Utilization of Organic Nitrogen Compounds by Marine and Freshwater Bacteria
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Abstract
Reduced nitrogen species represent the largest pool of nitrogen in many aquatic systems, yet little is known about the chemical composition of this pool and the metabolic pathways required for its remineralization. The long residence time of this material in aquatic systems suggests that it is very refractory and relatively resistant to degradation by direct metabolism or extra-cellular enzymatic attack. The two objectives of this project were (1) to identify organisms with the capability to degrade representative DON compounds, and (2) to examine the ability of ammonia-oxidizing bacteria to remineralize the nitrogen from the same DON substrates. The DON substrates included: urea, trimethylamine (TMA), N-acetyl glucosamine (NAG – for ammonia-oxidizer study only), uric acid, alanine and caffeine. Enrichments on all DON substrates were successful in sustaining microbial growth and colonies were observed and isolated on agar plates of most substrates. Liquid isolations were more successful but cannot be considered "pure" at this stage. Growth experiments were conducted with pure cultures of *Nitrosospira briensis* and *Nitrosococcus oceanii* on all DON substrates. Both ammonia oxidizers were able to grow on all DON substrates (except alanine). Surprisingly fast growth rates were observed for *Nitrosospira* growing on urea or uric acid. In conclusion, the ability of marine and freshwater bacteria to metabolize reduced organic nitrogen substrates has been vastly underestimated and merits further study.

Introduction
Reduced nitrogen species represent the largest pool of nitrogen in many aquatic systems, yet little is known about the chemical composition of this pool and the metabolic pathways required for its remineralization. The concentration of this pool is determined by analysis of total dissolved nitrogen and subsequent subtraction of the inorganic components (ammonia (NH_4^+) nitrite (NO_2^-) and nitrate (NO_3^-)). No investigations to date have been able to separate DON from the larger pool of organic matter for composition or structural analysis. The source of DON is presumed to bio-molecules such as peptidoglycan, a primary component of cell walls; proteins; amino-sugars and waste products such as urea. The long residence time of this material in aquatic systems suggests that it is very refractory and relatively resistant to degradation by direct metabolism or extra-cellular enzymatic attack. Nonetheless, this material must be degraded (albeit slowly) by microbes since the DON concentrations are not constantly increasing in the world's oceans and lakes. The primary organisms responsible for large-scale nitrogen oxidation are the ammonia-oxidizing bacteria, which use the energy from the conversion of ammonia (NH_3/NH_4^+) to nitrite (NO_2^-) to drive cellular function and biosynthesis. The amount of nitrogen required for energy production is significantly larger than that needed for protein or nucleic acid biosynthesis and so nitrogen-oxidizing bacteria could represent a large sink for reduced nitrogen. However, no bacteria have been isolated that oxidize reduced organic nitrogen for energy production (to my knowledge), although some ammonia-oxidizing bacteria have been shown to use urea as an energy source by conversion of urea to ammonia using urease. Few additional substrates were tested and so an examination of the ability of ammonia-oxidizing bacteria to remineralize a range of possible DON substrates was needed. The substrates used in this study could be divided into three groups of chemical nitrogen. First, urea and N-acetyl glucosamine (NAG) were chosen to represent amide-N. Urea is a common waste product and NAG is a

building block of peptidoglycan, the primary constituent of bacterial cell walls. Second, trimethylamine (TMA) and alanine were chosen to represent amine-N. TMA is a common waste product and alanine is a common amino acid. Lastly, caffeine and uric acid were chosen to represent aromatic-N. Both caffeine and uric acid are purine derivatives with four nitrogen atoms within two fused aromatic rings. Caffeine is commonly found in urban wastewater due to the high loading in urban sewage. Uric acid is a common component of bird guano and is routinely deposited on aquatic surfaces. In addition to testing the ability of ammonia-oxidizing bacteria ot degrade these compounds, I enriched seawater and freshwater samples with five of these compounds (i.e., all except NAG) to isolate novel bacteria that could remineralize DON. In general, these enrichments will select organisms that (1) use DON as a carbon source, (2) use DON as a nitrogen source for biosynthesis, (3) use DON as a nitrogen source for oxidation, or (4) use DON as both a carbon and nitrogen source. All six of these compounds are found in the two aquatic systems that were chosen for bacterial enrichment and thus should be representative of some of the compounds encountered by marine or lacustrine bacteria on Cape Cod.

