Occurrence of nitrification and nitrifying bacteria in encrusting sponges at the coral reefs of Curaçao

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Doctoral report
March 2004 - October 2004
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1 Abstract

From some open water sponges it is known that they possess nitrifying bacteria. Since in cavities an efflux of nitrate is found, these cryptic habitats can be very important as inorganic nitrogen source on coral reefs. In the present study encrusting cavity sponges as well as cavity sediment were used in enclosure experiments at the coral reefs of Curaçao to investigate possible nitrate effluxes. A nitrification inhibitor was added in some experiments in order to check if nitrate production was due to nitrification processes. CARD-FISH has been used to determine the presence of nitrifying bacteria inside sponges and in the water.

The sponge *Halisarca caerulea* showed clear nitrate effluxes (0.71 mmol m^{-2} d^{-1}). For two other sponge-species (*Mycale microsigmatosa* and *Merlia normani*) no clear nitrate effluxes were found. Nitrite and nitrate fluxes were lower when a nitrification inhibitor was added, indicating nitrate releases from sponges were due to nitrification processes. In these and 5 other sponge species (*Monanchora arbuscula, Clathria spp., Cliona spp., Hymeniacidon haeliophila* and *Diplastrella megastellata*) Nitrospira and β- and γ-Proteobacteria were detected, suggesting the presence of associated nitrifying bacteria in cavity sponges. Cavity sediment also showed nitrate effluxes (0.65 mmol m^{-2} d^{-1}).

All together it is demonstrated that sponges (by associated nitrifying bacteria) and cavity sediment release nitrate and explain therefore nitrate effluxes of cavities. This highlights the importance of cavities as an inorganic nitrogen source on coral reefs.

2 Introduction

Human activities have been affecting coral reefs and tropical ecosystems last decades. Increased run-off of terrestrial sediments and accompanying nutrients are considered one of the greatest threats to coral reefs. Coral reefs occur often in clear oligotrophic water (Bak, 2002b). It is thought that the appended nutrients can lead to eutrophication, in which algae flourish and the coral cover decrease. However too little is known about the nutrient cycle in coral reefs ecosystems to estimated the size of this threat. Cryptic habitats and microbial organisms tend to play a key factor in nutrient conversion, but did not get much attention yet (Bak, 2002a).

Corals and the reef framework harbour large cryptic habitats conducting of many holes and cavities. Cavities are according to Scheffers *et al.* (2003) defined as spaces and surfaces under rubble, the undersides of skeletal organisms, the shaded undersides of overhanging dead or live corals, and deep framework cavities. It is thought that these cavities play an important role in the nutrient dynamics of the reef water (Diaz and Rützler, 2001). In previous studies nitrate effluxes were found from cavities, suggesting a nitrate production by nitrification biota living in cavities (Scheffers *et al.*, 2004). From a few coral and sponge species on the outer reef is known that they excrete nitrate (Corredor *et al.*, 1988; Capone *et al.*, 1992; Diaz and Ward, 1997). Because cryptic habitats have a larger surface area than the front side of the reef (about 1.5 till 8 times as large), cavities appear to be more important for the nitrogen cycle than the frontal reef (Scheffers *et al.*, 2004). It is thought that the nitrate production of the coral and sponge species on the outer reef is due to nitrifying bacteria which these species possess (Capone *et al.*, 1992; Diaz and Ward, 1997). The hard substratum of cryptic habitats (about ⅔ of the total cavity surface area) is covered for 65% with sessile filter feeders (mainly sponges). The nitrate efflux of cavities can be explained by possible nitrifying bacteria associated with sponges. However other organisms and sediment can also contain bacteria. And there could be other processes causing a nitrate efflux, like a leakage of ground water. It is though more probable that it is caused by filter feeders (like sponges) in cavities, as also the bacteria concentration in cavities dropped quickly after cavities were closed (Scheffers *et al*, 2004).

Sponges (Porifera) are ancient filter feeders dating back more than 580 million years and are found all over the world from the deep sea to warm tropical seas and even in fresh waters. So far an estimated 15.000 species have been described, but the real diversity is probably much higher (Hooper and van Soest, 2002). As sessile filter feeders, sponges pump large volumes of water through a specialised canal system, termed the aquiferous system (see figure 1). This system is composed of many small inlet gaps (Ostia) and one or more larger outlet gaps (Oscula). The water is translocated by either pressure or specialised cells with flagella (choanocytes), causing a current inside the sponge. The filtration capacities of sponges are remarkably efficient (up to 24.000 litres kg^{-1} day^{-1}), leaving the expelled water essentially sterile. Sponges are therefore the most efficient filter feeders in the sea (Reiswig, 1971, 1974; Turon *et al.*, 1997). Food particles such as unicellular algae and bacteria are removed from the seawater by choanocyte cells in the choanocyte chambers. Accordingly they are transported into the mesohyl, the interior tissue of sponges. Other specialised cells, the archaeocytes, move freely through the mesohyl and digest the food particles by phagocytosis (Bergquist, 1978; Müller, 2003).

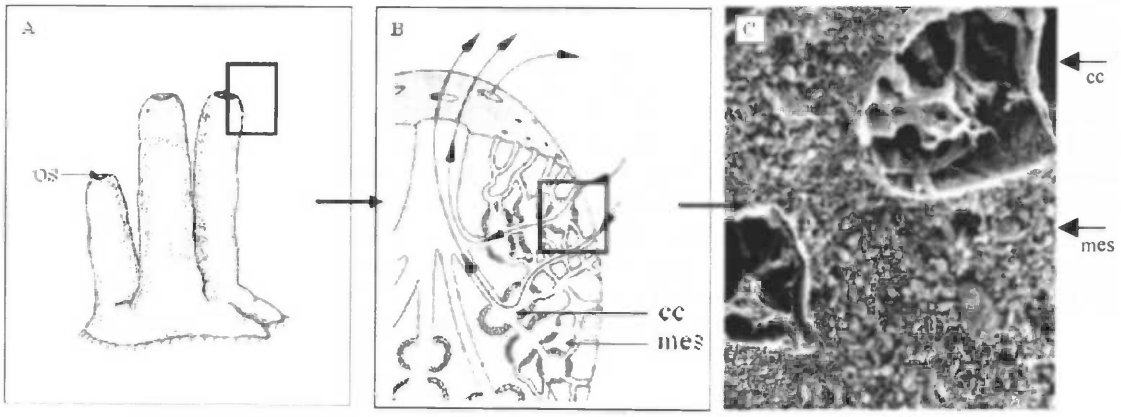

Figure 1. Schematic representation of the aquiferous sytem of a sponge. A) a tube sponge with three Oscula (Os), B) the canal system, with the choanocyte chambers (cc) and the mesohyl (mes), C) a scanning electron micrograph showing choanocyte chambers and mesohyl in more detail. (Müller, 2003)

Within the sponge mesohyl large amounts of micro organisms are present and bacteria can contribute up to 40% of the sponge biomass (equal to about 10^8 to 10^9 bacteria g of tissue^{-1}) (Müller, 2003). The bacteria are probably permanently associated with the host sponge unless they are disturbed by external stress factors. It is thought that some play a symbiotic function (Friedrich *et al.*, 2001; Thoms *et al.*, 2003). This is a strange fact since sponges also feed upon bacteria, yet it is unclear how this is regulated within the sponge. Nitrifying bacteria can have a function of removing toxic ammonium produced as a metabolic waste product by the sponge. Since ammonium is present it seems logic that nitrifying bacteria are living within sponges.
Sponges can have many features, from large round balls and tube forms to thin crusts. Even within one sponge species different features can appear depending of the circumstances. Cavity dwelling sponges are all of the encrusting type, some of which exclusively exist inside cavities while others are present on the whole reef. Since circumstances (light regime and chemical composition of the water) in cavities are different compared to the front reef, the kind of associating bacteria in sponges can also differ.

About a third of the cavity surface area is composed of sediment and this is also a possible nitrate producer (Scheffers *et al*, 2004). From coastal sediments in productive waters is known that these are important sites for cycling of nitrogen as degraded organic matter is deposited at the sediment surface. Accordingly ammonia, can be released from the sediment or nitrifying bacteria in the sediment can oxidize ammonia to nitrate. This results in net fluxes of dissolved inorganic nitrogen (DIN) to the overlaying waters (Capone *et al.*, 1992; Lohse *et al.*, 1993). However fluxes so far found indicate that sponges are larger producers of nitrate compared to the sediment and may be more important for the nitrogen cycle.

The nitrogen cycle controls the availability of nitrogenous nutrients and biological productivity in marine ecosystems and is therefore important for preserving inorganic N on the reef. Microbes play a crucial part in the nitrogen cycle by the catalyzation and conversion of nitrogenous compounds (Zehr and Ward, 2002). The nitrogen cycle is composed of oxidation-reduction reactions, many of which are used in the energy metabolism of microbes (Zehr and Ward, 2002). At the coral reef an important part of the nitrogen cycle is present, namely the nitrification. Nitrification is a chemoautotrophic pathway in which ammonia (NH_3) is conversed to nitrate (NO_3^{2-}) in a two-step process: the oxidation of ammonia into nitrite (NO_2^-) and accordingly the oxidation of nitrite into nitrate. Ammonia is present in water in equilibrium with ammonium (NH_4^+) and depending on the acidity of the water the equilibrium shifts towards more ammonia (pH>9) or ammonium (pH<9) (Ward and Carlucci, 1985; Lohse *et al.*, 1993; Bothe *et al.*, 2000; Madigan *et al.*, 2000; Zehr and Ward, 2002).

Ammonia oxidation:

$$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_2O$$
$$NH_2OH + H_2O + O_2 \rightarrow NO_2^- + 2H_2O + H^+$$

Sum: $NH_3 + 2O_2 + 2e^- + H^+ \rightarrow NO_2^- + 2H_2O$

Nitrite oxidation:

$$NO_2^- + \tfrac{1}{2}O_2 \rightarrow NO_3^{2-}$$

For each oxidation different bacteria are necessary, the ammonia oxidizing bacteria (AOB, e.g. *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*) for the first step and the nitrite oxidizing bacteria (NOB, e.g. *Nitrobacter*, *Nitrococcus*, *Nitrospira*) for the second step. So far, no autotrophic bacteria are known to oxidize ammonium directly to nitrate (Madigan *et al.*, 2000). The AOB and NOB are practically always present together. Since the oxidation of nitrite to nitrate is very quickly nitrite is hardly detectable in the reef water. Nitrifying bacteria obtain energy for growth from nitrification. They require reduced forms of inorganic nitrogen (ammonium, nitrite or hydroxyl amine), low light levels and oxygen. As a consequence, these bacteria are most active at the bottom of the photic zone of the ocean (50-100m), in cavities and in a narrow zone of surface sediment where oxygen is present (Ward and Carlucci, 1985; Vanzella *et al.*, 1990). Nitrification is an important step in the nitrogen cycle. Ammonia is poisonous for many organisms and nitrate is the most accessible source of nitrogen for plants, since nitrate can be absorbed directly (Ward and Carlucci, 1985).

The aim of the study was to investigate the origination of the nitrate efflux observed in cavities. Several enclosure experiments with common cavity dwelling sponges from the Caribbean coral reefs and cavity sediment were conducted to give an answer to this phenomenon. Subsequently the molecular technique CARD-FISH was used to visualise the presence of nitrifying bacteria inside sponges. Summarising, the objectives of this particular study are:

1. To assess nitrate release rates from different sponge species.
2. To assess nitrate release from cavity sediment.
3. To determine the presence of nitrifying bacteria in sponges.

The enclosure experiments were conducted in incubation chambers with pieces of encrusting sponge on calcareous substrate. Net dissolved inorganic nitrogen release and the removal of bacteria were investigated in time series. It is assumed that mineralization of consumed bacteria regenerates ammonia, which is converted to nitrite and subsequently nitrate by nitrifying bacteria associated with sponges. However to check if this is the only process of nitrate release from sponges respectively sediment, a nitrification inhibitor was added to several sponge enclosure experiments. The nitrification inhibitor, nitrapyrin, will prevent the ammonia oxidizing bacteria to do their function and therefore nitrite is not being produced (Lohse *et al.*, 1993). Subsequently the nitrite oxidizing bacteria lack nitrite and can not produce nitrate. If nitrifying bacteria are the only producers nitrate should not be found in these experiments, while ammonia is thought to be more present, since it could not be converted.

3 Materials & Methods

3.1 Sponge collection

Sponges were collected by SCUBA diving between March and May 2004 from the reef at Buoy 1, close to the institute of Caribbean Research and Management of Biodiversity CARMABI, on the island of Curaçao, Dutch Antilles (figure 2). Individual specimen of cavity sponges were chopped out of cavities between 10 and 20 meter depth. Attached substrate not covered by the sponge was scraped clean of epibionts. Sponge samples were transferred into and attached upside down a wire cage to protect them from predation and stored for recovery in a cavity at 14 meters depth for at least 7 days.

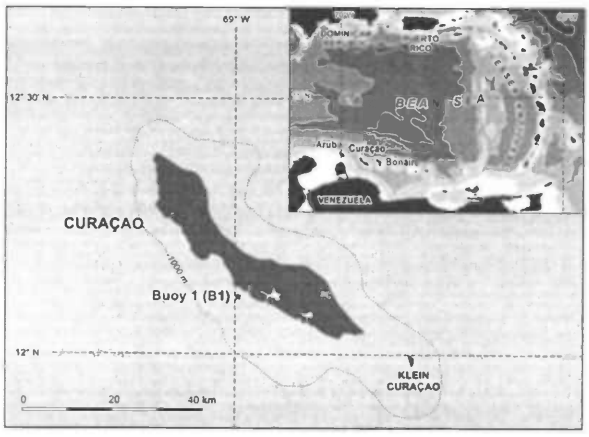

Figure 2. Location of Buoy 1 along Curaçao (Dutch Antilles), where experiments were conducted at 14 metres depth. The inset shows the position of Curaçao in the Caribbean Sea, north of Venezuela (Picture after Scheffers *et al.*, 2004).

3.2 Experimental design

3.2.1 Enclosure experiment with sponges

This experiment is executed to assess if cavity sponges excrete nitrate. Concurrent the removal of bacteria by these sponges was measured. A photograph of the sampling setup is given in figure 3 showing a standard with lead, a plastic bottle filled with air, the under lid of the container with a sponge placed on top and the upper part of the plastic 1.05 litres container. The underside of the container and the bottle were connected with a tube, which could be closed with a clamp. Another tube was attached to the container bottom at which water samples could be taken by a syringe. A rotator was present inside to stir the water. The containers were covered with alufoil to receive about the same light regime as in cavities. A small border was left to see if the sponges where still filtering. Lids of the container were sealed with a silicon ring.

The intention of this setup is to equal the pressure which arises when a water sample is taken out of the container. Pressure can influence the sponge and water samples under pressure are more difficult to take. Air from the bottle will ascend, but is inhibited by the clamp. When a water sample was taken, the clamp was loosened. Accordingly the air went into the container and replaced the water taken out. A hole at the under side of the plastic bottle caused a replacement of ambient seawater for the ascended air.

The experiments were done in the morning at 12 meters depth just above the cave containing the sponges for recovery. Gloves were put on to prevent exchange of bacteria between hands and sponge respectively water samples.

Prior to the experiment, pumping activity of the sponges was monitored visually and only active, healthy looking sponges were selected for the experiments. It is assumed that sponges are actively filtering water when their oscula are wide open. Sponges were transferred on the bottom of the container. For determination of bacteria groups in the expelled water of the sponge (by CARD-FISH) a water sample of 20 ml was taken directly above an oscula with a 100 ml syringe (see figure 4). The syringe was sealed with a silicon cap and stored in a dark bag to stop primary production. Subsequently the container was closed and directly the first water sample of 78 ml for bacterial counts and nutrient concentrations was taken (T=0).

Figure 3. Experimental set up showing a bottle filled with air connected to the bottom of the container at which the sponge *Halisarca caerulea* lies. With a syringe also connected to the container water samples were taken out when the chamber was closed.

Other water samples were taken after respectively 2 minutes (78ml), 4 (78ml), 10 (78ml) and 30 (100ml) minutes from starting point (figure 5). All water samples were stored in the dark bag.
Just before sampling 5ml was drawn trough the tube to refresh the water. In total 437ml water was taken out the container. The sponge remained submerged and did not come into contact with air.
After the experiment sponges were put in plastic bags containing surrounding seawater. Together with the syringes the sponges were transported to the laboratory of CARMABI in a cold container. Sample preservation occurred within 5 hours.
In total 12 experiments with the sponge *Halisarca caerulea*, 7 experiments with *Mycale microsigmatosa* and 3 with *Merlia normani* were executed. There were 3 control experiments with only bare substrate.

Figure 4. Before closing the chamber a water sample of the water directly above an oscula of the sponge (in this case *Halisarca caerulea*) is taken for CARD-FISH determination.

Figure 5. In a time series five water samples were taken out of the container by a syringe. Pressure was equalized by air elevating the second tube connected to a bottle (bottle not shown here).

3.2.2 Enclosure experiment with nitrification inhibitor

It is assumed that ammonia is converted to nitrite and nitrate within the sponge by nitrifying bacteria. However to determine if this is the only source of nitrate increase a nitrification inhibitor was added to the seawater inside the container of a comparable experiment as described above. The nitrification inhibitor nitrapyrin stops the working of ammonia oxidizing bacteria and accordingly the conversion of ammonia to nitrate (Henriksen, 1980). Prior to the experiment a nitrapyrin solution was made with 330mg nitrapyrin ($C_6H_3NCl_4$ 2-chloro-6-(trichloromethylpyridine)) solved in 80% ethanol with use of a sonicator (sonic dismembrator, model 300 with ¾" titanium tip). Subsequently the solution was filled to 100ml with bacteria free seawater. At the beginning of the experiment, just after the syringe for CARD-FISH determination was filled and after the container was closed, 5ml of the nitrapyrin solution (5-20mg/L) was injected with a needle through a rubber plug (figure 4). Accordingly water samples of 40ml respectively 60ml for the last time step were taken with 100ml syringes after 0, 4, 10 and 30 minutes. After the experiment the sponges were transferred into bags containing seawater and together with the syringes transferred to the lab and stored in a dark cold box until further investigation within 4 hours.

In total 4 experiments with the sponge *Monanchora arbuscula*, 3 experiments with *Halisarca caerulea*, 1 experiment with a *Clathria sp.* and 1 experiment with a *Cliona sp.* were carried out. Prior to the nitrapyrin experiment with the *Clathria sp.* also an experiment without nitrapyrin of the same piece of sponge was conducted as a control.

3.2.3 Enclosure experiment with sediment

This experiment is to determine if there are other systems apart from sponges present in cavities causing a nitrate release. Therefore the sediment of cavities was tested by placing a 1.05 litre container without the rubber lid upside down in the sediment, denoting that the rotator was at the top as well as the tube for water samples. Pressure was equalled by pushing the container gently into the sediment. Water samples were taken at 0, 10, 30 and 45 minutes of respectively 40, 40, 40 and 60ml. Before placing the container into the sediment a water sample of 10ml was taken for bacteria determination by CARD-FISH. The syringes were transported to the lab and stored in a dark, cold container until further investigation. Two cavities (estimated volume of $0.07m^3$) were used for 3 experiments each.

3.3 Sample preservation

For a detailed description of procedures and solutions see Appendix.

3.3.1 Water samples

Of every enclosure experiment (sponges, nitrapyrin and sediment) water samples were split in three parts in the lab. They received a treatment according to the purpose, respectively bacterial counts, bacterial composition with CARD-FISH and nutrient composition.

Sponges filter the reef water to take up bacteria, their food. To investigate whether the sponges filtered during the experiment bacteria of every water sample should be counted. Therefore bacteria in 10ml of all time step samples have been fixed with 0.57ml of 35% formaldehyde and coloured with 1ml Acridine-Orange. After 5 minutes the sample was filtered over a 0.22µm, 25mm Sudan black filter (Millipore), supported with a 0.45µm, 0.25mm cellulose nitrate HA filter (Millipore). Subsequently the black filter was placed on a microscope slide, with a drop of emersion oil and stored at -20°C until counting.

