COMMUNITY ANALYSIS OF 3-AMINO-1,2,4-TRIAZOL-5-ONE (ATO) AND 3-NITRO-1,2,4-TRIAZOL-5-ONE (NTO) DEGRADING MICROBIAL CULTURES

By

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COMMUNITY ANALYSIS OF

3-AMINO-1,2,4-TRIAZOL-5-ONE (ATO) AND

3-NITRO-1,2,4-TRIAZOL-5-ONE (NTO) DEGRADING

MICROBIAL CULTURES

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Abstract: The degradation of NTO is a growing concern to do its increasing popularity and persistence in the soil as ATO. There is concern over its environmental and health impact, particularly because contamination to water resources is likely due to its high mobility in soil. Understanding the microbial communities that reduce NTO and degrade ATO is important for the development of bioremediation techniques to remove these pollutants from the environment. A culture of ATO degrading microbes was diluted to extinction to reduce the culture to the species essential for ATO degradation. Quantitative polymerase chain reaction (qPCR) analysis showed that the number of species groups present was reduced from 19 to 9, with *Terrimonas* spp. and *Ramlibacter* – like spp. the most abundant. Metagenomic data from the culture was used to identify potential biomarker genes related to ATO degradation and qPCR primers were developed. A culture of NTO reducing microbes was measured throughout complete NTO transformation to identify the change in species abundance over time. qPCR analysis showed that *Geobacter* spp. were consistently found in the highest abundance in the community, and were slightly enriched after the end of the lag phase. The relative abundance of members in each community can give valuable insights into how the community reduces NTO and ATO. Examination of the key members of the community can be used to identify degradation pathways and bioaugmentation strategies for bioremediation. Potential ATO degradation biomarkers identified in this study can be used to monitor and track the *in-situ* bioremediation of contaminated sites.

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CHAPTER I

INTRODUCTION

The use of 3-nitro-1,2,4-triazol-5-one (NTO) in munitions has historically left traces of the chemical in natural systems where it is transformed into a soluble form, 3-amino-1,2,4-triazol-5-one (ATO), and remains in water systems. There is an increased interest in the biological degradation of this pollutant and methods to remove it from groundwater. This study aims to isolate the bacterial communities of NTO and ATO degrading cultures. In addition, the study also aims to identify genes associated with the biodegradation of ATO to develop biomarkers to monitor ATO degradation in situ.

Two distinct microbial communities that reduce NTO and degrade ATO independently were examined. Potential biomarkers for the degradation of ATO were developed for use in quantitative polymerase chain reaction (qPCR) methods. The relative abundance of the species that make up these two microbial communities were measured to provide insight into which groups of bacteria could be involved with NTO and ATO degradation. The change in the microbial community before and after a series of dilutions was measured in the ATO degrading culture. Biomarkers were identified from metagenomic information gathered from the final dilution of the ATO degrading culture. The abundance of each member of the NTO reducing microbial community was measured at several times during the complete reduction of NTO.

These experiments are part of a larger project centered on the biodegradation of NTO and ATO for use in remediation efforts of contaminated sites. The larger project is a result of collaboration between research groups from the University of Arizona, Georgia Institute of Technology, the University of West Florida, and Oklahoma State University. Members of the group have developed the two starting NTO and ATO degrading cultures used in my research. The research group conducted metagenomic and phylogenic analysis of the two microbial communities. The phylogenetic analysis was used to identify the community members present in each culture so PCR primers could be designed to measure their abundance. Information about the genes present in the ATO community was used in biomarker development.

The development of biomarkers is important in bioremediation efforts to remove ATO from contaminated sites. Biomarkers would be used to monitor and track the rate of removal and progress towards complete degradation. The ability to monitor a bioremediation treatment would drastically improve efficiency and allow greater control of the degradation process. The abundance of each member of the microbial community in these cultures can be used to identify which members are connected to ATO and NTO degradation. It can also help us to understand the mechanisms of degradation used by these communities and engineer better solutions. The results of these studies and the larger project as a whole can be used to asses the potential of biological remediation of ATO and NTO and develop methods such as biomarkers and bioaugmentation to enhance efficiency.

CHAPTER II

REVIEW OF LITERATURE

NTO Properties and Uses

NTO is a key ingredient in recently developed insensitive munitions (Halasz et al., 2018). NTO is notable for being much more thermally stable than competing chemicals, such as RDX (Rothgery et al., 1991). NTO can be produced economically at larger scales than other commercial chemicals and is less sensitive than RDX and TNT (Singh et al., 2001). NTO is a much safer and convenient compound than traditional munitions, which has lead to its growing popularity (Singh et al., 2001). Insensitive munitions contain a mixture of ingredients. For example, IMX101 is composed of NTO, 2,4-dinitroanisole (DNAN), and nitroguanidine (NQ) (Taylor et al., 2015). The solubility of the individual ingredients differs greatly and their dissolution creates a porous particle with more surface contact with water (Taylor et al., 2015). The gradient formed by the solubility differences may have a significant impact on the soil community and its ability to degrade the pollutants. The concentrations of different contaminants will change over time due to the gradient and exposure to NTO and the acidic conditions that accompany it may drastically affect the composition of soil microbial communities (Taylor et al., 2015). NTO's growing popularity has also prompted research into its environmental effects and engineering efforts to mitigate pollution of the chemical.

Origins in the Environment

NTO enters the environment through contamination from the use of Intensive High Explosives (IHE). Some of the ingredients in munitions remains unreacted after detonation and is deposited on the soil surface at sites where the insensitive munitions are used. A significant portion of these insensitive munitions is scattered after detonation and remains unreacted as it is spread across the immediate area (Dontsava et al., 2014). NTO on the soil surface can then be washed into the soil medium mainly through rainfall. The high solubility of NTO allows it to quickly travel through soil columns (Kim et al., 1998). NTO is more soluble than both TNT and RDX, traditional ingredients in munitions (Dontsava et al., 2014). NTO also has a low natural tendency towards adsorption to soils (Dontsava et al., 2014). pH appears to play a major role in NTO soil affinity and the acidity of a soil is the most important indicator for NTOs adsorption to soil surfaces (Dontsava et al., 2014). The dissolution of NTO into water entering the soil will affect the pH as well as composition of the solutions present (Dontsava et al., 2014). These two properties result in high mobility of the NTO contamination through soils before it is naturally reduced to ATO (Temple et al., 2018).

NTO can travel significantly before it is transformed, with previous studies measuring its half-life in soils to be between 1-72 days (Dontsava et al., 2014). However, the organic carbon content of a soil can impact this slow rate of transformation. The organic carbon percentage of a soil is a significant factor in the transformation rate of NTO in soils (Dontsava et al., 2014). NTO experienced very little resistance in laboratory soil columns, moving with similar velocity to water flowing naturally through the soil (Dontsava et al., 2014). The quick dispersal of this compound poses a threat to groundwater resources, which could be drastically impacted by nearby explosives testing ranges that do not account for the far reach of NTO residue.

Another concern is when NTO is transformed into ATO. ATO has been shown in a degradation study to be persistent in soils (Madeira et al., 2017). Once NTO is transformed into ATO, it can persist in the soil and remain a source of contamination for long periods. This leads to accumulation in soil and groundwater where there is potential for human and animal exposure. The conversion of NTO to ATO happens quickly but the mineralization of ATO is a much slower process. In one study, NTO in a pure culture had been transformed to ATO in 24 hours but the complete degradation of ATO required an additional 2 weeks (Richard et al., 2014).

Biodegradation

Some microbial communities possess the ability to reduce NTO to the intermediate compound, ATO, and then to innocuous compounds (Campion et al., 1999). NTO is generally reduced through the pathway of nitroreduction, producing cyctotoxic intermediates along the way (Campion et al., 1998). Some of these intermediates have been identified as ATO and HTO (Campion et al., 1998). 3-hydroxyamino-1,2,4-triazol-5-one (HTO) was measured as a brief intermediate in the transformation of NTO to ATO (Krzmarzick et al., 2015). HTO features a hydroxyl group in place of one of the oxygen molecules in the previously nitro group. The structure of the intermediate compound, HTO, provides valuable insight into the degradation pathway used by microbes (Krzmarzick et al., 2015). The nitro group in NTO is reduced to an amino group to become ATO. ATO is degraded through triazolone ring cleavage (Campion et al., 1998). As part of this study, the degradation products of ATO were examined and it was found that ATO is degraded to the completely mineralized compounds of nitrogen gas, ammonium and carbon dioxide (Madeira et al., 2019). From stoichiometric analysis, the nitrogen in ATO should be transformed in equal amounts to nitrogen gas and ammonium in the solution. In anaerobic conditions, NTO is transformed to ATO, but aerobic transformation of the compound was found

to produce mostly urazole (Campion et al., 1998). In agreement with the proposed pathway, energetics computations suggest the C-NO2 bond area of the NTO molecule to be the initial site of reduction (Meredith et al., 1998).

