Marine Heterotrophic nitrifiers

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ABSTRACT:

In order of searching and making new discoveries we tried to find if there are heterotrophic nitrifiers out there. The hypothesis was that if there are such bugs than they would grow microaerophilicly under carbon substrate like acetate. The indicator for such behavior was phenyl red which is a pH indicator that turn from purple in the alkaline pH to red in neutral to yellow in acidifying conditions. Few samples were taken from Sippewissete marsh and from MRC's water treatment systems assuming that there has to be some nitrifiers there. From oxygen gradient tubes and using a phenol red (pH indicator) it's indicating that there are heterotrophic nitrifiers. Another indication was to measure the nitrite in this tubes which revealed in good qualitative results also indicating ammonia oxidizing to nitrite. We didn't get to isolate them on plates because we couldn't maintain microaerophilic conditions and therefore the only colonies formed were assumed to be of true heterotrophs.

NTRODUCTION:

In nitrification, bacteria oxidize ammonia to nitrite and nitrate. These end products are easily drained out to the ground water, assimilated by plants and bacteria, or converted to gaseous N-compounds by denitrification. Classically, nitrifying bacteria are chemoautotrophs [2]. Relatively few of these bacteria can be grown under laboratory conditions, and their growth rates are usually low. In soil, nitrifying bacteria may represent an essential part of the unculturable population. Scattered in the literature are reports that many soil nitrifiers are not restricted to an autotrophic mode of life but can catabolize organic soil compounds. Such heterotrophic nitrifiers have been characterized only poorly, although some are culturable [1]. A newly discovered process by which ammonium is converted to dinitrogen gas under anaerobic conditions (the Anammox process) has now been examined in detail. The removal of ammonium is an important problem in modern wastewater treatment systems. Most denitrifying bacteria carry outthese reactions only under anaerobic conditions. Very recently, however, ammonium losses under anaerobic conditions were discovered to occur in a laboratory-scale denitrification reactor. Increased removal of ammonium was paralleled by increased disappearance of nitrate. This suggested that the following reaction was taking place (3):

$$5NH_4 + 3NO_3^- \longrightarrow 4N_2 + 9H_2O + 2H^+$$
$$\Delta G^{0'} = -1{,}483.5 \text{ kJ per reaction}$$

The overall reaction for this anaerobic ammonium oxidation (Anammox) process is exergonic and thus could, in theory, supply energy for growth. It was therefore postulated that the removal of ammonium observed to occur in the denitrifying reactor was carried out by bacteria using ammonium as an electron donor for nitrate reduction (3).

MATERIALS & METHODS:

Dilution series were made for 4 different samples taken from: #1: Sippewisset salt marsh near the upland, #2: a small pool right next to it, #3: Recirculation system in the MRC, #4: Raw SW from MRC.

Enrichment media: 3ml of 10mM DTT (dietholethionite) in 1% bacto difco agar were autoclaved and than added into 15ml glass tubes as a reluctant plug. A 6ml of 0.3% agar contained 1mM ammonium sulfate, Phenol red 0.1% (pH indicator), 0.01% nutrient broth 0.1% acetate, 50μM PO_4, and 2ml of widdle trace elements were added. Inoculation was done by stabbing the agar with a Pasteur pipette.

The agar plates were made without the DTT plug and with 10mM HEPES in order to keep the acidifying bacteria in neutral pH. The plates than were placed in anaerobic BBL chamber with microaerophilic campy-pack (5-12% O_2) and glass tube with NaOH as CO_2 trap.

PCR was conducted on colonies from the tube that was most interesting in performance and than PCR product was send to be sequenced.

Photos were taking by Sony digital camera.

Microscopy analysis was conducted by Zeiss model Axioplan2.

RESULTS:

The main assumptionof that project was that if there are heterotrophic nitrifiers they will use the oxygen gradient, acidify the acetate (for carbon source) and than create change in pH which will be indicated by change in color of the media. (pH 5 = yellow, pH 7 = red, pH = 9 purple).