CARD-FISH is a molecular technique to establish which kind of bacterial groups are present in the medium. Therefore 10ml water taken above the sponge oscula and 20ml water of the last time step sample were fixed with 0.57ml respectively 1.14ml 35% formaldehyde (end concentration of 2-4%) for 1-24 hours. After fixation the water was filtered over a 0,2 µm, 0.25mm white polycarbonate GTTP filter (Millipore), supported by a 0.45µm, 0.25mm HA filter. The dry GTTP filter was stored in a pony vial at -20° C until use.

A part of the remaining water sample was used to rinse glasswork and filters. Finally 20 ml was filtered over a 0.2µm, 0.25mm GTTP filter supported by a 0.45µm, 0.25mm HA filter. Two pony vials were rinsed two times each with filtered sample water and 2 x 5ml filtered water was stored in these pony vials at -20 °C until measuring for nutrient composition.

3.3.2 Sponge samples

Digital photographs were taken of all specimen used in the experiments with a Canon Ixus 500. Accordingly two pieces of $0.5cm^2$ each were cut of every sponge with a sharp scalpel and fixed for taxonomic research and CARD-FISH. Sponge tissues for determination were fixed in 5ml 80% ethanol.

For CARD-FISH sponge tissue was fixed in 3ml cold PFA (Paraformaldehyde fixation, see appendix) for 30 minutes up to 12 hours at 4°C. Subsequently tissues were washed twice with 1xPBS (Phosphate Buffered Saline) and stored in 5ml 1xPBS / 80% ethanol (1:1) at -20°C.
The remaining sponge tissue was scraped of the substrate and dry weight (DW, for 24h at 90°C) and ash free dry weight (AFDW, for 5h at 450°C) were determined. Volume of substrate was determined in a measuring cup with water.

3.4 Surface area of sponges

The total surface area of a sponge was measured by image analysis with the public domain software NIH-Image (http://rsb.info.nih.gov/nih-image/). Sponges were outlined manually with a digitizing pen to calculate the cover (cm^2) of each individual. Thickness of all individuals was measured under water with a calliper. However since all these encrusting sponges were less than 1mm thick, the measuring error would be too large to calculate body volume properly. Dry weight and ash free dry weight were recounted for the whole tissue surface, since $1cm^2$ of every sponge was cut for fixation.

3.5 CARD-FISH

For a detailed description of procedures and solutions see Appendix.

3.5.1 Dissociation of sponge tissue

To investigate the composition of the bacterial assemblage of sponges, with emphasis on nitrifying bacteria, from every sponge tissue fixed in PBS/ethanol the bacteria were separated. Therefore the tissue was crushed with a rubber stick specially made to fit in a reaction vial, which contained 200µl of Lysis T (Sigma) to demolish connection between cells. When all cells were loosened 200µl of artificial sea water (ASW) was added before the suspension was centrifuged at 5000 rpm for 30 seconds. Supernatant was poured into a 15 ml tube. The pellet was resolved in 800µl ASW, poured into a 15ml tube and diluted with 9.2ml ASW. Pellet dilution was filtered over 0.8µm, 0.25mm ATTP filter, to separate sponge cells from the bacteria. Filters were placed on a microscopic slide with a drop of DAPI-mix to colour possible retained bacteria and stored in the dark at -20°C. The filtered water was added to the supernatant which was further diluted to 10ml with ASW. Of this dilution 2.5ml was filtered over a 0.2µm, 0.25mm GTTP filter, supported by a 0.45µm, 0.25mm HA filter. The GTTP filters were air dried and stored at -20°C until CARD-FISH procedure started. It is thought that most bacteria are in the supernatant. But as a control pellet filters were made.

3.5.2 CARD-FISH procedure

Since both water samples and bacteria from sponges are fixed on a filter their procedures for CARD-FISH are the same. When a filter is mentioned it can be either come from a water sample or from a sponge. CARD-FISH is a molecular technique to colour specific type of organisms (i.e. Bacteria, Archaea and Eukaryotes) by the use of horseradish peroxidase (HRP) probes, which target 16S rDNA of organisms and the use of fluorescent tyramide tags (Pernthaler *et al.*, 2002). The technique described by Pernthaler *et al.* (2002) consists of several steps in which cells are secured on the filter (embedding), the cell walls are made permeable (permeabilisation), the probes are added (hybridisation) and a fluorescent dye is enclosed (amplification).

For embedding the filters were dipped in low-gelling-point 0.1% agarose on a petridish, dried upside down in a hybridisation oven at 37°C for 15 minutes and dehydrated in 95% ethanol. Subsequently filters were incubated either with lysozyme (for bacteria, 10mg/ml) or with proteinase-K (for Archaea, 0.2µl/ml) solution (0.1M Tris-HCl [ph 8.0], 0.05M EDTA) in a hybridisation oven at 37°C for 1 hour in order to make the cell wall permeable. Filters were washed three times with Milli-Q water and incubated in 0.01M HCl at room temperature for 20 minutes. This incubation in HCl is an important step to inactivate proteinase-K, otherwise the HRP-probe will not work properly (Teira, 2004). After incubation in HCl, filters were washed twice with Milli-Q water, dehydrated with 95% ethanol and dried at room temperature.
Filters were cut in sections for hybridisation with the HRP probes EUB338 (targeting *Bacteria*), BET42a (targeting *β-Proteobacteria*), GAM42a (targeting *γ-Proteobacteria)*, NSR1156 (targeting *Nitrospira*), Eury806 (targeting *Euryarchaea*) and Cren537 (targeting *Crenarchaea*) (table 1).
All nitrifying bacteria thus far examined are either α-, β-, γ- and δ- Proteobacteria except for Nitrospira which constitutes its own phylogenetic lineage. Ammonia oxidizing bacteria belong to the β- and γ-Proteobacteria and the nitrite oxidizing bacteria are found in the α-, γ- and δ-Proteobacteria (Hovanec

and DeLong, 1996; Schramm *et al.*, 1998; Friedrich *et al.*, 1999; Bothe *et al.*, 2000; Madigan *et al.*, 2000; Norton *et al.*, 2002). In the present study only β- and γ- Proteobacteria were examined, since most nitrifying bacteria are represented in these groups.

Archaea were investigated to get an impression about the distribution of Eubacteria and Archaea of the total counted DAPI. Also the effectiveness of the method was determined, since the sum of Archaea and Eubacteria counts should equal the DAPI counts. The probe NonEUB338 was used in a few cases to test whether the probe EUB338 (I) gave false fluorescence. Of all tests (four times) there were no counts of NonEUB338, concluding that all signals of EUB338 were Bacteria. A mix of EUB-probes (EUB338I, II and III) was used to test if this gave more signals than probe EUB338I. Nevertheless no differences in counts were established.

Since beta and gamma probes resemble each other a β-competitor was added to the γ-probe and likewise a γ-competitor was added to the β-probe. Three hundred microlitres of hybridisation buffer (containing 55% formamide for EUB338, β and γ, 30% formamide for NSR1156 and 20% formamide for Eury806 and Cren537) was dispensed into a 0.7ml reaction vial. The HRP-probe was added to a final DNA concentration of 0.28ng/µl (0.05µM) and filter sections were put in this reaction vial. Hybridisation was performed in the dark at 35°C for 14 hours in a hybridisation oven where reaction vials were gently spun round. Thereafter, filter sections were transferred into 50ml of prewarmed washing buffer (NaCl concentration depending on percentage formamide in hybridisation buffer, see appendix) at 37°C for 10 to 15 minutes. Sections were accordingly placed in 25ml phosphate-buffered saline (PBS) appended with 0.05% Triton X100 (PBS-T) at room temperature in dark for 10 to 15 minutes. After removal of excess buffer filter sections were immediately transferred to a 0.7ml reaction vial containing 493µl of amplification buffer and 3.4µl of tyramide-Alexa488 (0.7mg/ml, 1:150). Reaction vials were incubated in the dark at 37°C for 45 minutes in a hybridisation oven which gently turned the vials round. After amplification filters were washed in 25ml PBS-T at room temperature in the dark for 25 minutes, followed by a washing event in the dark with Milli-Q water. Finally filter sections were dehydrated in the dark with 95% ethanol. As soon as filters were dry, they were placed in a drop of DAPI-mix on a microscopic slide and stored at -20°C until counting.

Table 1. HRP-probes used for in situ hybridization

Probe	Sequence 5' → 3'	Form (%)	Specificity	Labelling	Reference
EUB 338	GCTGCCTCCCGTAGGAGT	55	most Eubacteria	Tyramide	Amann *et al.*, 1990
EUBmix		55	all Eubacteria	Tyramide	Daims *et al.*, 1999
NonEUB338	ACTCCTACGGGAGGCAGC	55	neg. control Eubacteria	Tyramide	Amann *et al.*, 1990
BET42a	GCCTTCCCACTTCGTTT	55	β-*Proteobacteria*	Tyramide	Manz *et al.*, 1992
GAM42a	GCCTTCCCACATCGTTT	55	γ-*Proteobacteria*	Tyramide	Manz *et al.*, 1992
NSR1156	CCCGTTCTCCTGGGCAGT	30	Nitrospira spp.	Tyramide	Schramm *et al.*, 1998
EURY806	CACAGCGTTTACACCTAG	20	Euryarchaeota	Tyramide	DeLong *et al.*, 1999
CREN537	TGACCACTTGAGGTGCTG	20	Crenarchaeota	Tyramide	DeLong *et al.*, 1999

3.5.3 Counting

Microscopic slides of CARD-FISH procedures, DAPI staining (sponge pellets) and Acridine-Orange were examined under a Zeiss Axioplan 2 imaging microscope equipped with a 100W Halogen lamp and appropriate filter sets for DAPI, Acridine-Orange and tyramides. To check if the tyramide signals were originating from auto radiation a green filter was used. The slides produced by CARD-FISH have both a DAPI signal and a specific probe signal. The DAPI of the EUB338 stained bacteria were counted. The other probes were not counted for DAPI, but checked if the bacteria were comparably distributed. In total about 200 bacteria per filter section were counted. The slides containing the sponge tissue pellets, were screened for bacteria coloured with DAPI. They were counted in order to get an indication about the effectiveness of the separation method and to recalculate the amount of bacteria in sponges. Pictures of DAPI-stained cells and of the different HRP-probes were made with a Zeiss AxioCam MRc5.

3.6 Determination of sponges

Sponges can look like each other and one sponge can also have different appearances. Sponges were characterised by the shape of minuscule silicate or carbonate skeleton structures, called spiculae, which are present in all kinds of shapes. Most sponges have one or more types of spiculae present in their tissue and these spiculae packages are uniquely for that sponge species.
From the sponge tissue fixed for taxonomic work, preparations were made to determine the sponge under the microscope for their characteristic spiculae. Since these encrusting sponges are so thin it was not possible to make standard preparations with a cross and longitudinal section. In stead a small piece was flattened between two microscope slides, subsequently dried, placed on one slide with a drop of Canada-balsam (Merck), covered with a cover glass and dried for at least 24 hour on a stove. Slides were searched for spiculae with a light microscope (Leitz Laborlux S) with a magnification of 40 times. Sponge species were determined with the help of taxonomic literature (Hooper and Van Soest, 2002) and personal communication (Van Soest).

3.7 Data analysis

3.7.1 Bacterial abundances

The bacterial density of reef water inside enclosures is given as the percentage decrease of the initial bacterial abundance compared to the amount bacteria after 30 minutes counted on the DAPI-stained filters made by CARD-FISH. Of three experiments bacterial abundances of the whole time series was investigated from the Acridine-Orange coloured filters. The abundance of specific bacterial groups visualised by CARD-FISH, was expressed as percentage of the total Eubacteria count. The amount of bacteria inside sponges was recalculated per millilitre water respectively square centimetre sponge.

3.7.2 Nutrient amounts and fluxes

Water samples (in duplicate) were analysed for the concentrations of ammonium, nitrite and nitrate. Duplicates were averaged to get the nutrient concentrations of that particular sponge at that time. Subsequently volume corrections were made, since at every time step water was taken out of the enclosure. Values not corresponding to the courses of the nutrient amounts were thought to be errors and were not taken into account. For example, an initial value of 1.9µmol l^{-1} NO_3 of the sponge *M. microsigmatosa* in experiment I. This value does both not correspond to the other initial values of NO_3 and to the nutrient course of that experiment.
Fluxes were calculated from the coefficient of a linear regression fit through all data points for each experiment. The rate was expressed as release rates of the nutrients in millimole per square meter sponge respectively sediment per day (mmol m^{-2} d^{-1}).

4 Results

4.1 Sponge species

The sponges used for the standard enclosure experiments were *Halisarca caerulea*, *Mycale microsigmatosa* and *Merlia normani* and for the nitrapyrin added incubation experiments the sponges *H. caerulea*, *Monanchora arbuscula*, *Clathria spp.* and *a Cliona spp.* has been used (see figure 6). From the sponge species *H. caerulea*, *M. microsigmatosa*, *M. normani*, *M. arbuscula*, *Clathria spp.*, *Cliona spp.*, *Hymeniacidon haeliophila* and *Diplastrella megastellata* samples were taken to investigate bacterial composition with CARD-FISH.

One sponge species used in all experiments was *Halisarca caerulea* (Vacelet, 1987). This sponge has no spiculae and could therefore not be determined at the regular way. However *Halisarca caerulea* has a very distinct appearance, purple with star-shaped exhalant channels, which could not be confused with other sponges. The other sponges are instead difficult to distinguish by visual observations, since they are all red and encrusting. These sponge species can solely be distinguished by their spiculae. Characteristics of the sponges used in enclosure experiments are shown in table 2.

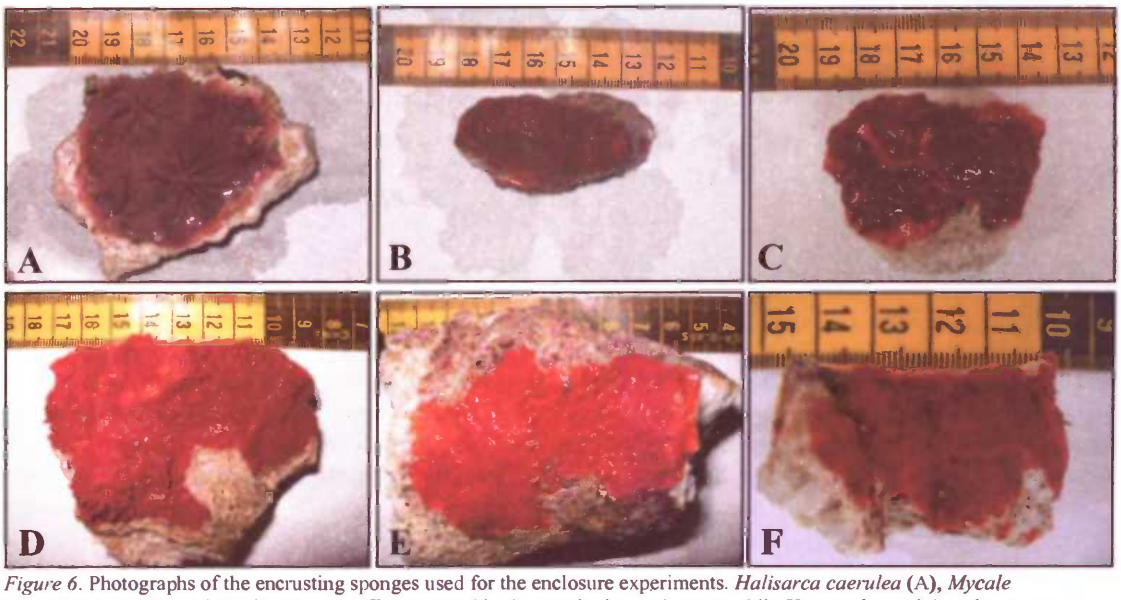

Figure 6. Photographs of the encrusting sponges used for the enclosure experiments. *Halisarca caerulea* (A), *Mycale microsigmatosa* (B) and *Merlia normani* (C) were used in the standard experiments, while *H. caerulea* and the other sponges *Monanchora arbuscula* (D), *Clathria sp.* (E) and *Cliona sp.* (F) were used in experiments with added nitrapyrin.

Table 2. Characteristics of sponges used in enclosure experiments

Sponge type	# exp	SA (cm^2)	Vol. substrate (ml)	DW (g)	AFDW (g)	Spiculae type
H. caerulea	I	43.0	20	1.12	0.18	-
	II	14.0	50	0.48	0.10	
	III	18.4	25	0.64	0.09	
	IV	27.8	15	0.83	0.15	
	V	16.9	35	0.57	0.12	
	VI	31.2	35	0.95	0.21	
	VII	28.0	55	0.74	0.17	
	VIII	22.2	95	0.51	0.08	
	IX	248	55	0.53	0.08	
	X	27.1	55	0.53	0.17	
	XI	30.6	40	0.92	0.20	
	XII	22.6	50	0.43	0.10	
	Nit I	38.8	15	1.98	0.29	
	Nit II	29.0	55	2.69	0.17	
	Nit III	15.0	5	0.34	0.08	
M. microsigmatosa	I	29.5	30	2.64	0.18	thick and thin (tylo)styles, sigma's
	II	28.8	65	2.38	0.17	
	III	14.0	20	0.62	0.06	
	IV	20.0	30	0.86	0.07	
	V	7.7	20	0.57	0.05	
	VI	14.0	20	0.62	0.06	
	VII	20.0	30	0.86	0.07	
M. normani	I	30.3	35	2.78	0.18	thin (tylo)styles, clavidiscs
	II	15.2	55	1.27	0.09	
	III	23.1	40	0.47	0.06	
M. arbuscula	Nit I	18.7	65	1.45	0.14	thick and thin styles, chela's
	Nit II	39.5	65	0.70	0.18	
	Nit III	15.8	15	0.39	0.06	
	Nit IV	35.0	20	0.93	0.11	
Clathria sp.	I	42.1	180	0.26	0.06	thick and thin (tylo)styles, chela's
	Nit I	42.1	180	0.26	0.06	
Cliona sp.	Nit I	10.6	15	0.3	0.04	thick tylostyles

4.2 Bacterial counts

Bacterial concentrations dropped quickly after closure of the incubation chambers with sponges (see figure 7). The average initial bacteria concentration of the reef water was $1.26x10^6$ bacteria ml^{-1}. After 30 minutes incubation time about $4.97x10^5$ bacteria ml^{-1} were left. A bacterial density decrease of on average 62%. There were although differences observed between the experiments. In the control experiments no clear decrease of bacterial abundance is observed.

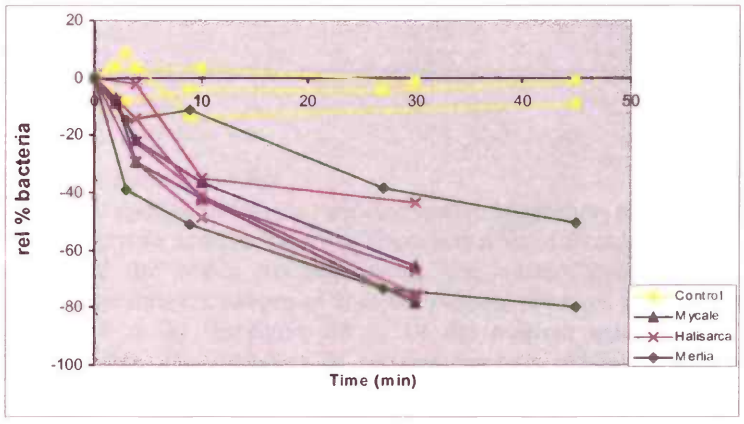

Figure 7. Graph showing the relative amount of bacteria in reef water during several incubation experiments with and without sponges.

4.3 Inorganic nitrogen dynamics

4.3.1 Control

Three control experiments with only bare substrate in the incubation chamber were conducted. The amounts of ammonium, nitrate and nitrite after volume correction are shown in time in figure 8 as the average of the three experiments. The inorganic nitrogen rates during incubation (fluxes) are shown in table 3. Fluxes were calculated from the coefficient of the linear regression lines shown in figure 8. For all experiments negative fluxes were observed, indicating that ammonium, nitrate and nitrite were net absorbed during incubation. However standard deviations were large, especially for the nitrate experiments. The other experiments are therefore shown with and without subtraction of the control fluxes.