Thus, this project represents an infinitesimally small step forward in the quest to identify organisms that can degrade DON and to identify the requisite physiology for these transformations. In brief, the two objectives of this project were:
1. to identify organisms with the capability to degrade representative DON compounds, and
2. to examine the ability of ammonia-oxidizing bacteria to remineralize the nitrogen from the same DON substrates.

Project #1:
Water samples were acquired from John's Pond (a freshwater pond - Mashpee MA) and Garbage Beach (Vineyard Sound seawater - Woods Hole MA). I set up enrichments for each environment with one DON substrate provided as both the carbon and nitrogen sources. Each seawater enrichment contained 75% autoclaved 0.2-µm filtered seawater, 0.1% trace metal mix, and 15uM PO_4^{-3}(all in sterile Milli-Q water). Each freshwater enrichment contained freshwater base (NaCl, $MgCl_2$, $CaCl_2$, KH_2PO_4, and KCl), 25uM SO_4^{-2} 10mM MOPS buffer (pH 7.2), 15uM PO_4^{-3} and 0.1% trace metal mix. The concentration of DON substrate was 5 mM in each enrichment. Each enrichment was started on the same day as the sample was collected. Each sample was diluted by 10X into eight tubes of 4.5mL of enrichment medium. All tubes were placed in racks and shaken at $27^{o}C$. After 9 days, agar plates were prepared for each enrichment by adding 1.5% agar to previously prepared media and then autoclaving. The enrichment dilution with the best evidence for growth was chosen for further purification (usually the 10^{-2} dilution for the freshwater enrichments and either the 10^{-2} or 10^{-3} dilutions for the seawater enrichments). Two 100uL aliquots of each dilution were transferred to appropriate agar plates for spreading and the plates were incubated at $30^{o}C$ in the dark. At the same time, three 10X dilutions were made of the same original enrichments into fresh media and incubated at room temperature in the dark.

After three days, colonies were observed on the seawater caffeine (SWC), alanine (SWA), TMA (SWT) and urea (SWU) plates. Likewise colonies were observed on the freshwater caffeine (FWC) and alanine (FWA) plates. For both the FWC and FWA plates, the colonies were quite small, suggesting that the bacteria may have been growing on the agar instead of on the DON substrate that was added. Colonies were streak-purified from each of these plates and incubated

again. At the same time, substantial growth was observed in the liquid cultures of all the DON treatments, with the most turbid cultures observed in the alanine treatments (FWA and SWA).
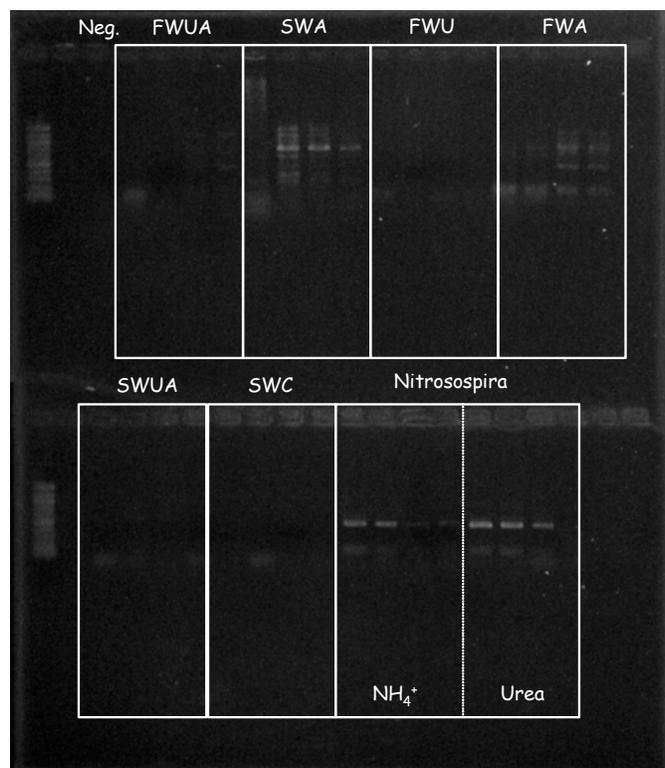

Figure 1. PCR products from *amoA* amplification of liquid DON isolates. Positive controls are represented by *Nitrosospira* cultures growing on either NH$_4^+$ or urea. Bands representing *amoA* are present in SWA and FWA.