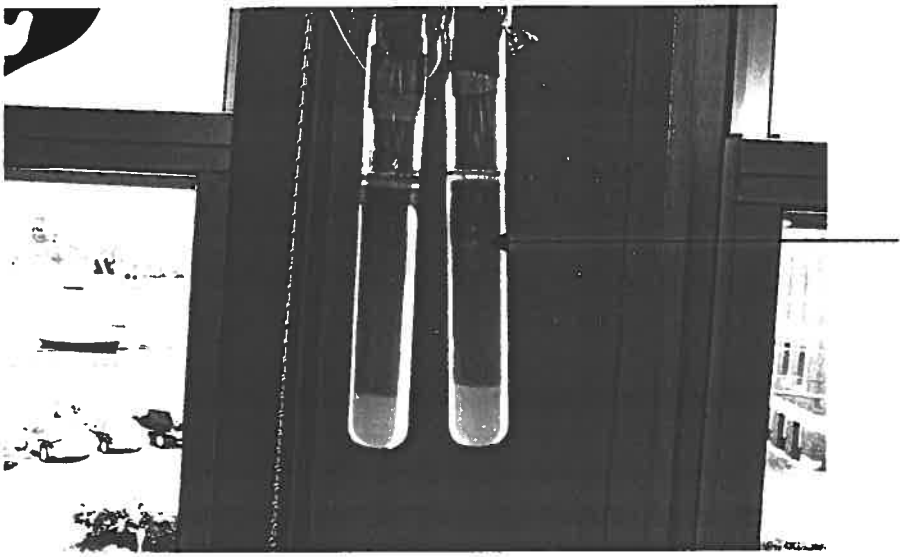
From picture #1 it's shown that the bacteria can grow in a microaerofilic environment and to use ammonia as sole electron donor. From nitrite analysis it revealed that there is nitrite production. This result indicate that ammonia is oxidize under such conditions (not shown).

Isolation on plate under microaerophilic conditions, observed by special (5-12% O_2) campi pack in anaerobic chamber, wasn't successful. Even though we got colonies they wouldn't make yellow hello around them so it seems that they are the regular heterotrophic bacteria using oxygen and acetate to breath.

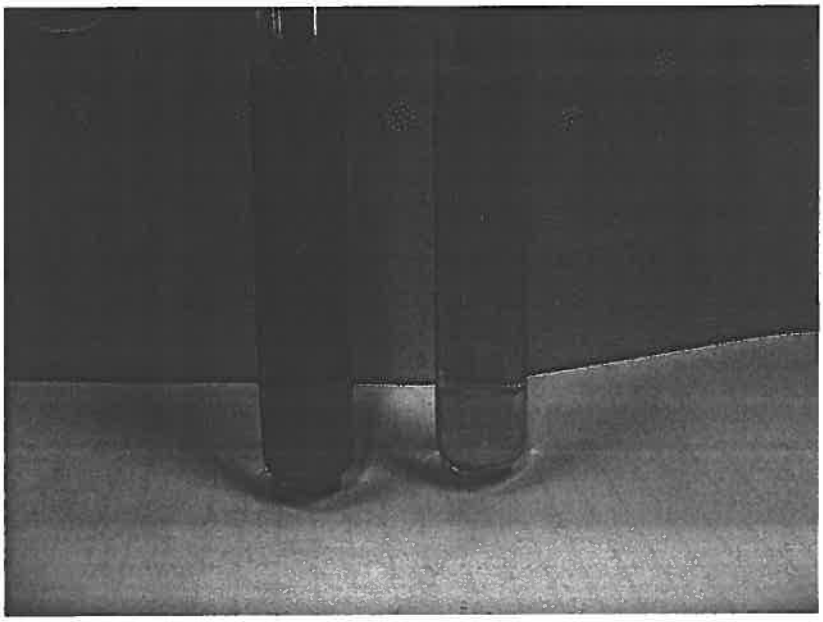
Microscopy analysis show that there are mainly rod shape bacteria and some large cocoid shape (slide).

16rRNA never came back from the sequence lab so we don't know yet what kind of bacteria we have.

Picture 1a: There is a sharp band on top indicating pure heterotrophs that use oxygen and probably acetate for growth. It's shown with arrows deeper colonies that assumed to be the heterotroph nitrifiers



Picture 1b: Acidified tube (right) as compared to a none (left).



