JCCC Honors Journal

Volume 7
Issue 1 *Fall 2015*

Article 3

Nitrosomonas eutropha: A Study of the Effects of Nitrosomonas on Pathogenic Bacterium and the Effects of Current Hygiene Habits on the Colonization of Nitrosomonas Within Our Normal Flora

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Abstract
Nitrosomonas eutropha is a motile, gram-negative, bacillus that metabolizes ammonia as its energy source. Nitrosomonas eutropha was isolated from a probiotic skin product and used to determine how it would function in the normal flora of the integumentary system, and how it potentially benefits the human body. The effects of current hygiene products and practices on the ability of the Nitrosomonas eutropha to form and maintain healthy colonies were also examined.

Cover Page Footnote
Dr. Ellyn R. Mulcahy, PhD, MPH was the Faculty Advisor for this Honors contract. The research of former students Heather Pointdexer, Araceli Hernandez Guerrero, Keith Kennedy, and Sarah Tonnies was utilized by the author to complete the research contained in this paper.

Introduction

The goals for the research included determining the effectiveness of the probiotic skin product. It was determined through research that the bacterium present in the solution was once a valuable part of the naturally existing colonization of beneficial bacteria found on our skin. Due to modern hygienic practices, including more frequent bathing, the use of harsher hygienic products, and antibacterial cleansers normal flora, such as *Nitrosomonas eutropha* is not present in beneficial amounts within the flora of the integumentary system. Additionally, reintroduction of *Nitrosomonas eutropha* would reduce the need for frequent bathing and harsh hygienic products. Additional research goals included determining how potentially pathogenic bacteria interacted with *Nitrosomonas eutropha*, as well as how various hygienic products available today effect the growth of *Nitrosomonas eutropha* and its colonization of the normal flora within the integumentary system (1, 2, 5 ,7).

Testing revealed that *Nitrosomonas eutropha* is a highly motile bacterium. It grows optimally in an acidic and dark environment at 23^0C. *Nitrosomonas eutropha* metabolizes urea, as well as citrate and is a facultative anaerobe, growing both aerobically and anaerobically. *Nitrosomonas eutropha* is a chemolithoautotroph, which is a classification of microbes meaning they use CO_2 for their primary carbon source and power their metabolism using inorganic compounds. In the case of *Nitrosomonas eutropha*, the inorganic compound is ammonia. *Nitrosomonas eutropha* also has the capability to change pigmentation depending on the environment. This is due to the release of chromophores when stressors are imposed upon the bacterium. Stressors can be sub-optimal temperatures, which produced a different pigmentation than *Nitrosomonas eutropha* grown in its optimal temperatures. The pH within the medium also influenced the pigmentation of the growing *Nitrosomonas eutropha* as demonstrated when grown while exposed to various soaps. When testing the susceptibility of various pathogenic bacteria, it was determined that many of them had significantly inhibited growth when *Nitrosomonas eutropha* are present. To explore why *Nitrosomonas eutropha* have become extinct from the normal flora of our skin, we examined the effects of various soaps, scrubs, and cleansers on *Nitrosomonas eutropha* bacteria. It was found that many of the more natural cleansers such as pure glycerin soaps, scrubs made with oatmeal, carrot, or other natural products either did not inhibit the growth at all or did so minimally, allowing for regrowth in a matter of a couple days. On the other hand, manufactured soaps such dial and dove more significantly inhibited their growth and did not allow for regrowth for longer periods (3, 6).

Materials and Methods

To begin the project it was required that first the bacterium, *Nitrosomonas eutropha,* found to be in the AO+ Biome samples, be isolated in order to determine that it contains a bacterium of which is beneficial to the integumentary system, and once a part of the normal skin flora. The method that allowed the bacterium to be isolated and grown used 1000 microliters of the original sample, which had been growing in an unknown nutrient solution incubated at 37^0C for ten days with no observable growth. Samples from this concentration were then from this original sample and used to inoculate incremental pH broth tubes of pH values of 2,4,6,8, and 10. Since the bacterium grows on human skin, as indicated by research, then it would prefer a more acidic environment. After two days, observable growth within the pH 6 broth tubes was seen. At this point, two more pH 6 broth tubes were inoculated from the original 1000-microliter tube. At the same time, growth in salinity environment was tested. Tubes with salinities of 0%, 5%, 10%, 15%, and 20% were then inoculated using growth from the initial pH 6 broth containing visible growth and incubated for five days at 37^0C (2, 7).

Once the optimal growing environment had been determined and several healthy colonies were available in pH 6 broth, the process of getting the *Nitrosomonas eutropha* to grow on Nutrient Agar plates began. The first growth of *Nitrosomonas eutropha* on a Nutrient Agar plate was established by using four loops of samples from the established growth in the pH 6 broth and incubated in the incubator at 37^0C for three days. Once healthy lawns of the *Nitrosomonas eutropha* were established*,* the inoculation of streak plates was begun in order to obtain and observe isolated colonies. At the same time, each week new pH 6 broth tubes and Nutrient Agar plates were inoculated utilizing samples from the growth established on the previously inoculated Nutrient Agar plate showing good growth. The inoculation of fresh pH 6 broth tubes and Nutrient Agar plates each week from previous week's growth continued throughout the time in the lab. It was found that the best way to obtain and view individual colonies on a streak plate was to use a sterile loop to inoculate streaks one, two, and three then use a sterile needle to inoculate streak four. A new sterile loop was used for each streak on the streak plate.

Due to time restrictions and need to preserve the organisms, some of the samples were placed in the refrigerator to slow down the metabolism and growth of the *Nitrosomonas eutropha*. It was after refrigerating the samples that pigmentation changes were noticed in the growth and determination was made that the *Nitrosomonas eutropha* produces a chromophore. Further research on the *Nitrosomonas eutropha* was conducted to determine why chromophores were being produced and what other environmental conditions could cause this effect. This research also yielded information regarding the *Nitrosomonas eutropha's* preference for lower growing temperatures that was put in place for future incubations (3, 6, 7).

After observable growth of the *Nitrosomonas eutropha* had been established both in broth media and on Nutrient Agar, slides were prepared with the best results in staining methods resulting from adjusting the traditional staining procedures. The ideal staining procedures to obtain the best views of the *Nitrosomonas eutropha* and its morphology as shown on Slide 1E (Figure 6), was using a sterile loop full of sample from a well-colonized lawn that was incubated at room temperature in the dark and transferring a heavy inoculation to the single drop of distilled H_2O on the slide and heat fixing it to the slide. Once the sample is heat fixed to the slide the stains were applied as follows: crystal violet for one minute, rinse with distilled H_2O, iodine for one minute, rinse with distilled H_2O, and then safranin for one minute, and then a final rinse with distilled H_2O before drying for viewing. Slide 2E (Figure 7) was prepared in the exact same manner as slide 1E with the only difference being the time each of the stains was allowed to remain on the slide. Instead of one minute per stain, two minutes per stain was used in order to create a more distinct image of the bacterial cells. The staining procedure for slide 2A (Figure 8) was as follows: used one loopful of sample from pH 6 broth tubes that had been in the refrigerator heat fixed to the slide with one drop of distilled H_2O; on top of that two drops of the same pH 6 broth containing the cultured *Nitrosomonas eutropha* was placed on top of the heat fixed sample using a sterile pipet and heat fixed on top of the first sample on the slide. Once heat fixed, the crystal violet was placed on the slide for one minute then rinsed with distilled H_2O, next was iodine for one minute then rinsed with distilled H_2O, and finally safranin for one minute then final rinse with distilled H_2O and air dried for viewing under the microscope.

Additional testing to determine metabolism, motility, and respiration of the *Nitrosomonas eutropha* were conducted. All testing was done using either a sterile loop or needle, taking samples from fresh healthy growth, and incubated at 23^0C in a dark environment. A control of the exact same media/test was used as the inoculated media/test and was incubated un-inoculated at the same time and in the same environment as the inoculated test to use for comparison of results. The Citrate test was inoculated using a sterile needle and sample from healthy growth on a lawn inoculated seven days prior, and then the Citrate tubes were allowed to incubate at room temperature in a darkened environment for five days. The same procedure that was used to inoculate the Citrate test was used to inoculate the Sulfide-Indole-Motility (SIM) tubes to test for motility, and utilization of sulfur, in addition the sample used for the SIMs test came from the same lawn that was used to provide the sample for the Citrate test. The rapid urea test was also inoculated in the exact same manner as the aforementioned tests. Tryptic Soy Agar (TSA) slant was inoculated using a sterile needle containing a sample of *Nitrosomonas eutropha* from a healthy lawn inoculated fourteen days prior to stab the center of the slant agar to just above the bottom of the slant tube and inoculating the upper part of the slant using a zig zag pattern. Phenol Red with Dextrose and Phenol Red with Lactose tests were inoculated using sterile

loops and healthy samples from lawn inoculated seven days prior. The Enterotube II was inoculated using healthy samples from a lawn of *Nitrosomonas eutropha* inoculated five days prior. Once the initial readings on the Enterotube II were made without the regents nine days after the inoculation of the Enterotube II, then Kovac's regents were added to the indicated space for the H_2S/Indole and additional readings taken. The Vogues-Proskauer (VP) testing was performed using a sterile loop to inoculate the VP broth tubes and the Nitrosomonas was allowed to grow in the VP broth for nine days. With good visible growth observed in the VP broth a sterile pipet was used to transfer 1 mL of the gently agitated VP broth into a new test tube and added five drops of Methyl Red regent. Observations were immediately made and recorded. A second new test tube was obtained and 1 mL of solution containing *Nitrosomonas eutropha* growth from the VP broth was placed into this tube using a sterile pipet. To this tube of 1 mL of VP broth containing *Nitrosomonas eutropha* growth fifteen drops of regent A (α-napthol) and regent B (KOH 40%) were added. Observations were taken and recorded on this tube every ten minutes for one hour. A Nitrate reduction test was conducted to show if and how *Nitrosomonas eutropha* utilized or reduced nitrate.

