

**Determination of Ammonia Oxidizing Bacteria and Nitrite
Oxidizing Bacteria in Wastewater and Bioreactors**

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Determination of Ammonia Oxidizing Bacteria and Nitrite Oxidizing Bacteria in Wastewater and Bioreactors

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The process of water purification has many different physical, chemical, and biological processes. One part of the biological process is the task of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Both play critical roles in the treatment of wastewater by oxidizing toxic compounds. The broad term is nitrification, a naturally occurring process that is carried out by AOB and NOB by using oxidation to convert ammonia to nitrite and nitrite to nitrate. To monitor this biological activity, bacterial staining was performed on wastewater contained in inoculum tanks and biofilm samples from bioreactors. Using microscopy and qPCR, the purpose of this experiment was to determine if the population of AOB and NOB in wastewater and membrane bioreactors changed depending on temperature and hibernation conditions to determine the optimal parameters for AOB/NOB culture to effectively clean wastewater.

Nomenclature

| | |
|-------------|--|
| <i>AOB</i> | = ammonia oxidizing bacteria |
| <i>JSC</i> | = Johnson Space Center |
| <i>NOB</i> | = nitrite oxidizing bacteria |
| <i>NSR</i> | = primer used for identification of NOB |
| <i>qPCR</i> | = quantitative polymerase chain reaction |
| <i>TTU</i> | = Texas Tech University |
| <i>rpm</i> | = revolutions per minute |
| <i>amoA</i> | = ammonia monooxygenase gene |

I. Introduction

Nitrification is an essential process in the treatment of wastewater. It is carried out by two types of bacteria. First, the AOB convert ammonia to nitrite, next the NOB convert nitrite to nitrate, and lastly the nitrate is reduced to elementary nitrogen – called denitrification. This project only focused on AOB and NOB; steps one and two.

- 1) AOB convert ammonia into nitrite:



- 2) NOB convert nitrite into nitrate:



- 3) Nitrate reduced to elementary nitrogen:



Wastewater is treated in water plants that use bacteria to treat the soiled water. These bacteria convert toxic wastes into elemental nitrogen that is not toxic to allow the water to be “clean” again. One component of wastewater is urine. Urine is composed of different components including ammonia NH_4^+ , the ammonia is then oxidized by bacteria into nitrite, the nitrite oxidizing bacteria then turn nitrite into nitrate which can then be reduced to nitrogen - an element that can be

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released into the environment. Several parameters influence the amount of AOB and NOB in wastewater including; temperature, pH, and environmental conditions.

One of the necessary means to sustaining life on another planet is having clean water to consume. The purpose of this project was to analyze “wastewater” and determine the presence of AOB and NOB bacteria maintained at various environmental conditions.

II. Materials and Methods

The inoculum tanks were filled with a 50/50 mixture of inoculum fluid from Johnson Space Center (JSC) and Texas Tech University (TTU). The fluid consisted of urine, shower water, and laundry fluid. The fluid was then filtered and placed into the bioreactors. A 3% urine feed was then fed to the bioreactors; the effluent was the fluid that came out of the bioreactors. Pre-hibernation was a 30 day steady state period. The two of the four bioreactors (013 and 014) had the liquid contained within them removed to create a dry environment and all bioreactors were put into hibernation at different conditions and temperatures; wet vs dry, 4°C vs 25°C.

Sampling and Bacterial Staining

Biofilm samples were taken from four aerated bioreactors stored in wet and dry conditions and at different temperatures in June 2014 (pre-hibernation) and in July 2014 (post hibernation); the biofilm samples were scraped from the bioreactor fibers and placed in a 1.5 ml microcentrifuge tube with 1 mL of ethanol. The biofilm samples were stored at -20°C for one week. Along with the biofilm samples, four other samples were taken from the inoculum tanks; the fluid contained in the inoculum tanks and fluid filtered from the tanks as well as the effluent and liquid drained from bioreactors 013 and 014.

The inoculum tank samples were mixed by placing 250mL of the liquid into a flask and poured the liquid back into the tank; this was repeated 3 times for each tank. 50 mL of inoculum fluid was taken from each of the two inoculum tanks. The samples were centrifuged at 5000 rpm for 10 min at room temperature. The 47 mL of the supernant was pipetted out of the centrifuge tube; the pellet was vortexed until mixed. The Nitri-vit kit (Vermicon Munich, Germany) was used for bacterial staining. Ten µl of sample was pipetted onto three slide wells ((+), (vit), and (-)) and the Nitri Vit kit® was used with minor modification; extension of the washing time from 30 min to 45 min in step 9. Once the staining was complete, the slides were analyzed using a Vit-adapted fluorescence microscope with a 100x oil immersion objective. The slides were analyzed under a Zeiss (Jena, Germany) florescent microscope using blue light, Zeiss filter 09 and green light, Zeiss filter 15. Due to the DNA probe in the dye, all viable cells will illuminate red, the AOB will illuminate red and the NOB will illuminate green. The negative control well will have no cell illumination. The slides were evaluated on a 0 to 5 scale indicating none, few, or an excessive amount of cells per vendor protocol (Figure 2). Twenty visual fields were scored using this method and a final score was calculated by averaging the fields.

DNA Extraction and qPCR

Genomic DNA was extracted using Ultraclean Microbial DNA Isolation Kit (Mobio Laboratories Inc, Carlsbad, Calif. USA) with no modifications. Fifty mL samples of inoculum from tanks one and two were placed into 50mL centrifuge tubes and centrifuged at 5000 rpm for 10 minutes at room temperature. The supernant was removed and 4 mL of distilled water added. 1.8 mL was pipetted into 2mL micro centrifuge tubes and the vendor suggested protocol was followed. The DNA concentration was determined on the Nano Drop (ThermoScientific Wilmington, DE). Tank one ratio of absorbance at 260/280nm was 1.74 with 35.8 ng/µl and tank 2 had a ratio of absorbance at 260/280 nm with 31.6 ng/µl. The samples were stored at -20°C to be used in qPCR.

