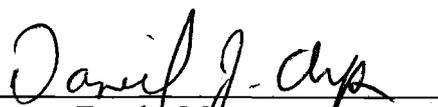


**Dormancy phase of *Nitrosomonas europaea*: The inducement
and temporary suspension of dormancy phase and the effects on
AMO and HAO.**

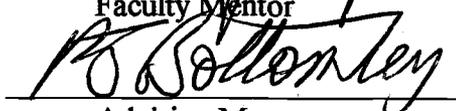
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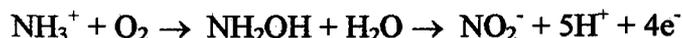
Abstract

Nitrosomonas europaea is an autotrophic ammonia-oxidizer that has been found to cometabolize various halogenated hydrocarbons. This ability to cometabolize various compounds (many of which are considered contaminants) has caused *N. europaea* to be considered a potential bioremediational organism. However, if *N. europaea* is to be used for bioremediation purposes, it will be necessary to understand *N. europaea*'s normal state in its natural environment, (thought to be a dormancy stage), and to understand how *N. europaea* may be activated to cometabolize desired compounds. *N. europaea* was shown to enter a dormancy phase under stress and starvation conditions. This dormancy phase was exhibited by an extended lag time needed to achieve full growth when inocula taken from cultures 11 and 12 days old. The lag time was found to be related to the loss of the enzyme ammonia monooxygenase, but not to the enzyme hydroxylamine oxidoreductase. Cells could be temporarily prevented from entering dormancy stage and exhibiting long lag times by resuspending in medium containing no growth substrate. This temporary suspension of the aging process could be maintained for up to 5 days.

Introduction

Nitrosomonas europaea is a lithoautotroph that uses ammonia as its only energy source, and utilizes CO₂ as its sole carbon source (2). *N. europaea* is an ubiquitous bacterium that is found in the first 10 cm of soil and the top layers of streams, sewage, and marine environments (2). The bacterium uses two main enzymes to metabolize ammonia. The first enzyme is ammonia monooxygenase (AMO), which initiates the ammonia-oxidizing reaction by oxidizing NH₃ to hydroxylamine (NH₂OH). Hydroxylamine is then oxidized to NO₂⁻ by the enzyme hydroxylamine

oxidoreductase (HAO), and four electrons are released. Two of the electrons are routed back to provide energy for the AMO reaction and the other two electrons enter the electron transport chain to provide energy for the bacterium. (Equation 1)



As an ammonia-oxidizing bacterium, *N. europaea* has historically been studied to find a way to develop ammonia-oxidizer inhibitors. This is due to the effect that autotrophic nitrifiers have had in agriculture. Autotrophic nitrifiers are largely responsible for significant losses of ammonia-based fertilizer applied to crops (4, 6). Although *N. europaea* has also been studied for its use in sewage plants where it plays a beneficial role in the removal of excess ammonia (6), the enzymology of *N. europaea* has mostly been studied to help design ammonia-oxidizer inhibitors.

More recently *N. europaea* has been found to oxidize a variety of halogenated hydrocarbons through the action of AMO, as AMO has a demonstrated lack of specificity (5). These compounds include trichloroethylene (a suspected carcinogen that has been widely spread by industry) (5), and other hydrocarbon substrates including alkanes, alkenes, ethers, thioethers, and aromatics (4). This ability to break down a wide variety of organic compounds has raised interest in trying to develop *N. europaea* for bioremediation uses.

If *N. europaea* is to be developed for bioremediation purposes, then it will be necessary to understand *N. europaea*'s normal state in its natural environment, and to understand how *N. europaea* may be activated to break down desired compounds.

In their natural environment bacteria are often starved for nutrients and exhibit very slow or no growth. When a source of nutrients becomes available, the bacteria recover out of their slow growth or no growth state and begin to grow rapidly and to replicate. This is commonly referred to as the feast-or-famine lifestyle (7). This slow growth or no growth phase is considered to be

the dormancy phase of a bacterium. In the life cycle of a bacterium, dormancy stage is entered into after reaching stationary phase. Stationary phase as defined by Kolter, et al (7) is the state when the number of bacterial cells in a culture ceases to increase. Dormancy phase is the when the bacterium has prepared itself to survive long periods of starvation. Bacteria such as *N. europaea* need a way to survive prolonged periods of stress and starvation. Previous experiments have proved that *N. europaea* is not a spore-forming bacterium and therefore *N. europaea* must enter a form of dormancy phase. The objective of this study was to examine the dormancy phase of *N. europaea* and the effects of dormancy phase on the production and activity of AMO and HAO.

