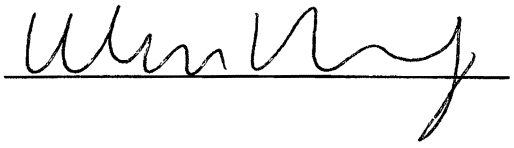
8-2014

Assessment of Nitrifying Bacteria in Massard Plant Using Molecular Tools

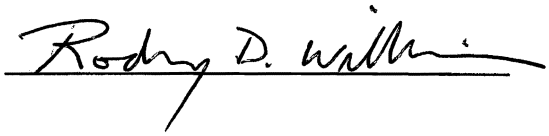
Jennifer Puanani Holmes-Smith
University of Arkansas, Fayetteville

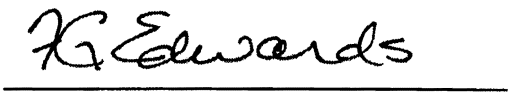
This thesis is approved.

Thesis Advisor:



Thesis Committee:





Assessment of Nitrifying Bacteria in Massard Plant Using Molecular Tools

An Undergraduate Honors College Thesis

in the

Department of Civil Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

Jennifer Puanani Holmes-Smith

Assessment of Nitrifying Bacteria in Massard Plant Using Molecular Tools

Jennifer Puanani Holmes-Smith

Advisor: Wen Zhang

Abstract

The discharge of nutrients (such as nitrogen and phosphorus) from wastewater treatment plants (WWTP) has become an increasingly important issue in the United States. Ammonia (NH_3) is a common contaminant found in domestic wastewater and agricultural runoff. It can cause toxicity in fish if left untreated. The United States Environmental Protection Agency (EPA) recommends low national criteria for ammonia limits in freshwater. With these greater restrictions, ammonia-nitrogen limit compliance (5 mg/L) has become an issue at the Massard WWTP in Fort Smith, Arkansas. The purpose of this research is to assess the ammonia removal in the Massard WWTP in order to improve the ammonia-nitrogen removal in the future. This purpose led to the testing of *Nitrosomonas europaea* in monitored wastewater samples. The addition of *N. europaea* in the wastewater did not result in improved ammonia removal, indicating inhibitions were present. When analyzing the activated sludge and trickling filter biomass using fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR), indigenous nitrifiers were found present, though not in great quantity. Future studies are needed to determine the identity of the nitrifying-inhibiting factors.

Introduction

Background

The excessive discharge of ammonia from individual WWTPs has become an increasingly important issue in the United States. Ammonia is a common contaminant found in domestic wastewater and agricultural runoff. Total Ammonia is the combination of normal ammonia (NH_3) and ionized ammonium (NH^{4+}). While ammonia is a nutrient needed in biological life, excessive ammonia (especially promulgated by industrial runoff and man-made factors) can cause toxicity in fish if left untreated. Aquatic organisms have difficulty excreting the toxicant, which leads to toxic build-up. Even slightly elevated ammonia levels create changes in the metabolism, hyperexcitability, increased heart rate and breathing exertion in aquatic life (Oram, 2014). Short exposures can cause eye and gill damage, as well as development and reproduction deficiencies and injury to internal organs. Extreme ammonia levels can cause comas or death (Randall et al., 2002). Because of these undesirable biological effects, the EPA has recommended low national criterion for ammonia limits in freshwater (EPA, 2013). With these higher restrictions, ammonia-nitrogen limit compliance has become an issue at the Massard WWTP in Fort Smith, Arkansas. The plant has a trickling filter (Figures 1 and 2) as its primary biological treatment unit, and a small activated sludge tank as a supplemental unit as seen in Figure 3. Both trickling filters and activated sludge systems are used to remove organic material from wastewater. The trickling filter functions as an aerobic treatment system using bacteria attached to a medium to remove organic matter from wastewater. These systems are known as attached-growth processes, in contrast to activated sludge systems where microorganisms are sustained in a liquid (EPA, 2014). Figure 1 and 2 show the biomass accumulated within the trickling filter basin at the Massard plant.

Figure 1 and 2: Trickling filter at the Massard Wastewater Treatment Plant (WWTP) in Forth Smith, Arkansas. Figure 1 is a close-up of the rock media with biofilm attached. Figure 2 is a broader view of the trickling filter with dimensions of 100 feet diameter and 8-10 feet depth.