The presence of ammonia-oxidizing bacteria was tested in the liquid cultures. Cells were isolated from the cultures by centrifugation of 1mL aliquots in 1.8mL Eppendorf tubes (13,000 rpm for 20 min at 20°C). The supernatant was removed and additional aliquots were added until a pellet was visible in the base of the Eppendorf tube. All cells were then washed in PBS:EtOH (1:1) and centrifuged again. The clean cells were resuspended in 20uL of lysis buffer (RNAse-free water with P-40 detergent) and boiled at 102°C for 5 min. The resulting suspension was used for direct PCR. To test for the presence of ammonia-oxidizing bacteria, primers optimized for *amoA*, the gene for ammonia monooxygenase, were used to amplify the extracted DNA. Positive results were obtained for two control cultures of *Nitrosospira briensis* growing on either NH$_4^+$ or urea. Positive results were also obtained for both the alanine treatments (SWA and FWA – Figure 1). PCR of the 16S gene (using 8F and 1492R) provided product for all treatments implying that there should have been ample template DNA in the PCR reaction for *amoA* amplification. Thus, it is possible to conclude that ammonia-oxidizing bacteria (or at least *amoA*-containing organisms) are present in the alanine amendments. Amplification of the 16S gene in these enrichments did not support this result in the sense that the most dominant organism in both the SWA and FWA enrichments were not known ammonia-oxidizers, but instead were members of the *Alteromonas* and *Pseudomonas* species, respectively. Identification of the *amoA*-containing organisms in these treatments will require more time and we intend to pursue the further purification of these organisms.

In the end, I was able to get 16S PCR products for 11 liquid enrichments or colonies and aligned the sequences with known organisms using ARB (Figure 2). Most sequences aligned quite closely to known representatives within the ARB database. Interestingly the SWC liquid isolate sequence aligned closely with a carbazole-degrading bacterium but the plate colonies were all members of the *Alteromonas* group. It is possible that the plate isolations selected for different species over those in the liquid cultures. Many of the organisms observed in the cultures were quite motile and may not have grown well in the relatively immobile medium of the agar plates.

