

Souriau

**Anaerobic oxidation of reduced nitrogen compounds by marine phototrophs in
Sippewissett marsh**

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Abstract

Enrichment cultures were prepared to investigate the anaerobic oxidation of reduced nitrogen compounds by bacterial communities from Sippewissett marsh, Woods Hole, Massachusetts. Anaerobic oxidation of these compounds is a part of the biological nitrogen cycle that is largely unexplored. The presence of phototrophs that can mediate these energetically expensive reactions is probable due to the abundant energy derived from light. The resultant reducing equivalents may be used to fix carbon dioxide and produce cell material. Either trimethylamine (TMA), hydroxylamine, or nitrite were sole electron donors, therefore the oxidation of these compounds was expected. Growth was observed in TMA, hydroxylamine, and nitrite enrichments incubated in broad spectrum light. Successful transfer of the TMA enrichment into a shake tube dilution series resulted in single colonies that were picked, sequenced and subcultured. Sequence analysis suggests the organism picked is closely related to *Rhodovulum sulphidophilum*, a purple nonsulfur bacterium. Hydroxylamine and nitrite enrichments have exhibited growth and have recently been successfully transferred. Further chemical analysis will be done to determine if oxidation of these reduced nitrogen compounds is occurring.

Introduction

Anaerobic oxidation of reduced nitrogen compounds is a part of the biological nitrogen cycle that is largely unexplored. Reduced nitrogen compounds such as trimethylamine are commonly found in marine environments as a result of decaying organic matter. With energy from light, phototrophs should be able to oxidize these compounds and use the resultant reducing equivalents to fix carbon dioxide. In this study, enrichments for purple nonsulfur bacteria with various reduced nitrogen compounds were done. Trimethylamine, hydroxylamine, and nitrite were used as sole electron donors in the enrichments.

Materials and Methods

Seawater based media (Table 1) with no nitrogen or electron donor was prepared in a widdel flask, autoclaved, and cooled under nitrogen gas to keep the solution anoxic. Media was dispensed into 50 ml Pfenig bottles and closed tightly. Trimethylamine, nitrite, and hydroxylamine were added as sole electron donors at 3mM, 500 μ M, and 500 μ M final concentration, respectively. Inocula were obtained from Sippewissett marsh. Mat 1 was an anaerobic mat which was brought back to the lab and kept in an airtight container. Mat 2 was maintained with seawater flowing across the surface. To inhibit cyanobacterial growth, some cultures were amended with 50 μ M DCMU, or were placed in an infrared light box. To account for sensitivity to oxygen some cultures were incubated with a small amount (few grains) of 97% Na-dithionite (Table2). All cultures were fed with 300 μ M (final concentration) neutralized H₂S solution.

SEAWATER PURPLE-NONSULFUR-PHOTOTROPH MEDIA

1x seawater base	2L
1M sulfate	1ml
MOPS 7.2	20ml
12-vitamin solution	2ml
B-12 vitamins	2ml
EDTA chelated trace metals	2ml
1M sodium bicarbonate	20ml (10mM final concentration)
TMA, hydroxylamine, or nitrite	3mM, 500 μ M, 500 μ M, respectively, final concentration

Table 1: For solution composition see microbial diversity 2000 media recipes compiled by Jared Leadbetter.

BOTTLE #	MAT	SUBSTRATE	+/- DCMU	+/- DITHIONITE	LIGHT
1	1	TMA	+	-	incandescent
2	1	TMA	-	-	incandescent
3	1	Nitrite	+	-	incandescent
4	1	Nitrite	-	-	incandescent
5	1	Hydroxylamine	+	-	incandescent
6	1	Hydroxylamine	-	-	incandescent
7	2	TMA	+	-	incandescent
8	2	TMA	-	-	incandescent
9	2	Nitrite	+	-	incandescent
10	2	Nitrite	-	-	incandescent
11	2	Hydroxylamine	+	-	incandescent
12	2	Hydroxylamine	-	-	incandescent
13	1	TMA	-	+	incandescent
14	1	Nitrite	-	+	incandescent
15	1	Hydroxylamine	-	+	incandescent
16	1	TMA	-	-	infrared
17	1	Nitrite	-	-	infrared
18	1	Hydroxylamine	-	-	infrared

Table 2: Enrichment conditions.

When growth was detected in bottles 13 (mat 1, dithionite, TMA) and 15 (mat 1, dithionite, hydroxylamine) a shake tube dilution series (10^{-0} to 10^{-9}) was prepared (Table 3). Shake tubes consisted of an H_2S /agarose plug overlain with low agar content seawater based media. These tubes were gassed with N_2/CO_2 to keep the head space anoxic and incubated in incandescent light. Colonies were picked for PCR amplification and subsequent DNA sequencing and were subcultured in liquid media. Colonies and cultures were viewed with light and fluorescent microscopy.