Quantitative polymerase chain reaction (qPCR) was used to amplify the AOB *amoA* gene region of the DNA extracted from wastewater samples. The forward primer set used to amplify the *amoA* gene in AOB was (*amoA* 21F; 5' – AGAAATCCTGAAAGCGGC) with a final concentration of 0.7 µM the reverse primer used was (*amoA* 187F; 5'- CAATGGTGGCCGGTTGT) with a final concentration of 0.7 µM (Invitrogen, Life Technologies Grand Island NY) and Roche 480 Master mix had a final concentration of 1x (Roche Diagnostics, Indianapolis IN USA). The samples were run on a Roche Light cycler at 95°C for 45s, 59°C for 30s, and elongation at 72°C for 30s. For NOB the forward primer used was (NSR 1156; 5'- GTAACCCGCCGACACTTA) and the reverse primer was (NSR 826; 5'- CCCGTTCTCTGGGCAGT). PCR will be completed on the NOB samples at a later date.

III. Results

Biofilm and Inoculum Samples

The vit key in the Nitri-Vit kit® provided a guide line on how to evaluate the biofilm/ inoculum samples (Fig. 1).

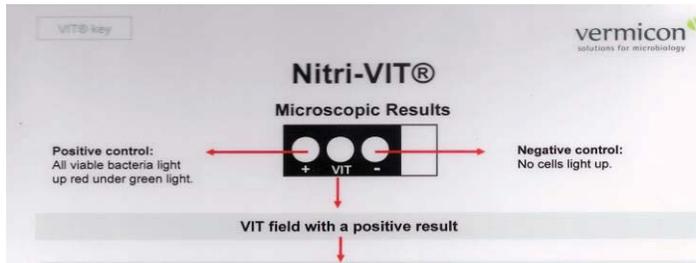


Figure 1. Nitri-Vit microscope slide showing (+), (vit), and (-) wells the 10 µl samples are pipetted into.

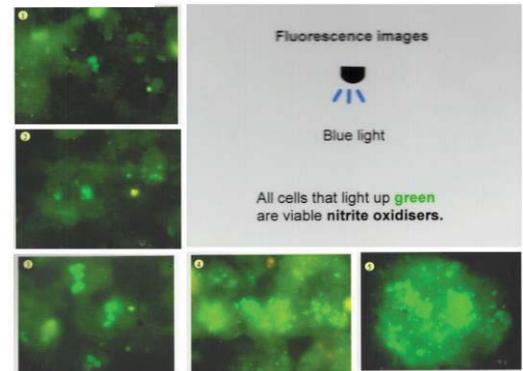
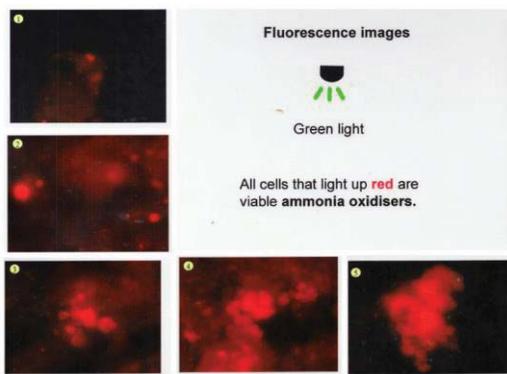
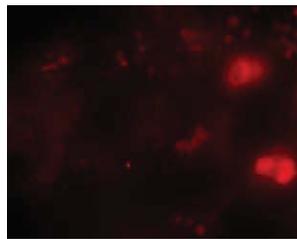
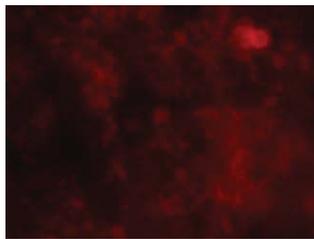


Figure 2. Vit Key. Provided with Nitri-Vit Kit® for proper evaluation of AOB and NOB.

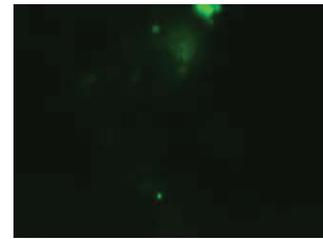
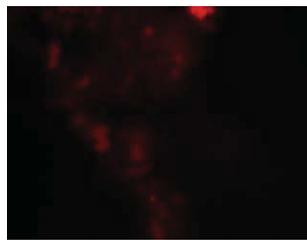
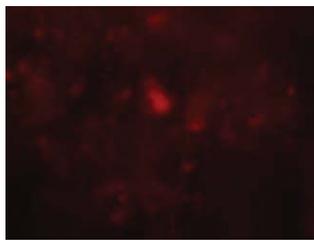


a)

b)

c)

Figure 3. Samples from inoculum tank 1 on July 1, 2014. a) Positive control all viable bacterial cells illuminate red. b) Red bacterial cell illumination signifies AOB presence. c) Green bacterial cell illumination signifies NOB presence.



a)

b)

c)

Figure 4. Samples from inoculum tank 2 on July 1, 2014. a) Positive control all viable bacterial cells illuminate red. b) Red cell illumination signifies AOB presence. c) Green cell illumination signifies NOB presence.



Figure 5. Samples from inoculum tank 1 on July 9, 2014. a) Positive control viable cells illuminate red. b) AOB bacterial cell illumination. c) Minimal AOB cell illumination.

