

# Molecular Community Analysis

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of the Falmouth Wastewater Treatment Facility

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**Microbial Diversity Course 2009**

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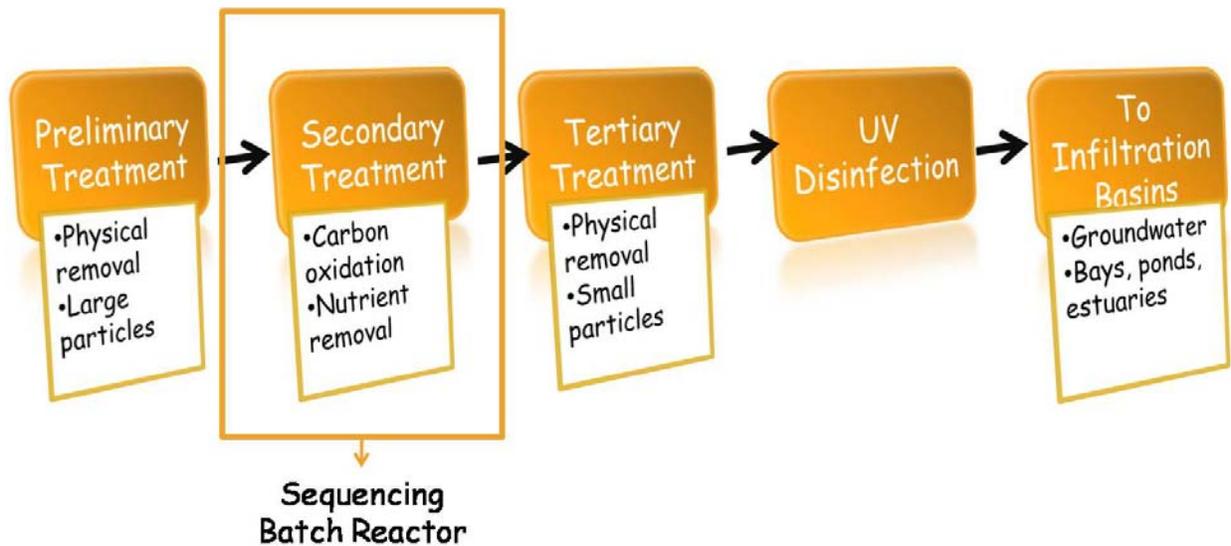
## Abstract

Excessive nitrogen originating primarily from wastewater discharge has led to coastal eutrophication in southeastern Massachusetts. The Falmouth Wastewater Treatment Facility recently upgraded to a biological nitrogen removal plant employing sequencing batch reactor (SBR) technology to meet a discharge limit of 3 mg/L total nitrogen. Wastewater treatment relies on the microbiological oxidation of carbon to reduce the oxygen demand of the influent wastewater along with the microbiological conversion of reduced nitrogen species (e.g., organic nitrogen and ammonia) to nitrogen gas. SBR operation selects for numerically abundant organisms and/or functionally important populations within the microbial community of the bioreactor, and the microbial community in turn determines how the bioreactor performs with respect to carbon oxidation and nutrient removal. In this study, molecular methods were used to examine the microbial community of the SRB at the Falmouth Wastewater Treatment Facility.

## Introduction

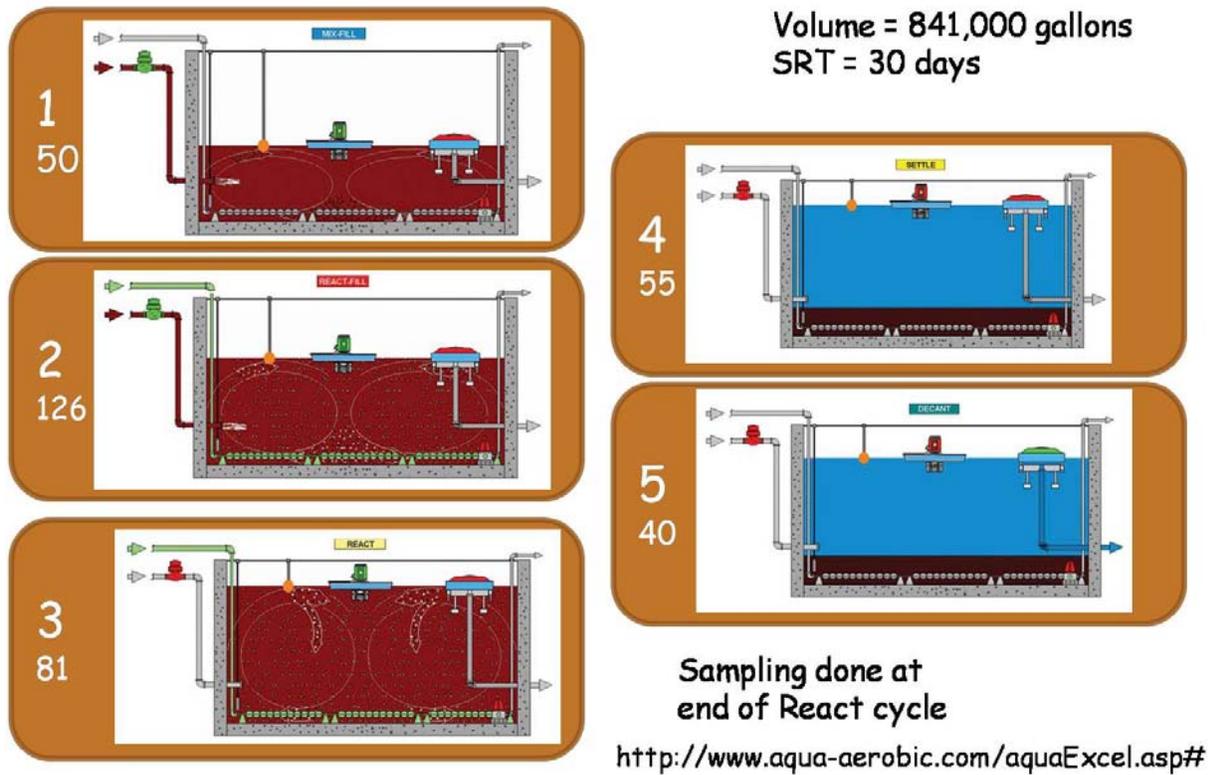
According to the Massachusetts Estuaries Program (MEP) of the Massachusetts Department of Environment Protection, MassDEP ([www.mass.gov/dep/water/resources/coastalr](http://www.mass.gov/dep/water/resources/coastalr)), excessive nitrogen originating primarily from wastewater disposal from both decentralized (e.g. septic systems) and centralized (e.g. wastewater treatment plants) treatment systems has led to a decrease in the quality of coastal rivers, ponds, and harbors in many communities in southeastern Massachusetts. It is believed that this trend in the reduced quality of the environment can be reverse if nitrogen inputs into these water bodies are decreased. Since nitrogen from wastewater is the primary source of nitrogen, reducing nitrogen inputs from wastewater sources should help improve the quality of coastal waters. In an effort to reduce nitrogen inputs from municipal wastewater plants, MassDEP analyzed the assimilative capacity of several bays, estuaries, and harbors and decided to set the total maximum daily load for nitrogen for the Falmouth Wastewater Treatment Facility (FWTF) to 3 mg/L total nitrogen. This means that FWTF's effluent should contain 3 mg/L or less of total nitrogen as mandated by MassDEP.

Wastewater treatment relies on the microbiological oxidation of carbon to reduce the oxygen demand of the influent wastewater along with the microbiological conversion of reduced nitrogen species (e.g., organic nitrogen and ammonia) to nitrogen gas. The FWTF needs to reduce its influent nitrogen concentration, measured as Kjeldhal nitrogen, and has a range of 20-45 mg/L to an effluent total nitrogen concentration of 3 mg/L or less. In 2005, the facility's upgrade from an aerated lagoon to a biological nitrogen removal plant employing sequencing batch reactor (SBR) technology was completed (Fig. 1), to meet this regulation.