SHAKE TUBE MEDIA

1x seawater base	1L
1M sodium bicarbonate	17mM final concentration
1M sodium sulfate	0.5 ml
12 vitamin solution	1ml
EDTA-chelated trace element solution	1ml
Selected N source	

Table 3: After heating medium to 42°C add 6ml aliquots to tubes containing 3 ml of washed, molten 3% agar (60°C). After inoculating gas with N_2/CO_2 .

Polymerase chain reaction with eubacterial primers was performed. Forward primer SDBact008F20 and reverse primer s*Univ1391 were used with the following conditions; One cycle of initial denaturation at 95°C for 5 minutes, 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, and 1 cycle of the final extension at 72°C for 5 minutes. Templates were sequenced by dideoxynucleotide sequencing with 519R (sequencing primer) and data was analyzed with ARB software, Munich Germany.

Results

Growth was detected in the hydroxylamine, dithionite bottle (number 15) after incubating in incandescent light for 10 days. The bottle was fed 500 μ M hydroxylamine at day 10 and had visibly more growth by the next day. I poured a shake series but no growth was detected after 11 days. Subcultures with and without dithionite amassed visible cell material within 3 days. The hydroxylamine bottle incubated in the infrared light box (18) has also begun to grow, but further analysis of this culture will be continued at a later date.

The TMA, dithionite bottle (13) exhibited the accumulation of cell mass after incubating in incandescent light for 12 days. The spectral analysis indicated the presence of bacterial chlorophyll a, which is consistent with purple nonsulfur bacteria. A shake tube dilution series was prepared for this enrichment and growth was seen within 3 days. Colonies were picked and viewed under the light microscope. Small, short rods with no autofluorescence were seen. Colonies were picked and subcultured in liquid media for further analysis in pure culture. Two colonies were picked, PCR amplified as described above and sequenced. The sequence aligned unambiguously to the purple non-sulfur *Rhodovulum sulfidophilum* (Figure 1). The TMA bottle incubated in the infrared light box exhibited significant growth after 15 days. Wet mounts of this enrichment were autofluorescent at factor 420 indicating the presence on archaeal methanogens. When this bottle was shaken a copious amount of gas bubbled to the top of the media. Though the

gas was not analyzed, the presence of F420 autofluorescence, and morphological characteristics consistent with methanogens suggests methane gas was evolving from the bottle.

The enrichment for nitrite with dithionite (14) exhibited growth after incubating in incandescent light for 17 days and responded (increase in cell mass) to daily feeding of 500 μ M nitrite. A wet mount of this enrichment showed an interesting morphology that is similar to *Hyphomicrobium sp.* The infrared nitrite enrichment (17) has exhibited growth, but time does not permit further examination currently.

All of the other enrichments have begun to show signs of growth (turbidity, wet mounts) but the time constraints on this project prevent further analysis at MBL. This research will be continued jointly at the University of Delaware and US Naval Research Lab, Washington DC.

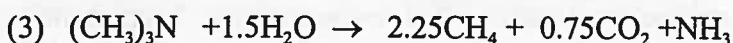
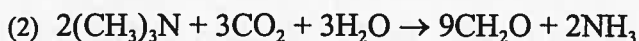
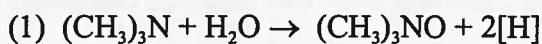
DISCUSSION

Anoxygenic phototrophs that can oxidize reduced nitrogen compounds are not well studied. These reactions are likely to occur with the light energy harvested by phototrophs, so it is suspected that organisms with this capability should be prevalent in the environment. Compounds such as TMA are commonly found in marine environments and microbes are known to reduce TMAO to TMA which suggests the reverse reaction should be possible.

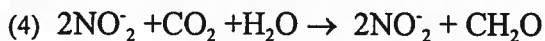
In trimethylamine enrichments there are at least three possible reactions occurring. Equation 1 shows the oxidation of TMA to TMAO and the production of reducing equivalents that may be used to fix CO₂. Without light energy, the potential of the reducing equivalents is too high to fix CO₂, but in these phototrophic enrichments it is feasible. Similarly, equation 2 takes advantage of energy from light to remove methyl groups and produce reducing equivalents for CO₂ fixation, but there is no oxidation of the nitrogen. Both equations 1 and 2 are anabolic light reactions and either is likely in these phototrophic enrichments where the only electron donor is TMA. The measurement of TMA and TMAO would be a good indication of which reaction is predominant but was not possible during the course. The more interesting of the two reactions would be

equation 1, the oxidation of TMA to TMAO since the anaerobic oxidation of reduced nitrogen compounds in general is poorly studied. Sequence analysis from shake tube isolates from incandescent enrichments indicated the presence of a purple nonsulfur bacterium closely related to *Rhodovulum sulphidophilum*. This result is consistent with microscopy and the specificity of the enrichment.