Since the product stated that the *Nitrosomonas eutropha* was beneficial bacterium for humans, present in the normal flora of the skin, the next step was to test how it reacted with various available possibly pathogenic strains of bacteria. The testing of the *Nitrosomonas eutropha's* ability to inhibit potentially pathogenic bacteria was tested using the following protocol: sterile Kirby Bauer disc were saturated in broth containing *Nitrosomonas eutropha* and then placed using sterile tweezers onto the center of a Nutrient Agar (NA) plate which was freshly inoculated in a with a pathogenic bacteria in a lawn pattern. Once the NA plate was inoculated with a pathogenic bacteria, and had a *Nitrosomonas eutropha* saturated disc placed in the center the NA plates were then incubated at 37^0C in the incubator, because this temperature is the pathogenic bacteria's optimal growing temperature. The possibly pathogenic bacteria species used for this experiment are *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, and *Staphylococcus epidermidis*. A blank un-inoculated disc was used for a control. For a comparison between the inhibition of the pathogenic bacterium with *Nitrosomonas eutropha* and inhibition of pathogenic bacterium with antiseptics, an experiment using disc soaked in bleach for one set of lawns innoculated with pathogenic bacterium, 70% isopropol alcohol for another set, and water on a third for control was conducted.

The next stage of this research project involves testing how various soaps and hygienic products effect the growth of *Nitrosomonas eutropha* and the healthy colonization of the normal flora of the human skin. It was determined that the best method was an adaptation of procedures used in another experiment by Jen Ruble by modifying it for sterile technique, the soaps being tested, as well as the use of Kirby

Bauer disc allowing for testing of the hygienic products to determine how much they inhibited the growth of the *Nitrosomonas eutropha.* The procedure followed when testing the soaps involved saturating a sterile Kirby Bauer disc in the liquid soap to be tested, using sterile tweezers to place a bank, sterile Kirby Bauer disc into a paper cup containing the liquid soap to be tested. Then using a newly sterilized pair of tweezers to pick up the saturated Kirby Bauer disc and placing it in the center of a newly inoculated lawn of *Nitrosomonas eutropha*, sealing the NA plate and incubating it at 23^0C in a dark environment. For the two soap bars that were tested, the procedure was to use a sterile scalpel to cut off a small sliver measuring 17 mm by 10 mm of each soap to be tested. Then using sterile tweezers or the sterile scalpel to transfer it to the center of a newly inoculated lawn of Nitrosomonas eutropha, seal the plate, and incubate it at 23^0C in a dark environment. Observations made, recorded, and pictures taken of results at seventy-two hours post inoculation and at one-hundred and twenty hours post inoculation. The soaps that tested are, with brand name first then product name, are as follows: Yes! To Carrots Daily Facial Cleaner, Dove Deep Moisture Liquid Body Wash, Yardley London Oatmeal and Almond Bar Soap, Nirvana Spa NSPA Real Fruit Goodness Exotically Creamy Coconut Rich Body Butter, Clearly Natural Pure Glycerin Soap (unscented), Freeman Charcoal and Black Sugar Polishing Mask, Yes to Tomatoes Clear Skin Acne Daily Pore Scrub, Tree Hut SHEA Sugar Scrub with Almond and Honey, SoftSoap Liquid Milk and Honey Hand Soap, and Dial Liquid Springwater Antibacterial Soap (4).

Results

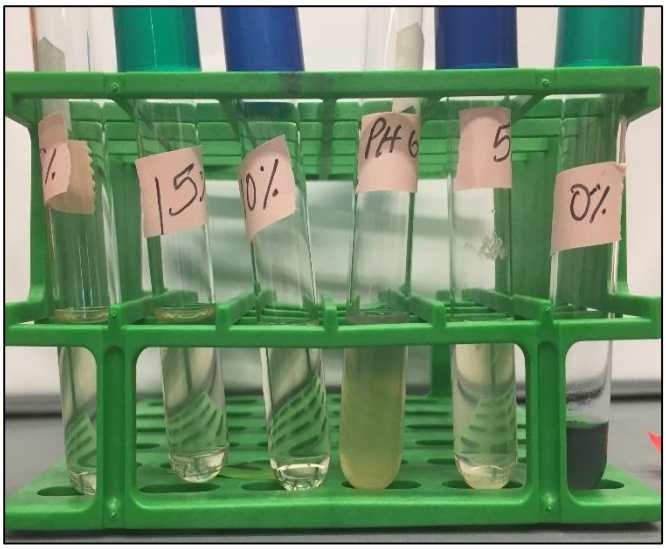
When testing the optimal environmental pH for the growth of the *Nitrosomonas eutropha*, the pH that yielded the best growth of *Nitrosomonas eutropha* was the broth with a pH level of 6 as seen in Figure 1.

Figure 1: Growth of *Nitrosomonas eutropha* in a pH 6 broth

Nitrosomonas eutropha in pH 6 broth on the left and control on the right. Note the turbid, pigmented broth in the tube on the left indicating the growth of the *Nitrosomonas eutropha*.

The results of testing the optimal salinity of the environment for the growth of *Nitrosomonas eutropha* concluded that the ideal salinity range was 0%-5% salinity as shown in Figure 2 below.

Figure 2: Optimal salinity for *Nitrosomonas eutropha* growth.



Note the pigmentation and turbidity changes in the 5% and 0% salinity tubes on the far right compared to the other salinity tubes indicating growth of the *Nitrosomonas eutropha*.

When working on establishing growth of the *Nitrosomonas eutropha* on a Nutrient Agar plate adequate growth was observed at 37^0C. However, it was later determined that optimal growth occurred at 23^0C in a dark environment. Growth on the Nutrient Agar plate can be described as a clear mucoid growth that changes the pigmentation of the media to a darker auburn color shown in Figure 3 below.

Figure 3: *Nitrosomonas eutropha* growth on a Nutrient Agar plate incubated at 37^0C

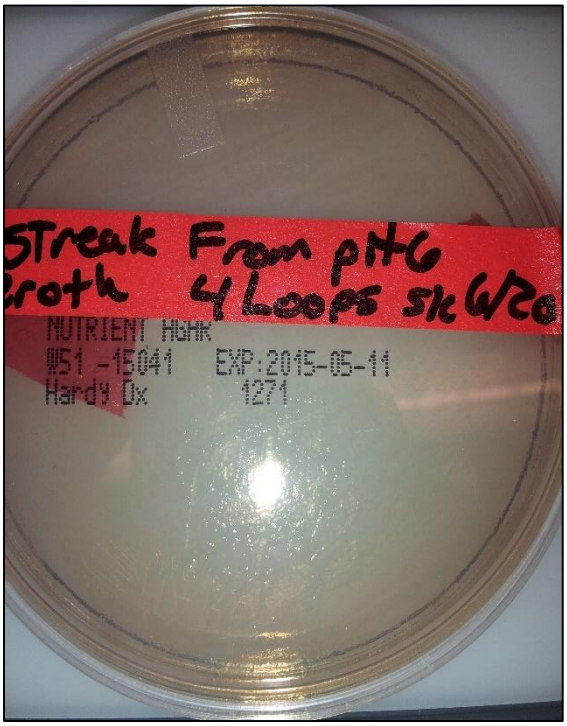


Figure 3 shows the first growth established on a Nutrient Agar plate viewed on a light box. This was obtained using four loops of sample from established growth in a pH 6 broth tube and incubated in the incubator at 37^0C for three days. Note the color change of the media and the translucent, mucoid growth.

Growth of *Nitrosomonas eutropha* on a Nutrient Agar plate incubated at room temperature can be seen in Figure 4 below. Note that the growth is still a mucoid consistency but the pigmentation of the growth has changed to a florescent greenish pigmentation.