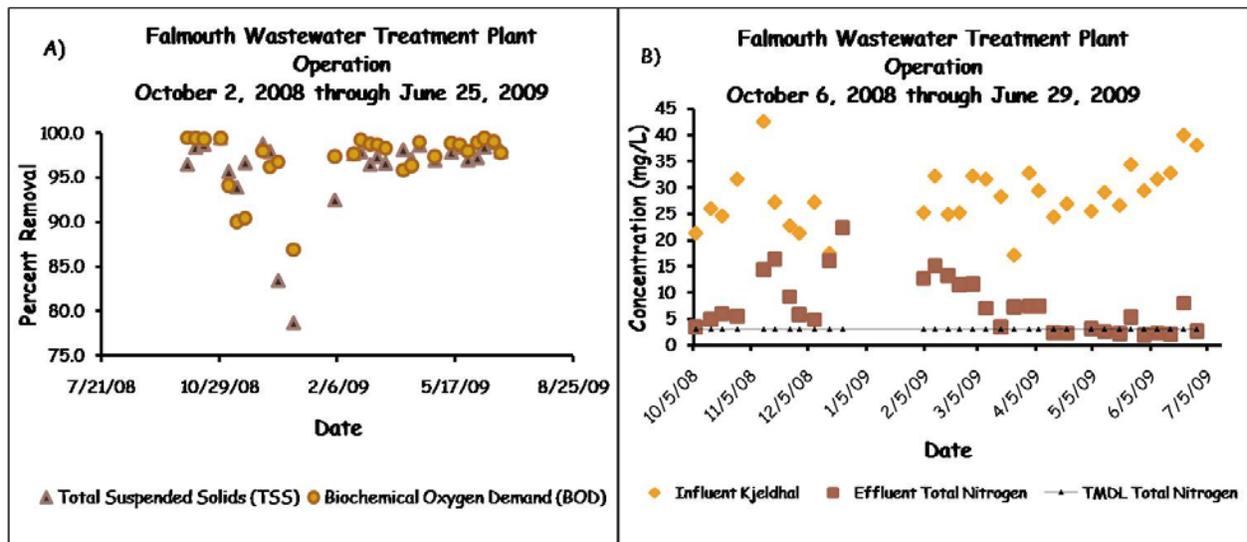
**Fig. 1.** Schematic flow diagram of the wastewater in the Falmouth Wastewater Treatment Facility. There are two sequencing batch reactors, which are run in parallel. Tertiary treatment consists of three denitrifying filters in parallel, and there are two open channel UV disinfections chambers. Treated wastewater is discharged to up to thirteen infiltration basins where the water is allowed to percolate to the groundwater and subsequently flow to local bays, ponds, or estuaries in Cape Cod.

SBR technology is a variation of the activated sludge process in which the anoxic and aerobic zones occur by changing the mixing condition in one large reactor, as opposed to varying the oxygen zones along the length of a long, narrow reactor (Fig. 2). In SBRs, nitrification, which is the conversion of ammonia to nitrate via nitrite, occurs during the aeration phases of the React Fill and React cycles; ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) require oxygen to convert ammonia to nitrite and nitrite to nitrate, respectively. Denitrification, the reduction of nitrate to nitrogen gas, takes place during the mixing, or anoxic, phases of the React Fill and React cycles.



**Fig. 2.** Sequencing batch reactor cycle description. The sequencing batch reactors used in this facility are the AquaExcel™ batch reactors designed by Aqua-Aerobic Systems, Inc. Cycle 1 is Mix Fill (50 minutes); influent enters reactor and is mixed without aeration. Cycle 2 is React Fill (126 minutes); influent continues to enter reactor and is mixed intermittently with air. Cycle 3 is React (81 minutes); there is no influent flow and reactor is mixed intermittently with air. Cycle 4 is Settle (55 minutes); solids separation phase where there is no mixing or aeration. Cycle 5 Decant/Waste (40 minutes); liquid volume removed, or decanted, by subsurface withdrawal, and solids are wasted near end of cycle. Nitrification and denitrification occur during the aeration and mixing phases, respectively, of the React Fill and React cycles.

Performance data for FWTF shows that while carbon oxidation, measured in terms of biochemical oxygen demand or BOD, was stable during October 2008 through June 2009 (Fig. 3A), nitrogen removal in the facility was unstable during the same period (Fig. 3B), particularly during the fall and winter. Possible reasons for this loss of nitrogen removal include loss of the nitrifying microbial population, most likely the ammonia oxidizing bacteria population, due to the temperature decrease or increase in dissolved oxygen concentration



**Fig. 3.** Operational data from the Falmouth Wastewater Treatment Facility. Data obtained from Pat Nieman, the staff laboratory technician, July 21, 2009. A) Plot of the percent removal of both total suspended solids (TSS) and biochemical oxygen demand (BOD) from October 2008 through June 2009; no data was received for January 2009. B) Plot of the influent Kjeldhal nitrogen (organic N + ammonia + ammonium), effluent total nitrogen (Kjeldhal nitrogen + nitrite + nitrate), and the target total nitrogen effluent concentration (total maximum daily load, TMDL, nitrogen).

SBR operation selects for numerically abundant organisms and/or functionally important populations within the microbial community of the bioreactor, and the microbial community in turn determines how the bioreactor performs with respect to carbon oxidation and nutrient removal. This is a cyclic interaction; changes in operation (e.g. increase/decrease aeration rate or increasing/decreasing the solids retention time) affect the structure of the microbial community within the reactor which determines if nitrification and/or denitrification are occurring. Knowledge of the microbial community structure within the SBR can help explain why the reactor is performing in a certain way. This is particularly important when the bioreactor is not achieving the desired performance (i.e. total nitrogen concentrations greater than 3 mg/L in the effluent). Information about the microbes of interest to the nitrogen removal process can help operators decide which operational parameters to change in order to obtain the desired performance.

In this study, I used molecular tools to examine the microbial community of the FWTF SRB. This approach involves taking sample(s) from the SBR, extracting DNA from a sample, and using the polymerase chain reaction (PCR) to amplify various genes (i.e. Bacteria 16S rRNA, Archaea 16S rRNA, ammonia monooxygenase A gene of AOB, etc). This PCR amplified DNA can then be used to identify members of the microbial community through cloning or used to examine changes in the community with a fingerprinting technique such as terminal restriction fragment length polymorphism (T-RFLP). In addition, bacterial or archaeal cells can be visualized in situ by hybridizing probes to their respective ribosomal RNA so that those cells fluoresce.

## Methods

**Sample collection.** Samples were collected from Falmouth Wastewater Treatment Plant (Falmouth, MA) sequencing batch reactor (SBR). Samples were collected from SBR1 and SBR 2 on July 7, 2009 and July 15, 2009, refer to Table 1 for a description. Ten samples were also collected during the react cycle (duration of 81 minutes) of SBR1 at 15 minute intervals on July 21, 2009; refer to Table 2 for a description.

Sample	Date	Location	Cycle	Volume	Type
1	7-Jul-09	SBR1	React air	250 mL	Grab
A	15-Jul-09	SBR1	Mix fill	250 mL	Grab
B	15-Jul-09	SBR2	React fill, air	250 mL	Grab
C	15-Jul-09	SBR2	React fill, no air	250 mL	Grab

Sample	Date	Time	Cycle	Volume	Type
D	21-Jul-09	10:10 AM	React, no air	250 mL	Grab
E	21-Jul-09	10:10 AM	React, no air	250 mL	Grab
F	21-Jul-09	10:25 AM	React, no air	250 mL	Grab
G	21-Jul-09	10:25 AM	React, no air	250 mL	Grab
H	21-Jul-09	10:40 AM	React, no air	250 mL	Grab
I	21-Jul-09	10:40 AM	React, no air	250 mL	Grab
J	21-Jul-09	10:55 AM	React, air	250 mL	Grab
K	21-Jul-09	10:55 AM	React, air	250 mL	Grab
L	21-Jul-09	11:10 AM	React, air	250 mL	Grab
M	21-Jul-09	11:10 AM	React, air	250 mL	Grab

**Phase-contrast Microscopy.** Wet mounts of sample collected on July 7, 2009 (Table 1A) were prepared and examined with phase-contrast microscopy using a Zeiss Axio Imager.M1 microscope (Carl Zeiss, Jena, Germany); Fig. 4.

**DNA Extraction.** All samples were stored at 4°C prior to analysis; all samples were analyzed within 24 hours of collection. DNA was extracted from 3 mL of activated sludge, which was centrifuged for 10 minutes at 13000 rpm to yield a 0.25 g pellet using an Ultraclean Soil DNA Extraction Kit (Mobio Laboratories, Solano Beach, CA). 20-60 ng/μL of genomic DNA was extracted from each sample, measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA); DNA extracts were diluted 10-fold, and all genomic DNA was stored at -20°C.