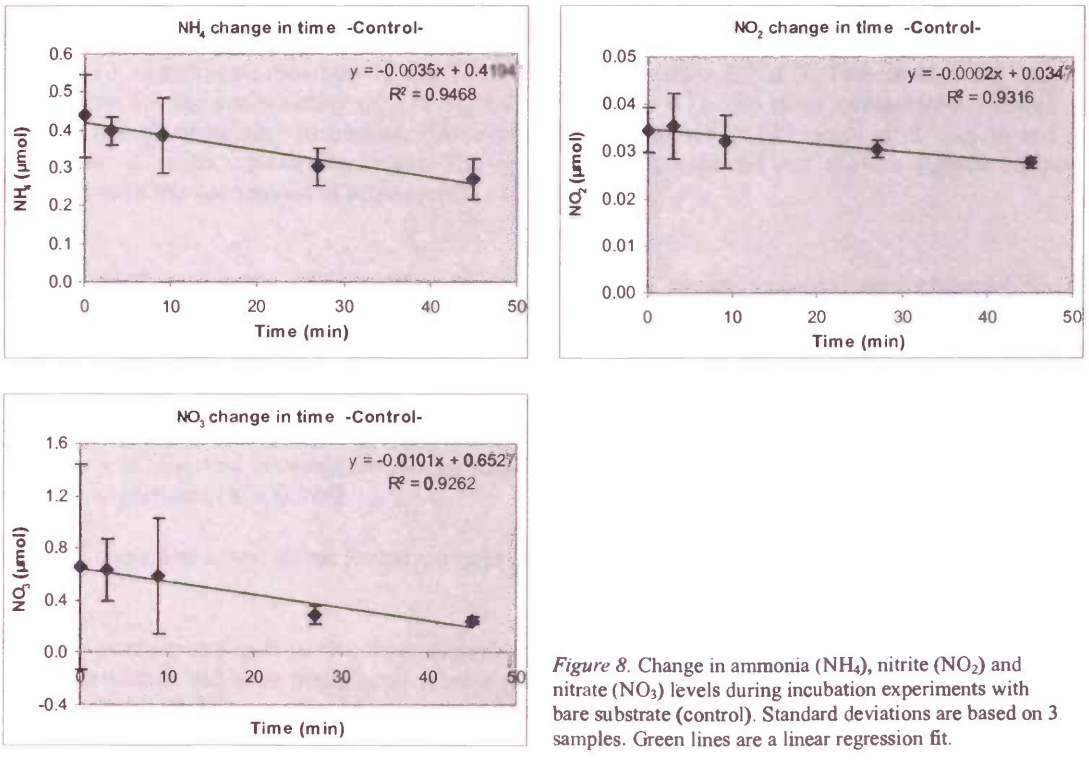

Figure 8. Change in ammonia (NH_4), nitrite (NO_2) and nitrate (NO_3) levels during incubation experiments with bare substrate (control). Standard deviations are based on 3 samples. Green lines are a linear regression fit.

Table 3. Fluxes of control experiments

# Experiment	Flux (mmol m^{-2} d^{-1})		
	NH_4	NO_2	NO_3
I	-3.01	-0.188	-15.78
II	-2.50	-0.056	-0.94
III	-1.13	-0.063	-4.63
Average	-2.21	-0.102	-7.12
SD	0.97	0.074	7.72

4.3.2 Halisarca caerulea

With the sponge *Halisarca caerulea* twelve incubation experiments were conducted without nitrapyrin and three with added nitrapyrin. There was a lot of fluctuation in the absolute nutrient amounts between days and also within one experiment. The nutrient fluctuations were therefore examined in time for each experiment. Changes of absolute nutrient amounts for ammonium, nitrite and nitrate are shown in figures 9 to 11. In figure 12 to 14 the nutrient amounts are displayed of the added nitrapyrin experiments. The increases in nutrient amounts indicate a net release. The decreases suggest a net absorption of nutrients. Fluctuations indicate that release as well as absorption occurs within the incubation period.

The inorganic nitrogen release rate respectively absorption rate during incubation (fluxes) are shown in table 4. Fluxes were calculated from the coefficient of the linear regression lines shown in figures 9-14.

Ammonium

Between the experiments differences were found in the patterns of ammonium amounts. Both increases and decreases occurred in the first minutes. In the last minutes ammonium increases were observed for most experiments (figure 9). Ten of the twelve experiments had a net release rate of ammonium after incubation (values ranging from -1.75 to 17.30 mmol m^{-2} d^{-1}). However, after correction for the control experiments all experiments showed a positive flux (values ranging from 0.46 to 19.51 mmol m^{-2} d^{-1}). The average ammonium flux is 5.97 mmol m^2 d^{-1} before and 8.18 mmol m^2 d^{-1} after control correction. A comparison of sponge surface area and the net amount of ammonium release did not show a significant positive relation (R^2=0.006).

Nitrite

Small increases and decreases of nitrite amounts are observed in the first minutes of incubation (figure 10). Overall an increase of in nitrite amount occurs for 10 experiments. Flux calculations show for 11 of the 12 experiments positive values (-0.235 to 0.319 mmol m^{-2} d^{-1}). This is also the case after correction for the control data (-0.132 to 0.421 mmol m^{-2} d^{-1}). So most experiments showed a net release rate of nitrite after incubation. The average nitrite flux was 0.133 mmol m^2 d^{-1} before and 0.235 mmol m^2 d^{-1} after control correction. The sponge surface area did not show a significant positive relation with the net amount of nitrite release (R^2=0.034).

Nitrate

Within the first minutes of incubation large variations in nitrate amounts were observed for some experiments (figure 11). Fluxes were positive for 7 experiments (values ranging from -17.59 to 6.14 mmol m^{-2} d^{-1}). But after correction for control experiments 11 of 12 experiments showed a net nitrate release (values ranging from -10.47 to 13.25 mmol m^{-2} d^{-1}). Experiment II appears to be an outlier compared to the values of the other experiments. Therefore this experiment was not taken into account for calculation of the average nitrate release (7.82 mmol m^{-2} d^{-1} after control correction). A positive relation was observed between the sponge surface area and the net amount of nitrate release although it was not significant (R^2= 0.074).

Overall there was a net release for all nitrogen species during incubation with the sponge *H. caerulea*.

Nitrapyrin

The ammonium amounts of the three experiments with added nitrapyrin increased linear (figure 12). All experiments had a net ammonium release rate between 1.82 and 8.46 mmol m^{-2} d^{-1} (average of 4.65 mmol m^{-2} d^{-1}). After correction for control data the average release rate was raised to 6.87 mmol m^{-2} d^{-}

$^{-1}$. This flux is lower than the average ammonium flux of the experiments without added nitrapyrin (8.18 mmol m^{-2} d^{-1}).
Nitrite amounts decreased during incubation with nitrapyrin (figure 13). The net release rates were therefore below zero; -0.334 to 0.000 mmol m^{-2} d^{-1} (average of -0.145 mmol m^{-2} d^{-1}). After correction for control data in experiment III a clear nitrite release was found (0.151 mmol m^{-2} d^{-1}). The average release rate, however, remained negative (-0.042 mmol m^{-2} d^{-1}). In the experiments without added nitrapyrin the fluxes were higher (0.235 mmol m^{-2} d^{-1}).
For two experiments with added nitrapyrin the nitrate amounts decreased during incubation (figure 14). Experiment III showed a small increase. The fluxes are in line with these patterns. Nitrate release rates varied from -1.86 to 1.44 mmol m^{-2} d^{-1}. Nevertheless, after correction for control data all experiments showed a positive net nitrate release rate (average of 6.61 mmol m^{-2} d^{-1}). Also for this inorganic nitrogen compound the fluxes were lower compared to the experiments without added nitrapyrin (7.82 mmol m^{-2} d^{-1}).

Summarising, there is a net ammonium and nitrate release and a nitrite absorption when nitrapyrin is added to the incubation chamber. Average fluxes of all the nitrogen species were lower than the average fluxes of the incubation experiments without added nitrapyrin.

Table 4. Fluxes of *Halisarca caerulea* with and without correction for fluxes of control experiment.

# Experiment	Flux (mmol m^{-2} d^{-1})					
	NH_4		NO_2		NO_3	
	Without correction	With correction	Without correction	With correction	Without correction**	With correction**
I	4.22	6.43	0.023	0.126	0.47	7.59
II	-1.75	0.46	0.206	0.308	(-17.59)	(-10.47)
III	9.78	12.00	-0.235	-0.132	-5.17	1.95
IV	-0.41	1.80	0.155	0.258	-1.23	5.87
V	17.30	19.51	0.256	0.358	6.14	13.25
VI	5.77	7.98	0.092	0.195	-2.31	4.81
VII	5.19	7.41	0.257	0.359	1.70	8.81
VIII	10.44	12.66	0.032	0.135	0.13	7.25
IX	13.18	15.39	0.058	0.160	3.25	10.37
X	2.44	4.66	0.319	0.421	0.01	7.13
XI	4.85	7.06	0.235	0.338	0.19	7.30
XII	0.64	2.85	0.191	0.293	4.59	11.70
Average	5.97	8.18	0.133	0.235	0.71	7.82
SD	5.73	5.73	0.151	0.151	3.17	3.17
Nit I *	8.46	10.68	-0.334	-0.232	-1.86	5.26
Nit II *	3.67	5.89	-0.099	0.003	-1.09	6.02
Nit III *	1.82	4.04	0.000	0.151	1.44	8.56
Average	4.65	6.87	-0.145	-0.042	-0.50	6.61
SD	3.43	3.43	0.171	0.171	1.73	1.73

* Nit denotes to the addition of Nitrapyrin.
** Values between ( ) are not used for calculation of the average

NH_4 change in time *-Halisarca caerulea-*

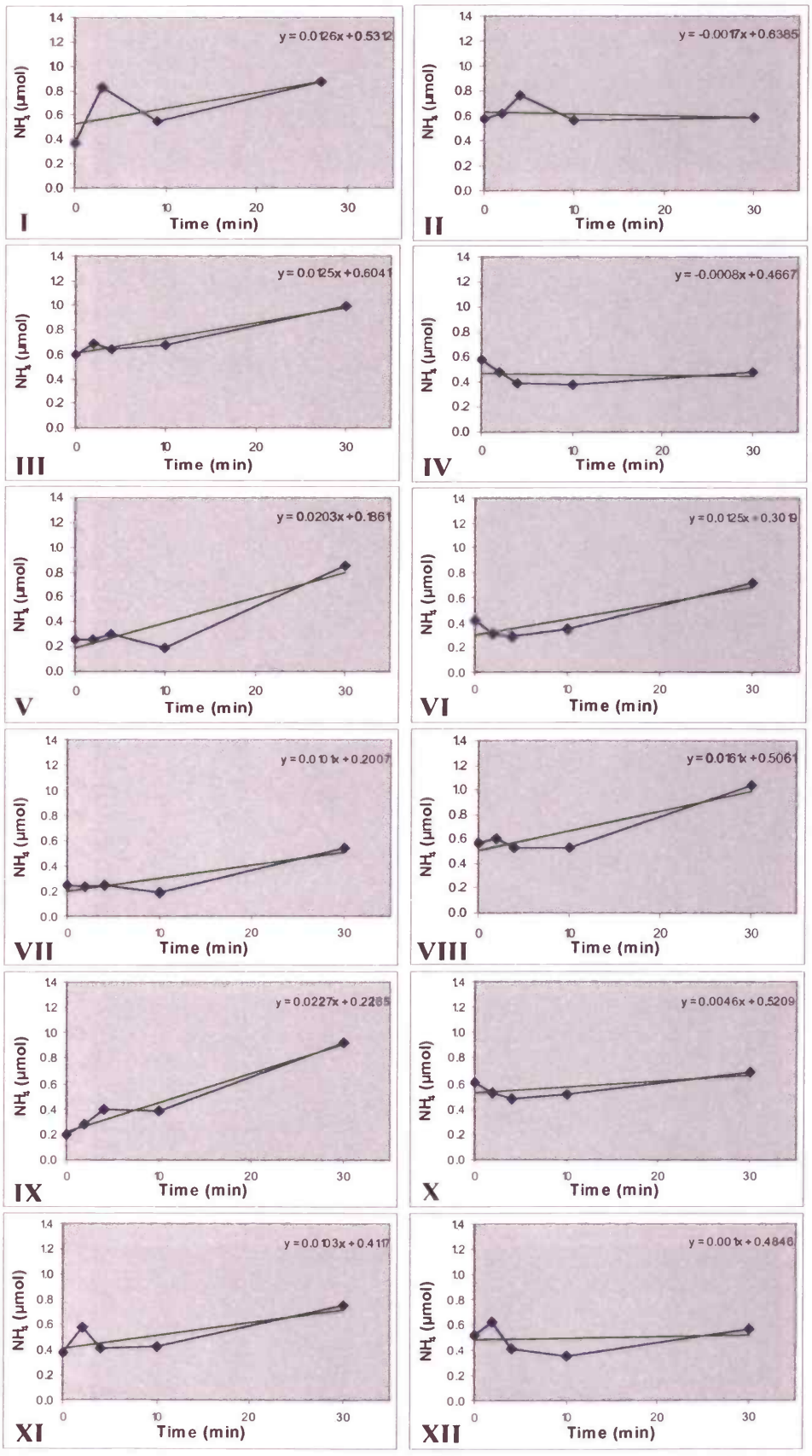

Figure 9. Changes in absolute ammonia amounts during incubation experiments with the sponge *Halisarca caerulea*. Each graph indicates a single experiment all with different pieces of sponge (see table 2). Green line is a linear regression.

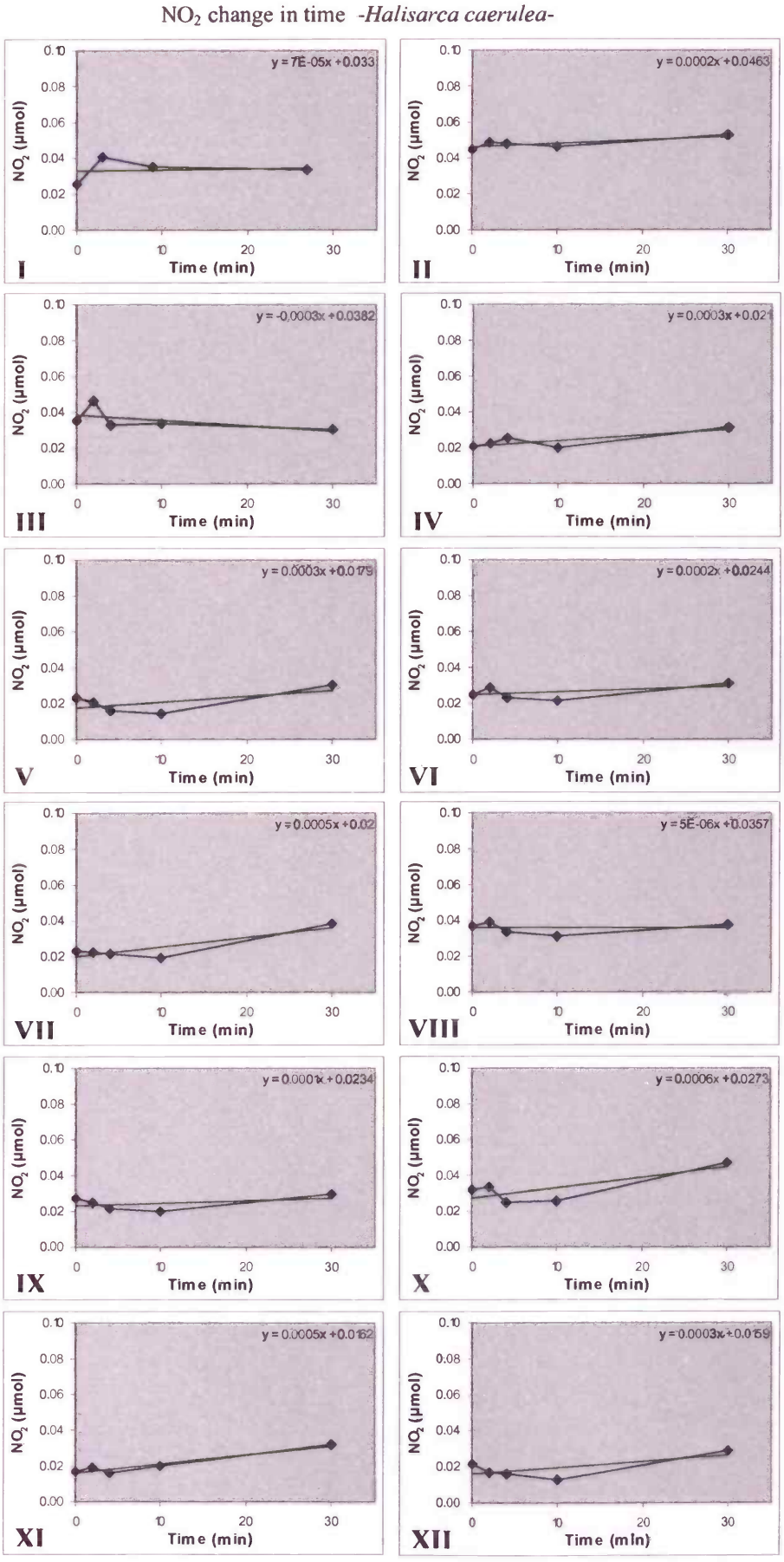

Figure 10. Changes in absolute nitrite amounts during incubation experiments with the sponge *Halisarca caerulea*. Each graph indicates a single experiment all with different pieces of sponge (see table 2). The green line is a linear regression.

NO_3 change in time *-Halisarca caerulea-*

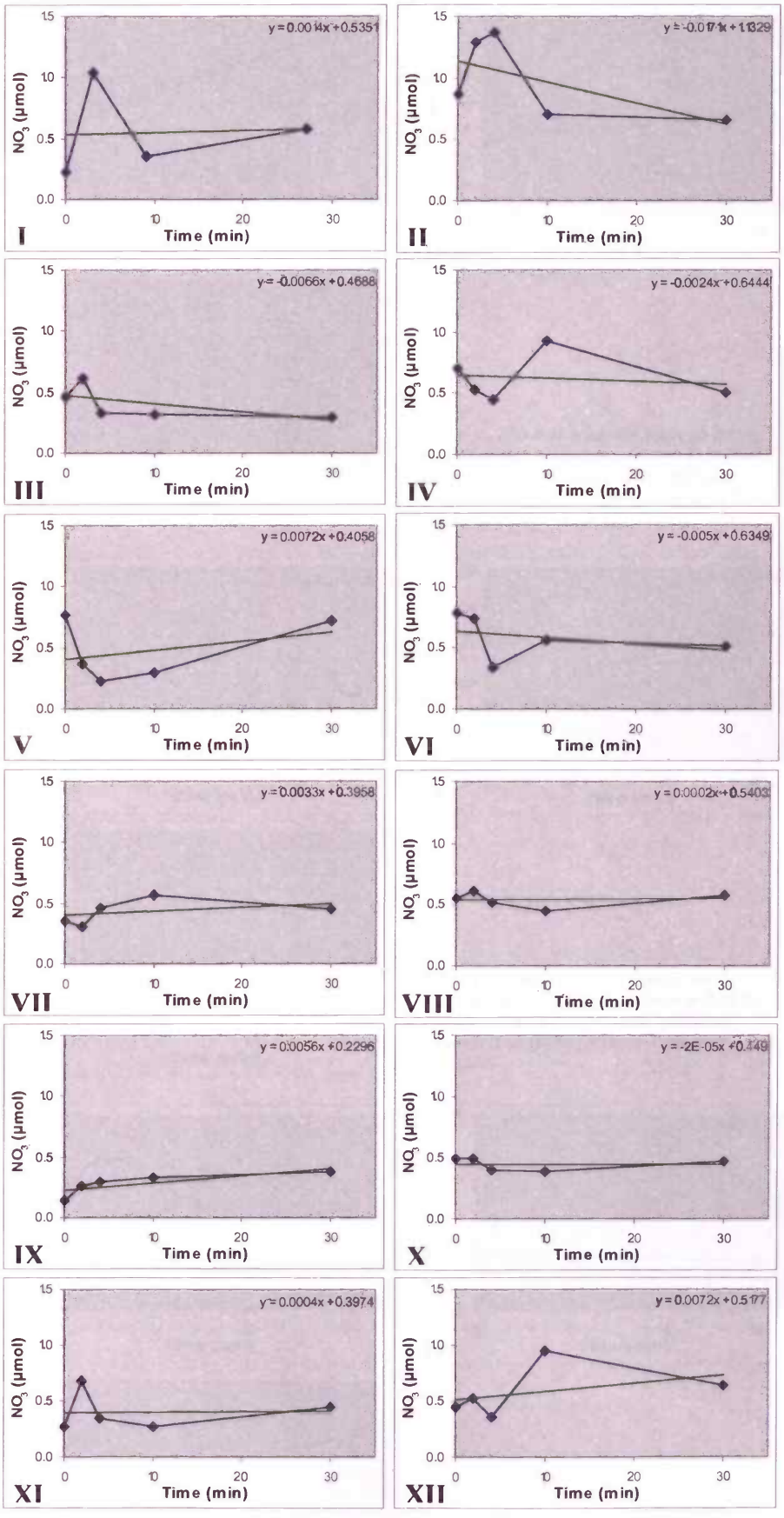

Figure 11. Changes in absolute nitrate amounts during incubation experiments with the sponge *Halisarca caerulea*. Each graph indicates a single experiment all with different pieces of sponge (table 2). The green line is a linear regression.

