DETECTION AND DISTRIBUTION OF AMMONIA-OXIDIZING BACTERIA OF COASTAL GEORGIA WATERS USING MOLECULAR-BASED METHODS

by

Kathryn Leigh Dudeck

(Under the Direction of ROBERT E. HODSON)

ABSTRACT

An *in situ* PCR/hybridization technique was developed to detect the *amoA* gene in marine ammonia-oxidizing bacteria. A degenerate primer set was used to amplify a region of the *amoA* functional gene that is common to a range of nitrifying bacteria. Concurrently, FISH techniques were utilized to detect a range of ammonia-oxidizers taxonomically via 16S rRNA homology to an oligonucleotide probe. Samples of coastal Georgia seawater were collected and size fractionated to retain organisms <1.0µm on 0.22µm filters. Filter-adhered bacterial cells were subjected to *in situ* PCR followed by *in situ* hybridization with a fluorescently-labeled internal probe. Replicate samples were subjected to 16S rRNA FISH. Filters were examined with epifluoresence microscopy to enumerate both total bacteria and ammonia-oxidizing bacteria. The percentage of AOB detected in the environment ranged from 3.4%-60.0% functionally and 1.2%-23.1% phylogenetically.

INDEX WORDS: Ammonia-oxidizing bacteria, AOB, Nitrification, *In situ* hybridization, FISH, *In situ* PCR, Georgia

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION

Purpose of this Project

Previously, molecular techniques to visualize individual bacterial cells involved in the process of ammonia-oxidation have been based on immunological (Ward 1982, Jones *et. al* 1988) and 16S rRNA investigations (Voytek and Ward 1995, Wagner *et. al* 1995, Stephen *et. al* 1996, Daims *et. al* 2001). However, these techniques provide a limited scope of the actual functional roles of the organisms involved. Additionally, the serological diversity and complex phylogeny of the microbes responsible for nitrification make it difficult to obtain a complete picture of the players using existing methodologies. Investigations of natural bacterial assemblages have frequently demonstrated that functionality is not necessarily related to phylogeny and vice-versa (Zehr and Capone 1996). To date, studies of nitrifying bacteria seem to point towards a discrete tie between phylogenetic and functional relationships in ammonia-oxidizing bacteria.

Sequence databases are currently limited in the amount of information they contain, and it cannot be completely ruled out that some bacterial species that are capable of nitrification are unrelated phylogenetically to the known group of ammonia-oxidizing bacteria. Also, in natural environments, cells may remain in extended inactive or less active states. Under such conditions, cellular ribosome numbers may decline, resulting in a decreased target for 16S rRNA studies, and thus could result in an underestimation of active ammonia-oxidizing bacterial cells by these procedures.

Therefore, this investigation examined the use of a functional gene, key to ammonia oxidation, which would provide a potentially more complete detection of ammonia-oxidizers in environmental samples. To that end, an *in situ* PCR/hybridization technique was employed in order to detect a wide taxonomic range of bacteria from coastal Georgia estuaries, and which was based on the functional capability of individual cells to oxidize ammonia. Concurrently, established 16S rRNA techniques were utilized in order to observe the distribution of these ammonia-oxidizers based on phylogenetic relatedness for comparison. This project is the first published discussion of the distribution of ammonia-oxidizing bacteria based on functional gene detection at the individual cell level.

Nitrification in the Marine Environment

In order to examine functional roles in nitrification, it is imperative to have an understanding of the complex interactions of the nitrogen cycle. The marine nitrogen cycle is comprised of a suite of reduction and oxidation (redox) processes that are carried out by microorganisms, with nitrogen existing in various oxidation states (Figure 1). Because it is an essential component of nucleic acids and proteins, nitrogenous compounds are required in one form or another for growth of all living organisms (Ward 1992). While molecular nitrogen (N_2) is the most common form in marine environments, most organisms cannot utilize it. Therefore, cyanobacteria convert N_2 into organic nitrogen, where it can then be taken up by organisms. Nitrite (NO_2^-), nitrate (NO_3^-) and ammonium (NH_4^+), the reduced form of ammonia, are assimilated by bacteria into amino

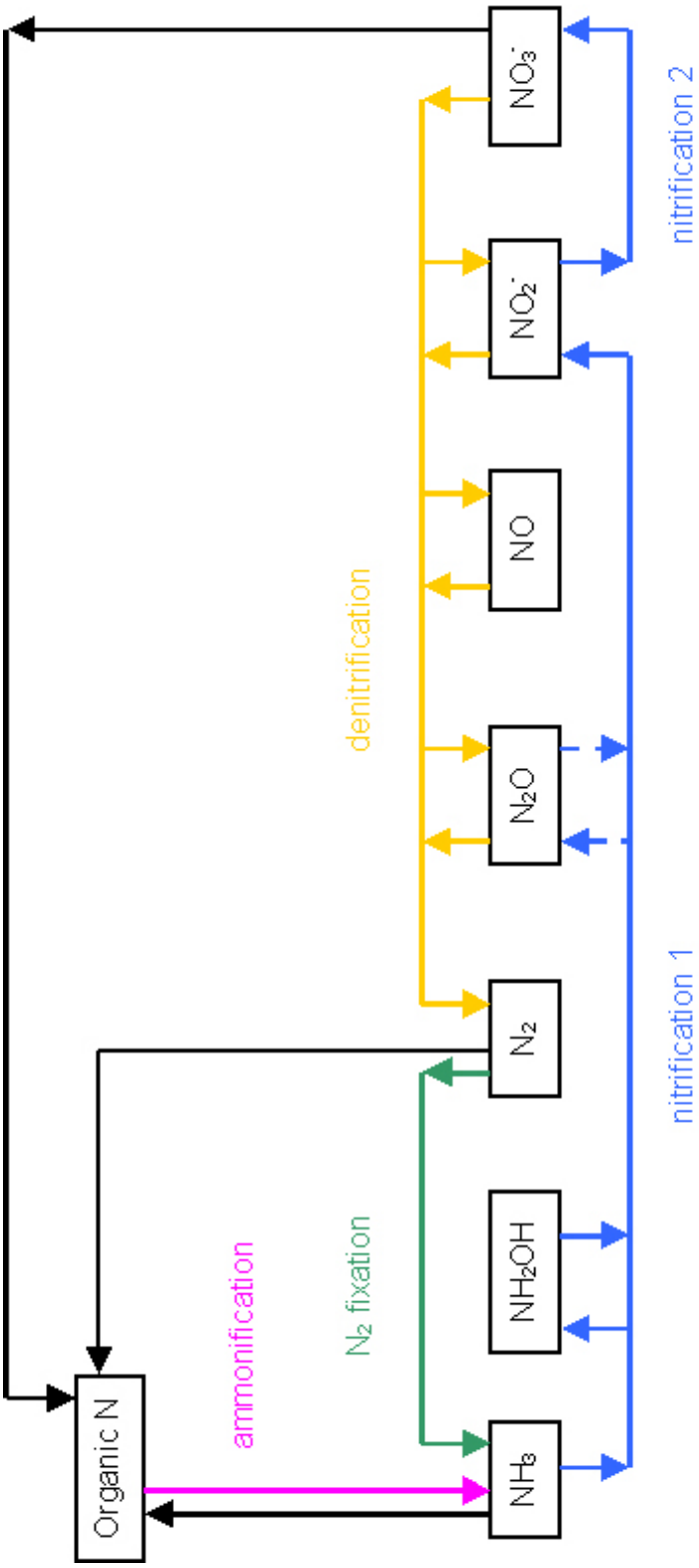

Figure 1. Schematic of the microbial nitrogen cycle. The hatched lines below nitrous oxide indicate that product if formed during nitrification, though how and why it is formed is unclear. Adapted from Valiela (1995).

acids, increasing biomass. Ammonification occurs when organic matter begins to decompose, and is the remineralization of organic nitrogen to ammonia (NH_3). This formation provides phytoplankton with the nitrogen necessary for primary production to occur. Nitrification, the oxidation of ammonia to nitrite and nitrate, is the pathway of interest to this study, and is explained in detail below. Denitrification is the reduction of nitrate to molecular nitrogen, and is considered the opposite pathway of nitrification (Ward 1993). It is commonly carried out in anaerobic environments, and shares many common intermediates with nitrification. When coupled with nitrification, denitrification helps to combat the common occurrence of eutrophication in aquatic systems, as well as reduce inorganic nitrogen concentrations in wastewater treatment plants (Bothe *et. al* 2000).

Ammonia-Oxidizing Bacteria

Nitrification is a central process in the Earth's nitrogen cycle, linking the most reduced and oxidized portions of the nitrogen cycle. It is a two-step process involving two distinct bacterial assemblages, ammonia-oxidizers and nitrite-oxidizers (Juretschko *et. al* 1998). Ammonia-oxidizing bacteria (AOB) oxidize ammonia to hydroxylamine then nitrite, generating nitrous oxide as a by-product. AOB are ubiquitous in marine, freshwater and terrestrial environments. These aerobic bacteria are divided into two distinct subclasses: γ-Proteobacteria, which is comprised of *Nitrosococcus oceani* and *Nitrosococcus halophilus*, and β-Proteobacteria, which includes the four remaining known genera of ammonia-oxidizers: *Nitrosomonas* (including *Nitrosococcus mobilis*), *Nitrosospira, Nitrosolobus* and *Nitrosovibrio* (Purkhold *et. al* 2000). However, Head *et.*

al (1993) have suggested that *Nitrosolobus* and *Nitrosovibrio* should be reclassified as members of the *Nitrosospira* genus. Most nitrifers are primarily obligate chemolithotrophic organisms, oxidizing inorganic ammonia as an energy source and utilizing carbon dioxide as the sole carbon source for building biomass. A few members, however, can simultaneously nitrify and denitrify (Bock *et. al* 1995). Likewise, other strains assimilate organic compounds such as acetate or pyruvate for mixotrophic growth (Bothe *et. al* 2000). The optimum growth temperature for AOB is approximately 30ºC, with a pH of ~8.2 (Ford and Churchwell 1980). Some AOB, however, are capable of persistence in extreme habitats. The marine bacterium *Nitrosomonas cryotolerans*, for example, was first isolated by Jones *et. al* (1988) from the surface of Kasistna Bay, Alaska, with a water temperature of 11ºC.

Enzymes Required for Ammonia Oxidation

Two key enzymes are involved in ammonia oxidation: ammonia monooxygenase (AMO), which is an integral membrane protein and hydroxylamine oxidoreductase (HAO), a periplasmic protein (Madigan *et. al* 1997). These codependent enzymes each generate the substrate and electrons required by the other (Bothe *et. al* 2000). The oxidation of ammonia to hydroxylamine and subsequently hydroxylamine to nitrite is catalyzed by AMO and HAO via the following pathways:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^-$$

Oxidation by HAO provides the above two required electrons from the oxidation of hydroxylamine to nitrite. One of the oxygen atoms in NO_2^- is derived from O_2, while the second O_2 is derived from H_2O. Two of the four electrons produced by HAO are reincorporated into the AMO reaction (Miller and Nicholas 1985). The remaining two electrons are transferred to the electron transport chain, though only minimal research has been conducted to characterize the carriers and intermediates of this pathway (Hooper *et. al* 1997).

The AMO enzyme of *Nitrosomonas europaea,* as well as many other AOB, possesses three subunits known as AMO-A, AMO-B and AMO-C. Each subunit is of a different size, structure and arrangement within the membrane of the bacterial cell. The *amo* operon encodes the genes *amoA*, *amoB* and *amoC* (Sayavedra-Soto *et. al* 1998; Alzerreca *et. al* 1999), and all three genes have been successfully cloned and sequenced from several genera of AOB.

The majority of AOB studies, including the one presented here, have focused on the *amoA* gene, which codes for the active site of the enzyme (Rotthauwe *et. al* 1997; Horz *et. al* 2000; Limburg *et. al* 2000). A region within this gene encoding the C-terminus is a suitable primer/probe site for distinguishing between the beta and gamma subclasses of these bacteria. This is because an intergenic spacer region of 163-445 bp is found between *amoA* and *amoC* in all β- and γ- AOB subdivisions, and its length is species-specific (Alzerecca *et. al* 1999).

Similarities of AMO to pMMO

The AMO enzyme of ammonia-oxidizing bacteria is highly homologous to the pMMO (particulate methane monooxygenase) enzyme of methanotrophic bacteria (Holmes *et. al* 1995; Bothe *et. al* 2000). Both enzymes are thought to be comprised of at least two membrane-associated polypeptides that are approximately 27-45 kDa and have similar sequences, suggesting a common evolutionary origin (Holmes *et. al* 1995). Greater similarities are seen between the AMO and pMMO enzymes of γ-Proteobacteria at both DNA and protein levels than are seen within the β- and γ-Subclasses of ammonia-oxidizing bacteria (Holmes *et. al* 1995; Alzerecca *et. al* 1999).

Additionally, methanotrophs also possess HAO, though it is not identical to HAO in ammonia-oxidizers (Zahn *et. al* 1994). This enzyme, coupled with pMMO, allows methanotrophs to oxidize ammonia to nitrite, though this cannot be used as a growth substrate (Madigan *et. al* 1997). Also, like AOB, methane-oxidizing bacteria are aerobic and ubiquitous in terrestrial and aquatic environments.

Distribution of Ammonia-Oxidizing Bacteria

To date, there have been no studies on the distribution of AOB utilizing functional gene-based methods on the per cell level. Therefore, all published values regarding ammonia-oxidizers in the environment or in man-made systems are based on phylogenetic assessments. Numerous investigations have focused on wastewater treatment plants and activated sludge, likely because these are "near pure" communities of nitrifiers and denitrifiers that are readily accessible. However, there is little agreement between published values in similar environments. Wagner *et. al* (1995) determined that

approximately 20% of the bacteria in activated sludge were β-Subclass AOB. Using probes Nso190, Nso1225, Nsm156 and NEU (all specific to β-Subclass AOB), Juretschko *et. al* (1998) calculated a similar 16-20% of the total cells in activated sludge to be ammonia-oxidizers. However, hybridizations conducted by Mobarry *et. al* (1996) on bacterial populations in a continuously-stirred reactor yielded abundance of AOB ranging from 31% (probe Nsm156) to 87% (probe Nso190) of the total population.

Ward (1988) conducted immunofluorescent assays on bacterial populations from three different aquatic sites. In Chesapeake Bay, AOB accounted for 1.0% of the population, while 0.01% of the bacteria off the Washington state coastline were determined to be AOB. Open ocean values from the Sargasso Sea led to a determination of 0.01% of the population to be ammonia-oxidizers. Stehr *et. al* (1995) investigated the distribution of ammonia-oxidizers in the Elbe River in Germany. The freshwater estuary yielded AOB abundance values of 0.9%, while the population of the brackish estuary was 0.001%. Additional studies by Ward *et. al* (1997) looked at two lakes in Germany. AOB accounted for 0.01% of the total bacterial population of the Belauer See and 0.04% of the Plußsee. A summary of these values is presented in Table 1.

In Vitro PCR Versus *In Situ* PCR

The polymerase chain reaction, or PCR, amplifies a desired DNA sequence millions of times within 2-3 hours using a series of heating and cooling steps in a thermal cycler (Mullis and Faloona (1987); Figure 2). The reaction is highly specific and able to amplify large amounts of DNA from small quantities of sample. PCR is based on a specialized polymerase, or enzyme, that can synthesize a complimentary strand for a

Table 1. Representative abundance values of ammonia-oxidizing bacteria in various environments.

Environment	Percent of Total Population	Reference
Chesapeake Bay	1.0%	Ward 1982
Washington coastline	0.01%	Ward 1982
Sargasso Sea	0.01%	Ward 1982
Freshwaster estuary	0.9%	Stehr *et. al* 1995
Brackish estuary	0.001%	Stehr *et. al* 1995
Belauer Sea, Germany	0.01%	Ward *et. al* 1997
Plußsee, Germany	0.04%	Ward *et. al* 1997
Bioreactor	31 - 87%	Mobarry *et. al* 1996
Wastewater treatment plant	20%	Wagner *et. al* 1995
Wastewater treatment plant	16 - 20%	Juretschko *et. al* 1998

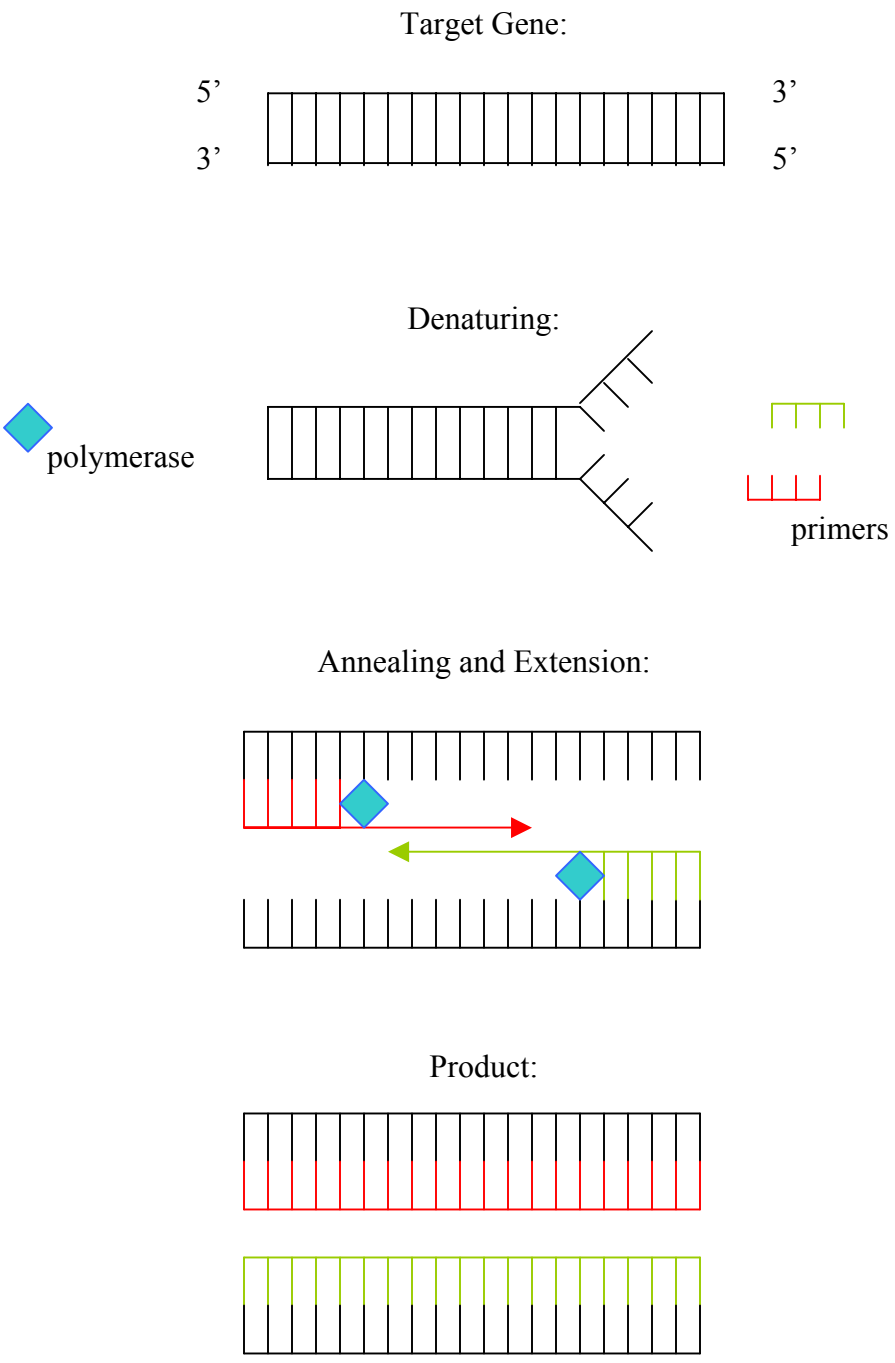

Figure 2. Schematic representation of the steps of one cycle of the polymerase chain reaction (PCR). After the product is formed, denaturing occurs again for 20-40 cycles to produce an exponential number of the target amplicon.

given DNA stand. Two short (17-22 bp) oligonucleotides called primers are designed based on the sequences of the region of interest. The oligonucleotides are then synthesized, containing complimentary sequences that flank the target. In *in vitro* PCR procedures, DNA from the cell is extracted and purified, and a "cocktail" is mixed containing the DNA, oligonucleotides, polymerase, buffer and dNTP. Magnesium chloride is also added as a binding cofactor for successful amplification of the intended product. The mixture is heated in order to denature (separate) the double-stranded DNA, and then cooled which allows the primers to bind to their complimentary regions in the gene. Additionally, the polymerase extends the oligonucleotides into complementary strands. This continual heating and cooling multiplies the PCR product exponentially for further analysis.

In situ PCR, also called whole-cell PCR, is a technique by which the target DNA is amplified within the cell, with no need to extract or purify the DNA (Hodson *et. al* 1995). It is performed very much as *in vitro* PCR, however the cells must first be fixed in paraformaldehyde, then minimally permeabilized to allow the chemical mixture to enter the cell for PCR to occur, while preventing product loss from inside the cell.

Fluorescent Oligonucleotide Labeling and *In Situ* Hybridization

Fluorescent *in situ* hybridization (FISH) was first utilized successfully with bacteria by Giovannoni *et. al* (1988). FISH relies on the specific binding of an oligonucleotide complimentary to a discrete region of a gene, and though these probes can be designed to bind with either DNA or RNA molecules, rRNA is commonly the desired target. This is because rRNA molecules are evolutionarily conserved and occur

in high copy numbers (1000-2000 ribosomes per active cell; Fegatella *et. al* 1998), organisms do not have to be cultured for exploration, and the vast 16S rRNA libraries now available lend themselves to straightforward probe design (Amann 1995a).

Probes are commonly labeled with a fluorescent dye for photometric verification of successful hybridization. Cy3 or Cy5 can be attached to a primary aliphatic aminolinker at the 5' end of the probe. Subsequently, fluorescein can be covalently bound to the 5' end (Amann *et. al* 1997). Each ribosome in a bacterial cell has one copy of 5S, 16S and 23S rRNA, and one probe molecule should bind to the target rRNA in each cell. Due to the high copy number of 16S rRNA, this technique is considered to be a natural signal amplification system for detecting specific bacteria (Pernthaler *et. al* 2001).

CHAPTER 2

MATERIALS AND METHODS

Oligonucleotide Primer and Probe Development

In order to detect the presence of this functional gene in marine samples, several primer sets were employed to ensure amplification of *amoA* in both β- and γ-Subclass ammonia-oxidizers. While amplification of β-Subclass AOB (*N. europaea*) was achieved with A189/A682 (Holmes *et. al* 1995), *N. oceani* was not amplified. The same results were obtained when PCR was conducted using *amoA*-1F/*amoA*-2R (Rotthauwe *et. al* 1997). Utilizing a "mixed" primer set led to more satisfactory results: while *amoA*-1F/A682 did not amplify γ-AOB, A189/*amoA*-2R (Table 2) successfully amplified a 675 bp region of the *amoA* gene in both Subclasses of ammonia-oxidizing bacteria.

Using GenBank, the amoA gene sequences of 8 β-Proteobacteria ammonia-oxidizing bacteria were compared (2 *Nitrosomonas eutropha* strains, *Nitrosomonas europaea*, 2 *Nitrosolobus multiformus* strains, *Nitrosovibrio tenuis*, and 2 *Nitrosospira* strains) (Appendix A). Based on the sequence alignment, a conserved region of 21 base pairs was determined to be optimal for use as probe β3b (Table 2).

Probe β3b labeled with CY3 was used as an internal probe to the amplicon for functional gene assessment. For the phylogenetic-based assessment, CY3 labeled probe NEU (Wagner *et. al* 1995; Table 2), targeting the 16S rRNA of β-Subclass AOB was employed for the June 2000 samples. June 2001 samples were subjected to hybridization

Table 2. Sequences and targets of primers and probes used in this study.

