Unraveling the black box of aquaria biofilter function:

FISHing for novel ammonia-oxidizing archaea associations

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Abstract

Nitrification, a biological link between the oxidized and reduced pools of inorganic nitrogen is typically limited by the first step, ammonia oxidation. Often controlled by ammonia oxidizing archaea (AOA), nitrification involves archaea within *Thaumarcheota* as well as bacteria from *Gammaproteobacteria* and *Betaproteobacteria*. This study examines how AOA associate with the ammonia oxidizing bacteria, and the specific spatial interaction between AOA/AOB and nitrite oxidizing bacteria through microscopy. Water and biofilm samples were collected from three seawater fish tanks and throughout the aquaria at the Marine Resources Center (MRC), Marine Biological Laboratory, Woods' Hole, MA to determine if nutrient availability or temperature affect these microbial assemblages through Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH) and confocal laser scanning microscopy. Within the closed loop aquaria, the microbial consortia present were significantly different at different points throughout the system due to the local nutrient and temperature conditions.

Introduction

Nitrification is the sole oxidative biological process connecting reduced and oxidized pools of inorganic nitrogen. Aerobic ammonia oxidation is the first, rate-limiting step of nitrification, a two-step process catalyzed by ammonia-oxidizing and nitrite-oxidizing microorganisms. Known ammonia-oxidizers include archaea within Thaumarcheota encompassing organisms previously classified in Crenarchaeota as well as bacteria from *Gammaproteobacteria* and *Betaproteobacteria* (Brochier-Armanet et al. 2008). Nitrite oxidation, the second step in nitrification, is performed by nitrite oxidizing bacteria (NOB). NOB are widely distributed throughout the proteobacteria, though they are concentrated within the gamma, beta, and deltasubdivisions (Teske et al. 1994). Ammonia-oxidizing archaea (AOA) tend to control ammonia oxidation in marine habitats due to their high specific affinities for nitrogen (Martens-Habbena et al. 2009, Stahl and de la Torre 2012). Novel archaeal ammonia-oxidizers were first detected in the nitrifying reactor system for the saltwater aquaria in Chicago's Shedd Aquarium. Therefore AOA could be controlling ammonia oxidation in other aquaria.

Past literature has indicated that the abundance and diversity of AOA and AOB community assemblages in aquaria are influenced by nutrient availability (Urakawa et al. 2008). However how AOA associate with the AOB, and the specific spatial interaction between AOA/AOB and NOB has yet to be visually explored. The objectives of this study were to i) evaluate the microbial consortia throughout an aquarium with microscopy; ii) identify AOA and AOB in marine biofilms; and iii) determine if nutrient availability or temperature affect these microbial assemblages through Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH) and confocal laser scanning microscopy.

Methods

Sample Collection

Water and biofilm samples were collected from three seawater fish tanks and throughout the aquaria at the Marine Resources Center (MRC), Marine Biological Laboratory, Woods' Hole, MA in July 2013. The MRC has a recycling water filtration system that is comprised of eight loops sourcing water from Great Harbor, MA to different parts of the aquaria. In each loop, recycled water first passes through a bag filter that

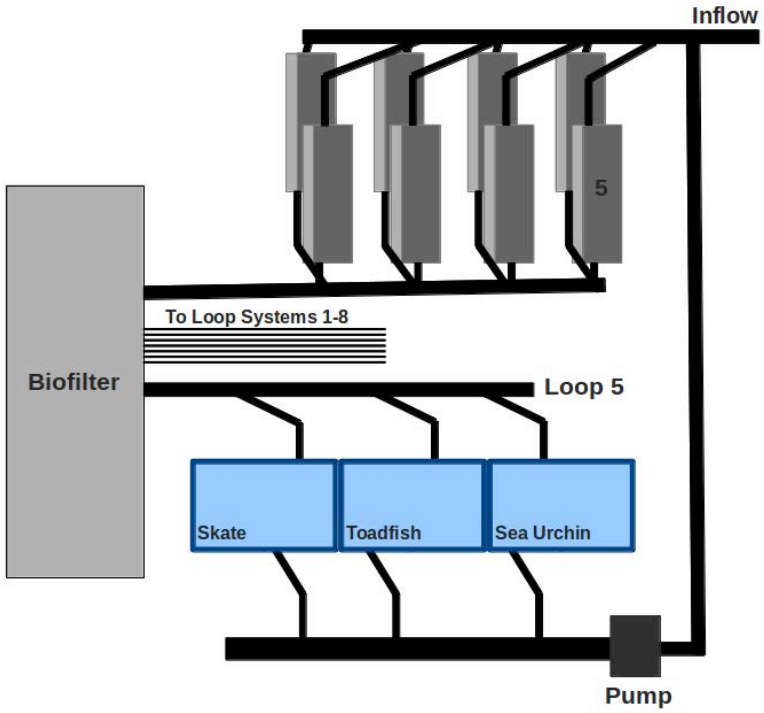

Figure 1. Simplified diagram of the cyclic water filtration system at the Marine Resources Center, the Marine Biological Laboratory, Woods' Hole, MA. This study will examine Loop 5, an unaltered recycling system with minimal new water input.

removes large particulate matter. The water then passes through a nitrifying biofilter to reduce ammonia and nitrite levels before it reaches the animal tanks. Loop 5 is a minimal water-loss system, and was selected for this analysis (Figure 1). Water and biofilm samples were taken from toadfish, skate, and sea urchin tanks, as well as at various locations throughout the system. These samples were taken from the inflow, side of the tank, and outflow of these locations (Figure 2). The toadfish and sea urchin tanks are kept at 22.5°C and 22.3°C, and the skate tank is kept at 18.3°C.

Water Composition Analysis

Water samples were filtered using a 0.2μm filter and analyzed in triplicate. The pH of the samples was determined using an Basic Accummet ABIT pH meter. Water samples were analyzed for ammonium with the salicylate-hypochlorite method (Bower and Holm-Hansen 1980). Nitrite and nitrate concentrations were determined after a second filtration (ThermoScientific OnGuard II Ag Cartridge) using ion chromatography (Dionex ICS-2100 Ion Chromatography Systems).