Figure 4: Nitrosomonas eutropha growth on a Nutrient Agar incubated at room temperature

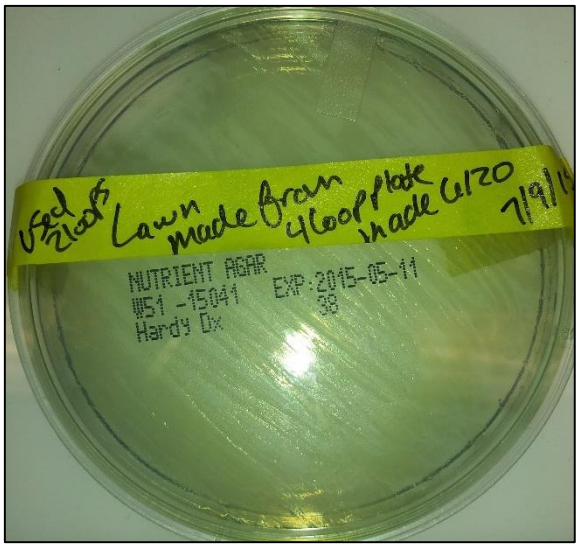


Figure 4 shows the growth of the *Nitrosomonas eutropha* on a Nutrient Agar plate that was incubated at room temperature in a dark environment for two days viewed on a white background. Note the growth is still a mucoid consistency but the pigmentation is a florescent green in color.

The streak plates showing individual colonies of the *Nitrosomonas eutropha* shown in Figure 5, shows mucoid growth that moves outward from the center of the colony. The center of the colony containing the “oldest” growth has a pigment to it while the outer edges lack pigmentation. The appearance of swarming from the center outwards suggest that the *Nitrosomonas eutropha* is a motile bacterium.

Figure 5: Streak Plate Containing Isolated Colonies of *Nitrosomonas eutropha*

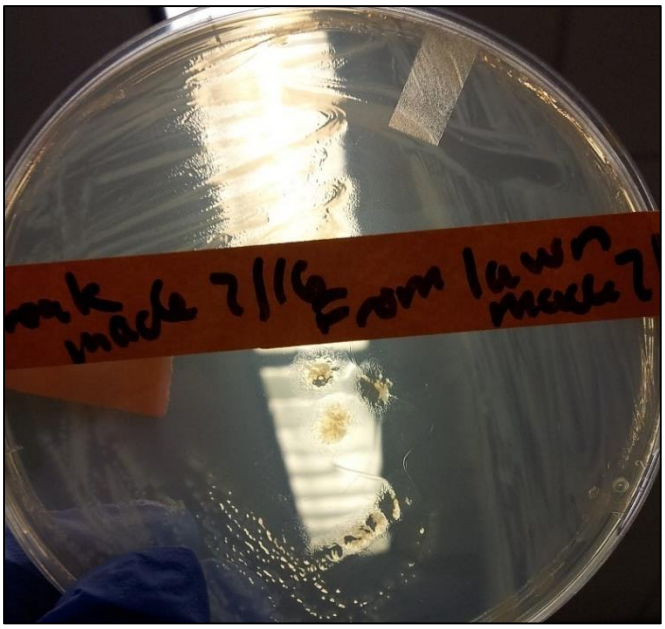
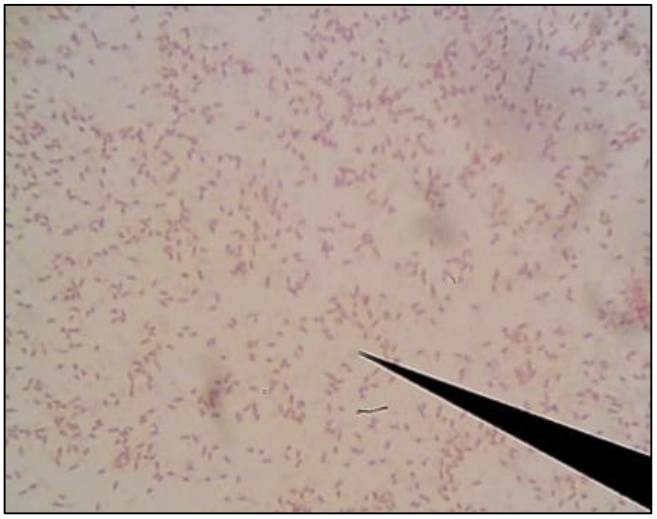


Figure 5 shows the streak plate as viewed with room lighting. Note how the growth in the individual colonies is mucoid in appearance, spreads out from the center, and the center of the isolated colony is pigmented while the outer edges remain clear.

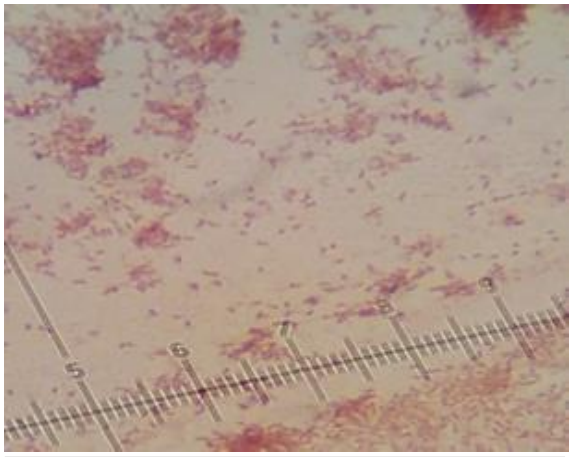
The slides that produced the best images are labeled as Slide 1E, 2E, and 2A. Each slide shows some unique features of the *Nitrosomonas eutropha*. Slide 1E shows basic morphology of the *Nitrosomonas eutropha* as being a Gram-negative, short rod bacterium seen in Figure 6. Slide 2E shows an improved view and contrast of the cell wall of the *Nitrosomonas eutropha* as shown in Figure 7 including the tendency of the bacteria to clump together. Slide 2A shows the changes that occur with the *Nitrosomonas eutropha* when the bacterium is exposed to a different temperature shown in Figure 8. The differences in appearance of the bacterial cells between slides 1 and 2 E and slide 2A can be directly attributed to the temperature and media that the bacteria grew in.

Figure 6: Slide 1E *Nitrosomonas eutropha*



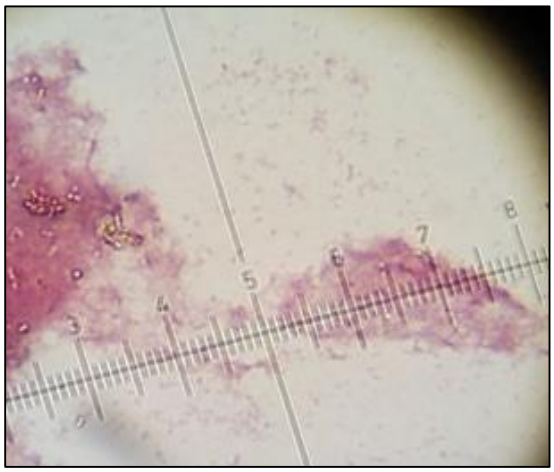
Slide 1E showing *Nitrosomonas eutropha* as a short rod, gram-negative bacillus at 100X and no zoom on the camera.

Figure 7: Slide 2E *Nitrosomonas eutropha*



Slide 2E showing an improved contrast of the cell walls of the *Nitrosomonas eutropha* as well as the clumping behavior of the bacteria. Seen at 100X magnification.

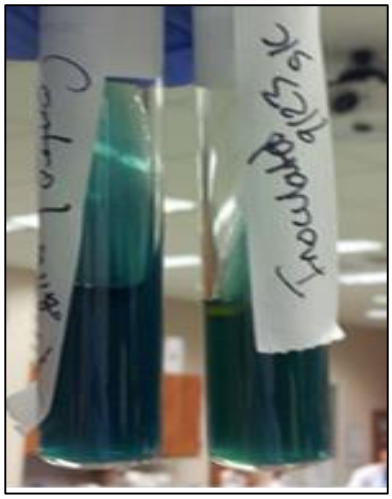
Figure 8: Slide 2A *Nitrosomonas eutropha*



Slide 2A showing in the middle far left of the picture what appears as a florescence to the cell wall of the *Nitrosomonas eutropha* when viewing the cells that were obtained from a sample that was refrigerated. This change illustrates the chromophore production capabilities of the *Nitrosomonas eutropha*. Seen at 100X

The Citrate test results show that *Nitrosomonas eutropha* does utilize citrate for a nutrient source as shown by the slight color change from the blue of the control tube to a more greenish color in the tube inoculated with *Nitrosomonas eutropha* in Figure 9.

Figure 9: Images of the Citrate Test



Citrate test shows a positive result in the *Nitrosomonas eutropha's* use of Citrate as a nutrient source. The inoculated tube on the right shows a change of color from the control tube on the left indicating the positive test result.

The results of the Sulfide-Indole Motility or SIMs testing shown in Figure 10, indicates that *Nitrosomonas eutropha* is highly mobile and does not utilize sulfur. It was also determined after the Kovac's regent was added that Nitrosomonas eutropha does not break down into indole and pyruvate as seen in Figure 11.

Figure 10: Sulfide-Indole Motility Test Prior to Kovac's Regent

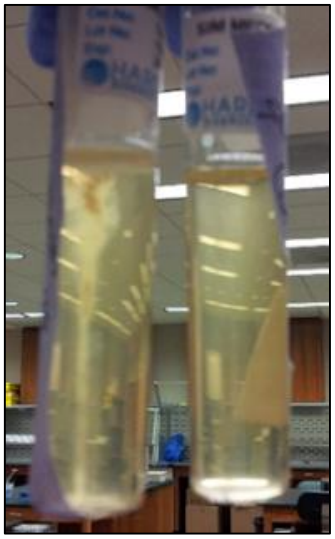


Figure 10 to the left shows the inoculated SIMs tube on the left of the image demonstrating *Nitrosomonas eutropha* to be highly motile. The control, un-inoculated tube on the right compared to the inoculated tube shows that no sulfur is utilized due to no color change in the media.