Oligonucleotide	Sequence (5' - 3')	Target Site	Reference
β3b	GAC CAC CAG TAG AAA CCC CAG	*amoA* of β- and γ-AOB	This study
NEU	CCC CTC TGC TGC ACT CTA	16S rRNA of *Nitrosomonas*	Wagner *et. al* 1995
Nso1225	CGC CAT TGT ATT ACG TGT GA	16S rRNA of all β-AOB	Mobarry *et. al* 1996
A189 *	GGN GAC TGG GAC TTC TGG	*amoA* 109-127 of *N. europaea*	Holmes *et. al* 1995
amoA-2R *	CCC CTC KGS AAA GCC TTC TTC	*amoA* 802-822 of *N. europaea*	Rotthauwe *et. al* 1997

* Though originally designed for only *N. europaea*, these primers were used in detection of all β-AOB for this study

with CY3 labeled probe Nso1225 (Mobarry *et. al* 1996; Table 2), also targeting the 16S rRNA of β-Subclass AOB. It should be noted that NEU is complementary to only two strains of β-AOB, *Nitrosomonas europaea* and *Nitrosmonas eutropha*. Therefore, while NEU is a useful probe for investigating AOB, it is not as general as Nso1225, which was designed for all β-Subclass AOB. For this reason, probe Nso1225 was employed for the second set of environmental samples.

Positive Controls

Nitrosococcus oceani (ATCC Strain 19707) was grown in ATCC 928 Nitrosococcus Medium. Three microliters of culture were added to 25 ml of medium in four replicate sterile screw-top 24 X 150 test tubes and placed in a shaking (100 rpm) incubator at 26°C. Morphological culture characteristics were observed under a microscope bi-weekly to ensure purity. After one month of growth, the contents of one tube were subjected to a DNA extraction using a QIAgen Blood Mini Prep Kit.

Nitrosomonas europaea (ATCC Strain 19718) was grown in ATCC Culture Medium 1573 Nitrosomonas europaea Medium. As with *N. oceani*, three microliters of culture were added to 25 ml of medium in four replicate sterile screw-top 24 X 150 test tubes and placed in the incubator with *N. oceani*. Morphological culture characteristics were observed under a microscope bi-weekly to ensure purity. After one month of growth, the contents of one tube were subjected to a DNA extraction using a QIAgen Blood Mini Prep Kit.

PCR and Hybridization Optimization

In order to optimize *amoA* amplification and minimize non-specific amplification, a gradient PCR was carried out. Annealing temperatures ranged from 45ºC-60ºC, with the strongest band visible at 56ºC. To ensure probe specificity in preparation for environmental samples, PCR with the extracted *N. oceani* DNA was carried out using primers A189 and *amoA*-2R, followed by hybridization with functional probe β3b.

Hybridizations with the functional probe were conducted with formamide concentrations ranging from 0%-40%, with 10% deemed optimal. After determining the most favorable formamide concentration, various hybridization temperatures were investigated, ranging from 45ºC-64ºC, with 60ºC considered most satisfactory. Once the formamide concentration and hybridization temperature were empirically determined, the most beneficial hybridization duration was investigated. Hybridizations were conducted lasting 1-3 hours, and it was ascertained that hybridizations greater than 1 hour led to only a minimal increase in successful hybridization of the target sequence.

Additionally, because probes NEU and Nso1225 are based on only β-Subclass AOB, it was necessary to ensure that they would successfully hybridize with the γ-Proteobacteria. Subsequent experiments with the two probes and *N. oceani* and *N. europaea* yielded successful hybridizations.

Negative Controls

DNA was extracted using a QIAgen Blood Mini Prep Kit from lab grown cultures of *Alcaligenes faecalis*, *Proteus vulgaris, Escherichia coli*, *Serratia marcescens*, *Streptococcus faecalis* and *Enterobacter aerogenes*. Standard *in vitro* PCR was

conducted in 0.6ml thin-wall tubes using the same protocol as for *in situ* PCR. The samples were run in a 1.5% agarose gel before staining with ethidium bromide. The gel was then viewed using an AlphaInnotech Gel Visualizer, and a photo taken. The absence of any bands on the gel showed that primers A189 and *amoA*-2R did not amplify any target sequence on these bacteria, and therefore the bacteria did not have a region homologous to the amoA of ammonia-oxidizing bacteria.

Additionally, separate hybridizations with *A. faecalis*, *E. aerogenes*, *E. coli*, and *P. vulgaris* were conducted using both probes Nso1225 and β3b to ensure probe specificity. The cells were fixed as above, using 10% paraformaldehyde for 2 hours at 4°C. Hybridization with Nso1225 consisted of 3 minutes at 94°C (in order to minimize any possible tertiary structures), followed by 1 hour at 46°C, while hybridization with β3b consisted of 3 minutes at 94°C, followed by 1 hour at 60°C. After hybridization, the slides were rinsed in their respective buffers and allowed to air dry.

The dried filter pieces were then counterstained with the total DNA stain YO-PRO (Molecular Probes, Eugene, OR) at a final concentration of 1.0 μM in dH_20 for 2 min in the dark at room temperature. Excess stain was removed with a 0.1% Nonidet P40 wash (Sigma Chemical Co., St. Louis, MO), filter quarters were rinsed in dH_20, and allowed to air dry before mounting in oil. Slides were then examined under epifluorescent microscopy with a high-resolution 100X UPlan oil objective lens (numerical aperture 1.35 to 0.50) on an Olympus BX40 microscope. Cells were viewed under orange light for CY3 excitation conditions (554 nm excitation, 568 nm emission), while YO-PRO stained cells were viewed under blue light (491 nm excitation, 509 nm emission). Samples were first exposed to the YO-PRO excitation conditions (to view

both total cells), and then exposed to the CY3 conditions (to view any successfully hybridized cells). A representative image of each species was acquired with a cooled charge-coupled (CCD) Sensys 1400 camera and processed with Oncor Image software package version 2.02 on a Power Macintosh. The image was first captured under YO-PRO excitation conditions, and then a second image of the same field was captured under CY3 conditions.

Though cells of *A. faecalis*, *P. vulgaris, E. coli* and *E. aerogenes* were all observed when stained with YO-PRO, no cells were visible under CY3 excitation conditions after *in situ* PCR/FISH with β3b or FISH with Nso1225 (Figures 3-6). This suggests that common contaminants did not bias the results, which would lead to falsely positive detection of AOB.

Due to the high similarities between AOB and MOB, it was necessary to ensure that methanotrophs were not being detected with these techniques. *Methylocystis parvus* OBBP, *Methylosinus trichosporium* OB3b, and *Methylobacter albus* BG-8 were all fixed in 10% paraformaldehyde, and subjected to hybridizations with β3b and Nso1225, using the above procedures, before being counterstained for examination with the same viewing conditions as the previous negative controls. None of the methanotroph cultures examined yielded positive results after *in situ* PCR/FISH or FISH, though cells were visible after staining with YO-PRO (Figures 7-9).

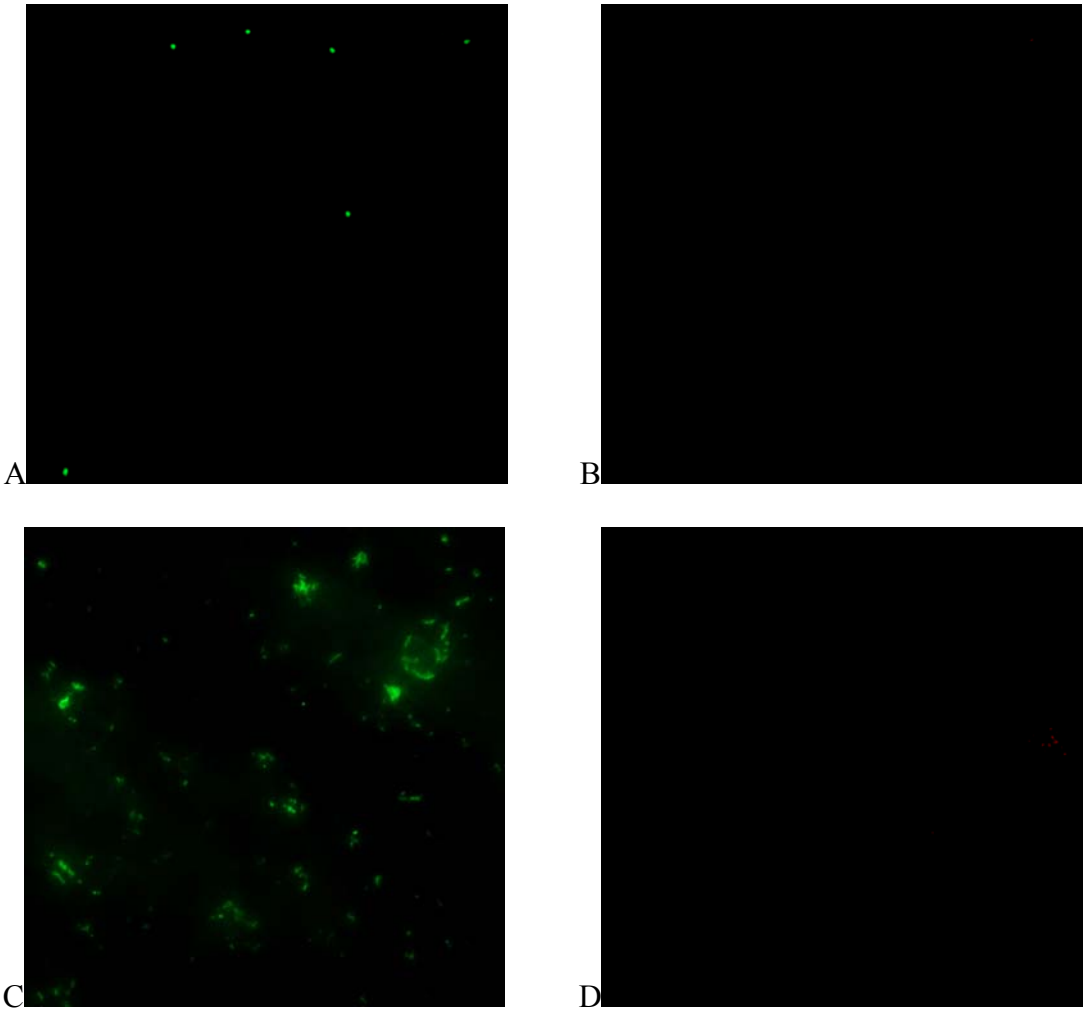

Figure 3. *Alcaligenes faecalis*. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

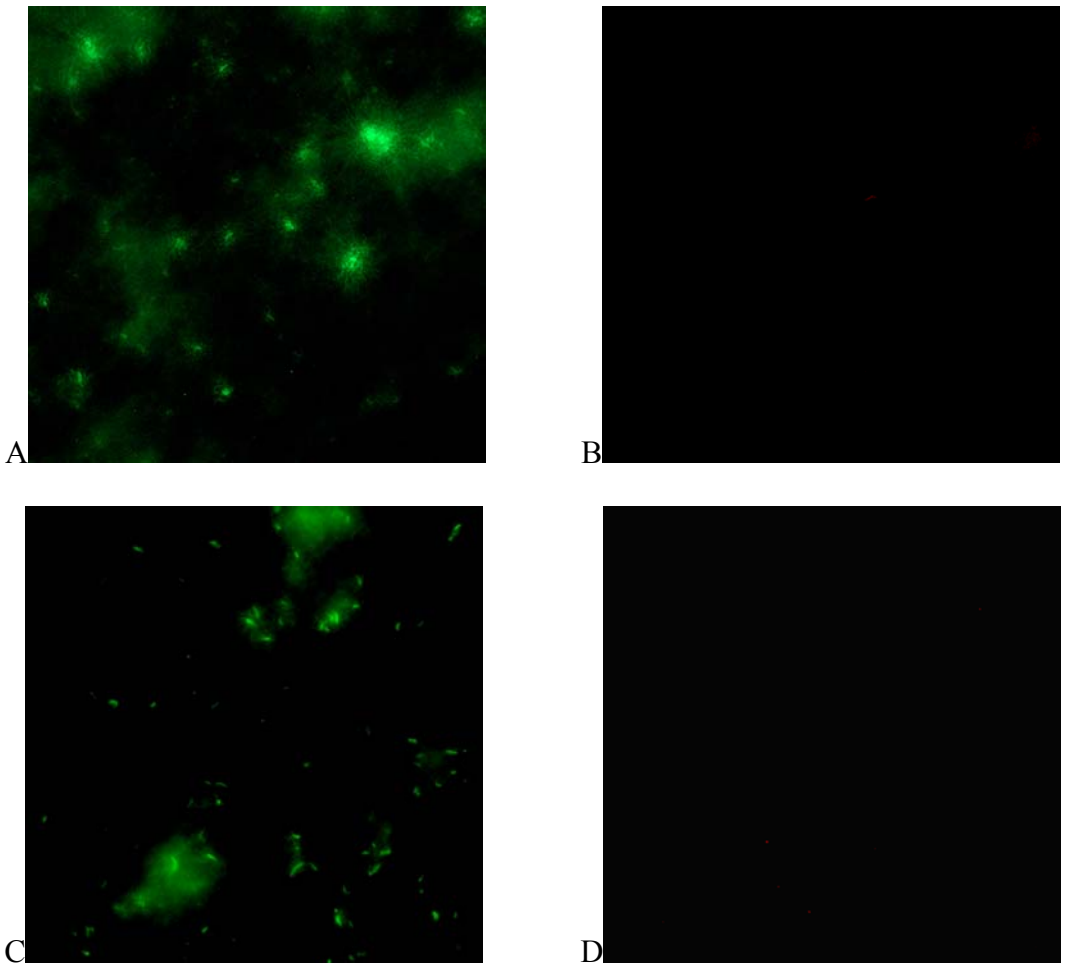

Figure 4. *Proteus vulgaris*. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

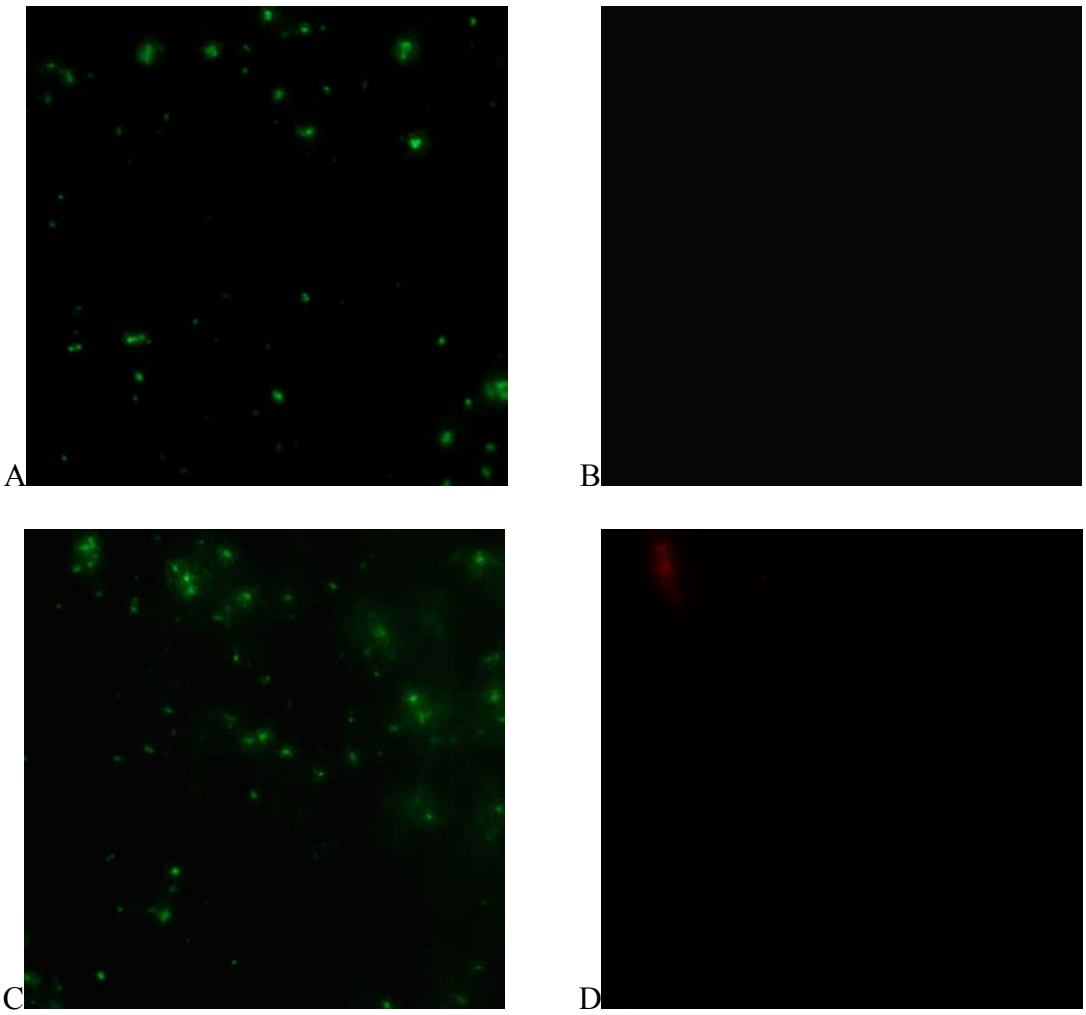

Figure 5. *E. coli*. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

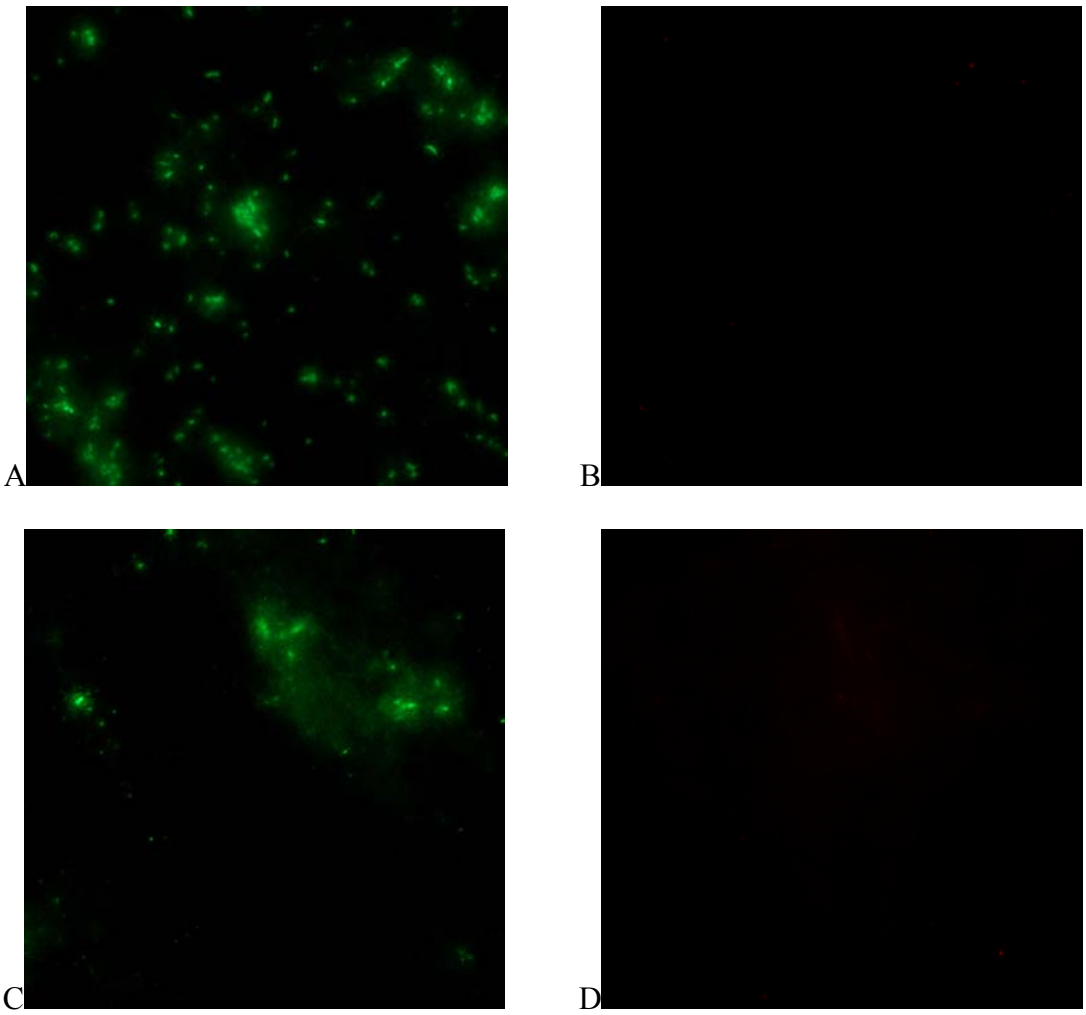

Figure 6. *Enterobacter aerogenes*. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

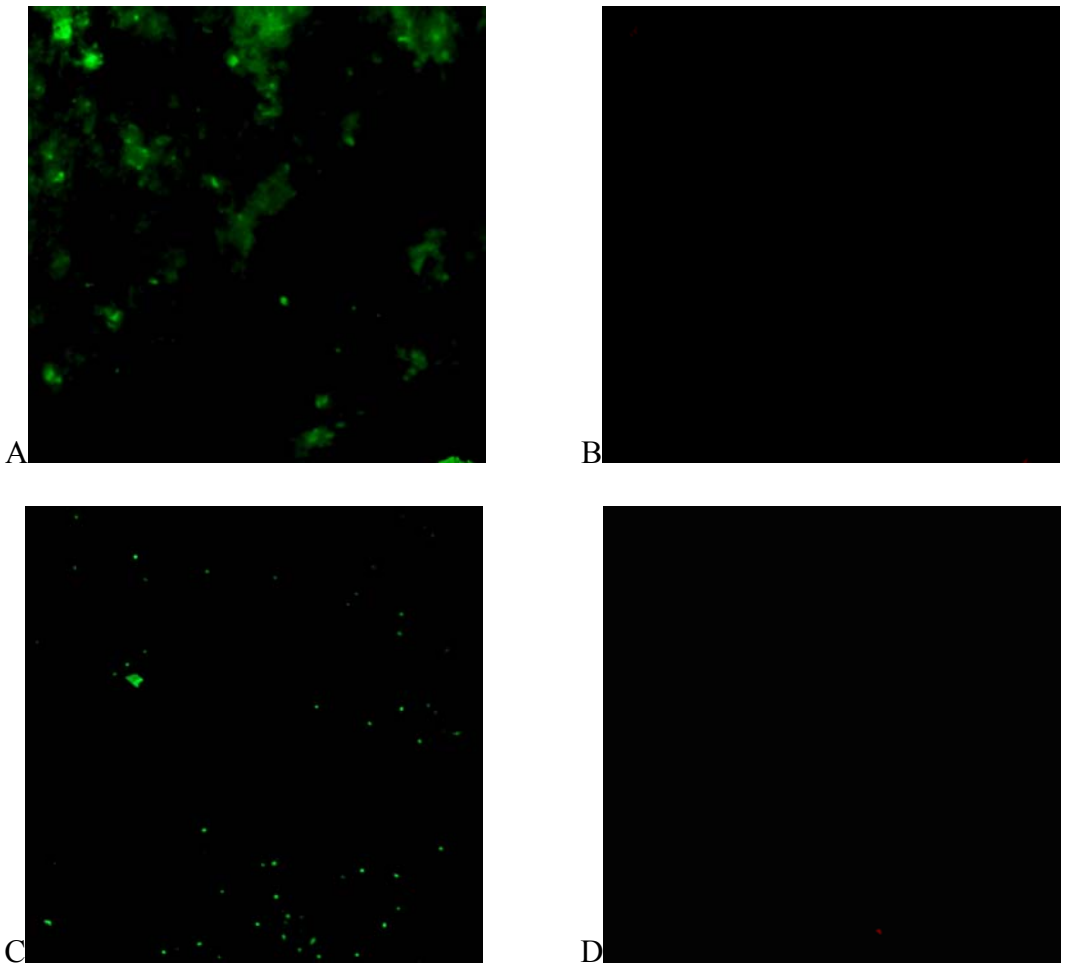

Figure 7. *Methylocystis parvus* OBBP. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