WWTPs use a biological process called nitrification to convert ammonia into a friendlier substance for the environment. This process uses the aerobic autotrophic bacteria *Nitrosomonas sp*. to convert ammonia to nitrite (NO^{2-}), and then *Nitrobacter sp*. to convert nitrite to nitrate (NO^{3-}). The incorporation of aeration (addition of dissolved oxygen) and the aerobic microorganisms in the biological processing unit enables the effective conversion of ammonia in wastewater by microorganisms.

Figure 3: Supplemental Activated Sludge Basin at Massard Plant

According to the data provided by the plant, the biological treatment system of Massard was found to have an ammonia removal efficiency of only 18.52%, which is very low compared to a nearby WWTP called P-Street, whose ammonia removal system has an efficiency of 70%. P-Street plant uses the modern activated sludge system, as opposed to the trickling filter system at the Massard plant. Multiple parameters can affect the efficiency of nitrifying bacteria and its presence in the activated sludge, including temperature, substrate composition, light, and chemical toxins. Nitrifying bacteria are slow-growing and sensitive to the environment, so seasonable variations can make an impact on the removal efficiency of ammonia. The Massard plant was found to achieve a removal efficiency higher than average in late May and June 2013 (higher temperature increases efficiency); however, the average ammonia-nitrogen concentration in the effluent in June 2013 was 6 mg/L and is still above the permitted ammonia-nitrogen EPA limit.

The presence of nitrifying bacteria is key in ammonia removal from wastewater. Multiple techniques are available to assess these bacteria in wastewater and activated sludge. Fluorescence *in situ* hybridization (FISH), is a relatively new technical for detecting and quantifying nitrifiers in activated sludge. FISH is a genetic technique using oligonucleotide probes labeled with fluorescent dyes. The probes are able to bind to a specific genetic sequence of interest, in this case with nitrifying bacteria. This is especially useful in connection to multi-species biofilm, where it can be extremely difficult to differentiate various bacteria species under fluorescence microscope. After hybridization, target species emitting fluorescence enables the identification and topographical visualization in a multispecies biofilm and activated sludge samples (Wang

et al., 2012). Other techniques such as polymerase chain reaction (PCR) can also be used to detect nitrifying bacteria in wastewater and biomass samples. PCR is a biochemical process that takes extracted DNA samples and amplifies a specific strand of DNA to an amount of several orders of magnitude using the appropriate primer. With primers that target genes in nitrifying bacteria, the presence of these genes can be confirmed through gel electrophoresis, and gives a broad indication of the presence or absence of nitrifiers in the samples.

The object of this study was to assess ammonia removal in Massard WWTP through the investigation of nitrifying bacteria in the biological treatment system, and ultimately improve the ammonia removal in the Massard treatment plant.

Materials and Methods

Nitrosomonas europaea Winogradsky (ATCC 25978) purchased from ATCC (Manassas, Virginia) was grown in lab-made ATCC-2265 medium (see Table 1) at 25ºC

Table 1: ATCC-2265 *Nitrosomonas europaea* medium

Solution 1:
$(NH_4)_2SO_4$ (for 50 mM NH_4^+) .....4.95 g
KH_2PO_4 .....0.62 g
$MgSO_4 . 7H_2O$ .....0.27 g
$CaCl_2 . 2H_2O$ .....0.04 g
$FeSO_4$ (30 mM in 50 mM EDTA at pH 7.0) .....0.5 ml
$CuSO_4 . 5H_2O$.....0.2 mg
Distilled water .....1.2 L
Filter sterilized.
Solution 2:
KH_2PO_4 .....8.2 g
NaH_2PO_4.....0.7 g
Distilled water ..... 300.0 ml
Bring to pH 8.0 with 10N NaOH. Filter sterilize.
Solution 3 (buffer):
Na_2CO_3 anhydrous .....0.6 g
Distilled water .....12.0 ml
Filter sterilized.
Complete medium:
Solutions 1, 2 and 3 are combined. Dispensed aseptically into desired aliquots.

for 12 days. OD measurements at 620 nm wavelength were used to monitor the growth of the bacteria, and ammonia measurement within the media was performed three times per week to confirm the growth.