Figure 11: Sulfide-Indole Motility Test After Addition of

Figure 11 to the right shows the inoculated tube on the left and the un-inoculated control tube on the right. The SIMs test after the addition of Kovac's regent shows that *Nitrosomonas eutropha* does not break down Indole into pyruvate due to the lack of color change in the media.

Figure 12: Rapid Urea Test

Positive result on the Rapid Urea test as shown by the color change in the inoculated media on the right compared to the control on the left.

Results of the rapid urea testing determined that the *Nitrosomonas eutropha* rapidly hydrolyzes urea and has a strong production of urease in the presence of urea. This is shown by the color change in the inoculated media compared to the control as seen in Figure 12.

The Tryptic Soy Agar (TSA) testing for motility and respiration confirms that *Nitrosomonas eutropha* is highly motile, capable of respiring both anaerobically and aerobically as shown in Figure 13. The TSA test also confirms that the *Nitrosomonas eutropha* is not in the *Enterobacteriaceae* family.

Figure 13: Tryptic Soy Agar Test

Figure 13 to the right shows the control for the TSA test on the left and the inoculated TSA tube is on the right as evidenced by the growth into the media and outward from the stab inoculation with *Nitrosomonas eutropha*. This growth demonstrates the bacterium's ability respire anaerobically and the growth on the upper portion of the slant demonstrates the ability to respire aerobically.

Phenol Red with Dextrose testing, shown in Figure 14 resulted in an orange color and tiny bubbles in the Durham tube giving a slightly positive result for dextrose fermentation and gas production. The Phenol Red with Lactose test, shown in Figure 15, is orange in color with no bubble indicating again a very slight positive result for lactose fermentation and no gas production during the metabolism and fermentation of lactose.

Figure 14: Phenol Red with Dextrose

Figure 14 to the left shows the color change in the tube on the left inoculated with *Nitrosomonas eutropha* to an orange color compared to the control on the right, which remains red.

Figure 15: Phenol Red with Lactose

Figure 15 to the right shows the color change in the tube to the right inoculated with *Nitrosomonas eutropha* to an orange color while the control is on the left remains red.

The results of the Enterotube II after nine days of incubation are shown in Table 1 below. Images for the Enterotube II results prior to the addition of the Kovac's regent and after the addition of the regent are shown in Figures 16, 17, and 18 on the following page.

Table 1: Results of Enterotube II Inoculated with *Nitrosomonas eutropha*

Test	Positive or Negative	What Result Means
Glucose	Negative	Does not ferment glucose
Lysine	Negative	Does not decarboxylate lysine
Ornithine	Negative	Does not decarboxylate ornithine
H_2S/Indole	Positive/Negative	Reduces sulfur and does not produce Indole
Adonitol	Negative	Does not ferment Adonitol
Lactose	Negative	Does not ferment Lactose
Arabinose	Negative	Does not ferment Arabinose
Sorbitol	Negative	Does not ferment Sorbitol
VP	Negative result	Did have a greenish beige color which was different than expected but NOT a positive result

Figure 16: Enterotube II Inoculated with *Nitrosomonas eutropha* Prior to Addition of Kovac's Regent with Labels Visible (below)

Figure 17: Enterotube II Inoculated with *Nitrosomonas eutropha* Prior to Addition of Kovac's Regent Showing the Backside of the Individual Cells with Room Light in Background (below) Note: Image is the reverse of Figure 16

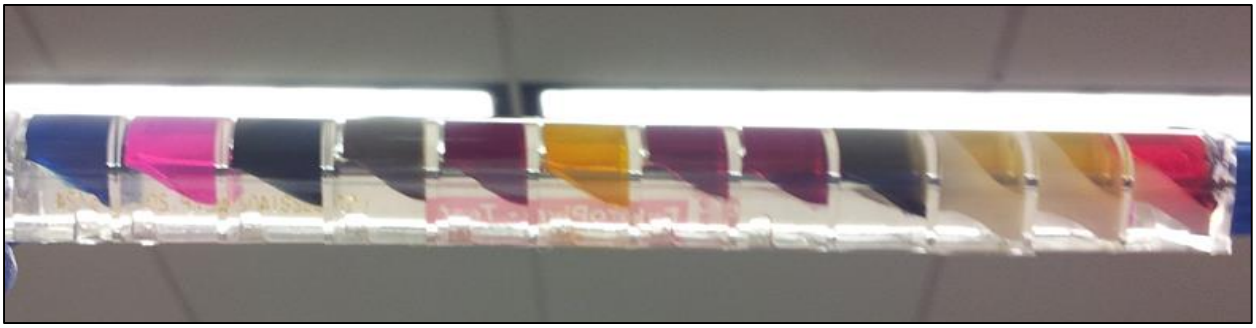
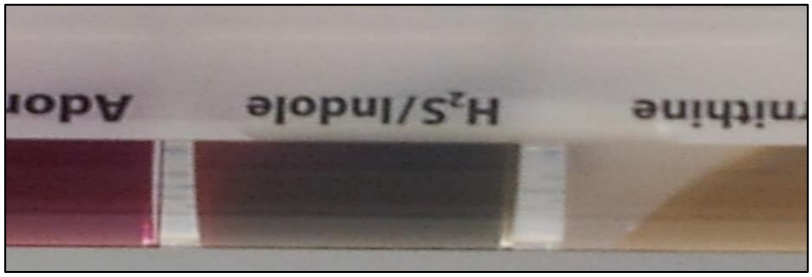


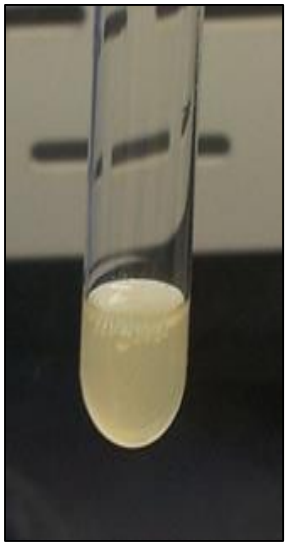
Figure 18: Enterotube II H_2S/Indole Cell After Kovac's Regent is Added (below)



The Vogues-Proskauer broth that is used for the Methyl Red test and the Vogues-Proskauer (VP) test shows good growth of *Nitrosomonas eutropha* as seen in Figure 19. No color change as seen in Figure 20 on the following page, once the Methyl Red was added to the tube containing 1 mL of VP broth colonized with *Nitrosomonas eutropha* demonstrates no mixed acid fermentation took place. The Vogues-Proskauer (VP) testing revealed that there is no mixed acid fermentation during the metabolic processes of *Nitrosomonas eutropha.* This is evidenced by the lack of notable color change once fifteen drops of α-napthol regent and fifteen drops of KOH 40% regent were added to the VP broth containing *Nitrosomonas eutropha* shown in Figure 21, and there continued to be no color change checked every ten minutes over a

period of one hour shown in Figure 22. Therefore, giving a negative result on the VP test reinforcing that it is not of the *Enterobacteriaceae* family.

Figure 19: VP Broth Showing Good Growth of *Nitrosomonas eutropha* Before Any Regents Added

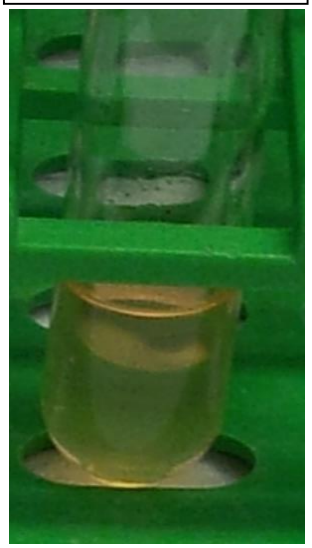


Notice the turbidity of the broth with slight pigmentation indicating growth *of Nitrosomonas eutropha*

Figure 20: VP Broth with *Nitrosomonas eutropha* After Methyl Red is Added

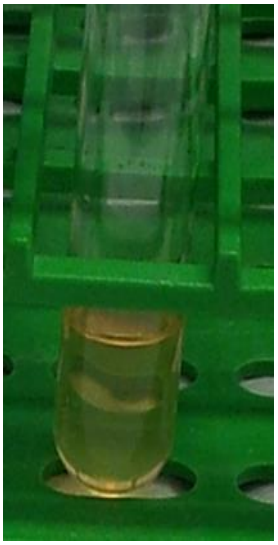
Notice the color did not change to red; this is a negative indication of mixed acid fermentation.

Figure 21: VP Broth with *Nitrosomonas eutropha* Ten Minutes After α-napthol and KOH 40% are Added



Notice no color change after α-napthol and KOH 40% regents were added indicating a negative result.

Figure 22: VP Broth with *Nitrosomonas eutropha* Sixty Minutes After α-napthol and KOH 40% are Added



Notice that there is still no color change after sixty minutes reinforcing the negative result.