**Cloning, Clone Screening, and Sequencing.** Primer sets 8F/1492R, 109F/934b, Pla46f/Amx820, and amoA-1F/amoA-2R were used for amplification of bacterial 16S rRNA, archaeal 16S rRNA, *Planctomycetes* 16S rRNA, and bacterial amoA genes, respectively, via

PCR (Table 2). PCR mixtures amplifying 16S rRNA contained 12.5  $\mu$ L Promega GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 400 nM of each primer (Integrated DNA Technologies, Coralville, IA), and 1  $\mu$ L 10-fold diluted template DNA in a final volume of 25 $\mu$ L. Bacterial amoA gene fragments were amplified with PCR mixtures containing 12.5  $\mu$ L Promega GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 800 nM of each primer (Integrated DNA Technologies, Coralville, IA), and 3  $\mu$ L 10-fold diluted template DNA in a final volume of 25 $\mu$ L. DNA amplification was performed with a PTC 200 Thermal Cycler (MJ Research, Ramsey, MN), refer to Table 3 for thermal cycling conditions. Amplified DNA was verified by electrophoresis of 3  $\mu$ L of PCR mixtures in 1% agarose in 0.5X TBE. All PCR products were cloned directly using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Only the *Planctomycetes* and bacterial amoA clones were screened by colony PCR using the vector primers M13F/M13R. Clones were sequenced with an automated sequencer at the Josephine Bay Paul Center Sequencing Facility (Marine Biological Laboratory, Woods Hole, MA).

Target	Primer	Primer name	Sequence (5'→3')	Reference
Bacteria 16S rRNA	Forward	8f	AGA GTT TGA TCM TGG CTC AG	Lane (1991)
	Reverse	1492R	GGT TAC CTT GTT ACG ACT T	
Archaea 16S rRNA	Forward	109f	ACK GCT CAG TAA CAC GT	Grosskopf et al. (1998)
	Reverse	934b	GTG CTC CCC CGC CAA TTC CT	
<i>Planctomycetes</i> 16S rRNA	Forward	Pla46f	GGA TTA GGC ATG CAA GTC	Egli et al. (2001)
	Reverse	Amx820	AAA ACC CCT CTA CTT AGT GCC C	
Bacterial amoA gene	Forward	amoA-1F	GGG GTT TCT ACT GGT GGT	Rotthauwe et al. (1997)
	Reverse	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	

Step	8F/1492R		109F/934b		Plaf/Amx820		amoA-1F/amoA-2R	
	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)	Temp. (°C)	Time (sec)
1. Initial denaturation	95	300	94	300	95	240	95	300
2. Denaturation	95	30	94	60	95	45	94	30
3. Annealing	46	30	52	60	56	45	48	60
4. Extension	72	90	72	75	72	60	72	60
5. Repeat step 2-4	30		30		35		30	
6. Final extension	72	300	72	360	72	180	72	300

**Phylogenetic Analyses.** Nucleotide sequences were screened for quality and then analyzed. Sequences were aligned with the NAST alignment tool available [greengenes.lbl.gov](http://greengenes.lbl.gov) (DeSantis, *et al.*, 2006a). Once aligned sequences were checked for chimeras using the Bellerophon (version 3, (DeSantis, *et al.*, 2006b)) tool at the Greengenes website ([greengenes.lbl.gov](http://greengenes.lbl.gov)). The aligned, non-chimera sequences were classified using the classification tool at the Greengenes website

(greengenes.lbl.gov). The classified sequences are displayed graphically by domain in Fig. 6 (Bacteria) and Fig. 7 (Archaea).

The Arb program package ([www.arb-home.de](http://www.arb-home.de); (Ludwig, *et al.*, 2004) was used to generate the phylogenetic tree in Fig. 8. Nucleotide sequences from clones targeting the *Planctomycetes* 16S rRNA gene were imported into Arb and aligned using the fast aligner tool. Aligned sequences were then manually refined and added to a prealigned 16S rRNA gene database. After selecting a subset of sequences closely related to the *Planctomycetes* group, a phylogenetic tree was calculated with the maximum-likelihood method using AxML + FastDNA ML; the results are displayed as a neighbor-joining tree. Only *Escherichia coli* positions 1789-22467, i.e. between primers Pla46f and Amx820, were used to calculate the 16S rRNA gene-based tree.

**T-RFLP.** Bacteria 16S rRNA genes were amplified with the primer set 8F/1492R. The 8F primer was fluorescently labeled at the 5' end with 6-carboxyfluorescein (6-FAM). Reaction mixtures for PCR were done in triplicate for each sample; each reaction mixture contained 12.5  $\mu$ L Promega GoTaq® Green Master Mix (Promega Corporation, Madison, WI), 400 nM of each primer (Integrated DNA Technologies, Coralville, IA), and 1  $\mu$ L 10-fold diluted template DNA in a final volume of 25  $\mu$ L. DNA amplification was performed with a PTC 200 Thermal Cycler (MJ Research, Ramsey, MN) by using the following the same program as listed for 8F/1492 primer set in Table 3. Amplified DNA was verified by electrophoresis of 3  $\mu$ L of PCR mixtures in 1% agarose in 0.5X TBE.

Fluorescently labeled PCR products from the for each sample were pooled (72  $\mu$ L) and purified using a QuickStep™2 PCR Purification Kit (Edge Biosystems, Gaithersburg, MD). Amplified 16S rRNA gene products were digested separately with HhaI (GCG' C) and RsaI (GT' AC). The digestion mixtures consisted of 0.4  $\mu$ L BSA (only for HhaI), 4  $\mu$ L of 10X buffer 4, 0.75  $\mu$ L enzyme (HhaI), 1  $\mu$ L enzyme (RsaI), and 300 ng amplified PCR products in a final volume of 40  $\mu$ L. Both digestion reaction conditions were as follows: incubation at 37°C for 3 hours and denaturation for 20 minutes at 80°C. Restriction digests were cleaned using an iso-propanol assay and then analyzed on a 3730XL capillary sequencer, DNA analyzer (Applied Biosystems, Foster City, CA). Fragments were resolved by using an Applied BioSystems GeneScan 500 Liz marker (Josephine Bay Paul Center Sequencing Facility, Marine Biological Laboratory, Woods Hole, MA). The GeneMarker software (available at [www.softenetics.com](http://www.softenetics.com)) was used to generate an electropherogram for each sample; an overlay of all samples by enzyme was generated (Fig. 9, RsaI overlay not shown).

T-RFLP data was processed and analyzed with the T-REX software (Culman, *et al.*, 2009) available at [trex.biohpc.org](http://trex.biohpc.org). Raw data files that contained the peak information for samples, divided by restriction enzyme, were tabulated with GeneMapper software (version 4; Applied Biosystems). The data was subjected to quality control procedures: T-RF Alignment (clustering threshold =0.5), Noise Filtering (peak area, standard deviation multiplier = 1), and elimination of samples with less than 20 T-RFs. Processed data were subjected to AMMI analysis with T-REX using a data matrix constructed based on the presence/absence of peaks in several ways for each enzyme (HhaI, Figs. 10-13; RsaI, data not shown):

1. All samples defined as un-replicated (variability between all samples),
2. Samples from SBR1 taken on July 7 and July 21 with the July 21 samples grouped as replicates (variability in samples from one reactor over time),

3. Only samples from July 21 grouped as replicates (variability in samples collected during one cycle of one reactor), and
4. Only samples from July 21 not grouped as replicates (variability across samples taken during one cycle in one reactor).

With replicated data, the AMMI analysis provides a calculation interaction pattern and noise which provides a more resolute picture of the strength of the interaction term (Culman, *et al.*, 2009).

**CARD-FISH.** A cell pellet from the sample collected on July 7, 2009 (Table 1A) was formed by centrifuging 1.5 mL of activated sludge for 10 minutes at 13000 rpm. The cell pellet was re-suspended in 1% paraformaldehyde and fixed at room temperature for 1 hour. After washing with 1X PBS, the fixed sample was stored in 1X PBS: ethanol (1:1) at -20°C until further processed.