Equation 3 is likely occurring in the TMA enrichment that was incubated in the infrared box. This equation describes methanogenesis, a catabolic dark reaction. While methane measurement would be useful, other evidence such as F 420 fluorescence and morphology suggests that archaeal methanogens are present. This is also not surprising since methanogens are known to degrade TMA. This reaction is not as likely to occur in the incandescent light because methanogens are known to be inhibited by light



Nitrite enrichments are likely to carry out at least one anabolic light reaction (4). These enrichments are particularly interesting because nitrite is the sole electron donor and the nitrogen must be oxidized to generate reducing equivalents to produce cell material. The other possible electron donor is the HEPES buffer in the media. Measuring the $\text{NO}_2^- / \text{NO}_3^-$ concentrations would indicate which electron donor is being used. The use of buffer like MOPS and HEPES as electron donors is not common but should be considered and ruled out before further work is done.



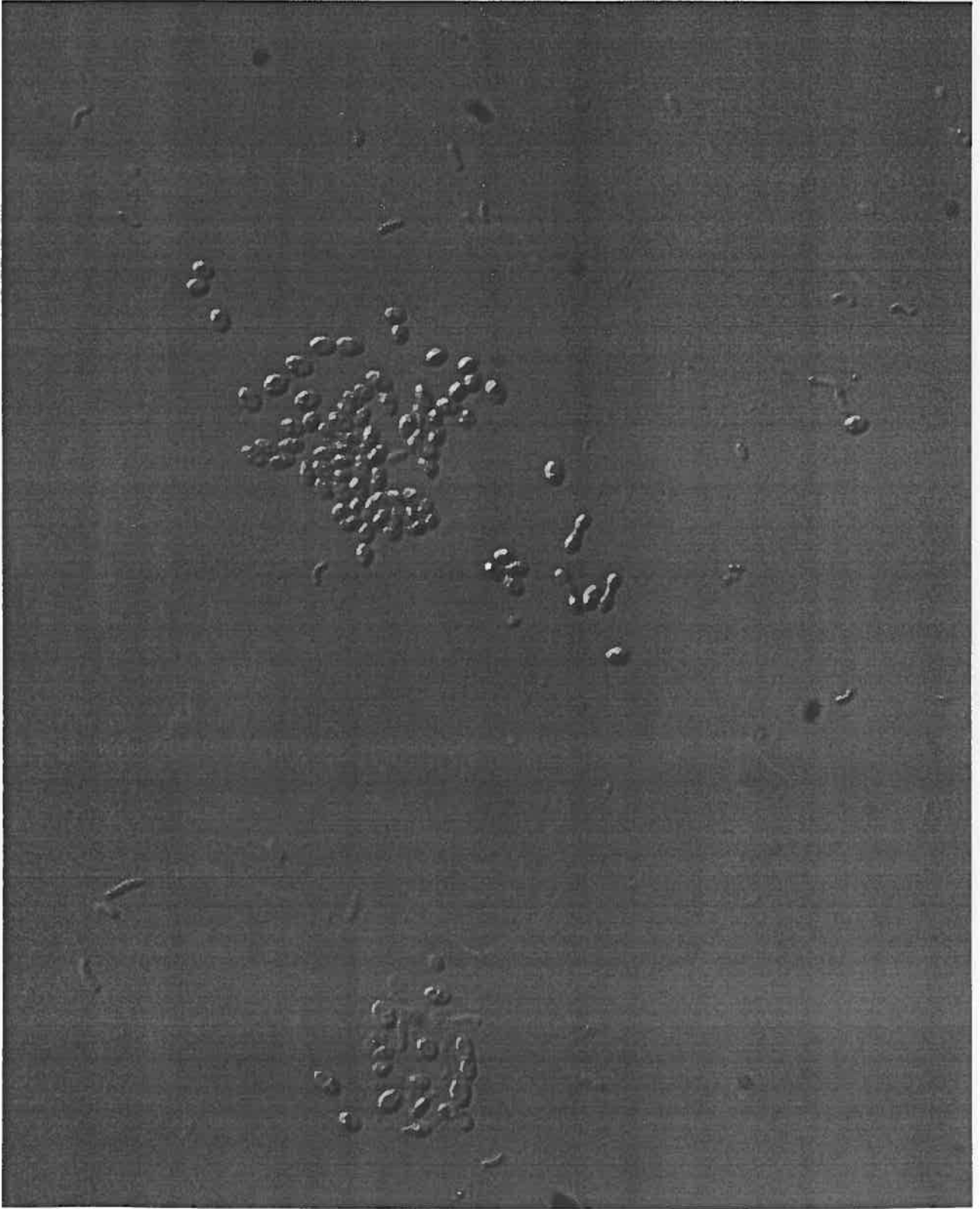
Hydroxylamine enrichments have similar possible reactions. Equation 5 depicts the anabolic light reaction allowing the fixation of CO_2 . Equation 6 shows the dark