CARD-FISH and Confocal Laser Scanning Microscopy

Biofilm samples were analyzed for distinct community structure and composition using dual-hybridization CARD-FISH techniques (Prenthaler et al. 2002 and Ishii et al. 2004). The protocols presented were utilized with modification after several optimizing experiments. Biofilm samples of 0.05mg were immediately fixed with 1% paraformaldehyde for 1h in the dark. Samples were centrifuged and diluted with a 1:1 phosphate buffer saline (PBS)/ethanol mixture. Samples were twice resuspended by vortex, centrifuged, and replaced with a 1:1 phosphate buffer saline/ethanol mixture. Samples were then resuspended by vortex, and homogenized gently several minutes by hand. 50μL of resuspended sample was added to 10mL of 1x PBS and filtered onto a 0.2μm polycarbonate membrane filter overlaying a 0.4μm cellulose nitrate support filter. Membrane filters were dried at room temperature and embedded using 0.1% agarose. All membrane filters underwent two permeabilization steps: lysozyme followed by hydrochloric acid. A third permeabilization step utilizing Proteinase K was applied to the filter from the skate tank sample to improve the crenarchaeal signal resolution. The first hybridization followed the protocol given in the literature (Pernthaler et al. 2002 and Ishii et al. 2004), and then the protocol was repeated from the inactivation step for dual-hybridization.

Three dual-hybridization experiments were conducted targeting various spatial scale community organizations. First broad probes for bacteria (Eub I-III) and archaea (Arch915) were applied to biofilms from the biofilter, bag filter, skate, sea urchin, and toadfish systems. The second experiment utilized CARD-FISH probes to identify specific groups within all the samples. The single lineage of marine archaea affiliated with *Crenarchaeota* is thought to comprise more than 40% of all marine archaea (Stahl and de la Torre 2012). Therefore, in the second experiment, specific AOA was first targeted using the *Crenarchaeota* (Cren 554) probe. AOB was targeted with AOA by utilizing the betaproteobacterial ammonia-oxidizing bacteria (NSO1225) probe. The third experiment sought to determine if another specific group had a stronger community association with *Crenarchaeota* (Cren554) than the reported relationship with the betaproteobacterial ammonia-oxidizing bacteria (Martens-Habbena et al. 2009). Associations of *Crenarchaeota* (Cren554) and Marine Euryarchaeota (Eury806), Planctomycetales (Pla46),

Gammaproteobacteria (Gam42a with competitor Bet42a), or Betaproteobacteria (Bet42a with competitor Gam42a) were explored using CARD-FISH dual-hybridization on a sample dense with archaea. This sample was biofilm from the skate tank. Community assemblages for the three experiments were imaged using a Zeiss Axio Imager.M2 microscope at 100x magnification and through confocal laser scanning microscopy with a Zeiss LSM 700 microscope at 63x magnification.

Statistical Methods

Biogeochemical data was analyzed using multiple two-way ANOVAs. The significance of sampling location within the chosen tank systems and resulting nutrient data were assessed using *R* (R Core Development Team 2008).

Results & Discussion

Water Analysis Using Two-Way ANOVAs

Ammonium and nitrate concentrations within water undergo changes throughout the loop system (Figure 2; see also Supplementary Information for water values). Specific biological processes within the tank aquaria are exerting an important biogeochemical impact on the individual tank systems. Sampling location of the tank (inflow vs. outflow) shows a significant effect on the observed ammonium concentrations, indicating points throughout the tank system are unique. ANOVA results (see Supplementary Information) also indicate there is a significant interaction effect between the location (inflow/outflow) and the tank sampled.

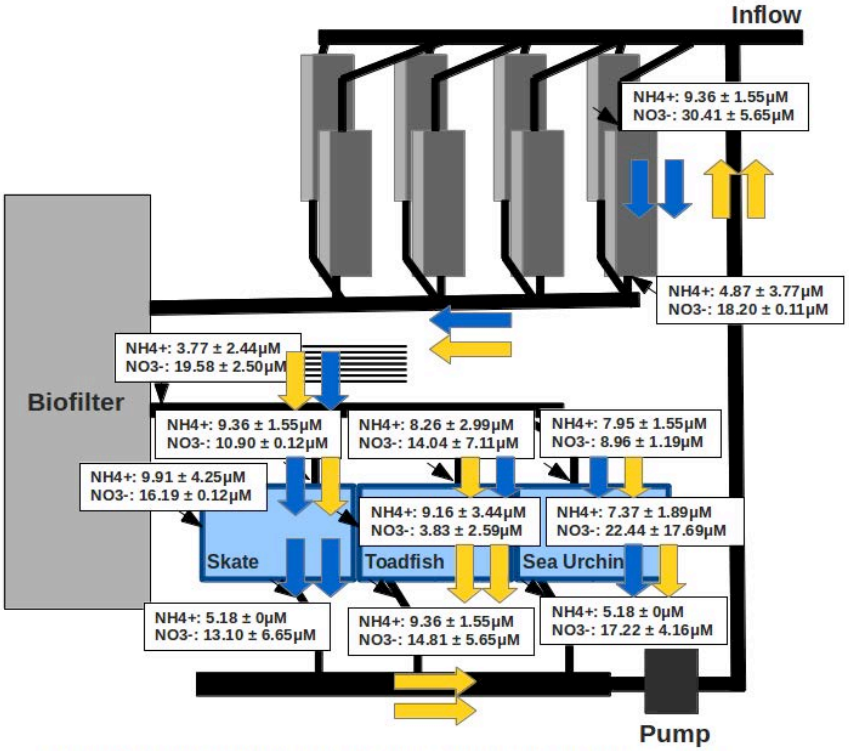

Figure 2. Nutrient Map of Loop 5. Black arrows mark points sampled. The colored arrows indicate nutrient level changes for ammonium (NH4+) values (top or left arrow) and nitrate (NO3-) values (bottom or right arrow) between sampling points. Yellow indicates increasing values, and blue indicates decreasing values.

CARD-FISH Analysis

General bacterial and archaeal probes were applied to biofilm samples throughout Loop 5 to investigate suspected community differences and to determine how archaeal and bacterial aggregates differed throughout the loop. Small to medium sized bacterial aggregates were common within the bag filter, and AOB made up approximately half of each aggregation seen (Figure 3). There were fewer but larger bacterial aggregates within the biofilter that seemed to be independent. Within the tank systems, bacterial aggregates were small to medium and had greater associations with Archaea. Each tank system observed showed unique community structures and were subsequently investigated with higher specificity probes, as reported below.