Sample filtration onto membrane. Prior to hybridization planktonic cells were filtered onto membrane filters. A moistened support filter (0.45 µm pore size, cellulose nitrate, 47 mm diameter) and a membrane filter (0.2 µm pore size, white polycarbonate, 47 mm diameter) were placed into a filtration tower with the glossy side of the membrane filter facing up. Due to the high number of cells in the fixed sample, 10 µL and 20 µL of sample were diluted in 10 mL of 1X PBS, respectively, to dilute the cell number. The samples were then filtered by applying a vacuum to the filter tower. After filtration the membrane filters were air dried.

Hybridization. Samples were processed as previously described (Pernthaler, *et al.*, 2002, Ishii, *et al.*, 2004) with the following modifications (see Table 4A, 4B); 1:500 dilution of tyramide was used. Probes Eub(I-III), Non338, Arch915, and Euk516 were used in this study (Table 5, (Amann & Fuchs, 2008)).

Microscopy. Microscopic images of hybridized samples were recorded on a Zeiss Axio Imager.M1 (Carl Zeiss, Jena, Germany) using an AxioCam MRc camera and the AxioVision software (version 4.7); Fig. 5.

TABLE 4A. Summary of steps for CARD-FISH of activated sludge from SBR1 July 7, 2009

Stage	Step no.	Description <sup>a</sup>
Embedding	1	Prepare 0.2% (w/v) low-gelling-point agarose (Metaphor agarose) in milliQ water. Boil agarose; cool to 35-40°C
	2	Dip both sides of filters in agarose; place filters face down onto a glass slide. Dry at 46°C for at least 15 min.
	3	Peel filters from glass slide after letting them stand in 2 mL of 80% (v/v) ethanol (30 sec, RT).
	4	Air dry filters. <sup>b</sup>
Permeabilization and inactivation of peroxidases	5	Cut filters into sections and label with a pencil.
	<i>For bacterial probe sections follow steps 6-7. For archaeal and eukaryal probe sections follow steps 6Ai-8Ai or 6Aii-8Aii</i>	
	6B	Incubate in fresh lysozyme solution (60 min, 37°C).
	7B	Wash filters in milliQ water (1 min, RT).
	8B	Wash filters in milliQ water (1 min, RT).
	6Ai	Incubate in 0.15% (v/v) hydrogen peroxide in methanol (30 min, RT).
	7Ai	Wash filters in 10 mL 0.1M hydrochloric acid (1 min, RT).
	8Ai	Wash filters in 50 mL 1X PBS (5 min, RT).
	6Aii	Wash filters in 10 mL 0.1M hydrochloric acid (1 min, RT).
	7Aii	Wash filter in 50 mL 1X PBS (1 min, RT).
	8Aii	Incubate filters in 10 mL 3% (v/v) hydrogen peroxide in milliQ water (10 min, RT).
9	Wash filters in milliQ water (1 min, RT).	
10	Wash filters in 100% (or 200 proof) ethanol (1 min, RT).	
11	Air dry filters. <sup>b</sup>	
<sup>a</sup> RT, room temperature.		
<sup>b</sup> Preparations may be stored at -20°C for several days to weeks without an apparent loss in signal		

Hybridization	12	Place filter sections in reaction vial (0.5 ml, 10 to 20 sections per vial).
	13	Mix 400 µl of hybridization buffer and 4 µl of probe working solution, and add to filter sections.
	14	Incubate sections at 46°C for at least 3 h.
	15	Wash sections in prewarmed washing buffer (1 min, 48°C); immediately transfer filters to second vial of washing buffer.
	16	Wash sections in prewarmed washing buffer (15 min, 48°C); do not air dry filter sections after washing them.
	Tyramide signal amplification	18
20		Incubate filters in substrate mix (1 part Cy3-tyramide, 10 parts of amplification diluent) (30 min, 46°C, in the dark).
21		Dab filter on blotting paper.
22		Wash sections in 50 mL 1µ PBS (pH 7.6) for 15 min at RT (in the dark)
23		Wash filter in 10 ml of milliQ water (1 min, RT, in the dark).
24		Wash filter in 10 ml of 96% (v/v) ethanol (1 min, RT, in the dark).
25		Air dry preparations. <sup>b</sup>
26	Counterstain filters with DAPI. <sup>b</sup>	
<sup>a</sup> RT, room temperature.		
<sup>b</sup> Preparations may be stored at -20°C for several days to weeks without an apparent loss in signal		

Probe name	Specificity	Sequences (5'-3')	FA <sup>a</sup> (%)	T <sub>h</sub> <sup>b</sup> (°C)
Eub(I-III)	Bacteria	Mixture of Eub338, Eub338P, Eub338V	35	46
Eub338	Most Bacteria	GCT GCC TCC CGT AGG AGT		
Eub338P	<i>Planctomyces</i>	GCA GCC ACC CGT AGG TGT		
Eub338V	<i>Verrucomicrobium</i> spp.	GCT GCC ACC CGT AGG TGT		
Non338	Antisense of EUB338	ACT CCT ACG GGA GGC AGC	35	46
Arch915	Archaea	GTG CTC CCC CGC CAA TTC CT	35	46
Euk516	Eukarya	ACC AGA CTT GCC CTC C	0	46
<sup>a</sup> Formamide concentration in the hybridization buffer.				
<sup>b</sup> Hybridization temperature.				

## Results and Discussion

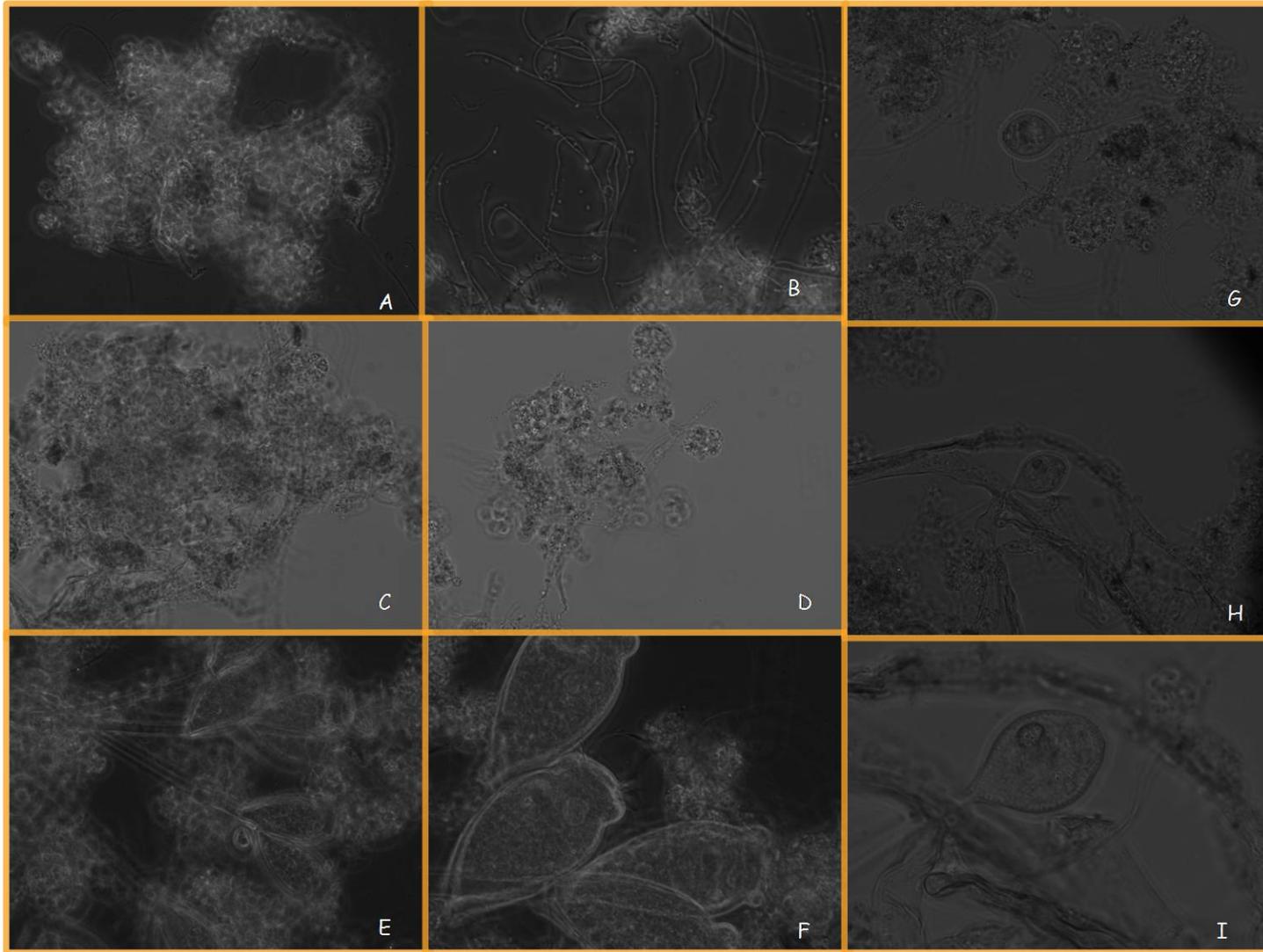
Samples were collected from the SBRs at the Falmouth Wastewater Treatment Facility (FWTC) on July 7, July 15, and July 21, 2009. Only the sample collected on July 7 was analyzed in depth. This analysis included phase contrast microscopy (Fig. 4), CARD-FISH (Fig. 5), cloning and sequence classification (Fig. 6-7), and a phylogenetic tree of *Planctomycetes* clones (Fig. 8).