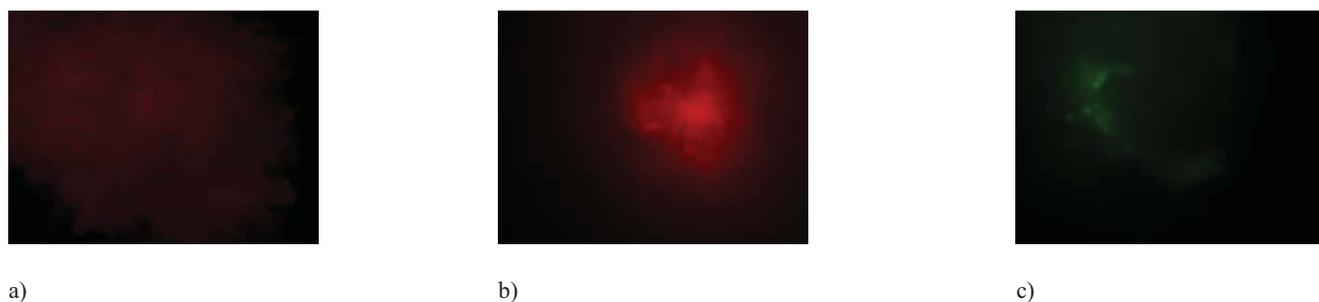


Figure 6. Samples from inoculum tank 2 on July 9, 2014. a) Positive control, all viable cells illuminate red. b) Bright red cell illumination signifies strong AOB presence. c) Green cell illumination although slight illumination signifies AOB presence

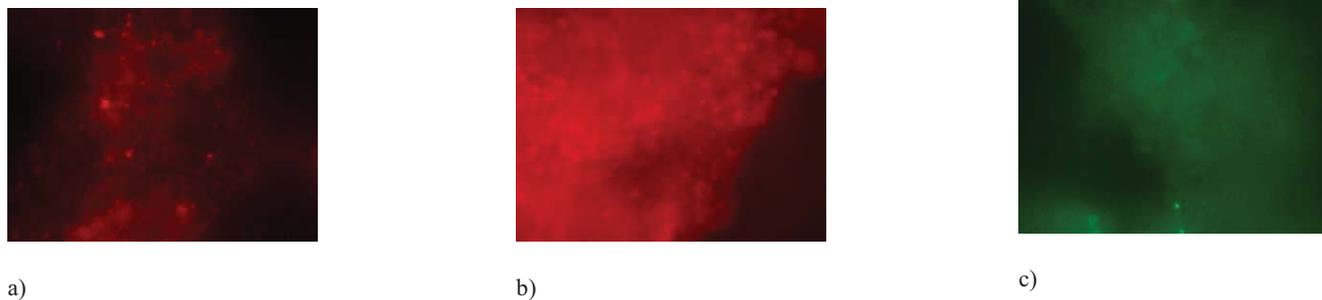


Figure 7. Samples from tank 1 filter. Bacterial stain applied on July 9, 2014. a) Positive control, viable cells illuminate red. b) Bright red cell illumination with small circular regions signifying presence of micro colonies. c) Bright green dot is a single AOB bacterial cell.

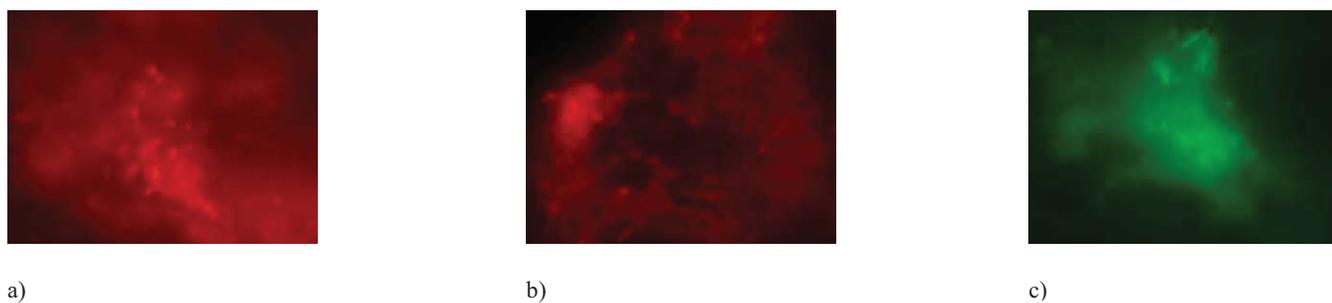


Figure 8. a) Positive Control, viable cells illuminate red. b) Red cell illumination. AOB presence micro colonies are visually seen. c) AOB culture present.

| | |
|---|---|
| <p>Evaluation Analyse 20 arbitrary visual fields using the VIT® key: each visual field must be assigned a value according to the amount of cells present (1-5). The numeric average is calculated by dividing the total sum of the values by the number of evaluated visual fields.</p> <p>For example: $(6 \times \text{group 4}) + (5 \times \text{group 3}) + (6 \times \text{group 2}) + (3 \times \text{group 1}) = 24 + 15 + 12 + 3 = 54$; $54/20 = 2.7$ According to the VIT® system, the level of nitrite oxidisers in this sample has a value of 2.7.</p> | <p>Amount of cells:</p> <ul style="list-style-type: none"> ① Few ② Some ③ Many ④ Abundant ⑤ Excessive |
|---|---|

Figure 9. Evaluation. Provided with the Nitri-Vit kit®, shows how to score visual fields.

