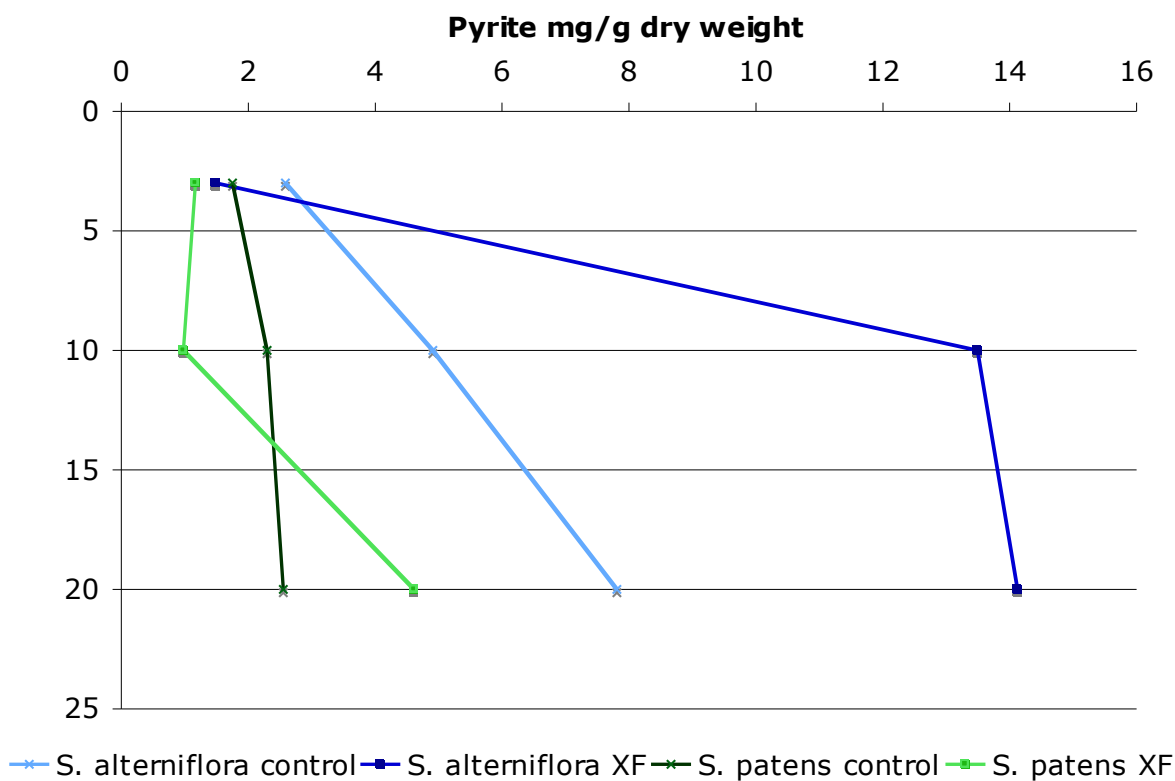


Figure 3. Graph of average pyrite concentrations obtained by the digestion of sediments in nitric and hydrochloric acid. Data is in units of mg pyrite per gram of dry weight.

Control	<i>S. alterniflora</i> 1	<i>S. alterniflora</i> 2	<i>S. patens</i> 1	<i>S. Patens</i> 2
3	1.975	3.2	1.55	1.95
10	4.69	5.13	3.21	1.38
20	7.17	8.45	1.72	3.39
LF	<i>S. alterniflora</i> 1	<i>S. alterniflora</i> 2	<i>S. patens</i> 1	<i>S. Patens</i> 2
3	4.47	4.39	7.83	10.4
10	13.66	8.9	11.66	11.38
20	8.33	16.86	15.79	0.96
XF	<i>S. alterniflora</i> 1	<i>S. alterniflora</i> 2	<i>S. patens</i> 1	<i>S. patens</i> 2
3	1.49	1.47	1.15	1.19
10	3.98	23	1.04	0.92
20	14.4	13.85	5.54	3.69