Within a few hours of sample collection, a wet mount of the sample was prepared and examined with phase contrast microscopy. Various sized cell aggregates, filamentous microorganisms, and protozoa were visible at 20X or 40X magnifications (Fig. 4). Wet mounts were also prepared for samples collected July 15 and July 21; however, micrographs were only taken for the July 7 sample.

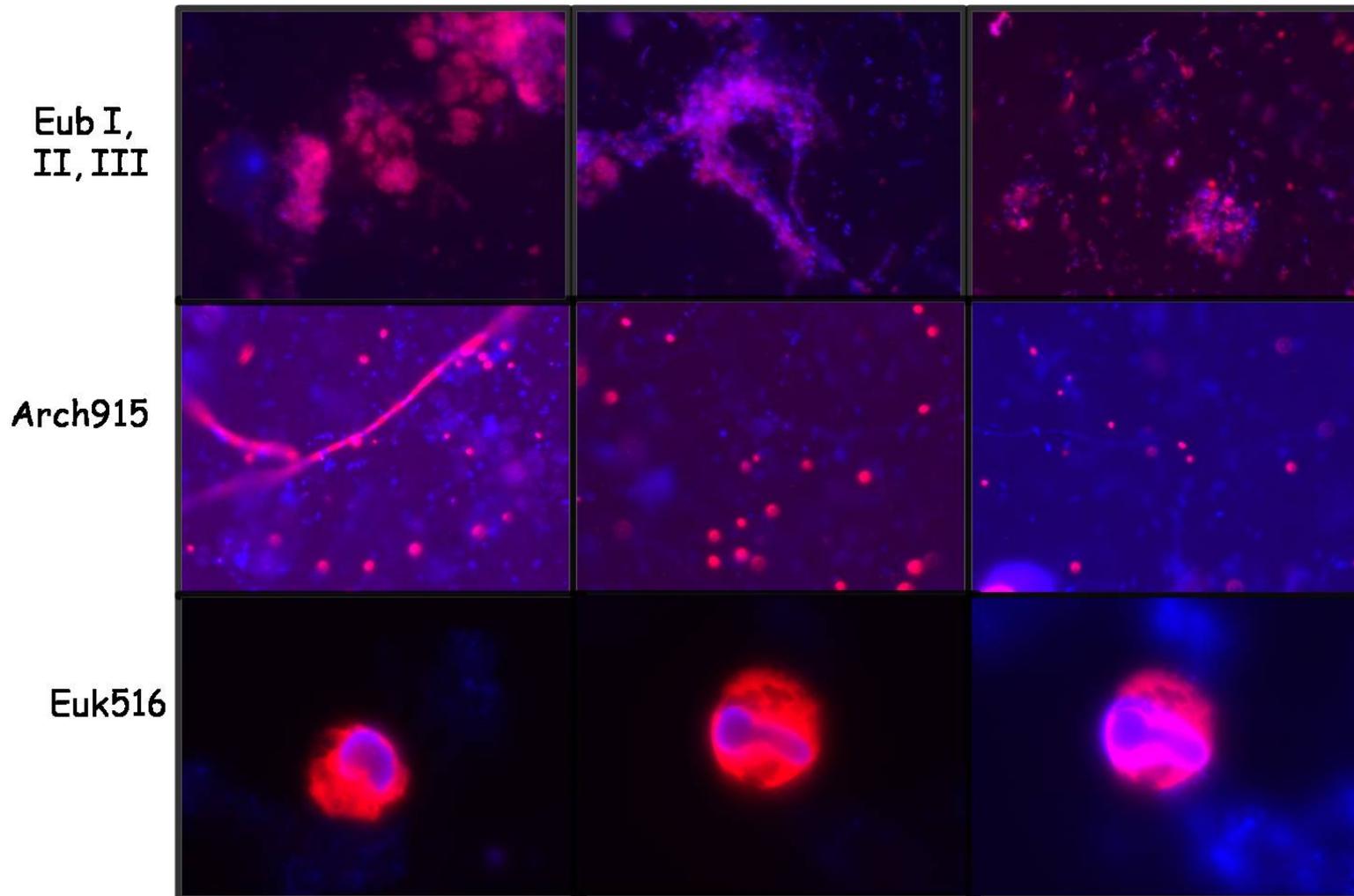
Fluorescence in situ hybridization with catalyzed reporter deposition (CARD-FISH) was used to identify cells in the sludge aggregates. DAPI was used to stain all DNA in the aggregates blue, while the CARD-FISH probes bound to specific rRNA; the probe combination Eub(I-III) was used to target nearly all the Bacteria, the probe Arch915 was used to target all Archaea, and the probe Euk516 was used to target all Eukarya (Fig. 5). From observation of the filter sections Bacteria are the dominant microbes in the sludge microbial community. The Archaea are also abundant within the community, while the protozoa make up a small fraction of the community. Quantification of cell numbers was not possible because of the high cell density on the filter sections made it difficult to take high quality images for image analysis software.

A 16S rRNA gene clone library was made to identify abundant microorganisms in the sample collected on July 7. The sequences were aligned, chimera-checked, and classified using Greengenes. Fig. 6 is the graphical representation of the numerical composition of the bacterial 16S rRNA clones at the phylum level. 13% of the clones were from the *Nitrospirae* group and were related to *Nitrospira*, which are known nitrite oxidizing bacteria; only 1 clone was related to a known ammonia oxidizing bacteria. An attempt was made to amplify the bacterial *amoA* gene; however, sequences from these clones were of poor quality and not included in subsequent analysis. Ammonia oxidizing bacteria and nitrite oxidizing bacteria are present within the microbial community, so I would expect that nitrification is taking place within the reactor at this time. Fig. 7 is the graphical representation of the numerical composition of the archaeal 16S rRNA clones at the phylum level. All of the clones were members of the *Euryarchaeota*. It is interesting to note that several of the bacteria and archaeal clones are related to known thermophilic organisms; I did not obtain temperature data on the reactor, so it is not possible to say if the temperature in the reactor is high enough to sustain the growth of thermophilic organisms.