A nitrate reduction test was performed resulting in a positive test result showing *Nitrosomonas eutropha* growth and air bubbles in the Dextrose tubes seen in Figure 23. This indicates that the *Nitrosomonas eutropha* does reduce Nitrate to produce Nitrogen and non-gaseous Nitrogenous products.

Figure 23: Nitrate Reduction Test

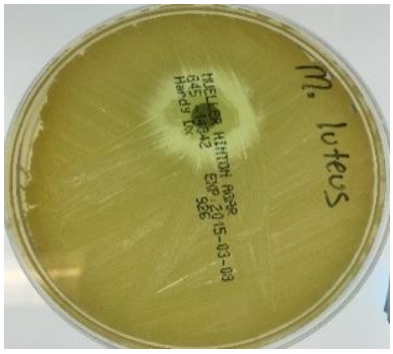
Figure 23 to the right shows *Nitrosomonas eutropha* growth as shown by turbidity and a positive test result as indicated by the bubble in the tube.

The results of the testing of whether or not the *Nitrosomonas eutropha* inhibited the growth of potentially pathogenic bacteria showed that the disc soaked in the *Nitrosomonas eutropha* did indeed inhibit the growth of all of the species of bacterium tested as seen in Figures 24-27. The inhibition zones are shown in Table 2.

Figure 24: *Staphylococcus aureus* Lawn with *Nitrosomonas eutropha* Saturated Disc

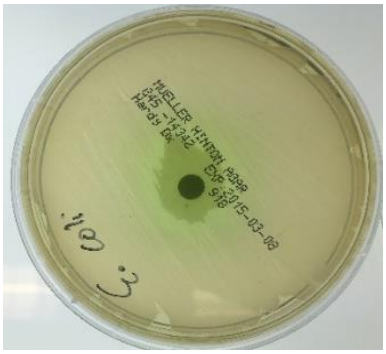
The Zone of Inhibition measures 26 mm and a florescent green pigmentation is observed where it appears the *Nitrosomonas eutropha* is growing into the *Staphylococcus aureus*.

Figure 25: *Micrococcus luteus* Lawn with *Nitrosomonas eutropha* Saturated Disc



The Zone of Inhibition measures 24 mm and an auburn pigmentation is noted. There appears to be a area of no growth of *Micrococcus luteus* with regrowth around the disk.

Figure 26: *Escherichia coli* Lawn with *Nitrosomonas eutropha* Saturated Disc



The Zone of Inhibition measures 30 mm and a florescent green pigmentation is noted. The *Nitrosomonas eutropha* appears to be growing into the *Escherichia coli*.

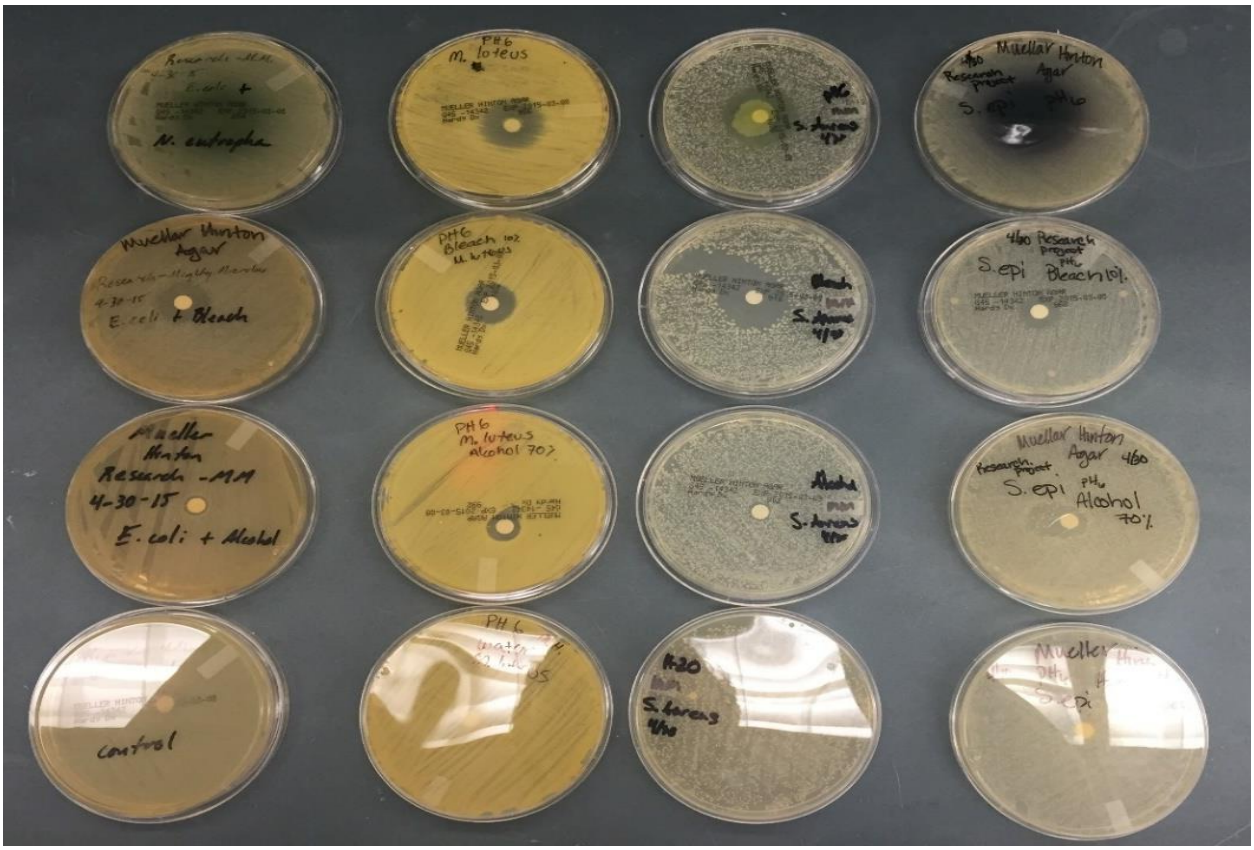
Figure 27: *Staphylococcus epidermidis* Lawn with *Nitrosomonas eutropha* Saturated Disc

The Zone of Inhibition for the Figure to the left measures 36 mm and a florescent green pigmentation is observed. It appears that the *Nitrosomonas eutropha* is growing into the *Staphylococcus epidermidis*.

Table 2: Zones of Inhibition for Pathogenic Bacteria

Organism	ZOI in mm	Pigmentation
Staphylococcus aureus	26 mm	Florescent Green
Micrococcus luteus	24 mm	Auburn
Escherichia coli	30 mm	Florescent Green
Staphylococcus epidermidis	36 mm	Florescent Green

Figure 28: Comparison of the Inhibition of Pathogenic Bacterial Species between the Use of Antiseptics and Nitrosomonas eutropha with a Control Shown



In Figure 28 above, a comparison is shown in how the potentially pathogenic bacteria is inhibited in one of three ways with the control on the bottom row. The top row is the pathogenic bacterial lawn with the *Nitrosomonas eutropha* saturated Kirby Bauer disc. The second row from the top is the pathogenic bacterial lawn with a bleach soaked Kirby Bauer disc. The third row down is pathogenic bacterial lawn with a 70% isopropyl alcohol saturated Kirby Bauer disc. Again, the very bottom row is the control, a pathogenic bacterial lawn with a sterile Kirby Bauer disc in the center. The pathogenic bacterial laws are from right to left: *Escherichia coli, Micrococcus luteus, Staphylococcus aureus,* and *Staphylococcus epidermidis.*

The results for the comparison between how *Nitrosomonas eutropha* inhibits pathogenic bacterica and how antiseptics such as bleach and isopropol alcohol inhibit the growth of the same pathogenic bacterium is shown in Figure 28 at the bottom of the previous page.

The pigmentation changes that are seen in the pictures throughout the paper are the result of the fact that *Nitrosomonas eurtropha* produce a chromophore and exhibit pigmentation changes when subjected to tempurature, pH, and other enviornmental stressors. Pigmentation can range from a greenish color to a yellow, or even deep auburn.

The results of the different soaps ability to inhibit the growth of the *Nitrosomonas eutropha* are shown by a Zone of Inhibition Table at seventy-two hours out from inoculation that includes descriptions and active ingredients for each soap shown as Table 3, and a second Table showing Zones of Inhibition and descriptions at one-hundred twenty hours post inoculation shown in Table 4. In addition Figures 29–38 show the interaction between the soaps and the *Nitrosomonas eutropha* at seventy-two hours and Figures 39-48 show the interaction between the soaps and the *Nitrosomonas eutropha* at one-hundered twenty hours post inoculation.

The results of the testing of the soaps revealed additional chemolithoautotrophic characteristics in the form of a wider range of pigmentation even though the temperature and other environmental conditions remained constant. This leads to the hypothesis that the environmental stressors of the *Nitrosomonas eutropha* are due to the pH of the soaps as well as the ingredients found in the soaps and their chemical reactions with the *Nitrosomonas eutropha*. This was evidenced by the most drastic color changes taking place with soaps that were high in sugars or other carbohydrates such as the Sugar scrubs and Oatmeal scrubs which yielded a more auburn pigment in the growth around the disc saturated with soap, while other soaps resulted in a white to a yellowish-green pigmentation. The fewer the ingredients and the more natural the ingredients overall the less inhabitation of the *Nitrosomonas eutropha* was observed and if inhibited at all the faster it grew back into the inhibition zone. In addition, the more natural and fewer the ingredients in the soaps the lighter and less pigmentation was observed in the *Nitrosomonas eutropha* growth.