Four grab-samples were taken from the Massard WWTP on March 16, 2014, and 1 mL of nitrifying bacteria culture grown in the lab was added to the wastewater collected from trickling filter effluent and activated sludge basin effluent. Nitrifier growth and ammonia removal were monitored during an 11-day period. Hach kits (ammonia salicylate reagent Cat. 23952-66 and ammonia cyanurate reagent Cat. 23954-66) were utilized to measure the ammonia-nitrogen in the wastewater and removal efficiencies were calculated and evaluated.

Another set of grab-samples were retrieved from the Massard WWTP of activated sludge basin and secondary clarifier in July 2014. At the same time, rock media within the trickling filter basin was also collected to retrieve biofilms. These samples were analyzed using FISH and PCR to detect the presence of nitrifiers. FISH procedure took the following steps: cell fixation, hybridization, and visualization. Activated sludge samples and biomass from trickling filters were fixed with a 4% glutaraldehyde and PBS solution for 4 hours at 4°C. 100 μL of fixed cells were spotted on glass slides and dried for 2 hours at 37°C. A duplicate was made for each sample. Each fixed sample was dehydrated in 70% ethanol and then 100% ethanol for 3 minutes each. All *in situ* hybridizations were performed by using the procedure described by Amann (1990), Manz et al. (1992), and Moberry et al. (1996). NEU probe (5'-CCCCTCTGCTGCACTCTA-3') with a FAM dye attached to the 5' is used to target gene sequence in *N. europaea*. The probe was purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). Two

sets of FISH testing were done. The first was done with the NEU oligonucleotide probe and DAPI as dual staining (similar to Hicks et al. 1992*)*, and the second set was conducted without DAPI presence. Microscope slides with fixed samples were hybridized with 200 μL of hybridization buffer [(35%) formamide (0.9 M NaCl, 20mM Triss Hydrochloride pH 7.2, 0.01% Sodium Dodecyl Sulfate)] and 2 μL of NEU oligonucleotide probe. Slides were incubated for 1.5 hours at 46°C, and 50 μL of DAPI were added for the final 30 minutes of incubation. The secondary FISH process skipped the DAPI step in the 2-hour hybridization. Subsequently, a stringent washing step was conducted, slides were immersed for 20 minutes in 50 mL wash solution (56 mM NaCl, 20 mM Tris/HCl (pH 8.0) and 0.01% SDS)] at 48°C, rinsed with double-distilled H_2O and air-dried overnight (or for the second set, air-dried for 2 days in a humid environment) before microscope viewing.

A Nikon Ni-E fluorescence microscope (Melville, NY) was used to examine the FISH specimens. The slides were viewed with a FITC filter cube in 100X magnification. Eight to ten views of microscope images were taken of each slide. Images were processed using Nikon software.

PCR was performed following DNA extraction. DNA was extracted from activated sludge sample, trickling filter biomass sample, and secondary effluent sample using Qiagen QIAamp DNA Stool Mini Kit (Germantown, MD). PCR was performed on the extracted DNA using amoA-1F (5'-GGGGTTTCTACTGGTGGT – 3') and amoA-2R primers (5'-CCCCTCKGSAAAGCCTTCTTC-3') for 30 cycles. The primers were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). Gel Electrophoresis was performed afterwards to view the PCR product.

Results and Discussion

Tables 2 shows the ammonia removal efficiency following the addition of lab-grown nitrifiers in the wastewater samples taken in March 2014. A decrease in ammonia levels in all samples (including the control growth medium) was observed. However, the performance of a t-test on the wastewater control and wastewater with added bacteria

Table 2: Hach kit results and removal efficiencies

	Control (ATCC)	Tricking Filter Control	Trickling Filter + Bacteria	Secondary Effluent Control	Secondary Effluent + Bacteria
	mg/L NH3-N	mg/L NH3-N	mg/L NH3-N	mg/L NH3-N	mg/L NH3-N
20-Mar	96.10	5.91	5.47	5.85	5.16
		12.77*	6.23	5.79	6.35
21-Mar	92.58	30.50*	6.35	57.86*	4.78
		28.36*	8.49	5.03	5.79
24-Mar	88.18	5.47	5.03	4.84	6.16
		6.35	5.91	4.47	10.50*
26-Mar	90.63	5.66	4.21	5.35	4.59
		4.15	4.28	1.95	5.35
28 -Mar	87.92	4.15	4.59	4.40	6.79
		4.84	4.40	4.09	4.47
31-Mar	76.67	3.65	4.34	4.28	2.14
		3.58	7.30*	3.40	4.03
Removal Efficiency	**20%**	**39%**	**21%**	**42%**	**22%**