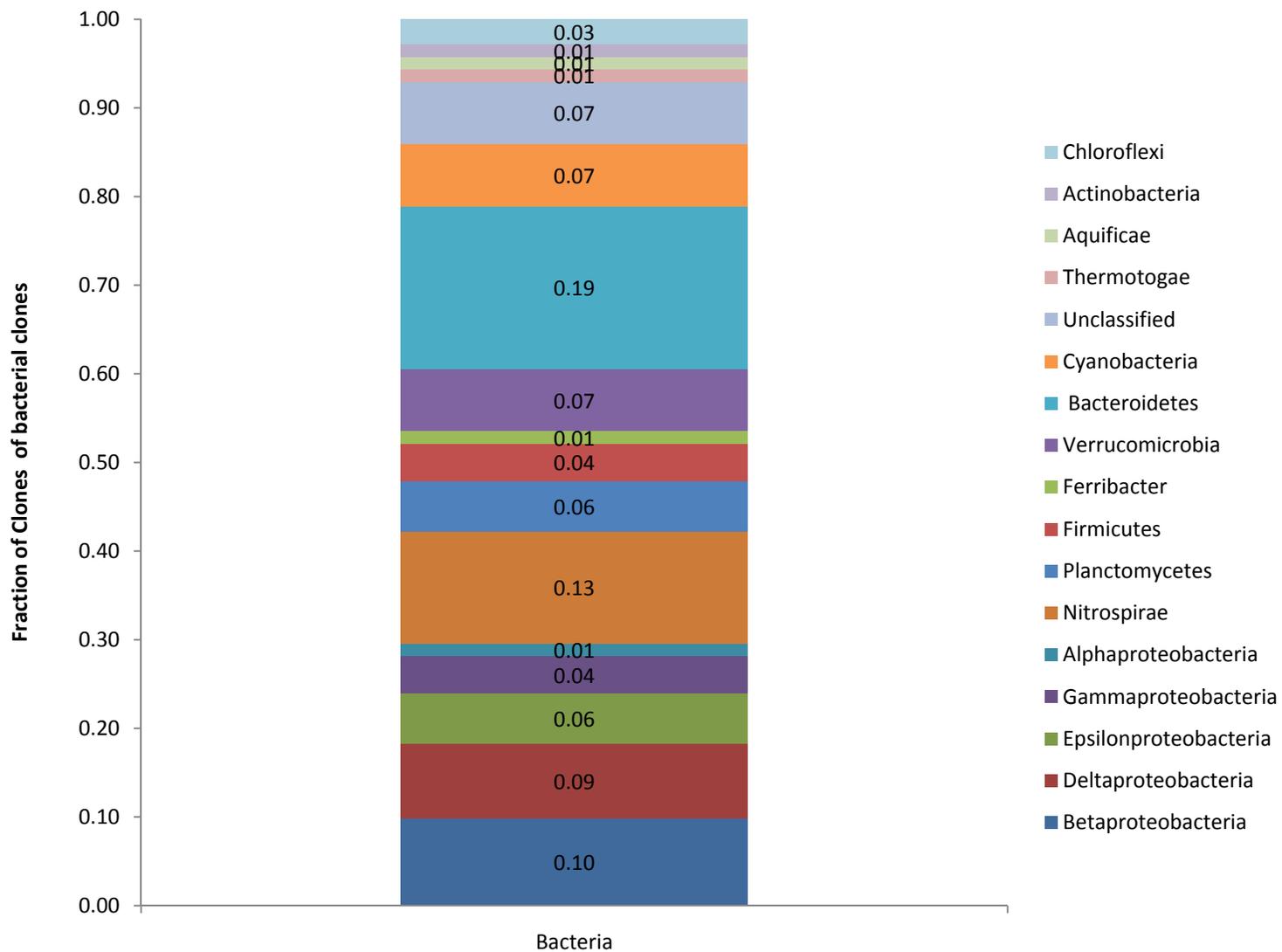
It is thought that anaerobic ammonia oxidation (anammox) occurs in all wastewater treatment plants (personal communication J. Gijs Kuenen). A *Planctomycetes* 16S rRNA specific forward primer (Pla46f) and an anammox specific reverse primer (Amx820) were used to detect potential anammox organisms in FWTF. The sequences from these clones were aligned in Arb and a phylogenetic tree was generated (Fig. 8). The clones from FWTF cluster together and are close enough, from an evolutionary perspective, to the known anammox bacteria to warrant further investigation into their potential anammox activity.



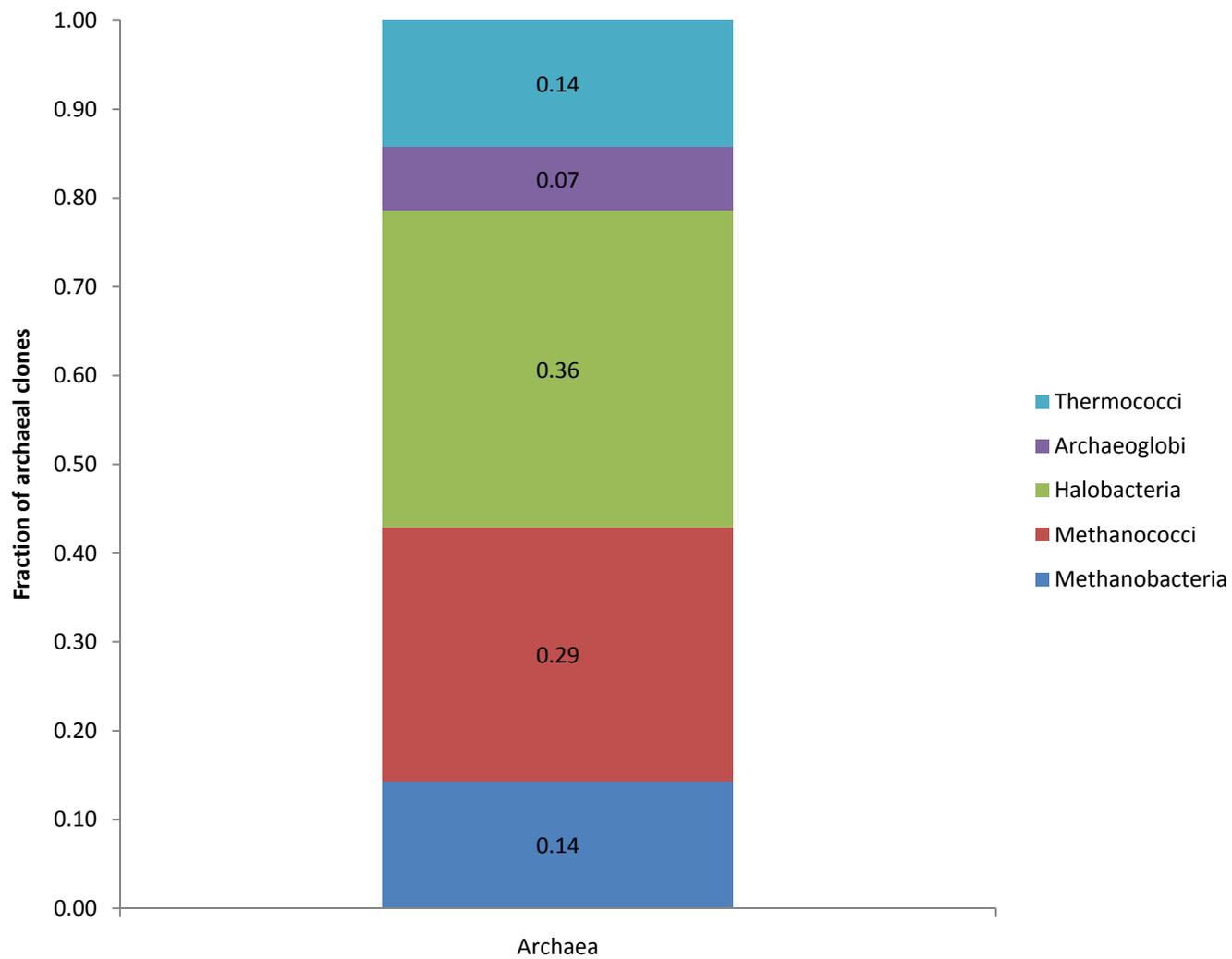
**Fig. 4.** Phase contrast images of sample collected July 7, 2009. Images: A) sludge aggregate, B) filamentous microorganisms, C) sludge aggregate, D) sludge aggregate, and G)-I) various protozoa present within the microbial community. Magnification varies from 20X to 40X.



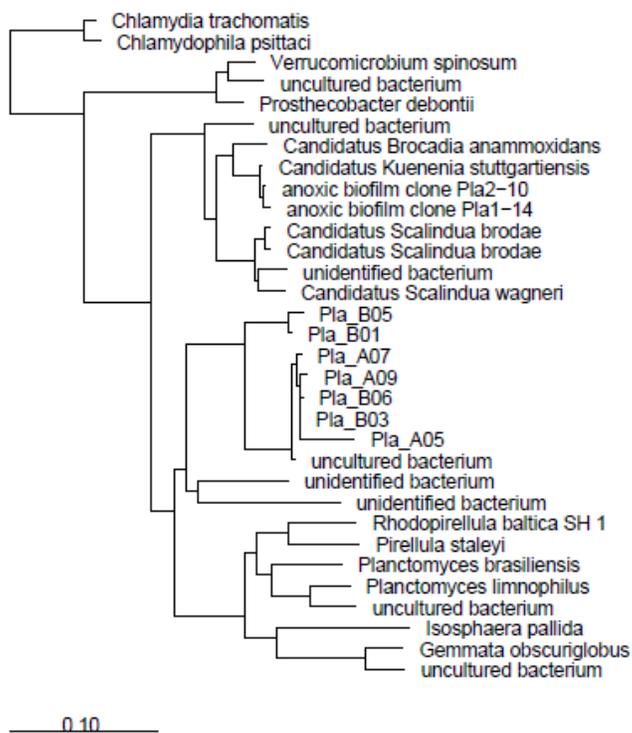
**Fig. 5.** CARD-FISH images from all three probes used in this study. The rows correspond to the probes used in the hybridization; three images per probe. Red fluorescence is from the probe hybridized to rRNA of Bacteria, Archaea, and Eukarya, respectively; blue fluorescence is from DAPI, which binds to DNA. The sludge microbial community is dominated by bacteria (Row 1); the majority of the cells in the aggregates fluorescence red. Archaea are also abundant within the community (Row 2), and while protozoa are present (Row 3) they make up a small fraction of the community.