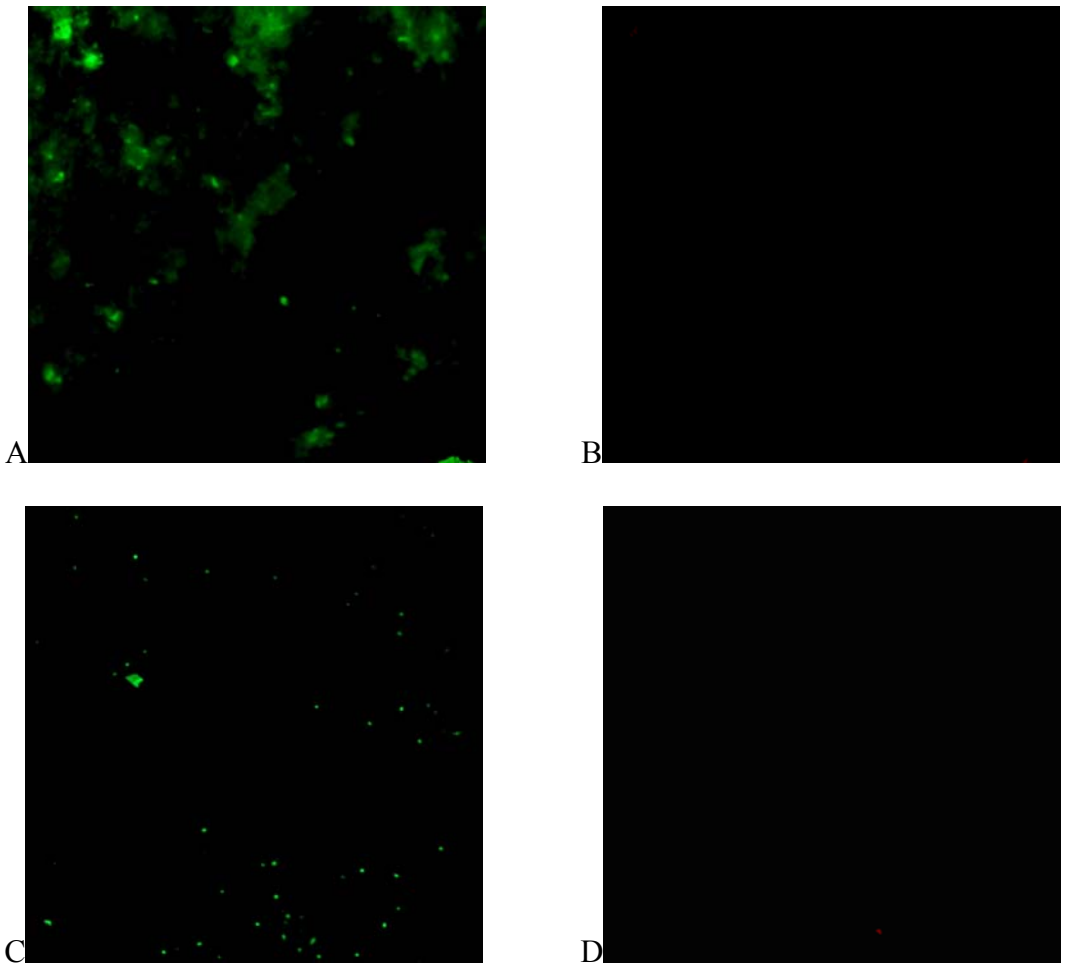

Figure 8. *Methylocystis parvus* OBBP. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

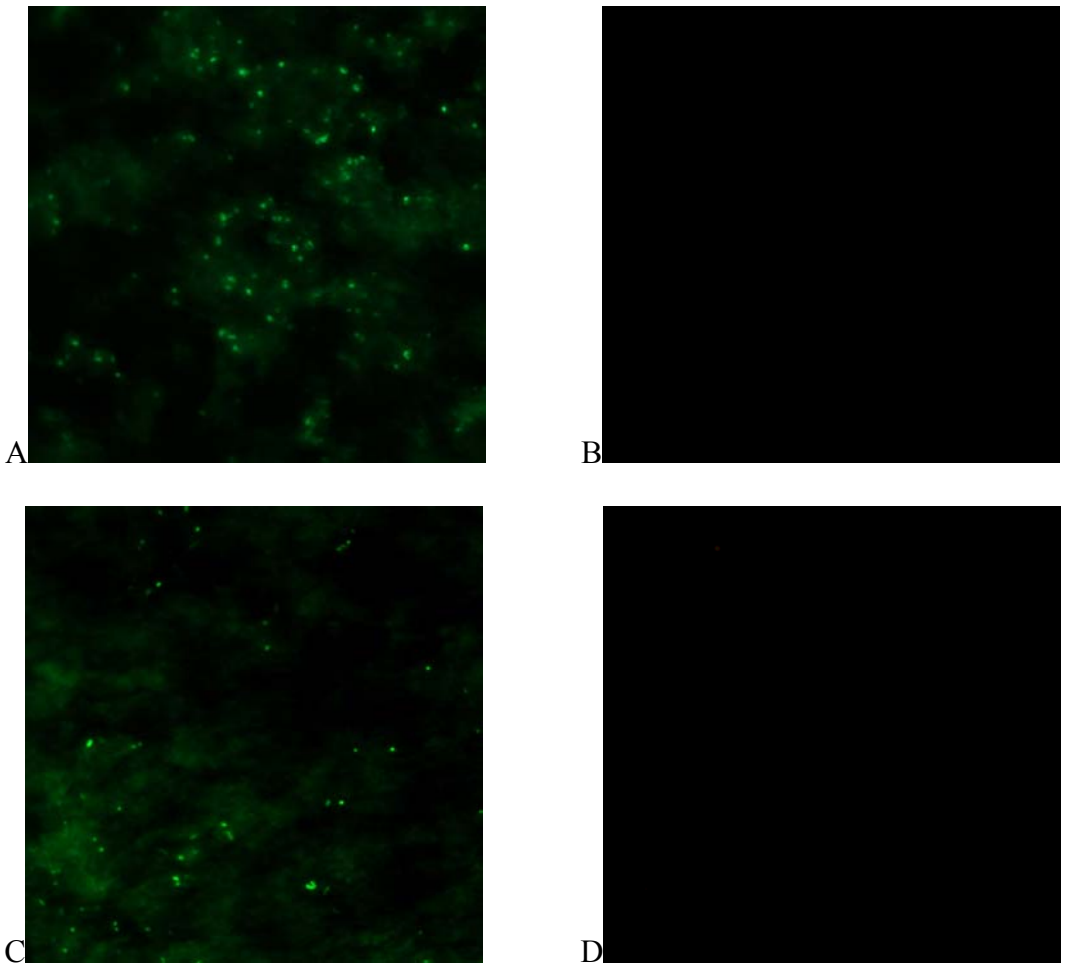

Figure 9. *Methylobacter albus* BG-8. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells.

Mixed Positive and Negative Controls

In addition to the positive and negative controls that were investigated separately, an artificially mixed environment was created. Cultures of *N. europaea*, *E. coli* and *M. parvus* OBBP were combined, as well as cultures of *N. oceani*, *E. coli* and *M. parvus* OBBP. These mixtures were fixed in paraformaldehyde and concentrated onto filter quarters as previously described. One filter quarter from each combination was subjected to *in situ* PCR followed by hybridization with β3b, and a second quarter was hybridized with probe Nso1225. For both artificial mixtures, the AOB were seen to positively hybridize, while the negative controls did not exhibit any signal under the CY3 viewing conditions (Figures 10 and 11).

Environmental Samples and Sample Concentration

Surface seawater samples were collected from several estuaries and tidal creeks on the coast of Georgia during June 2000 (Dean Creek, Doboy Sound, Long Tabby, Lumber Dock and Marsh Landing) and June 2001 (Bourbon Field, Cabretta, Duck Pond, Eulonia, High Point, Hunt Camp, Hunt Camp Creek, Lumber Dock, Meridan, and UGAMI) (Figure 12). All samples were transported on ice back to the University of Georgia Marine Institute (UGAMI) laboratory on Sapelo Island and stored at 4°C until processing. Samples were agitated vigorously to ensure uniform distribution of bacteria, and salinities were measured with a hand-held refractometer. Samples were then passed through successive sterile 3.0 μm pore size and 1.0 μm pore size syringe filters to remove large particles. Fractionated samples were transported on ice to the UGA Athens laboratory and fixed (1 part sample : 3 parts 10% paraformaldehyde in PBS) at 4°C for 2

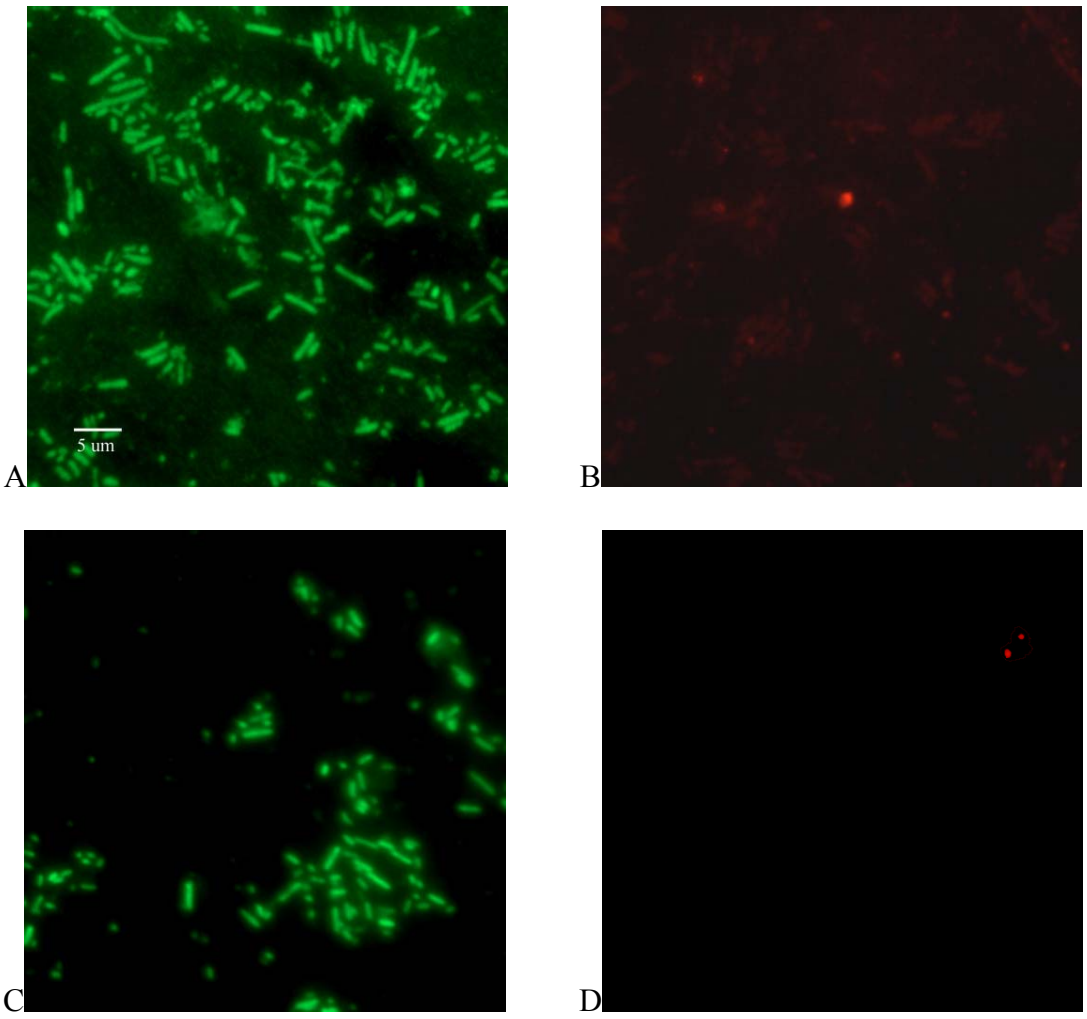

Figure 10. *N. europaea*, *E. coli* and *Methylocystis parvus* OBBP mixed community. A) PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH with Nso1225 showing total cells; D) FISH with Nso1225 showing all CY3-visible cells.

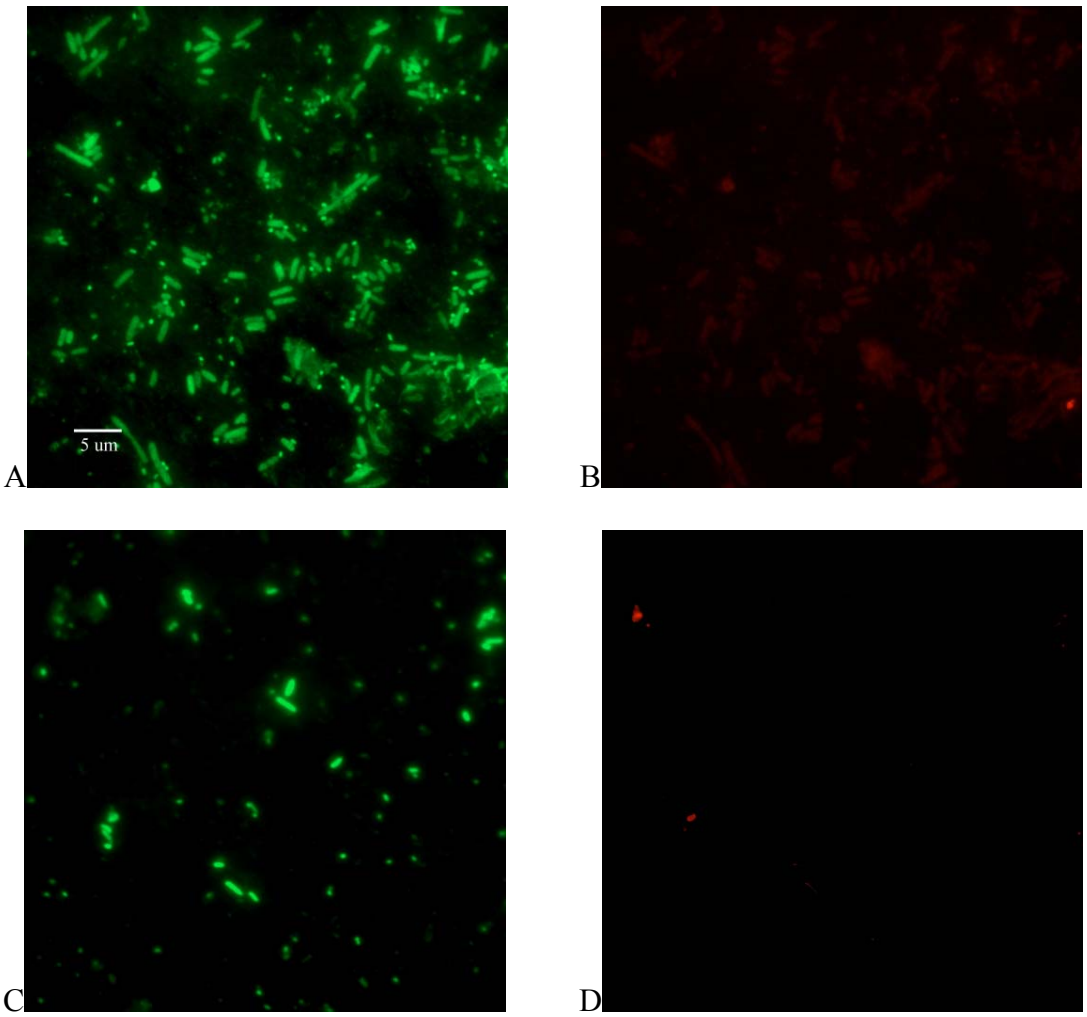

Figure 11. *N. oceani*, *E. coli* and *Methylocystis parvus* OBBP mixed community.
A) PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH with Nso1225 showing total cells; D) FISH with Nso1225 showing all CY3-visible cells.

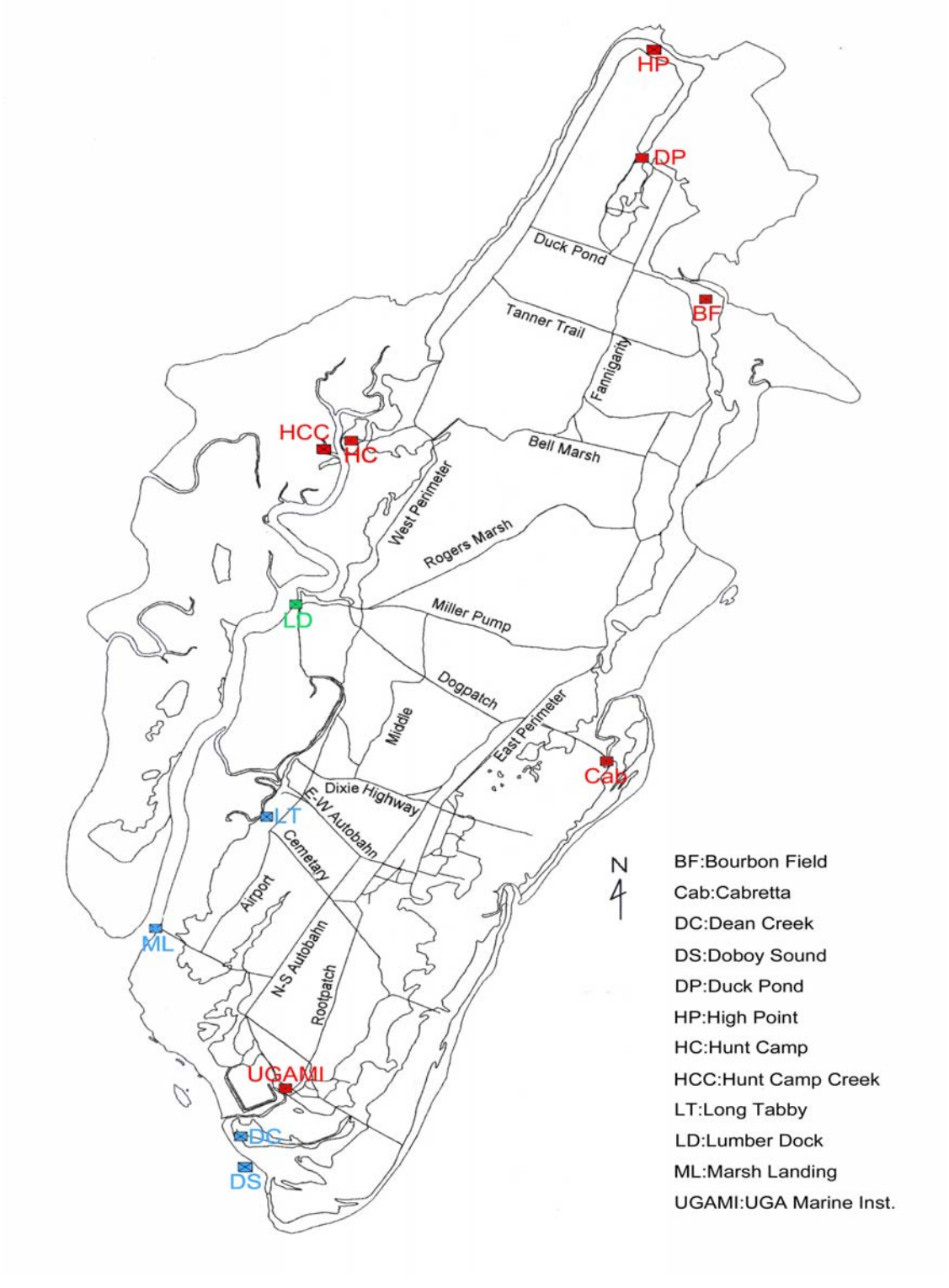

Figure 12. Map of Sapelo Island, Georgia showing sample sites used for this project. Sites indicated in blue were visited in June 2000 and sites in red from June 2001. The green site Lumber Dock was visited during both sample trips. Eulonia and Meridian are mainland sampling sites, and not shown on this map.

hours. After fixation, samples were concentrated onto 0.22 μm pore size polycarbonate filters using a 25 mm glass filter tower (d=15 mm) with a vacuum of 300 mbar. Filters were then rinsed copiously with PBS and given a dH_2O final rinse before being placed in sterile tissue culture dishes and allowed to air dry. The air-dried filters were then stored at -20°C until FISH or *in situ* PCR/FISH was performed.

Four replicate filters were prepared for each sample site, and then cut into quarters. One quarter of each replicate was left untreated to serve as an assessment of the original number of filter-adhered cells (as observed by YO-PRO staining) prior to the *in situ* PCR/FISH or FISH technique, another quarter was used for *in situ* PCR/FISH, a third quarter was subjected to *in situ* hybridization with NEU (June 2000) or Nso1225 (June 2001), and the remaining untreated quarter was kept in reserve.

In Situ PCR/FISH for *amoA*

In situ PCR was carried out using modifications of established methods (Hodson *et. al* 1995). Replicate filter quarters were treated with 1.0 mg ml^{-1} lysozyme in DEPC treated lysozyme buffer (100 mM Tris, 50 mM EDTA [pH 8.0]) for 10 minutes at room temperature to permeabilize the cell walls. PCR of the filter-adhered cells was conducted in Frame Seal-Chambers (MJ Research, Watertown, MA) in an MJ Research PTC-200 Thermocycler. Experimental conditions were as follows: 94°C (2:00 min); 94°C (1:00 min); 56°C (1:00 min); 72°C (1:00 min); n=35; 56°C (5:00 min); 4°C (holding temp). Upon completion of PCR, filters were rinsed with 0.22 μm filtered PBS to remove any chemicals remaining on the samples. Filters were placed in new Frame-Seal Chambers with 62 μl of 10% formamide hybridization buffer at 60°C with β3b probe (5 ng ml^{-1}

final concentration). Hybridization with β3b was carried out for 3 minutes at 94°C (in order to minimize any possible secondary structures), followed by 1 hour at 60°C in the thermocycler. After hybridization, filters were lightly rinsed in 10% formamide hybridization buffer at 60°C, placed in tissue culture dishes, and allowed to air dry. It was determined that washing in hybridization buffer colder than 60°C led to high background color. Most likely, the DNA coiled onto itself because of the cooler temperature, trapping unattached probe molecules.

FISH with NEU (June 2000 Samples)

Experimental protocols were followed as per Wagner *et. al* (1995). Filters with collected marine bacteria were placed in Frame-Seal Chambers with 62 µl of 46°C 40% formamide hybridization buffer with probe NEU (5 ng ml^{-1} final concentration). Hybridization with NEU consisted of 3 minutes at 94°C (in order to ensure identical treatments with all FISH protocols), followed by 1 hour at 46°C in an MJ Research PTC-200 Thermocycler. After hybridization, filters were lightly rinsed with 46°C 40% formamide hybridization buffer, placed in tissue culture dishes, and allowed to air dry.

FISH with Nso1225 (June 2001 Samples)

Experimental protocols were followed as per Mobarry *et. al* (1996). Filters were placed in Frame-Seal Chambers with 62 µl of 35% formamide hybridization buffer at 46°C with probe NSO1225 (5 ng ml^{-1} final concentration). Hybridization with Nso1225 consisted of 3 minutes at 94°C (in order to ensure identical FISH conditions), followed by 1 hour at 46°C in an MJ Research PTC-200 Thermocycler. After hybridization, filters

were lightly rinsed with 46°C 35% formamide hybridization buffer, placed in tissue culture dishes, and allowed to air dry.

Enumeration and Digital Image Analysis

As discussed previously, filters were subjected to dual staining with YO-PRO in order to visualize all cells present. Eight random fields on each of the four replicates were observed, and the numbers of visible cells in each of the 32 fields were recorded. Target cells labeled with CY3 were viewed under orange light (554 nm excitation, 568 nm emission), while YO-PRO stained cells were viewed under blue light (491 nm excitation, 509 nm emission). Samples were first exposed to the YO-PRO excitation conditions (to view both positive and negative cells), and then exposed to the CY3 conditions (to view positive cells only). Additionally, representative images from the sample filters were acquired with a cooled charge-coupled (CCD) Sensys 1400 camera and processed with Oncor Image software package version 2.02 on a Power Macintosh. The image was first captured under YO-PRO excitation conditions, and then a second image of the same field was captured under CY3 conditions.

CHAPTER 3

RESULTS AND DISCUSSION

In situ PCR/FISH and FISH techniques successfully detected AOB both functionally (DNA) and phylogenetically (rRNA) in natural bacterial communities. In order to insure that the probes utilized had no effect on the abundance of the total detected cells as an artifact of the techniques, a general linear model (GLM) using a one-way analysis of variance (ANOVA) was calculated using $\alpha = 0.05$. No statistically significant differences were determined when analyzing the total number of cells as a function of the probe utilized. Thus, the probes themselves did not contribute to an artifact of the technique. Additionally, these same statistical tests were administered to determine whether the functional probe (ß3b) led to significant results in AOB detection in comparison to the phylogenetic probes (NEU or Nso1225). These results are discussed in detail below.