Nitrapyrin added nutrient change in time -*Halisarca caerulea*-

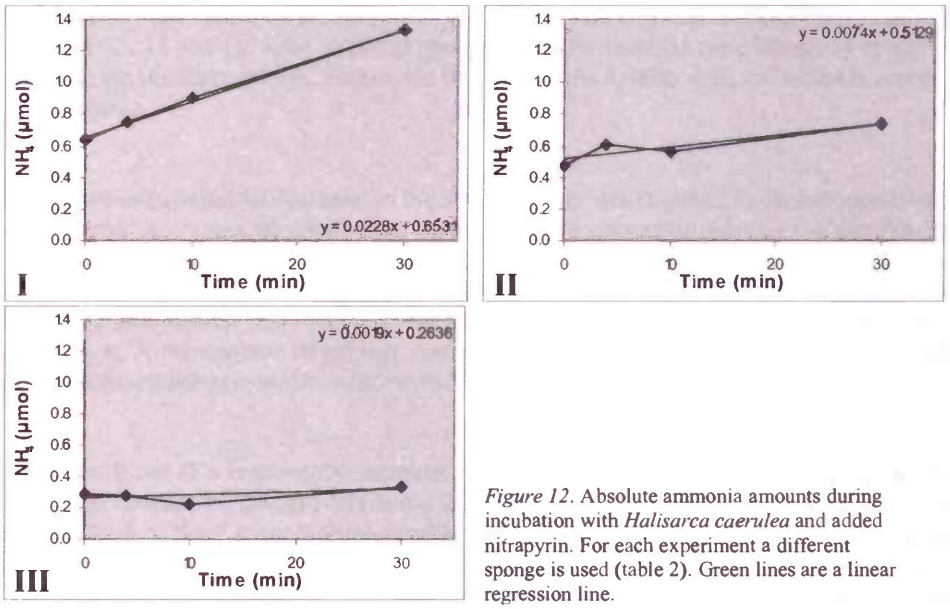

Figure 12. Absolute ammonia amounts during incubation with *Halisarca caerulea* and added nitrapyrin. For each experiment a different sponge is used (table 2). Green lines are a linear regression line.

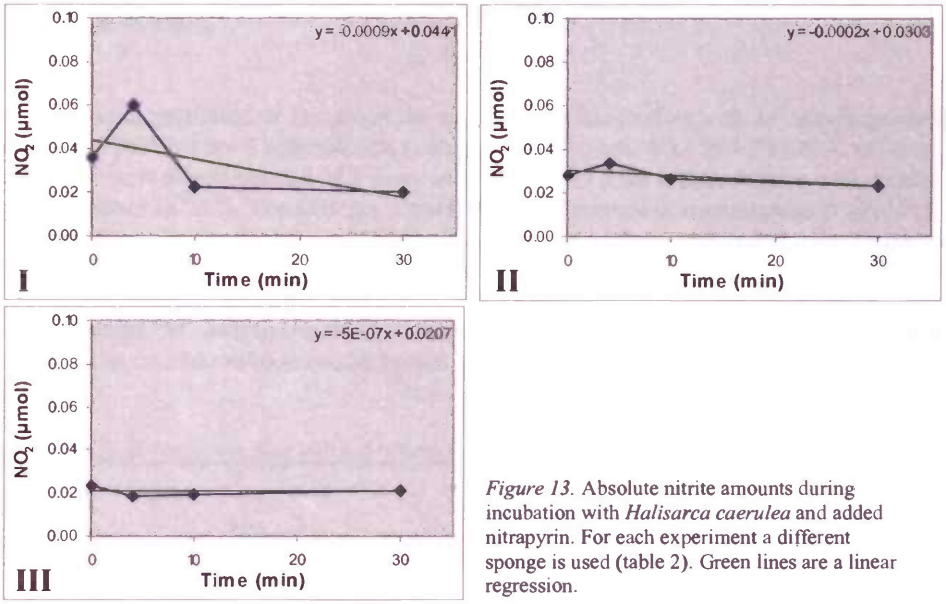

Figure 13. Absolute nitrite amounts during incubation with *Halisarca caerulea* and added nitrapyrin. For each experiment a different sponge is used (table 2). Green lines are a linear regression.

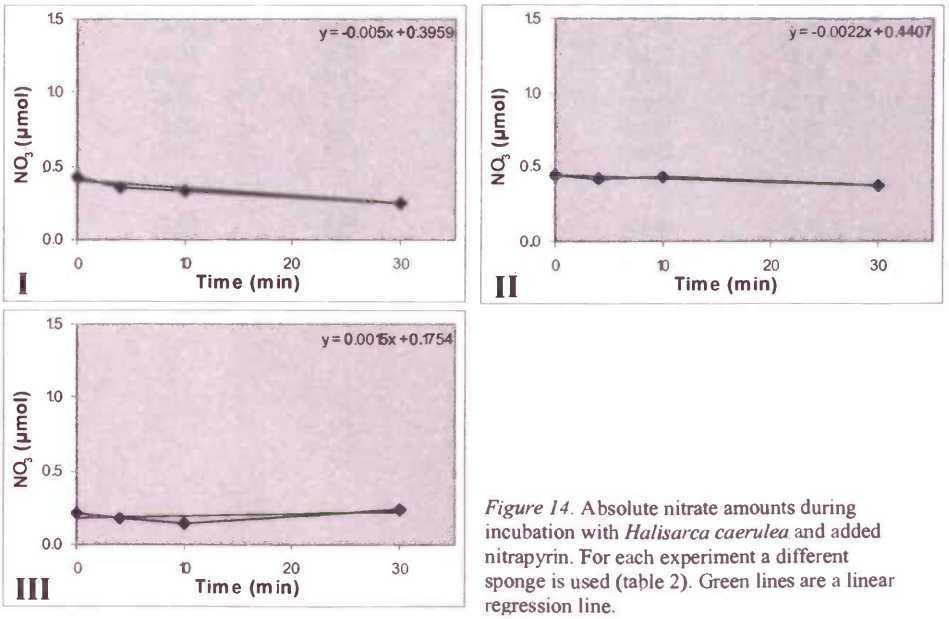

Figure 14. Absolute nitrate amounts during incubation with *Halisarca caerulea* and added nitrapyrin. For each experiment a different sponge is used (table 2). Green lines are a linear regression line.

4.3.3 Mycale microsigmatosa

Seven experiments with the red encrusting sponge *Mycale microsigmatosa* were conducted, which are shown in figure 15, 17 and 19. Also with this sponge large fluctuations were observed in nutrient amounts during the incubation time. Fluxes are shown in table 5, both with and without correction for control experiments.

Ammonium

Ammonium amounts tended to decrease in the first few minutes (figure 15). Subsequently an increase was usually found. So fluxes of ammonium appeared to be generally positive (values ranging from -4.16 to 10.15 mmol m^{-2} d^{-1}). After correction for the control experiments almost all experiments showed a positive flux (except for experiment II). Values were ranging from -1.94 to 12.37 mmol m^{-2} d^{-1}. The average ammonium flux was positive: 2.03 mmol m^2 d^{-1} before and 4.25 mmol m^2 d^{-1} after control correction. A comparison of sponge surface area and the net amount of ammonium release did not show a significant positive relation (R^2=0.013).

Nitrite

For experiments I and II a reasonable decrease in nitrite amount was observed during the incubation (figure 17). Experiments III, IV and VII had a small increase of nitrite in the last minutes. The fluxes were negative for 5 of the 7 trials (values ranging from -0.301 to 0.029 mmol m^{-2} d^{-1}). After correction for control there were 4 positive, 2 negative and 1 neutral fluxes. The average nitrite flux was negative (-0.102 mmol m^{-2} d^{-1}) before and neutral (0.000 mmol m^{-2} d^{-1}) after control correction. The sponge surface area tended to have a negative correlation with the net amount of nitrite release. It was although not significant (R^2=0.681).

Nitrate

The nitrate amounts decreased or remained the same during incubation with *M. microsigmatosa* (figure 19). Fluxes were positive for 3 experiments (values ranging from -8.21 to 4.75 mmol m^{-2} d^{-1}). But after correction for control experiments 6 of 7 experiments showed a net nitrate release (values ranging from -1.10 to 11.87 mmol m^{-2} d^{-1}). The average nitrate flux after control correction was positive (5.40 mmol m^{-2} d^{-1}). A negative trend was observed between the sponge surface area and the net amount of nitrate release although it was not significant (R^2= 0.051).

Overall, the sponge *M. microsigmatosa* showed on average a net ammonium and nitrate efflux. Whereas for nitrite no clear release or absorption was observed.

Table 5. Fluxes of *Mycale microsigmatosa* with and without correction for fluxes of control experiment.

# Experiment	Flux (mmol m^{-2} d^{-1})					
	NH_4		NO_2		NO_3	
	Without correction	With correction	Without correction	With correction	Without correction	With correction
I	5.23	7.44	-0.196	-0.093	1.03	8.14
II	-4.16	-1.94	-0.301	-0.198	-8.21	-1.10
III	-0.51	1.70	0.009	0.112	-3.19	3.93
IV	10.15	12.37	0.029	0.131	4.75	11.87
V	-0.56	1.65	-0.009	0.093	-3.74	3.38
VI	2.06	4.27	-0.103	-0.001	-2.78	4.34
VII	2.02	4.23	-0.144	-0.042	0.14	7.26
Average	2.03	4.25	-0.102	0.000	-1.71	5.40
SD	4.61	4.61	0.121	0.121	4.13	4.13

4.3.4 Merlia normani

With another red encrusting sponge, *Merlia normani*, three incubation experiments were conducted shown in figure 16, 18 and 20. Fluxes are shown in table 6, both with and without correction for control experiments.

Ammonium

The ammonium amounts tended to decrease in the first minutes, but increased at the end of the incubation with *M. normani* (figure 16). Both for and after correction of control data 2 out of 3 experiments showed positive fluxes. Values after correction were ranging from -0.94 to 8.96 mmol m^{-2} d^{-1}, with an average of 3.75 mmol m^{-2} d^{-1}. A comparison of sponge surface area and the net amount of ammonium release did not show a significant positive relation (R^2=0.039).

Nitrite

For experiments I and II a reasonable decrease in nitrite amount was observed during the incubation (figure 18). Experiment III showed after a decrease in the first few minutes an increase in nitrite amount. The fluxes were negative for all experiments, both for and after correction for control data. The average nitrite flux before correction was -0.268 mmol m^{-2} d^{-1} and after control correction -0.166 mmol m^{-2} d^{-1}. So a nitrite absorption occurred. The sponge surface area tended to have a negative correlation with the net amount of nitrite release. It was although not significant (R^2=0. 531).

Nitrate

In the second experiment with M. normani very high values (1.5 resp. 3.1 µmol) for the first two time steps were obtained (figure 20). Since these high values are not in line with the other starting nitrate amounts, it was decided to leave them out. In experiments I and II mainly a nitrate decrease is observed. The third experiment has small variations in nitrate amounts. Fluxes were all negative (values ranging from -4.76 to -1.03 mmol m^{-2} d^{-1}). But after correction for control experiments all experiments showed a net release of nitrate (values ranging from 2.82 to 6.09 mmol m^{-2} d^{-1}). The average nitrate flux after control correction was 3.75 mmol m^{-2} d^{-1}. A negative trend was observed between the sponge surface area and the net amount of nitrate release (R^2= 0.987).

So, with the sponge *M. normani* there is overall a net ammonium and nitrate release and a nitrite absorption found during incubation.

Table 6. Fluxes of *Merlia normani* with and without correction for fluxes of control experiment.

# Experiment	Flux (mmol m^{-2} d^{-1})					
	NH_4		NO_2		NO_3	
	Without correction	With correction	Without correction	With correction	Without correction	With correction
I	6.75	8.96	-0.196	-0.093	-4.30	2.82
II	-3.16	-0.94	-0.301	-0.198	-4.76	2.36
III	1.03	3.24	-0.309	-0.206	-1.03	6.09
Average	1.54	3.75	-0.268	-0.166	-3.36	3.75
SD	4.97	4.97	0.063	0.063	2.03	2.03

NH_4 change in time -*Mycale microsigmatosa*-

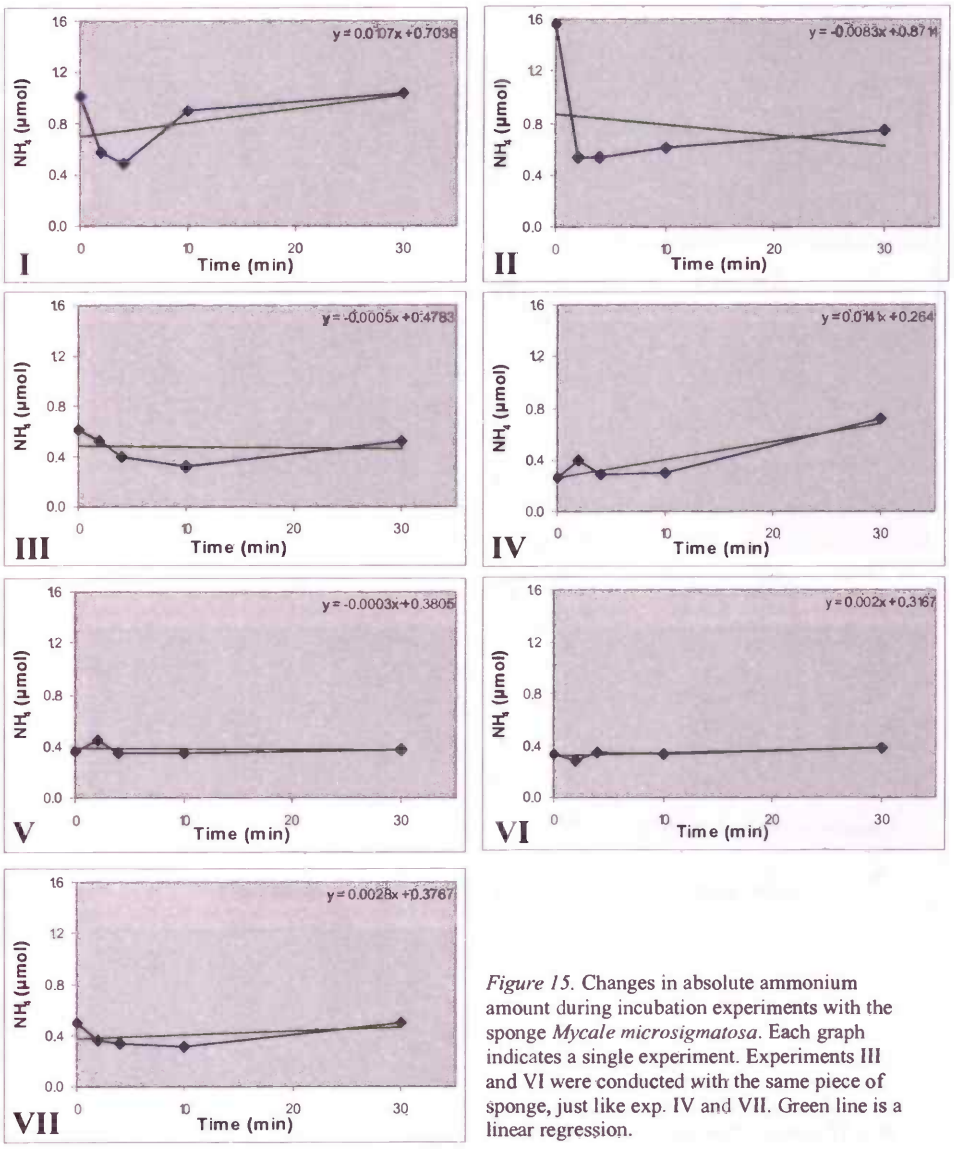

Figure 15. Changes in absolute ammonium amount during incubation experiments with the sponge *Mycale microsigmatosa*. Each graph indicates a single experiment. Experiments III and VI were conducted with the same piece of sponge, just like exp. IV and VII. Green line is a linear regression.

NH_4 change in time -*Merlia normani*-

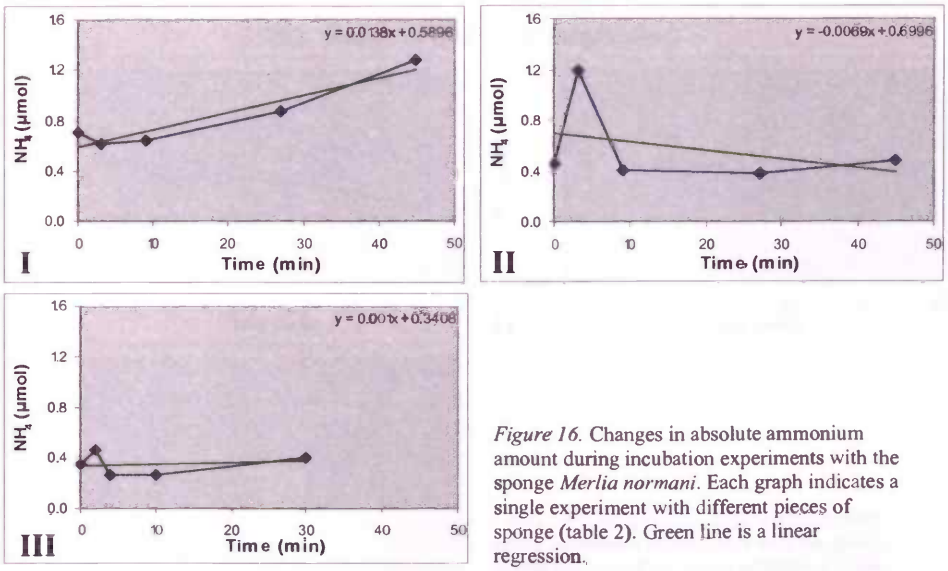

Figure 16. Changes in absolute ammonium amount during incubation experiments with the sponge *Merlia normani*. Each graph indicates a single experiment with different pieces of sponge (table 2). Green line is a linear regression.