**Fig. 6.** Classification of aligned, non-chimera, bacterial 16S rRNA sequences using the NAST aligner, Bellophon, and classification tool at the Greengenes website (greengenes.lbl.gov). Total number of clones = 69. Numbers in graph indicate fraction of clones at the phylum level.

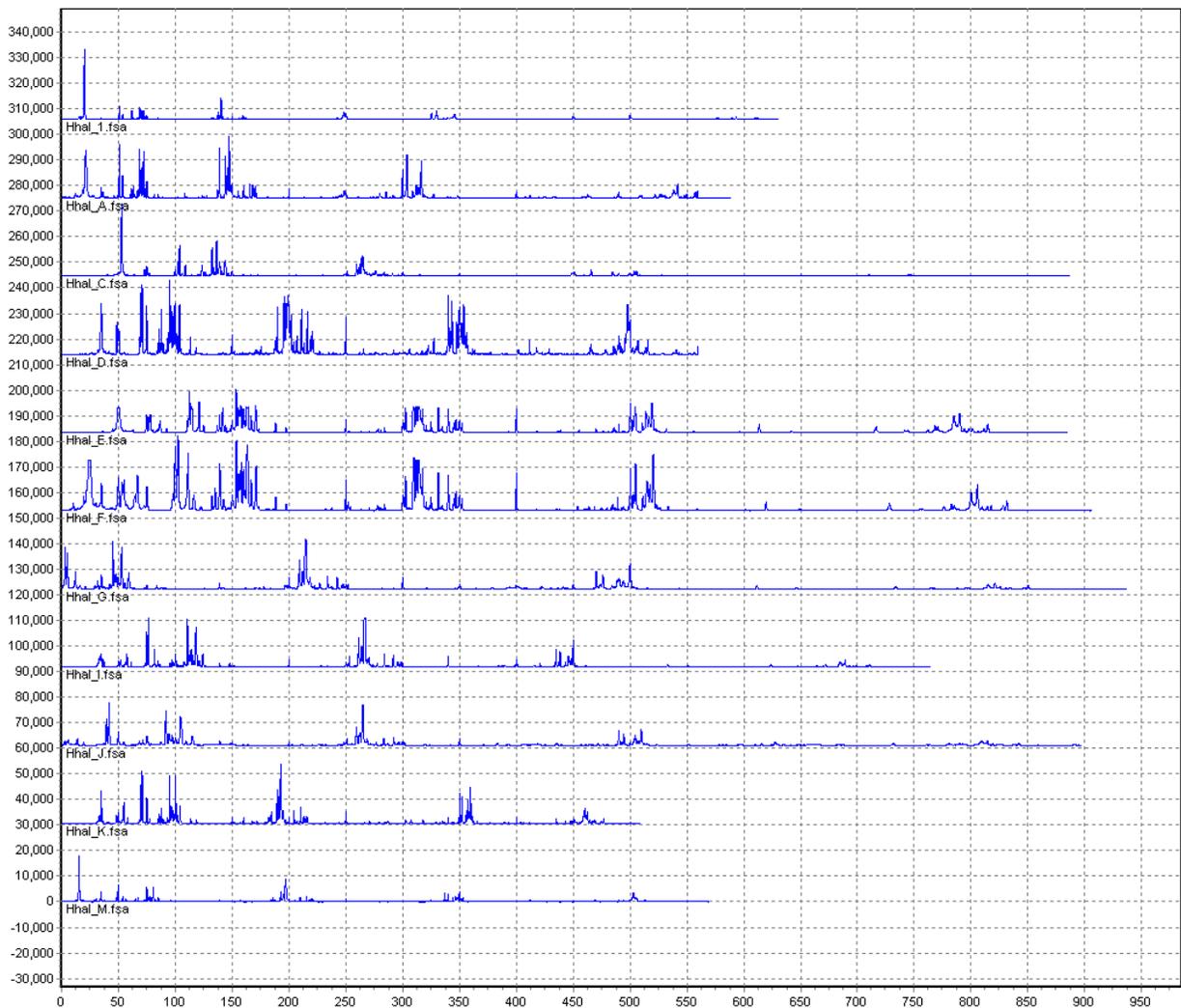


**Fig. 7.** Classification of aligned, non-chimera, archaeal 16S rRNA sequences using the NAST aligner, Bellophon, and classification tool at the Greengenes website (greengenes.lbl.gov). Total number of clones = 14. Numbers in graph indicated fraction of clones at phylum level.



**Fig. 8.** Phylogenetic tree (neighbor joining method) of partial 16S rRNA gene (653 bp) of *Planctomycetes* clones. Sequences or sequence groups retrieved within this study are identified as “Pla...” Scale bar =10% sequence difference.

The DNA fingerprinting technique terminal restriction fragment length polymorphism (T-RFLP) was used to analyze the microbial community in several samples. Samples were collected on three days and at several time points within the React cycle of the SBR for a total of 14 samples. All 14 samples were digested with two restriction enzymes HhaI (GCG’C) and RsaI (GT’AC). The HhaI digestion reaction yielded more T-RFs than RsaI, consequently only results from the HhaI digestion are shown in this report. Fig. 9 shows the electropherograms of all 14 samples on one graph, which is an overlay. The peak heights in some of the graphs distorted the data because of too much DNA in the digest; therefore, the samples were analyzed only the basis of the presence or absence of T-RFs (Fig. 10-13). The T-REX software program was used to analyze the T-RFLP data. Four analyses are presented. In the first analysis (Fig. 10), all of the samples collected are treated as individual samples without replicates to examine the variability between all samples collected. The samples taken during the aerobic phase of the React cycle on July 21 (E11, E12, E13, E14) cluster in quadrants III and IV; samples E13 and E14 cluster close together in quadrant III, which is expected as they were collected at the same time point. Of the samples collected at the same time point only E5 and E6 cluster together. This data suggests that the variability between the samples is high. In the second analysis (Fig. 11), the July 7 sample and the July 21 samples are compared with the July 21 samples treated as replicates (see Table 2) to examine the variability in one reactor over time. Relative to the sample collected on July 7, the samples July 21 are more closely related to each other, suggesting variability over time in this reactor. In the third analysis (Fig. 12), samples collected at the same time point on July 21, during the React cycle in SBR1, are treated as replicates to examine the variability during one cycle. There is no clustering between the environments which indicates a high degree of variability between the samples collected when they are treated as replicates.