Materials and Methods

Bacteria and Materials

Nitrosomonas europaea was grown as described previously (3) in 2 liter batches and also in 250 ml batches. All cultures were kept on a shaker in the dark, at 30°C throughout all experiments.

AMO activity assay. *N. europaea* was grown as described above. Cell culture (100 ml) was centrifuged at 8,000 rpm for 10 minutes. Cells were resuspended in 10 ml phosphate buffer (50 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 7.8) and centrifuged for 8,000 rpm for 10 minutes. Cells were resuspended in 300 μl phosphate buffer. Three glass vials (10 ml), were set up as follows: 1) 20 μl of 0.5 mM $(\text{NH}_4)_2\text{SO}_4$, 880 μl phosphate buffer. 2) 900 μl phosphate buffer. 3) 20 μl of 0.5 mM $(\text{NH}_4)_2\text{SO}_4$, 870 μl phosphate buffer, and 10 μl allylthiourea. Vials were placed in a water bath at 30°C and samples allowed to reach 30°C, 100 μl of cells were then

added to each vial and allowed to react for 30 min. Samples (10 μ l) were taken from each vial and a colorimetric nitrite assay was run on each sample as described previously (1).

Determination of lag times. *N. europaea* was grown as described above. After the culture obtained maximum growth (at 3 days) inocula were taken daily and added into new growth media. The time each culture needed to obtain maximum growth was measured.

Fluorescent staining. Fluorescent dyes were Live/Dead *Baclight*TM Bacteria Viability Kit (L-7012) from Molecular Probes Inc. Cells were centrifuged at 10,000 rpm for 10 minutes. Cells were washed by resuspending in 1ml ddH₂O, and centrifuged for 3-5 min. at 14,000 rpm. The wash step was repeated. Cells were resuspended in 1ml ddH₂O and a 200 μ l sample taken. Cells were stained with the Live/Dead dyes, and fluorometer readings taken as described by Molecular Probes, Inc.

AMO and HAO assay on the oxygen electrode. Cell culture (10 ml) was centrifuged at 10,000 rpm for 10 minutes. Cells were resuspended in 1ml phosphate buffer (50 mM NaH₂PO₄·H₂O, 2 mM MgSO₄·7H₂O, pH 7.8) and centrifuged for 3-5 min at 14,000 rpm. Cells were then resuspended in 100 μ l of phosphate buffer. AMO and HAO O₂ uptake rates were measured in the chamber of an O₂ electrode. AMO was measured after the addition of 10 mM NH₃. AMO was then inhibited with 100 μ M allylthiourea, and HAO activity was measured after the addition of 300 μ M hydrazine.

Resuspension assay to check affects of pH and nitrite on cell growth. *N. europaea* cells were grown up in 250 mL flasks.. Growth was monitored for all cultures by optical density to determine the amount of nitrite production. The following calculation was used to determine mM of nitrite produced: $(\lambda_{352} - \lambda_{400}) \div 225$ (2). All cultures were considered full grown after producing 18-20 mM of nitrite. As a culture reached the desired age (8,9,10, 11, or 12 days), a

control was made from it with 1% inoculum. The culture was then centrifuged at 8,000 rpm for 10 min. Cells were resuspended in 3 ml of supernant. Cells (1 ml) were transferred to a 250 ml flask containing growth media made as previously described with the following changes: 3.5 mM nitrite was added in place of the NH_3 , (named Medium 8). Cell suspension (1 ml) was added to a 250 ml flask containing growth media at a pH of 6, with 3.5 mM nitrite, and without NH_3 , and 1 ml of cell suspension was added to a 250 ml flask containing growth media at a pH of 6 without NH_3 . Each flask and its respective media was placed on a shaker, and 1% inoculums were taken from each flask, at 24 h and at 48 h, and transferred into normal growth media. All cultures were monitored for growth by nitrite production.