Table 3: Inhibition of *Nitrosomonas eutropha* growth at 72 hours with soaps

Soap	Zone of Inhibition In mm	Pigmentation	Top Five Ingredients
Softsoap Milk and Honey hand soap	3-5 mm	None	Water, Sodium C14-16 Olefin Sulfonate, Laureth-3, Cocamidopropyl Betaine, Glycol Stearate…
Dial Liquid Springwater Antibacterial soap	2 mm	Solid white-auburn ring	**Active:** Benzethonium Chloride 0.10% **Inactive Top Five:** Water, Cetrimonium Chloride, Glycerin, Lauramine Oxide, Sodium Chloride…
Dove Deep Moisture Liquid Body Wash	0 mm	Cream colored	Water, Cocamidopropyl Betaine, Sodium Hydroxpropl Starch Phosphate, Lauric acid, Sodium Lauroyl Glycinate…
Yes! To Tomatoes Clear Skin Acne Daily Pore Scrub	0 mm	None	**Active:** Salicylic acid 2% **Inactive Top Five:** Water, Stearic Acid, Propanediol, Sodium Stearate, Glycerin…
Charcoal and Black Sugar Polishing Mask	0 mm	Florescent Green	Sucrose, Propylene Glycol, Carbon (activated charcoal), Kaolin, musa sapientum (banana)…
SHEA Sugar Scrub with Almond and Honey	2-5 mm	Auburn pigment furthur from the disc	Sucrose, glycerin, polysorbate 20, silica, honey…
Exotically Creamy Coconut Rich Body Butter	0 mm	None	Water, cetearyl alcohol, Paraffinum liquidum (mineral oil), Cyclopentasiloxane, Glyceryl stearate SE…
Yes! To Carrots Daily Facial Cleanser	0 mm	None to cream colored	Water, Glycerin, Disodium Cocomphodipropionate, stearic acid, glyceryl…

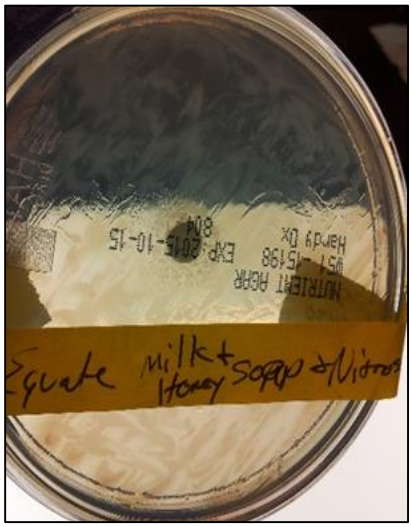
Clearly Natural Pure Glycerin Bar Soap (unscented)	Irregular 1-5 mm	Translucent green to an auburn pigment	Glycerin, propylene glycol, sodium stearate, Pecyl Glucoside, Sorbitol…
Yardley London Oatmeal and Almond Bar Soap	Irregular 1-5 mm	Translucent green to auburn pigment	Sodium Tallowate, water, sodium cocoate, glycerin, Fragrance…

Table 4: Inhibition of *Nitrosomonas eutropha* growth or evidence of regrowth with soaps at 120 hours

Soap	Zone of Inhibition in mm	Pigment	Regrowth observed
Softsoap Milk and Honey hand soap	4-6 mm	Cream to Auburn colored	Small specs of what appears as individual colonies around a disc of white in the form of tiny white specs
Dial Liquid Springwater Antibacterial soap	2 mm	Solid white to auburn	None
Dove Deep Moisture Liquid Body Wash	Up to 5 mm	auburn	Shows new growth up to 5 mm from disc with a greenish pigment
Yes! To Tomatoes Clear Skin Acne Daily Pore Scrub	0 mm	Cream to light auburn	None
Charcoal and Black Sugar Polishing Mask	0 mm	Translucent to deep auburn	None
SHEA Sugar Scrub with Almond and Honey	0 mm	Deep auburn to purplish color	Growth has moved into the ring observed at 72 hours
Exotically Creamy Coconut Rich Body Butter	0 mm	Slight auburn to translucent	None
Yes! To Carrots Daily Facial Cleanser	0 mm	Cream colored to translucent	None

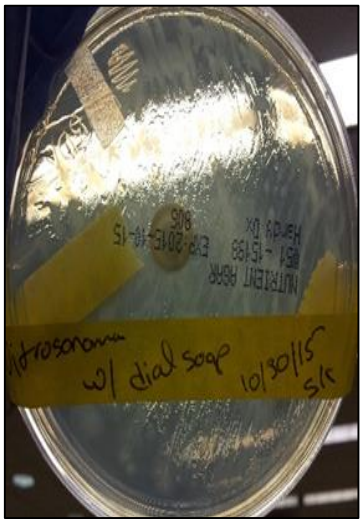
Clearly Natural Pure Glycerin Bar Soap (unscented)	2-4 mm	Darker auburn pigmentation then at 72 hours.	Appears to have a ring of concentrated growth around the sliver of soap measuring 2-4 mm out
Yardley London Oatmeal and Almond Bar Soap	1-2 mm	Translucent to greenish pigmentation	There appears to be white mucoid growth on the sliver of soap and 1-2 mm of heavy growth around the soap.

Figure 29: *Nitrosomonas eutropha* Lawn with a Softsoap Milk and Honey Kirby Bauer Disc at 72 Hours



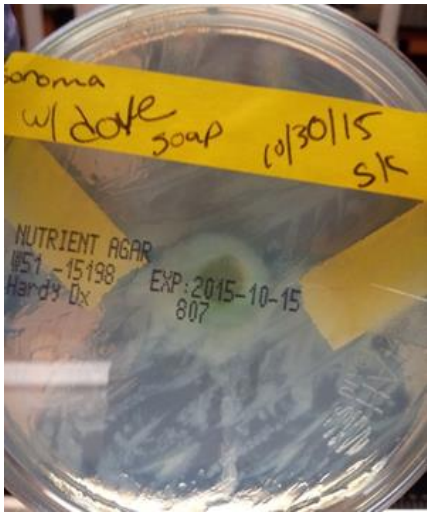
Good growth is observed up to approximately 3 mm from the disc when the growth is more sporadic or absent. Notice the auburn pigment of the growth.

Figure 30*: Nitrosomonas eutropha* Lawn with a Liquid Dial Kirby Bauer Disc at 72 Hours



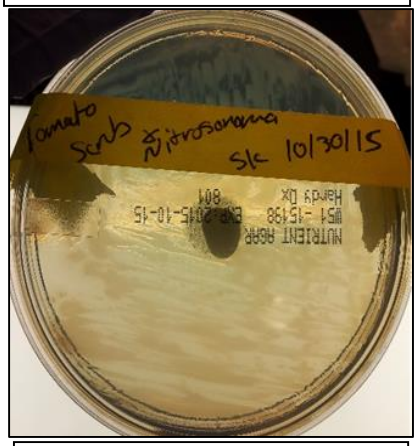
Good growth is observed up to the 2 mm wide solid white-auburn ring around the disc

Figure 31*: Nitrosomonas eutropha* Lawn with a Kirby Bauer Disc saturated with Dove Liquid soap at 72 Hours



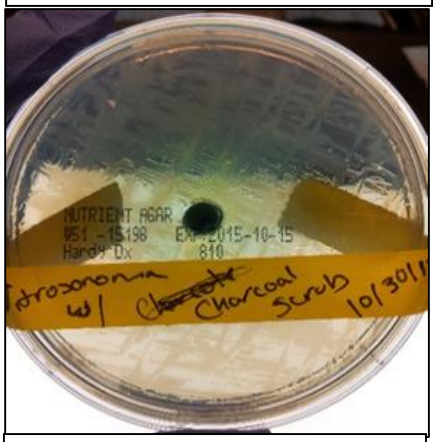
Good growth is observed throughout the plate having a cream-colored pigmentation with a thick area of growth around the disc

Figure 32: *Nitrosomonas eutropha* Lawn with a Yes to Tomatoes Kirby Bauer disc at 72 hours



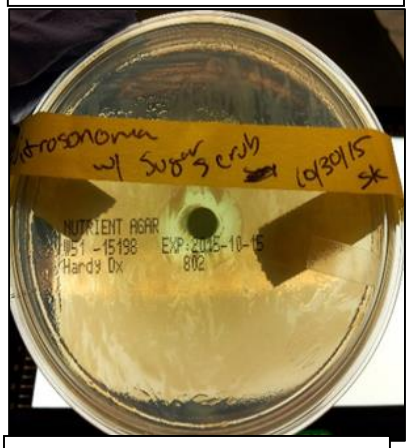
Notice the excellent auburn tinted growth throughout the plate, even right up to the disc saturated in Tomato scurb