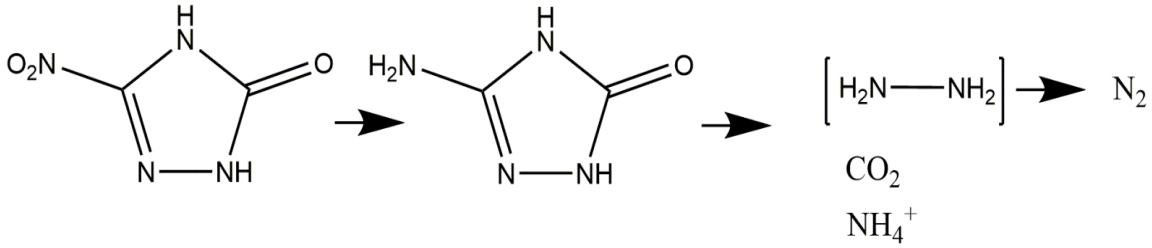

Figure 1. Proposed NTO degradation pathway through ATO and mineralization end products (Krzmarzick et al., 2015).

NTO is only reduced under anaerobic conditions and an electron donor, such as acetate, is required for efficient biotransformation of NTO to ATO (Krzmarzick et al., 2015). However, aerobic conditions are needed for ATO mineralization (Krzmarzick et al., 2015). Because of the different environmental conditions, a two-step approach can be used to remediate NTO contamination. Methods of remediation would require creating an anaerobic phase in the soil to transform NTO to ATO and then allowing an aerobic phase to completely mineralize the ATO that has then accumulated (Krzmarzick et al., 2015). In another possible method NTO was completely mineralized in an aerobic reactor because NTO reduction occurred in anaerobic biofilms that formed while the resultant ATO was degraded in the aerobic conditions of the bulk solution of the reactor (Madeira et al., 2017). Transcriptome analysis has been performed on an NTO reducing culture and found various genes that might be important in the transformation of NTO. Specifically, N-ethylmaleimide reductase and multiple deaminase genes were upregulated in the culture (Weidhaas et al., 2018).

Multiple genera present in the ATO degrading culture have been shown to have wide degradation ability among aromatic compounds and components in their degradation pathway. A species of *Ralstonia* possesses genes that encode for an enzyme involved in the final degradation

steps of aromatic compounds (Gan et al., 2012). *Ralstonia* sp. Strain PBA was found with *Hydrogenophaga* sp. Strain PBc as well (Gan et al., 2012). These two species together may enable the biodegradation of an aromatic amine, 4-aminobenzenesulfonate (Gan et al., 2012). A bacterium closely related to *pseudomonas* species was found to degrade s-triazine herbicides, such as simazine as the sole nitrogen source (Hernandez et al., 2008). The similar S-triazine aromatic structure means results could indicate *Pseudomonas* plays a role in ATO degradation pathway (Hernandez et al., 2008). *Pseudomonas* species are active in aerobic degradation of a wide variety of compounds including aromatic hydrocarbons and pesticides (Hernandez et al., 2008). *Mesorhizobium* sp. F28 showed the ability to convert acetonitrile into acetamide and acetic acid (Feng and Lee, 2009). The culture used in the study had a two-step pathway of first degrading acetonitrile to acetamide and then degradation to acetic acid and ammonia (Feng and Lee, 2009). The degradation of these nitrogen-containing compounds to ammonia is relevant to the possible ATO degradation pathways. A species of *Ramlibacter*, *Ramlibacter Tataouinensis*, can degrade toluene, which is an aromatic ring compound as well as the amino acids valine and leucine (KEGG, 2019). A species from the *Sphingopyxis* genus was found capable of degrading 5 microcystin variants (Maghsoudi et al., 2016). However, the activity was reduced at acidic conditions, which are usually a result of NTO contamination (Maghsoudi et al., 2016).

Degradation of Azoles and Triazoles in the Environment

Triazoles can take long periods of time to degrade naturally in soils. In high temperature and moisture soil conditions, some triazole compounds have a half-life of multiple years (Bromilow et al., 1999). It can be expected to take even longer for them to degrade in real conditions due to low temperatures and droughts that will significantly slow NTO degradation. Triazoles have been found in previous studies to be persistent in soils, with little measurable

degradation by natural processes (Li et al., 2012). Azole compounds, in general, were not significantly removed from water systems by normal wastewater treatment plant system sludge (Kahle et al., 2008). Traditional wastewater treatment fails to remove azole compounds from water systems, allowing these compounds to be distributed to a wide area once in the water system (Kahle et al., 2008). Treatment plants do little to mitigate the effects of contamination of azoles broadly and possibly NTO or ATO specifically.

There are various microbes that have properties indicating they may possess the ability to reduce NTO or ATO. The ability to reduce nitro or amino groups as well as the ability to degrade similar compounds, such as azoles or triazoles, are particularly relevant. A strain of *Shinella* demonstrated an ability to degrade a triazole compound used in pesticides (Wu et al., 2016). The strain was able to degrade the triazole as the sole carbon and energy source for growth (Wu et al., 2016). However, it was found that degradation could be accelerated through the addition of other carbon sources (Wu et al., 2016). This was exhibited by only a single strain of *Shinella*, but it should be considered in any culture of triazole degrading organisms. The ability to increase degradation by using an alternate carbon source in addition to the triazole could be seen in other triazole degrading species and should be considered when developing biodegradation techniques.

The exact details of NTO and ATO transfromation by microbes are not clear at this time. Enzymes known to act on other triazoles, can be examined to infer clues about which ones may play a role in the transformation of NTO and ATO. Cyanuric acid is a similar compound that is acted on by cyanuric acid hydrolase produced by microbes (Seffernick et al., 2016). The ring is opened in the compound and the nitrogen transformed to ammonia (Seffernick et al., 2016). A *Pseudomaonas* was also found to metabolize cyanuric acid with cyanuric acid hydrolase in addition to producing two other similar enzymes (Fruchey et al., 2003). The prevalence of these enzymes suggests Cyanuric acid metabolism is widespread and shared by many species (Fruchey et al., 2003).

Toxicity and Health Effects

NTO has been found to cause negative health effects in rats, including reduced body mass and food consumption as well as a decrease in testes mass (Crouse et al., 2015). The effect on the rats' reproductive organs was seen in doses of 500 mg/kg-d and sperm counts were greatly decreased by the exposure (Crouse et al., 2015). There is concern that these results could apply to humans as well and that sites contaminated with it are a public health risk. NTO was also found to exhibit neurotoxicity in Japanese quail in doses of 500 mg/kg-d. Oral exposure of NTO in this dosage affected neuromuscular function and resulted in mortality (Jackovitz et al., 2018). Toxic effects on the environment from NTO contamination have been demonstrated previously (Dontsava et al., 2014). NTO is toxic to methanogenic bacteria, which are typically found in wastewater treatment plant sludge (Madeira et al., 2018). NTO and ATO are harmful to some aquatic species, such as Zebrafish embryos and the microcrustaceans, *Daphnia magna* (Madeira et al., 2018). Exposure to adequate amounts of ATO could be exceptionally harmful to their population and ecosystems.

Alternate Methods of Degradation

An alternate method of reducing aqueous solutions of NTO is photo-transformation. A complete reduction of NTO within a matter of days when exposed to certain wavelengths of light (Becher et al., 2019). A concern with this method is that the eco-toxicity of NTO increases with it due to the intermediate products that are formed when the NTO is transformed (Becher et al., 2019). The NTO was mostly mineralized but some intermediate products could have high toxicity. Other issues with this process are that it is energy-intensive from the light use and the water must be treated away from the site and lacks the benefits of an in-situ method that would remediate the source of the NTO contamination.

Other methods facilitate NTO and ATO oxidation by adsorption to metal oxide surfaces. It was found that NTO sorbed to ferrihydrite, but the oxidation was ineffective (Khatiwada et al., 2018). The study did find ATO to be rapidly degraded by birnessite interaction (Khatiwada et al., 2018).

An NTO remediation strategy that focuses on ATO degradation is in situ chemical oxidation. This has been demonstrated with the addition of potassium permanganate to oxidize compounds (Madeira et al., 2019). NTO is resistant to oxidation by this chemical so NTO was first transformed biologically into ATO (Madeira et al., 2019). The ATO was much more susceptible to oxidation by the potassium permanganate treatment and was degraded quickly (Madeira et al., 2019).

In a much different method, wastewater sludge is used to treat the NTO. The microbial community of NTO reducing wastewater reactor sludge was analyzed and quantified (Eberly et al., 2016). A large number of species where present existing in a complex network of species (Eberly et al., 2016). However, this study's goal is to isolate a small core community of microbes that are directly active in the reduction of NTO.

CHAPTER III

METHODOLOGY

ATO Degrading Culture Enrichment

A community of microbes capable of degrading ATO was enriched in a culture through dilution to extinction. The initial reactor was inoculated with a sample from an ATO degrading microbial culture developed earlier by the University of Arizona research group. The reactor was then serially diluted to obtain a culture enriched with microbes associated with ATO degradation.

The reactor was composed of a 100 ml solution of mineral media containing 1 mM ATO as the sole carbon and energy source. For each dilution, the reactor was inoculated with a 0.1% by volume sample of the previous ATO degrading culture. The reactor was sealed with a cotton cover that allowed the transfer of air into the culture. The culture was incubated at 30 °C and continuously mixed at 130 rpm on an orbital shaker. A 0.1% dilution of the culture to fresh mineral media was performed after more than 50% of the ATO in the reactor had degraded. The ATO concentration in the culture was determined by measuring the ammonium concentration, which is a product of ATO degradation. To measure the ammonium concentration, an Ammonium Ion Selective electrode (Cole-Parmer, Vernon Hills, IL) was used on a 5 ml sample taken from the culture. The culture was diluted 7 times in total over a period of 142 days.