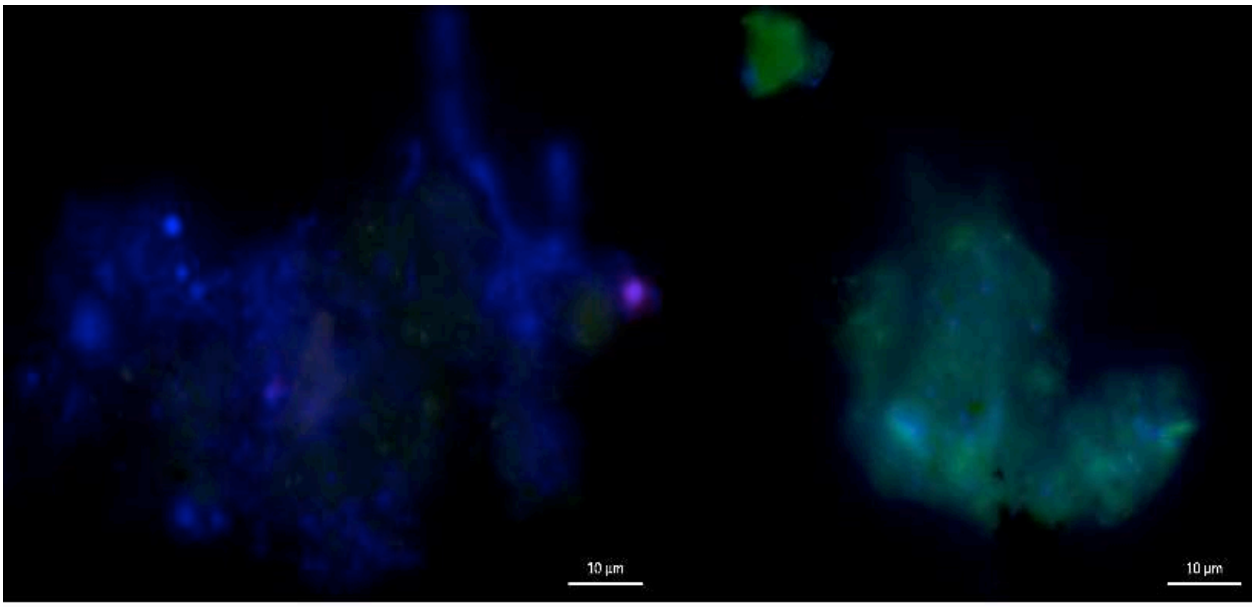

Figure 3. Left: A common, medium-sized aggregate from the bag filter of Loop 5. Betaproteobacteria (green) make up about half of the aggregate. Crenarchaea are shown in red and the DAPI stain is shown in blue. Right: Two small, independent bacterial aggregates from the biofilter of the MRC. Bacteria(green) were the dominant members of the biofilter.

Toadfish Tank System

When first examined with the general archaea and bacteria probes, the inflow of the system was characterized by small aggregates that seemed to be linked via by bacterial filamentous chains (Figure 4). Archaea were present but appeared to be free-living. The bacterial aggregates present within the tank were commonly more than 50µm across and larger than any other aggregates seen throughout the aquaria. Most fish produce ammonia as their sole nitrogenous waste, however toadfish are also capable of excreting urea through their gills (Walsh et al. 2000). This