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Ferrous iron (Fe^{II}) oxidation by marine chemolithoautotroph denitrifiers.

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ABSTRACT:
The ability to oxidize ferrous iron anaerobically with nitrate at approximately pH 7 appears to be a widespread capacity among mesophilic denitrifying bacteria. Since nitrate-dependent iron oxidation closes the iron cycle within the anoxic zone of sediments and aerobic iron oxidation enhances the reoxidation of ferrous to ferric iron in the oxic zone, both processes increase the importance of iron as a transient electron carrier in the turnover of organic matter in natural sediments.
A chemolithoautotroph conditions were set with Ferrous iron as sole electron donor, nitrate as sole electron acceptor and CO_2 as sole carbon source in sea water base media. In 3 of the 4 incubated samples (#1= black matt, #2= red matt, #3=red rusty mud, #4=red covered rock), there was growth which was indicated by nitrate consumption, change in color from gray to orange and from microscopy observation.

INTRODUCTION:

Iron is one of the most abundant elements in the Earth's crust and is the second most abundant metal. Due to their low water solubility, iron oxides accumulate in aquatic habitats in higher amounts only in sediments, as complex amorphous or crystalline structures (Cornell and Schwertmann 1996). Ferric iron is reduced by heterotrophic bacteria to ferrous iron (Lovley 1991). Ferrous iron can be reoxidized in the presence of oxygen at low pH by acidophilic bacteria such as Thiobacillus ferrooxidans (Blake et al. 1993) or at a pH of approximately 7 by bacteria such as *Gallionella ferruginea* (Hallbeck et al. 1993), both of which obtain growth energy from this redox reaction. The standard redox potential (E0) of the Fe 3+ /Fe 2+ couple is +770 mV, but the actual redox potential in natural habitats depends strongly on the prevailing pH (Widdel et al. 1993). At pH 7 in the presence of bicarbonate, the redox transition of FeOOH/FeCO3 dominates with an E value of approximately +200 mV. With this low redox potential, ferrous iron could also serve as an electron donor for redox processes in anoxic habitats. Electrons released at +200 mV could also be used for microbial dissimilatory nitrate reduction. Such a metabolism has been reported recently for mesophilic bacteria (Straub et al. 1996) This process would connect the iron cycle with the nitrogen cycle, and Introduction thus, would further increase the importance of iron as an electron acceptor in anoxic microbial habitats. The present study trying to find whether there is a marine chemolithoautotroph that can reduced nitrate with ferrous iron as a sole electron donor. There are some bacterial strains reported to do so bur all of htem used co-substrate as acetate as precursor for the process of denitrification to begin while we tried to isolate bacteria that capable of using CO_2 only as a carbon source.

MATERIALS & METHODS:

Enrichment culture: SW based media contained (per Liter): 20g NACl, 15g $MgCl_2$, 0.15g $CaCl_2$, 0.2g KH_2PO_4, 0.5g KCL autoclaved and cooled under N_2 and than 2ml Widdle base trace elements, 0.5ml vitamins, 0.5ml B_{12}, 30mM $CaCO_3$, 32mM $FeSO_4$ and 2mM NO_3 were added. Nitrate served as the only electron acceptor and $FeSO_4$ as the only electron donor. CO_2 was the sole carbon source.

After addition of that media into 100ml serum bottles inoculation was conducted from matt samples from salt marsh. Cells were incubated in 30^0c. Microscopy observation, nitrate determination, amplifying bacteria DNA from the enrichment culture and sequencing conducted.

Nitrate was measured spectrophotometricly at 210nm.

Changes in colors from gray to orange, indicating that iron is oxidized, observed visibly.

RESULTS & DISCUSSION:

Changes in the medium color from gray to orange indicated ferrous iron oxidation (fig 1). Nitrate determination: measuring nitrate in #1, 2,4 and #1 transfer samples indicated that there is a net reduction of nitrate (fig 2). Microscopy analysis revealed that there are mainly rods and vibrio like bacteria (slide).