June 2000 Sample Results

All five sample sites showed statistically more ammonia-oxidizers detected via *in situ* PCR/FISH versus FISH alone (Figure 14, Table 3). Increased detection via PCR/FISH ranged from 8.0% of the total cell count at Dean Creek to 51.0% at Marsh Landing. The mean recovery efficiencies of bacterial communities were 94.6% with FISH and 99.3% with *in situ* PCR/FISH, respectively, as calculated with the YO-PRO only stained filters (Table 4). This suggests that only a negligible proportion of the cells

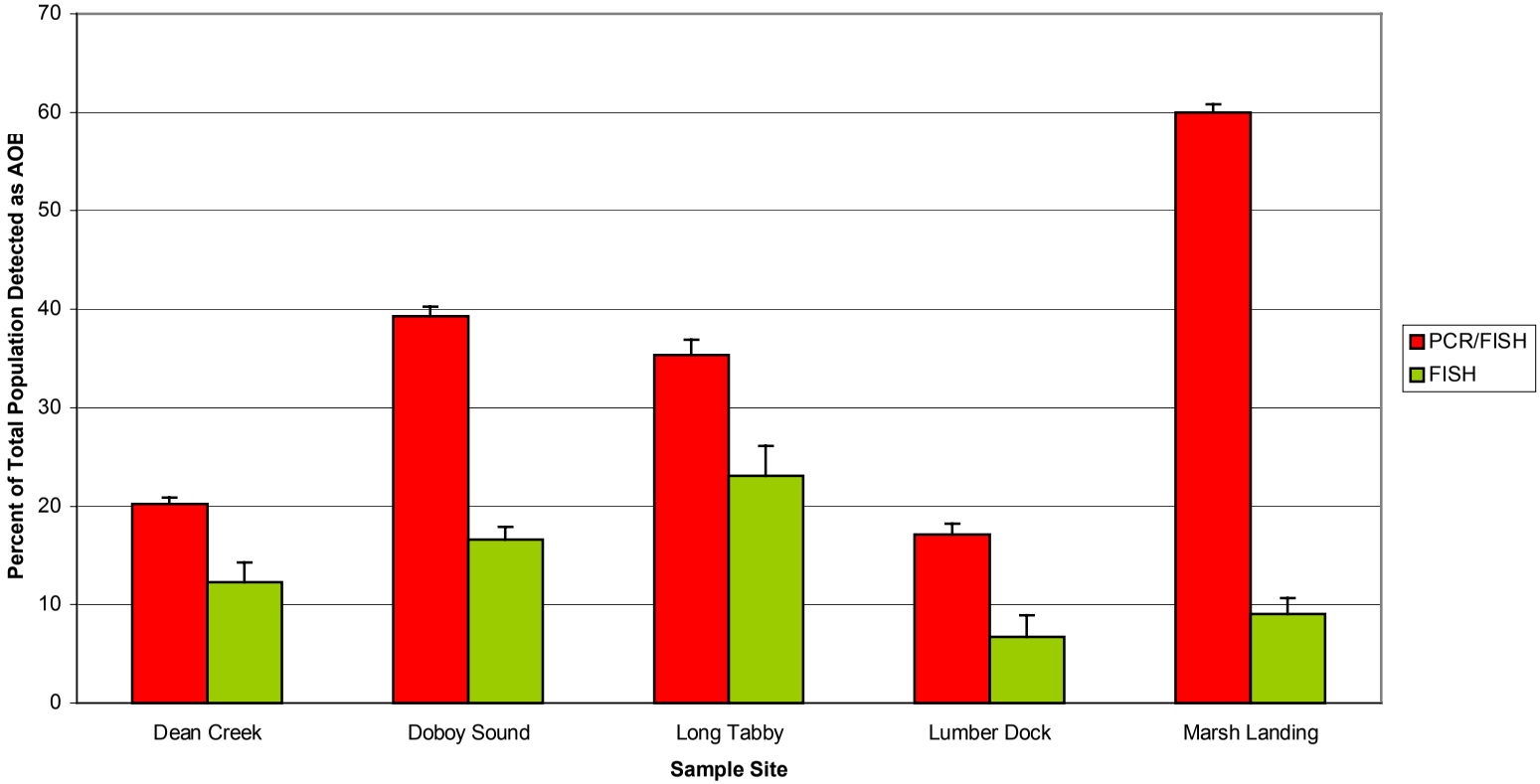

Figure 14. Percent of sample detected as AOB by each technique utilized for 2000 sampling sites. The first column of each site represents *in situ* PCR of the amoA region followed by *in situ* hybridization with the β3b probe (PCR/FISH). The second column represents *in situ* hybridization of 16S rRNA with the NEU probe (FISH). These values have been corrected for autofluorescence, and are +/- standard deviation.

Table 3. Mean percentage of the total population detected as ammonia-oxidizing bacteria at each sample site via FISH or *in situ* PCR/FISH. These values have been corrected for autofluoresence.

Sample Site	Salinity (ppt)	Phylogenetic Probe	FISH	*in situ* PCR/FISH	Difference
Dean Creek	25	NEU	12.29	20.22	+ 7.93
Doboy Sound	28	NEU	16.59	39.27	+ 22.68
Long Tabby	34	NEU	23.10	35.38	+ 12.28
Lumber Dock	32	NEU	6.70	17.12	+ 10.42
Marsh Landing	31	NEU	9.02	59.99	+ 50.97
Bourbon Field	34	Nso1225	3.43	7.26	+ 3.83
Cabretta	33	Nso1225	15.82	15.65	- 0.17
Duck Pond	32	Nso1225	2.10	4.47	+ 2.37
Eulonia	26	Nso1225	2.19	21.57	+ 19.38
High Point	5	Nso1225	7.61	10.62	+ 3.01
Hunt Camp	27	Nso1225	1.16	3.67	+ 2.51
Hunt Camp Creek	27	Nso1225	4.08	4.74	+ 0.66
Lumber Dock	27	Nso1225	3.47	3.97	+ 0.50
Meridian	25	Nso1225	3.25	3.42	+ 0.17
UGAMI	28	Nso1225	4.56	3.99	- 0.57

Table 4. Average percentage of total bacteria showing autofluorescence, and percent recovery of total bacteria after *in situ* hybridization with NEU or Nso1225 probes, or *in situ* PCR followed by ß3b hybridization.

Sample Site	Phylogenetic Probe	Autofluorescence	FISH	*in situ* PCR/FISH
Dean Creek	NEU	7.18	90.54	100.52
Doboy Sound	NEU	1.76	102.63	106.11
Long Tabby	NEU	1.54	105.55	114.76
Lumber Dock	NEU	6.83	93.93	93.01
Marsh Landing	NEU	1.05	95.31	100.23
Bourbon Field	Nso1225	6.16	101.77	98.95
Cabretta	Nso1225	7.56	99.74	99.66
Duck Pond	Nso1225	1.01	84.83	96.03
Eulonia	Nso1225	5.51	104.45	106.76
High Point	Nso1225	9.62	101.89	98.73
Hunt Camp	Nso1225	6.43	98.84	101.29
Hunt Camp Creek	Nso1225	2.47	95.19	106.04
Lumber Dock	Nso1225	2.38	98.54	96.53
Meridian	Nso1225	2.59	99.79	96.01
UGAMI	Nso1225	2.35	105.5	104.18

were lost during the extensive manipulations involved in the techniques. Additionally, low levels of autofluorescent cells were seen in all YO-PRO samples, ranging from 1.1% at Marsh Landing to 7.2% at Dean Creek (Table 4). When corrected for these false positives, the Figure 14 illustrates that on average, AOB accounted for 17.1%-60.0% of the bacterial community by *in situ* PCR/FISH of the functional gene, and 6.7-23.1% of the community by FISH phylogenetic markers in the <1.0 μm size fractionation. Photomicrographs of each sample site can be seen in Figures 15-19.

Functional-based detection values of AOB at Marsh Landing are a cause for concern due to the extremely high value obtained (60.0% of the total population). This site is a marina, as well as part of the Intracoastal Waterway. It is probable that disturbances of the sediment and water by passing boats suspended AOB that normally would not be present in the surface of the water column. Also, samples were obtained during high tide, increasing the potential for terrestrial AOB to be dislodged into the water column and subsequently collected.

June 2001 Sample Results

While eight of the ten sites sampled in 2001 showed more AOB detected via functional means than phylogenetic, only four of these sites (Bourbon Field, Eulonia, Duck Pond and Hunt Camp) reflected a statistically significant increase (Figure 15, Table 3). The increased detection via *in situ* PCR/FISH ranged from 0.2% at Meridian to 9.4% at Eulonia (Figure 15, Table 3). As in the 2000 samples, low levels of autofluorescent cells were observed in all sites examined. These values ranged from 1.0% of the total cells detected via YO-PRO at Duck Pond, to 9.6% of the cells from High Point (Table 4).

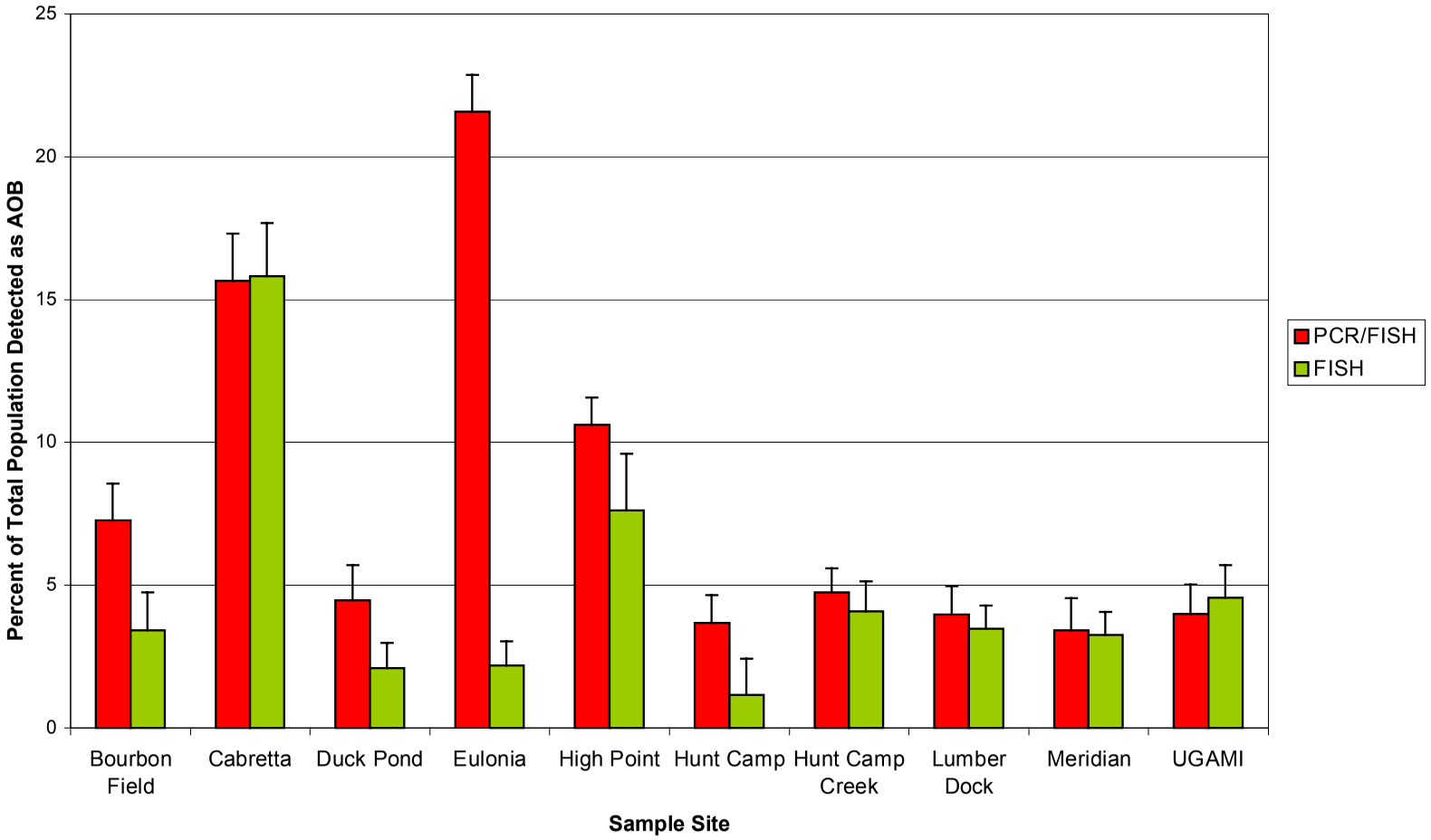

Figure 15. Percent of sample detected as AOB by each technique utilized for 2001 sampling sites. The first column of each site represents *in situ* PCR of the amoA region followed by *in situ* hybridization with the β3b probe (PCR/FISH). The second column represents *in situ* hybridization of 16S rRNA with the Nso1225 probe (FISH). These values have been corrected for autofluorescence, and are +/- standard deviations.