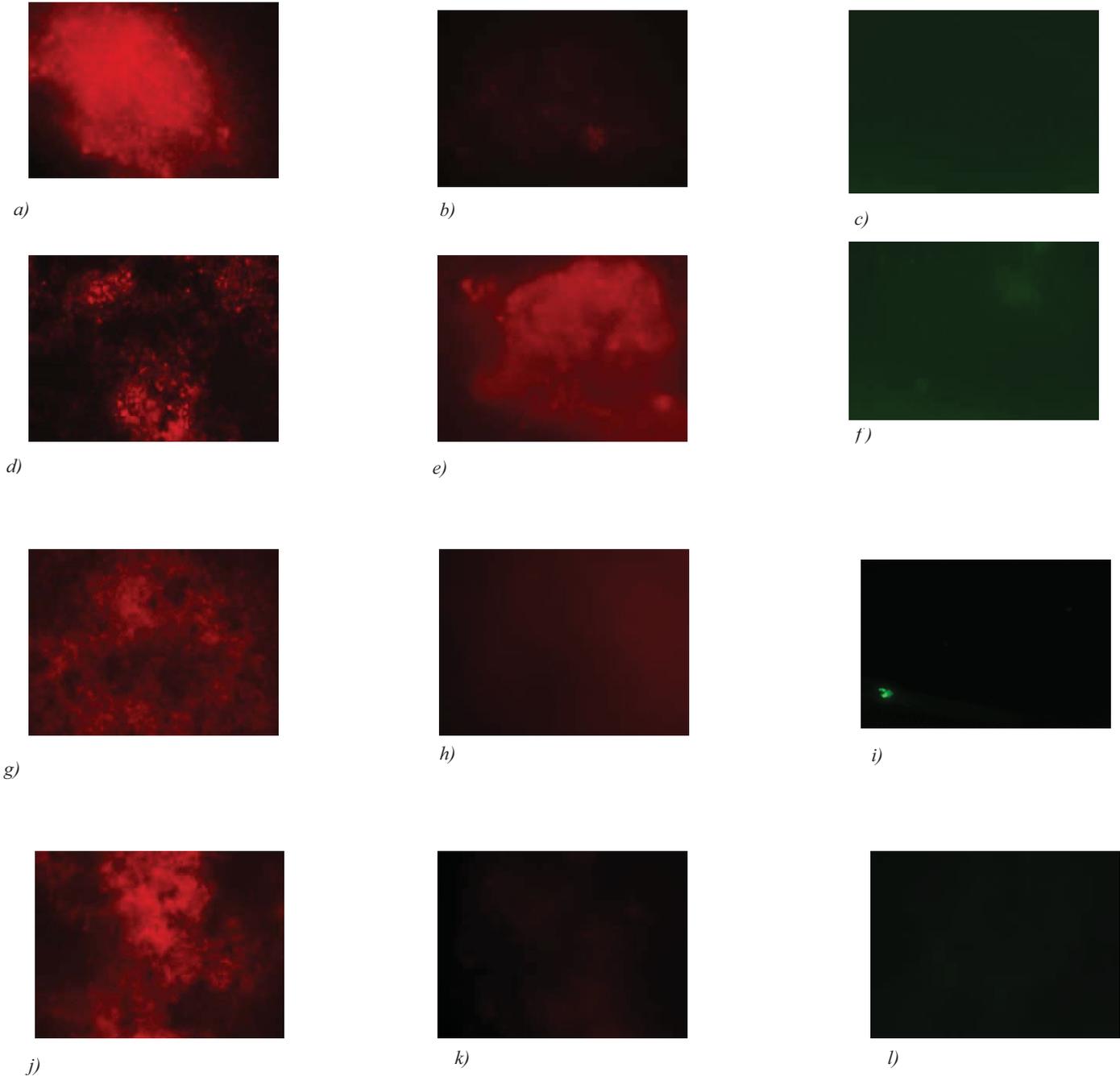


Figure 10. Pictorial depictions of the liquid contained in the bioreactor and effluent samples from bioreactors 013 and 014 after reaching steady state. a) Bioreactor 013 liquid positive control. b) Bioreactor 013 liquid AOB. c) Bioreactor 013 liquid NOB. d) Bioreactor 013 effluent positive control. e) Bioreactor 013 effluent AOB. f) Bioreactor 013 effluent NOB. g) Bioreactor 014 liquid positive control. h) Bioreactor 014 liquid AOB. i) Bioreactor 014 liquid AOB. j) Bioreactor 014 effluent positive control. k) Bioreactor 014 effluent AOB. l) Bioreactor 014 effluent AOB.

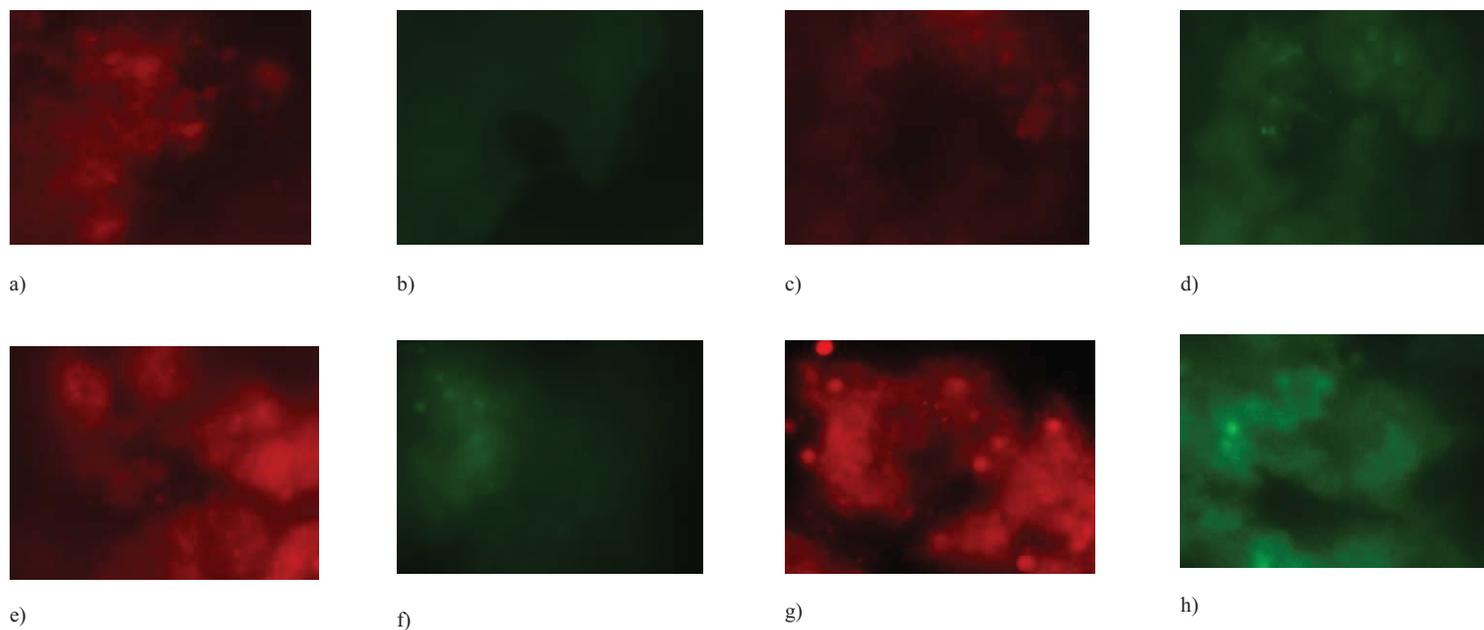


Figure 11. Pre-Hibernation samples from biofilm 6.5.14 after 30 days of steady state. a) Bioreactor 11 AOB depiction. b) Bioreactor 11 NOB depiction. c) Bioreactor 012 AOB depiction. d) Bioreactor 013 NOB depiction. e) Bioreactor 013 AOB depiction. f) Bioreactor 013 NOB depiction. g) Bioreactor 014 AOB depiction. h) Bioreactor 014 NOB depiction.