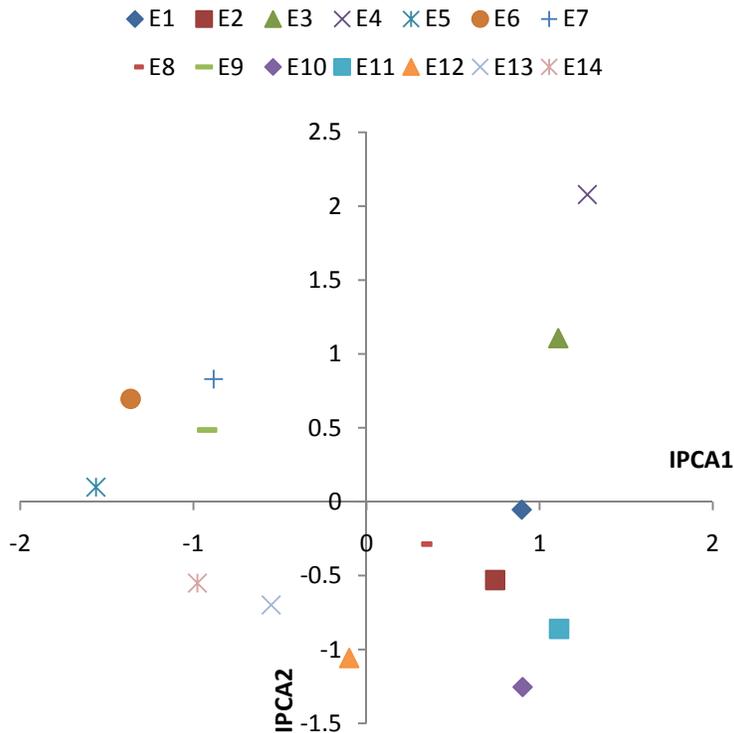


**Fig. 9.** Overlay of electropherograms of all samples digested with restriction enzyme HhaI; x-axis is size (number of bp) in fragment, y-axis is peak height. Valid range of fragment sizes is 50-500 bp. Graph generated with GeneMarker software ([www.softgenetics.com](http://www.softgenetics.com)).

In the fourth analysis (Fig. 13), the July 21 samples are treated as individual samples without replication to examine variability of samples collected during one cycle in the same reactor. The only “replicates” that cluster together when treated as individual samples are E13 and E14, the aerobic samples collected at the last time point.

The T-RFLP analysis shows that there is a high degree of heterogeneity in this system. However, this is a small sample population, and discernible patterns within the microbial community may develop when more samples are included in the analysis.

**Hhal: 533 T-RFs, 14 Environments**

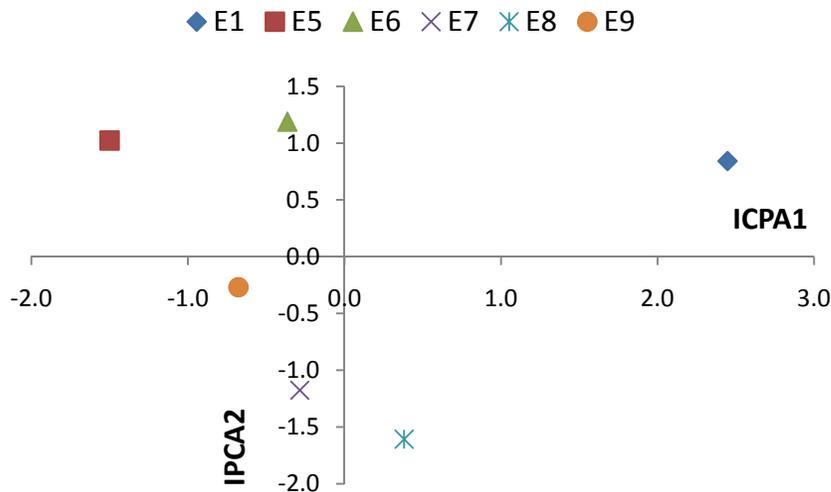


**Fig. 10.** T-RFLP profiles with each sample treated as an individual replicate to examine the variability between all samples collected.

Environments:

1. E1-Sample 1
2. E2-Sample A
3. E3-Sample B
4. E4-Sample C
5. E5-Sample D
6. E6-Sample E
7. E7-Sample F
8. E8-Sample G
9. E9-Sample H
10. E10-Sample I
11. E11-Sample J
12. E12-Sample K
13. E13-Sample L
14. E14-Sample M

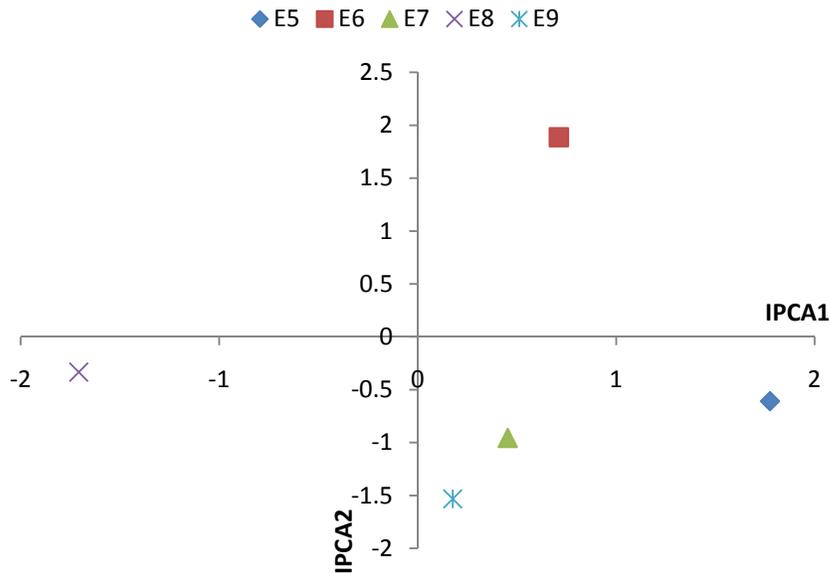
**Hhal: 495 T-RFs, 6 Environments**



**Fig. 11.** T-RFLP profile with July 7 sample with July 21 samples as replicates to examine the variability in one reactor over time. Environments:

1. E1-Sample 1
2. E5-Samples D, E
3. E6-Samples F, G
4. E7-Samples H, I
5. E8-Samples J, K
6. E9-Samples L, M

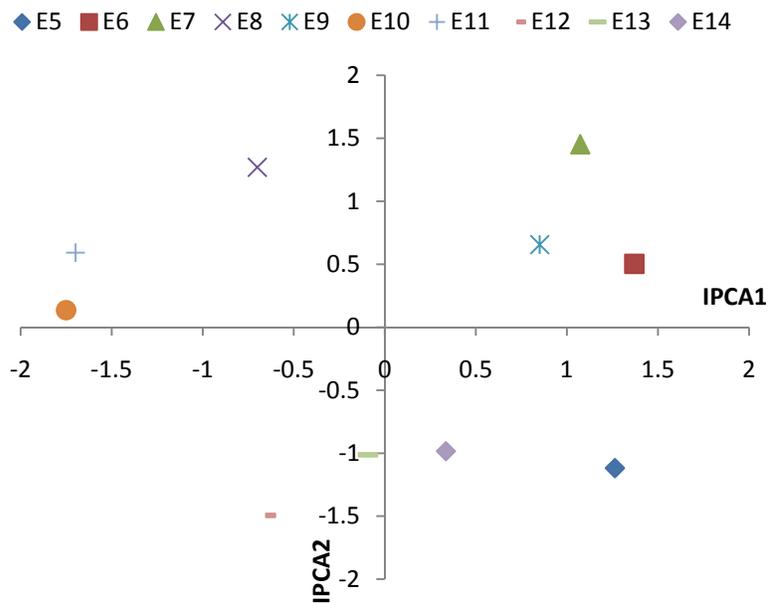
### Hhal: 485 T-RFs, 5 Environments



**Fig. 12.** T-RFLP profile of samples collected July 21 during react cycle in SBR1. Samples collected at the same time point were treated as replicates to examine the variability during one cycle.

- Environments:
- 7. E5-Samples D, E
  - 8. E6-Samples F, G
  - 9. E7-Samples H, I
  - 10. E8-Samples J, K
  - 11. E9-Samples L, M

### Hhal: 484 T-RFs, 10 Environments



**Fig. 13.** T-RFLP profile with July 21 samples treated as individual samples without replication to examine variability of samples collected during one cycle in the same reactor.

- Environments:
- 1. E5-Sample D
  - 2. E6-Sample E
  - 3. E7-Sample F
  - 4. E8-Sample G
  - 5. E9-Sample H
  - 6. E10-Sample I
  - 7. E11-Sample J
  - 8. E12-Sample K
  - 9. E13-Sample L
  - 10. E14-Sample M

This study demonstrated that organisms involved in the nitrification, the first step of the conversion of reduced nitrogen to nitrogen gas, are present in FWTF, and that the microbial community is dominated by bacteria although both archaea and eukarya are members of the community. Microbes related to anammox bacteria are also present in the system; however, their anammox activity was not determined. The performance data from FWTF shows that nitrogen removal is unstable in the reactor, particularly during the fall and winter. If microbial community structure and operation can be linked to performance over time, then reliable performance with respect to nitrogen removal can be achieved in the FWTF.

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