Resuspension assay. *N. europaea* cells were grown up in a 2L batch (referred to as Starter), culture was kept on a shaker in the dark at 30°C throughout experiment. Controls were made by inoculating 250 ml cultures with a 1% inoculum from the Starter. Controls were made at 7,8,9,10,11, and 12 days, and growth was monitored as above by the amount of nitrite produced. Samples (100 ml) were taken from Starter at days 9, 10, 11, and 12. Samples were washed in 1 ml phosphate buffer (described above) and resuspended in 1 ml of Medium 8 (growth media containing no ammonia, and 3.5 mM NO_2^-). Cells were pipetted into 250 ml flasks containing 100 ml of Medium 8. After 24 h, 250 ml flasks containing normal growth media were inoculated with 1% inoculum taken from the cells in Medium 8. Every 24 h, cultures were monitored for growth by nitrite production as described above.

Length of time that the aging process can be suspended in Medium 8. The resuspension assay above was repeated with the following changes: cell sample (100 ml) was taken at 10 days only. After 10 day old cells were resuspended in Medium 8, 1% inoculum was taken from

Medium 8 at 24 h, 48 h, 4, 5, and 6 days and pipetted into 250 ml flasks containing normal growth media. Growth of all cultures were monitored same as described above.

Results

Lag time of *N. europaea*. *N. europaea* normally takes 3 days to obtain full growth. In this study, the levels of nitrite produced were used to measure growth, as 18-20 mM of nitrite is the typical amount of nitrite produced in a culture. Once the amount of nitrite levels off, the culture is no longer growing since nitrite is a by-product of energy metabolism.

Inocula of *N. europaea* cells taken daily from an aging culture (3 day through 9 day) had normal growth times. All of these cultures obtained full growth in 3 days. At 10 days *N. europaea* needed 4 days to produce 18 mM nitrite, at 11 days *N. europaea* needed 6-8 days, and at 12 days *N. europaea* needed 10-15 days to produce 18 mM nitrite. (Figure 1). After *N. europaea* is 12 days old it becomes extremely difficult to get any inocula to grow. One exception was a 16 day old sample that grew up in 12 days.

Live/Dead assay. There were two fluorescent dyes used in this assay. One is a cytoplasmic dye that enters a healthy cell and is cleaved by cytoplasmic enzymes, allowing the dye to fluoresce green. The other is a dye that enters cells with damaged membranes, and reacts with nucleic acids to fluoresce red. As *N. europaea* cannot be grown on agar or by the other usual methods of obtaining population counts, the use of the Live/Dead dyes gave a good estimate of the amount of viable cells in a given sample. Live/Dead assays were done on cultures starting at day 3 and continued through day 19. The results showed an average Live/Dead ratio of 10.3 was maintained through days 4-12. After day 12, the cells exhibited a steady decline. (Figure 1).

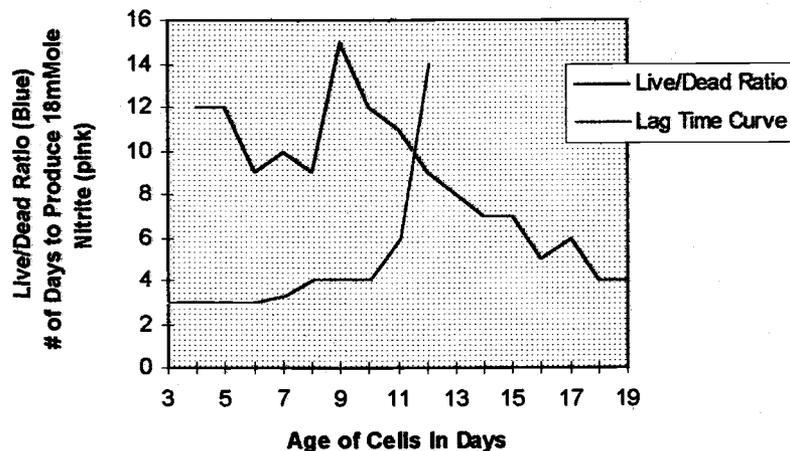


Figure 1: Live/Dead ratios of an aging cell culture, with an overlay of the observed lag times in older inoculums, which shows that lag time is caused by the cells entering dormancy stage, and not from cell death.

AMO activity/ Nitrite assay. The activity rates of AMO, showed that AMO steadily decreased as *N. europaea* aged. By the time the cells were 8 days old they had lost all AMO activity. (Figure 2).