Figure 4. Table of raw pyrite data in mg pyrite per gram dry weight

	Control		XF	
	<i>S. Alterniflora</i>	<i>S. Patens</i>	<i>S. Alterniflora</i>	<i>S. Patens</i>
3	42	48	70	46
20	46	39	53	34

Figure 5. DAPI counts of bacteria per 100 μm^2 in slide preparations of 20 μl

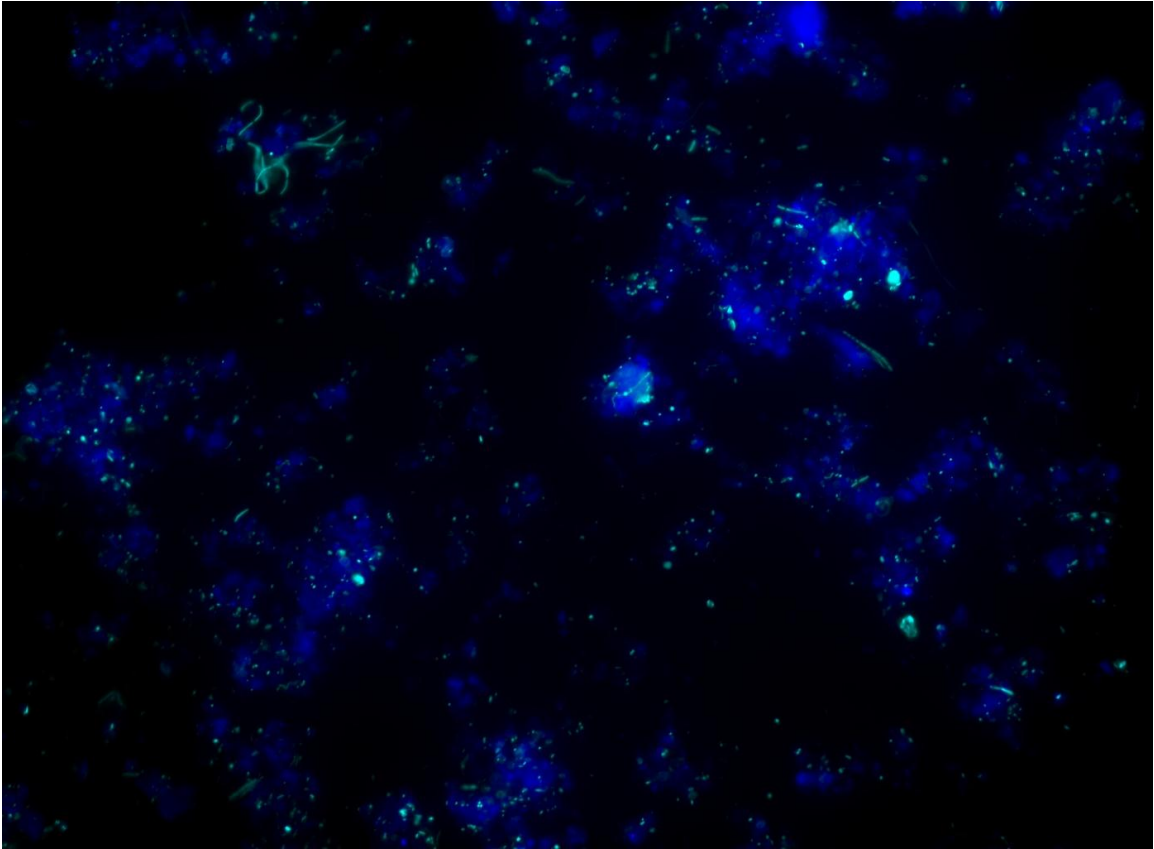


Figure 6. 3 centimeter sample taken from the XF plot near *Spartina alterniflora*. Image was taken at a wavelength of 400nm (DAPI excitation) and enhanced with the nuance interface to isolate the microbes from autofluorescence. Color was artificially imposed on a grayscale image. Detritus is dark blue, and microbes are labeled cyan.

