

## Nitrification in intertidal sponge *Cinachyrella cavernosa*

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### Abstract

The nitrification process in different sections of the sponges remains unresolved, despite several studies on the nitrogen cycling pathways in the tissues of temperate and Arctic sponges. In this study, the abundance, diversity and activity of the associated nitrifying organisms in intracellular, intercellular, extracellular and cortex of a tropical intertidal sponge, *Cinachyrella cavernosa*, were investigated using most probable number, next-generation sequencing and incubation method, respectively. The nitrification rate and the abundance of nitrifying bacteria showed significant difference among different sections. The nitrification rate in *C. cavernosa* was 2–12x higher than the reported values in other sponge species from temperate and Arctic regions. Nitrification rate in sponge cortex was 2x higher than intercellular and extracellular sections. Ammonia and nitrite oxidisers ranged from  $10^3$  to  $10^4$  CFU g<sup>-1</sup> in the sponge with a high number of ammonia and nitrite oxidisers in the cortex. Nitrifiers belonging to *Nitrosomonas*, *Nitrospira*, *Nitrospina*, *Nitrobacter* and *Nitrosopumilus* were present in different sections of the sponge, with nitrifying archaea dominating the intracellular section and nitrifying bacteria dominating other sections. This study reports for the first time the nitrification inside the sponge cells. The study also suggests that the intertidal sponge, *C. cavernosa* harbours metabolically active nitrifiers in different sections of the sponge body with different rates of nitrification. Thus, nitrifiers play an important role in ammonia detoxification within the sponge and also contribute to the nitrogen budget of the coastal ecosystem.

**Key words:** Nitrification, Ammoniumoxidiser, Nitrite oxidiser, intertidal sponge, *Cinachyrella* sp., Next generation sequencing

## Introduction

Sponges evolved in the Precambrian era and represent one of the early lineages of metazoans (Muller 1998; Sperling et al. 2007; Philippe et al. 2009; Worheide et al. 2012; Moroz et al. 2014). Their evolutionary success is likely to be dependent on their association with microorganisms (Hentschel et al. 2006). Sponges are classified into 2 groups based on the density of associated microorganisms. “High microbial abundance (HMA) sponges” carry up to  $10^8 - 10^{10}$  microorganisms per gram of sponge wet weight, whereas “low microbial abundance (LMA) sponges” carry lower microbial abundance of less than  $10^6$  microorganisms per gram of sponge wet weight (Vacelet and Donadey 1977; Hentschel et al. 2006; Weisz et al. 2008; Hentschel et al. 2012; Gloeckner et al. 2014). These microorganisms are phylogenetically diverse (Erwin et al. 2011) and are presumably involved in different functional roles, such as production of bioactive compounds (Dharmaraj and Sumantha 2009), photosynthesis (Thacker 2005), sulphate reduction (Hoffmann 2003) etc. Sponges play a crucial role in the biogeochemical cycling in the coastal environment and also in benthic-pelagic coupling (Reiswig 1971; Pile 1997; Gili and Coma 1998; Ribes et al. 1999, 2005; Pile and Young 2006). This is mainly due to their high abundance, ability to filter large volume of water (Weisz et al. 2008), heterogeneous diet and their association with large number of microorganisms. A few studies have described the nitrogen cycling pathways in sponges, like nitrogen fixation (Wilkinson and Fay 1979), nitrification process and presence of 16S rRNA gene sequences of nitrifiers (Jimenez and Ribes 2007; Bayer et al. 2007, 2008; Hoffmann et al. 2009; Schlappy et al. 2010; Radax et al. 2012; Ribes et al. 2012; Morganti et al. 2017). Nitrifiers may also contribute to the carbon fixation as 1 mol of carbon dioxide is fixed for every 9 mol of ammonium oxidised to nitrate (Feliatra and Bianchi 1993). Thus, sponges not only contribute to the local nitrogen budget (Maldonado et al. 2012) but also to the carbon cycling. Most of these studies were on sponge tissues from temperate (Bayer et al. 2007, 2008; Schlappy et al. 2010), and cold water (Hoffmann et al. 2009; Radax et al. 2012).