**Asterisk represents outliers due to measurement error*

shows the p-value to be below 0.5, meaning the addition of bacteria did not significantly change the ammonia levels. Even in the samples collected (before the addition of nitrifiers), there is no significant difference in ammonia levels comparing trickling filter effluent and activated sludge effluent, indicating activated sludge process was not working properly. This could be caused by the size of the activated sludge basin, as it can only take in a quarter of the plant's average flow, which limited its impact in ammonia removal. The results from Table 2 indicate there could be inhibiting factors in the

wastewater for *N. europaea* growth to occur.

To evaluate if the inhibition present in the wastewater is strong enough to prevent nitrifiers to survive, FISH and PCR were performed on the samples collected in July 2014. Figures 4-7 show the microscopy pictures of samples stained by DAPI and hybridized by NEU probe. DAPI binds with nucleic acid in the samples, and the images showed all bacteria present. Through hybridization, the FAM dye should only bind with N. europaea, however, the amount of NEU probe added to the sample was very high, and it caused unspecific binding to the sample as well. In the images, sometimes the FAM fluorescence completely overlapped with the DAPI signal, which rendered the FISH method unsuccessful (such as Fig 6 and 7). When the effect of unspecific binding was not strong, the difference shown in the images are quite distinct. In Fig. 4 and 5, the green fluorescence by FAM was clearly different from the blue color by DAPI, and FISH captures the presence of *N. europaea* in the activated sludge.

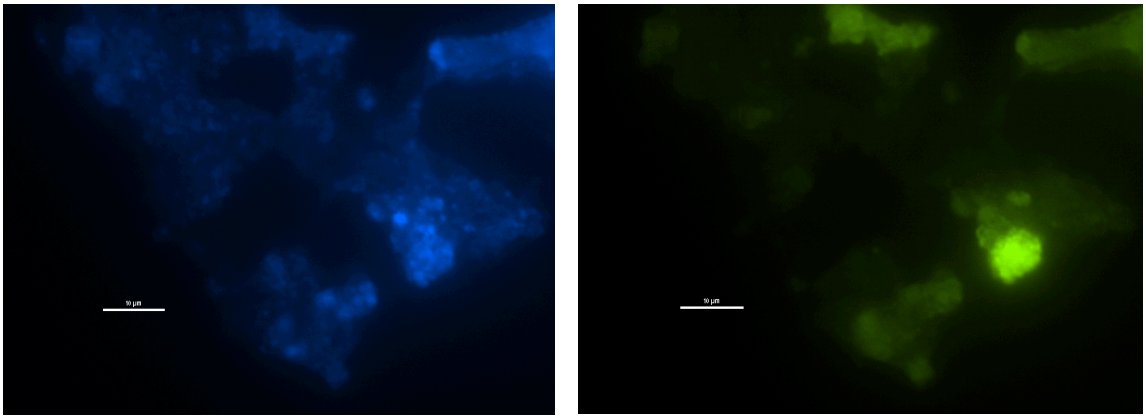


Figure 4 and 5: Activated Sludge sample stained by DAPI (left) and hybridized by NEU probe (right).