NO_2 change in time *-Mycale microsigmatosa-*

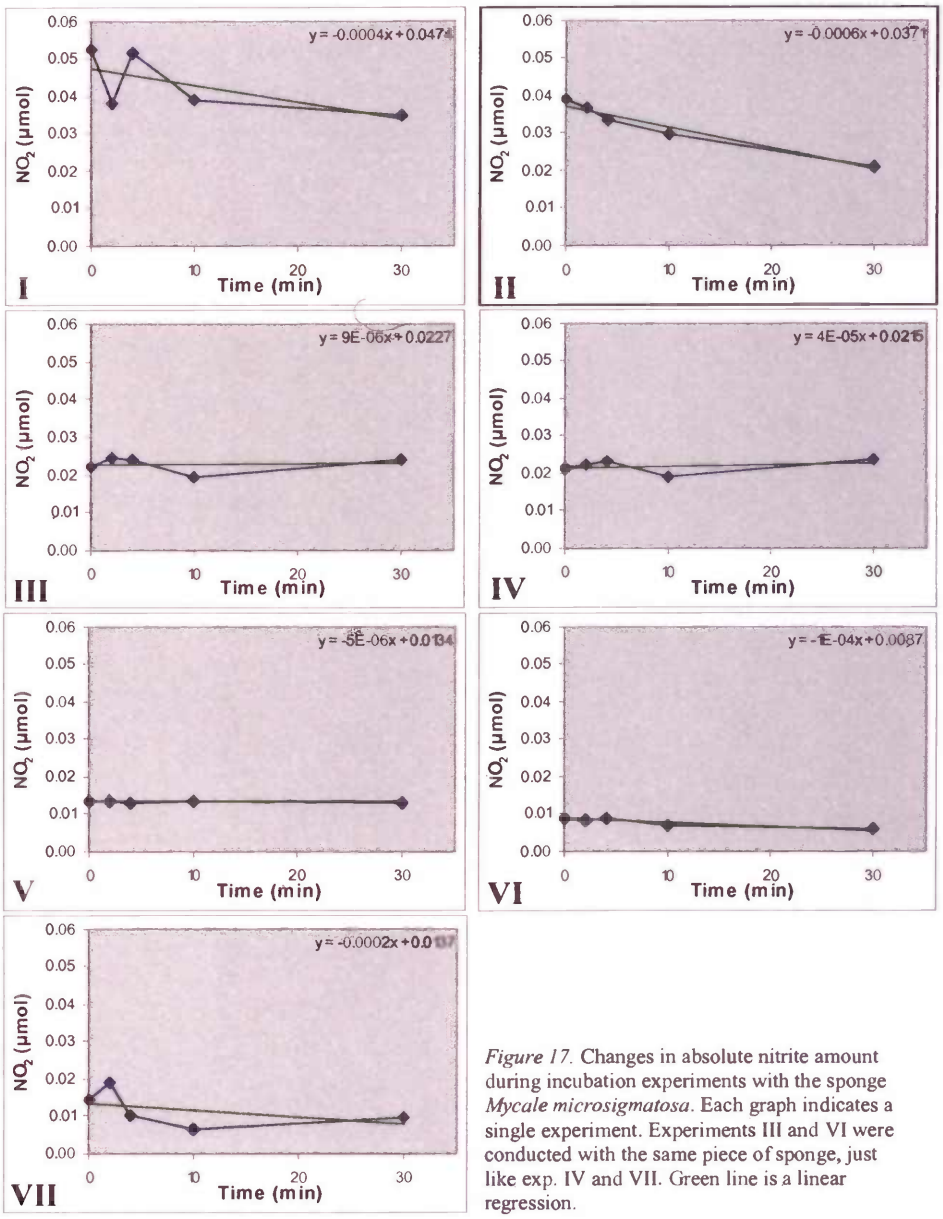

Figure 17. Changes in absolute nitrite amount during incubation experiments with the sponge *Mycale microsigmatosa*. Each graph indicates a single experiment. Experiments III and VI were conducted with the same piece of sponge, just like exp. IV and VII. Green line is a linear regression.

NO_2 change in time *-Merlia normani-*

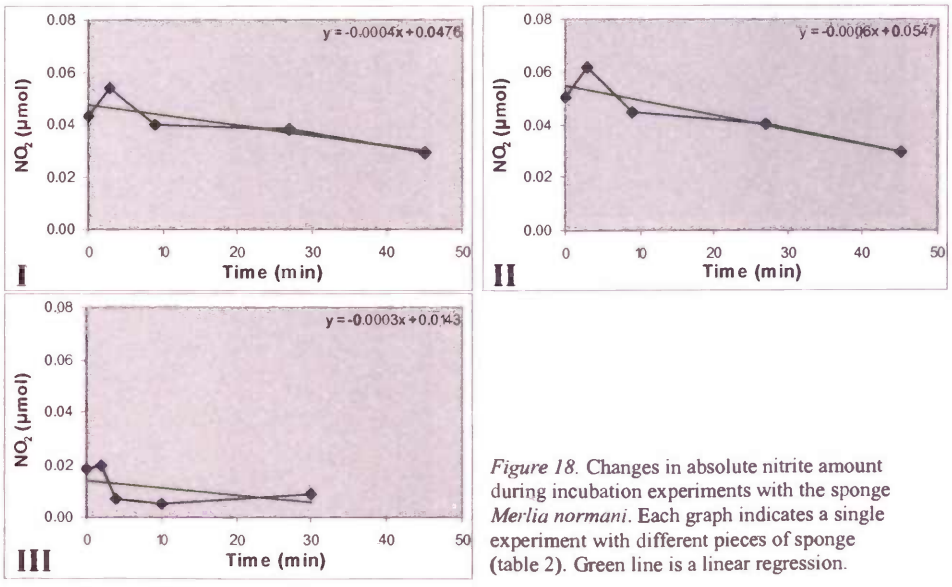

Figure 18. Changes in absolute nitrite amount during incubation experiments with the sponge *Merlia normani*. Each graph indicates a single experiment with different pieces of sponge (table 2). Green line is a linear regression.

NO_3 change in time -*Mycale microsigmatosa*-

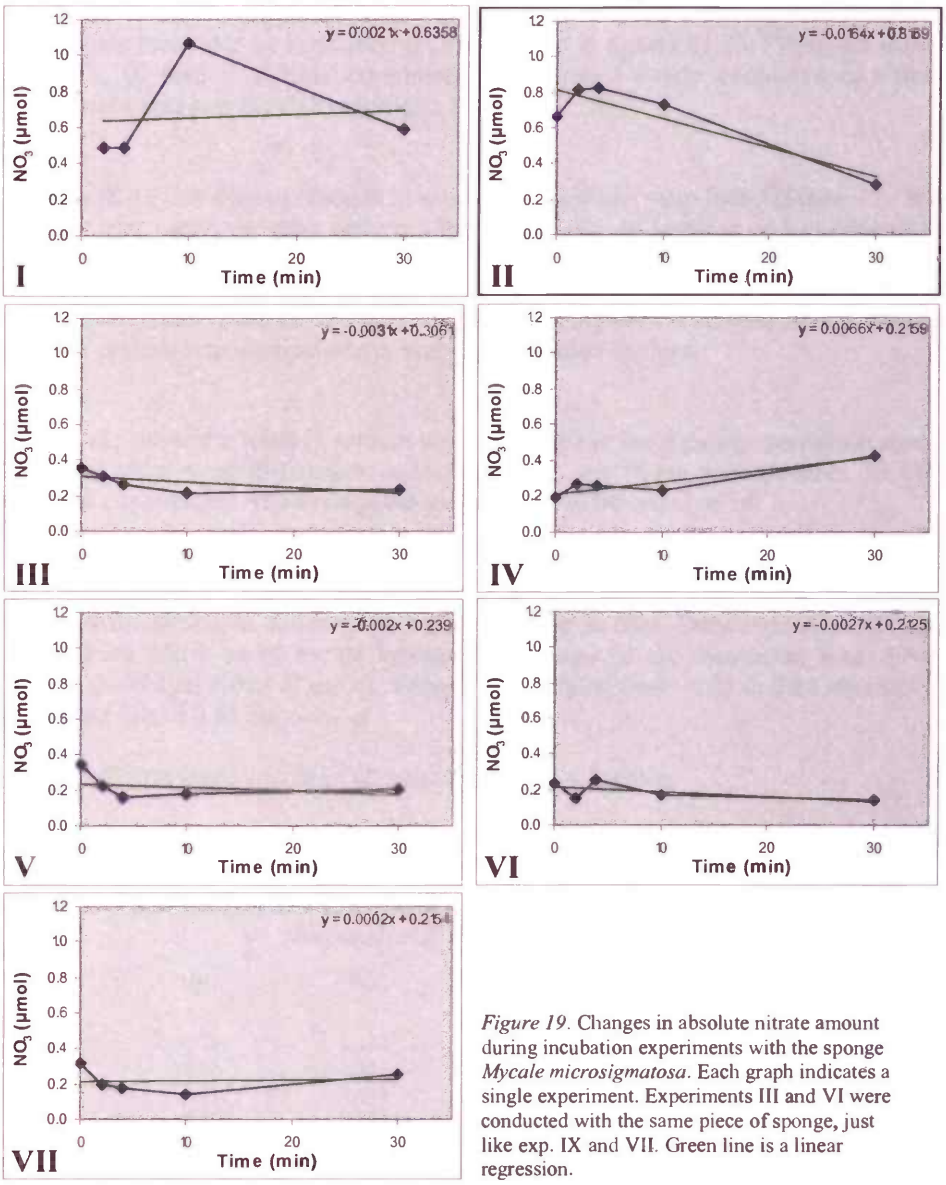

Figure 19. Changes in absolute nitrate amount during incubation experiments with the sponge *Mycale microsigmatosa*. Each graph indicates a single experiment. Experiments III and VI were conducted with the same piece of sponge, just like exp. IX and VII. Green line is a linear regression.

NO_3 change in time -*Merlia normani*-

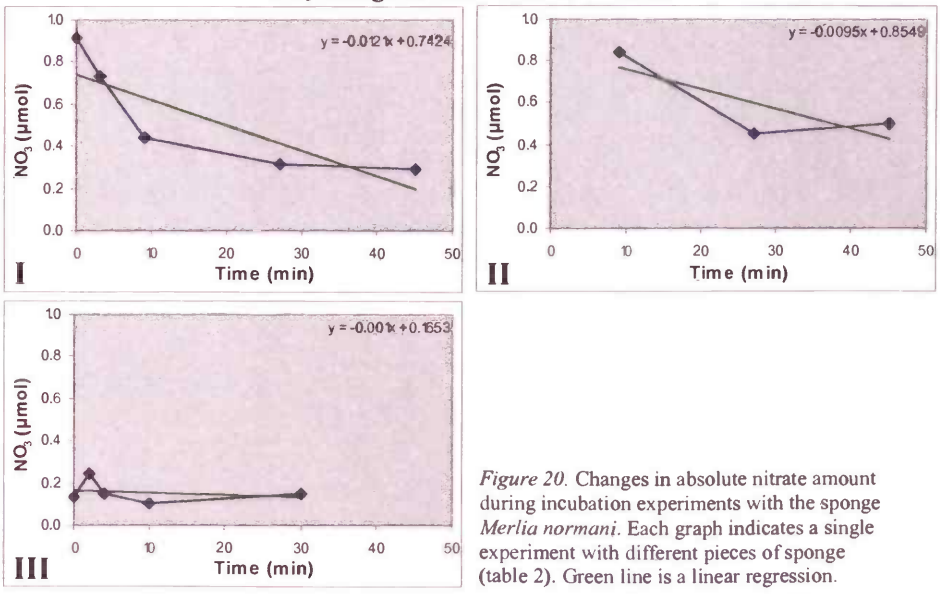

Figure 20. Changes in absolute nitrate amount during incubation experiments with the sponge *Merlia normani*. Each graph indicates a single experiment with different pieces of sponge (table 2). Green line is a linear regression.

4.3.5 Sediment

Two cavities at 15 meters depth were used to perform 6 enclosure experiments above sediment. The nutrient amounts found during incubation time are shown in figures 21-23. Fluxes are shown in table 7. Experiments I, III, and V and the experiments II, IV and VI were conducted in different cavities. Sediment surface area was for all experiments 81.7 cm^2.

Ammonium

In three trials (II-IV) no distinct changes in ammonium amounts were found (figure 21). Whereas in the first and last trial variations were observed beginning with an increase and in experiment V with a decrease. In four experiments there was on average an ammonium release net from the sediment, with fluxes ranging from -0.74 to 1.41 mmol m^{-2} d^{-1}. The average flux was 0.27 mmol m^{-2} d^{-1}. Although the experiments were done in two cavities no differences were observed between the cavities. Variations in fluxes within cavities were comparable to variations between cavities.

Nitrite

All experiments showed a roughly smooth increase in nitrite level during incubation time (figure 22), although there were some differences in the steepness. All fluxes were positive, so nitrite was net released in all experiments. The average release rate was 0.094 mmol m^{-2} d^{-1}.

Nitrate

The nitrate amounts were variable within the experiments (figure 23). In five trials an increase was observed after the enclosure, followed by a decrease later in time. Only experiment III had a decrease in the beginning and a small nitrate increase at the end of the incubation time. Five of the six experiments showed an efflux of nitrate. Fluxes were ranging from -0.35 to 2.24 mmol m^{-2} d^{-1}, with an average release rate of 0.65 mmol m^{-2} d^{-1}.

On average sediment was a source of all inorganic nitrogen species.

Table 7. Fluxes of cavity sediment with a surface area of 81.7 cm^2.

# Experiment	Flux (mmol m^{-2} d^{-1})		
	NH_4	NO_2	NO_3
I	0.78	0.123	1.16
II	0.16	0.053	0.69
III	0.14	0.053	-0.35
IV	-0.14	0.035	0.16
V	-0.74	0.053	0.01
VI	1.41	0.247	2.24
Average	0.27	0.094	0.65
SD	0.75	0.081	0.94

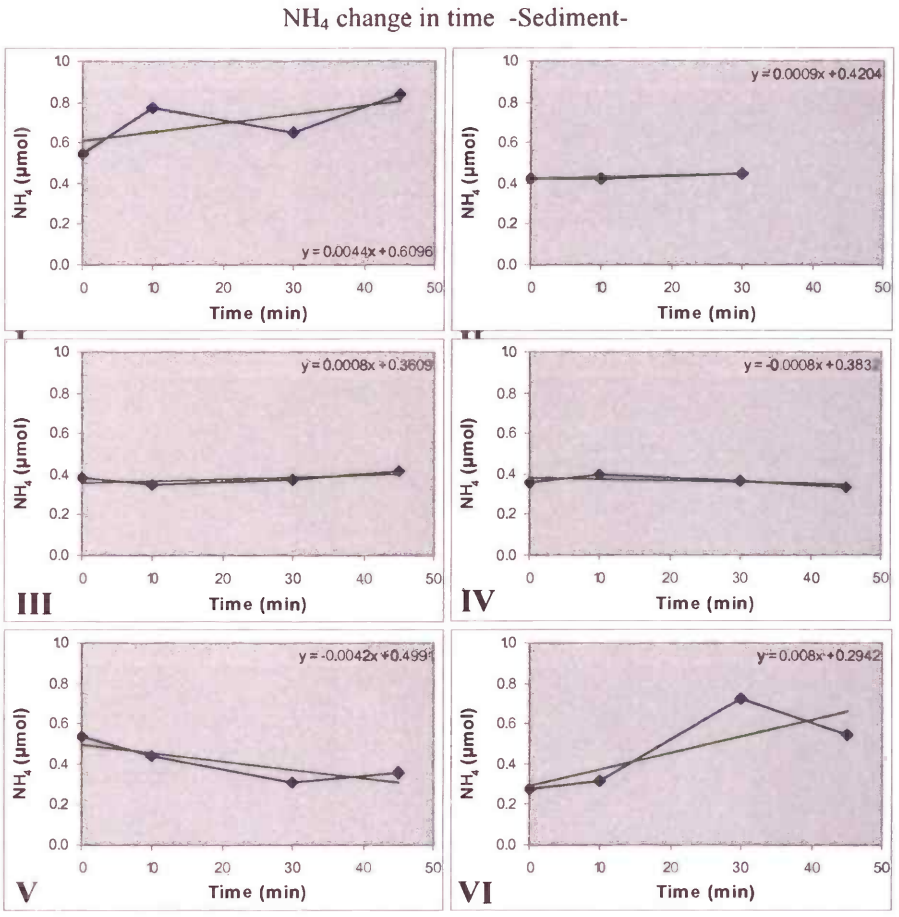

Figure 21. Changes in absolute ammonium amount during enclosure experiments above sediment. Each graph indicates a single trial of which experiments I, III and V and experiments II, IV and VI were conducted in different cavities at 15 meters depth. The green line is a linear regression.

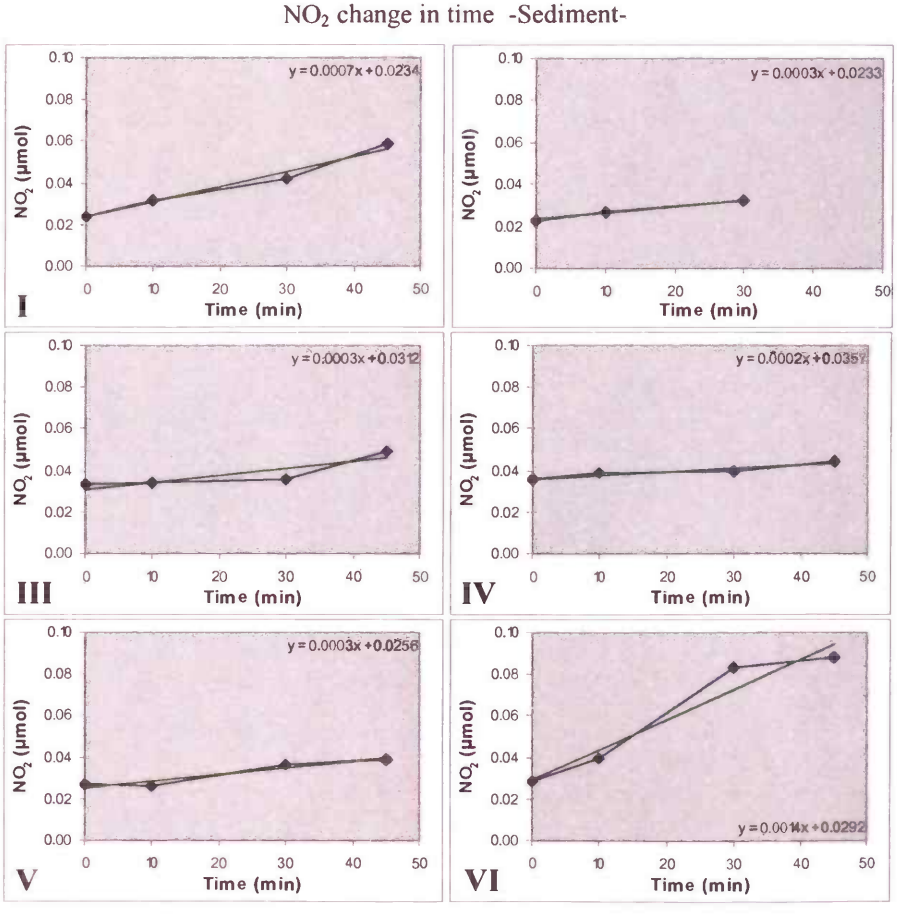

Figure 22. Changes in absolute nitrite amount during enclosure experiments above sediment. Each graph indicates a single trial of which experiments I, III and V and experiments II, IV and VI were conducted in different cavities at 15 meters depth. The green line is a linear regression.