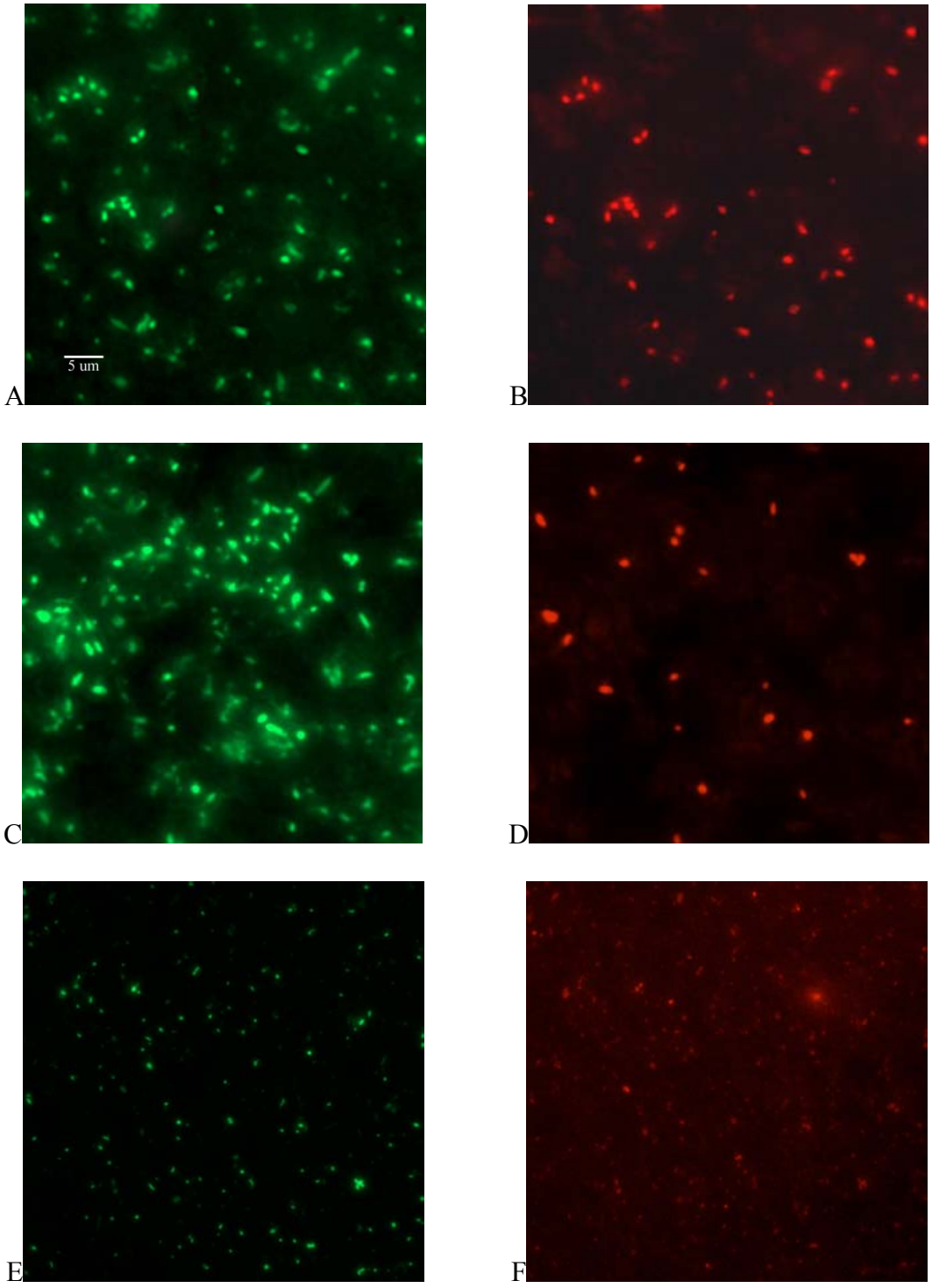

Figure 15. Dean Creek. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

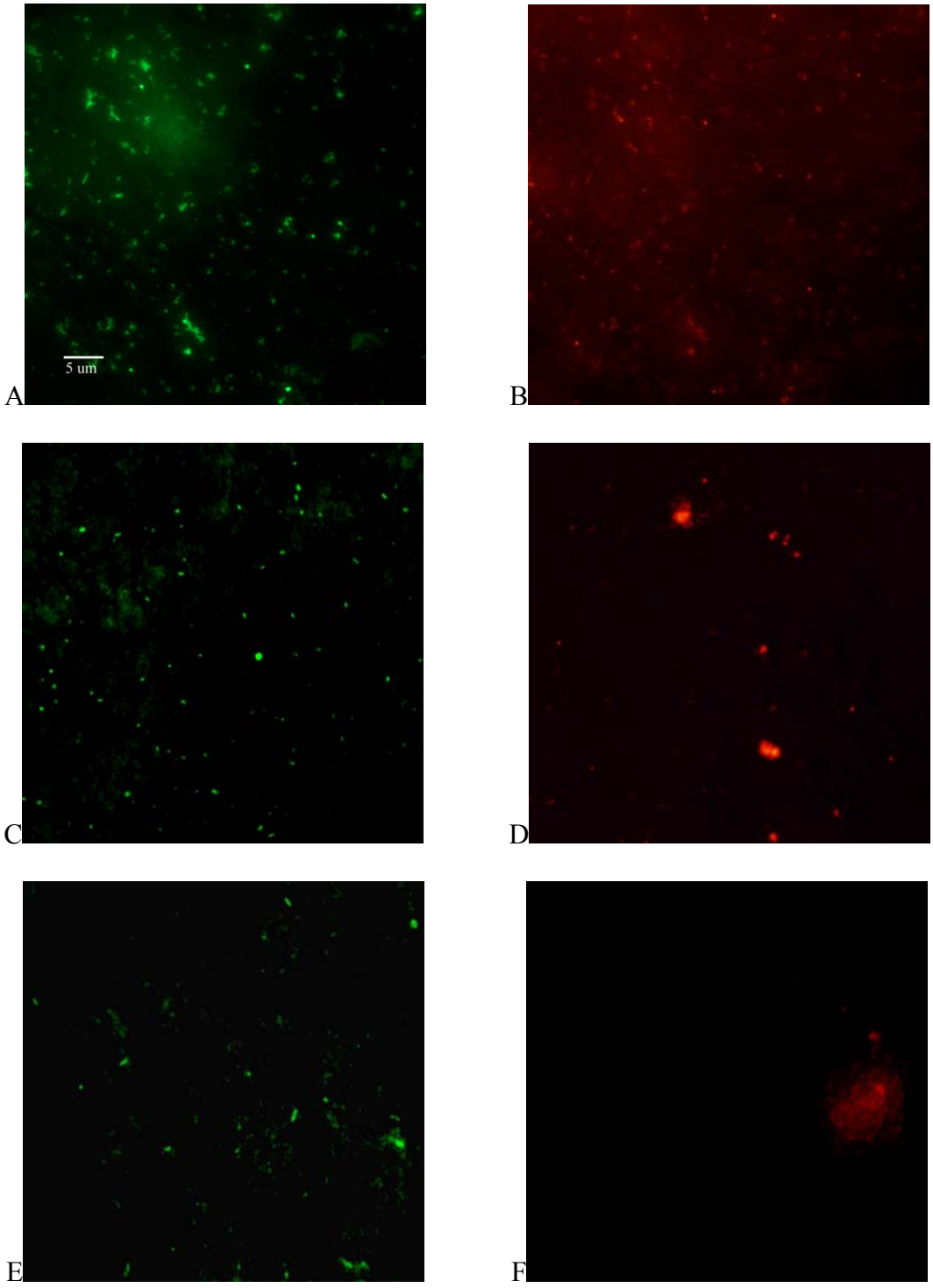

Figure 16. Doboy Sound. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

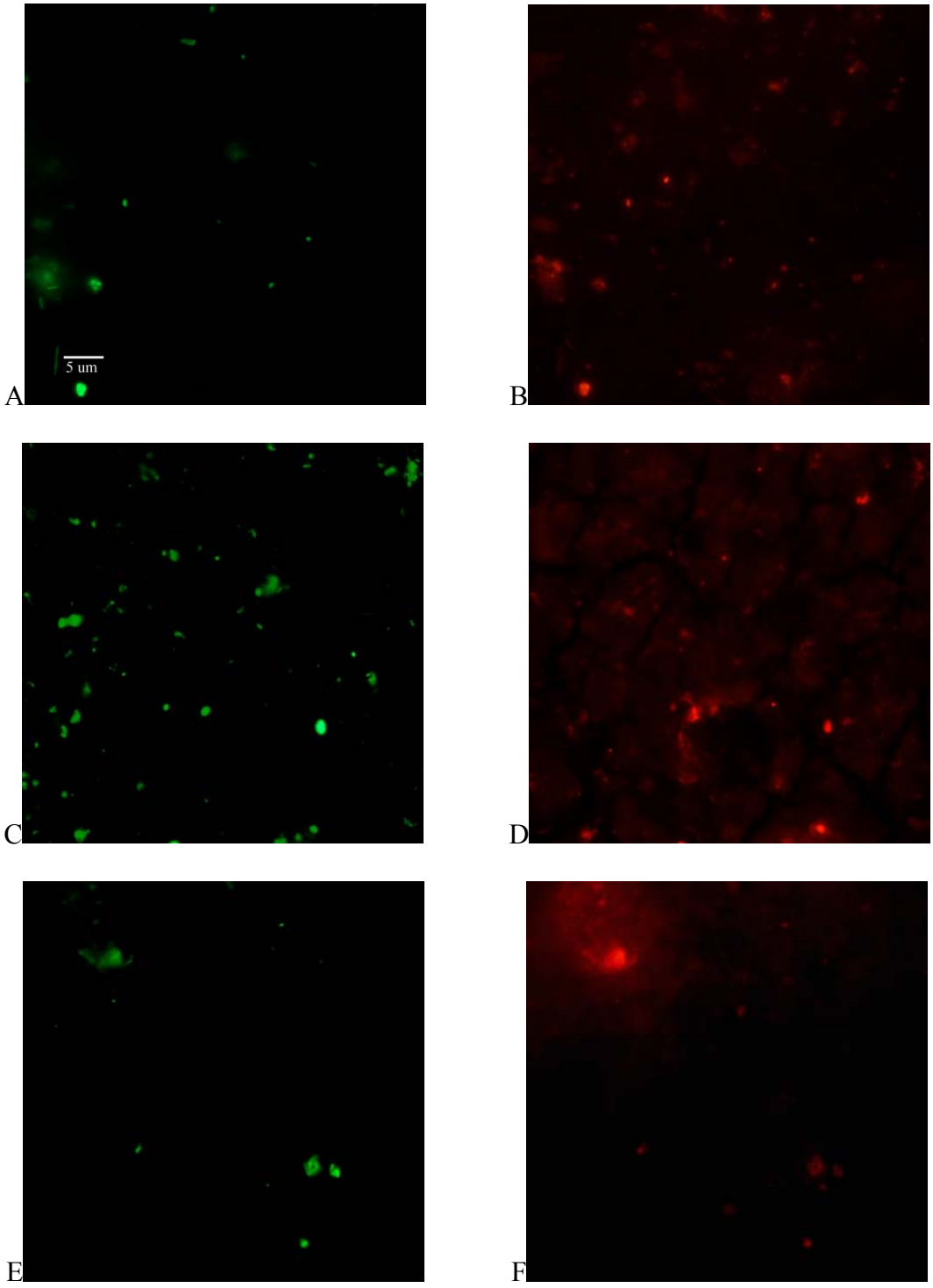

Figure 17. Long Tabby. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

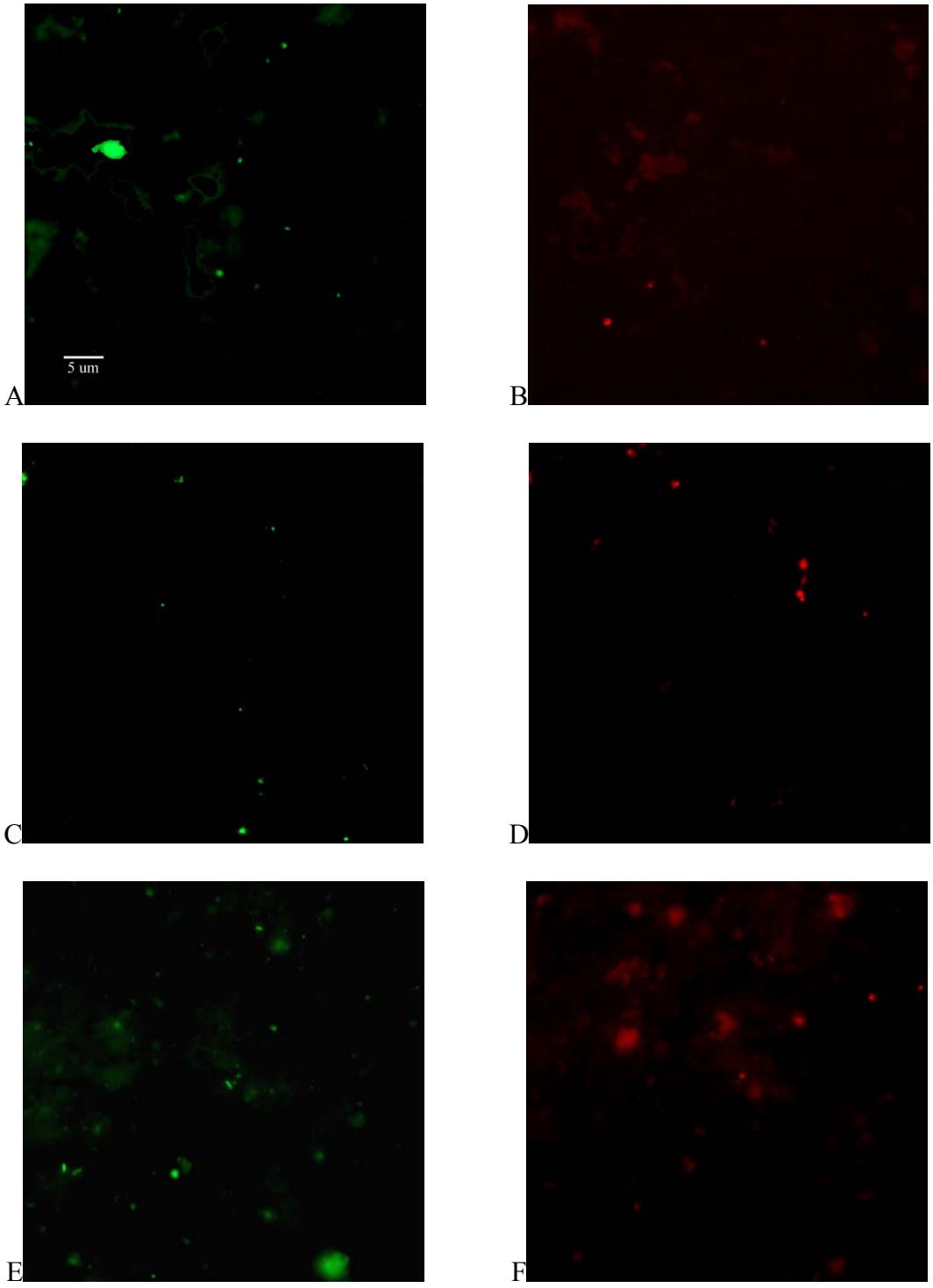

Figure 18. Lumber Dock 2000. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

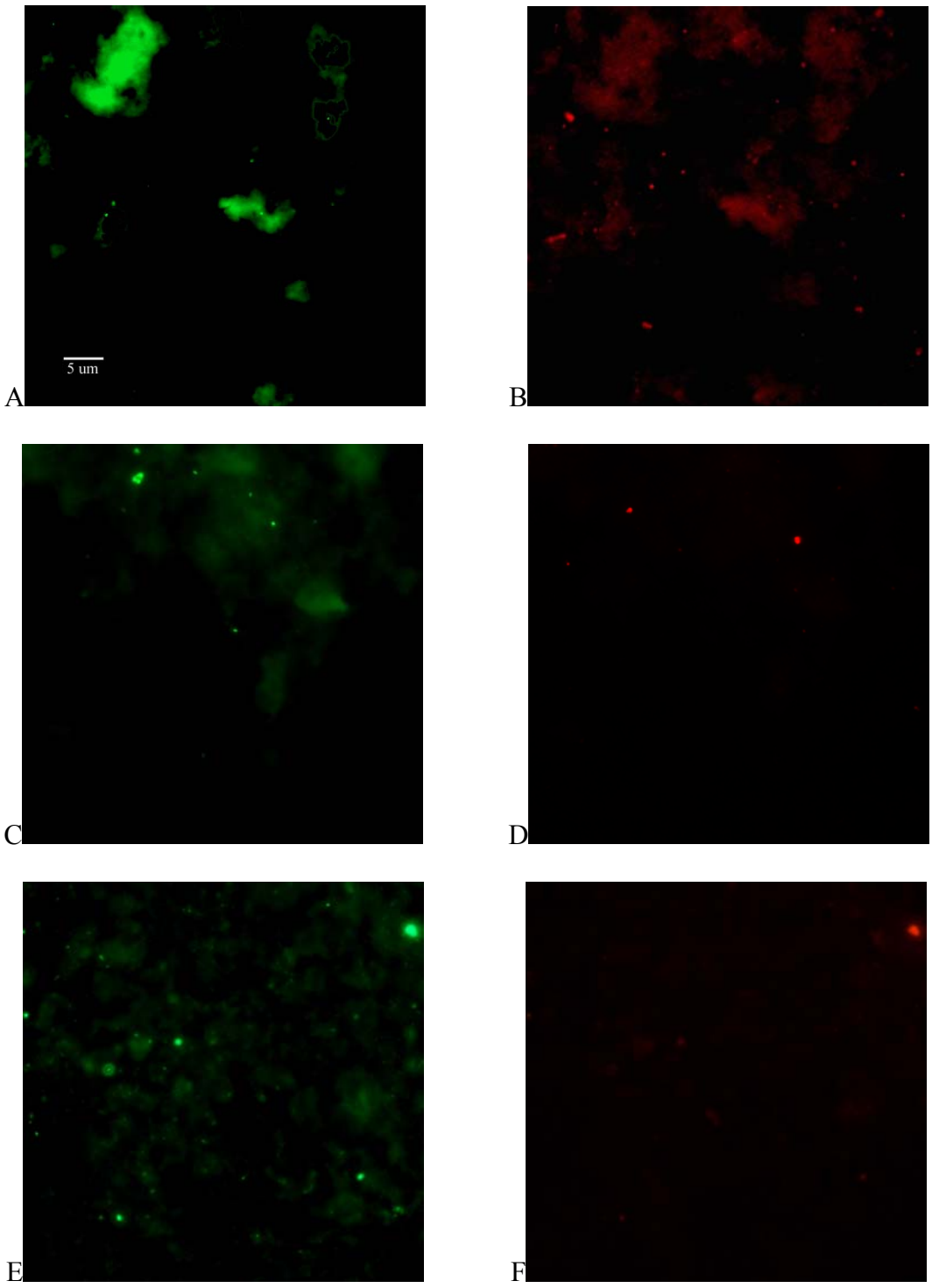

Figure 19. Marsh Landing. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

Mean recovery efficiencies of the bacterial communities were 99.1% with FISH and 100.4% with *in situ* PCR/FISH, respectively (Table 4). When corrected for autofluoresence, Figure 20 illustrates that on average, AOB accounted for 3.4%-21.6% of the bacterial community by *in situ* PCR/FISH of the functional gene, and 1.2%-15.8% of the community by FISH phylogenetic markers in the <1.0 μm size fractionation. Photomicrographs of each sample site are seen in Figures 21-30.

AOB functional detection from Eulonia (21.6% of the total population) is another concern, due to the high percentage of AOB detected in population. Large amounts of pollution were seen around the site, and a green algal mat covered the surrounding banks. This site is located directly off of a main roadway where heavy equipment was present due to building construction. Because this site was sampled after a brief rain shower, it is possible that flushing of the nearby soils into the sample site introduced additional AOB that were not originally present in the water.

As with Marsh Landing from June 2000, samples from Cabretta (15.7% of the total population) were also taken during high tide. This increase in the terrestrial-aquatic interface could have easily acted to artificially "spike" the collected water, dislodging commonly-terrestrial AOB into the water column that were subsequently detected in this study.

Implications of this Study

The high net detection rate of ammonia-oxidizing bacteria in this study could be caused by a number of issues. Greater sensitivity to *in situ* PCR/FISH is probable due to the increased copy numbers of the target sequence. While active cells commonly have