It is now well established that the microbiota of sponge has different association such as intracellular (specific inside the cells), intercellular (specific in the mesohyl), extracellular (non-specific that are loosely associated with the canals and chambers) (Vacelet and Donadey 1977; Liu et al. 2011) and in the cortex (outer thick layer of sponge) (De Vos et al. 1991), due to the difference in filtering by canals with different apertures and different interior environments (Gerce et al. 2011; Yang and Li 2012). Depending on the site in the sponge, the abundance and functions of the microbiota may differ due to different nutritional strategies (Vacelet and Donadey 1977). Schlappy

(2008) has suggested that different tissue types have different abilities in the uptake and maintenance of sponge-associated microbes. Also, HMA and LMA sponges show different feeding strategies. HMA species mainly rely on the associated microbes for energy inputs (Weisz et al. 2007; Freeman and Thacker 2011). They can also remove ammonium from seawater and excrete nitrate and nitrite (Corredor et al. 1988; Southwell et al. 2008; Ribes et al. 2012). In contrast, LMA species obtain nutrients mainly by filtering particulate organic matter (Ribes et al. 1999; Schlappy et al. 2010; Freeman and Thacker 2011). LMA sponges excrete dissolved compounds such as ammonium, phosphate and dissolved organic matter (Southwell et al. 2008; Maldonado et al. 2012; Ribes et al. 2012). The sponge *C. cavernosa* comes under HMA category as it contains  $>10^9$  microorganisms per gram of sponge tissue (Subina 2017). This intertidal demosponge has neither been explored for its microbial association or its link in nitrogen cycling. Hence, in the present study, we assessed the nitrification rate and its link with the nitrifying bacterial abundance in different body sections *viz.*, intracellular, intercellular, extracellular and cortex of *C. cavernosa*.

## **Materials and methods**

### *Sample collection and processing*

The sponge (*C. cavernosa*) samples were collected from the rocky intertidal region of Anjuna beach, Goa, India (15° 34' 0" N, 73° 44' 0" E) during the low tide. Sponges of similar body mass (~16 g) showing a visually healthy appearance were used for all the experiments. The sponges were washed with filtered, autoclaved seawater till they were visibly clean of any debris and sediment. The sponge "cortex" tissues were carefully removed using a sterile scalpel and the intracellular, intercellular and extracellular sections were separated according to Liu et al. (2011). Briefly, after removing the cortex, the sponge tissues were cut into small pieces and washed using artificial seawater, with shaking at 150 rpm and sieved through a 300-mesh sieve. The resulting seawater was centrifuged at 15000X g to separate "extracellular microbiota". The resulting sponge tissues were disintegrated in Ca<sup>2+</sup>- and Mg<sup>2+</sup>- free artificial seawater at 110 rpm. The supernatant obtained after centrifugation at 300X g was further centrifuged at 15000X g to obtain "intercellular microbiota". The pellet was washed several times with Ca<sup>2+</sup>- and Mg<sup>2+</sup>- free artificial seawater to remove bacterial contamination from other sections and was seen free of bacteria under the microscope. This pellet was used to obtain "intracellular microbiota".

### *Abundance and diversity of Nitrifiers*

Ammonia oxidisers (AOB) and nitrite oxidisers (NOB) from different sections of the sponge were enumerated by the most probable number (MPN) method using the media formulated by

Elbanna et al. (2012) with phenol red as pH indicator (Table S1). The inoculated tubes were incubated for 2–3 weeks and the number of nitrifiers was calculated based on McCready's table.

Next-generation sequencing (NGS) technique was used to understand the diversity of nitrifiers present in different sections of the sponge. DNA from different sections of the sponge was extracted using MOBio soil DNA kit, according to manufacturer's instruction. The DNA was used as a template for nested PCR to amplify the V6 region of 16S rRNA gene. Metagenomic analysis of V6 region of the total bacterial community was carried out using Illumina technology (Genotypic Technologies, Bangalore). Raw DNA sequence reads were downloaded from Illumina Base Space platform and processed in Quantitative Insights into Microbial Ecology (QIIME v1.9.0) (Caporaso et al. 2010). Operational taxonomic units (OTUs) were assigned using uclust (Edgar 2010) with 97% similarity. The sequences were aligned using the greengenes reference alignment (DeSantis et al. 2006). The paired-end sequences of samples were submitted to NCBI Sequence Read Archives (SAMN06226897, SAMN06226899, SAMN06226900 – SAMN06226902, SAMN06226904 – SAMN06226906).