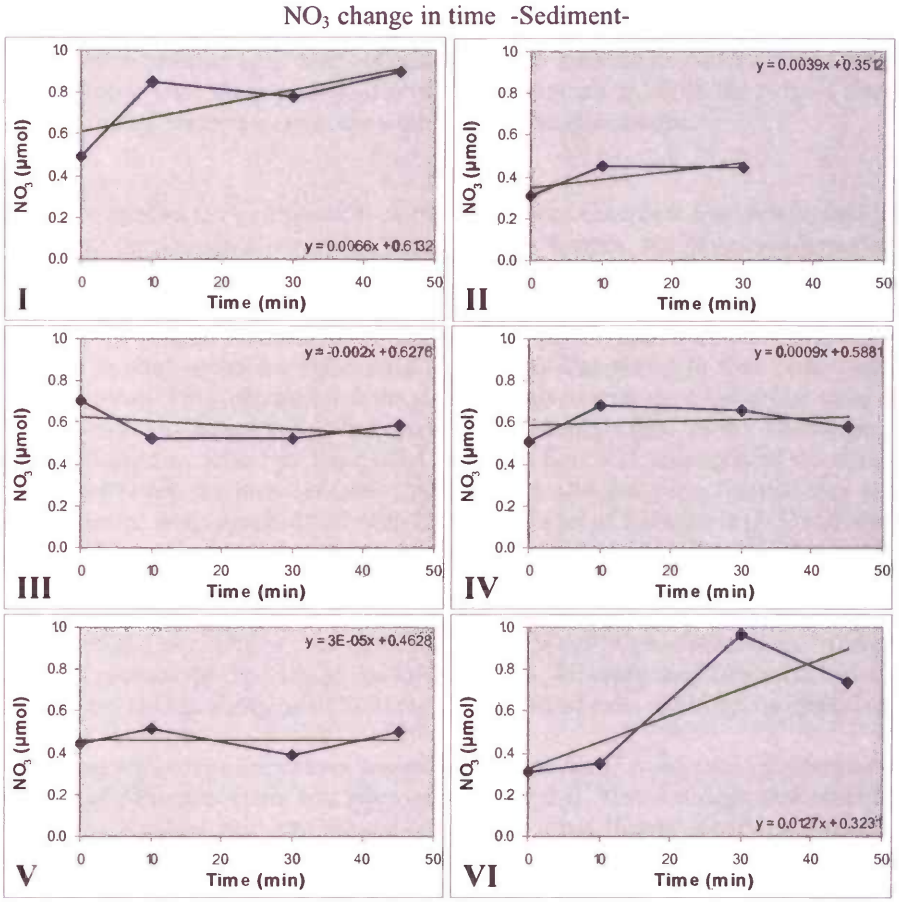

Figure 23. Changes in absolute nitrate amount during enclosure experiments above sediment. Each graph indicates a single trial of which experiments I, III and V and experiments II, IV and VI were conducted in different cavities at 15 meters depth. The green line is a linear regression.

4.4 Bacterial communities

The bacterioplankton compositions in the water before closure of the incubation chamber and at the end of the incubation time were determined. Also bacteria associated with sponges were established. Both especially for the presence of nitrifying communities. With the help of fluorescent probes and a DAPI staining, bacteria were made visible under the microscope.

4.4.1 Water

The bacterioplankton composition in the incubation chambers was determined before closure and at T=30. For the sponge *H. caerulea* this was done 7 times, for *M. microsigmatosa* 2 times and for *M. normani* once. Also the bacterioplankton compositions of all added nitrapyrin experiments (9 times) were determined. Further, samples were checked for the bacterial composition in the water above the sediment (2 times). Bacteria were stained with DAPI and with particular fluorescent probes. The negative control probe for Eubacteria (NonEUB) was tested in four water samples and all gave a negative result. This indicated that the signals received with the EUB-probe were indeed all Eubacteria. A EUB-mix was tested twice, but gave a comparable signal as the EUB-I probe alone. So further samples were only tested for the EUB-I probe. In figure 25 photographs are shown of bacteria present in the reef water, the blue cells are DAPI stained, and the green fluorescence ones are tagged with a specific probe and a tyramide. Picture 25B shows a lot of Eubacteria ($5.53x10^5$ bacteria ml^{-1}), although not as much as the DAPI-staining shows (picture 25A; $1.26x10^6$ ml^{-1}) of the same place on the filter. The β-Proteobacteria had a contribution of $8.13x10^3$ bacteria ml^{-1} (25D), their were $4.11x10^4$ γ-Proteobacteria ml^{-1} on average on the filters (25F) and $1.62x10^2$ Nitrospira ml^{-1} (25H). Archaea were also present; $1.40x10^4$ Euryarchaeota ml^{-1} and $3.56x10^2$ Crenarchaeota ml^{-1} (no pictures shown). This group of prokaryotes had 1% of the DAPI counts. Whereas the Eubacteria had an average percentage of 44 of the DAPI counts. So 55% of the DAPI stained cells could not be identified.

More than 90% of the Eubacteria present in the initial reef water could not be identified with the probes for β- and γ-Proteobacteria and Nitrospira (figure 24). This indicates that other bacterial groups were far more dominant than the groups of, among other things, nitrifying bacteria. Forty filters were counted (both before closure and after 30 minutes closure) of which 18 were treated with Nitrapyrin. However no differences were found between the counts with and without nitrapyrin.
The γ-Proteobacteria were with an average of 7.4% of the Eubacteria the most abundant group of the tested bacterial communities. β-Proteobacteria were for 1.5% present in the initial reef water and Nitrospira were very rare with only 0.03%. After 30 minutes incubation time there were small changes compared to the initial composition. About 3% more γ-Proteobacteria were present of the total Eubacteria and a half percent more β-Proteobacteria, while the amount of Nitrospira was comparable.

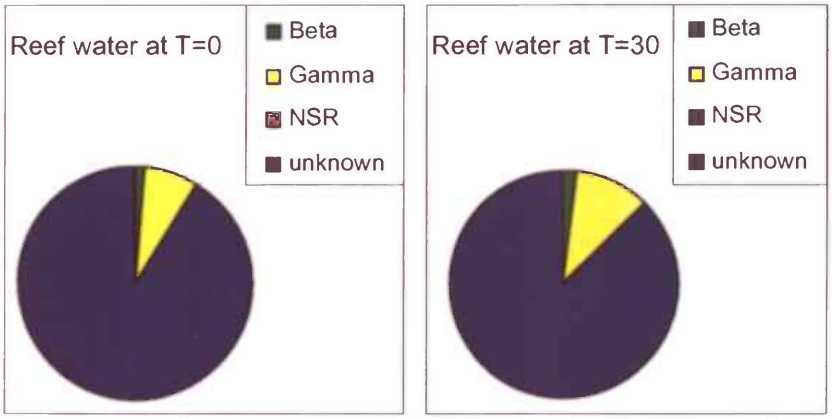

Figure 24. Diagrams showing the relative amount of three bacteria groups as percentage of the total Eubacteria in the initial reef water (left) and after incubation time of 30 minutes (right). Green are β-Proteobacteria, yellow are γ-Proteobacteria and red are Nitrospira. Blue parts are Eubacteria counts which could not be identified with any of the just mentioned probes.

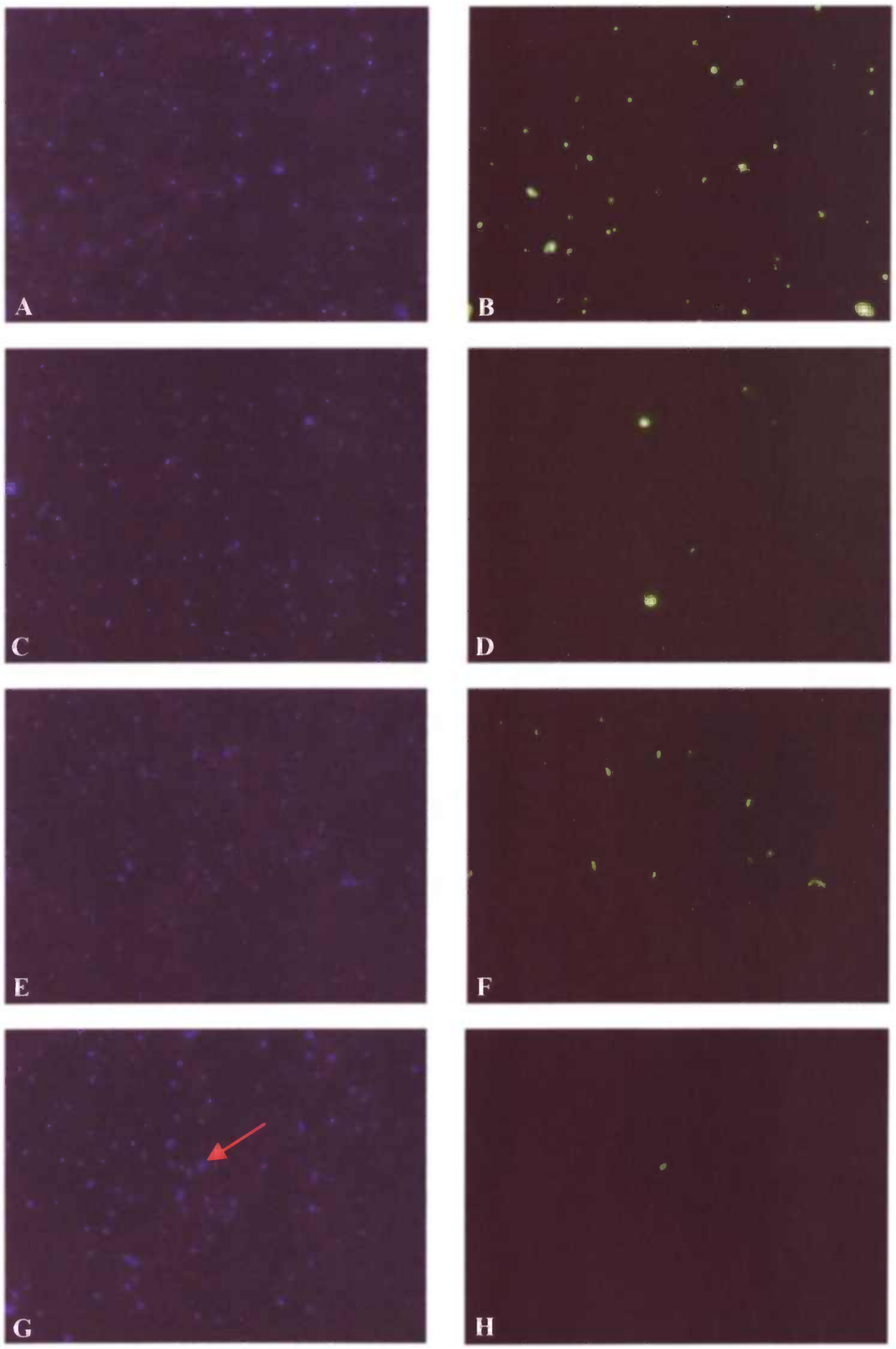

Figure 25. Epifluorescence micrographs of bacteria from reef water visualized with DAPI-staining (A,C,E,G) and Alexa tyromide labelled HRP-probes (B,D,F,H) on polycarbonate filters. Reef water samples were taken before incubation (A-F) or after (G-H) incubation with the sponge *H. caerulea*. Bacteria were hybridized with the EUB338 probe (A and B; Eubacteria), the BET42a probe (C and D; β-Proteobacteria), the GAM42a probe (E and F; γ-Proteobacteria) and the NSR1156 probe (G and H; Nitrospira). Red arrow (G) points at the Nitrospira shown in H.

4.4.2 Sponges

Of eight sponge species the associated bacterial communities were determined. The absolute Bacteria and Archaea numbers are depicted in table 8. Comparable pictures were found of bacteria associated with sponges as depicted in figure 25 (water samples). With the remark that there was more background signal derived from the sponge tissue. Only 62% of the DAPI counts could be identified by either Eubacteria (61%) or Archaea (1%).

Most Bacteria were found in the sponge species *H .caerulea* with an average of $3.78x10^8$ bacteria cm^2 sponge and *H. haeliophila* had the fewest bacteria associated ($5.92x10^6$). For every sponge Nitrospira, β- and γ-Proteobacteria were found, although numbers differed a bit. Of *H. caerulea* 7 pieces of sponge were examined, of which three got in contact with nitrapyrin. These three showed less abundance of most examined groups, suggesting that nitrapyrin influenced the bacteria.

To get an idea about the bacterial composition in each examined sponge the used probes were portrayed as average percentage of the total associated Eubacteria (figures 26). The green pieces are β-Proteobacteria, the yellow pieces are γ-Proteobacteria and the red pieces are Nitrospira. While the blue part of the pie is the residual amount of Eubacteria not detected by a more specific probe. *H. caerulea* had very few bacterial groups containing nitrifiers relative to the total Eubacteria indicating other bacteria groups were more dominant. The *Cliona sp.* contained relatively most of the examined groups. The γ-Proteobacteria were in most sponges more present compared to the other investigated groups, except for *M. normani* and *H. haeliophila*. The relative amount of Nitrospira was very different between the sponge types.

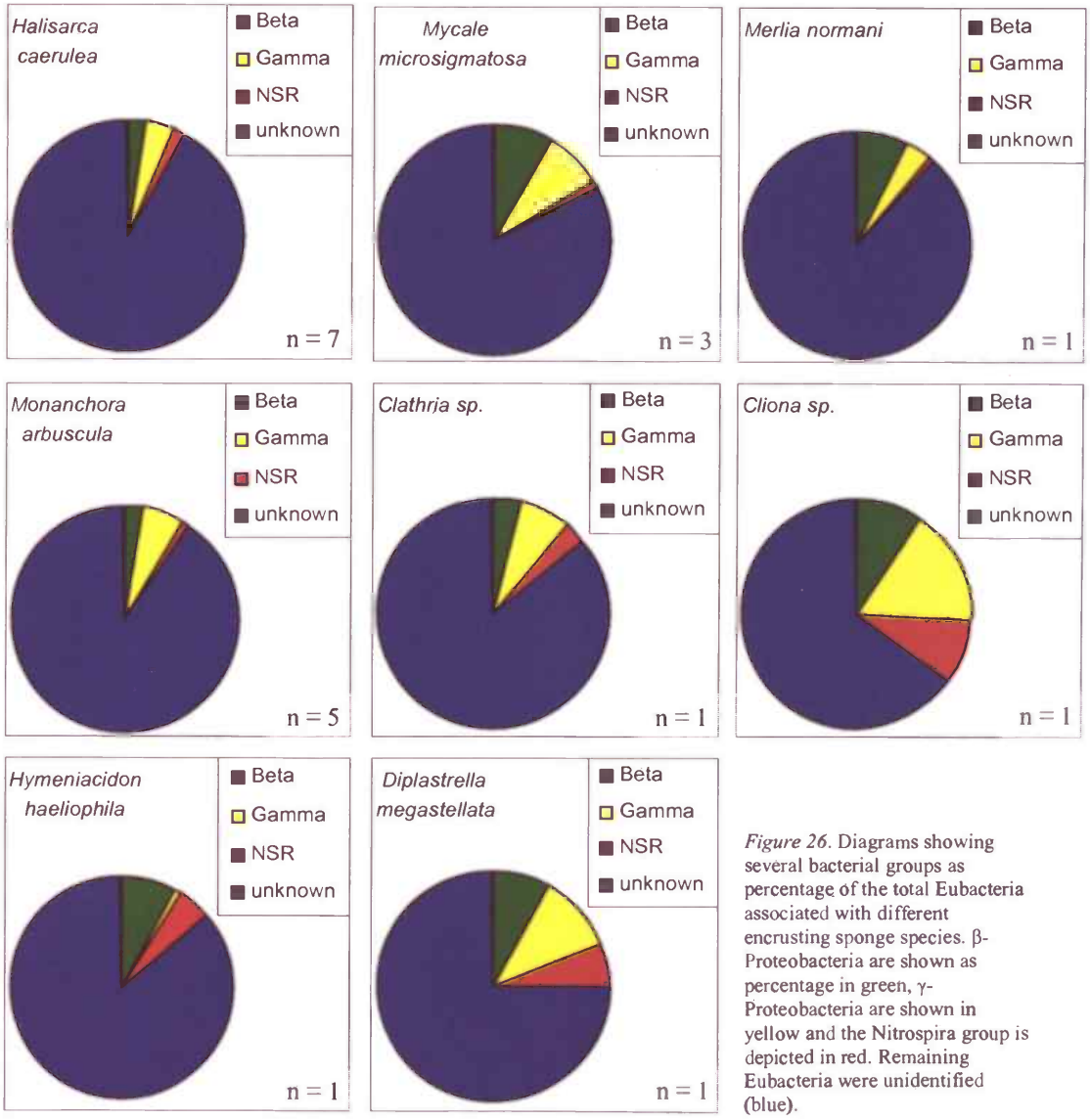

Figure 26. Diagrams showing several bacterial groups as percentage of the total Eubacteria associated with different encrusting sponge species. β-Proteobacteria are shown as percentage in green, γ-Proteobacteria are shown in yellow and the Nitrospira group is depicted in red. Remaining Eubacteria were unidentified (blue).

Table 8. Average numbers of Bacteria and Archaea per cm^2 sponge surface area.
n is the amount of examined sponges of that species.

Sponge species	*n*	DAPI	Eubacteria	β-Proteobact.	γ-Proteobact.	Nitrospira	Euryarchaea	Crenarchaea
H.caerulea (- Nit)	4	$5.13x10^8$	$4.17x10^8$	$1.04x10^7$	$1.68x10^7$	$7.11x10^6$	$4.23x10^6$	$3.21x10^6$
H.caerulea (+ Nit)	3	$5.97x10^8$	$3.25x10^8$	$7.89x10^6$	$1.13x10^7$	$5.01x10^6$	$4.79x10^6$	$4.51x10^6$
M.microsigmatosa	3	$5.36x10^8$	$3.48x10^8$	$2.94x10^7$	$2.74x10^7$	$4.28x10^6$	$2.58x10^6$	$4.01x10^6$
M.normani	1	$3.71x10^8$	$2.17x10^8$	$1.65x10^7$	$7.44x10^6$	$2.08x10^6$	$1.91x10^6$	$9.93x10^5$
M.arbuscula	5	$4.59x10^8$	$2.03x10^8$	$4.93x10^6$	$1.28x10^7$	$1.79x10^6$	$7.44x10^5$	$3.45x10^6$
Clathria sp.	1	$5.15x10^8$	$3.15x10^8$	$1.24x10^7$	$2.28x10^7$	$9.48x10^6$	$1.32x10^6$	$3.21x10^6$
Cliona sp.	1	$4.13x10^8$	$2.14x10^8$	$2.03x10^7$	$3.57x10^7$	$1.88x10^7$	$1.47x10^7$	$1.19x10^7$
H.haeliophila	1	$9.74x10^6$	$5.92x10^6$	$4.84x10^5$	$4.01x10^4$	$2.91x10^5$	$2.96x10^4$	$1.05x10^5$
D.megastellata	1	$1.60x10^7$	$1.26x10^7$	$1.01x10^6$	$1.36x10^6$	$7.57x10^5$	not examined	not examined

5 Discussion

5.1 Filter rates of the sponges

The coral reef water contained in these experiments on average about $1.26x10^6$ bacteria ml^{-1}. Compared to the normal seawater values (1 to $5x10^6$ bacteria per ml) this reef water belongs to the 'clean' regions of the sea (Reiswig, 1974). However when compared to reef overlaying water the found concentration is rather high (Gast *et al.*, 1999).

The average decrease of bacterial concentration of 62% after 30 minutes incubation time indicates that the sponges filtered well. Especially in the first minutes the sponges filtered a lot of bacteria out the water, within 4 minutes an average decrease of 15% is observed. In the remaining time series fewer bacteria were consumed. So the observed exponential decrease of bacteria suggests the sponges had constant filter rates. The average disappearance rate over 30 minutes incubation was $2.53x10^4$ bacteria ml^{-1} min^{-1}, somewhat higher than observed during cavity closure experiments ($1.43x10^4$ bacteria ml^{-1} min^{-1}; Scheffers *et al.*, 2004). Also the percentage bacterial disappearance was higher after 30 minutes closure; 62% in sponge experiments and 50-60% for cavity closure (Scheffers *et al.*, 2004). These differences are small, but can be explained by the sponge surface - water volume proportion. The mean surface area of the sponges in the incubation chambers was 24.3 cm^2. Accordingly there was 0.24 m^2 sponge surface area l^{-1} enclosed reef water in contrast to a sponge water relation in cavities of 0.09 m^2 sponge surface area l^{-1} (Scheffers *et al.*, 2004). This means that the water in the incubation chambers passed the sponge more times compared to the natural situation in cavities and accordingly the water was cleared more times for bacteria.

One incubation experiment (*H. caerulea* nit III) differs from the others with added nitrapyrin, as in the first minutes no bacterial decrease is detected (see figure 7). This could be an effect of the added nitrapyrin which may influence the sponge, causing disturbance of the filtering activity in the first minutes.

5.2 Inorganic nitrogen dynamics

5.2.1 Sponge experiments

Sponges mineralize organic matter to inorganic compounds of nitrogen, phosphor and carbon. Ammonium is excreted by the sponge as a waste product. It was suggested that this ammonium and the ammonium already present in the reef water is being converted to nitrite and nitrate by nitrifying bacteria associated with the sponges. So during the incubation experiments with sponges it was expected to observe a nitrate increase in time.