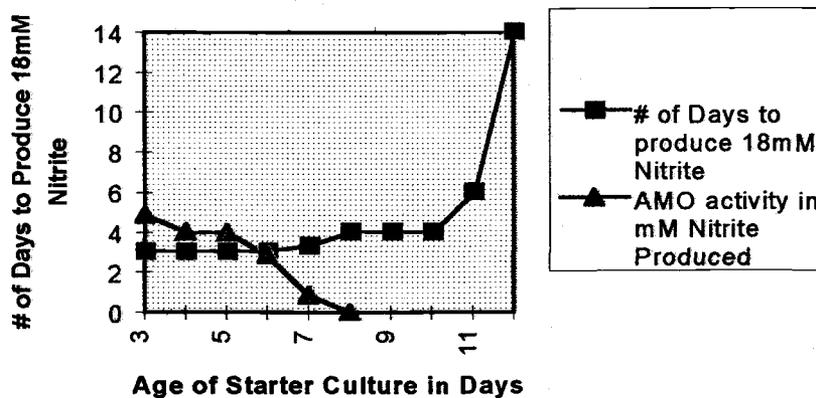


Figure 2: AMO activity over time overlaid with the lag time observed in inoculums taken from aging cells.

AMO activity was measured by O_2 electrode to see if there was any correlation between loss of AMO activity and increased lag time. AMO activity was determined on the O_2 electrode to verify the above AMO assay results. We chose O_2 electrode because it is more

sensitive than the above AMO assay. AMO activity was measured for five separate cultures. Readings were taken every day starting at day 3, and continued until all AMO activity was gone. All five cultures had very high activity on day 3 with a range from 130-201 nmoles of O₂ consumed per minute. On day 4, all five cultures showed a substantial drop in activity, down to 47-107 nmoles O₂ consumed. After day 4, all five cultures showed a steady decrease in activity until all activity was lost. Two cultures lost all AMO activity at day 9, while the other three cultures lost all AMO activity at day 12. (Figure 3). The two cultures that lost activity at day 9 were tested for lag time. Incula from both cultures exhibited lag times of 10 days, a lag time typical of 12 day old cells.

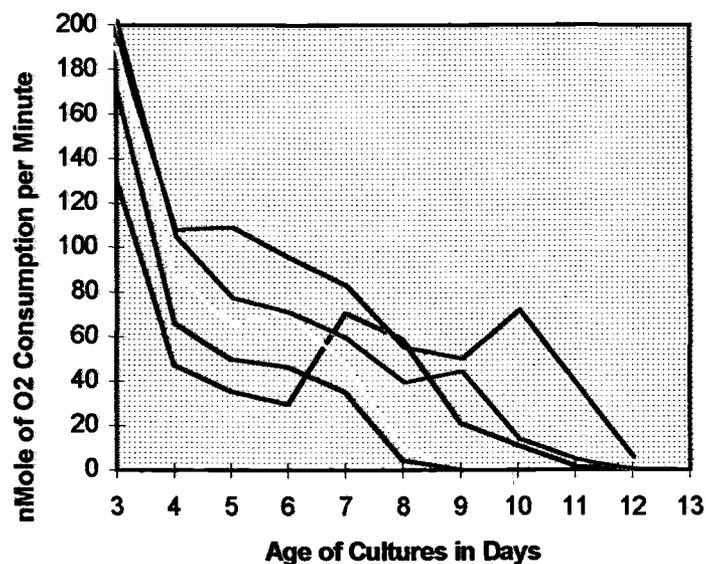


Figure 3: AMO activity as measured by the O₂ electrode.

HAO activity was measured by the O₂ electrode to see if there was any correlation between HAO and lag time. HAO activity was measured on the same five cultures that were used for measuring AMO activity. On day 3 the cultures showed an activity that ranged from 25-40 nmoles of O₂ consumed per minute. From day 3 to day 19 the cultures slowly lost HAO

activity. Despite all efforts, the HAO activity readings had a lot of variability, this variability was observed until day 11, when a fairly steady overall decrease of HAO activity was observed.

(Figure 4). Although four of the five cultures were measured up to 19 days, HAO activity was never completely lost.