A 20 ml liquid sample of the culture was taken before each dilution for use in DNA analysis. The organic content was concentrated by centrifuging 1ml of the sample at a time in a microcentrifuge tube at 10,000×g for 60 seconds and then decanting and repeating until a pellet of material had formed. The DNA from the pellet of organic material was extracted using a DNeasy PowerSoil extraction kit (Qiagen, Hilden, Germany). The extracted DNA was used in qPCR analysis to identify the relative abundance of 16S rRNA genes present in the culture.

ATO Culture Biomarker Development

The University of West Florida collected metagenomic data from the serially diluted ATO culture. This data on the newly simplified culture was used to identify various genes that were likely to be expressed in the ATO degradation process. Five of these genes were selected as potential biomarkers and a PCR primer was designed for each. To design the primers, the gene sequence was run through the National Center for Biotechnology Information (NCBI) primerBLAST database to obtain forward and reverse primer sequences for that gene. These primer sequences were then run through the National Center for Biotechnology Information (NCBI) nucleotide BLAST database to identify what genes they would likely interact with. If the primer was not specific to the gene of interest, another forward and reverse primer sequence was chosen and tested to avoid false positives. The five chosen primers were ordered from thermofisher (Waltham, Ma) for use in PCR.

The specificity of these primers to each gene of interest was tested using a PCR method on the isolated DNA from previous cultures of the microbial community. 1.25 uL of each of the forward and reverse primer, 1 uL isolated DNA, 31.25 uL of PCR water, 10 uL 5x green GoTaq flexi buffer (Promega, Madison, WI), 2.5 uL of a 20 mg/ml BSA solution, 2 uL 25mM MgCl2, 0.4 uL dNTPs, and 0.25 uL GoTaq G2 flexi DNA (Promega, Madison, WI) polymerase were

mixed for each primer. A control without DNA extract was created for each primer as well. The PCR was performed with thermal cycles using a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, Ca). The samples were heated to a temperature of 94°C for 5 minutes. Then the samples went through 35 cycles of denaturing at 92°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 2 min. After the cycles, the samples were heated to 72°C for 5 minutes.

Any DNA segments the primer was genetically similar enough to react with were amplified in the PCR method. Gel Electrophoresis was used to separate the amplified strands of DNA by size. For each sample, 3 uL blue/orange loading dye (Promega, Madison, WI) and 7 uL PCR product were added to a 1.5% agarose gel along with a 100 bp ladder (Promega, Madison, WI) and run on a MUPID-exU (Mupid, Tokyo, Japan) submarine electrophoresis system for 25 minutes at 100 V. Afterwards, the gel was added to a 1x SYBR Gold dye solution (Promega, Madison, WI) for 40 minutes and imaged in a Gel Doc XR+ (Bio-Rad Laboratories, Hercules, Ca) using Image Lab software. The performance of the primers was evaluated by viewing the fluorescent bands. This verified the PCR reaction had amplified only the gene of interest. Primers that showed a single clear band indicated that they were specific to a single gene and could be used as potential biomarkers.

NTO Reducing Culture Analysis

An enriched culture of an NTO reducing culture was developed and sampled by the University of Arizona research group. Triplicate reactors containing the culture were grown under anaerobic conditions in a mineral media containing NTO and acetate as an electron donor. The culture had been enriched through periodic serial dilution and measurements were taken during the latest phase of NTO transformation. A total of 6 samples were taken from each reactor over a

period of 32 hours during the complete transformation of NTO. The samples were shipped to the OSU lab for microbial community analysis. DNA was extracted from the community samples using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany). The concentration of DNA in the extracted samples was measured using Quantus Fluorometer (Promega, Madison, WI) and QuantiFluor dsDNA System (Promega, Madison, WI). The DNA was then used in a qPCR method to identify the relative quantity of each of the 4 microbial groups in the community.

Multiple primers were designed using the phylogenic data from the larger research group to identify the different microbial community members. 4 groups of microbes were identified from a previous phylogenetic analysis of the community. Primers were designed based on the 16S rRNA genes of each group.

qPCR Analysis

A qPCR method was used to quantify the amount of a specific gene in a sample. The method used a PCR reaction to amplify the gene of interest in an unknown sample and measure the number of copies produced using a fluorescent dye. A standard curve was created by using a PCR reaction on a series of samples with known starting concentrations of the gene. The amount of starting gene in the unknown sample was then calculated using the standard curve. A solution of 1uL sample DNA, 5 uL 2X SYBR Green (Bio-Rad Laboratories, Hercules, Ca), 0.5 uL of a 20 mg/ml BSA solution, and 0.15 uL each of forward and reverse primer was used in the qPCR reaction in a CFX Connect Real-Time System (Bio-Rad Laboratories, Hercules, Ca), which measured the fluorescence in each sample and compared it to the standard curve to obtain the relative amount of the chosen gene in the sample. The method used by the system called for samples to be heated to a temperature of 95 °C for 3 minutes. Then the samples went through 39 cycles of denaturing at 95 °C for 15 seconds and annealing at 59 °C for 30 seconds. After the

cycles, the samples were heated to 94°C. qPCR analysis was performed using each of the primers developed for the culture to quantify the abundance of each microbial community member.

A set of qPCR standards was developed for each gene using the corresponding primers and a DNA sample from the culture. For each gene, a PCR reaction was performed on the DNA sample using the specific primers. The PCR product, now with a large number of copies of the gene, was cleaned using a PCR Cleanup Kit (Qiagen, Hilden, Germany) to eliminate contaminants. The concentration of DNA in the sample was measured using a Quantus Fluorometer (Promega, Madison, WI) and QuantiFluor dsDNA System (Promega, Madison, WI). The sample was then diluted by a factor of 10 multiple times to produce 9 standards of different gene concentration. The concentration of DNA in the unknown samples was measured and used to normalize qPCR results per nanogram of DNA extract.

Phylogenetic Tree Creation

Phylogenetic trees were created to examine the relationship of community members and identify groups of similar organisms. Trees were created for the ATO degrading culture after dilution, the original and most recent NTO reducing cultures, and the genes identified as potential biomarkers for ATO degradation. Metagemomic data of the species present in the ATO and NTO cultures were obtained from the group at the University of West Florida. Evolutionary analysis was conducted on the metegenomic data of the cultures in MEGA6 to identify the relationship between species and create phylogenetic trees(Tamura et al., 2013). The Neighbor-Joining method was used to infer the evolutionary history (Saitou and Nei, 1987). A bootstrap test (1000 replicates) created replicates of the tree, and next to each branch is the percentage of replicate trees in which the members were grouped together (Felsenstein, 1985). The tree was drawn to scale with branch lengths used to illustrate evolutionary distance between members. The

evolutionary distances of the ATO and NTO cultures were created using the Maximum Composite Likelihood method and are in units of base substitutions per site (Tamura et al., 2004). Ambiguous positions were removed for each sequence pair. The evolutionary distances of the potential ATO biomarker genes were created using the Poisson Correction method and are in units of amino acid substitutions per site (Zuckerkandl and Pauling, 1965). Alignment was done with amino acid sequences in MUSCLE (Edgar, 2004).

CHAPTER IV

RESULTS

ATO Degrading Community Analysis

The conductivity of the ATO degrading cultures shows the increase in ammonium concentration over time. This indicates a decrease in ATO concentration due to biological transformation into ammonium. A lag phase was observed after dilutions were performed and the degradation rate increased drastically after the lag phase ended. It was observed that by the 7th dilution, the lag phase had grown shorter on average. The initial lag phases could last more than a week but it decreased until it was only approximately 3 days long after the last dilution.

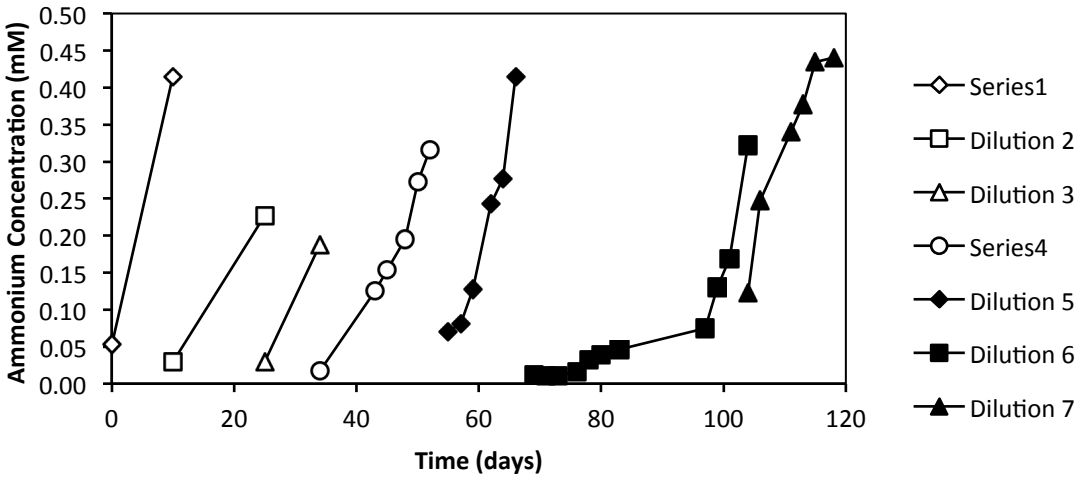

Figure 2. The change in conductivity of ATO degrading culture during each serial dilution.