In all experiments the sponges were attached to a piece of dead coral. To investigate whether this substrate, the container or possibly the reef water influenced the experiment several controls were conducted with only a piece of dead coral. All inorganic nitrogen species showed a decrease in time, indicating that more uptake of the nutrients occurred than was released. The results of the sponge experiments can therefore not only be ascribed to the sponges. Instead, they are a combination of processes of the sponge and of the substrate, container and/or reef water. The sponge results were therefore corrected for the control experiments. Accordingly, the fluxes of al inorganic nitrogen species became substantial larger. This indicates that in reality more nutrients were released than the basic data suggest. However since only three control experiments were conducted and variations were found between these controls the results both with and without control correction will be discussed.

The sponge *Halisarca caerulea* showed after control correction for all experiments an ammonium efflux. For nitrite and nitrate the fluxes of 11 of the 12 trials were positive after control correction. The results before control correction also suggest an average inorganic nitrogen efflux. So overall a net release of all inorganic nitrogen species was observed. These results are in line with the expectations, and suggest the presence of nitrifying bacteria.

For *Mycale microsigmatosa* similar results were found after control correction. Ammonium and nitrate fluxes were in 6 of the 7 experiments positive, indicating the presence of nitrification. However, for nitrite no clear release was observed, with 4 positive fluxes, two negative and 1 neutral fluxes. When not corrected for control experiments other conclusions can be drawn. The negative fluxes for nitrite and nitrate suggests no presence of nitrification. For this sponge species no clear conclusions can given about the presence of nitrate effluxes.

Merlia normani showed in 2 of the 3 experiments an ammonium efflux and for nitrate in all experiments effluxes were observed after control correction. Nitrite fluxes were although negative for all trials after control correction. As well as the sponge *M. microsigmatosa* also *M normani* showed other results when not corrected for controls. In all experiments negative nitrate fluxes were observed before control correction. So also for this sponge species no clear conclusions can given about the presence of nitrate effluxes.

Large fluctuations in inorganic nitrogen courses were observed during most sponge incubation experiments. These fluctuations indicate that the inorganic nitrogen species were either released or absorbed. So at least two processes played a role. At one moment the release was higher and at the other moment the uptake of ammonium was dominant. The observed inorganic nitrogen courses were therefore a product of more processes. A release of ammonium can be declared by the fact that organisms (sponges and micro organisms associated with sponges) excrete ammonium as a metabolic waste product. Uptake of ammonium is possible by micro organisms associated with the sponges. Nitrite is produced by the ammonia oxidizing bacteria and converted to nitrate by the nitrite oxidizing bacteria. Nitrate can accordingly be taken up by bacteria and algae. All bacteria need nitrogen for their growth and they sequester it in the form if ammonium, nitrate and/or amino acids (Tungaraza *et al.*, 2003). Algae prefer nitrate as a nitrogen source, it is however assumed that they are hardly present in cavity sponges, since algae need light for photosynthesis. From open reef sponges it is although known that algae are living inside sponges. Open reef water was used for the experiments and here are algae present, so they can also contribute to a nitrate decrease.

Especially in the first minutes of most incubation experiments (large) peaks are found for the inorganic nitrogen species. This is probably an effect of the changing circumstances, as the incubation chamber is closed. The sponge might react on it and accordingly the micro organisms inside them. Of some bacteria it is known that under limiting food supply their metabolic enzymes are absent. Accordingly when they are placed into an enriched environment it takes them minutes to hours to produce these enzymes and digest the organic matter (Madigan *et al.*, 2000). This could be an explanation for the peaks during the first minutes of incubation. However for nitrifying bacteria the enzyme activity stays 8 to 72 hours stable while the bacteria are starved (no ammonium supply) (Bothe *et al.*, 2000). It is not probable that the bacteria were so long under starved conditions since sponges always produce ammonium as a waste product.

The AOB and NOB, so far discussed, are chemolitho-autotrophic bacteria. However these nitrifying bacteria are not the only groups that can produce nitrate (Focht and Verstraete, 1977; Robertson *et al.*, 1988). From some heterotrophic bacteria it is known that they can also oxidize ammonium and organic components to nitrite and nitrate. Heterotrophic nitrifiers are known for their ability to nitrify and denitrify simultaneously (Robertson *et al.*, 1988). Yet also was shown that the autotrophic AOB *N. europaea* and *N. eutropha* (and probably other AOB) could nitrify and denitrify simultaneously when grown under oxygen limitation. So in that situation no nitrate release is observed at all (Bock *et al.*, 1995). However sponges constantly filter and supply associated bacteria with oxygen rich water and food. So it is assumed that the AOB associated with sponges have no oxygen limitation and it is therefore not probable that they denitrified as well.

5.2.2 Comparison of sponge experiments with and without nitrapyrin

The experiments with added nitrapyrin were conducted to investigate whether an observed nitrate efflux within the normal experiments was due to nitrification processes. Nitrapyrin inhibits namely the oxidization of ammonium to nitrite. There are large differences between the experiments with the sponge *H. caerulea* both with and without added nitrapyrin. However a trend is observed that with the added nitrapyrin no nitrite and nitrate production occurred, when not corrected for control data (figure 27, table 4). This indicates that the nitrapyrin inhibited the ammonium oxidation and that the released nitrite and nitrate are indeed caused by nitrification processes. After correction for controls the nitrapyrin added nitrate flux was positive, although the fluxes were indeed lower than the flux without added nitrapyrin. Probably the time period was too short to measure a clear nitrate decrease.
The ammonium fluxes were not as the hypothesis explained in the introduction. It was expected that with added nitrapyrin more ammonium would be released, because of the inhibited ammonium oxidation. This was however not the case. The average ammonium fluxes of the experiments without nitrapyrin were larger. Probably the nitrapyrin not only stops ammonia oxidation but also other processes, where ammonia is produced as a waste product.

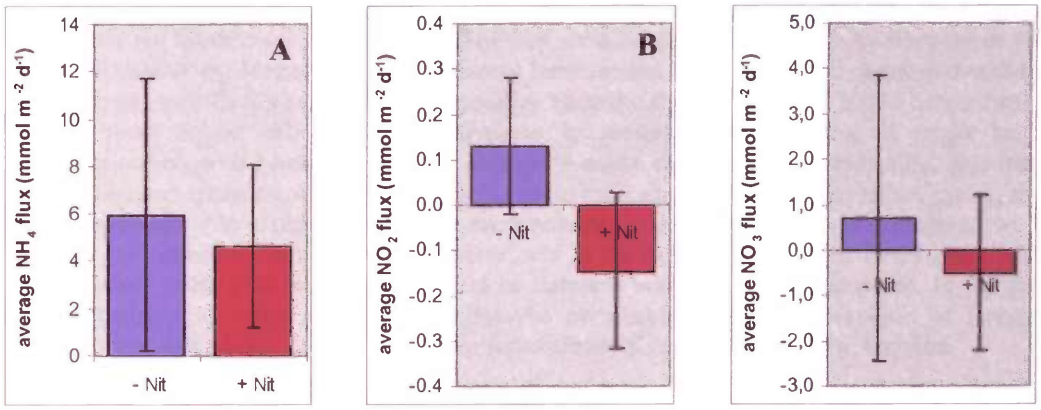

Figure 27. Average fluxes of ammonia (A), nitrite (B) and nitrate (C) of experiments with the sponge *H. caerulea* without (blue bars) and with (red bars) added nitrapyrin (before control correction). Standard deviations shown are based on twelve experiments without and three experiments with added nitrapyrin.

5.2.3 Sediment experiments

The DIN levels of the experiments conducted above cavity sediment do indicate that nitrifying processes were taken place in the sediment. In two experiments (# I and VI) clear nitrate effluxes were observed. The nitrate amounts are although lower than those of the sponge *H .caerulea*. This suggests that in the sponges more nitrate is formed per unit of time than in the sediment. However there were no nitrapyrin treated experiments done to examine the origin of nitrate. Also no bacterial samples for CARD-FISH were taken to investigate the presence of nitrifying bacteria in the sediment. Instead nitrate could already have been present in the sediment or originated from ground water. On the other hand for nutrient rich coastal waters is known that sediments contain nitrifying bacteria, so it is likely that these bacteria are present in cavity sediment as well. So when explaining the efflux of nitrate from cavities, it should not be ignored that a nitrate increase was observed from the sediment.

5.3 Bacterial communities in reef water and sponges

With DAPI-staining more bacteria were counted when compared to the Archaea and Eubacteria HRP-probes of CARD-FISH together, while one expect them to be equal. For the water samples an average of 45% Eubacteria and Archaea of the DAPI stained cells was found and for the sponge samples this was 62%. So 55% of the DAPI-stained cells in the water and 38% of DAPI-stained cells in sponges could not be identified with specific HRP-probes.

An explanation of the difference between DAPI-staining and CARD-FISH could be that although CARD-FISH gives a better signal than FISH, this method is still not optimal. Pernthaler *et al.* report (2002) that with a tyramide concentration of 1:10 CARD-FISH shows about 86% of the DAPI-stained cells. Since in the present study a tyramide concentration of 1:150 was used it would be plausible that a less percentage of CARD-FISH signal was obtained in comparison to DAPI. However this does not explain the difference between the signal percentages of the water and the sponges, since the same concentration of tyramide was added. In the water another bacteria population is present than in sponges, and since every bacteria is different they can also react different on the CARD-FISH method. Small bacteria and bacteria with a thick cell membrane are more difficult to detect (Madigan *et al.*, 2000).

Another explanation for the difference in signal between DAPI and CARD-FISH is the distinct colouring method. With a DAPI-staining nucleonic acids are coloured from any organism, both death and alive, in contrast to FISH which only stains organisms which were active (Madigan *et al.*, 2000). Sponge samples have been filtered over a 0.8µm filter, so most organisms other than bacteria are removed. Water samples however were not filtered. At these filters other organisms can be present indeed, like algae. Most algae are larger than bacteria and have other shapes. But there is a possibility that some of them were mistakenly counted as bacteria.

Moreover most of the difference will be made by the dead bacteria, which are not detected by FISH, yet are stained by DAPI. This could lead to an overestimation of the DAPI counts. However also an underestimation of the Eubacteria is possible. In pure cultures it has been shown that FISH detects specific organisms with a high rRNA content, but may miss non-growing or starving cells (Eilers *et al.*, 2000). Since from the marine pelagic environment is known that not all bacteria are growing (Gasol *et*

al., 1995), FISH may not show the real abundances of the targeted organisms. This raises the question of whether the bacteria are not present or that they were simply not detectable by fluorescent probes.
The last possible explanation for the difference between the DAPI and FISH count is that the protocol used was not specifically tested for gram-positive bacteria (Pernthaler *et al.*, 2002). Since these bacteria have a more robust cell wall in comparison to gram-negative bacteria, it might be that the permeabilisation with lysozyme was not enough to make the membrane permeable. Accordingly the HRP-probe and tyramide were not able to penetrate the cell and attach to the rRNA giving the cell its signal. Although it is assumed the marine environment is composed mostly of gram-negative bacteria, the few gram-positive bacteria can also contribute to the difference between DAPI and CARD-FISH signals, since gram-positive bacteria should be detected with the Eubacteria probe. In the future it is recommendable to make gram-positive bacteria permeable with a combination of lysozyme and achromopeptidase (Sekar *et al.*, 2003) or with proteinase-K, normally used for Archaea.

Most of the bacteria in the reef water could not be identified with the probes for β- and γ-Proteobacteria and Nitrospira, indicating that other bacterial groups, like α-Proteobacteria, Acidobacteria and Bacteroides, are probably far more dominant. Most nitrifying bacteria belong to the examined groups, meaning that they are hardly present in the reef water. Since coral reef water is oligotrophic, not much dissolved inorganic nitrogen is present in the water for the nitrifying bacteria. It seems therefore logic that these bacteria do not live in the reef water, as low concentrations of DIN can limit the rate of productivity in the reef water (Zehr and Ward, 2002). Sponges produce ammonium as a metabolic waste product and inside sponges are indeed more (nitrifying) bacteria found compared to the surrounding water. The bacteria are here always certain of food supply and the sponges have the advantage that their poisonous waste product is removed.
In some experiments relatively more Nitrospira were found at the end of the incubation time, while in others no difference or a decrease was found. However these data are not very reliable, because there were very few Nitrospira found per observed filter section. For the β- and γ-Proteobacteria there were somewhat higher bacteria percentages found after incubation time, indicating the sponge had expelled these bacteria.

Between the sponge species differences in bacterial communities were found. The sponge *Halisarca caerulea* has relatively not much nitrifying bacteria, yet in the absolute amounts it has indeed many Nitrospira (table 8). The nitrate effluxes indicate that nitrifying processes have taken place inside this sponge type, so this is probably due to these nitrifying bacteria. *Mycale microsigmatosa* and *Merlia normani* have less Nitrospira and within the incubation experiments no clear nitrate effluxes were observed. This raises the question whether there were not enough nitrifying bacteria, that they were inactive or that absorption exceeded release of nitrate. It is unlikely that there were not enough nitrifying bacteria, since they also belong to the β- and γ-Proteobacteria, which were good represented in these sponge types. The initial ammonium concentrations were of the same order as within the *H. caerulea* experiments, it is therefore not realistic to think that the nitrifying bacteria were inactive due to starvation. Most probable explanation would be that the nitrate absorption exceeded the nitrate release making it more complex to examine this part of the nitrogen cycle.

Experiments with added nitrapyrin suggested an inhibition of nitrification. Eubacteria counts of these pieces of sponge show less bacteria as obtained from sponges without contact to nitrapyrin (see table 8). Probably the nitrapyrin did not only inhibit the nitrifying bacteria, but also killed many (others) as the DAPI-counts are about the same.

Cliona sp. contained most Nitrospira (table 8) and has probably most associated nitrifying bacteria. This was not expected, as *Cliona* species are bio-eroding sponges and not common in cavities (Van Soest, pers. comm.). *Diplastrella megastellata* also has relatively large amounts of Nitrospira and because it is thought that these sponges are exclusively for cavities and present in large amounts (Van Soest, pers. comm.) this sponge may have a considerable contribution to the nitrate efflux of cavities.

Bacterial counts of the sponges were corrected for the remaining bacteria of the pellet (see materials & methods). It is assumed that the bacteria composition of the pellet is identical to the observed composition obtained from the supernatant. However this is not examined and it is conceivable that larger and heavier bacteria remained more in the pellet.

5.4 Comparison with other studies

5.4.1 Inorganic nitrogen release rates

In cavity closure experiments nitrite and nitrate concentrations increased significantly in time, with average efflux rates of 0.03mmol m^{-2} cavity surface area (TSA) d^{-1} for nitrite and 1.41mmol m^{-2} TSA d^{-1} for nitrate. Ammonium concentrations were variable and did not show a consistent change over time in the experiments (values ranging from -2.00 to 0.34 mmol m^{-2} TSA d^{-1}) (Scheffers *et al.*, 2004).

When sponges account for the nitrate effluxes out of cavities, in the present study the same order of fluxes should be found.

The fluxes of *H. caerulea* were more or less in the same order (table 4 and 9). Ammonia and nitrite fluxes were somewhat higher, whereas nitrate fluxes were on average lower in the sponge experiments before control correction. After control correction all inorganic nitrogen fluxes were on average higher in sponges compared to the fluxes out of cavities. The other sponges, *M. microsigmatosa* (table 5) and *M. normani* (table 6), both showed somewhat higher ammonia fluxes and lower nitrite and nitrate fluxes before control correction compared to cavity fluxes (table 9). After control correction these sponges also showed higher nitrate fluxes.

Overall can be said that all inorganic nitrogen fluxes of the sponges are of the same order as the fluxes of cavities. So sponges and especially *H. caerulea* explain partially the nitrate efflux of cavities. The other part of cavity effluxes can be explained by nitrate effluxes from cavity sediment (table 9).

About one third of a cavity is composed of sediment and two third with filter feeders, like sponges. From this data approximately cavity fluxes can be calculated from the fluxes found in the present study. Accordingly the approximately cavity fluxes can be compared with the real found cavity fluxes (Scheffers *et al.*, 2004). A third of the average value of the sediment fluxes and two third of the average value of the sponge fluxes (not corrected for control experiments) was taken to calculate cavity fluxes. An efflux for ammonium (2.83mmol m^{-2} TSA d^{-1}) and nitrite (0.034mmol m^{-2} TSA d^{-1}) was obtained and an influx of nitrate (-0.24mmol m^{-2} TSA d^{-1}) as cavity values. Values are of the same order of magnitude. This suggests that the examined processes are in accordance with the results found after cavity closure. However after real cavity closure lower ammonia and higher nitrate fluxes were found. The sponge data used for this calculation are without control correction. When controls are taken along, a nitrate efflux is found when cavity closure is calculated, just like the real found fluxes. Besides there are more sponge species in cavities not examined in this study, which could have more or less nitrification.

Table 9. Minimum / maximum and average () fluxes of NH_4 and NO_x from cavities (Scheffers *et al.*, 2004), some sponge species and cavity sediment (present study). Fluxes of sponge species are without correction for controls.

	Flux (mmol m^{-2} d^{-1})				
	Cavity	*H .caerulea*	*M. microsigmatosa*	*M. normani*	Sediment
NH_4	-2.00 / 0.34 (-0.75)	-1.75 / 17.30 (5.97)	-4.16 / 10.15 (2.03)	-3.16 / 6.75 (1.54)	-0.74 / 1.41 (0.27)
NO_x	0.43 / 3.88 (1.42)	-5.40 / 6.39 (0.83)	-8.51 / 4.78 (-1.82)	-5.06 / -1.34 (-3.63)	-0.30 / 2.49 (0.74)

For many Caribbean reef sponges also NO_x effluxes are found. Especially the sponges *?Pseudaxinella zeai* and *Chondrilla nucula* show very high production rates, respectively 211 to 396mmol m^{-2} d^{-1} and 242 to 413mmol m^{-2} d^{-1}. These are the most active nitrification rates yet reported from the tropics (Diaz and Ward, 1997). Other nitrate rates known from sponges so far are 96mmol m^{-2} d^{-1} for *Chondrilla nucula* (Corredor *et al.*, 1988) and 0.48mmol m^{-2} d^{-1} for *Anthosigmella varians* (Corredor *et al.*, 1988). Also *Ircinia felix* and *Ircinia strobilina* had high rates of DIN release (Pile, 1997).

Kötter (2002) observed releases of all inorganic nitrogen species during enclosure experiments with several Caribbean reef sponges. She found a Total Inorganic Nitrogen (TIN) of 0.51 µmol g $AFDW^{-1}$ h^{-1}. The nitrate part of the TIN was about 20%. No significant difference of nitrate release between epi-reefal and cavity dwelling sponges was found. Therefore Kötter (2002) suggested that microbial sponge associations are no major nitrification pathway in framework cavities. In the present study larger nitrate fluxes were found with *H. caerulea* (0.66 µmol g $AFDW^{-1}$ h^{-1}). Also the presence of nitrifying bacteria in this sponge type indicates the occurrence of nitrification. Possibly there are differences between sponge species in nitrification amounts or the enclosures experiments influence the sponges too much to see nitrate increases.

All these results indicate that there are large differences found between sponge species. None of the sponges in the cited references have been tested for the presence of nitrifying bacteria, so this would be very interesting. Since in the present study no clear nitrate effluxes were found for *M. microsigmatosa* and *M. normani*, while nitrifying bacteria were present, questions can be drawn for the reliability of their fluxes. It could be that the sponges were affected to much by the enclosures or that other processes, like uptake of nitrate or denitrification were more dominant. However al these results together show that there are nitrifying bacteria present in sponges which could produce nitrate.

5.4.2 Microbial diversity in sponges

Bayer (2004) also looked at the presence of nitrifying bacteria in sponges and used the Mediterranean sponge *Aplysina aerophoba* for investigation. From this sponge is known that there is an efflux of nitrite and nitrate, so it was expected to find nitrifying bacteria in it. However she did not find any signal of the presence of these bacteria, while she did FISH as well as PCR. In the present study the nitrifying bacteria Nitrospira were found with CARD-FISH. Different methods and probes were used. So possibly there are Nitrospira in *A. aerophoba* when tested with suitable probe. It would although be strange if there are indeed no nitrifying bacteria in this type of sponge since it releases nitrate. Other processes with nitrate release are not known from sponges.