reaction that may also occur when hydroxylamine is the electron donor. Without further chemical analysis it isn't possible to know which of these reactions is most prevalent.



The observation of growth in these enrichments should be considered a success despite the final results of the project. Aside from the use of buffer as an electron donor, these reduced nitrogen compounds should be oxidized. The growth in nitrite and hydroxylamine enrichments is interesting because the only source of reducing equivalents is the oxidation of nitrogen. In contrast, organisms in TMA enrichments may not be oxidizing the nitrogen, but rather the methyl groups. Further chemical analysis of all enrichments is necessary to determine the reactions that are occurring. I will continue this research at my home institution in attempt to gain a better understanding of these processes which have not been well-studied but should be occurring in nature.

100x DIC, wet mount of *Nitella* enrichment



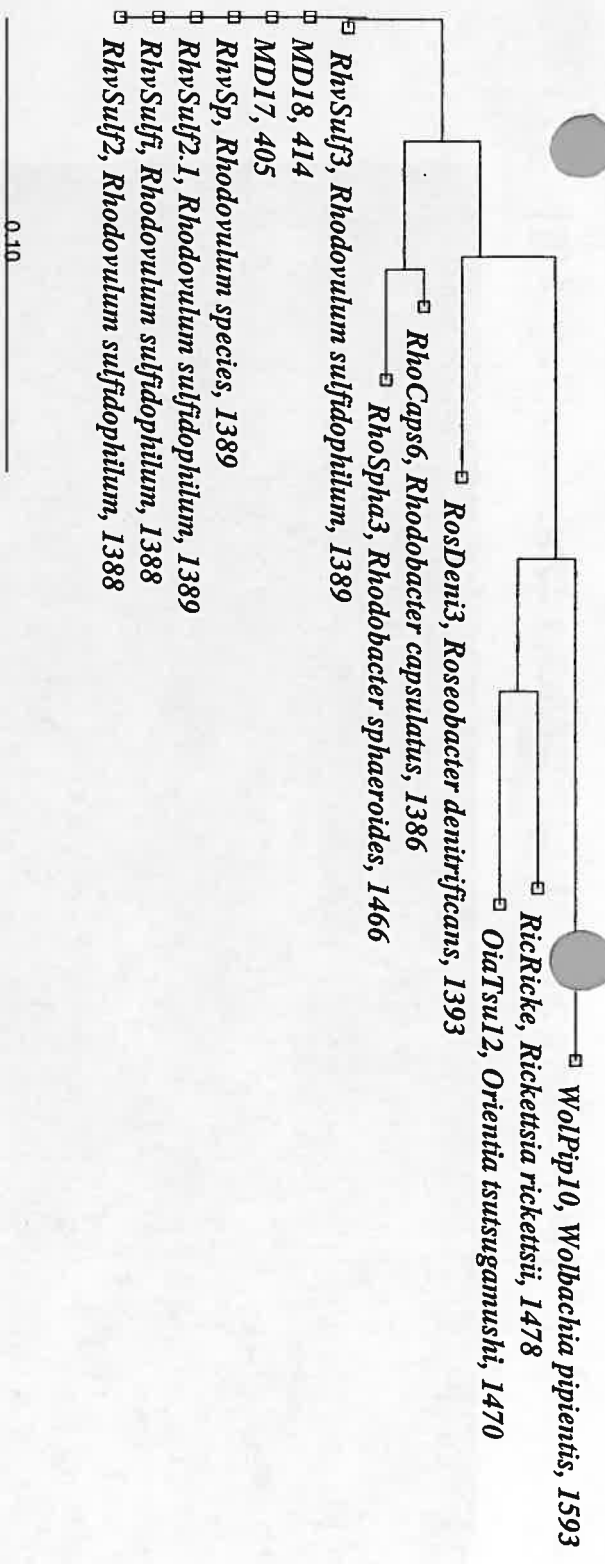


Figure 1: MD18, MD17 are TMA shake tube colonies