However, the 6th dilution experienced a much longer lag phase. This is likely attributed to nearly all of the ATO being degraded in the 5th dilution. The cultures have been previously observed to develop a significant lag phase if the culture is denied adequate ATO since there is no other carbon or nitrogen source. Measurement of an increase in ammonium in the solution as ATO was degrading indicates that the community is completely mineralizing ATO as they degrade it. Microbial growth was observed as ammonium, nitrogen gas, and carbon dioxide were measured as ATO degradation products (Madeira et al., 2019). Evidenced by these results, members of the ATO degrading community use ATO as a sole nitrogen and carbon source and growth. This is the first study to identify the complete mineralization of ATO by a culture of soil microbes.

The serial dilutions allowed for the growth of the essential ATO degrading community members and excluded any species not involved in the degradation due to ATO being the only carbon source. Table 3 shows the results of qPCR analysis of the abundance of microbial groups in the ATO degrading community before and after serial dilution. Table 1 lists the qPCR primers developed for species quantification. The culture had previously consisted of a large number of species but, after serial dilution, the number of significant groups was reduced to just 9. Figure 3 shows the phylogenetic tree of the simplified culture after the final dilution. Results of qPCR analysis indicate *Terrimonas* spp. and *Ramlibacter* – related spp. were enriched by the dilutions and existing in abundance in the culture. These two groups were found in the greatest numbers in the community with $10^{7.02}$ and $10^{6.82}$ 16S rRNA genes/mL, respectively. Various other microbial groups, such as *Mesorhizobium* spp., *Hydrogenophaga* spp., *Ralstonia* spp., *Pseudomonas* spp., and *Ectothiorhodospiraceae*, comprised a significant amount of the culture but much less than the main two microbial groups. *Ralstonia* spp. and *Pseudomonas* spp. in particular were enriched to greater numbers by the dilutions. *Sphingopyxis* spp. and *Taonella* spp. were relatively low in abundance compared to all other microbial groups in the culture. The presence of these 9

members remaining after numerous dilutions suggests they may all play an important role in the microbial community and removing a seemingly unimportant member might reduce the functionality of the community.

Results of qPCR analysis found that *Terrimonas* spp., *Ramlibacter*-related spp., *Mesorhizobium* spp., *Hydrogenophaga* spp., *Ralstonia* spp., *Pseudomonas* spp., *Ectothiorhodospiraceae*, and *Sphingopyxis* spp. were enriched from the conditions with ATO as the sole carbon and nitrogen source. The enrichment and high abundance of *Terrimonas* spp. and *Ramlibacter* – related spp. in the ATO degrading culture suggests they were important in the degradation of ATO. They likely possess an ability to degrade ATO or play a critical role in the degradation. There also exist multiple other community members that were enriched in the culture despite repeated dilution. These members appear to be critical to the function of the community as a whole, but their role is not currently known. They could play a direct role in the degradation process or they may act in support roles that benefit the main ATO degraders in some way. For example, they could provide support to the ATO degrading members by removing their waste products as they grow. It is likely that, if they were removed, the culture would lose its ability to effectively degrade ATO. The PCR primers developed for the ATO degradation biomarkers can be used in further research to verify if the genes are expressed when the community degrades ATO.

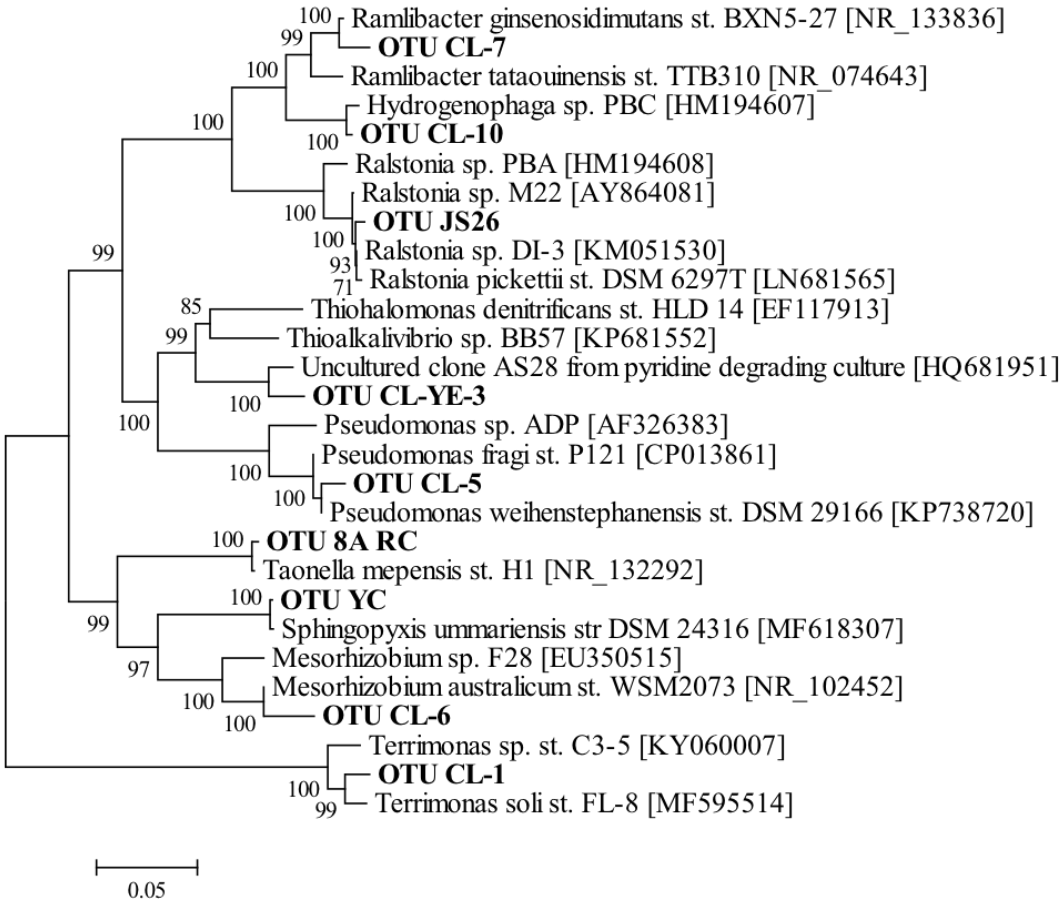

Figure 3. Phylogenetic tree of ATO degrading culture after seven 0.1% dilutions.

Table 1. List of PCR primers used in ATO degrading culture analysis.

qPCR target	Primers	Amplicon Size (bp)	Standard range (log (copies)/µL)
Ectothiorhodospiraceae	5'-GCGCGTAGGCGGTTTGATAA-3' 5'-CGCACCTCAGCGTCAGTATT-3'	189	1.68-8.68
Pseudomonas spp.	5'-GCTCAACCTGGGAACTGCAT-3' 5'-CGCATTTCACCGCTACACAG-3'	82	1.47-8.47
Hafnia spp.	5'-CACGCAGGCGGTTGATTAAG-3' 5'-AGCGTCAGTCTTTGTCCAGG-3'	180	2.02-7.02
Stenotrophomonas spp.	5'-TGAGCCGATGTCGGATTAGC-3' 5'-CGTCATCCCAACCGGGTATT-3'	256	2.25-6.25
Pseudoxanthomonas spp.	5'-GAGTGTGGTAGAGGGATGCG-3' 5'-CCACGGATGTTCCTTCCGAT-3'	72	1.56-6.56
Nitrosospira spp.	5'-GAAACTGCCGGTGACAAACC-3' 5'-CTTCTGGTGGAACCCACTCC-3'	282	2.50-8.50
Ralstonia spp.	5'-GCATACGACCTGAGGGTGAAA-3' 5'-TGTGGCTGATCGTCCTCTCA-3'	139	1.84-6.84
Polaromonas spp.	5'-GTGCCCAATTGTGGGGGATA-3' 5'-GTGGCTGGTCGTTCTCTCAA-3'	183	1.89-6.89
Ramlibacter-related spp.	5'-GATTGAACGCTGGCGGAATG-3' 5'-CTGGGCACGTTCCGATGTAT-3'	107	2.38-8.38
Hydrogenophaga spp.	5'-CGGGTGAGTAATGCATCGGA-3' 5'-CCCCGCTTTCATCCACAGAT-3'	83	3.41-8.41
Sphingopyxis spp.	5'-AAGTCAGAGGTGAAAGCCC-3' 5'-TTGTCCAGTCAGTCGCCTTC-3'	152	1.39-6.39
Sphingomonadaceae	5'-TGGGTTCGGAATAACTCGCC-3' 5'-AGCTAATCTTACGCGGGCTC-3'	97	1.08-7.08
Brevundimonas spp.	5'-ATGACGGTACCCGGAGAAGA-3' 5'-GAGCTCTGGGATTTCACCCC-3'	142	2.42-7.42
Shinella spp.	5'-CTTCAGTTAGGCTGGACCGA-3' 5'-CCCCTTAGAGTGCCCAACTG-3'	134	1.51-7.51
Mesorhizobium spp.	5'-GGCAGGCTTAACACATGCAA-3' 5'-CAGCTATGGATCGTCGCCTT-3'	218	2.67-8.67
Xanthobacter spp.	5'-GAGAGGTTGGTGGAACTCCG-3' 5'-GCGTCAGTATCGAGCCAGTT-3'	84	2.55-8.55
Hyphomicrobium spp.	5'-CTCAACCTCGGAACTGCCTT-3' 5'-GCCACCGGTGTTCTTCCTAA-3'	109	1.21-8.21
Uncultured *Acidobacteria* spp.	5'-TGGGGGATGAAGGTCTTCGG-3' 5'-CCCGGGATGATTAGCTCCCA-3'	73	1.61-7.61
Gemmata-related spp.	5'-TGCAAAGTCCGGGGTGAAAT-3' 5'-TTTCACCGCTCCACCCATAG-3'	106	2.14-7.14
Mycrocystis-related spp.	5'-CACGTGAACAATCTGCCGTC-3' 5'-AGGTTTCCTCCCCACCTGAT-3'	97	1.21-7.21
Pseudonocardia spp.	5'-TGCAGCTTAACTGTGGGCTT-3' 5'-CTCCTCAGCGTCAGTATCGG-3'	152	1.62-6.62
Mycobacterium spp.	5'-GTTTGTCGCGTTGTTCGTGA-3' 5'-GGAATTCCAGTCTCCCCTGC-3'	93	1.9-7.9
Terrimonas spp.	5'-AGCGTGGGGATCAAACAGG-3' 5'-TTGCGATCGTACTTCCCAGG-3'	131	2.3-7.3
Flavobacterium spp.	5'-CACATGCAAGTCGAGGGGTA-3' 5'-TCAGTACCAGTGTGGGGGAT-3'	269	2.21-7.21
Leadbetterella-related spp.	5'-ACGTAGGTGGCGAGCGTTATC-3' 5'-GGAAATTCCATCTGCCTCCCAAT-3'	148	2.47-8.47