Figure 33: *Nitrosomonas eutropha* Lawn with a Charcoal and Black Sugar disc at 72 hours



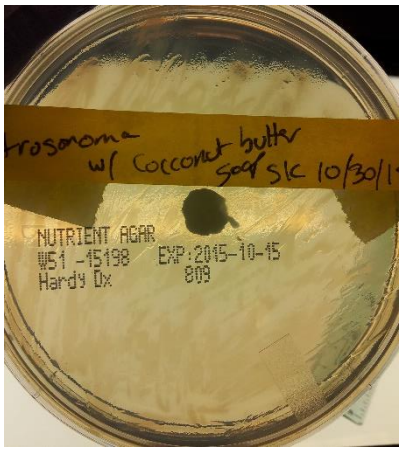
Notice the heavy pigmentation ranging from deep green to a deep auburn in the growth on the media and heavy growth up to the disc

Figure 34: *Nitrosomonas eutropha* Lawn with a SHEA Sugar Scrub disc at 72 Hours



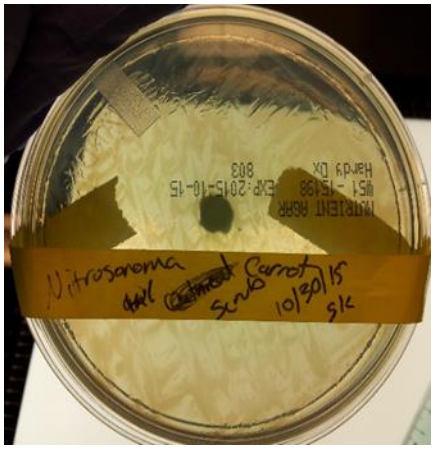
Notice the good growth throughout the plate with auburn pigmentation of growth further from the disc and greenish pigmentation closer to the disc.

Figure 35: *Nitrosomonas eutropha* Lawn with a Coconut Body Butter disc at 72 Hours



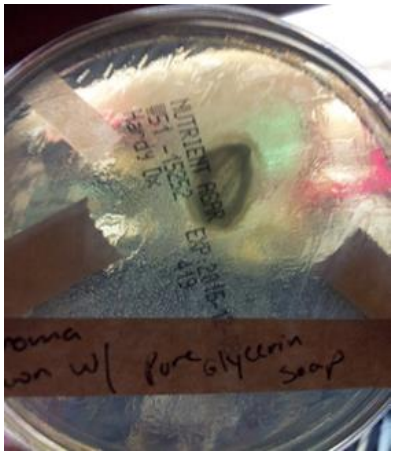
Notice the excellent growth with no pigmentation to a slight cream color even right up to the disc

Figure 36*: Nitrosomonas eutropha* Lawn with a Yes to Carrots disc at 72 Hours



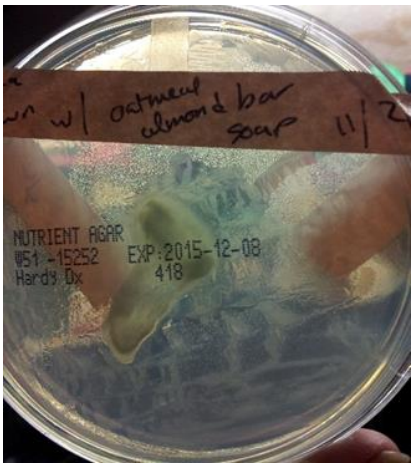
Notice the uninterrupted growth right up to the disc that has no pigmentation to a slight cream color

Figure 37: Nitrosomonas eutropha Lawn with a sliver of Pure Glycerin Bar soap at 72 Hours



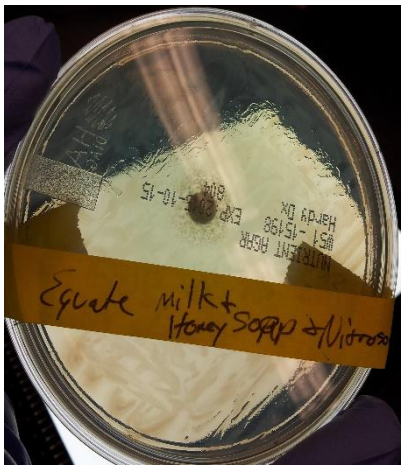
Notice the thick healthy green to auburn growth extending from the sliver of soap

Figure 38: *Nitrosomonas eutropha* Lawn with a sliver of Oatmeal and Almond Bar soap at 72Hours



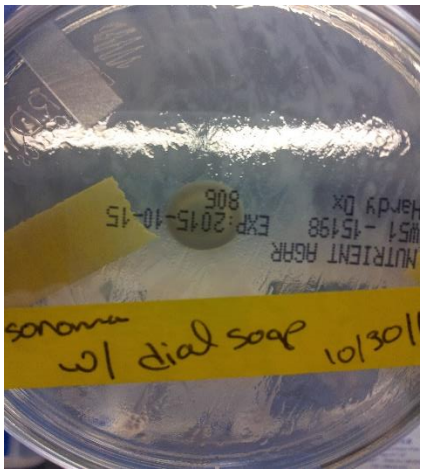
Notice the irregular heavy growth around the sliver of soap, which has a green to auburn pigmentation depending on the distance from the soap sliver.

Figure 39*: Nitrosomonas eutropha* Lawn with Softsoap Milk and Honey disc at 120 Hours



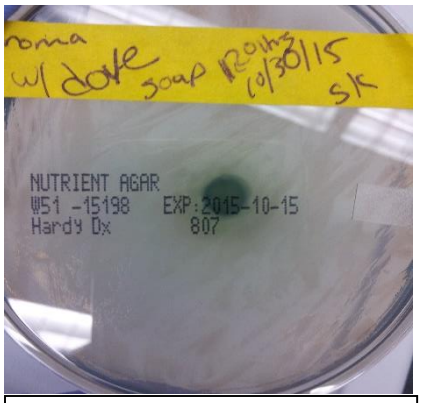
Notice continued good growth throughout the plate with auburn pigmentation and new growth forming in a ring around the disc

Figure 40: *Nitrosomonas eutropha* Lawn with Liquid Dial disc at 120 Hours



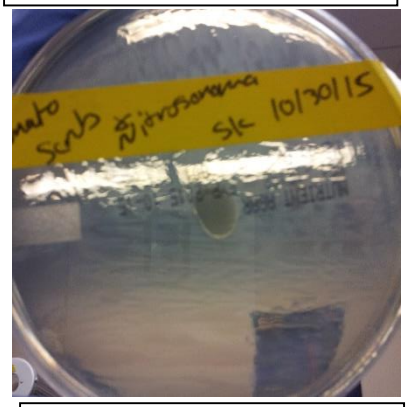
Notice solid white to auburn tinted ring around the disc, continued growth at outer edges of the plate with no pigmentation in the growth beyond and no new growth observed

Figure 41*: Nitrosomonas eutropha* Lawn with Liquid Dove disc at 120 Hours



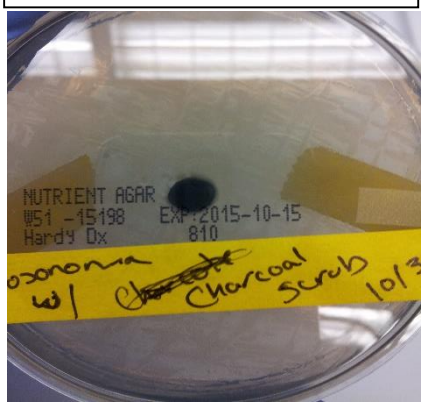
Notice the green pigmentation in the new growth around the disc and the auburn pigmentation in the growth further out

Figure 42: *Nitrosomonas eutropha* Lawn with Tomato Scrub disc at 120 Hours



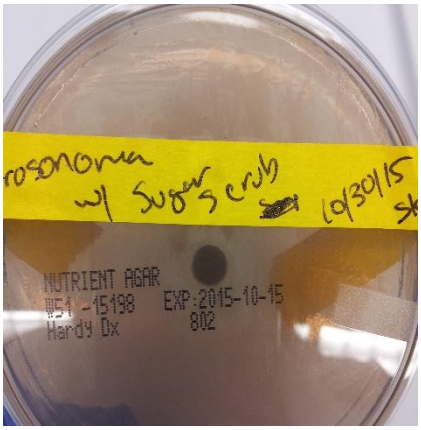
Notice the healthy mucoid growth with no pigmentation changes even against the disc containing the tomato scrub

Figure 43: *Nitrosomonas eutropha* Lawn with Charcoal and Black sugar disc at 120 Hours



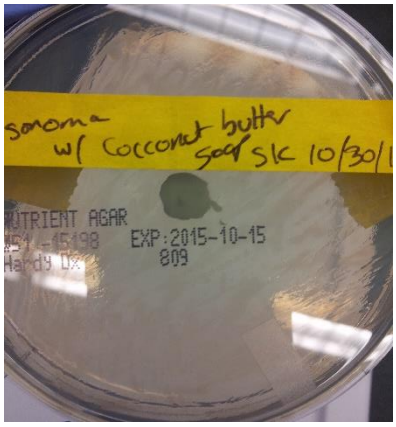
Notice the health growth of the throughout the plate, even right up to the disc also note the darker auburn pigmentation

Figure 44: *Nitrosomonas eutropha* Lawn with SHEA Sugar scrub disc at 120 Hours



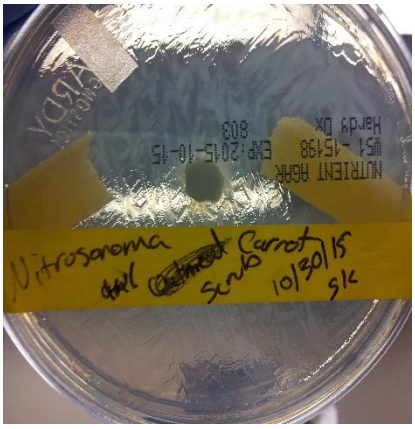
Notice the thick growth and dark auburn pigmentation. There appears to be regrowth into the area that was considered the ZOI at 72 hours.