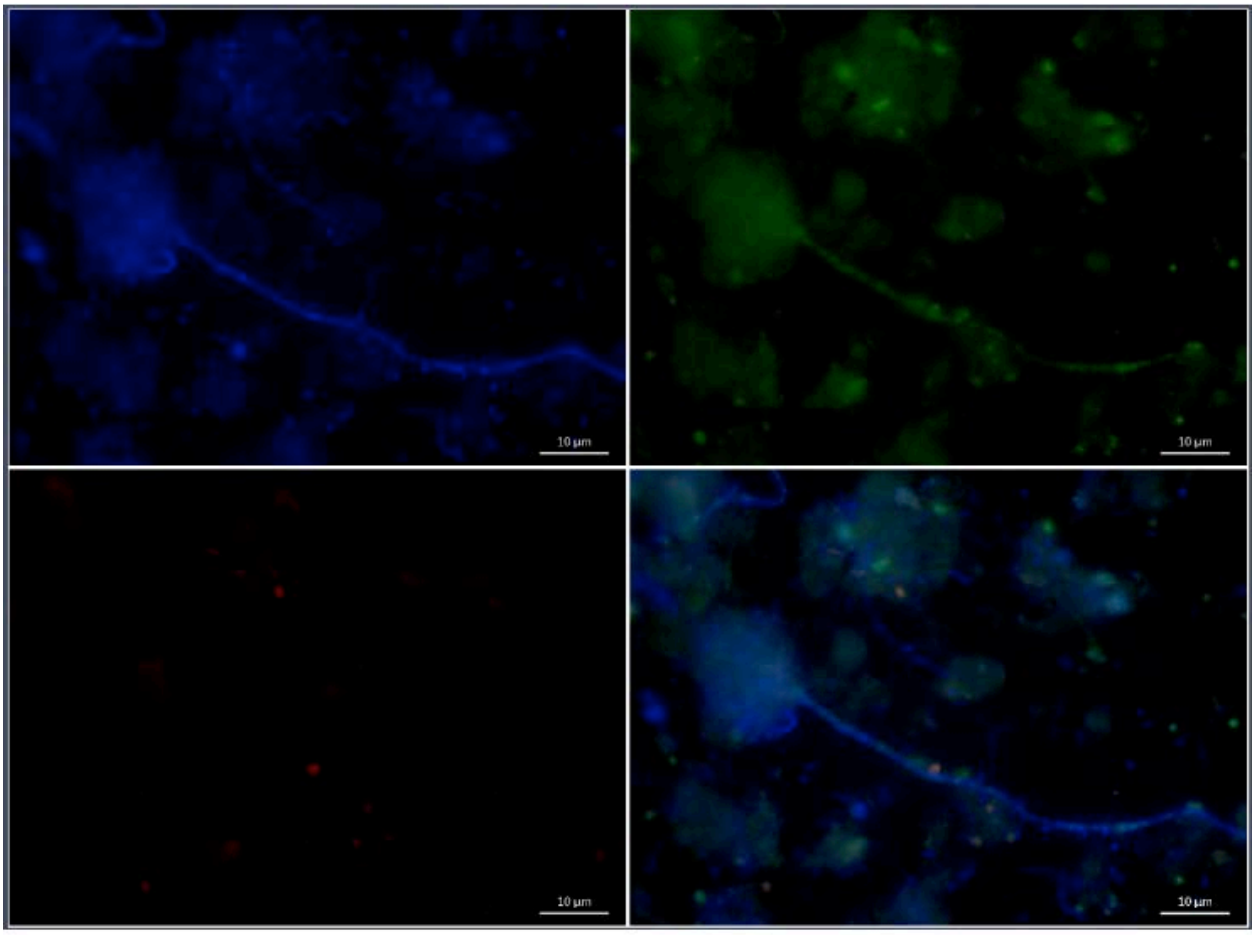

Figure 4. Panel of microbial assemblages from the Toadfish tank inflow. Small to medium aggregates are linked by filamentous chains. These chains may be sulfur oxidizing bacteria. The various panels display the DAPI(blue), bacteria(green), archaea(red), and the overlay of the dual-hybridization .

adaptation introduces a different nitrogen source in addition to ammonia potentially creating a distinctive nutrient environment for microorganisms that could be responsible for the large bacterial assemblages seen within the tank. Large archaeal aggregations were also seen on the larger bacterial assemblages, however they were not illuminated with the *Crenarchaeota* probe. These large archaeal aggregates could be *Crenarchaeota* the Cren554 probe may have not hybridized to or they may be members of *Thaumarchaeota* who have the ability to utilize urea as well as ammonia (Tourna et al. 2011). This study did not include DNA sequencing and cannot confirm the aforementioned claim, however the morphologies observed support the hypothesis of *Thaumarchaeota*. Overall within the tank system, is it clear there is a strong association between archaea and bacteria, however finer resolution of which members has yet to be determined.

Sea Urchin Tank System

Though this system had a similar temperature to the toadfish tank system, the community structures throughout the system differed. At the inflow, wispy hair-like filaments were prominent throughout the sample and crenarchaeal aggregates were few in number. AOB were not observed. Within the tank, smaller aggregates were visible in conjugation with small rod-like

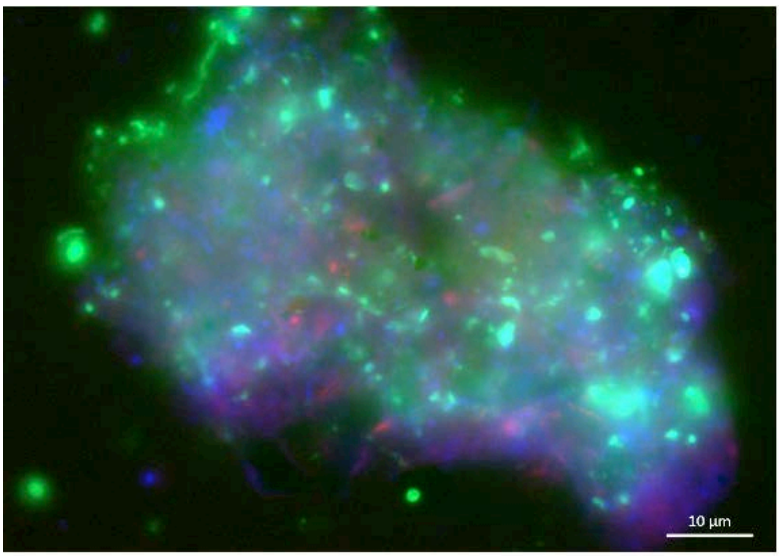

Figure 5. An aggregate found within the toadfish tank comprised of both archaea(red) and bacteria(green). Toadfish produce urea as well as ammonia and may be reseponsible for the large, bright consortia observed.

bacteria present with crenarchaea and betaproteobacteria that were few in number. However in the outflow of the system, long chains of square shaped cells and cocci formed filaments were the predominant structures found (Figure 7). These chain-forming organisms did not fluoresce and based upon their morphology it is suspected they may be sulfur bacteria or small eukaryotes.

Skate Tank System

Within the skate tank system, crenarchaeota and betaproteobacteria were common at the inflow and within the tanks.

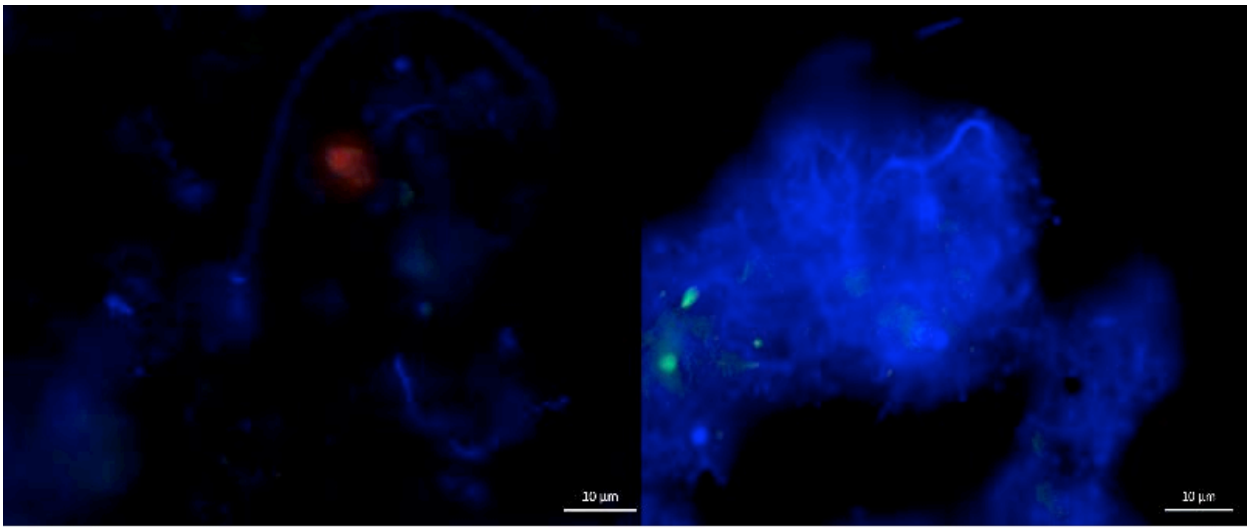

Figure 6. CARD-FISH images from the sea urchin tank's biofilm are shown from the inflow (left) and from within (right). Crenarchaea associated with other crenarchaea are present in low numbers throughout the community aggregate. In the tank, betaproteobacteria(green) are present in low numbers.