Table 2. qPCR analysis of the culture in the first 4 dilutions after significant ATO degradation.

	16S rRNA genes (log units / mL)				
Phylogenetic Group	Original	Dilution 1	Dilution 2	Dilution 3	Dilution 4
Ectothiorhodospiraceae	6.00	4.35	4.54	3.80	5.16
Pseudomonas spp.	4.42	4.30	4.66	5.33	5.80
Hafnia spp.	4.43	4.39	4.36	4.38	4.35
Stenotrophomonas spp.	BDL	BDL	BDL	BDL	BDL
Pseudoxanthomonas spp.	3.21	BDL	BDL	BDL	BDL
Nitrospira spp.	3.38	3.11	3.75	4.19	4.52
Ralstonia spp.	2.94	4.76	5.90	6.41	6.12
Polaromonas spp.	3.32	2.22	2.91	3.35	3.38
Ramlibacter-related spp.	6.50	6.40	7.53	8.04	7.69
Hydrogenophaga spp.	5.76	4.47	4.19	3.41	3.46
Sphingopyxis spp.	4.77	4.35	4.70	5.19	5.26
Sphingomonadaceae	BDL	BDL	BDL	BDL	BDL
Brevundimonas spp.	4.70	3.93	3.87	3.78	3.70
Shinella spp.	3.40	3.56	3.23	3.03	3.47
Mesorhizobium spp.	6.38	5.45	6.07	6.52	6.63
Xanthobacter spp.	4.10	3.28	BDL	BDL	BDL
Hyphomicrobium spp.	4.98	3.84	3.85	3.44	4.23
Uncultured Acidobacteria spp.	5.22	BDL	BDL	BDL	BDL
Gemmata-related spp.	BDL	BDL	BDL	BDL	4.31
Mycrocystis-related spp.	BDL	BDL	BDL	BDL	BDL
Pseudonocardia spp.	BDL	BDL	BDL	BDL	BDL
Mycobacterium spp.	BDL	BDL	BDL	BDL	BDL
Terrimonas spp.	5.86	5.58	6.56	6.98	6.89
Flavobacterium spp.	4.67	BDL	BDL	BDL	BDL
Leadbetterella-related spp.	BDL	BDL	BDL	BDL	BDL

Table 3. Results of qPCR analysis of the ATO degradation culture.

Phylogenetic Group	16S rRNA genes (log units / mL)		Closest isolate	16S rRNA genes per genome of genus
	Start	End		
Terrimonas spp.	5.86	7.02	98% identity to T. "soli" sp. FL-8 [MF595514]	1
Ramlibacter-related spp.	6.50	6.82	98% identity to R. *ginsenosidimutans* st. BXN5-27 [NR_133836]	1 - 2
Mesorhizobium spp.	6.38	5.89	98% identity to *M. australicum* strain WSM2073 [NR_102452]	1 - 2
Hydrogenophaga spp.	5.76	5.52	99% identity to *Hydrogenophaga* sp. PCB [CP017311]	1
Ralstonia spp.	2.94	5.45	99% identity to *Ralstonia* sp. M22 [AY864081]	2 - 4
Pseudomonas spp.	4.42	5.36	99% identity to P. *weihenstephanensis* st. DSM 29166 [KP738720]	4 - 7
Ectothiorhodospiraceae	6.00	5.10	91% identity to *Thioalkalivibrio* sp. BB57 [KP681552]	1 - 2
Sphingopyxis spp.	4.77	4.89	99% identity to *S. ummariensis* st. DSM24316 [MF618307]	1 - 2
Taonella spp.	4.01	2.99	98% identity to *T. mepensis* st. H1 [NR_132292]	Unknown
Uncultured *Acidobacteria*	5.22	BDL	91% identity to Vicinamibacter silvestris st. AC_5_C6 [KP761690]	1
Hyphomicrobium spp.	4.98	BDL	98% identity to Hyphomicrobium sulfonivorans st. WDL6 [AF538931]	1 - 2
Brevundimonas spp.	4.70	BDL	99% identity to Brevundimonas bullata st. IAM 13153 [NR_025831]	1 - 2
Flavobacterium spp.	4.67	BDL	99% identity to Flavobacterium suncheonense st. NBRC 106391 [AB682407]	1 - 6
Hafnia spp.	4.43	BDL	98% identity to Hafnia sp. CBA7124 [AP017469]	8
Xanthobacter spp.	4.10	BDL	99% identity to Xanthobacter sp. st. VII/1 [MH368459]	1 - 2
Shinella spp.	3.40	BDL	99% identity to Shinella kummerowiae st. IRNB-274-1 [LC177119]	1 - 3
Nitrospira spp.	3.38	BDL	99% identity to Nitrospira sp. NI5 [AY123812]	1
Polaromonas spp.	3.32	BDL	98% identity to Polaromonas aquatica st. CCUG 39797 [AM039831]	1 - 2
Pseudoxanthomonas spp.	3.21	BDL	99% identity to Pseudoxanthomonas indica st. PT-C1 [KX682026]	1 - 2

ATO Culture Biomarker Primers

Table 4 shows the primer pairs designed for the biomarkers in the ATO degrading community. The forward and reverse primers, as well as the amplicon size of the biomarker gene are listed. The primers were evaluated for specificity to the biomarker gene using gel electrophoresis.

Table 4. List of PCR primers developed for ATO biomarker analysis.

Target Gene	Primers	Amplicon Size (bp)
Gene 3654 Cyanuric Acid Amidohydrolase	5'-CAAGATCCATCGCATCCCCA-3' 5'-CCGAAGATGGCGAGGATACC-3'	105
Gene 1361 Dihydropyrimidinase D-hydantoinase	5'-CTGCTGGTCGACGATTCAGT-3' 5'-CCGTTCGGGATCTTGGTGAA-3'	212
Gene 1108 Nitrous-oxide reductase	5'-TGTTTGCCAATGACAAGGCG-3' 5'-ACGTACCCGGTCTTGGGATA-3'	139
Gene 1618 Nitrous-oxide reductase	5'-CAGTGCCGGTAACCACTCTT-3' 5'-GGGGAACACTGAAGCGTGTA-3'	161
Gene 3851 Nitrous-oxide reductase	5'-AAGAACGCGAAAGTACGTGGT-3' 5'-AGCGGTCCTTGGAGAACTTG-3'	133

Figure 4, 5, and 6 are phylogenetic trees of the genes selected as biomarkers and their relationship to the nearest sequences found from the protein-Blast database. Cyanuric acid amidohydrolase genes were found to be closely related to other ring-opening amidohydrolase genes from the *Xanthobacter* genus and also more distantly from the *Azorhizobium* genus. dihyropyrimidinase D-hydantoinase genes had the closest resemblance to the *Parvibaculum* genus and *Rhodospirillaceae*. The nitrous-oxide reductase genes formed 3 groups of relation in

the phylogenetic tree. Nitrous-oxide reductase Gene 3851 was observed to be related to other cultured *Ralstonia* genus species. This suggests the *Ralstonia* members of the microbial community express this gene in the ATO degrading culture. The other two nitrous-oxide reductase genes had more varied results with various separate genera represented in the sequences most closely related to them. Gene 1618 was similar to multiple *Sphingobacteriales* sequences in particular. Gene 1108 was very similar to a *Hyphomicrobium* sequence as well as two from *Bosea*.