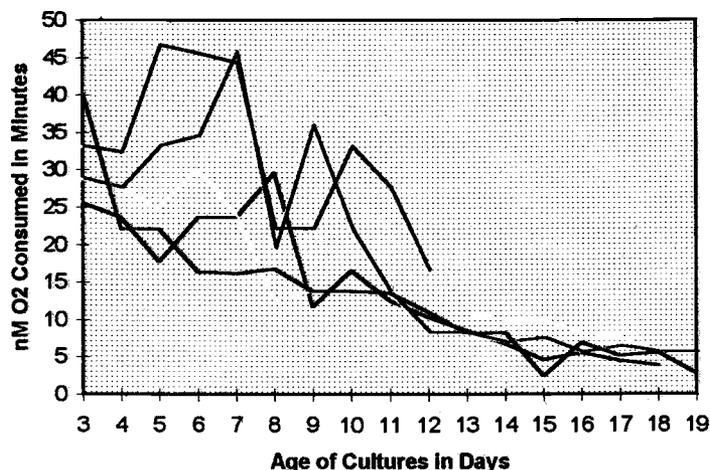


Figure 4: HAO activity as measured on the O₂ electrode over time.

Live/Dead readings were taken on the same five cultures used above.

For the two cultures that lost AMO activity at day 9 an average of 9.7 was maintained for the Live/Dead ratio for the days 4-9. Between day 9 and 10 these two cultures showed a large decline, going from a ratio of 11 at day 9 to a ratio of 4-6 at day 10. (Figure 5).

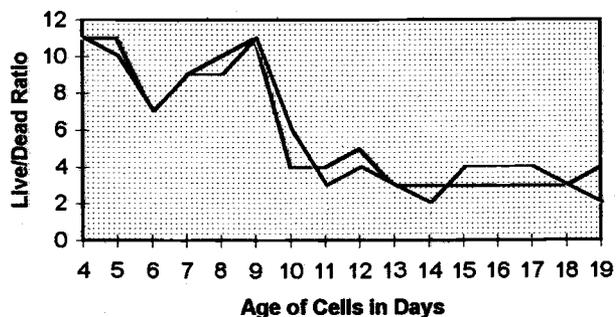


Figure 5: Live/Dead ratios over time. The two cultures that lost AMO activity at day 8 instead of at day 12 as expected, showed a marked decrease in live cells between the ages of 8 and 10 days.

For the other three cultures that did not lose AMO activity until day 12, an average Live/Dead ratio of 10.3 was maintained through days 4-12. After day 12, the cells exhibited a steady and non-recoverable decline. (Figure 6).

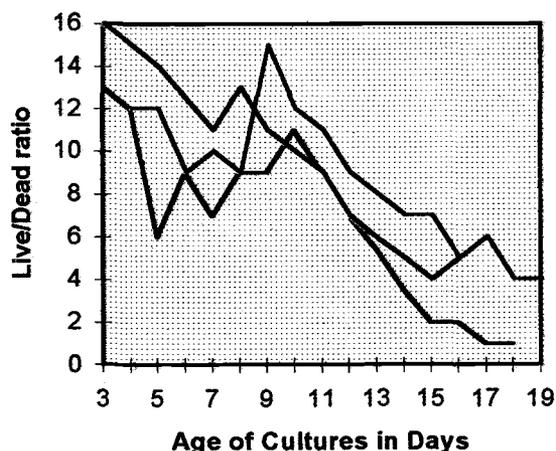
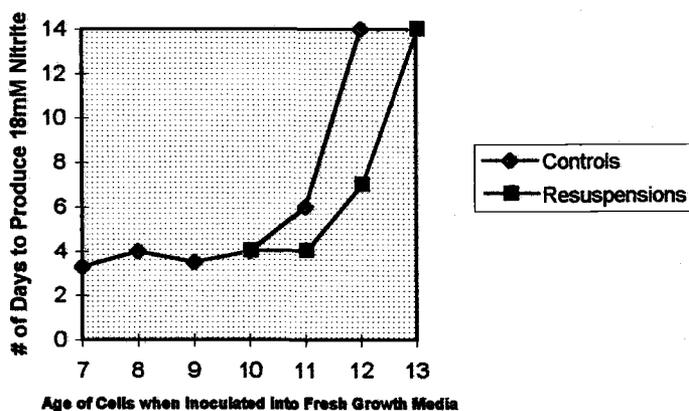


Figure 6: Live/Dead ratio over time. Three cultures that lost AMO activity at day 12.