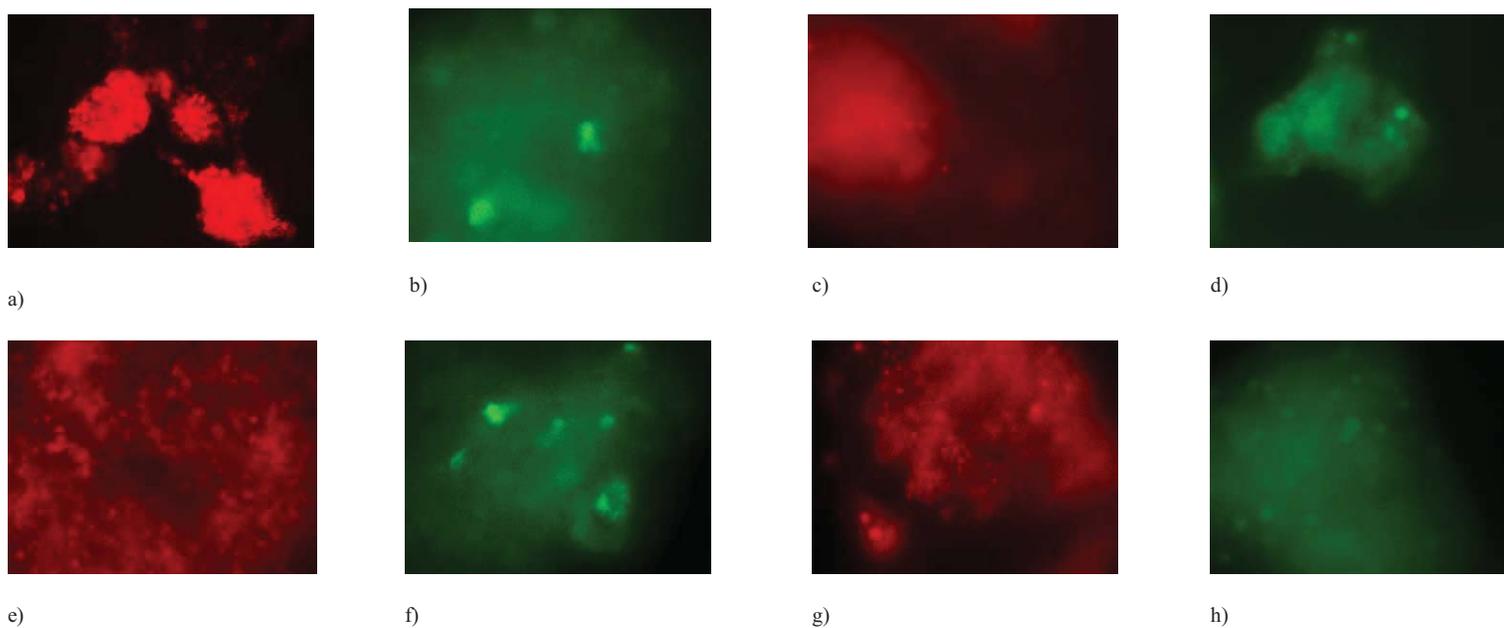


Figure 12. Post Hibernation samples from biofilm, 30 hibernation. 7.9.14 a) Bioreactor 11 AOB depiction hibernated wet, 4°C. b) Bioreactor 11 NOB depiction hibernated wet, 4°C. c) Bioreactor 012 AOB depiction hibernated wet, 25°C. d) Bioreactor 013 NOB depiction hibernated wet, 25°C. e) Bioreactor 013 AOB depiction hibernated dry 4°C. f) Bioreactor 013 NOB depiction hibernated dry 4°C. g) Bioreactor 014 AOB depiction hibernated dry 25°C. h) Bioreactor 014 hibernated dry 25°C.

The state of storage of the bioreactors were compared in this study, to see which method is the best at re-starting the growth of AOB and NOB cultures in the bioreactors after hibernation. As shown in the graphs below, the results indicate that overall the post hibernation contained more AOB and NOB in the biofilm samples.

Bioreactor 011

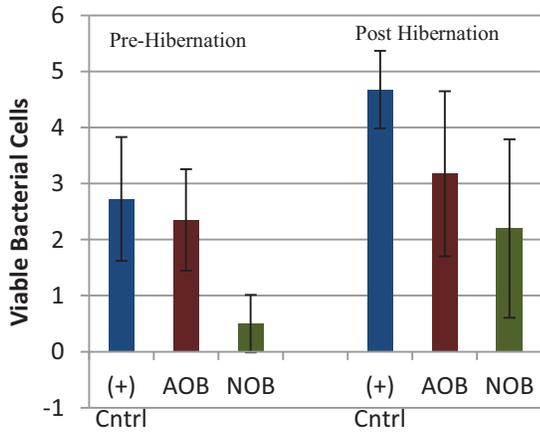


Figure 13. Conditions hibernation wet, refrigerated. Error bars represent standard deviation.

Bioreactor 012

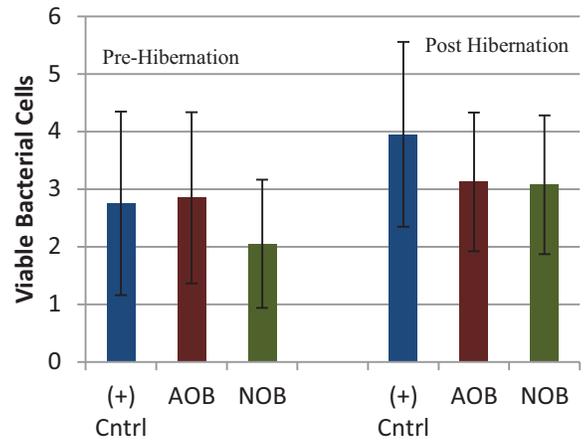


Figure 14. Conditions hibernation wet, room temperature. Error bars represent standard deviation.