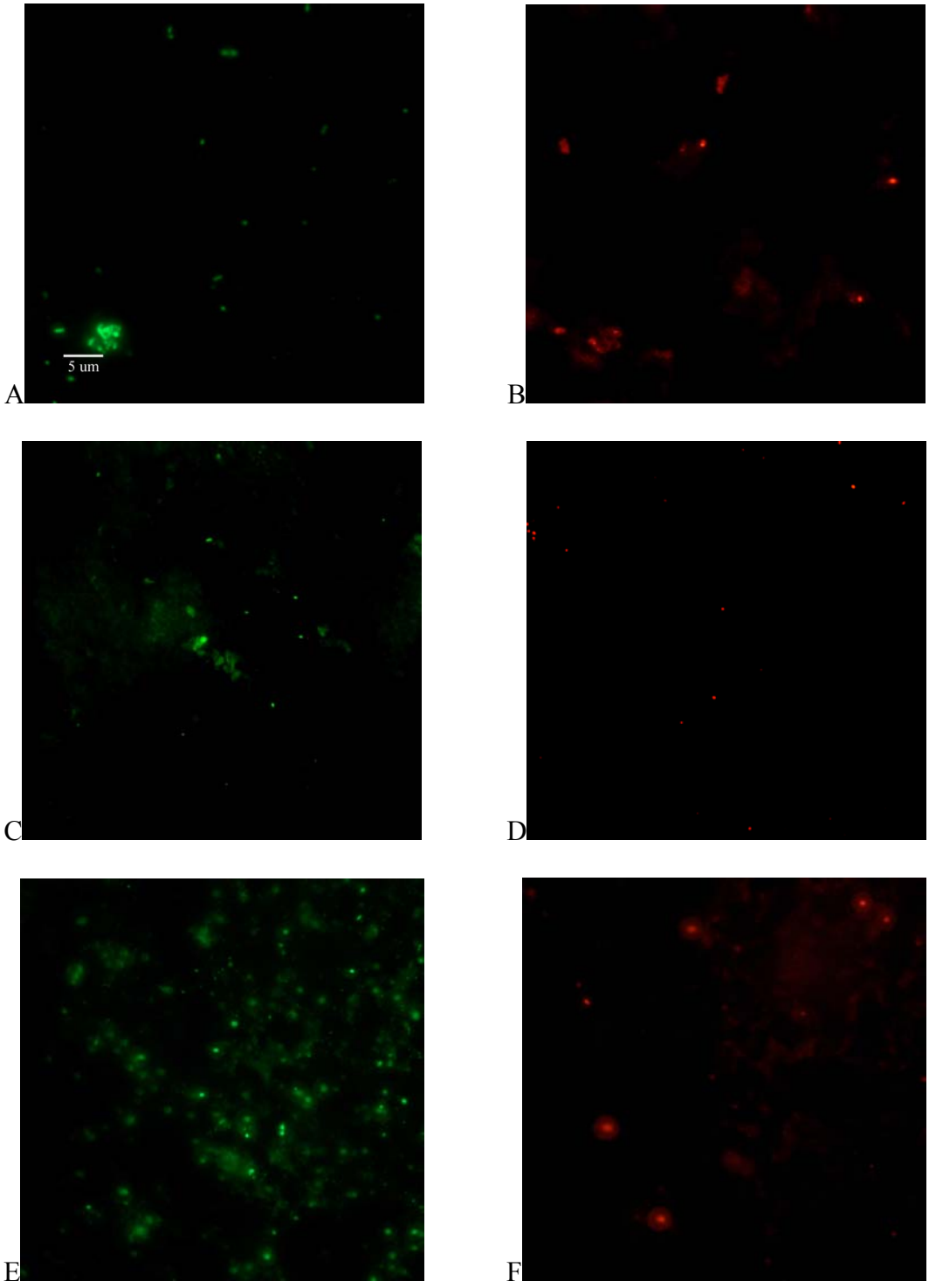

Figure 21. Bourbon Field. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

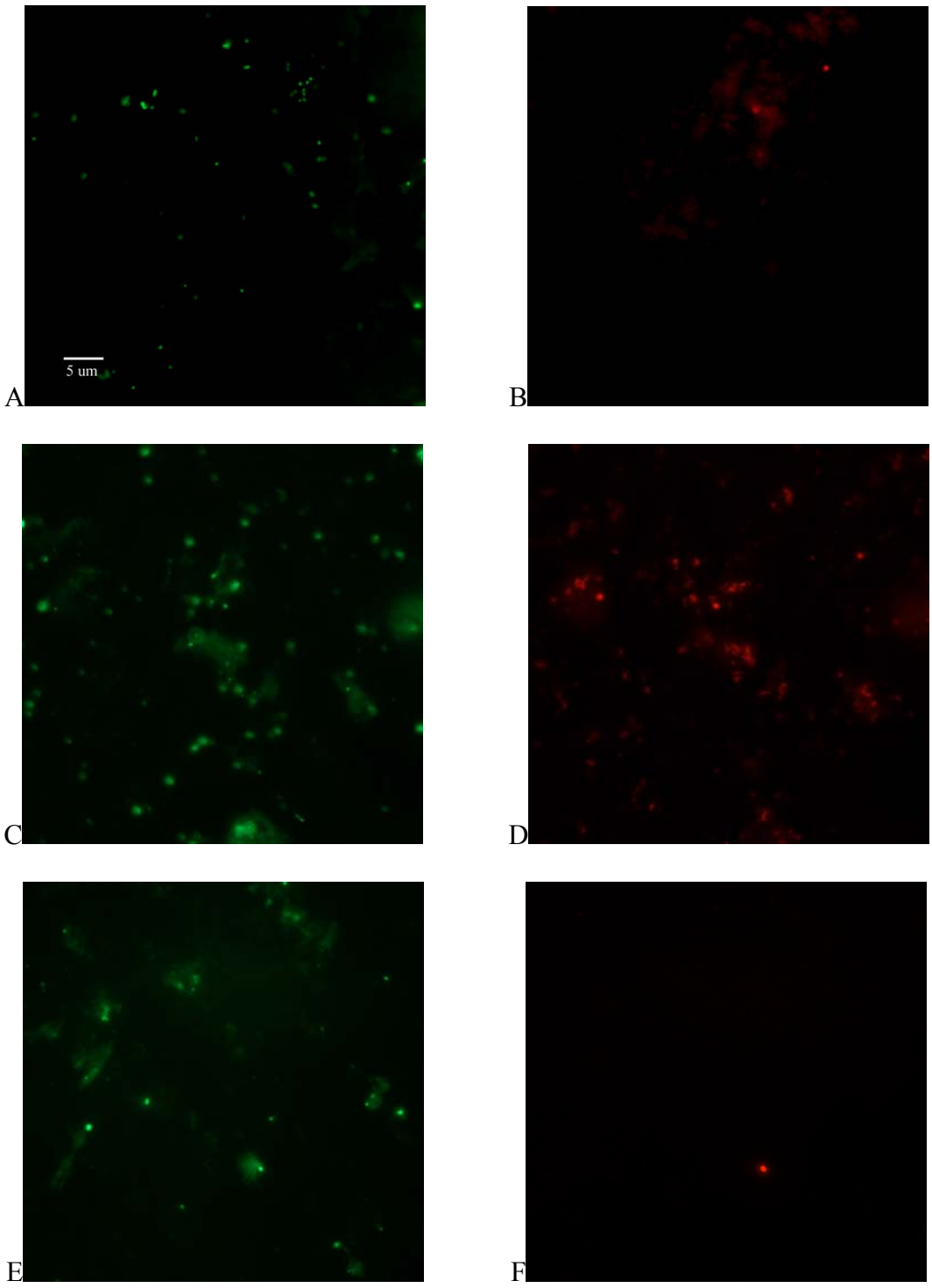

Figure 22. Cabretta. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

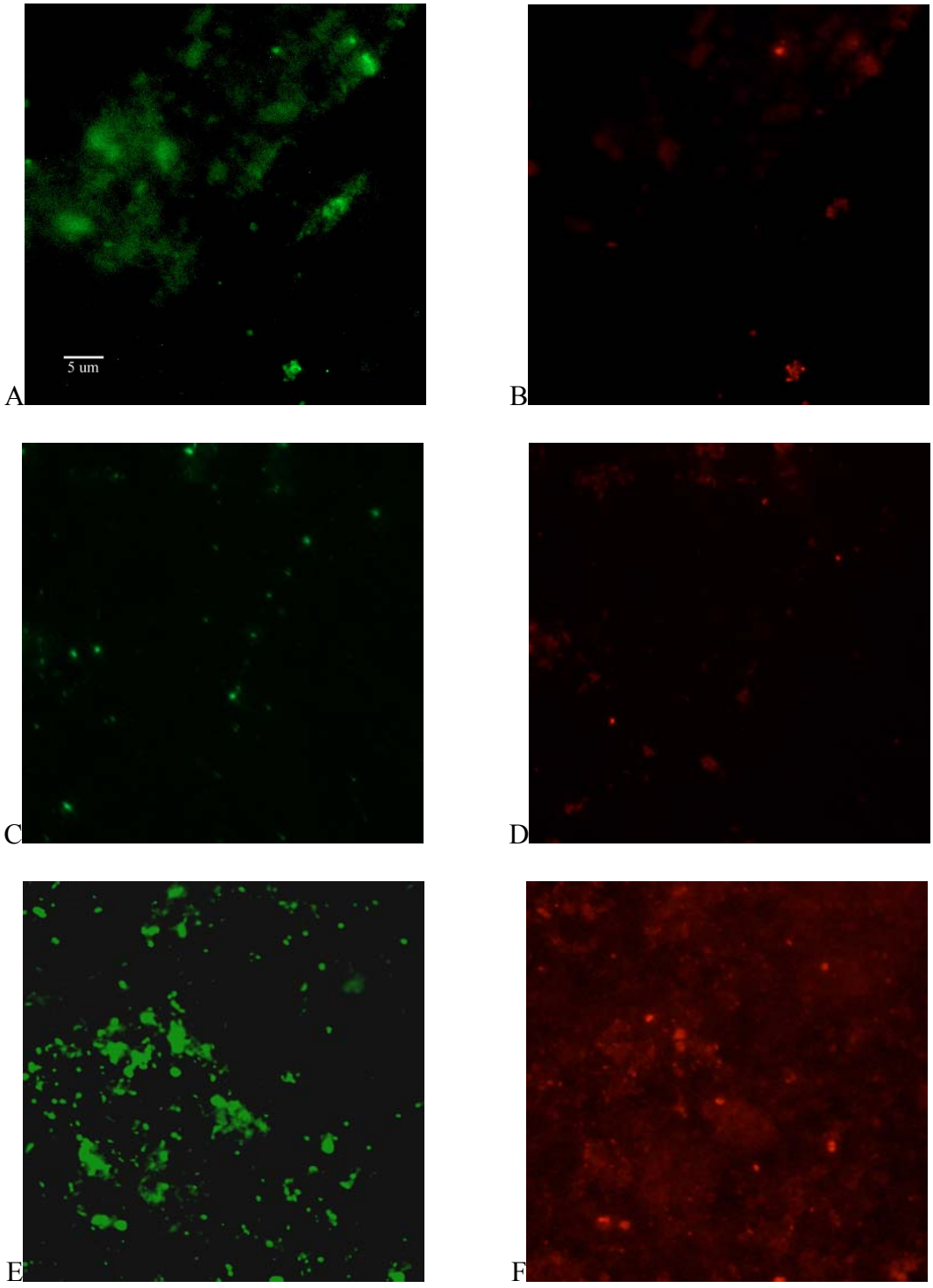

Figure 23. Duck Pond. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

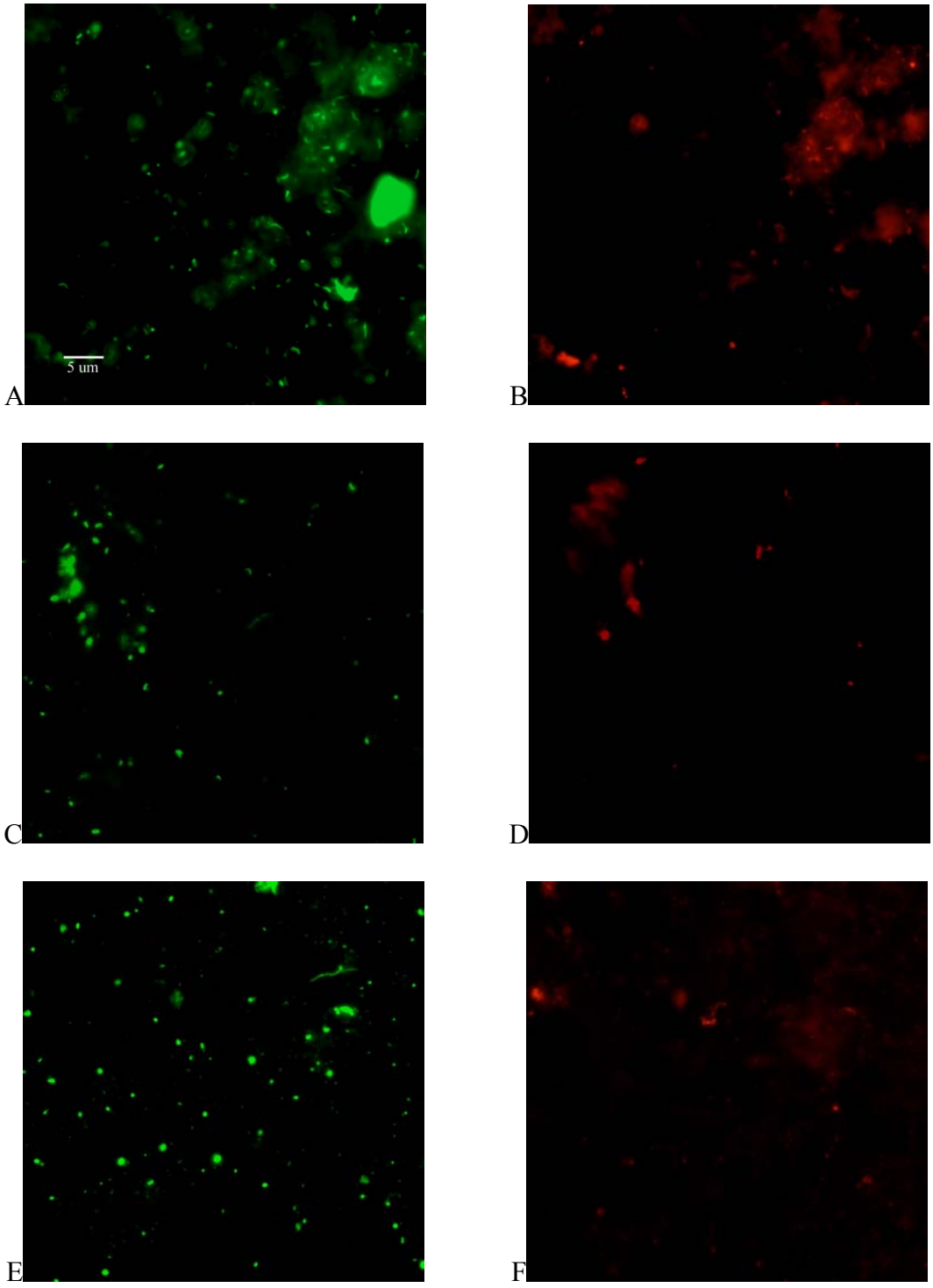

Figure 24. Eulonia. A A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

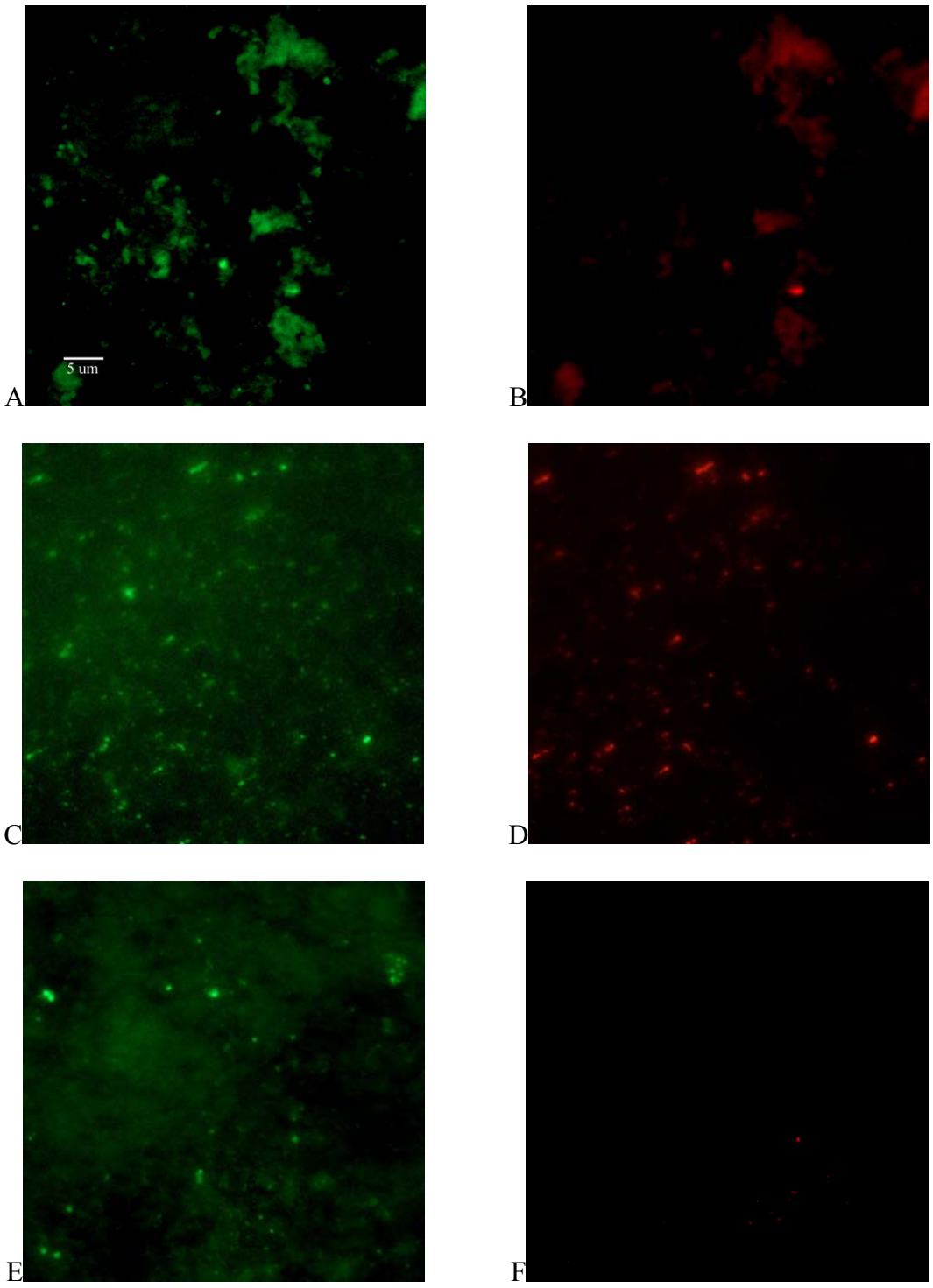

Figure 25. High Point. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

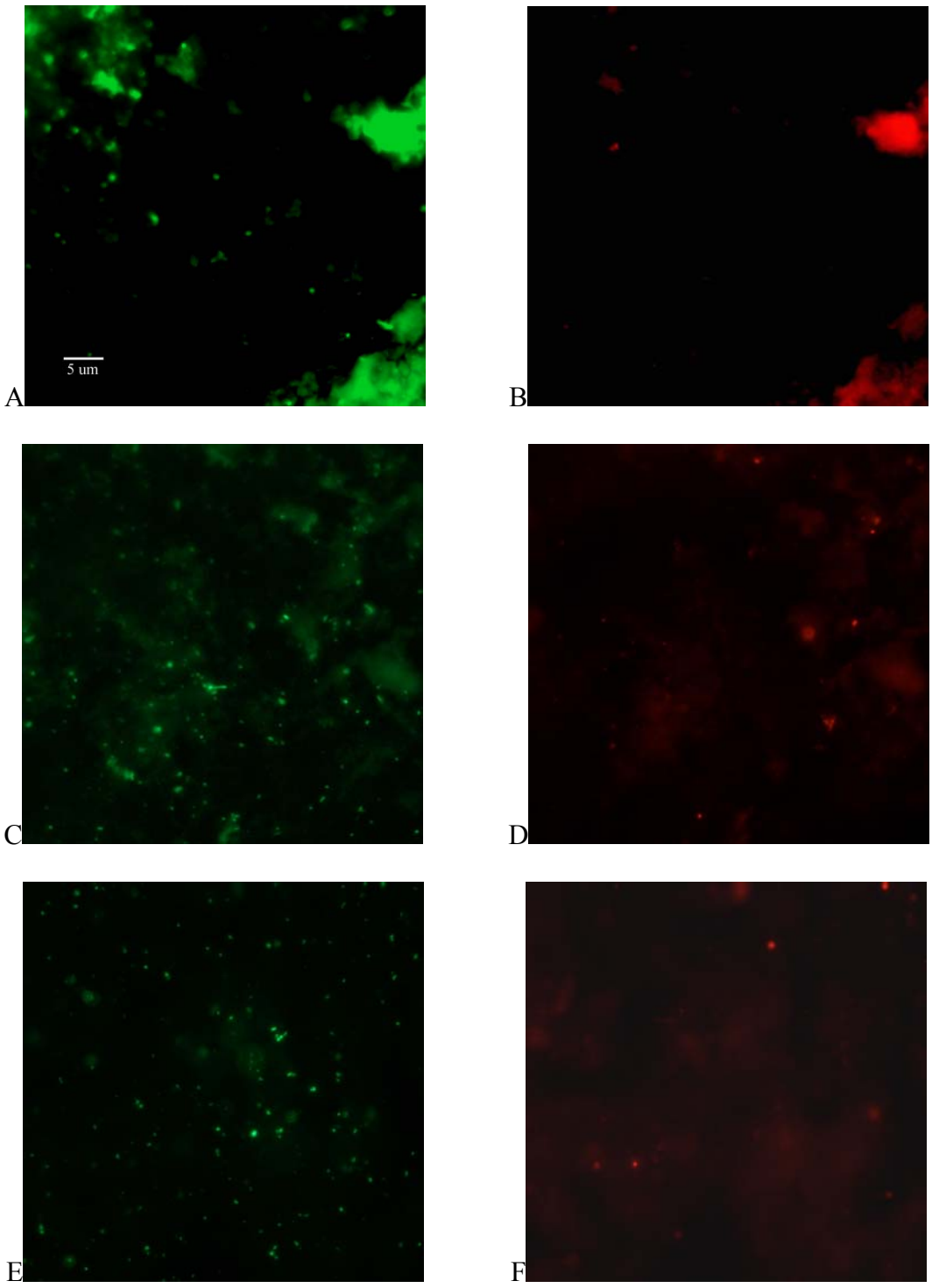

Figure 26. Hunt Camp. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

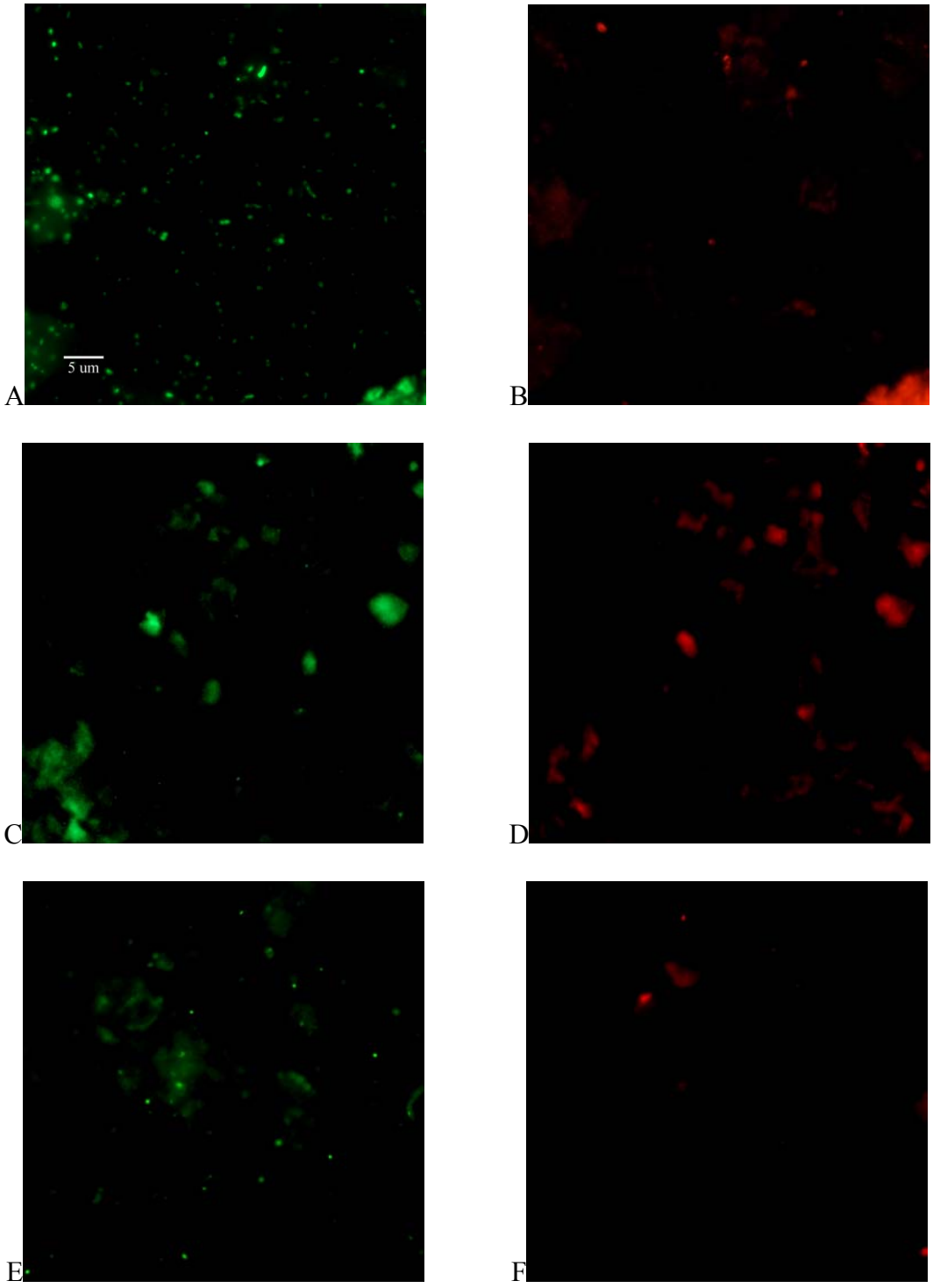

Figure 27. Hunt Camp Creek. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

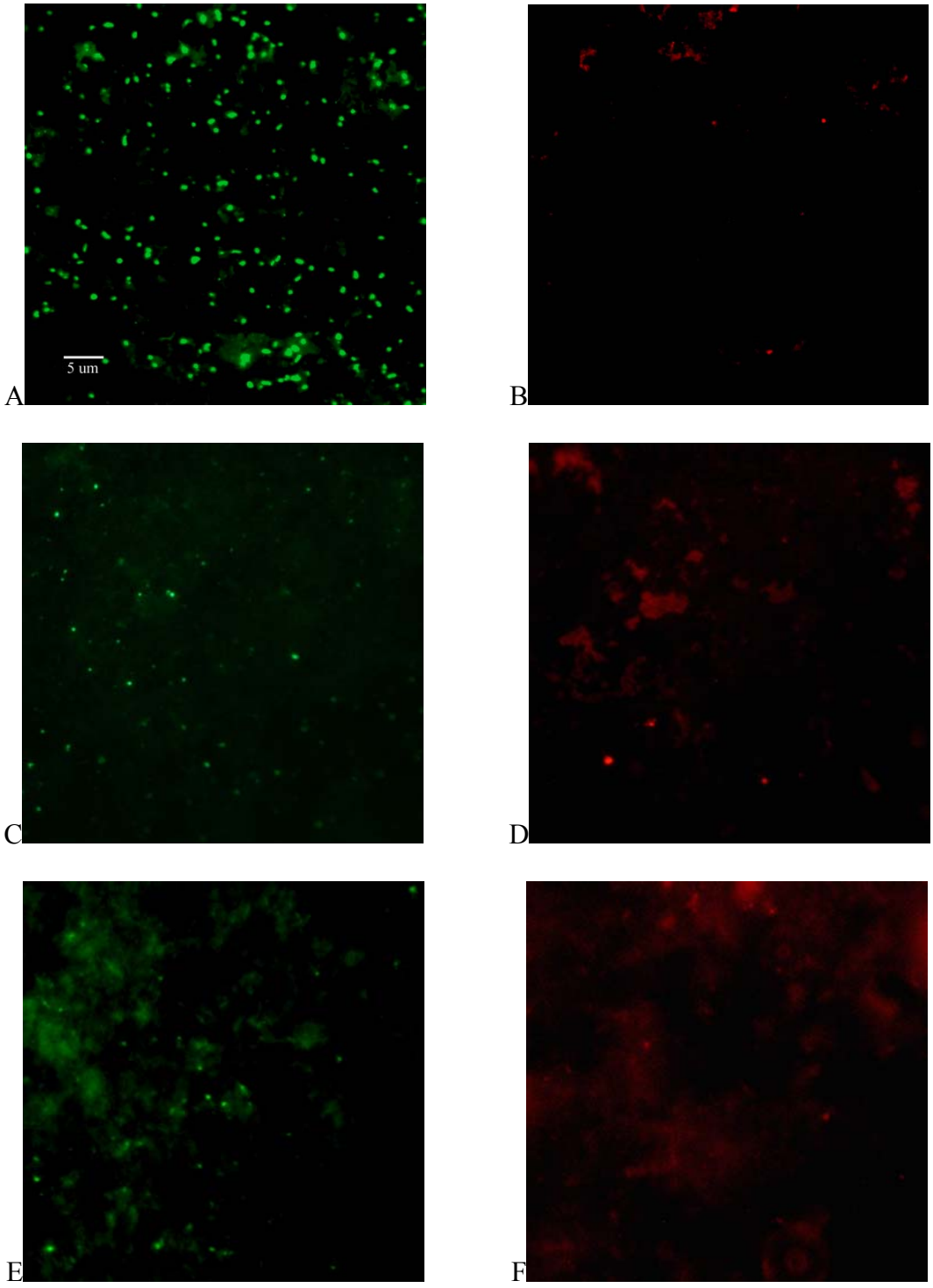

Figure 28. Lumber Dock 2001. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

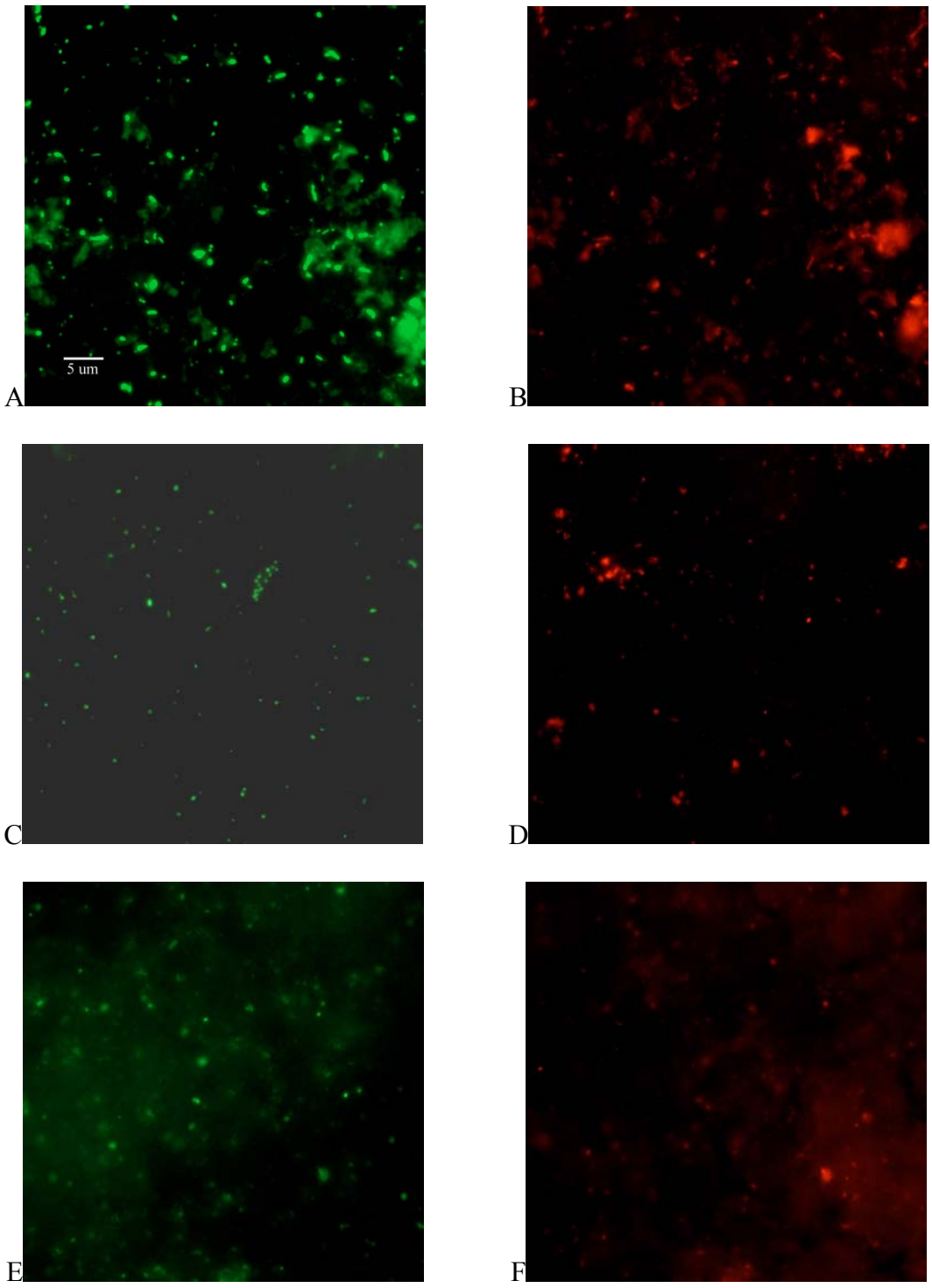

Figure 29. Meridian. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

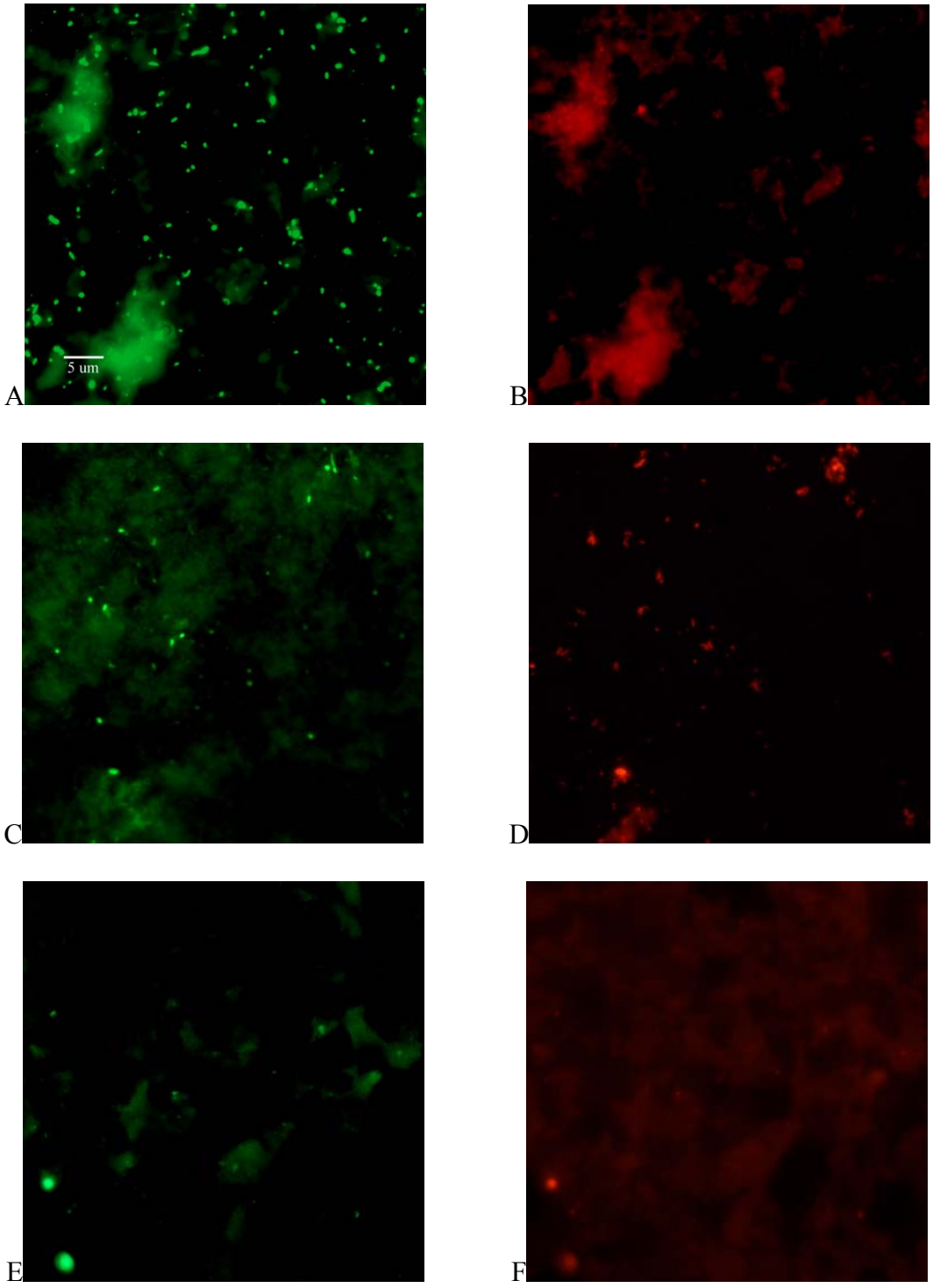

Figure 30. UGAMI. A) *in situ* PCR/FISH treatment showing total cells; B) *in situ* PCR/FISH treatment showing all CY3-visible cells; C) FISH treatment showing total cells; D) FISH treatment showing all CY3-visible cells; E) no treatment showing total cells; F) no treatment showing all cells autofluorescent under CY3 wavelength.

1000–2000 ribosomes (Fegatella *et. al* 1998), with one copy of 16S rRNA present on each ribsome to serve as a target, PCR increases the target sequence exponentially, potentially resulting in a much greater number of targets for hybridization. This would lead to higher detection with the functional gene approach utilizing PCR followed by FISH than with the phylogenetic approach using FISH only. Additionally, a higher rate of total bacteria was expected to be detected as AOB with probe NEU than with Nso1225. While NEU is based on 16S rRNA of only 2 genera of β-Subclass AOB, Nso1225 is based on the 16S rRNA of all known β-Subclass ammonia oxidizers. Thus, it is more inclusive than NEU for detecting AOB.