From all of this results it appears that there is a chemolythoautotroph out there that can use ferrous iron as sole electron donor and use CO_2 as sole carbon source for growth. Further investigation needed to be conducted on isolation of these bacteria the and on the physiological properties they posses.

FIGURES:

Fig 1: Changes of color from gray to orange indicating Fe^{II} oxidation.

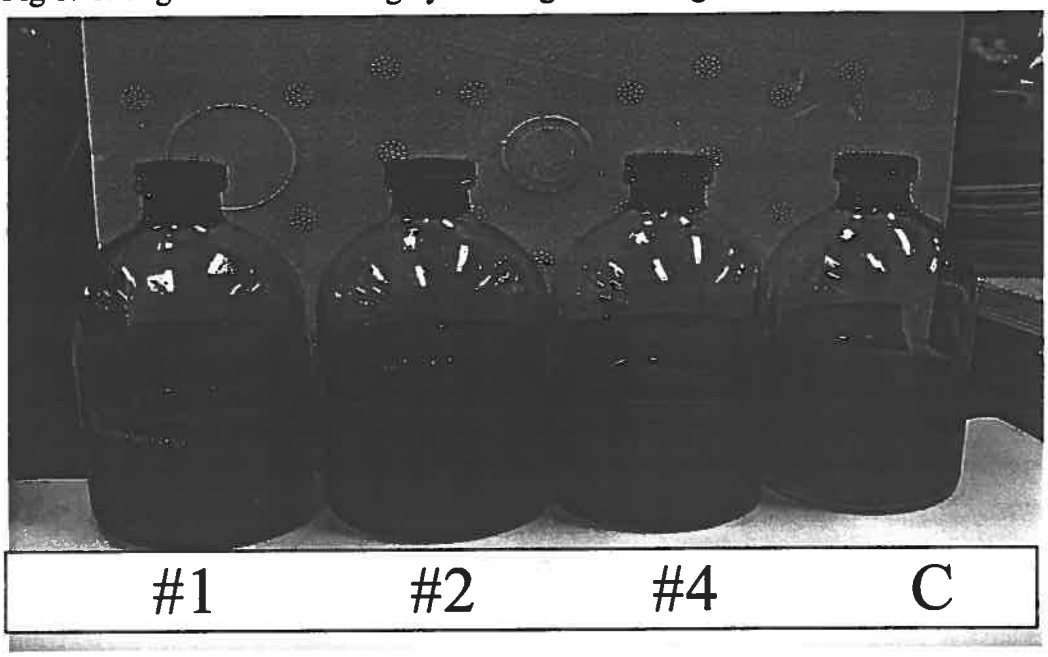
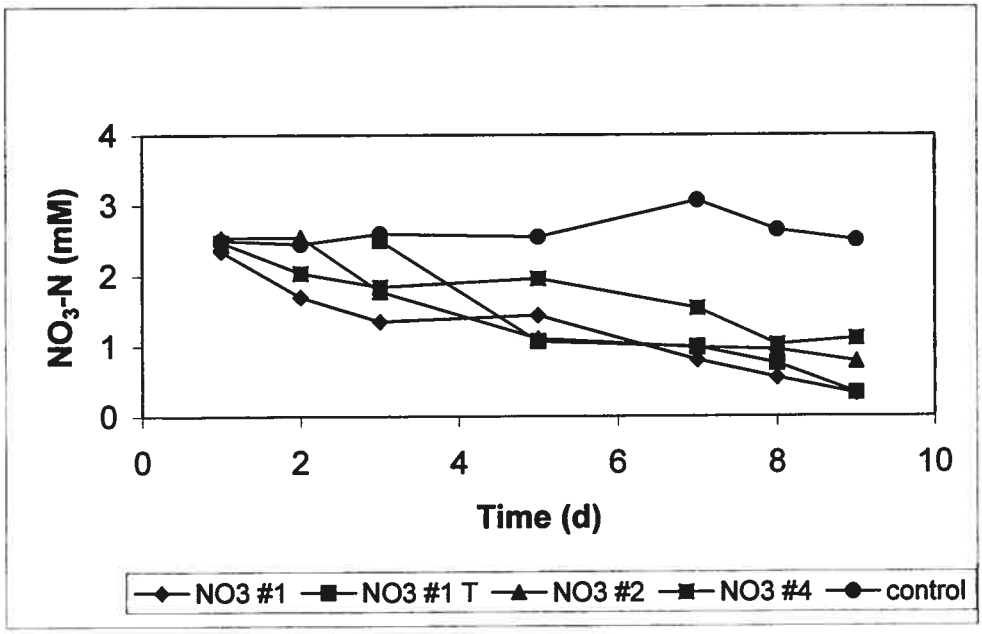


Fig 2: Changes in nitrate during incubation period of 9 days.