Bioreactor 013

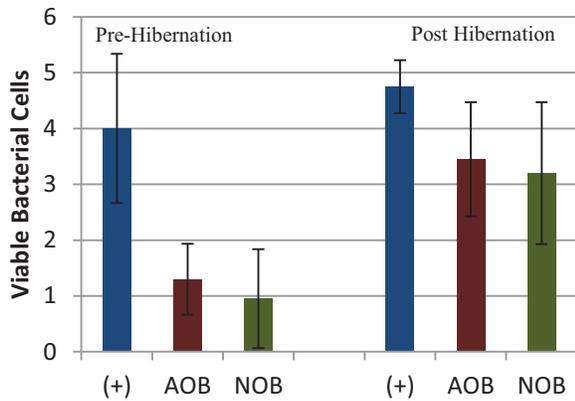


Figure 15. Conditions hibernation, dry, refrigerated. Error bars represent standard deviation.

Bioreactor 014

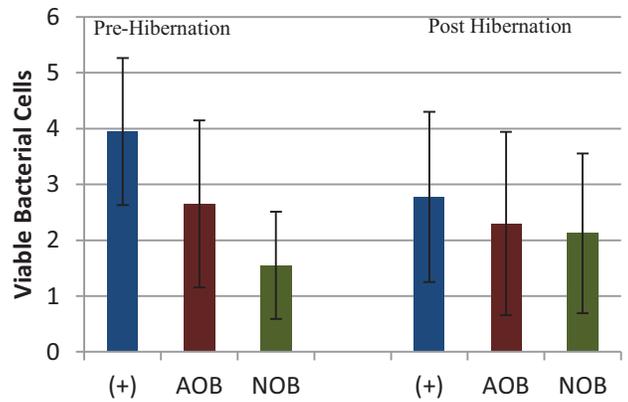


Figure 16. Conditions hibernation dry, room temperature. Error bars represent standard deviation.

Bioreactor 013 Liquid vs Effluent

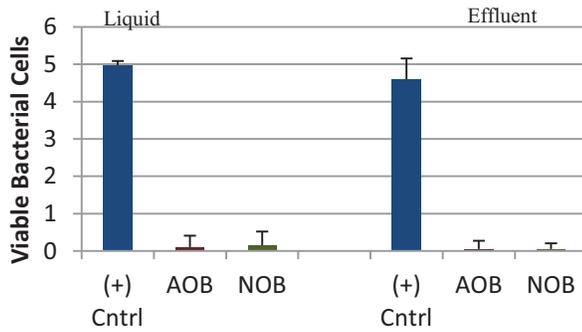


Figure 17. Comparison of bioreactor 013 liquid and effluent in bioreactor 013.

Bioreactor 014 Liquid vs Effluent

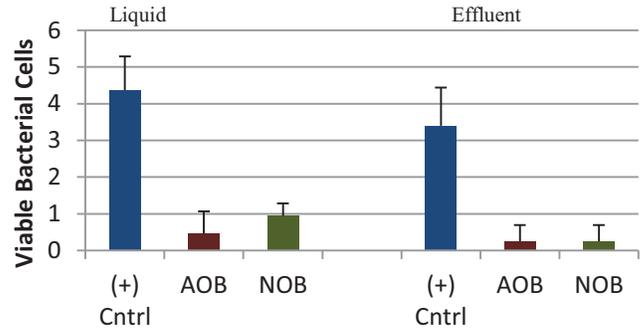
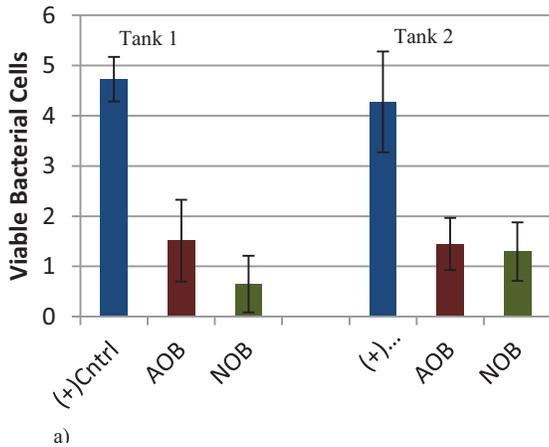


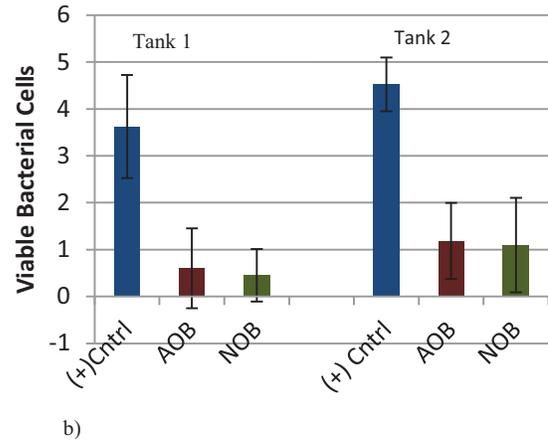
Figure 18. Comparison of bioreactor 014 liquid and effluent of bioreactor 014.