For all of these isolations, further work is necessary to purify these organisms. I would like to extend this work by examining the metabolism of these compounds in the presence of the respective isolates to understand the physiology that leads to the remineralization of these DON substrates.
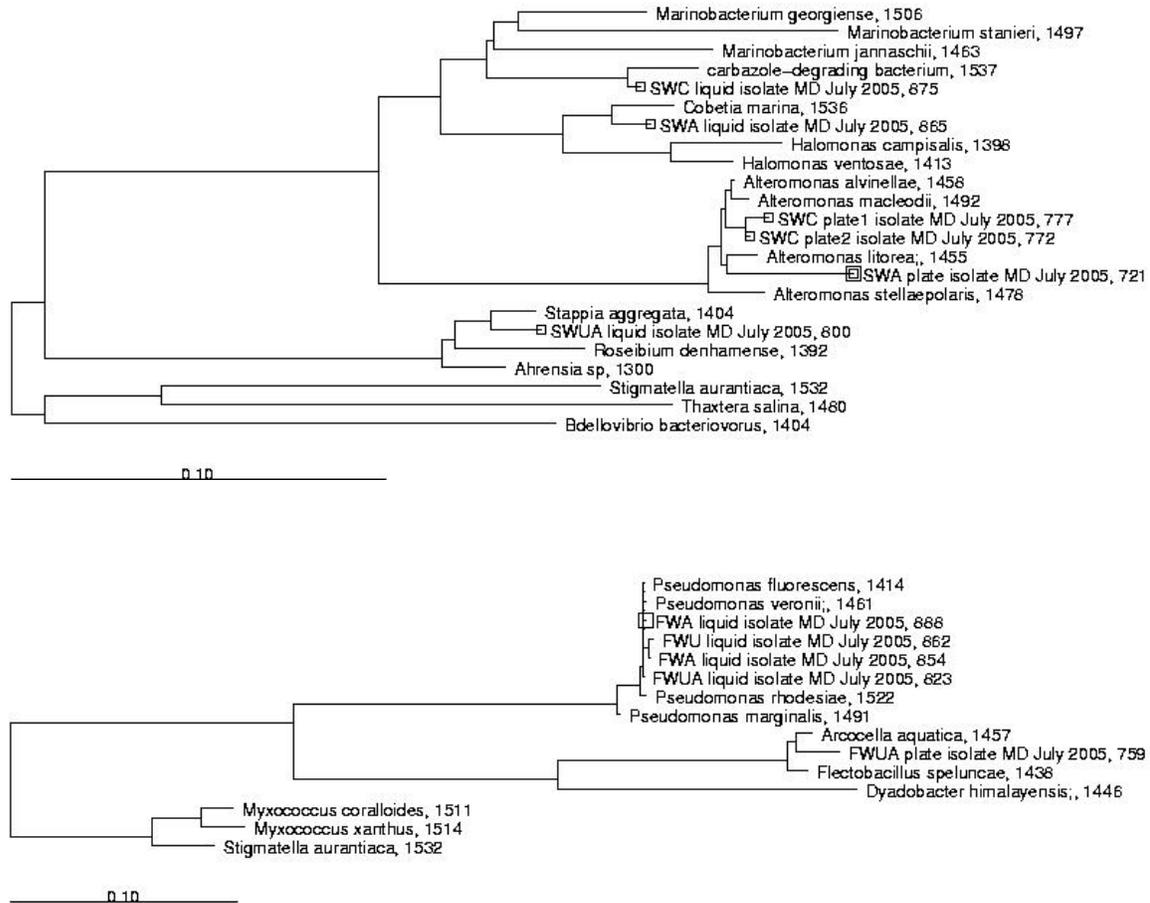

Figure 2. 16S sequences from seawater isolates (top) and from freshwater isolates (bottom). Both trees were constructed using known sequences with $\geq$ 1400 bp via the neighbor-joining calculation. A Felsenstein correction was used but no positional filter. The outgroup was chosen in both cases as organisms that could exist in a similar environment but were not present in the 16S sequences of the isolates.

Project #2:

I obtained four cultures of different ammonia-oxidizing bacteria from the collection of John Waterbury and Freddy Valois: *Nitrosococcus oceanii* (a marine ammonia-oxidizer), *Nitrosomonas europaea*, *Nitrosolobus multiformis*, and *Nitrosospira briensis* (3 freshwater ammonia-oxidizers). I isolated cells from approximately 200mL of culture by centrifugation at 13000 rpm for 15 min. The supernatant was removed and fresh N-free media was added. The cells were re-suspended and then centrifuged again. After the supernatant was removed the second time, the cells were re-suspended in approximately 10mL N-free media and split into 20 5-mL tubes of media with 5mM DON-N. All substrates were added so that the concentration of N was the same in all treatments (thus caffeine with 4 N's per molecule was ¼ as concentrated as

trimethylamine with 1 N per molecule). Two of the tubes contained 1mM NH_4^+ as a control. Each of the DON growth experiments were performed in triplicate. The tubes were incubated vertically in the dark for a week. After this time, the production of NO_2^- was tested in all the cultures. Since NH_4^+ is converted to NO_2^- during ammonia oxidation, the production of the end-product can be used as a proxy for cellular activity and growth. NO_2^- presence was tested by combining a drop of culture with a 1:1 mixture of 1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylene diamine/ HCl. In the presence of NO_2^-, the mixture turns pink and this can be quantified by spectrophotometry with 543nm-wavelength light. I was shocked to find that all *Nitrosospira* cultures turned dark pink, regardless of the DON substrate. For the other ammonia-oxidizers, all cultures turned the same color, though the pink was much less pronounced in the *Nitrosococcus* and *Nitrosolobus* cultures and practically non-existent in the *Nitrosomonas* cultures. Given this startling result, I set up another growth experiment with *Nitrosospira* and *Nitrosococcus* to examine the daily production of NO_2^- on the various DON substrates.