When investigating natural samples, it is impossible to remove all organisms and compounds that can bias results without losing some of the organism of interest. Though samples were size-fractionated, cyanobacteria, as well as the bacteria of interest, were retained in the collected sample fractions. Thus, in this study, it is possible that autofluoresence by phycoerythrin pigments of cyanobacteria contributed to some overestimation in both techniques. These pigments have an excitation spectrum of 490-565 nm and emission spectrum of 575-578 nm, which is very near the emission spectrum of 568 nm for the orange light used with CY3-probed bacteria (Schutte 2002). Fluorescence by phycoerythrins may have acted to falsely increase the detection rates of AOB functionally and phylogenetically, though correcting the detected values for autofluoresence minimized this effect.

Successful hybridization of previously undiscovered AOB could also contribute to these results. Because of the slow-growth of ammonia-oxidizers, it is possible that previous studies of AOB have overlooked additional genera or species when analyzing

the community structure and interactions of these organisms. Potentially inactive cells with a lower ribosome content than those that are actively growing may lead to underestimations of AOB in the environment when utilizing 16S rRNA techniques.

Primer and probe specificity issues are always a concern when utilizing these techniques in an attempt to elucidate natural communities. As additional bacterial sequences are deciphered and included in the databases, more class-, genus- and possibly species-specific primers and probes can be designed, leading to a more thorough investigation of the natural bacterial assemblages.

When investigating the ability of oligonucleotide probes to bind to 16S rRNA of *E. coli*, Fuchs *et. al* (1998) discovered that the probe intensities varied depending upon where they were bound to the ribosome. Because all bacterial 16S rRNA has identical structures, this information can be extrapolated for use with AOB. At the site on the ribosome where probe NEU initially binds (653), the fluorescence intensity is only 0-5% of the maximum intensity measured. Also, while probe Nso1225 (1225) binds to a site that exhibits an intensity of 41-60%, it attaches to an area that is folded back upon itself. It is probable that the 94ºC denaturing step acted to minimize secondary structures of the ribsosome. Therefore, both probes NEU and Nso1225 had more opportunity to bind to the 16S rRNA of AOB, resulting in an increased detection of these bacteria in comparison to other detection values attained with these probes.

Additionally, when probe β3b is compared to the *amoA* sequence of *N. oceani*, no alignment can be made. This is also true with the two phylogenetic probes NEU and Nso1225 and the 16S rRNA sequence of this γ-AOB. However, all three of these probes

did successfully hybridize with the bacterium. Therefore, it is likely that the *N. oceani* culture was contaminated with a β–Subclass ammonia-oxidizer.

Climatic conditions may have also impacted the results of this study. Since May 1998, Georgia has been experiencing one of the worst droughts of the century (Drought in Georgia). The estuaries on Sapelo Island are controlled by tidal creek flow, and Stooksbury and Knox (2002) noted that record-lows of stream flow have been recorded for the Altamaha River and several smaller creeks that feed many of the estuaries sampled in this study.

For the 10 days previous to the sampling times in June 2000, only 0.35 inches of rain was recorded on the Georgia, and this rainfall all occurred on one day. However, a total of 0.67 inches of rain was measured on the coast for the 10 days previous to the 2001 sampling trip, and this rainfall occurred over 3 days (Georgia Automated Environmental Monitoring Network). Because of the lack of tidal flushing in June 2000, eutrophication likely occurred. This subsequent break down of organic matter could conceivably lead to an increase in substrate for AOB, reflected in the significant increase in AOB detected functionally at all sites during the 2000 sampling season. Conversely, if these samples were obtained during the beginning of a eutrophic event, it is possible that the growth of algae yielded increased oxygen levels. Because AOB are obligately aerobic, increased detection is possible. Prinčič *et. al* (1998) observed that ammonia-oxidizing bacteria were more prevalent in wastewater reactors that were aerated with a 21% oxygen mixture, in comparison to reactors with lower amounts of oxygen present. Additionally, the 2001 sites that showed a significant increase in AOB detected functionally are internal to Sapelo Island. Though an increase in rainfall was recorded,

which would lead to greater flushing in the estuaries, eutrophication was likely possible at the island's internal sampling sites, providing either increased oxygen levels (at the beginning of the event) or increased substrate (at the end of the event) for AOB growth.

Finally, it should be noted that the results based on functional detections are simply a survey of bacteria that are capable of ammonia-oxidation, though they might not be actively oxidizing it. While this is not a likely occurrence, it must be considered.

Future Directions

The obvious next step in this project is to culture and/or clone the environmental samples obtained. Once this is accomplished, sequences of the bacteria can be acquired. While most bacterial investigations have shown that functionality is not related to phylogeny, the opposite seems to be true for ammonia-oxidizing bacteria. Therefore, it is imperative that sequences of these environmental samples are obtained in order to discover whether or not the AOB detected via functional methods are phylogenetically related to known ammonia-oxidizing bacteria.

It has long been known that ammonia-oxidizers have a tendency to adhere to surfaces (Prosser 1989). Detection of particle-bound AOB has been fairly difficult for the molecular ecologist to quantify. Due to the highly dense flocs formed, coupled with extraneous DNA from natural contaminants, these bacteria cannot be examined via *in situ* PCR/FISH and a compound microscope; instead, a scanning confocal laser microscope is commonly employed. While utilizing *in situ* PCR to investigate biofilms, Mobarry *et. al* (1996) noted that the nitrifying bacteria were not evenly distributed, and that the majority of ammonia-oxidizers formed dense clusters. These clusters were also observed by

Wagner *et. al* (1995) on trickle filters of wastewater treatment plants and Hoshino *et. al* (2001) on the surface of biofilms.

Studying the spatial and temporal distribution of AOB will lead to a better understanding of the importance of ammonia-oxidizing bacteria to the nitrogen cycle in natural populations. While little investigation has been given to temporal distribution, numerous studies of spatial distribution have been conducted. In the Artic Ocean, Bano and Hollibaugh (2000) detected greater numbers of AOB in samples from 55-133 meters, with fewer of the bacteria observed in waters from both 5 meters and 235 meters. Because a pycnocline exists between 30 to 200 meters in this portion of the ocean, it is possible that organic matter accumulates here, eventually increasing the amount of substrate available to the bacteria, thus contributing to a higher population.

To increase the sensitivity of phylogenetic detection of ammonia-oxidizing bacteria, more specific 16S rRNA probes must be developed. There are various probes available for β-Proteobacteria that have successfully been used to detect AOB. However, there are only two probes for the γ-Proteobacteria ammonia-oxidizers, NOC1 and NOC2 (Voytek 1996). This group is comprised of only two species of AOB, making it much more difficult to design a probe complimentary to all AOB because of the limited scope of γ-AOB sequences.

Finally, the combination of functional and phylogenetic detection methods, coupled with biogeochemical analyses such as ammonia, nitrate and nitrate concentrations, will be an asset to molecular ecologists attempting to unravel the closely tied interactions between ammonia-oxidizing bacteria in the marine environment.