Friedrich *et al.* (1999, 2001) report a microbial diversity in the sponge *A. aerophoba* dominated by δ-Proteobacteria, followed by the γ-Proteobacteria, while no signals were obtained to the domain of Archaea, α- and β-Proteobacteria. In the present study δ-Proteobacteria were not examined and a high percentage of unidentified bacteria were observed. A substantial part of the unidentified bacteria may well be δ- and α- Proteobacteria. There are some nitrifying bacteria known, which belong to these Proteobacteria groups (Diaz, 1997; Hovanec and DeLong, 1996). However the majority of the bacteria in these groups are not capable of nitrification. So probably the α- and δ-Proteobacteria have a minor contribution to the nitrogen cycle.

Bacterial numbers in the tissue of the tube sponge *A. aerophoba* revealed $6.4x10^8$ bacteria per gram sponge (Friedrich *et al.*, 2001). Since 1 gram sponge is comparable with $1cm^2$ sponge in the present study (data not shown), bacterial abundance in the observed sponges are in the same range. This is again an indication that bacterial concentrations in sponges exceed those of seawater by two or three orders of magnitude.

5.4.3 Sediment

Dissolved inorganic nitrogen amounts of sediment have been studied before in different kind of sediments. Capone *et al.* (1992) report an ammonium efflux of Australian coral reef sediment of 0.03 to 0.009mmol m^2 d^{-1}, which was relatively high compared to previous studies. Two experiments showed higher rates (about 1.2 to 16.9 and 0.84mmol m^2 d^{-1}), but were conducted with anoxic respectively dark conditions. Sediment in coral reef cavities also show relatively high ammonium release rates compared to Capone *et al.* (present study: average of 0.27mmol NH_4 m^2 d^{-1}), although not as high as at anoxic conditions. The results of the study with dark conditions are actually in the same order of magnitude. This is expected since cavities have less light compared to the open reef. Rasheed *et al.* (2002) examined sediment inside cavities in the Red Sea and found efflux rates of 0.06mmol NH_4 m^2 d^{-1}, 0.01mmol NO_2 m^2 d^{-1} and 0.03mmol NO_3 m^2 d^{-1}.

For sediment in temperate coastal areas nitrogen rates have been found of 0.072 to 0.672mmol m^2 d^{-1} for ammonium and 0.204 to 0.552mmol m^2 d^{-1} for nitrate (Lohse *et al.*, 1993). In the present study some nitrate effluxes have been found of the same order of magnitude. The nutrient rich coastal areas are at this point similarly to cavity sediment, stressing the importance of the cavity sediment as a source of inorganic nitrogen for the oligotrophic reef water.

5.5 Recommendations

The sponge surface - water volume proportion in the enclosures of the present experiment was not according to the natural situation. In cavities the sponge cover is lower in relation to the available water. Since sponges in the enclosure filtered the water quickly their food supply decreased considerable. For future experiments smaller pieces of sponge or larger enclosures should be applied in order to keep the sponges at natural circumstances.
Nutrient concentrations were very low and accordingly measure errors could occur. Especially when sponge surface - water volume proportion is according to the natural circumstances, increases in inorganic nitrogen species will be hard to detect. An experiment with added ammonia can be conducted to solve this problem. The changes in inorganic nitrogen concentrations will accordingly be easily to detect. Furthermore, nitrifying bacteria have replete substrate for their production of nitrite and accordingly nitrate. So nitrification will probably not be limited and occur at maximum speed.
The time steps for sampling can be set at more distance, especially in the beginning of the incubation time. Probably large fluctuations occur in the beginning due to changing circumstances. A longer time series would also be interesting. At the end of the incubation time in the present experiments most nutrient levels increased, however it was often lower compared to the initial level. Probably nitrification occurs mostly when conditions are stable.

Enclosure experiments over cavity sediment demonstrated a nitrate efflux. It was however not examined if there were nitrifying bacteria present in this sediment so it would be interesting to do this the in future. Other processes like nitrate release of lower sediment layers could also play a role.

The sponge *Diplastrella megastellata* showed many Nitrospira inside, however no enclosure experiments have been done with this sponge species. Since this sponge is common in cavities it would be interesting to know if it also shows nitrate effluxes. When this is true it supports the suggestion that cavities are very important as a source of inorganic nitrogen on the coral reefs.

5.6 Conclusions

The present study showed variations in the inorganic nitrogen fluxes of sponges and sediment. Both influxes and effluxes occurred for ammonium, nitrite and nitrate. The sponge *Halisarca caerulea* showed clear effluxes of nitrate. For the other two sponges *M. microsigmatosa* and *M. normani* no clear nitrate releases could be detected. Nitrapyrin experiments showed that net nitrite and nitrate fluxes are due to nitrification processes. Lower nitrite and nitrate amounts were found compared to the experiments without nitrapyrin. In all investigated sponge species, including the three named above, nitrifying bacteria were found. Nitrospira, which oxidises nitrite to nitrate, were found in all species. Also β- and γ-Proteobacteria, groups containing most nitrifying bacteria, were present in all sponge species. So nitrification processes in sponges can be ascribed to nitrifying bacteria
Cavity sediment also showed nitrate effluxes, although they were smaller than of the sponge *H. caerulea*. It is yet unclear if these nitrate effluxes are due to nitrification processes.
All together it is demonstrated that sponges and to lesser extent cavity sediment are important sites for nitrate release and explain nitrate effluxes of cavities. Subsequently cavities are very important sites on the coral reef as a source of inorganic nitrogen.

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7 Acknowledgements

The work described here was the second research carried out for my doctoral exam at the Rijksuniversiteit Groningen. Special thanks go to my supervisors for there encouragement and advice; Ir. Jasper de Goeij, who learned me being creative when doing research under special conditions (e.g. under water and in a minimal laboratory) and Dr. Fleur van Duyl for her advices and for providing me the possibility of carrying out this study at the royal NIOZ. I also acknowledge the staff of the CARMABI foundation for the accommodation and research facilities at Curaçao and especially Brian and Carlos for there great help in all aspects. I am Dr. Rob van Soest of the University of Amsterdam very thankful for his support with taxonomy of sponges and with preparations of slides for spiculae identification. Arjan Smit (NIOZ) receives my gratitude for introducing me in the molecular technique CARD-FISH. Karel Bakker, Jan van Ooyen and Evaline van Weerlee (NIOZ nutrient lab) are thanked for the nutrient analyses. Govert van Noort (NIOZ) receives my gratitude for his help with and advice about microscopes. Finally many thanks go to my fellow students Hubert Eijkelenboom and Herbert van den Berg and supervisor Jasper de Goeij for their help and of course the really nice moments we experienced together at Curaçao and at the Potvis.

8 Appendix

8.1 Protocols

8.1.1 Fixation of water samples

Bacterial counts

- Put 10ml 0.2µm filtered seawater in a Petri dish and add 0.57ml 36% formaldehyde
- Take a 0.45µm, 0.25mm HA support filter and place in Petri dish
- Put 10ml sample water into a 15ml tube
- Add 0.57ml 35% formaldehyde solution (formalin)
- Add over an acrodisc 1ml acridine orange
- Wait for 5 minutes
- Filtrate sample water over a Sudan black filter (0.22µm, 0.25mm) and the support filter
- Place the Sudan black filter with emersion oil on a microscope slide
- Store at -20°C

CARD-FISH for water samples

- Put 10ml (T=begin) respectively 20ml (T=end) in a tube (so ~10^6cells will be filtered)
- Add 35% formaldehyde solution to a final concentration of 2-4% (respectively 0.57µl and 1.14µl)
- Fix 1-24 hours
- Filtrate sample on white polycarbonate (0.2µm, 25mm, GTTP) filter with cellulose nitrate support filter (0.45µm, 25mm, HA)
- Air dry GTTP filter
- Store filter with bacteria in a vial at -20°C until further processing

Nutrient analysis

- Put a 0.2µm, 0.25mm GTTP filter on a filter arrangement
- Clean filter glasswork and filters twice with 10ml 0.1M HCl
- Clean filter glasswork and filters twice with 10ml MQ
- Clean filter glasswork and filters twice with 10ml sample water
- Filter about 20ml sample water
- Rinse two pony vials twice with half the filtered sample water
- Put 5ml filtered sample water in each pony vial
- Store at -20°C until further processing

8.1.2 Fixation of sponge tissue

For determination

- Fix piece of sponge tissue (about 1cm^2) in 5ml 80% ethanol in a pony vial
- Store in dark at room temperature until further processing

For CARD-FISH

- Add 3 volumes of cold PFA to 1 volume of sample
- Fix sponge tissue for 30 minutes up to 12 hours at 4°C
- Wash sponge tissue twice with 1xPBS
- Store sponge tissue in pony vial in PBS/ethanol (1:1) at -20°C until further processing

8.1.3 Preparation of sponges for determination

- Cut with a scalpel a very thin piece of the upper layer of the sponge
- Place thin piece on a microscope slide
- Cut a very thin cross-section of the sponge tissue
- Place on a microscope slide
- (With encrusting sponges tissue is too thin and all tissue can be placed on a microscope slide)
- Flatten tissue pieces in between two microscope slides
- Dry in stove
- Put a large drop of Canada-balsam on piece of sponge tissue and cover with cover glass
- Dry in stove for at least two days
- Examine spiculae under light microscope (40x)

8.1.4 Separation of bacteria from sponge tissue

- Put piece of fixed sponge tissue into an eppendorf vial
- Add 200µl of Lysis-T (Sigma)
- Crush the sponge tissue with a clean rubber stick to dissociate cells
- Add Artificial Seawater (ASW) to dilute (about 200µl)
- Centrifuge at 5000rpm for about 30minutes
- Put supernatant into 15ml tube
- Resolve pellet in ASW (about 800µl)
- Pour resolved pellet into 15ml tube and fill to 10ml with ASW
- Filter 2x 5ml resolved pellet over 0.8µm, 0.25mm ATTP filters
- Place filters on a microscope slide
- Put a drop of DAPI-mix on the filters and store at -20°C
- Add filtered pellet water to supernatant
- Filter 2.5ml (or 5ml depending on the amount of cells) of supernatant water over a 0.2µm, 0.25mm GTTP filter supported by a 0.45µm, 0.25mm HA filter
- Dry GTTP filters by air and store at -20°C till CARD-FISH procedure start

8.1.5 CARD-FISH procedures

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1. Sample Fixation

1. Fix samples with 35% formaldehyde solution (formalin) to water sample to final concentration of 2-4% and fix for at least 1 but not longer than 24h.
2. Filtrate sample (for ~10^6 cells/ml 10ml will do) on white polycarbonate (25 mm 0.2 µm) filter with cellulose nitrate support filter (0.45 µm).
3. After sample filtration, wash with 5-10 ml of MQ.
4. Air-dry filters.
5. Store at –20°C until processing. Filters can be stored frozen for several months.

2. Embedding

1. Warm up 0.1% Agarose and pipette 30µl on petri dish. Dip filter with both sides in Agarose and place filters shiny side down on drop.
2. Let filters dry at 37°C in hybridization oven without lid for 10-15min.
3. Pour ethanol (95%) in petri dish and carefully remove filters.
4. Dry filters on blotting paper, now they can be stored at -20°C.

3. Permeabilization

1. Prepare 10ml of lysozym (Bacteria)/proteinase-K (Archaea) mix; after preparation put on ice.

Stock reagent	Volume (µl)	Final
Lysozym	100mg	10mg/ml
1M Tris-HCl	1000	0.1M
0.5M EDTA	1000	0.05M
MQ water	8000	

Stock reagent	Volume (µl)	Final
Proteinase-K	2	0.2 ul/ml
1M Tris-HCl	1000	0.1M
0.5M EDTA	1000	0.05M
MQ water	8000	

Tris pH 8.0

2. Pour lysozyme/proteinase-K in petri dish and place filters upside down into it incubate for max 1h at 37°C in hybridization oven.
3. After 1h wash filters in MQ (for proteinase-K, 3x in excess MQ).
4. Place filters for 10min. in 0.01M HCL at RT (20 min for proteinase-K).
5. Wash filters 2x in excess MQ.
6. Shortly dip them into Ethanol (95%).
7. Dry filters on blotting paper, now they can be stored at -20.

4. Hybridization

1. Prepare hybridization buffer (mind % formamide).
2. Cut 25mm filters in up to 8 sections and place them into 0.5ml Eppi (labeled).
3. Mix 300µl hybridization buffer + 15µl HRP-probe on ice. Probe to buffer ratio: 1:20. Freeze probes just once, after thawing store them at 4°C for up 0.5 years.
4. Hybridize at 35°C for 2-14h (overnight), stick darkened Eppis around rotor and shake slowly.
5. Prepare washing buffer:

Washing Buffer for 35°C Hybridization Buffers

Stock reagent	Volume (µl)	Final
1M Tris-HCl (pH 7.5)	1000	20mM
0.5M EDTA	500	5mM
Than add:		
• For Hybr. Buffer with 55% Formamide:		
5M NaCl	30	3mM
MQ water	48420	
10% SDS	50	0.01%
• For Hybr. Buffer with 30% Formamide:		
5M NaCl	1120	112mM
MQ water	47330	
10% SDS	50	0.01%
• For Hybr. Buffer with 20% Formamide:		
5M NaCl	1350	135mM
MQ water	47100	
10% SDS	50	0.01%

6. Pre warm buffer at 37°C in water bath.
7. After hybridization quickly transfer filter sections 15min in washing buffer in 37°C water bath, afterwards pour in to buechner funnel.

5. Amplification

1. Prepare PBS-T-Mix, store in dark:

Stock reagent	Volume (µl)	Final
1xPBS	49750	
10% Triton x100	250	0.05%

PBS should have pH of 7.4 -7.6 (better peroxidase turnover)

2. Incubate filters in 25ml PBS-T-Mix at RT in dark for 10-15min.
3. Prepare Substrate mix on ice with prepared amplification buffer (AMP) and 30% H2O2:
4. Dilute 30% H2O2 to final concentration of 0.0015% (Step A to B; all Volumes in µl). Ad tyromide (choose best conc; 2.5, 3.4 or 5.0µl) to 493µl AMP. and 5µl of A. Keep in dark and put on ice.

	AMP	H2O2	Tyr	Ratio	
A	200	1 from 30%			
B	493	5 from A	2.5	1:200	A555
B	493	5 from A	3.4	1:150	A488
B	493	5 from A	5.0	1:100	A488

5. Dab filters on blotting paper (don't dry), place sections on top of each other and place in 0.7 ml Eppi. Keep in dark.
6. Incubate in substrate mix for 45 min at 37°C in hybr. oven in the dark. (max 20 filters per eppi)
7. After incubation in Substrate mix dab filters on blotting paper.
8. Wash in 25ml PBS-T mix at RT for 10-45 min in the dark. Afterwards pour into beuchner funnel.
9. Wash in MQ (in dark).
10. Wash in Ethanol 95% (in dark).
11. Dry on blotting paper and mount in DAPI mix.
12. Place on microscope slide.
13. Store at -20°C in dark.

8.1.6 CARD-FISH solutions

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- **Hybridization Buffers, 10ml**

Store at -20°C for up to 1 year; if in use keep on ice!

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	1g	10%
5M NaCl	1800	900mM
1M Tris-HCl (pH 8.0)	200	20mM
10% Triton X100	50	0.05%
Bring in solution at 60°C in water bath, takes about 4h then cool down on ice. Then add:		
For Buffer with 55% Formamide: (EUB, ALF, BET, GAM, CF)		
Formamide	5500	55%
10% Blocking	1000	1%
Sigma Water	1455	
For Buffer with 30% Formamide: (NSR)		
Formamide	3000	30%
10% Blocking	1000	1%
Sigma Water	3955	
For Buffer with 20% Formamide: (Archaea; Eury, Cren)		
Formamide	2000	20%
10% Blocking	1000	1%
Sigma Water	4955	

- **Amplification Buffer, 10ml**

Store at +4°C

Stock reagent	Volume (µl)	Final conc.
Dextran Sulfate	1g	10%
5M NaCl	4000	2M
10% Blocking	100	1%
1x PBS	5900	

- **DAPI mix, 2ml**

Store in dark at +4°C

Stock reagent	Volume (µl)	Final
DAPI 50µg/ml	40	1 µg/ml
1xPBS	140	0.5
Vectashield	280	1
Cititfluor	1540	5.5

- **Blocking Reagent 10%, 100ml**

1. Prepare Maleic acid buffer

a) 100mM Maleic acid
MW: 116.08g/mol
11.608g/l

b) 150mM NaCl
MW: 58.44g/mol
8.766g/l

c) Adjust pH with a lot of solid NaOH to 7.5

2. Add Boehringer Mannheim Blocking reagent to a final concentration of 10%
10mg with 100ml Maleic Acid buffer
3. Dissolve reagent on heating plate at around 60°C with stirring for about 1h, do not boil
4. Autoclave solution
5. Aliquot to 10ml parts and store them frozen at -20°C; According to the manufacture solution should be good for a few days at 4°C and many months if stored frozen.

- **10x PBS, pH 7.7, 1L**

1. 20mM NaH_2PO_4
MW: 138g/mol
2.76g/l → weigh 2.76g

2. 80mM Na_2HPO_4
MW: 178g/mol
14.24 g/l → weigh 14.24g

3. 1.3M NaCl
MW: 58.44 g/mol
75.97 g/l → weigh 75.97g

4. Fill up to 1L with MQ
5. Adjust to pH 7.7 with HCl
6. Autoclave

- **1x PBS pH 7.7, 1L**

1. Take 100ml 10x PBS
2. Add 900ml MQ
3. Adjust to pH 7.7
4. Autoclave

- **0.5M EDTA, pH 8.0, 1L**

1. MW: 372.24g/mol
186.12g/l
2. Add NaOH pastilles, only at pH 8 EDTA will dissolve!
3. Fill up to 1 litre with MQ
4. Adjust to pH 8.0
5. Autoclave

- **10% SDS (Sodium Dodecyl Sulphate), 500ml**

1. 50g SDS to 500ml MQ
2. Do not autoclave!
3. Dissolve at 40°C in water bath

- **1M Tris-HCL, 1L**

1. MW: 121.14g/mol
121.14g/l
2. Adjust to pH 7.5 resp. 8.0 with 6M HCl
3. Fill up to 1 litre with MQ

- **5M NaCL**

1. MW: 58.44g/mol
292.09 g/l
2. Autoclave

8.2 Chemicals/solutions

- Artificial sea water (ASW), S=35.8 g/l; 30.5‰

  For 1L:
  - 20.76g NaCl
  - 3.48g Na_2SO_4
  - 0.58g KCl
  - 3.48g Na_2SO_4
  - 0.58g KCl
  - 0.17g $NaHCO_3$
  - 0.09g KBr
  - 0.02g H_3BO_3
  - MQ

- DAPI-mix, 2ml:
  - 40µl DAPI (4'6'-diamidino-2-phenylindole)
  - 140µl 1xPBS
  - 280µl Vectashield
  - 1540µl Cititfluor
  - Store at 4°C in dark

- Lysis-T (Sigma)

- Lysozym (Sigma; catalogue number 7651)

- Nitrapyrin (N-serve)
  $C_6H_3NCl_4$ 2-chloro-6-(trichloromethylpyridine), FW 230.9

  Stock solution, 100ml:
  - 330mg Nitrapyrin
  - 4ml of 80% ethanol
  - Solve with help of a sonicator
  - Fill to 100ml with bacteria free seawater

- PBS (Phosphate Buffered Saline), pH 7.2

  3xPBS, 1L:
  - 22.8g NaCl
  - 1.16g NaH_2PO_4
  - 3.8g Na_2HPO_4
  - 800ml MQ
  - Adjust pH to 7.4
  - Fill to 1L with MQ
  - Autoclave

  1xPBS:
  - Dilute 3x with MQ

- PFA-fixative, 100ml:
  - 4g Paraformaldehyde
  - 90ml 1xPBS
  - Stir at 60°C until dissolution (takes about 6h)
  - Fill to 100ml with 1xPBS
  - Filter over 0.2µm GTTP filter
  - Store at 4°C

- Proteinase-K (Fluka; catalogue number 82456)