Figure 45: *Nitrosomonas eutropha* Lawn with Coconut Body Butter disc at 120 Hours



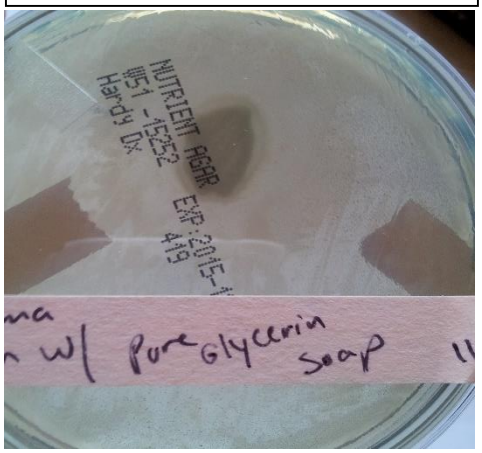
Notice the continued healthy growth even up to the disc. The pigmentation has changed slightly in some areas to a slight auburn pigmentation

Figure 46: *Nitrosomonas eutropha* Lawn with Carrot scrub disc at 120 Hours



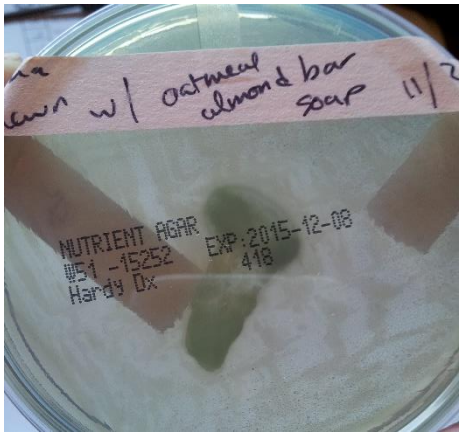
Notice the continued healthy growth even up to the disc and no pigmentation changes.

Figure 47: *Nitrosomonas eutropha* Lawn with sliver of Glycerin Bar Soap at 120 Hours



Notice the darker auburn pigment and loss of the green pigment in the growth compared to 72 hours. Also, notice the heavier growth seen around the sliver of soap extending out 2-4 mm from the sliver of soap

Figure 48: Nitrosomonas eutropha Lawn with sliver of Oatmeal and Almond soap at 120 Hours



Notice that the growth further from the sliver of soap is a slight auburn pigmentation while closer to the sliver it is a greenish pigment. Note increased growth 1-2 mm out from the sliver.

Discussion

All testing, research, and experimentation leads to the conclusion that the AO+ Biome product does indeed contain the bacteria *Nitrosomonas eutropha,* which are a highly motile, gram-negative, short rod-shaped, ammonia oxidizing bacteria. *Nitrosomonas eutropha* have chromolitoautophic characteristics and is able to respire in both anaerobic and aerobic environments. *Nitrosomonas eutropha* prefers a slightly acidic environment with a pH of 6, it grows well in salinities of 0-5%, and grows the best in temperatures between 20^0C and 24^0C. *Nitrosomonas eutropha* is a nitrate oxidizing bacteria producing nitrogen and non-gaseous nitrogenic byproducts. *Nitrosomonas eutropha* hydrolyzes urea and produces urease in the presence of urea. *Nitrosomonas eutropha* does utilize citrate as a nutrient source, and will ferment arabinose, a naturally occurring monosaccharide found in the products of the hydrolysis of various wood-based materials or other biomasses. This is fitting as *Nitrosomonas eutropha* naturally lives in the soil as well as within the normal flora of our skin.

Nitrosomonas eutropha significantly inhibits the growth of the potentially pathogenic bacterium *Staphylococcus epidermidis*, and *Staphylococcus aureus*, and a moderate inhabitation of *Escherichia coli* and *Micrococcus luteus*. Further research and experimentation with additional species of bacteria could expand on the list of potentially pathogenic bacteria that *Nitrosomonas eutropha* inhibits. This leads to the conclusion that the *Nitrosomonas eutropha* is indeed beneficial to the natural flora of the human skin.

When testing some of the soaps available on the market today, including some of the more natural glycerin soaps used in previous generations, it was found that the soaps with the most ingredients, that were more chemically based, and had antibacterial ingredients inhibited the growth of the *Nitrosomonas eutropha* the greatest with the slowest regrowth rate of the *Nitrosomonas eutropha*. Soaps that contained the fewest and most natural ingredients such as the tomato scrub, Dove, and Oatmeal scrub, etc., had little to no effect on the growth of the *Nitrosomonas eutropha* and if any initial interruption of growth did occur, the regrowth of the *Nitrosomonas eutropha* was rapid and abundant. When looking at the results of the soap testing and looking at Tables 3 and 4, one must keep in mind that people wash their hands throughout the day ad nauseum, and most people bathe daily or at the least every other day. The experiment done on this small list of available soaps shows that those soaps that do inhibit the growth of *Nitrosomonas eutropha* initially generally require an average of 2-4 days to regrow and repopulate the areas that were initially inhibited due to the soap used. The idea of reintroducing the *Nitrosomonas eutropha* after bathing or washing one's hands through an artificial method such as a spray, cream, or other vehicle as

being beneficial over having to wait for the *Nitrosomonas eutropha* to repopulate the skin naturally, is a very sound one. Most likely, there will not be enough time for the *Nitrosomonas eutropha* to grow back on its own between bathing and handwashing to provide a protective benefit as was shown when testing how the soaps inhibit the growth of *Nitrosomonas eutropha* and regrowth was observed in period of 72 to 120 hours depending on the soap.

It would be advantageous to encourage further study on the benefits of *Nitrosomonas eutropha* to humans via the repopulation and reintroduction of *Nitrosomonas eutropha* to the normal flora of human skin. Researching the possible significant benefits in recolonization of the human flora with the *Nitrosomonas eutropha* such as the reduction of infections within the general population, infections caused by drug resistant pathogens, and infections in immunocompromised individuals, such as those who have cancer, immature or weakened immune systems due to autoimmune disease, medication or other pathophysiological conditions that render the immune system less than fully functional. Additionally, the reduction in infections among agricultural workers both crop and livestock farmers because of their constant contact with *Escherichia coli* and other pathogenic bacteria within their occupation, with a possibility of also reducing the spread of disease within a heard on a farm where individuals utilize products containing *Nitrosomonas eutropha* before, after, and in between handling individual animals, could yield some important breakthroughs in public health.

References

1. “Nitric Oxide Functions in Humans.” *AOBiome.* Web. March 2015. http://aobiome.com/nitric-oxide-function-humans

2. “The Human Microbiome.” *AOBiome*. Web. March 2015. http://aobiome.com/human-microbiome

3. Hommes, Norman G., Luis A. Sayavedra-Soto, and Daniel J. Arp. “Chemolithooganotrophic Growth of *Nitrosonomas europaea* on Fructose.” *Journal of Bacteriology* 185.23 (2003): 6809-6814. Web. 18 June 2015. http://jb.asm.org/content/185/23/6809.full

4. Rubel, Jan. “Comparing the Effects of Commercial Antibacterial Soaps on Bacteria.” 24 Feb 1997. Web. 26 Oct 2015. http://web.horacemann.org/academics/science/expbio/webpages/rubel/bacteria.html

5. Schmidt, Ingo, Rob J. M. van Spanning, and Mike S. M. Jetten. “Denitrification and Ammonia Oxidation by *Nitrosonomas eruopaea* Wild Type, and NirK-and NorB Deficient Mutants.” *Microbiology* 150.12 (2004): 4107-4114. Web. 18 June 2015. http://mic.sgmjournals.org/content/150/12/4107.long

6. Schmidt, Ingo. “Chemooranoheterotrophic Growth of *Nitrosomons europaea* and *Nitrosomonas eutropha”.* Current Microbiology 59.2 (2009): 130-138. *ProQuest*. Web. August 2015. http://dx.doi.org/10.1007/s00284-009-9409-8

7. Stein, L.Y., et al. “Whole-Genome Analysis of the Ammonia-Oxidizing Bacterium, *Nitrosonomas europha* C91: Implications for Niche Adaptation.” *Environmental Microbiology* 12 (2007): 2993-3007. *PubMed.* Web. 18 June 2015. http://www.ncbi.nlm.nih.gov/pubmed/17991028