500uL of old culture was inoculated into 4.5mL fresh media containing 0 N, 1mM NH_4^+ or 5mM DON-N. Cultures were transferred into media of the same DON substrate to avoid cross contamination of different substrates. The original substrate was further diluted by making a second 10X dilution into the same media. The no-N control did not work well because the cells were inoculated using the NH_4^+ positive control from the previous experiment and so the media had residual N at the start of the experiment. NO_2^- concentrations were monitored as a proxy for growth of the ammonia-oxidizers in all treatments. NO_2^- concentrations were quantified with the above colorimetric method against a six-point standard curve ranging from 10uM to 150uM $NaNO_2$. Phenol red was added as a pH indicator and sterile 0.1M K_2CO_3 was added when the cultures turned yellow due to acid production during nitrification. *Nitrosococcus* cultures were started 3-4 days after the *Nitrosospira* cultures so less data is available for this organism.
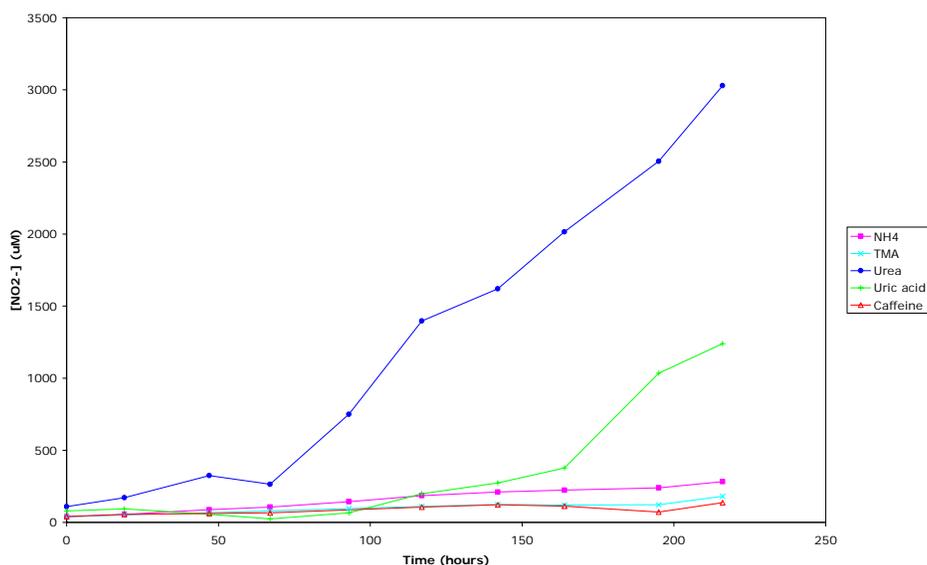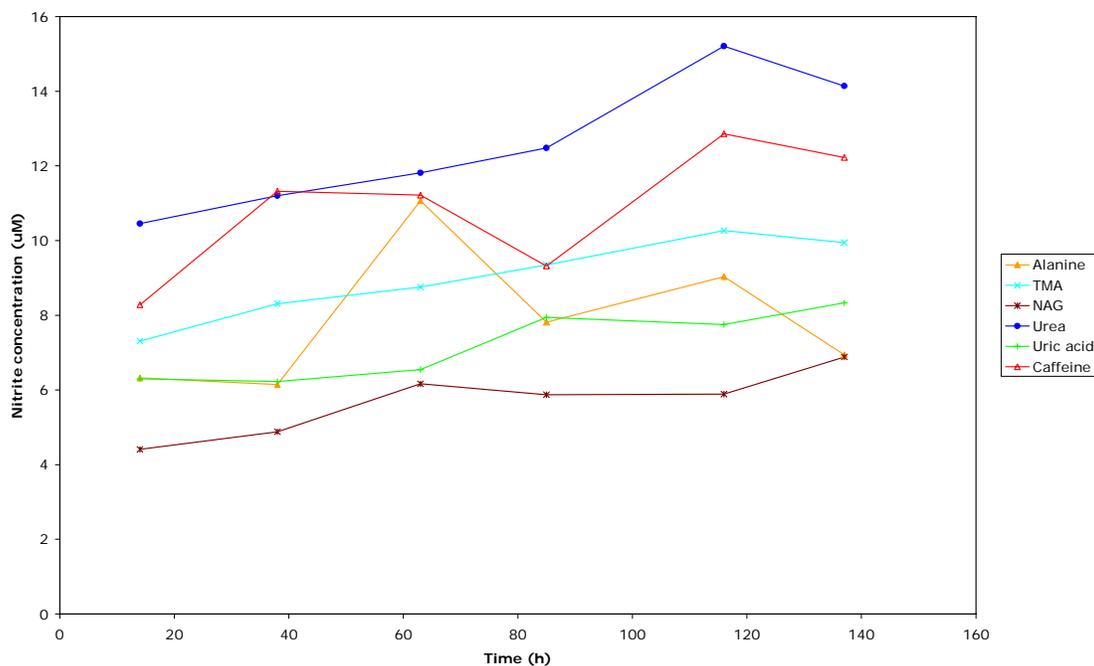