At the inflow, the microbial communities were comprised of small, round crenarchaeota and betaproteobacteria aggregates. Within the tank, the crenarchaeota were greater in number and formed larger loose aggregations (Figure 8). These dense numbers of crenarchaeota and large aggregations within the skate tank inspired a third experiment to elucidate the members of the community consortia in association with the ammonia-oxidizing archaea. Probes targeting the *Planctomycetales* phylum, *Gammaproteobacteria* class, *Betaproteobacteria* class, and *Marine*

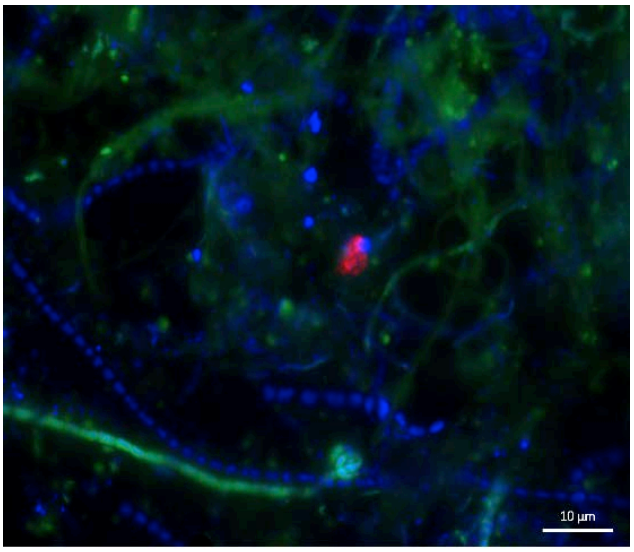

Figure 7. A dual-hybridized sample from the sea urchin outflow exemplifying the various filamentous bacteria found. The sample has been DAPI stained (blue), hybridized with the archaeal probe (red) and the bacteria probe (green).

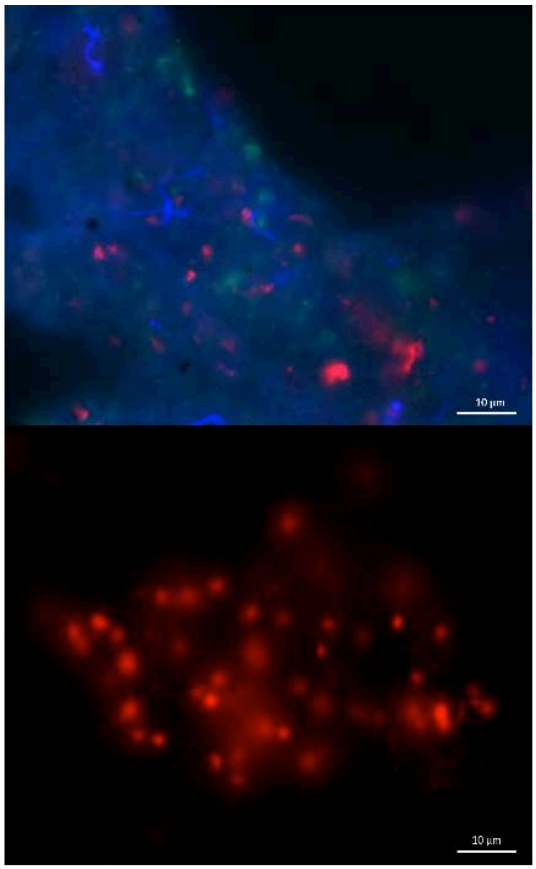

Figure 8. CARD-FISH image of a biofilm samples obtained from a skate tank. Top: Sampled from the inflow of the skate tank, the archaeal aggregates (shown in red) are small but present and involved in the consortium. DAPI is shown in blue and bacteria are shown with green. Below: Sampled from the skate tank itself, the archaeal aggregates are larger and prevalent throughout the sample. The aggregations seem to be purposefully spaced and associated with one another or with a bacterial partner.

Euryarchaeota class were each hybridized independently with the crenarchaeal probe. From these images (see supplementary Information), it appears that gammaproteobacteria and betaproteobacteria have the closest association with crenarchaea. As mentioned, members of *Gammaproteobacteria* and *Betaproteobacteria* are known nitrite-oxidizing bacteria that use the nitrite produced by AOA or AOB. However AOA also produces an inhibiting secondary metabolite (Stahl and de la Torre 2012). These two facts together may explain the loose but deliberate spatial association of *Gammaproteobacteria* and *Betaproteobacteria* members with members of *Crenarchaeota* observed via confocal laser scanning microscopy and CARD-FISH.

Conclusions & Future Research

This study set out to explore microbial marine biofilm consortia throughout several aquaria with a focus on nitrogen processes within a closed loop system. This study determined that the microbial consortia throughout the system (Loop 5) vary through space, regardless of the specific tank system analyzed. The presence of AOB and AOA were recognized in the marine biofilms through qualitative analysis and microscopy. Based upon the biogeochemical data and the microscopy results, it was determined the microbial consortia present were significantly different at different points throughout the system. Based on the morphological results and the significance value of the ammonium concentrations, this study also suggests that the specific species of nitrogen and the nutrient concentrations may influence community associations.