#### *Nitrification experiment*

The nitrification experiment was carried out according to Hoffmann et al. (2009). The microbiota of different sections of sponge (as explained earlier) was placed in individual flasks with 500 ml of natural, unfiltered and autoclaved seawater amended with 10  $\mu\text{M}$   $(\text{NH}_4)_2\text{SO}_4$ . Three parallel incubations and controls (autoclaved seawater with 10  $\mu\text{M}$   $(\text{NH}_4)_2\text{SO}_4$  and without sponge) were used. Flasks with aerators and magnetic stirrers were closed and placed in a temperature-controlled room at 25°C in the dark. Water samples were removed at 2 hours interval for estimating nutrient concentration (ammonium, nitrite and nitrate) using Continuous-Flow Autoanalyser (SKALAR) according to the method by Grasshoff et al. (1983) over a time-course of 24 hours. The results were expressed as  $\mu\text{M}$  concentration  $\text{g}^{-1}$  (wet weight).

#### *Statistical analysis*

Analysis of variance (ANOVA), t-test and correlation analysis were carried out (Statistica 8) to test the difference in the abundance of nitrifying bacteria, nutrient concentration and variation of nitrification rate among different sections of the sponge, and to relate the abundance of nitrifiers and nutrient concentrations, respectively. Analysis of similarity (ANOSIM) and cluster analyses (dendrogram) were carried out using Primer 6. Canonical Correspondence Analysis (CCA) was performed by MVSP.

## Results

### *Nutrient dynamics*

The net increase in the nitrite and nitrate concentrations in the incubation experiment demonstrated active nitrification in the sponge. Figure 1a-c shows the trends in the ammonium, nitrite and nitrate concentrations during the incubation experiment. In the case of ammonium, its consumption though exceeded the production in the first 4 to 10 hours by the microbiota from different sections of sponge, there was a net production of ammonium thereafter (Figure 1a). In the case of nitrite, the concentration was low and increased after 8 hours and there was no detectable increase in nitrite production in cortex microbiota (Figure 1b). Figure 1c shows the trend in the nitrate concentration in the different sections. Nitrate concentration was 10x more than nitrite concentration. At the end of the experiment, the net production of ammonium ( $1.06 \pm 0.26 \mu\text{M g}^{-1}$ ) in intracellular microbiota was approximately 2x higher than cortex microbiota ( $0.53 \pm 0.01 \mu\text{M g}^{-1}$ ) whereas high net production of nitrite was recorded in intercellular ( $1.02 \pm 0.03 \mu\text{M g}^{-1}$ ) section and low production ( $0.07 \pm 0.02 \mu\text{M g}^{-1}$ ) was in the cortex (Figure 1b). Unlike ammonium and nitrite, the highest production of nitrate was noted in the cortex ( $3.30 \pm 0.62 \mu\text{M g}^{-1}$ ) which was ~6x higher than intercellular ( $0.42 \pm 0.06 \mu\text{M g}^{-1}$ ) and extracellular ( $0.51 \pm 0.12 \mu\text{M g}^{-1}$ ) sections and 2x higher than intracellular ( $1.80 \pm 0.58 \mu\text{M g}^{-1}$ ) section (Figure 1c). ANOVA clearly showed a significant difference in the concentration of nutrients produced by microbiota from different sections ( $p < 0.05$ ).

The average net production of nutrients were calculated by dividing the net production by hours of incubation and expressed as  $\mu\text{M g}^{-1} \text{h}^{-1}$ . As seen in the table 1, the average net production of ammonium in the different sections did not show any significant difference, whereas the rates of nitrite and nitrate production in different sections varied significantly. Based on nitrite and nitrate production, it was observed that there was significant variation in the nitrification rate among different sections ( $t = 5.0$ ;  $p < 0.05$ ). ANOSIM also showed a significant difference in the nutrient production and nitrification rate among different sections (Global R=0.165;  $p < 0.001$ ). Among the different sections, nitrification rate was higher in the cortex ( $0.15 \pm 0.04 \mu\text{M g}^{-1} \text{h}^{-1}$ ) and lower in the extracellular microbiota ( $0.06 \pm 0.03 \mu\text{M g}^{-1} \text{h}^{-1}$ ). Dendrogram showed that cortex was different in nutrient production and net nitrification than the other sections and intercellular and extracellular sections were similar as they formed one cluster (Figure S1).