Resuspension assay to check effects of pH and nitrite on cell growth. The growth times of the cultures in the three different media, were all about the same, and showed that pH 6 versus pH 8 made no difference in the growth time. Neither did the presence or lack of nitrite in the medium.

Resuspension assay. The growth time of the controls made on days 7-10 produced 18 mM nitrite in 3-4 days. The control made on day 11 required 6 days, and the 12 day control required 14 days to produce 18 mM nitrite. The inocula taken from the cells that were resuspended in Medium 8 for 24 h, followed the same growth curve produced by the controls, only the curve was delayed one day. The inocula taken 24 h after cells had been resuspended in Media 8, at day 9 and day 10, produced 18 mM nitrite in 4 days. The inoculum taken from the 11 day resuspended culture required 6-8 days, and the 12 day old required 13-15 days to produce 18 mM nitrite. (Figure 7)

A



B

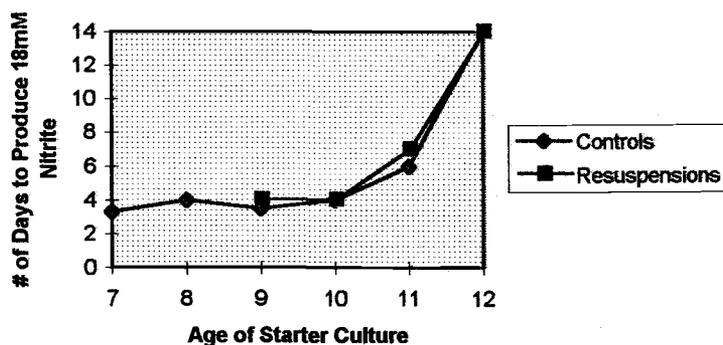


Figure 7: Resuspension assay, showing growth time of the controls and growth time of the inocula taken from cell suspension in medium 8, 24 h after the cell suspension was made. Graph A shows the growth times of the age of the cells when inoculated into fresh growth media, and graph B shows a superposition of the two growth curves.

Length of time the aging process can be suspended in Medium 8. Inocula were taken from a culture of medium 8 that contained 10 day old cells at 24 h, 48 h, and 4, 5, 6, and 12 days. The inocula required 4 to 5 days to produce 18 mM nitrite, up to the age of 6 days, where the inoculum required 7 days to grow (Figure 8). At the age of 12 days the inoculum had not yet started to grow after 7 days. The culture containing the 10 day old cells in Medium 8 was monitored for growth. No changes were observed in the optical density or in the amount of

nitrite contained in the culture, proving that the *N. europaea* cells were not growing while in Medium 8. (Data not shown.)

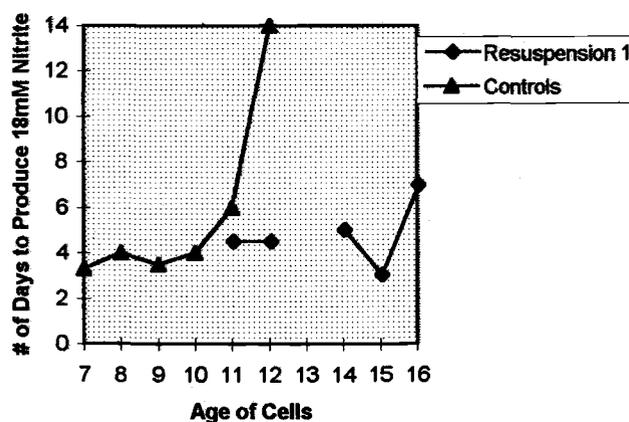


Figure 8: Growth of cells resuspended in Media 8, compared to the growth of cells left in the original culture.

Discussion

The first objective of this project was to determine whether *N. europaea* entered a form of dormancy phase, or if *N. europaea* simply died off when in a stressful environment. Our hypothesis was that *N. europaea* entered a form of dormancy phase, based on the common dogma that bacteria spent most of their life in a starvation state, and therefore *N. europaea* must have a way to maintain viability when there is no food source available for long periods of time. The results of the lag time experiment show that *N. europaea* needs between 3 to 4 days to achieve full growth from inocula 3 to 10 days old. *N. europaea* normally requires 3 days to produce 18-20 mM of nitrite when the inoculum is taken from a fresh culture. At 11 days of age the cells require 6-8 days to grow, and at 12 days of age the cells require 10-15 days to reach maximum growth. This observed increase in the time needed to achieve full growth could be caused by two things: 1) The cells are dying as they age in the original culture, and the increasing growth times are caused by the inocula containing fewer viable cells. This decrease in the number of live cells

forces the cells to have to replicate many more times before growth can be detected, causing a lag time to be observed. 2) The cells have entered a form of dormancy phase and the lag time observed is a result of the cells needing to recover from dormancy phase before they can begin replicating.