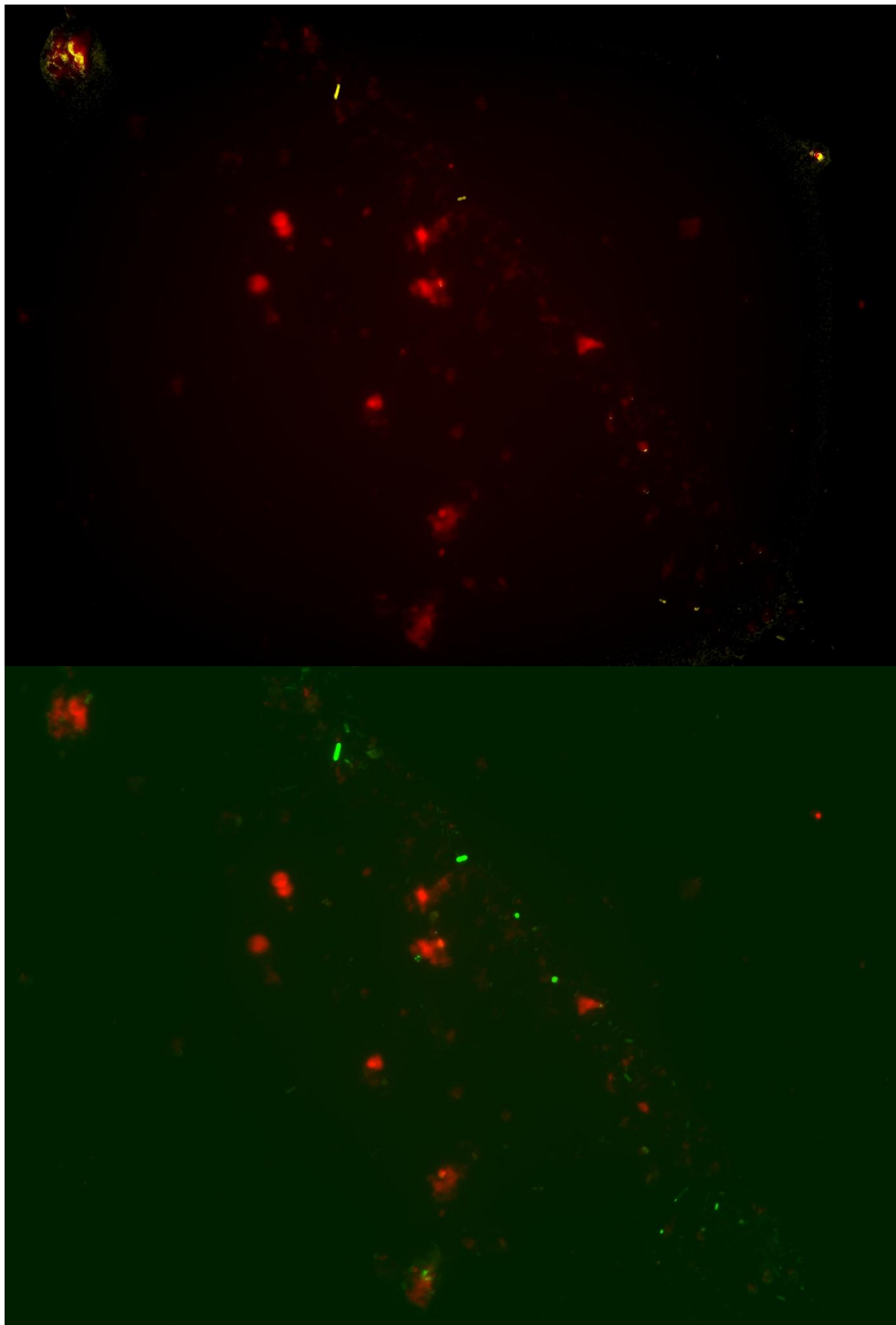


Figure 7. 3 centimeter sample taken from the control plot near *Spartina patens*. Image was taken at 470 nm wavelength (top) which depicts excitation of Alexa 488, and 570 nm (bottom) which depicts excitation of Alexa 647. Images were enhanced with the nuance interface from a grayscale image and color was artificially imposed. Microbes are green in the left image, and yellow in the right image. Detritus is labeled red. The microbes visible in the left image are stained with a eubacterial probe, while those labeled in the right image depict probe conferred fluorescence for G123T (*Thiotrix spp.*).