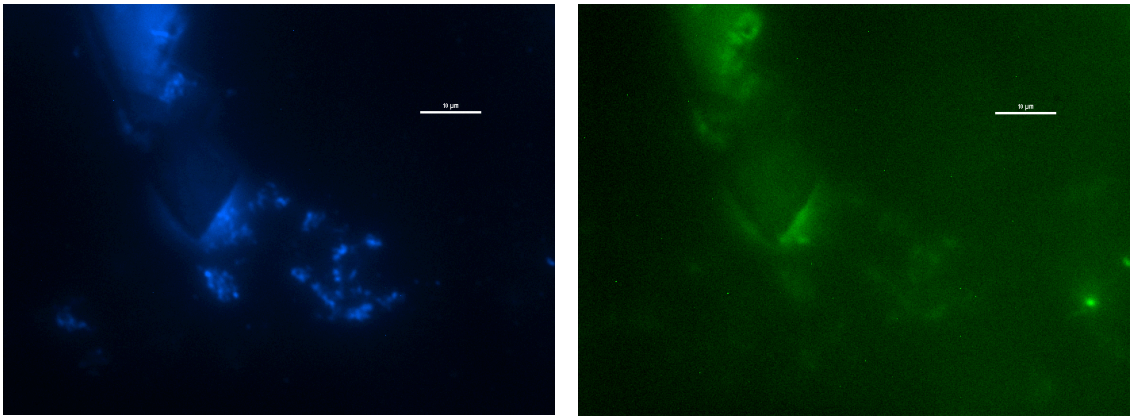


Figure 6 and 7: Trickling Filter sample stained by DAPI (left) and hybridized by NEU probe (right)

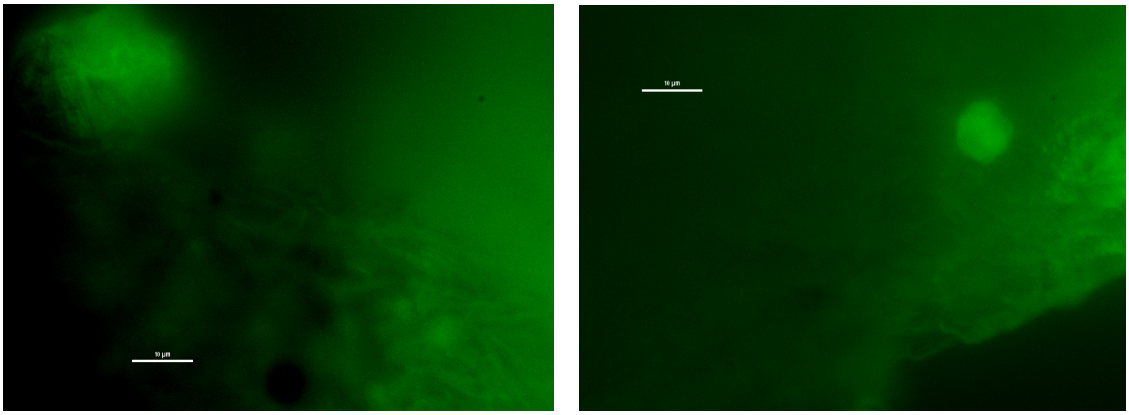


Figure 8 and 9: Trickling filter sample fluorescing with FAM.

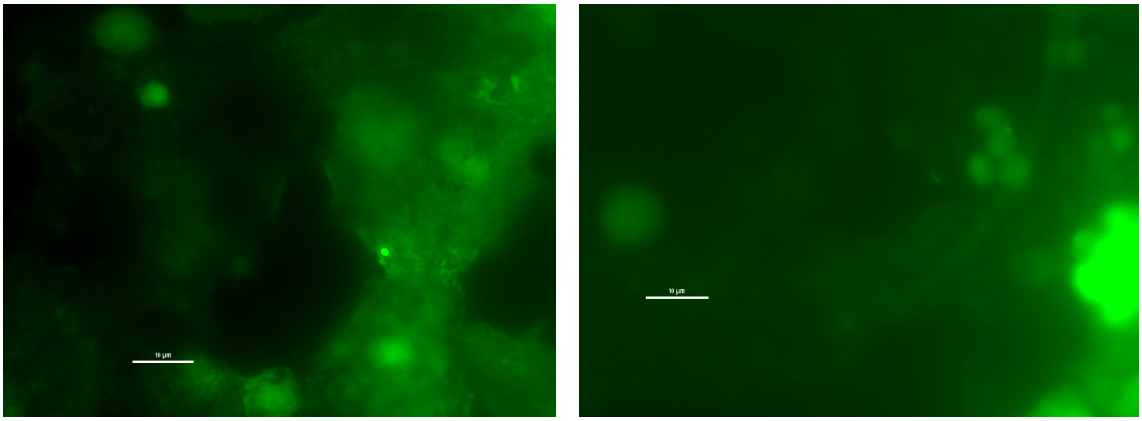


Figure 10 and 11: Activated sludge sample fluorescing with FAM.

The duplicate samples showed similar results (Figures 8-11). It can be concluded that indigenous nitrifiers are present in the activated sludge basin, but it's not clear in the trickling filter biomass.