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APPENDICES

Appendix A. Alignment of the *amoA* genes from 8 β-Subclass AOB. *Nitrosomonas eutropha* (U51630 and U72670), *Nitrosomonas europaea* (L08050), *Nitrosolobus multiformus* (U91603 and U15733), *Nitrosovibrio tenius* (U76552), *Nitrosospira* sp. (U38250), and *Nitrosospira* briensis (U76553). PCR primers 1F and amoA-2R are outlined in black, and probe β3b is shaded in gray.

```
PileUp of: @Amoa.Fil

Symbol comparison table:      GenRunData:Pileupdna.Cmp      CampCheck: 6876
                              GapWeight: 5.000
                              GapLengthWeight: 0.300
 Amoa.Msf         MSF:831   Type: N   August 30, 1997       14:40 Check: 7954

 Name:   U51630        Len: 831       Check:   765   Weight: 1.00
 Name:   U72670        Len: 831       Check:  1777   Weight: 1.00
 Name:   L08050        Len: 831       Check:  6462   Weight: 1.00
 Name:   U91603        Len: 831       Check:  3280   Weight: 1.00
 Name:   U15733        Len: 831       Check:  3496   Weight: 1.00
 Name:   U76552        Len: 831       Check:  1881   Weight: 1.00
 Name:   U38250        Len: 831       Check:  8299   Weight: 1.00
 Name:   U76553        Len: 831       Check:  3111   Weight: 1.00


           1                                                    50
    U51630     GTGAGTATAT TTAGAACAGA AGAGATCCTG AAAGCGGCCA AGATGCCGCC
    U72670     GTGAGTATAT TTAGAACAGA AGAGATCCTG AAAGCGGCCA AGATGCCGCC
    L08050     GTGAGTATAT TTAGAACGGA AGAAATCCTG AAAGCGGCCA AGATGCCGCC
    U91603     ......ATGA GCAGAACAGA TGAAATACTG AAGGCGGCGA AGATGCCGCC
    U15733     ......ATGA GCAGAACAGA TGAAATACTG AAGGCGGCGA AGATGCCGCC
    U76552     ......ATGA GCAGAACAGA TGAGATACTA AAGGCGGCGA AGATGCCGCC
    U38250     ......ATGA GCAGAACAGA CGAAATATTA AAGGCGGCCA AGATCCCGCC
    U76553     ......ATGA GCAGAACCGA TGAAATACTG AAGGCGGCCA AGATGCCGCC

           51                                                   100
    U51630     GGAAGCGGTC CATATGTCAC GCCTGATTGA TGCGGTTTAT TTTCCGATTC
    U72670     GGAAGCGGTC CATATGTCAC GCCTGATTGA TGCGGTTTAT TTTCCGATTC
    L08050     GGAAGCGGTT CATATGTCAC GTCTGATTGA TGCAGTTTAT TTTCCAATTC
    U91603     CGAAGCGGTA AAGATGTCCA GGATGATAGA CGTGATTTAC TTCCCGATTC
    U15733     CGAAGCGGTA AAGATGTCCA GGATGATAGA CGTGATTTAC TTCCCGATTC
    U76552     GGAAGCAGTA AAGATGTCCA GGATGATAGA TGCGGTGTAT TTCCCGATTC
    U38250     GGAATCGGTA AAGATGTCCA GGATGATAGA CGCGATCTAT TTTCCGATTT
    U76553     GGAAGCAGTA AAGATGTCCA GGATGATAGA CGCGGTATAT TTCCCGATTC

           101                                                  150
    U51630     TGGTTGTTCT GTTGGTAGGT ACCTACCATA TGCATTTCAT GTTGTTGGCA
    U72670     TGGTTGTTCT GTTGGTAGGT ACCTACCATA TGCATTTCAT GTTGTTGGCA
    L08050     TGATTATTTT GCTGGTGGGT ACCTACCACA TGCACTTTAT GCTGCTGGCA
    U91603     TATGCATCCT GCTGGTTGGA ACCTACCACA TGCACTTCAT GCTGCTGGCG
    U15733     TATGCATCCT GCTGGTTGGA ACCTACCACA TGCACTTCAT GCTGCTGGCG
    U76552     TATGCATACT GCTGGTTGGA ACCTACCACA TGCACTTCAT GCTGCTGGCG
    U38250     TATGCATCCT GCTGGTAGGC ACCTACCACA TGCACTTCAT GCTGCTGGCG
    U76553     TATGTATCCT CCTGGTTGGA ACCTACCACA TGCACTTCAT GCTGCTGGCA


           151                                                  200
    U51630     GGTGACTGGG ATTTCTGGAT GGACTGGAAA GATCGTCAAT GGTGGCCTGT
    U72670     GGTGACTGGG ATTTCTGGAT GGACTGGAAA GATCGTCAAT GGTGGCCTGT
    L08050     GGTGACTGGG ATTTCTGGAT GGACTGGAAA GATCGTCAAT GGTGGCCGGT
    U91603     GGTGACTGGG ACTTCTGGCT TGACTGGAAG GACCGGCAAT GGTGGCCTGT
    U15733     GGTGACTGGG ACTTCTGGCT TGACTGGAAG GACCGGCAAT GGTGGCCTGT
    U76552     GGAGACTGGG ACTTCTGGCT GGACTGGAAA GACCGTCAAT GGTGGCCGGT
    U38250     AGCGACTGGG ACTTCTGGCT TGACTGGAAA GACCGCCAAT GGTGGCCGGT
    U76553     GGCGACTGGG ACTTCTGGCT GGACTGGAAA GACCGCCAAT GGTGGCCGGT
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            201                                                 250
  U51630    AGTAACACCT ATTGTGGGCA TTACCTATTG CTCGGCAATT ATGTATTACC
  U72670    AGTAACACCT ATTGTGGGCA TTACCTATTG CTCGGCAATT ATGTATTACC
  L08050    TGTAACGCCA ATCGTGGGGA TCACCTACTG TTCGGCAATC ATGTATTACT
  U91603    GGTAACCCCA ATTGTAGGGA TCACCTACTG TGCCGCGATC ATGTACTACC
  U15733    GGTAACCCCG ATTGTAGGGA TCACCTACTG TGCCGCGATC ATGTACTACC
  U76552    GGTAACCCCG ATTGTGGGGA TCACCTATTG TGCCGCCATC ATGTACTACC
  U38250    TGTCACCCCC ATTGTAGGCA TCACCTACTG TGCCACCATC ATGTACTACC
  U76553    AGTGACCCCC ATCGTGGGCA TCACCTATTG CGCCGCCATC ATGTACTACC

            251                                                 300
  U51630    TGTGGGTCAA CTACCGTCAA CCATTTGGTG CGACTCTGTG CGTAGTGTGT
  U72670    TGTGGGTCAA CTACCGTCAA CCATTTGGTG CGACTCTGTG CGTAGTGTGT
  L08050    TGTGGGTCAA CTACCGCCAA CCGTTTGGTG CAACGTTGTG TGTGGTGTGT
  U91603    TGTGGGTGAA CTACCGCCTG CCGTTTGGAG CCACACTGTG CATCGTGTGC
  U15733    TGTGGGTGAA CTACCGCCTG CCGTTTGGAG CCACACTGTG CATCGTGTGC
  U76552    TGTGGGTGAA CTACCGGCTG CCGTTTGGGG CCACACTGTG CATCGTTTGC
  U38250    TGTGGGTGAA CTACCGCCTG CCGTTTGGCG CGACGCTATG TATCGTCTGC
  U76553    TGTGGGTCAA CTACCGGCTG CCCTTCGGCG CCACCCTCTG CGTCGTCTGC

            301                                                 350
  U51630    TTGCTGATAG GTGAGTGGCT GACACGTTAC TGGGGTTTCT ACTGGTGGTC
  U72670    TTGCTGATAG GTGAGTGGCT GACACGTTAC TGGGGTTTCT ACTGGTGGTC
  L08050    CTGCTGATTG GTGAGTGGCT GACACGTTAC TGGGGATTCT ACTGGTGGTC
  U91603    CTTCTGGTGG GTGAATGGCT GACCCGTTTC TGGGGTTTCT ACTGGTGGTC
  U15733    CTTCTGGTGG GTGAATGGCT GACCCGTTTC TGGGGTTTCT ACTGGTGGTC
  U76552    CTGCTGGCGG GTGAATGGCT GACACGCTTC TGGGGTTTTT ACTGGTGGTC
  U38250    CTGCTGGTAG GCGAATGGCT GACCCGCTTC TGGGGTTTCT ACTGGTGGTC
  U76553    CTGCTGACCG GCGAATGGCT GACCCGCTAC TGGGGCTTCT ACTGGTGGTC

351                                                             400
  U51630    ACACTATCCA CTCAATTTTG TAACCCCAGG TATCATGCTC CCGGGTGCAT
  U72670    ACACTATCCA CTCAATTTTG TAACCCCAGG TATCATGCTC CCGGGTGCAT
  L08050    ACACTACCCC ATCAACTTCG TAACACCGGG CATTATGCTT CCGGGTGCGC
  U91603    GCACTACCCC ATGAACTTTG TATTCCCCTC CACCATGATT CCCGGCGCGC
  U15733    GCACTACCCC ATGAACTTTG TATTCCCCTC CACCATGATT CCCGGCGCGC
  U76552    GCACTACCCC ATGAGCTTCG TGTTTCCCTC CACCATGATA CCGGGTGCGC
  U38250    GCACTACCCG ATCAACTTCG TCCTGCCCTC CACCATGATT CCGGGCGCGC
  U76553    GCACTACCCC ATCAACTTCG TCTTCCCCTC CACCATGATA CCGGGCGCAC

            401                                                 450
  U51630    TGATGTTGGA TTTCACAATG TATCTGACAC GTAACTGGTT GGTGACTGCA
  U72670    TTATGTTGGA TTTCACAATG TATCTGACAC GTAACTGGTT GGTGACTGCA
  L08050    TGATGCTGGA CTTCACGCTG TATCTGACAC GCAACTGGCT GGTGACGGCT
  U91603    TGGTGATGGA CACCGTCCTG CTTCTGACGC GCAACTGGAT GATCACGGCA
  U15733    TGGTGATGGA CACCGTCCTG CTTCTGACGC GCAACTGGAT GATCACGGCA
  U76552    TGGTGATGGA CACCGTCATG CTGCTCACGC GCAACTGGAT GATCACCGCC
  U38250    TCATCATGGA CACCGTCATG CTGCTCACGC GCAACTGGAT GATCACGGCC
  U76553    TGGTCATGGA CACCGTCATG CTGCTCACCC GCAACTGGAT GATCACAGCC

451                                                             500
  U51630    TTGGTTGGAG GTGGATTCTT TGGCCTGATG TTTTACCCGG GTAACTGGCC
  U72670    TTGGTTGGAG GTGGATTCTT TGGCCTGATG TTTTACCCGG GTAACTGGCC
  L08050    CTGGTTGGAG GTGGATTCTT CGGTCTGCTG TTCTATCCGG GTAACTGGCC
  U91603    CTGGTTGGCG GCGGCGCCTT TGGTTTGTTG TTCTATCCTG GCAACTGGAC
  U15733    CTGGTTGGCG GCGGCGCCTT TGGTTTGTTG TTCTATCCTG GCAACTGGAC
  U76552    CTGGTAGGCG GGGGCGCCTT CGGGTTATTG TTCTACCCTG GCAACTGGCC
  U38250    CTGGTAGGCG GTGGCGCATT TGGCCTGCTG TTTTATCCGG GCAACTGGCC
  U76553    CTGGTTGGCG GAGGCGCATT CGGACTCCTG TTCTACCCGG GTAACTGGCC
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          501                                                       550
U51630    AATCTTTGGC CCGACCCATC TGCCAATCGT TGTAGAAGGA ACACTGTTGT
U72670    AATCTTTGGC CCGACCCATC TGCCAATCGT TGTAGAAGGA ACACTGTTGT
L08050    GATTTTTGGA CCAACCCATT TGCCAATCGT TGTAGAAGGC ACATTGCTGT
U91603    CATCTTCGGG CCGACCCACC TGCCGCTGGT GGCAGAAGGC GTGCTGCTCT
U15733    CATCTTCGGG CCGACCCACC TGCCGCTGGT GGCAGAAGGC GTGCTGCTCT
U76552    CATTTTTGGC CTGACCCACC TGCCGCTGGT CGTGGAAGGC GTACTGCTGT
U38250    GATATTCGGA CCGACGCACC TGCCGCTGGT GGCCGAAGGC GTTCTGCTGT
U76553    CATCTTTGGC CCGACCCACC TGCCGCTGGC AGCCGAAGGC GTATTGTTGT

          551                                                       600
U51630    CGATGGTTGA CTACATGGGT CATCTGTATG TTCGTACGGG TACACCTGAG
U72670    CGATGGCTGA CTACATGGGT CATCTGTATG TTCGTACGGG TACACCTGAG
L08050    CGATGGCTGA TTACATGGGA CATCTGTATG TTCGTACAGG TACACCCGAG
U91603    CGGTAGCCGA CTACACGGGC TTTCTGTATG TCCGTACCGG CACCCCTGAG
U15733    CGGTAGCCGA CTACACGGGC TTTCTGTATG TCCGTACCGG CACCCCTGAG
U76552    CGGTAGCCGA CTACACGGGC TTCCTGTATG TGCGTACCGG CACGCCTGAA
U38250    CGCTGGCTGA CTACACCGGC TTCCTGTATG TCCGCACGGG CACCCCGGAA
U76553    CGGTTGCTGA CTACACCGGC TTCCTGTACG TTCGCACCGG CACCCCCGAG

          601                                                       650
U51630    TATGTTCGTC ATATTGAACA AGATTCATTA CCTACCTTTG GTGGTCACAC
U72670    TATGTTCGTC ATATTGAACA AGGTTCATTA CGTACCTTTG GTGGTCACAC
L08050    TATGTTCGTC ATATTGAGCA AGGTTCACTG CGTACCTTTG GTGGTCATAC
U91603    TACGTGCGAC TGATCGAACA AGGGTCACTG CGCACCTTTG GCGGTCACAC
U15733    TACGTGCGAC TGATCGAACA AGGGTCACTG CGCACCTTTG GCGGTCACAC
U76552    TACGTCCGCA ATATCGAACA AGGCTCGCTG CGCACGTTTG GAGGCCACAC
U38250    TACGTACGGC TGATCGAACA AGGCTCCCTG CGCACCTTTG GTGGTCACAC
U76553    TACGTACCCA ACATCGAACA AGGCTCACTC AGAACCTTTG GCGGGCACAC

          651                                                       700
U51630    CACAGTTATT GCAGCATTCT TCGCTGCGTT TGTATCCATG CTGATGTTTG
U72670    CACAGTTATT GCAGCATTCT TCGCTGCGTT TGTATCCATG CTGATGTTTG
L08050    CACAGTTATT GCAGCATTCT TCTCTGCGTT CGTATCAATG TTGATGTTCA
U91603    CACCGTTATC GCCTCCTTCT TCTCCGCGTT CGTCTCCATG CTCATGTTCA
U15733    CACCGTTATC GCCTCCTTCT TCTCCGCGTT CGTCTCCATG CTCATGTTCA
U76552    CACCGTCATT GCCGCATTCT TTGCCGCGTT CATCTCCATG CTCATGTTCA
U38250    CACGGTGATT GCGGCCTTCT TCTCCGCCTT CGTCTCCATG CTCATGTTCT
U76553    CACCGTCATC GCCTCATTCT TTGCCGCCTT CGTCTCCATG CTCATGTTCT

          701                                                       750
U51630    CAGTCTGGTG GTATCTTGGA AAAGTTTACT GCACAGCCTT CTTCTACGTT
U72670    CAGTCTGGTG GTATCTTGGA AAAGTTTACT GCACAGCCTT CTTCTACGTT
L08050    CCGTATGGTG GTATCTTGGA AAAGTTTACT GTACAGCCTT TTTCTACGTT
U91603    CCGTCTGGTG GTACTTTGGC AAGGTCTACT GCACCGCCTT CTACTATGTC
U15733    CCGTCTGGTG GTACTTTGGC AAGGTCTACT GCACCGCCTT CTACTATGTC
U76552    CCATCTGGTG GTACTTTGGC AAACTCTACT GCACCGCATT CTTCTACGTG
U38250    GCGTCTGGTG GTACTTTGGC AAACTCTACT GCACCGCGTT CTACTACGTC
U76553    GCCTCTGGTG GTACTTCGGC AAACTTTACT GCACCGCATT CTTCTACGTC

          751                                                       800
U51630    AAAGGTAAAA GAGGACGTAT CGTACAGCGC AATGATGTTA CGGCATTTGG
U72670    AAAGGTAAAA GAGGACGTAT CGTACAGCGC AATGATGTTT CGGCATTTGG
L08050    AAAGGTAAAA GAGGTCGTAT CGTACATCGC AATGATGTTA CCGCATTCGG
U91603    AAGGGCGCGC GCGGCCGTGT CAGCATGAAG AACGACGTGA CAGCATTTGG
U15733    AAGGGCGCGC GCGGCCGTGT CAGCATGAAG AACGACGTGA CAGCATTTGG
U76552    AAAGGCCATC GCGGCCGCGT CACCATGAAG AACGACGTTA CCGCATTTGG
U38250    AAAGGCCCGC GTGGCCGGGT TACCATGAAG AACGACGTCA CCGCCTATGG
U76553    AAGGGAACCC GTGGCCGTGT CACCATGAAG AACGATGTCA CCGCATTTGG
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          801                              831
U51630    TGAAGAAGGT TTTCCAGAGG GGATCAAATA A
U72670    TGAAGAAGGT TTCCCAGAGG GGATCAAATA A
L08050    TGAAGAAGGC TTTCCAGAGG GGATCAAATA A
U91603    CGAAGAAGGC TTTGCCGAGG GGATCAAATA A
U15733    CGAAGAAGGC TTTGCCGAGG GGATCAAATA A
U76552    CGAAGAAGGC TTTCCAGAGG GGATCAAATA A
U38250    CGAAGAAGGG TTTCCGGAGG GGATCAAATA A
U76553    GGAAGAAGGC TTCCCGGAGG GGATCAAATA A
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Appendix B. Gross Bacterial Counts. Dean Creek.

	NEU				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	36	41	47	41	42	52	57	43	39	47	41	53
Cy3 #	9	7	6	10	13	16	11	12	2	5	3	4
Total #	42	44	36	50	39	47	44	53	45	54	53	41
Cy3 #	7	9	5	12	15	16	14	18	3	4	5	2
Total #	48	45	37	39	56	49	37	66	41	44	52	56
Cy3 #	10	7	9	7	15	14	12	15	3	4	3	3
Total #	37	51	43	46	41	38	49	47	43	47	42	57
Cy3 #	9	11	8	9	9	9	17	14	1	3	1	7
Total #	44	41	49	36	46	46	61	39	40	59	46	54
Cy3 #	11	8	10	5	11	11	19	11	2	6	3	4
Total #	43	36	51	53	51	61	54	59	51	54	38	55
Cy3 #	8	6	12	10	16	15	12	16	3	3	3	6
Total #	39	40	43	45	32	52	37	52	48	45	39	42
Cy3 #	7	6	9	9	9	15	9	11	3	2	4	3
Total #	49	38	55	39	47	64	33	46	55	47	46	58
Cy3 #	9	8	12	5	10	18	6	13	5	2	3	5

Appendix B (cont.). Gross Bacterial Counts. Doboy Sound.

	NEU				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	21	25	38	27	31	25	24	25	25	28	33	30
Cy3 #	4	4	6	5	11	8	13	15	1	1	1	0
Total #	18	35	24	20	28	24	32	31	32	24	21	29
Cy3 #	3	6	5	2	9	13	15	14	0	2	0	0
Total #	26	31	19	34	24	27	26	29	21	20	29	21
Cy3 #	5	5	5	6	8	11	12	12	1	0	0	0
Total #	29	21	26	28	37	25	34	20	29	31	25	18
Cy3 #	4	3	4	5	13	9	14	9	1	1	0	0
Total #	30	28	29	29	25	21	29	27	22	27	18	36
Cy3 #	5	4	5	3	11	8	18	13	0	0	0	1
Total #	23	26	26	23	23	29	21	30	18	23	22	29
Cy3 #	6	4	6	6	9	10	9	12	0	1	0	0
Total #	19	23	21	35	31	32	18	24	27	29	27	21
Cy3 #	4	5	5	9	9	11	8	8	1	1	1	1
Total #	25	20	16	28	22	26	22	29	19	16	26	26
Cy3 #	3	4	3	7	8	9	10	10	0	0	0	0

Appendix B (cont.). Gross Bacterial Counts. Long Tabby.

	NEU				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	74	67	63	76	72	65	76	67	72	52	63	73
Cy3 #	18	16	18	16	31	28	33	22	11	9	5	11
Total #	73	74	74	81	63	73	84	72	76	55	71	68
Cy3 #	16	9	16	20	26	26	41	20	12	10	11	7
Total #	56	68	56	50	68	79	69	61	65	48	52	63
Cy3 #	19	14	20	14	28	31	28	16	10	4	4	10
Total #	61	51	79	64	75	61	76	84	76	63	63	54
Cy3 #	20	21	20	13	32	22	29	23	14	9	13	9
Total #	52	76	55	72	81	62	63	79	67	71	58	61
Cy3 #	14	18	15	18	35	25	21	26	12	8	9	9
Total #	57	53	74	53	71	76	65	65	59	56	66	71
Cy3 #	12	16	18	16	24	29	24	26	9	6	10	14
Total #	74	61	64	65	79	81	71	73	61	51	53	57
Cy3 #	17	12	12	17	26	28	27	28	10	10	8	8
Total #	71	70	73	73	62	72	68	81	59	67	69	59
Cy3 #	15	11	18	21	21	24	23	24	12	11	13	10

Appendix B (cont.). Gross Bacterial Counts. Lumber Dock.

	NEU				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	68	77	53	91	78	87	68	74	82	94	85	94
Cy3 #	9	11	7	9	16	21	18	19	5	5	6	6
Total #	91	84	82	63	84	70	94	70	93	99	73	78
Cy3 #	12	10	10	10	19	16	26	16	6	6	4	5
Total #	84	61	74	84	67	78	83	68	76	83	93	71
Cy3 #	11	9	6	12	15	17	22	18	6	4	7	4
Total #	74	88	67	73	81	62	76	78	78	76	86	87
Cy3 #	6	12	11	10	20	16	19	19	4	5	5	6
Total #	61	96	79	68	71	91	71	93	98	85	97	81
Cy3 #	8	15	12	11	18	23	17	20	6	7	10	5
Total #	84	85	84	71	83	84	64	79	69	62	69	77
Cy3 #	10	12	11	8	19	12	15	16	5	5	4	6
Total #	63	76	69	79	71	75	69	64	76	68	74	68
Cy3 #	11	8	14	9	22	14	17	16	6	6	5	5
Total #	79	74	76	88	69	72	72	76	89	91	78	74
Cy3 #	14	9	11	13	19	16	17	22	7	6	5	6

Appendix B (cont.). Gross Bacterial Counts. Marsh Landing.

	NEU				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	24	27	34	23	26	32	23	32	29	38	18	26
Cy3 #	1	6	1	2	18	19	14	24	0	1	0	0
Total #	25	23	22	23	33	20	26	28	33	19	21	29
Cy3 #	3	4	2	1	21	12	16	16	1	0	0	0
Total #	17	28	35	38	27	24	31	23	18	23	39	34
Cy3 #	2	5	2	4	18	15	15	10	0	0	1	1
Total #	19	27	28	26	29	28	29	20	21	28	32	18
Cy3 #	0	3	1	1	18	18	16	12	0	0	0	0
Total #	15	32	23	27	30	31	33	28	26	34	23	36
Cy3 #	2	10	2	4	21	19	20	17	1	1	0	1
Total #	13	14	20	18	24	26	27	31	34	31	27	19
Cy3 #	0	4	1	0	16	19	11	14	0	0	0	0
Total #	19	37	33	32	23	22	19	27	15	25	31	21
Cy3 #	1	2	6	2	14	13	12	15	0	0	1	0
Total #	27	31	28	25	28	29	24	22	26	27	24	28
Cy3 #	1	3	3	3	18	16	16	19	0	1	0	0

Appendix B (cont.). Gross Bacterial Counts. Bourbon Field.

	Nso1225				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	148	126	138	134	126	132	128	122	130	126	138	136
Cy3 #	9	12	10	5	17	16	16	14	11	9	8	7
Total #	132	134	132	126	132	130	122	114	122	120	132	144
Cy3 #	10	16	15	11	22	17	13	11	8	7	7	9
Total #	120	122	136	130	128	124	126	126	124	128	130	128
Cy3 #	7	13	14	10	18	16	15	14	7	8	7	8
Total #	134	132	138	128	134	132	134	128	138	122	124	142
Cy3 #	12	15	16	13	20	18	17	14	10	7	5	7
Total #	136	138	144	134	126	130	124	138	114	146	134	126
Cy3 #	16	15	16	14	17	18	16	21	8	9	7	6
Total #	138	130	136	124	128	138	130	132	120	142	136	124
Cy3 #	14	13	15	11	18	22	18	18	9	9	8	6
Total #	128	134	140	136	132	134	136	128	110	134	142	134
Cy3 #	11	16	17	15	21	19	23	16	7	8	9	10
Total #	134	124	128	132	124	132	128	130	132	126	136	132
Cy3 #	13	12	9	12	15	18	19	17	10	7	10	9

Appendix B (cont.). Gross Bacterial Counts. Cabretta.

	Nso1225				3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	131	150	142	156	150	142	147	138	145	137	140	151
Cy3 #	39	36	31	39	33	30	35	29	9	8	9	12
Total #	150	154	156	148	147	149	140	144	152	146	144	137
Cy3 #	38	38	36	31	35	34	30	31	1	10	13	9
Total #	133	136	158	173	143	138	153	149	139	141	149	146
Cy3 #	37	31	35	40	32	37	37	35	10	9	12	13
Total #	128	148	136	142	140	151	148	154	143	148	153	142
Cy3 #	34	33	31	30	38	42	32	36	9	11	17	11
Total #	142	143	140	148	146	127	146	142	146	150	145	148
Cy3 #	36	30	35	36	35	28	31	30	12	16	12	11
Total #	129	145	144	154	135	148	141	147	138	143	141	147
Cy3 #	28	32	30	38	37	32	30	34	1	14	1	12
Total #	148	127	139	147	144	146	150	138	145	149	148	151
Cy3 #	36	26	27	35	30	32	37	31	13	14	16	10
Total #	142	146	145	153	139	153	148	146	141	144	151	145
Cy3 #	37	34	36	28	32	41	35	33	12	13	17	14

Appendix B (cont.). Gross Bacterial Counts. Duck Pond.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	205	240	185	180	210	240	260	280	215	230	310	275
Cy3 #	8	6	6	3	13	10	14	13	2	0	5	0
Total #	230	205	210	160	225	250	56	285	255	240	285	260
Cy3 #	7	9	7	3	11	13	15	17	4	2	4	1
Total #	235	195	215	195	255	245	245	255	235	225	250	270
Cy3 #	6	6	9	4	18	11	10	14	2	3	2	3
Total #	240	185	200	230	275	255	250	240	265	230	275	285
Cy3 #	7	7	8	6	20	12	11	11	3	2	3	3
Total #	255	170	195	200	185	240	265	260	245	220	245	275
Cy3 #	9	3	5	6	11	10	13	15	3	3	1	2
Total #	210	200	180	165	240	270	255	255	235	235	255	265
Cy3 #	8	7	6	3	15	14	13	16	3	4	3	4
Total #	315	240	190	195	215	280	230	245	230	220	240	250
Cy3 #	12	7	6	4	12	15	10	13	1	3	2	2
Total #	360	230	220	185	220	265	240	230	260	250	255	255
Cy3 #	14	8	7	5	14	17	11	11	3	2	4	2

Appendix B (cont.). Gross Bacterial Counts. Eulonia.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	114	112	98	104	114	106	108	106	108	104	96	102
Cy3 #	7	8	11	8	31	27	26	26	3	4	3	5
Total #	104	106	96	100	110	110	104	108	102	110	106	98
Cy3 #	8	7	9	7	29	31	28	26	5	7	4	3
Total #	110	116	104	98	106	112	110	102	110	112	104	110
Cy3 #	7	9	8	7	27	32	27	29	6	5	3	5
Total #	98	118	94	106	116	104	102	112	96	106	14	108
Cy3 #	5	10	6	11	38	25	26	32	5	6	6	6
Total #	112	124	106	102	118	114	110	108	110	98	112	106
Cy3 #	10	12	9	10	36	33	28	29	7	6	8	5
Total #	116	114	108	112	114	108	112	106	108	106	92	100
Cy3 #	9	8	9	9	34	31	30	27	7	9	5	3
Total #	108	110	102	106	110	106	116	110	112	110	106	112
Cy3 #	9	6	6	7	35	28	32	28	9	8	4	7
Total #	102	120	98	110	116	112	106	108	110	108	110	96
Cy3 #	7	10	7	8	33	30	28	27	10	4	7	6

Appendix B (cont.). Gross Bacterial Counts. High Point.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	78	85	74	69	76	73	75	72	71	75	73	76
Cy3 #	11	24	12	10	17	16	13	14	3	6	5	7
Total #	77	78	81	73	75	77	78	74	74	83	75	73
Cy3 #	15	20	15	13	15	19	17	15	7	9	6	5
Total #	79	83	89	74	69	74	73	76	79	77	79	75
Cy3 #	12	28	11	11	12	13	14	17	11	8	9	4
Total #	81	85	77	68	73	76	74	80	78	74	75	79
Cy3 #	14	23	14	9	15	17	15	19	9	5	7	8
Total #	76	84	79	79	77	75	71	77	82	78	79	72
Cy3 #	12	11	9	10	16	15	12	15	12	7	8	6
Total #	78	79	72	72	75	72	77	79	77	73	80	77
Cy3 #	7	10	17	14	14	13	13	18	8	6	11	7
Total #	70	75	75	76	73	79	76	75	78	75	74	76
Cy3 #	5	13	14	16	14	16	17	16	11	10	6	5
Total #	77	76	78	81	71	82	74	73	74	79	73	69
Cy3 #	10	11	10	16	12	18	15	14	7	12	5	4

Appendix B (cont.). Gross Bacterial Counts. Hunt Camp.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	200	270	235	225	255	225	230	240	230	235	240	220
Cy3 #	14	21	18	17	27	21	24	26	14	11	13	15
Total #	255	235	225	245	265	235	235	220	245	210	230	235
Cy3 #	19	19	17	16	26	23	26	23	18	16	12	18
Total #	170	215	260	235	240	215	220	210	255	235	215	225
Cy3 #	16	18	21	20	24	20	23	20	18	12	13	17
Total #	225	240	235	245	235	230	250	235	210	240	245	230
Cy3 #	17	22	18	19	22	22	26	23	14	17	16	14
Total #	205	210	195	210	245	210	240	225	220	230	235	240
Cy3 #	18	17	14	15	22	21	25	24	17	12	15	16
Total #	215	230	215	220	230	255	220	235	215	245	225	215
Cy3 #	22	15	14	17	23	26	21	26	15	18	16	12
Total #	180	260	220	215	230	235	230	215	240	220	240	220
Cy3 #	12	20	15	13	25	24	23	20	13	14	17	14
Total #	230	245	240	250	240	230	235	220	235	225	205	230
Cy3 #	14	18	17	18	25	23	25	22	15	13	12	15

Appendix B (cont.). Gross Bacterial Counts. Hunt Camp Creek.

	Nso1225				B3b				Yo-Pro			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	130	195	130	170	170	180	195	175	185	190	160	200
Cy3 #	9	15	9	11	12	13	12	14	6	5	4	5
Total #	200	140	125	220	180	190	205	180	170	170	180	190
Cy3 #	13	9	8	13	14	11	12	13	3	4	5	4
Total #	210	155	200	210	185	200	190	175	205	180	145	165
Cy3 #	14	10	15	13	15	12	11	13	7	5	2	4
Total #	155	165	170	225	190	180	165	185	175	145	175	180
Cy3 #	9	13	11	14	13	15	13	12	5	2	6	5
Total #	130	150	160	185	210	215	175	215	180	165	205	170
Cy3 #	8	9	10	12	14	16	17	14	4	3	7	3
Total #	135	170	130	145	200	185	205	180	160	155	175	205
Cy3 #	9	11	8	10	18	13	15	12	3	4	4	5
Total #	185	185	185	175	190	195	190	170	190	200	160	175
Cy3 #	11	13	13	11	12	14	13	15	4	4	3	6
Total #	190	145	145	225	215	190	180	200	210	215	180	155
Cy3 #	12	10	9	14	16	16	14	13	5	5	5	4

Appendix B (cont.). Gross Bacterial Counts. Lumber Dock.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	225	195	185	205	240	220	215	230	215	225	210	215
Cy3 #	13	11	9	13	16	15	11	16	6	7	4	5
Total #	230	220	205	210	220	230	220	215	225	210	230	240
Cy3 #	14	12	11	12	15	14	13	12	5	6	5	8
Total #	220	210	220	230	210	215	225	235	235	215	235	220
Cy3 #	12	14	13	15	16	13	15	17	6	4	8	4
Total #	200	230	220	220	235	235	240	225	205	240	215	255
Cy3 #	12	14	12	13	18	16	17	16	4	6	5	7
Total #	245	250	210	235	195	210	215	215	210	230	220	230
Cy3 #	15	16	11	14	13	11	14	13	3	4	5	7
Total #	230	235	240	230	215	205	210	215	230	210	235	240
Cy3 #	14	14	15	12	14	11	13	14	5	3	6	6
Total #	215	205	240	210	220	215	220	200	245	235	225	210
Cy3 #	11	12	14	11	15	13	13	11	7	5	4	4
Total #	220	225	255	240	205	200	205	210	235	250	215	205
Cy3 #	13	14	15	15	12	12	11	12	6	9	5	3

Appendix B (cont.). Gross Bacterial Counts. Meridian.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	225	195	185	205	215	210	200	195	210	235	225	190
Cy3 #	13	11	9	13	12	11	12	10	5	6	6	4
Total #	235	220	205	210	230	200	220	205	220	225	240	220
Cy3 #	14	12	11	12	15	12	13	12	7	5	7	6
Total #	220	215	225	230	225	215	210	220	215	210	225	240
Cy3 #	12	14	13	15	14	13	14	15	7	5	4	7
Total #	200	230	220	225	210	225	220	235	225	235	235	230
Cy3 #	12	14	12	13	10	16	16	18	6	8	5	4
Total #	245	250	210	235	220	205	215	200	235	215	240	235
Cy3 #	15	16	11	14	15	10	11	10	8	6	6	6
Total #	230	235	240	230	200	215	235	210	230	200	215	220
Cy3 #	14	14	15	12	11	12	15	11	9	5	4	5
Total #	215	205	240	210	230	225	220	205	215	220	195	225
Cy3 #	11	12	14	11	15	16	13	13	6	4	3	7
Total #	220	225	255	240	220	210	205	210	225	240	210	245
Cy3 #	13	14	15	15	12	12	10	13	5	7	4	8

Appendix B (cont.). Gross Bacterial Counts. UGAMI.

	Nso1225				B3b				YO-PRO			
	1	2	3	4	1	2	3	4	1	2	3	4
Total #	225	240	235	250	265	225	230	210	225	235	240	215
Cy3 #	17	15	16	19	14	15	13	15	4	8	7	4
Total #	240	235	220	245	245	235	220	225	210	225	220	230
Cy3 #	18	16	15	15	12	13	15	13	3	6	5	5
Total #	220	215	235	235	250	255	215	235	230	210	235	205
Cy3 #	15	14	17	14	14	17	12	19	7	3	6	3
Total #	265	230	250	230	235	225	240	220	220	230	245	235
Cy3 #	19	17	18	20	13	16	19	16	5	5	7	9
Total #	245	250	220	230	240	215	225	215	200	220	210	225
Cy3 #	18	18	15	13	16	13	16	14	4	5	4	7
Total #	255	245	215	260	255	230	220	240	215	240	220	210
Cy3 #	20	19	15	20	19	15	13	15	5	6	5	4
Total #	235	260	230	240	265	250	245	220	225	215	240	225
Cy3 #	16	20	14	11	16	15	15	12	6	4	8	4
Total #	215	255	235	220	230	240	230	235	235	230	235	230
Cy3 #	15	16	12	17	15	14	13	18	4	5	6	5

Appendix C. Calculations utilized in this study for detecting ammonia-oxidizing bacteria.

$$\%\ \text{Recovery after FISH} = \frac{\text{mean of YO-PRO 16S rRNA}}{\text{mean of YO-PRO only}}$$

$$\%\ \text{Recovery after } \textit{in situ} \text{ PCR/FISH} = \frac{\text{mean of YO-PRO } \beta\text{3b}}{\text{mean of YO-PRO only}}$$

$$\%\ \text{Autofluorescence} = \frac{\text{mean of CY3 (YO-PRO only)}}{\text{mean of YO-PRO only}}$$

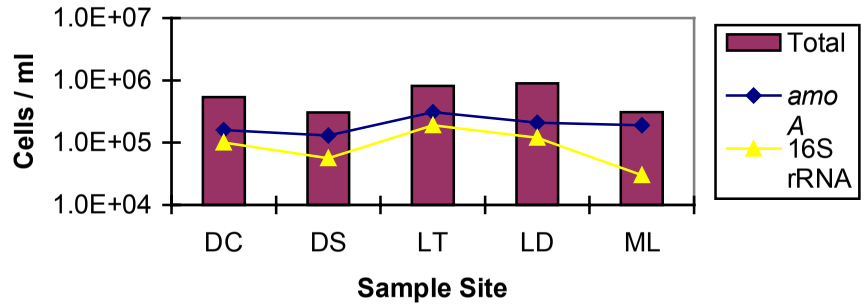
$$\%\ \text{Detected by FISH} = \frac{\text{mean of CY3 16S rRNA probe}}{\text{mean of YO-PRO 16S rRNA probe}}$$

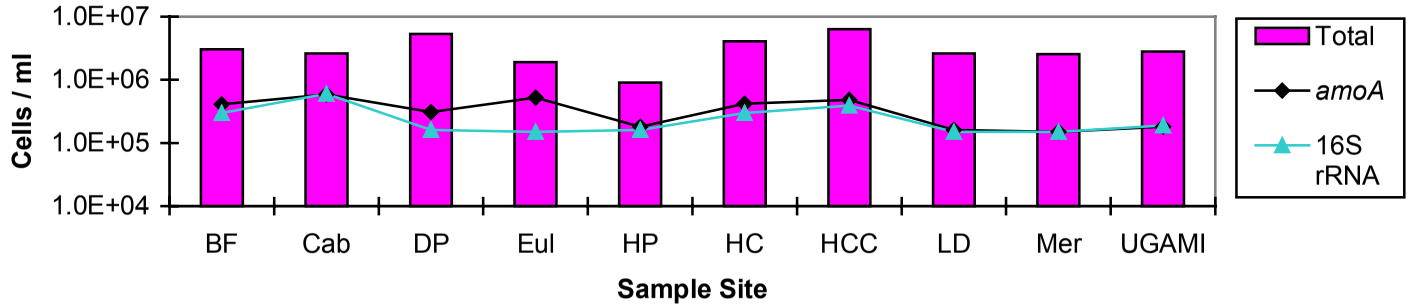
$$\%\ \text{Detected by } \textit{in situ} \text{ PCR/FISH} = \frac{\text{mean of CY3 } \beta\text{3b}}{\text{mean of YO-PRO } \beta\text{3b}}$$

$$\text{Cells per ml} = \frac{\text{avg \# of cells in field} \times 1.77 \times 10^{4}}{\text{\# ml filtered}}$$

Appendix D. Average number of AOB and total cells detected per milliliter of water at each site via *in situ* PCR/FISH or FISH alone.

Sample Site	*in situ* PCR/ FISH ($x10^6$)	Total Cells Detected ($x10^6$)	Phylogenetic Probe	FISH ($x10^6$)	Total Cells Detected ($x10^6$)
Dean Creek	0.2	0.6	NEU	0.1	0.5
Doboy Sound	0.1	0.3	NEU	0.06	0.3
Long Tabby	0.3	0.8	NEU	0.2	0.8
Lumber Dock	0.2	0.9	NEU	0.1	0.9
Marsh Landing	0.09	0.4	NEU	0.03	0.4
Bourbon Field	0.4	3.0	Nso1225	0.3	3.1
Cabretta	0.6	2.6	Nso1225	0.6	2.6
Duck Pond	0.3	5.7	Nso1225	0.2	5.0
Eulonia	0.5	1.9	Nso1225	0.1	1.9
High Point	0.2	0.9	Nso1225	0.2	0.9
Hunt Camp	0.4	4.1	Nso1225	0.3	4.0
Hunt Camp Creek	0.5	6.7	Nso1225	0.4	6.0
Lumber Dock	0.2	2.6	Nso1225	0.2	2.6
Meridian	0.2	2.5	Nso1225	0.2	2.6
UGAMI	0.2	2.8	Nso1225	0.2	2.8





Appendix D (cont.). Average number of AOB per ml detected via functional (*amoA*) and phylogenetic (16S rRNA) methods as compared to total number of bacteria detected per ml.