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SRB's biofilm formation under denitrifying conditions with different carbon source concentration.

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Abstract

The distribution of sulfate-reducing bacteria (SRB) in anoxic biofilms grown in a biofilm chamber was investigated by con focal microscopy. Two different conditions were investigated, 0.5 and 10mM lactate as sole electron donor. NO_3- was measured with colorimetric method in order to make sure that the denitrifying bacteria have enough electron acceptor. SO_4 reduction was measured in order to show that there is a community of SRB's in the biofilm. It was found that there is a better reduction of nitrate under the condition of 10mM lactate which is folowing the Michaelis-Menten kinetic. No H_2S founded in both outlet of the chambers, but a white material apeared in the chambers as well as in the pipes wall asuming to be S^0. My interpatation is that SO_4 was reduced in the biofilm when it became totally anaerobic but as soon as the H_2S produced it been oxidized by denitrifiers as a source of electron donor. The confocal microscopy revealed that there is a larger biofilm producing in the 10mM chamber than in the 0.5mM.

INTRODUCTION:

Although sulfate reduction is thought to be an anaerobic process, sulfate-reducing bacteria (SRB) are also important in aerobic environments if they can proliferate in anaerobic zones. For example, in marine sediments (4,5) and in aerobic wastewater treatment systems (6), sulfate reduction accounts for up to 50% of the mineralization of organic matter. Furthermore, sulfate reduction strongly stimulates microbially enhanced corrosion of metals (2,3). Therefore, the detection of sulfate reducers and sulfate-reducing activity in sediments, wastewater treatment plants, and fouling biofilms is of great practical and scientific relevance. Conventional microbial techniques based on selective culturing are of limited usefulness for quantification and characterization of environmental populations, as it is now well recognized that most strains do not grow in vitro, either because cultivation media poorly resemble natural growth conditions or because different strains of microorganisms are interdependent. Techniques based on the analysis of bacterial DNA and RNA may complement the conventional microbiological approach and nowadays are routinely used to determine the presence and distribution of individual bacterial species, including SRB, in complex communities such as those in bacterial biofilms (1).

So far, these studies of microbial communities, i.e., bacterial biofilms, have mainly focused on the exploration of bacterial diversity and on the detection of individual bacterial taxa by molecular techniques. Studies relating community structure to community function are scarce, partially because of difficulties in monitoring microbial activities. Within biofilms, the convection of compounds is hindered, and consequently mass transfer to the cells often limits conversion rates. Because of this resistance to mass transfer, biofilms develop various microenvironments, which differ from the bulk liquid (6). This complicates the interpretation of community function analysis, because extrapolation of community behavior to that of individual cells is impossible without knowledge of their microenvironment.

In this research we are trying to find whether there is SRB's creating a biofilm under denitrifying conditions in two different electron donor (lactate) concentrations (0.5 and 10mM).

MATERIALS & METHODS:

Biofilms were cultivated in 2-channel flow cells with individual channel dimensions of 1 by 4 by 40 mm supplied with a flow of . The substratum consisted of a microscope glass cover slip attached and sealed with aquarium silicon. The secret is to lay a lair of silicon with a syringe and 200µl tip around the chamber, than put the cover slip on and cover the edges with the silicon. Flow cells were inoculated with an 45min of black sw matt. Medium flow was then started, and the substrate was pumped through the flow cells at a constant rate of 1ml/20min using a Watson Marlow 205S peristaltic pump. Anaerobic media (SW based) contained (per Liter): 20g NACl, 15g $MgCl_2$, 0.15g $CaCl_2$, 0.2g KH_2PO_4, 0.5g KCL autoclaved and cooled under N_2 and than 2ml Widdle base trace elements, 0.5ml vitamins, 0.5ml B_{12}, 30mM $CaCO_3$, 28mM $Na(SO_4)_2$, 2mM NO_3 and either 0.5 or 10mM of sodium lactate were added. Nitrate served as the only electron acceptor and sodium lactate as the only electron donor. The chamber was incubated for 6 days and than was harvested washed with acridine orange 0.01% and visualized under the con focal microscopy.