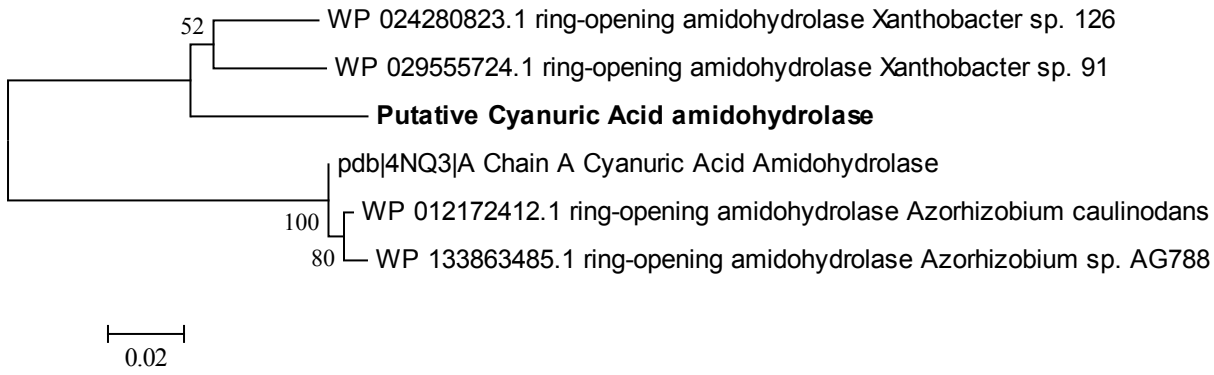

Figure 4. Phylogenetic tree of Cyanuric acid amidohydrolase with nearest 5 homologous sequences from protein-Blast using alignment of amino acids.

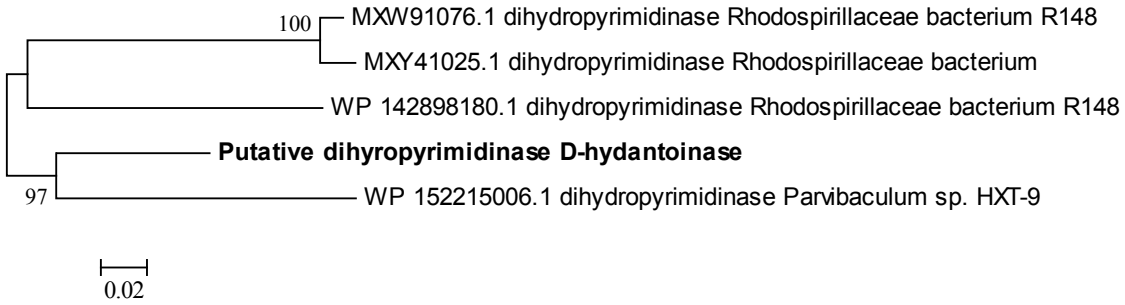

Figure 5. Phylogenetic tree of dihyropyrimidinase D-hydantoinase with nearest 4 homologous sequences from protein-Blast using alignment of amino acids.

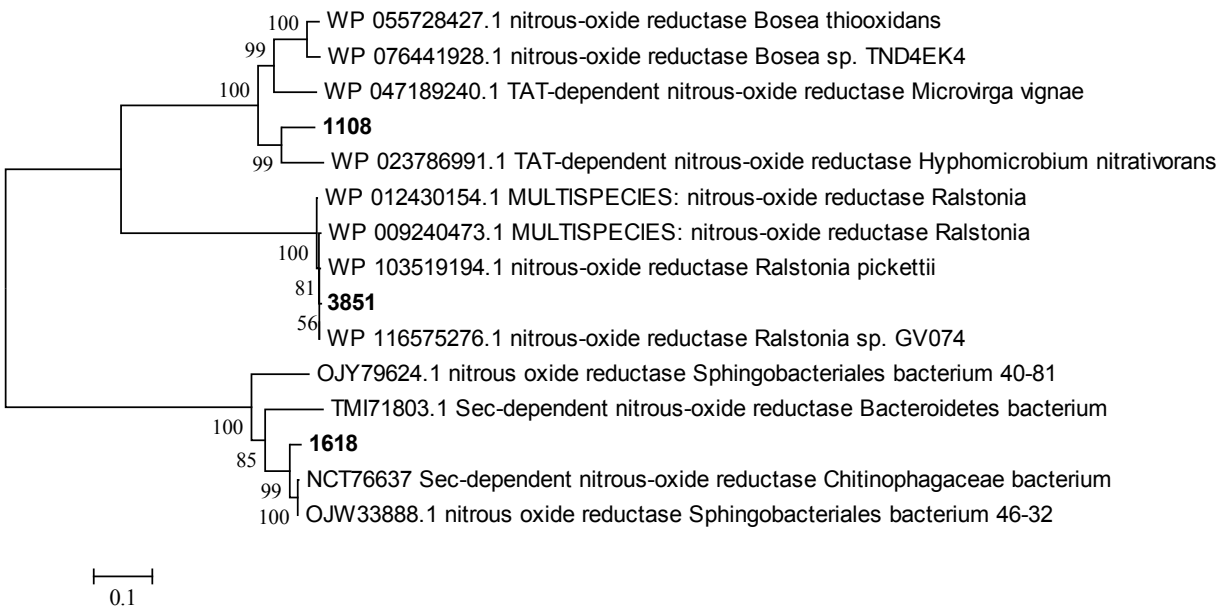

Figure 6. Phylogenetic tree of nitrous-oxide reductase genes with nearest homologous sequences from protein-Blast using alignment of amino acids.

NTO Reducing Community Analysis

The reduction of NTO to ATO and the microbial growth of the culture were measured in figure 7. Figures 8, 9, 10, and 11 show the relative abundance of each microbial group from qPCR analysis of the NTO reducing community. The enriched culture contained a majority of *Geobacter* spp. throughout the complete reduction of NTO. The *Geobacter* spp. group was typically found in the greatest numbers in the cultures throughout the experiment. *Thauera* spp. was the second most abundant microbial group, about 1-2 magnitudes less than *Geobacter* spp. In the last two remaining groups, *Soehngenia* spp. and *Pseudomonas* / *Pseudoxanthomonas* spp. were found in the least abundant numbers in the culture. These two groups were measured in approximately the same abundance, about 3-4 magnitudes less than *Geobacter* spp.

The abundance of *Geobacter* spp. in the NTO reducing culture suggests it is important and likely possesses the ability to transform NTO. The community reducing NTO, with only 4 main species groups, is much smaller and simpler in comparison to the ATO degrading culture. An increase in the growth of a microbial group during the NTO reducing phase of the reactor would strongly indicate a role in the transformation. Some of the microbial groups in the rectors seamed to be slightly enriched during the phase of active NTO reduction. The *Geobacter* group becomes slightly enriched after the end of the lag phase, during NTO reduction. The *Thaura* group is also enriched somewhat but at a steady rate over the entire experiment. According to the OD600 measurements, the starting culture had a relatively high initial concentration and this may have led to lower overall growth of the triplicate reactors. The low growth could be impacting the results and could lead to only subtle increases in community member abundance. Future studies could likely obtain clearer results by analyzing an NTO culture that began with a much lower starting concentration of the NTO reducing culture and had much more overall growth.

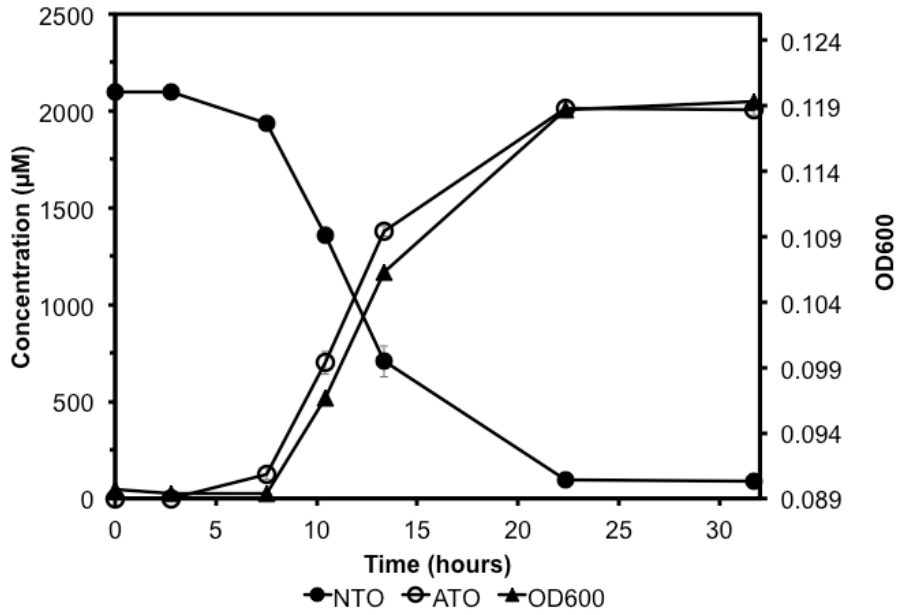

Figure 7. Concentrations of NTO and ATO compared to the measured OD600 over 32 hours in the NTO reducing culture (Camilla Madeira, Unpublished).

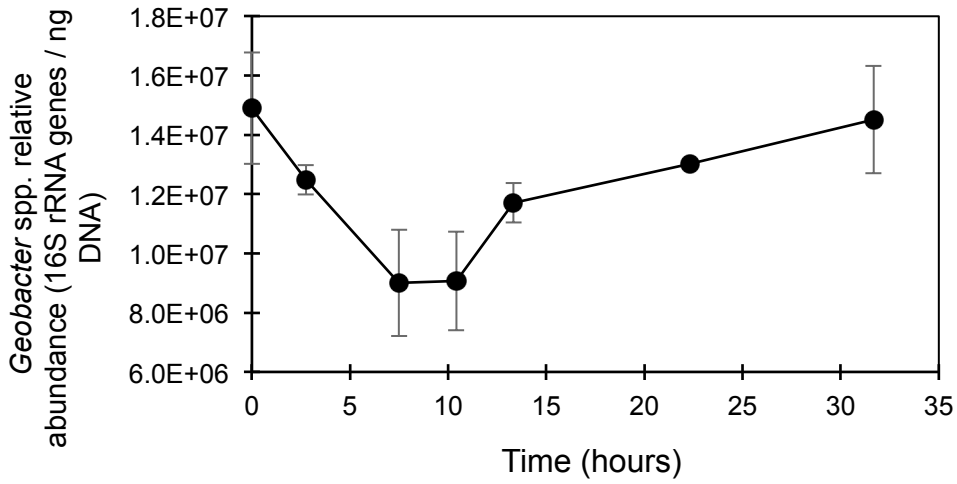

Figure 8. Relative abundance of *Geobacter* spp. 16S rRNA gene copies in the NTO reducing culture over a period of 32 hours.