The MRC maintains biofilms within their biofilters that are cleaned at various points throughout the year. Optimizing recolonization of these biofilters is crucial for the MRC staff for animal maintenance. Currently, the MRC has specific issues initially establishing a robust nitrite oxidizing bacteria population following a disturbance. The MRC would like help from microbiologists to address this issue. Now that a general large-scale understanding has been established, looking at the loop system in finer resolution is next. This suggests three major projects: i) identification of the key aspects of community development and its response to specific variables; ii) development of a microbial metric for evaluating system health; iii) cultivation of a NOB population in anticipation of biofilter reseeding.

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Supplementary Information

a) Summary of Biogeochemical Data

Samples	**NH_4^+ (μM)**	**NO_3^- (μM)**	**pH**
BAG1	9.36 ± 1.55	30.41 ± 5.65	7.68
BAG3	4.87 ± 3.77	18.20 ± 0.11	7.63
BF3	3.77 ± 2.44	19.58 ± 2.50	7.66
U1	7.95 ± 0.89	8.96 ± 1.19	7.78
U2	7.37 ± 1.89	22.44 ± 17.69	7.74
U3	5.18 ± 0	17.22 ± 4.16	7.79
T1	8.26 ± 2.99	14.04 ± 7.11	7.77
T2	9.16 ± 3.44	3.83 ± 2.59	7.73
T3	9.36 ± 1.55	14.81 ± 15.06	7.72
S1	9.36 ± 1.55	10.90 ± 0.12	7.70
S2	9.91 ± 4.25	16.19 ± 0.12	7.65
S3	5.18 ± 0	13.10 ± 6.65	7.70

b) Two-way ANOVA Statistical Results

Analysis of Variance Table

Response: Ammonium Levels (μM)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Tank	2	15.829	7.9146	3.2725	0.07341
Location	1	17.091	17.0906	7.0664	0.02086*
Tank:Location	2	22.322	11.1608	4.6146	0.03262*
Residuals	12	29.023	2.4186		

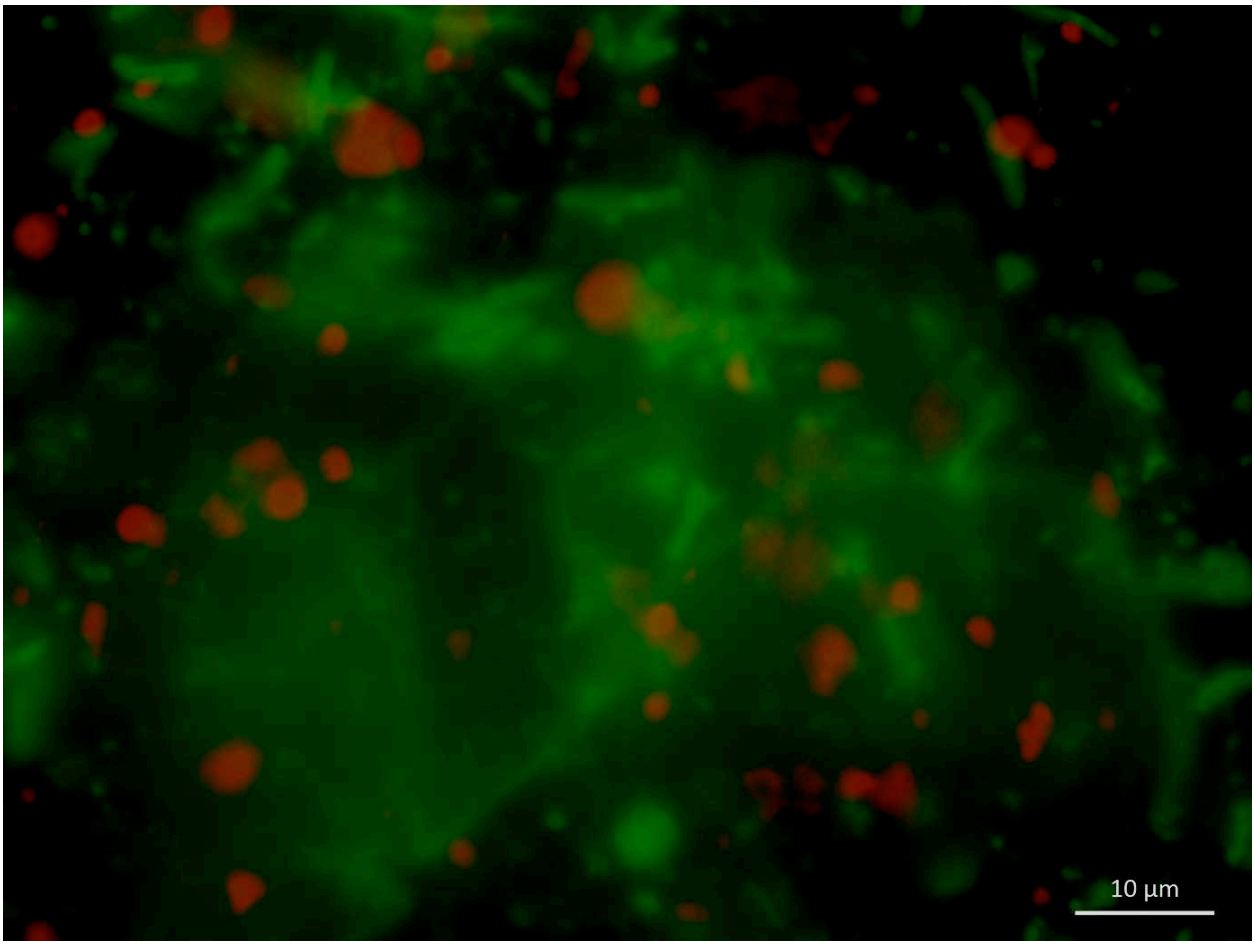
Analysis of Variance Table

Response: Nitrate Levels (μM)