PCR product were made with SRB's specific primers resulted in no product which might mean that there weren't SRB's but further analysis is needed in order to verify this point. NO_3 determination was according to (Cawse, P.A. 1967. Analyst: **92**:311-317).

RESULTS:

It's shown clearly from the con-focal pictures that the bio-film formation in the 10mM lactate is thicker than the one in the 0.5mM lactate (fig 1A,B respectively).
From nitrate analysis it shown that under conditions of 10mM lactate the amount of nitrate reduced is significantly higher than that under the 0.5mM lactate (fig 2).
This results are shown just to show that there is a biofilm formation under these conditions and I wanted to use the con-focal microscopy. It assumed that in the 10mM treatment the reduction of nitrate to 0 will lead to SRB's formation in the down layers which will produce H_2S. No H_2S was found in the outlet of both chambers according to Schnell test. There are 2 possibilities for that:

1. Chemical oxidation of H_2S in the outlet of the biofilm chamber.
2. denitrifiers in the upper layers consuming the H_2S and producing S^0.
   This was confirmed by mash white layer producing on the pipe walls as well as in the chambers.

Fig 1A: Con-focal microscopy image of 10mM lactate SRB's denitrifers biofilm

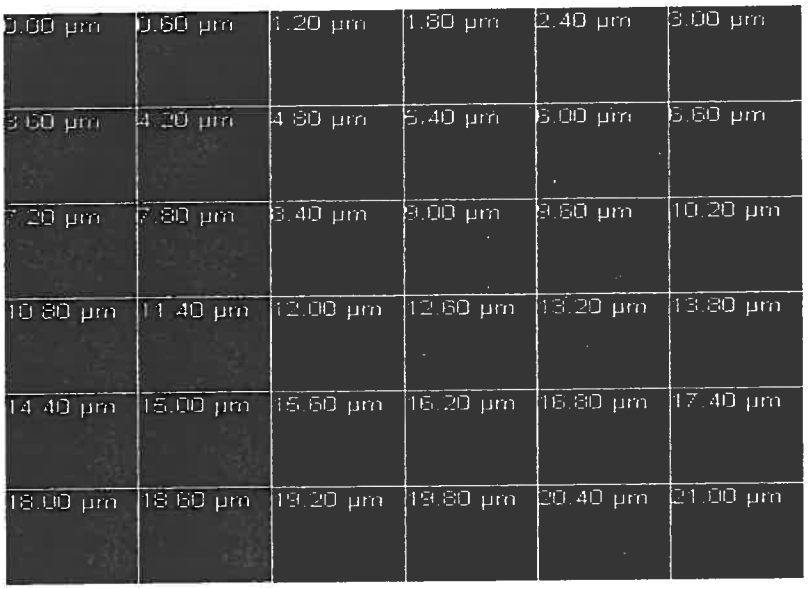


Fig 1B: Con-focal microscopy image of 0.5mM lactate SRB's denitrifers biofilm

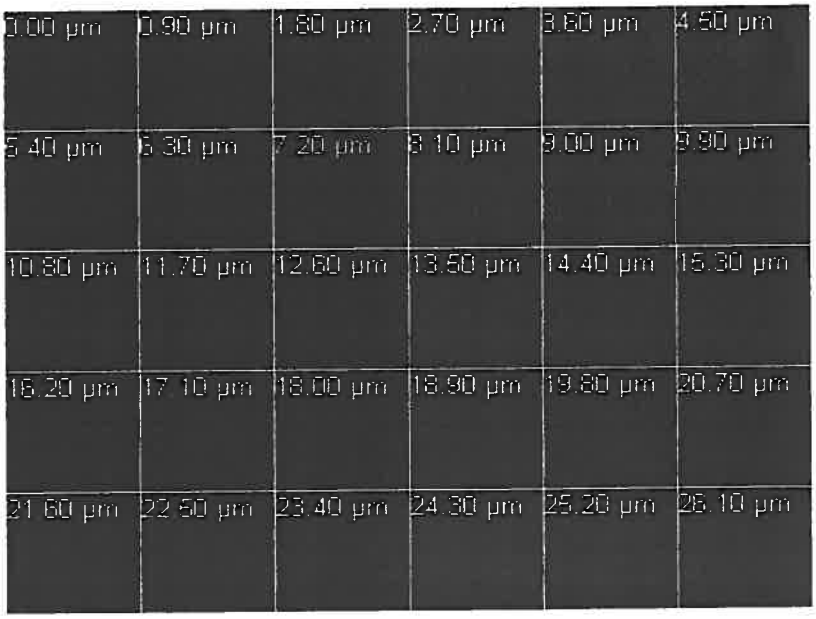
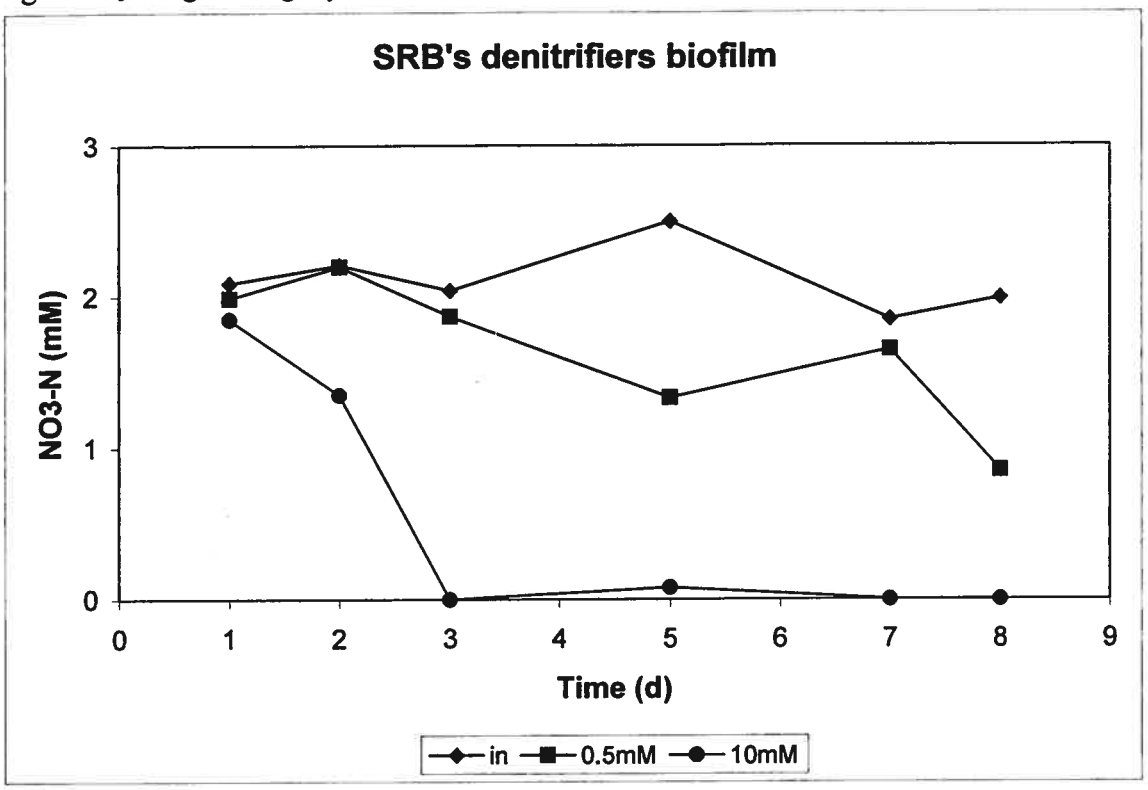


Fig 2: NO_3 changes during days of bio-film incubation.



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