Figure 3. Growth curves for *Nitrosospira* on different DON substrates.
Growth is not normalized for initial cell number.

The *Nitrosospira* cultures grew on all the DON substrates provided, albeit at different rates (Figure 3 and Table 1). The alanine and NAG treatments were contaminated by heterotrophs

early in the experiment and so these treatments were dropped from further study. No heterotrophic contamination was observed in the other treatments (noted by the relative clarity of the cultures). The highest growth rates were observed in the urea and uric acid treatments. These results are somewhat unexpected due to the isolation conditions of these organisms. It is perplexing to consider the mechanism by which these organisms grow on compounds such as caffeine with specific abilities to break the purine ring. Furthermore, these organisms are autotrophic, meaning that they use CO_2 as a carbon source. It is not clear in these experiments whether the organism is incorporating the carbon from the organic nitrogen or whether it is also fixing CO_2 into cellular carbon. Further work is needed to distinguish between these two mechanisms.

Substrate	*Nitrosospira*	*Nitrosococcus*
NH_4^+	0.0087 ± 0.0009	0.0180 ± 0.0060
Urea	0.0160 ± 0.0010	0.0029 ± 0.0005
TMA	0.0059 ± 0.0006	0.0026 ± 0.0004
Alanine	*	*0.0015 ± 0.0023*
NAG	*	0.0031 ± 0.0008
Uric acid	0.0150 ± 0.0030	0.0026 ± 0.0005
Caffeine	0.0044 ± 0.0011	0.0025 ± 0.0013

Table 1. Growth rates for the two ammonia-oxidizing bacteria on different N substrates. Growth rates were calculated from the least-squares fit of the $\ln([NO_2^-])$ versus time. All growth rates are presented in units of h^{-1}.

Figure 4. Growth curves for *Nitrosococcus* growing on different DON substrates. No correction is made for initial cell number.

The *Nitrosococcus* cultures also grew on all the DON substrates provided, with the notable exception of alanine (Figure 4 and Table 1). This is interesting given the presence of *amoA* in the alanine enrichments discussed above. The growth rates of *Nitrosococcus* were lower than those

of *Nitrosospira* and the highest growth rates were observed for ammonia rather than the other DON substrates. The result from the *Nitrosococcus* cultures matched our predictions more closely since the growth rates on DON were approximately 10X lower than on NH_4^+. Nonetheless it is interesting to note that statistically significant growth did occur on these substrates. Further work is needed to confirm these results and to elucidate the mechanisms that allow this metabolism.

Conclusions and Reflections

It is clear from both the DON enrichments and the ammonia-oxidizer study that the ability to remineralize DON substrates is present in both marine and freshwater systems. The hypothesis that this metabolic activity existed has been confirmed, although we lack many important details in elucidating this process. This project highlights the need to use many different enrichment substrates for isolating bacteria with novel capabilities. I am not convinced that the dominant organisms in my DON enrichments are all using the DON substrates without help from other heterotrophic organisms. However, we can build on this work by further purifying these isolates and attempting new enrichments with more complex (and potentially more representative) DON substrates such as peptidoglycan, poly-amino-saccharides and other N-containing macromolecules. I am convinced that we will find interesting microbes with novel metabolisms that can degrade these compounds. Furthermore, the physiology of these organisms will elucidate the required gene (or genes) for critical steps in this metabolism and may shed light on the numerous "hypothetical" genes and proteins emerging from marine genomes and fosmid libraries.