Inoculum Tanks 7.1.14



a)

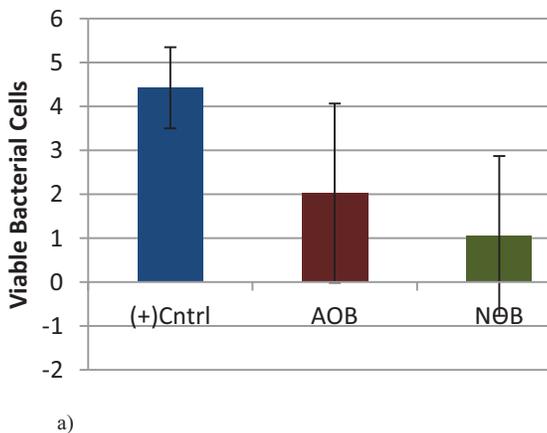
Inoculum Tanks 7.9.14



b)

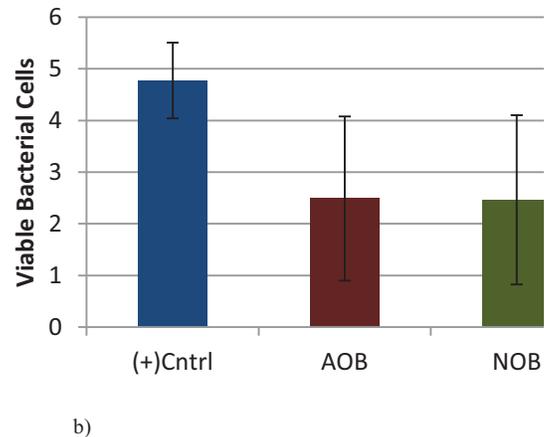
Figure 19. Comparison of inoculum (a) tank 1 and (b) 2 on July 1, 2014 and July 9, 2014.

Tank 1 Filtrate



a)

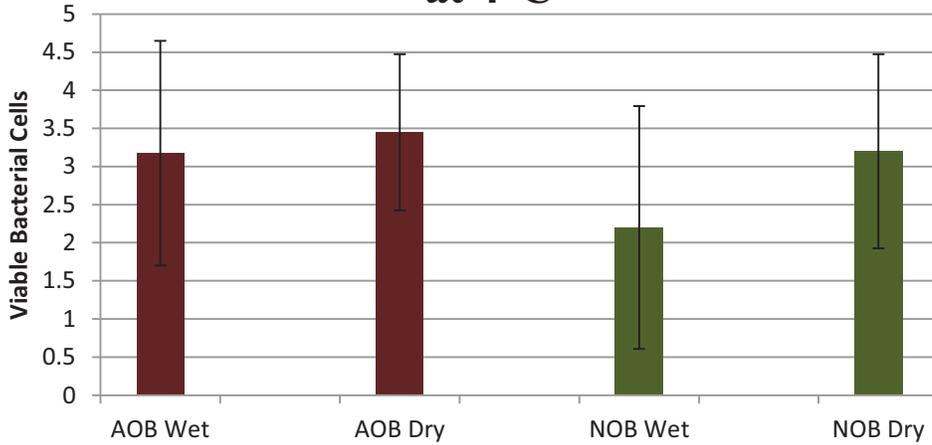
Tank 2 Filtrate



b)

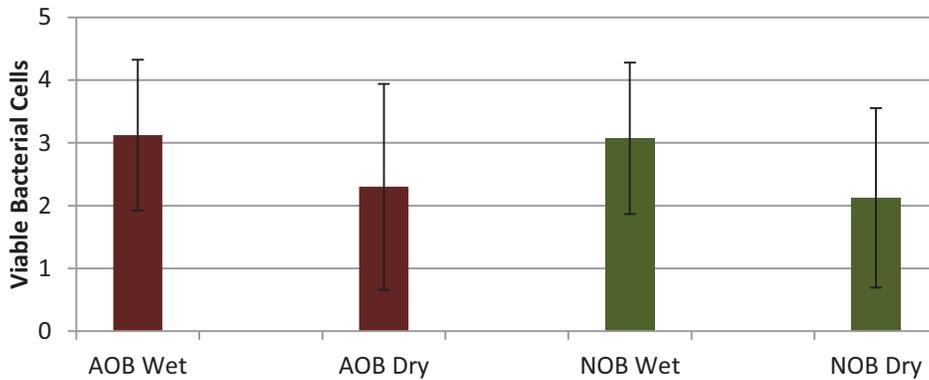
Figure 20. Comparison of AOB and NOB in (a) Tank 1 and (b) Tank 2 filter stained on July 9, 2014.

AOB Wet vs AOB Dry & NOB Wet vs NOB Dry at 4°C



a)

AOB Wet vs AOB Dry & NOB Wet vs NOB Dry at 25°C



b)

Figure 21. a) Comparison of post hibernation conditions of a wet vs dry environment at a constant temperature of 4°C from bioreactors 011 and 012. b) Comparison of hibernation conditions of a wet vs dry environment at a constant temperature of 25°C from bioreactors 013 and 014.

IV. Discussion

This study analyzed the presence of AOB and NOB throughout pre and post hibernation of bioreactors and the presence of the two different types of bacteria in the inoculum tanks. The interpretation of the data above suggests that there is not a significant difference between the presence of AOB and NOB cultures in any of the samples due to Nitri-Vit kit® evaluation of the microscopy pictures and the over lapping of +/- standard deviation bars indicated on the graphs.

Microscopy

The bacterial staining provides a quick way of determining whether there is a sufficient amount of AOB and NOB present. The amount of AOB and NOB in the wastewater and biofilm samples were determined by the Nitri-Vit kit® recommended evaluation formula (Figure 1). Each of the 20 visual fields were evaluated on a 0 to 5 scale then were averaged to give a final score for each sample; the positive control well illuminated red, this indicated the presence of all bacterial cells not just AOB or NOB (Figures 3 (a), 4 (a), 5(a), 6(a), 7(a), 8(a)). The nitrifiers were determined by a colored dye which contained a DNA probe that inserted into the bacterial DNA and either illuminated red for AOB or green for NOB. The negative control well did not illuminate bacterial cells. Figure 1 shows a picture of the Nitri-Vit slide. Each of the samples were stained and evaluated shown in the Figures above. Due to the lack of cell illumination of a green color, it can be concluded that there are not as many NOB as AOB in any of the inoculum or biofilm samples.