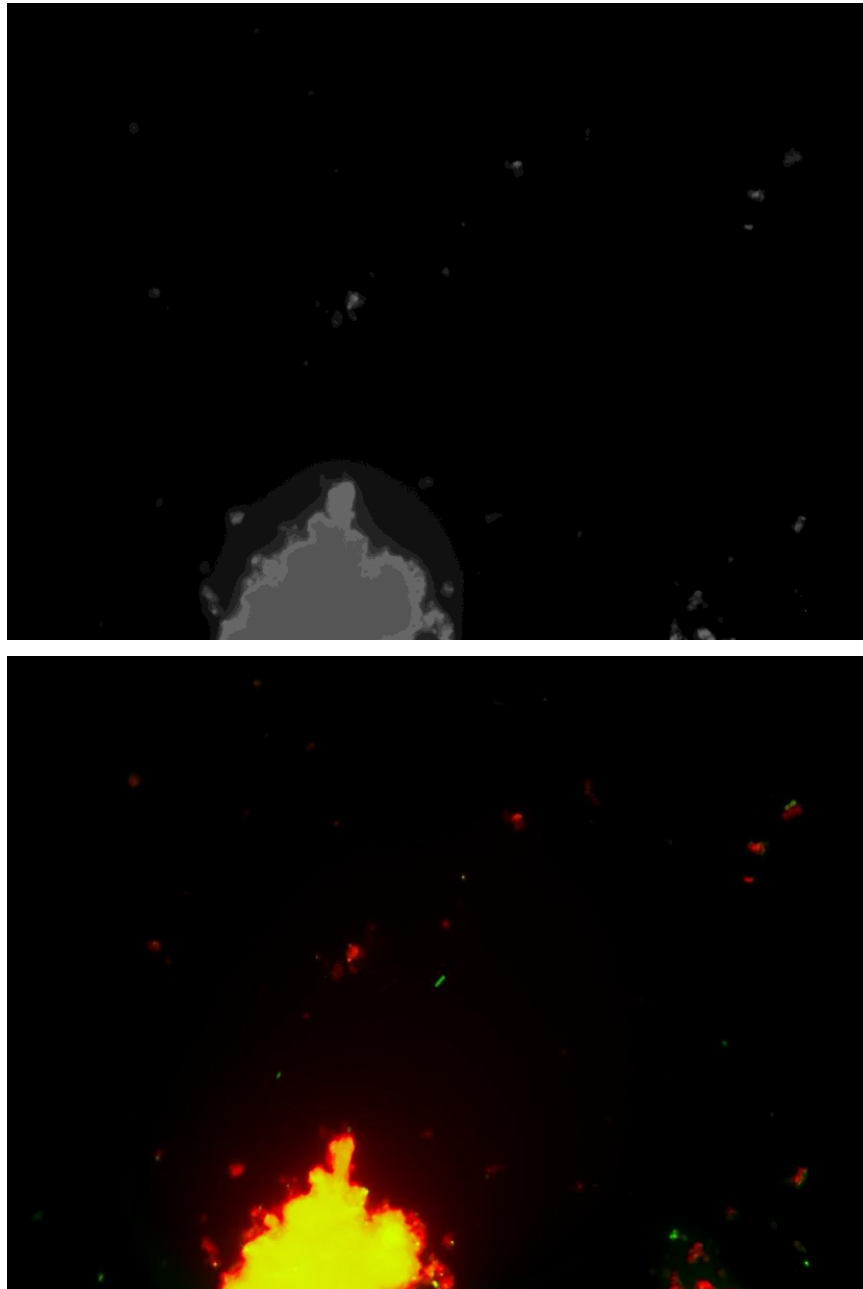


Figure 8. 3 centimeter sample taken from the XF plot near *S. patens* at 470 nm (Alexa 488 excitation). Original grayscale image (top) was altered using the Nuance interface, and microbes were isolated and color artificially imposed. Microbes are green, and detritus remains red.

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