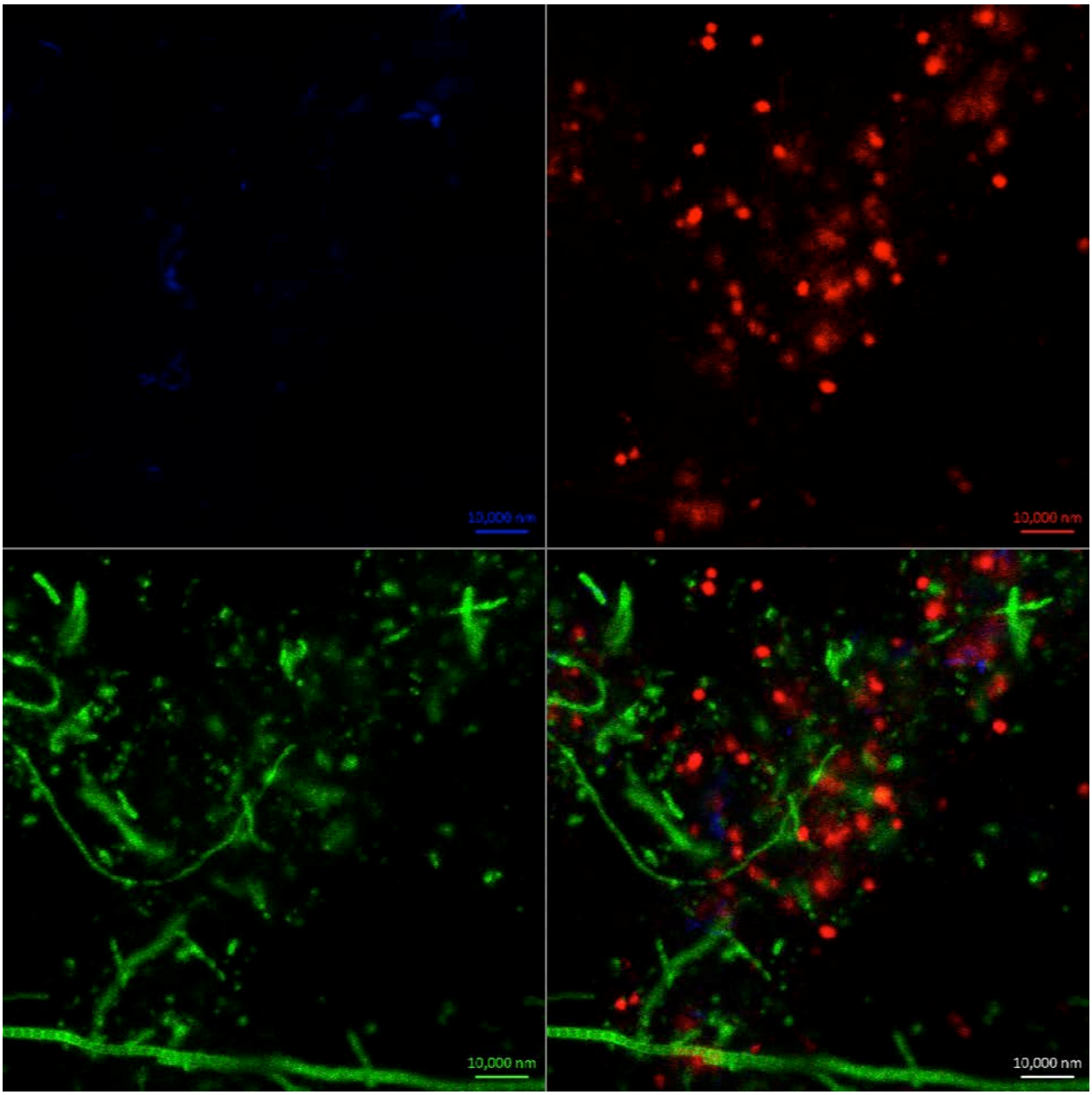
	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Tank	2	18.86	9.432	0.1662	0.8488
Location	1	48.81	48.814	0.8602	0.372
Tank:Location	2	30.98	15.492	0.273	0.7657
Residuals	12	680.95	56.746		

c) CARD-FISH of Gammaproteobacteria associated with Crenarchaeota.

Archaea(red), Bacteria(green)