To confirm the presence of N. europaea in the samples collected, DNA extraction and PCR were performed on the activated sludge, biomass from trickling filter, and secondary effluent. Fig. 12 shows the gel electrophoresis image. According to the genes shown on each lane, the indigenous nitrifying bacteria are present in all samples, with the least amount found in the secondary effluent. Even though they are present in the activated sludge and trickling filter biomass, the quantity is rather small, which explains why the ammonia removal was inefficient in the Massard plant. The inhibitions exhibited from the ammonia addition experiment could also be one of the reason the nitrifiers are not abundant in the biological removal unit, but future analysis is required to evaluate thes inhibitions.

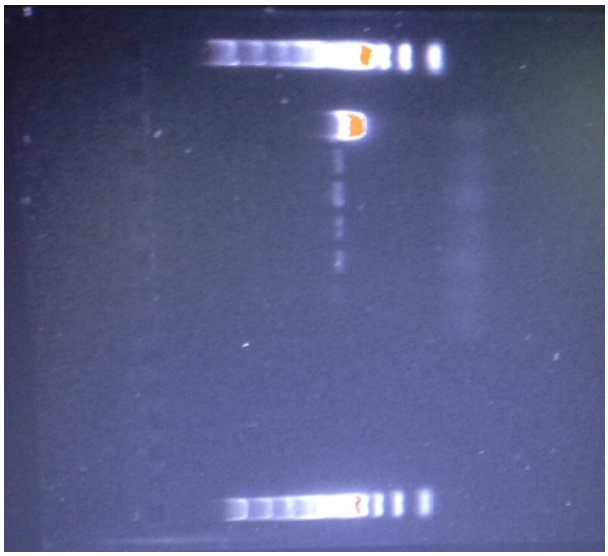


Figure 12 : Gel Electrophoresis with a top and bottom ladder as well as a positive control. Lanes from top to bottom: negative control, positive control, trickling filter, activated sludge samples 1 and 2, and secondary clarifier samples 1 and 2.

Conclusion

According to the results from FISH and PCR, indigenous nitrifiers are indeed present in the activated sludge at the Massard WWTP, though not in high concentrations. The addition of nitrifers did not improve ammonia removal from wastewater collected from the Massard WWTP significantly, indicating unknown inhibitions are present in the wastewater. Future studies are needed to confirm these inhibitions.

Acknowledgements

The authors thank the Arkansas Department of Higher Education for a Student Undergraduate Research Fellowship (SURF), the Garden Club of America for providing a Summer Environmental Studies Grant, the research lab support of Connie Moloney, and David Shelley from the Massard Wastewater Treatment Plant.

References

Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Caldwell, D. E., D. R. Korber, and J. R. Lawrence. 1992. Confocal laser microscopy and digital image analysis in microbial ecology. Adv. Microb. Ecol. 12, 1–67.

EPA, Office of Water. August 2013. Aquatic Life Ambient Water Quality Criteria for Ammonia – Freshwater. United States Environmental Protection Agency

EPA, Office of Water. September 2014. Wastewater Technology Fact Sheet: Trickling Filters. United States Environmental Protection Agency.

Hicks, R., Amann, R. and Stahl, D.A. 1992. Dual staining of natural bacterioplankton with 4', 6-diamidina-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. 58, 2158-2163.

Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.

Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl. 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 6, 2156–2162.

Okabe, S., Satoh, H., and Watanabe, Y. 1999. In situ analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. Appl. Environ. Microbiol. 65, 3182–3191.

Oram, Brian. Ammonia Nitrogen Fish Toxicity Surface Water. Water Research Center. Web. 24 July 2014. <http://www.water-research.net/index.php/ammonia-in-groundwater-runoff-and-streams>.

Randall, D. J., Tsui, T.K.N. Ammonia toxicity in fish. 2002. Marine Pollution Bulletin. 45, 17-23

Wagner, M., Rath, G., Amann, R., Koops, H.-P. and Schleifer, K.-H. 1995. In situ identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. 18, 251–264.

Wang, D. O., Matsuno, H., Shuji, I., Nakamura A., Yanagisawa, H., Hayashi, Y., Okamoto, A. 2012. A quick and simple FISH protocol with hybridization-sensitive fluorescent linear oligodeoxynucleotide probes. RNA Society. 18:166-175.