Pre-hibernation

The only significant differences in the pre-hibernation period are shown in Fig. 13 and 15. Figure 19 shows a significant difference between the presence of all viable bacteria and the presence of NOB in bioreactor 011. Figure 15 displays bioreactor 013 positive control having significantly more viable bacteria than specific cultures of AOB or NOB in this bioreactor. It is interesting to note, the only significant difference between bacterial cultures in bioreactors 011- 014 (Fig. 13-16) occurred during the pre-hibernation phase. Bioreactors 013 and 014 were drained prior to hibernation, meaning the liquid in the bioreactor was removed to allow for dry storage for hibernation; samples were taken from the liquid removed from the bioreactor and the effluent. According to Fig. 11 and 12 the majority of AOB and NOB stayed within the bioreactor due to the low number of AOB and NOB culture within the samples (Fig. 10 h).

Culture Response Post Hibernation

Bioreactors 011 and 012 were hibernated “wet” with filtered inoculum fluid. Even though both of these reactors were hibernated with inoculum, bioreactor 011 was incubated at 4°C and bioreactor 012 was at 25°C. Bioreactor 013 was put into hibernation dry and at 4°C while bioreactor 014 was dry at 25°C (Fig. 21). These different parameters were put in place to see what would be the best environment for the AOB and NOB cultures to thrive in. Referring to the graphs above, bioreactor 013 performed the best after hibernation; this is interesting because it was the worst of the four bioreactors pre-hibernation. The dry and cold condition of bioreactor 013 proves to be the best at re-starting the growth of cultures. In figure 21 the dry performed better than the wet at 4°C. While the wet performed better than the dry at 25°C. Even though there is not a significant difference between the wet vs dry storage at different temperatures, there is a slight difference between when the AOB and NOB cultures grow depending on temperature.

There does not seem to be a significant difference between any of the AOB NOB cultures when solely comparing post hibernation within the same bioreactor nor when comparing between different bioreactors, this indicates that the storage of the bioreactors only has a slight affect on the presence of AOB and NOB cultures. However, there is significance between the AOB NOB cultures when compared to pre-hibernation. The only occurrence is in bioreactor 013 (Fig. 15). Bioreactor was hibernated dry and at 4°C, the specific cultures of AOB and NOB responded best to this type of condition. Figures 21 demonstrate that there is no significant difference between the storage of the bioreactors. There was only slight difference between the presence of AOB and NOB cultures with a cold and wet hibernation.

Inoculum Tanks

As seen in the bioreactors, there was not a significant difference between the AOB or NOB cultures between the dates of July 1, 2014 and July 9, 2014 but there was a significant difference between the presence of all types of bacteria when compared to the AOB and NOB cultures in both tanks one and two (Fig. 19). Figure 20 compares the filtrate of tank one and tank two which is the fluid placed into the bioreactors. Even though it is not a significant difference, the filtrate does contain more AOB and NOB culture than the inoculum tanks (Fig. 19 and 20).

V. Conclusion

The work reported here concludes that AOB cultures were more prevalent among two of the samples; the inoculum tank liquid and the biofilms while the bioreactor liquid and the effluent contained more NOB culture, although a significantly less amount. This could be due to the fact that NOBs are slow growing and usually have a doubling time of 12-32 hours, while the AOBs usually have a doubling time of 8.5 hours. Most bacteria have a much shorter doubling time within minutes, which also demonstrates why there were so more viable bacterial cells in the positive control. Also, some of the NOB culture is being removed through the effluent before hibernation; this could signify why there is a slight increase in AOB than NOB pre- hibernation.

VI. Acknowledgments

Special thanks to my mentor Dr. Christina Khodadad and her colleague Mary Hummerick for their patience, guidance, and time.

VII. References

- ¹Alawi, M., S. Off, M. Kaya, and E. Spieck. "Temperature Influences the Population Structure of Nitrite-oxidizing Bacteria in Activated Sludge." *Environmental Microbiology Reports* 1.3 (2009): 184-90.
- ²Gómez-Silván C, Vilchez-Vargas R, Arévalo J, Gómez MA, González-López J, Pieper DH, Rodelas B. *Bioresour Technol.* 2014 Jul 2;169C:126-133. doi: 10.1016/j.biortech.2014.06.089
- ³Hoshino, T., N. Noda, S. Tsuneda, A. Hirata, and Y. Inamori. "Direct Detection by In Situ PCR of the AmoA Gene in Biofilm Resulting from a Nitrogen Removal Process." *Applied and Environmental Microbiology* 67.11 (2001): 5261-266.
- ⁴Hu, Ziye, T. Lotti, R. Kleerebezem, M. Loosdrecht, J. Kruit, M. Jetten, and B. Kartal. "Nitrogen Removal by a Nitritation-Anammox Bioreactor at Low Temperature." *Applied and Environmental Microbiology* 79.8 (2013): 2807-812.
- ⁵Li, Baikun, Shannon Irvin, and Katherine Baker. "The Variation of Nitrifying Bacterial Population Sizes in a Sequencing Batch Reactor (SBR) Treating Low, Mid, High Concentrated Synthetic Wastewater." *Journal of Environmental Engineering and Science* 6.6 (2007): 651-63.
- ⁶P. W. J. J. Van Der Wielen, S. Voost, and D. Van Der Kooij. "Ammonia-Oxidizing Bacteria and Archaea in Groundwater Treatment and Drinking Water Distribution Systems." *Applied and Environmental Microbiology* 75.14 (2009): 4687-695.
- ⁷Sinigalliano, C. D., D. N. Kuhn, and R. D. Jones. "Amplification of the AmoA Gene from Diverse Species of Ammonium-oxidizing Bacteria and from an Indigenous Bacterial Population from Seawater." *Applied and Environmental Microbiology* 61.7 (1995): 2702.
- ⁸Wang J, Zhang C, Rong H. Analysis and succession of nitrifying bacteria community structure in sequencing biofilm batch reactor. *Appl Microbiol Biotechnol.* 2014 May;98(10):4581-7. doi: 10.1007/s00253-014-5537-6.
- ⁹Zeng J, Zhao D, Yu Z, Huang R, Wu QL. Temperature responses of ammonia-oxidizing prokaryotes in freshwater sediment microcosms. *PLoS One.* 2014 Jun 24;9(6):e100653. doi: 10.1371/journal.pone.0100653.