The results of the Live/Dead assays clearly showed that the observed lag time was caused by the cell's need for time to exit out of dormancy phase, and not by cell death. As the cells exhibited increasing lag times before there was a significant increase in cell death. (Figure 1.)

In an attempt to figure out what was happening to cells as they became older and more stressed, and to see if AMO activity has any correlation with the observed lag time, we looked at what was happening to AMO activity as the cells aged. AMO activity was first monitored by an AMO activity/nitrite assay. The results of this assay showed that AMO activity decreases as the cells age and is totally lost at day 9. When the loss of AMO activity is compared to the observed lag times (see Figure 1), it becomes apparent that lag time is not observed until after the AMO activity was lost.

To insure that the above results were accurate, AMO activity was measured on an oxygen electrode, a more sensitive method of measuring AMO activity. The oxygen electrode measures the amount of O₂ uptake by an enzyme, when the required substrate is provided. By using the oxygen electrode, we were able to measure both the AMO and HAO activity of the same sample. The results showed that AMO activity decreased as the cells aged, but was not completely lost until day 12. (See figure 3.) Day 12 is usually the last day that *N. europaea* inocula could be grown when inserted into fresh medium. (Figure 1.) An exception is the two cultures that lost AMO activity on day 9. Inocula from these cultures were tested for lag time, and both cultures showed lag times of 10 days, a lag time typical of 12 day old cells. Therefore, these two cultures

had lost AMO activity early, and prematurely entered dormancy phase. The loss of AMO activity in a culture indicates that a long lag time, of 10 or more days, will be observed.

Unlike AMO, the presence of HAO seemed to have no correlation with exhibited lag times. HAO did slowly decrease as the cells aged, and a relationship between HAO activity and the Live/Dead ratio was observed. As long as there was HAO activity there were more live cells than dead in the culture. It is interesting that AMO is lost as the cells enter stationary phase, but HAO is not lost. As AMO is the first enzyme in the energy metabolism reaction, one would expect AMO to be more valuable to the cell than HAO.

The Live/Dead tests showed variability in the ratio of live to dead cells, during cell ages 3 day through 10 day. However, after day 10 there was a steady decline of live cells. The variability of the live to dead ratio is a most likely caused by two things: 1) The result of losing an unknown portion of dead cells during the multiple washing processes that were required to use the fluorescent dyes, as dead cells lose their adhesiveness and are very easily lost during washing. 2) The fact that the dyes depend on membrane integrity to differentiate between dead and live cells. This allows for a live but damaged cell to be dyed both green and red. If a damaged cell later recovers it will dye only green and will change the ratio of live to dead.

When the Live/Dead ratios are compared to the AMO activity, it is observed that on the day AMO is completely lost there are still a large portion of live cells. Two days after AMO is lost, the amount of live cells had drastically decreased and slowly continued to decrease. There is very little variability observed in the slope after AMO is lost. This suggests that once AMO is lost the cells can no longer maintain themselves in their stressed environment and cannot recover even if placed in fresh medium.

In summary, *N. europaea* exhibits a increasing lag time as the cells age. This lag time is not a result of cell death in the original culture, but is caused by the cells entering dormancy phase and needing time to recover out of this phase. The loss of AMO appears to have a negative effect on the cells ability to maintain viability, and loss of AMO is related to the cells entering a deep stationary phase. Oddly, HAO is not lost like AMO is; HAO appears to have no effect on the viability of the cells, other than to indicate live cells, as no live cells have been observed that did not have HAO activity. Once *N. europaea* enters dormancy phase, the cells require long lag times to recover out of dormancy. This entrance into dormancy can be temporarily prevented by resuspending washed cells in medium containing no growth substrate. This suspension of the aging process can be maintained for up to five days, after which the cells start showing signs of aging.

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