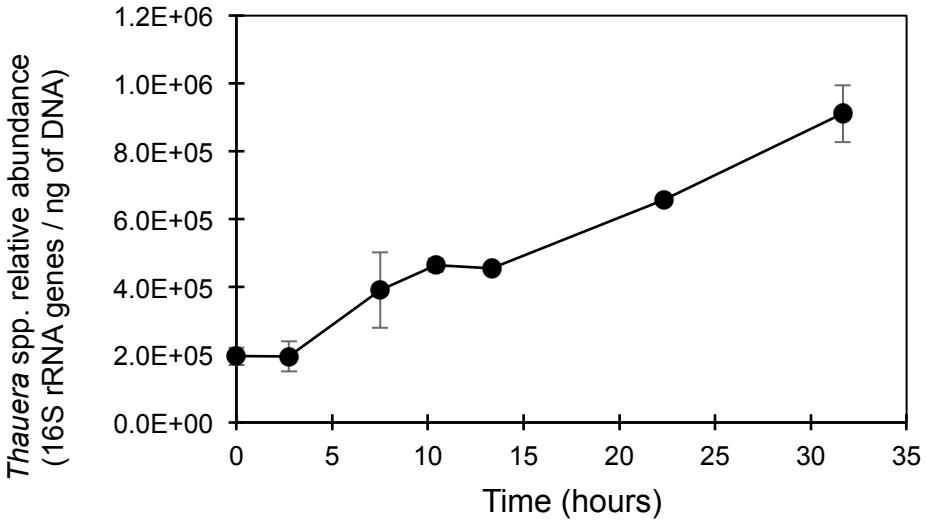

Figure 9. Relative abundance of *Thauera* spp. 16S rRNA gene copies in the NTO reducing culture over a period of 32 hours.

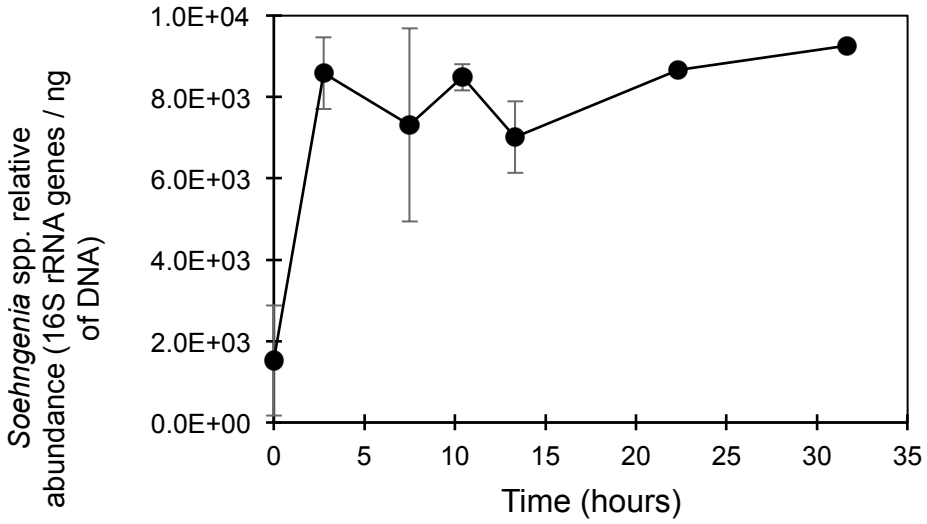

Figure 10. Relative abundance of *Soehngenia* spp. 16S rRNA gene copies in the NTO reducing culture over a period of 32 hours.

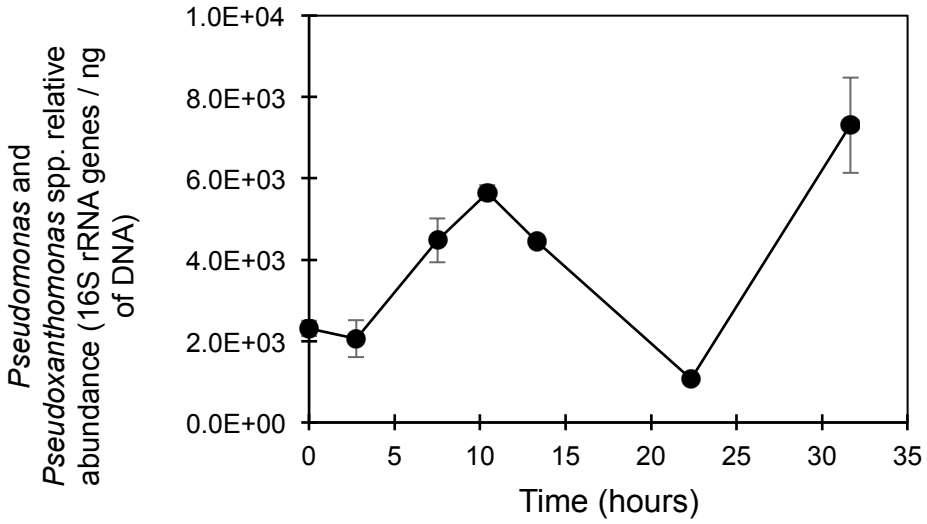

Figure 11. Relative abundance of *Pseudomonas* and *Pseudoxanthomonas* spp. 16S rRNA gene copies in the NTO reducing culture over a period of 32 hours.

The phylogenetic tree in figure 12 was used while designing qPCR primers to determine what groups of microbial species were present in the NTO reducing culture. The tree was produced from data collected from the original NTO reducing culture produced by the research group at the university of Arizona. The tree shows that there are 4 major groups of microbe species present in the culture. Multiples species in the *Thauera* genus and other related species were put into one group. Species related to the *Pseudomonas* and *Pseudoxanthomonas* genera comprise another group of related species. Species in the *Geobacter* genus or closely related to it are grouped together to form another group. The last group is made up of the *Soehngenia* genus species and a closely related species.

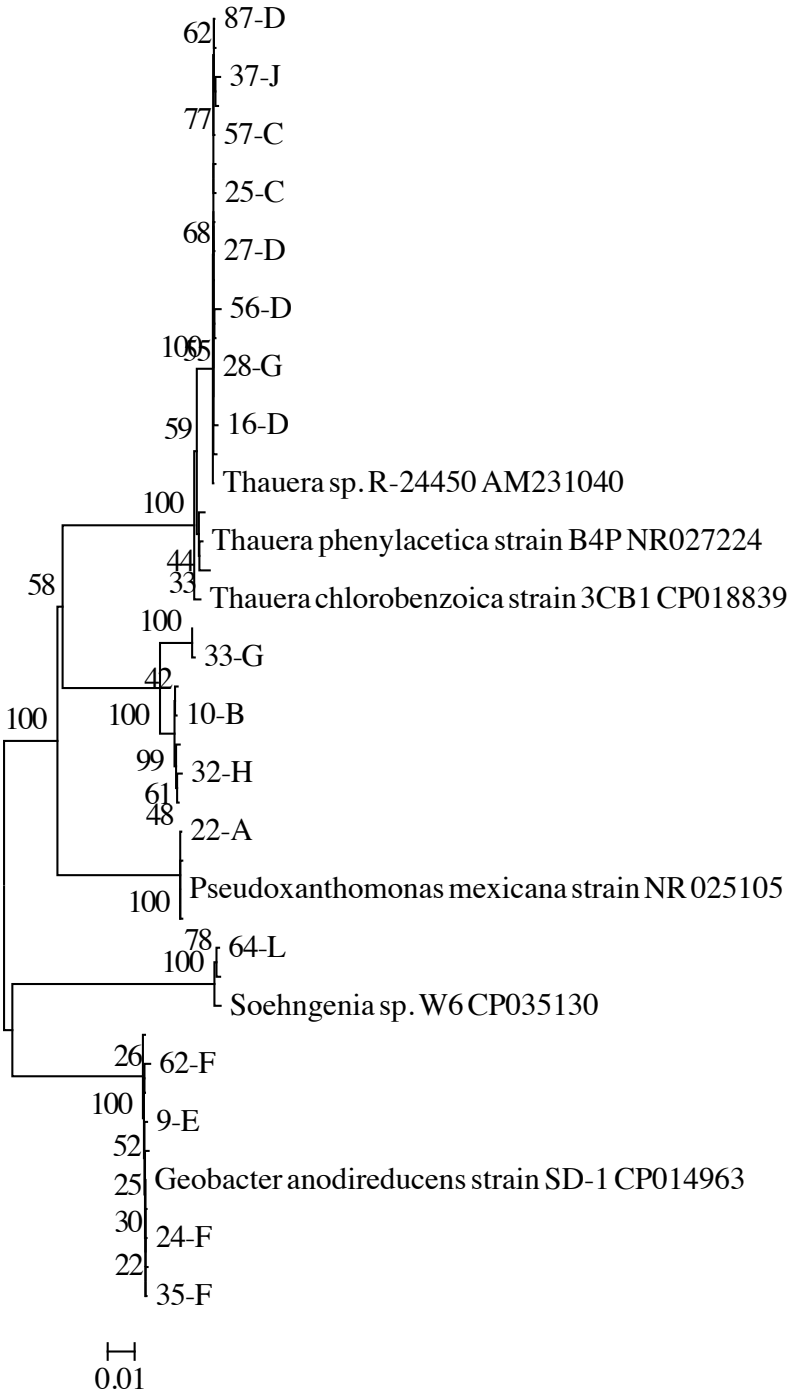

Figure 12. Phylogenetic tree of the original NTO reducing culture.