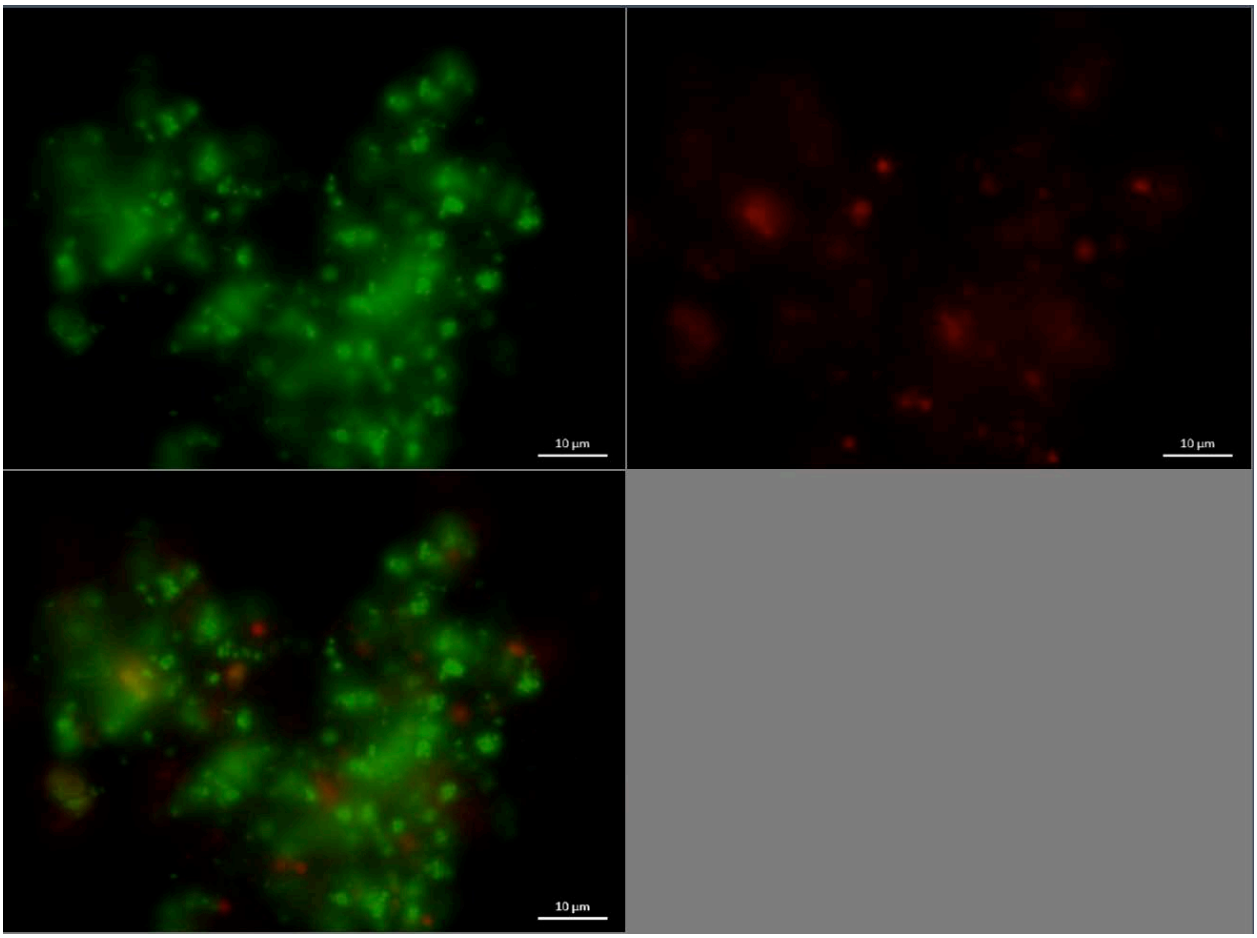


d) Confocal Laser Scanning Microscopy Panel of Gammaproteobacteria associated with Crenarchaeota; DAPI(blue), Archaea(red), Bacteria(green)

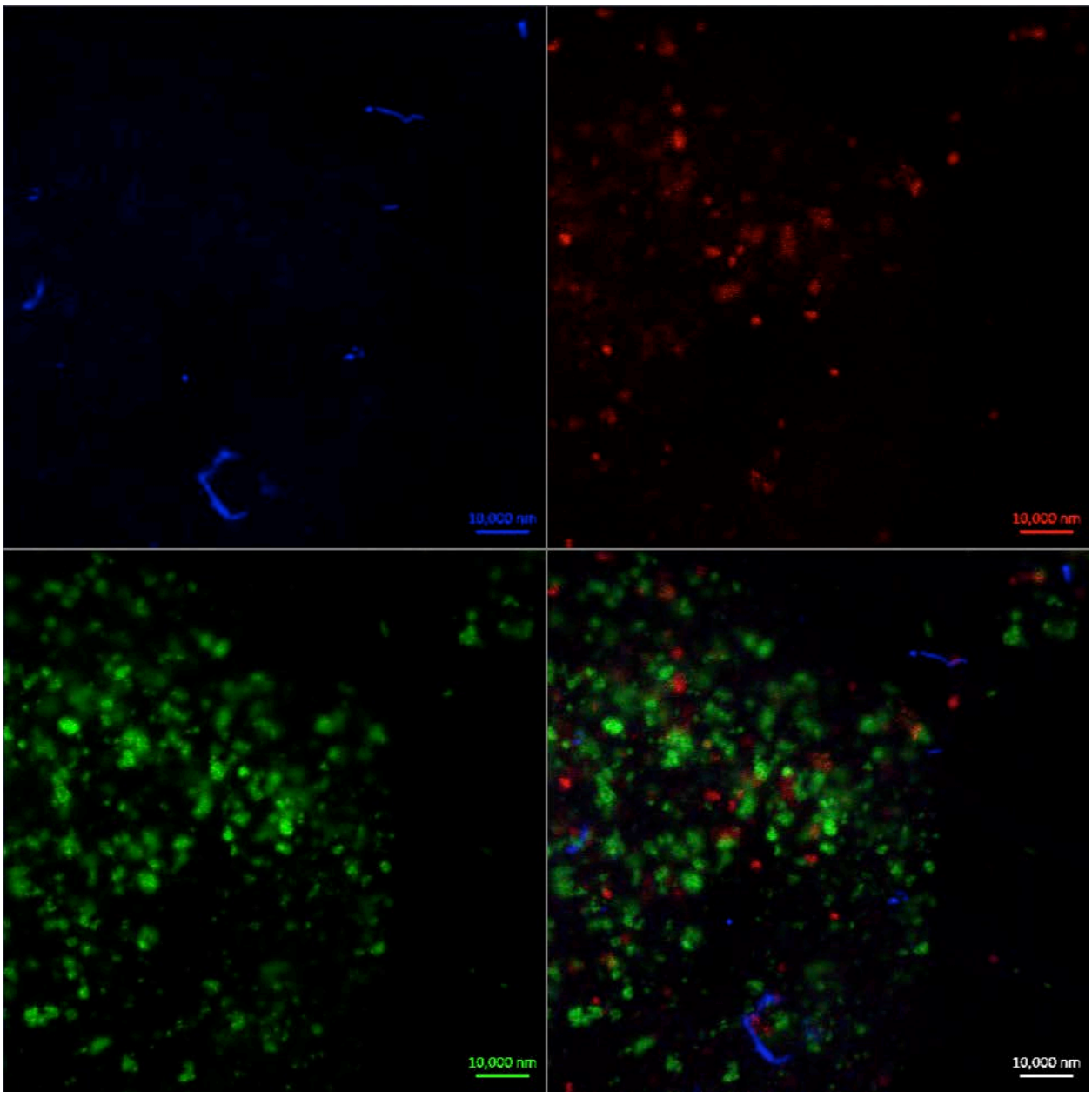


e) CARD-FISH panel of Betaproteobacteria associated with Crenarchaeota

DAPI(blue), Archaea(red), Bacteria(green)



f) Confocal Laser Scanning Microscopy Panel of Betaproteobacteria associated with Crenarchaeota; DAPI(blue), Archaea(red), Bacteria(green)



G)

List of Specific Probes Utilized

Archaea (Arch 915)

Bacteria (Eub I-III)

Crenarchaeota (Cren 554)

Betaproteobacterial ammonia-oxidizing bacteria (NSO1225)

Crenarchaeota (Cren 554)

Marine Euryarchaeota (Eury 806)

Planctomycetales (Pla 46)

Gammaproteobacteria (Gam 42a with competitor Bet 42a)

Betaproteobacteria (Bet 42a with competitor Gam 42a)

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