The Phylogenetic tree in figure 13 was created from samples collected after the final transformation trial of NTO measured in this study. The results show a change in community members, but the most recent samples of the culture retained the species belonging to the *Geobacter* genus and *Thauera* genus. A similarity between the two trees is that they both contain approximately 4-5 distinct groups of organisms in the community. This suggests *Geobacter* spp. or *Thaurea* spp. could be critical for NTO reduction because not only are they the most prevalent in the culture but they are also retained as major groups in the phylogenetic trees of both the original culture and the recent culture dilution. *Pelomonas*, *cutibacterium*, and *sediminibacterium* related organisms form 3 other distinct groups in this phylogenetic tree but are absent from the original culture analyisis.

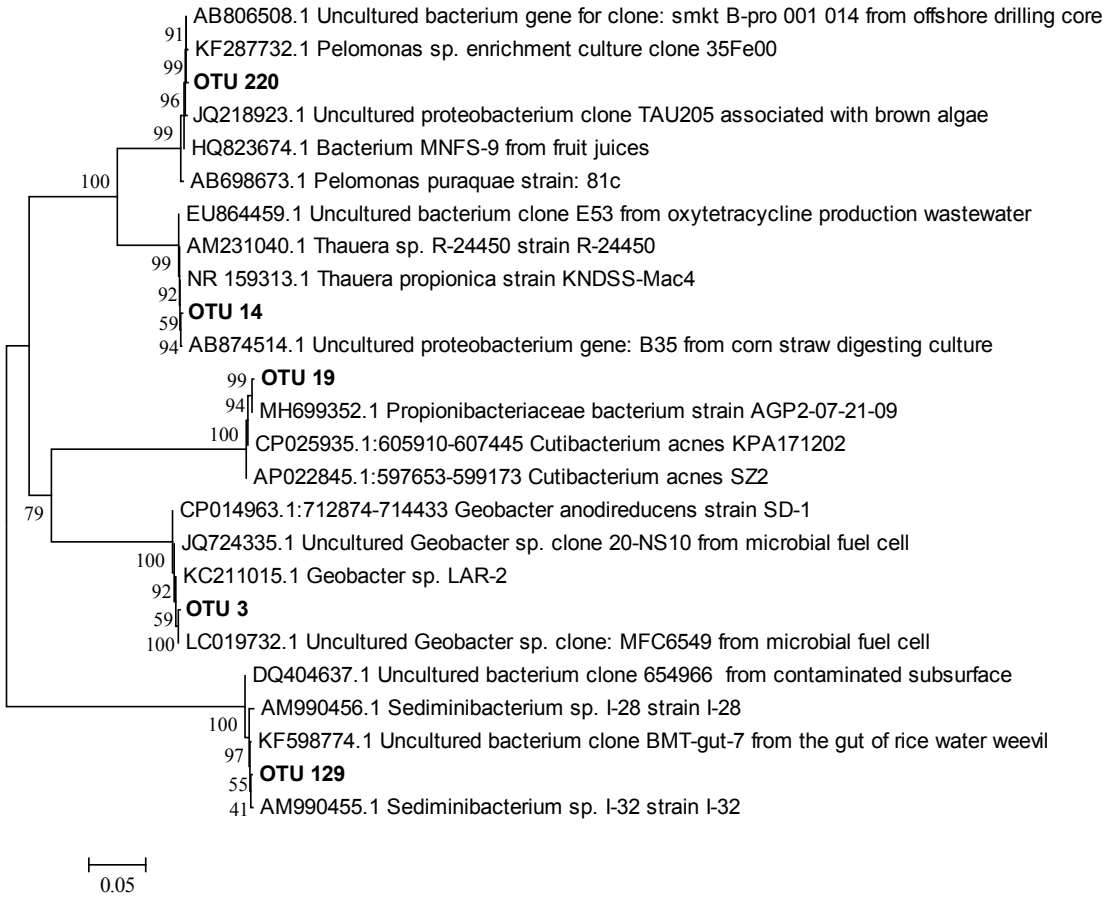

Figure 13. Phylogenetic tree of the most recent dilution of NTO reducing culture.

Table 5 lists the primer pairs used to amplify 16S rRNA genes of NTO community members with the qPCR method. The forward and reverse primer pairs are listed along with the length of the gene they produce in the PCR reaction. Gel electrophoresis indicated that each primer is specific to a single group's 16S rRNA genes without any interference.

Table 5. List of PCR primers developed for NTO community analysis.

Phylogenetic Group	Primers	Amplicon Size (bp)	Standard Range (Log(copies)/uL DNA extract)
Thauera spp.	5'-TGGATGACGGTACCGGACTA-3' 5'-GGTTAAGCCCGGGGATTTCA-3'	148	0.94 – 5.25
Pseudomonas / *Pseudoxanthomonas* spp.	5'-ACGTCAAGTCATCATGGCCC-3' 5'-TAAGCTACCTGCTTCTGGTGC-3'	256	1.02 – 6.07
Soehngenia spp.	5'-CGAAGGCGACTTTCTGGACT-3' 5'-CCTGGTAAGGTTCTTCGCGT-3'	259	0.84 – 4.85
Geobacter spp.	5'-CGAGGTGGAGCCAATCTCAG-3' 5'-TCCCCTACGGCTACCTTGTT-3'	254	1.99 – 5.89

CHAPTER V

CONCLUSION

An increase in ammonium concentration was measured during ATO degradation, supporting the hypothesis that ATO is completely mineralized by the microbial culture. The number of community members in the culture decreased from 19 to only 9 members after 7 dilutions. *Terrimonas* spp., *Ramlibacter*-related spp., *Ralstonia* spp., and *Pseudomonas* spp. were enriched by the dilutions. *Terrimonas* spp. and *Ramlibacter*-related spp. were found in the highest abundance with $10^{7.02}$ and $10^{6.82}$ 16S rRNA genes/mL, respectively. Their high abundance suggests they play a major role in the degradation of ATO and possibly use it as a carbon and nitrogen source for growth. *Ralstonia* spp. could also be involved in ATO degradation due to the close relationship of the nitrous-oxide gene 3851 to the *Ralstonia* genus. Other members of the community, including *Mesorhizobium* spp., *Hydrogenophaga* spp., *ectothiorhodospiraceae*, *Sphingopyxis* spp., and *Toanella* spp., persisted in the culture despite dilution, indicating they are important in some way to the function of the community.

Several potential ATO degradation biomarkers were developed for use in monitoring the rate of degradation. 5 potential biomarker genes were identified and qPCR primers were developed to track their expression in the culture. The genes selected were cyanuric acid amidohydrolase, dihydropyrimidinase D-hydantoinase, and 3 nitrous-oxide reductase genes.

The members of the NTO reducing culture generally maintained a constant concentration during the reduction, but some groups showed slight enrichment. *Geobacter* spp. were found in the highest abundance in the NTO reducing culture, approximately 10^7 16S rRNA copies/ng DNA extract. *Geobacter* spp. were enriched slightly after the lag phase ended and NTO reduction had started. The second most abundant members in the ATO culture were *Thauera* spp. with approximately 10^6 16S rRNA copies/ng DNA extract. *Thauera* spp. were slightly enriched throughout the entire experiment. Two groups, *Geobacter* spp. and *Thauera* spp., were present in both the phylogenetic trees of the original NTO culture and the most recent dilution. These groups are also found in the largest abundance in the culture. This suggests these two groups, particularly *Geobacter* spp., are directly involved in the transformation of NTO. The other members of the NTO reducing community were found in much smaller numbers that were multiple orders of magnitude less than the most abundant two groups. These members also varied greatly in the phylogenetic tree analysis, suggesting they are not critical members of the NTO reducing community. There was no obvious growth of any species during the transformation of NTO. An explanation for this could be the initial inoculation of the reactor with the NTO reducing culture was likely too high and left too little room for growth, which led to a stable concentration of the most abundant members of the community.

The species makeup of the NTO reducing and ATO degrading communities provides useful information about what species are likely involved in the transformation. It also revealed that multiple species are likely to play critical roles in the community. Further investigation into these communities could be used to further identify what genes are active in the degradation and lead to a better understanding of the degradation pathway. The potential ATO biomarkers identified could be useful in increasing bioremediation efficiency and allow monitoring of the rate of degradation. Future studies should involve testing of the qPCR primers performance with ATO bioremediation treatments to verify their accuracy. The examination of both NTO reducing

and ATO degrading culture and identification of the major community members can be used in future bioaugmentation techniques. Results from this study, as well as from researchers at the University of Arizona, supporting the hypothesis of complete ATO mineralization by the community can help improve understanding of degradation pathways and how they can be used at large scale in bioremediation

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