### *Abundance and diversity of nitrifiers*

The abundance of AOB and NOB showed significant variation among different sections of the sponge ( $p < 0.001$ ). Among the different sections, the number of AOB ranged from  $3.0 \times 10^3$  to

$6.1 \times 10^3 \text{CFU g}^{-1}$ . The highest number of AOB ( $6.1 \pm 0.7 \times 10^3 \text{CFU g}^{-1}$ ) was noted in the cortex and the lowest number in the intercellular section ( $3.0 \pm 0.8 \times 10^3 \text{CFU g}^{-1}$ ). The highest number of NOB was also noted in the cortex ( $2.4 \pm 0.02 \times 10^4 \text{CFU g}^{-1}$ ), which was 4 times higher than AOB abundance. The intracellular section harboured the lowest number of NOB ( $1.3 \pm 0.7 \times 10^3 \text{CFU g}^{-1}$ ), which was 3.5 times less than AOB counts of  $4.6 \pm 0.9 \times 10^3 \text{CFU g}^{-1}$  (Figure 2). NGS showed that a total of 46 bacterial and 5 archaeal OTUs of nitrifiers were present in the different sections of sponge. Cortex showed a higher number of OTUs (39 bacterial and 1 archaeal OTU) than other sections. Though extracellular and intracellular sections harboured 19 bacterial and 2 archaeal OTUs, the number of reads of OTUs differed. Unlike other sections, the intracellular sections showed 5 archaeal OTUs as against 1 bacterial OTU.

*Nitrospira*, was the dominant nitrifying bacteria in the cortex, extracellular and intercellular sections (Figure 3). *Nitrospira* which is known to carry out complete nitrification (Ammonia and nitrite oxidation) was found to be the dominant taxa as well as a biomarker group for the sponge microbiome (Reveillaud et al. 2014). The other nitrifiers found in the different sections of sponge were, *Nitrosomonas*, *Nitrospina*, *Nitrobacter* and *Nitrosopumilus*. The abundance of ammonium oxidising bacteria, *Nitrosomonas* and nitrite oxidising bacteria, *Nitrospina* was 1–2 orders higher in the cortex (108 reads and 133 reads, respectively) than in the other sections. Another nitrite oxidising bacteria, *Nitrobacter* was present only in the cortex. Though the bacterial abundance varied among different sections, the diversity of the nitrifying bacteria did not show any difference. Ammonia oxidising archaea, *Nitrosopumilus* was present in all the sections, with high abundance (3784 reads) in the intracellular section. Cluster analysis on the abundance of nitrifiers showed that extracellular and intercellular sections of the sponge clustered together, which was similar to that observed on the nutrient production (Figure S2). It further shows that the abundance of nitrifiers varied between the intracellular section and other sections.

Correlation analysis and CCA showed that both the abundance and type of nitrifiers determine the rate of production of inorganic nitrogen in the sponge, irrespective of the sections. *Nitrospira* showed a negative correlation with ammonium concentration ( $r = -0.596$ ,  $p < 0.05$ ) whereas the abundance of *Nitrosopumilus* increased with ammonium concentration ( $r = 0.949$ ,  $p < 0.005$ ). All nitrifying bacteria showed positive correlation with nitrate concentration and negative correlation with nitrite concentration (Table S2). Further, CCA clearly (Figure 4) showed that the rate of nitrification was controlled by *Nitrospira* and *Nitrospina* in extracellular and intercellular sections, whereas in the cortex, it was *Nitrosomonas* and *Nitrobacter*.

## Discussion

In the intertidal sponge *Cinachyrella cavernosa*, active nitrification was observed as nitrite and nitrate were produced during our incubation experiment. The bulk nitrification rate in this study can be considered as net rate as Hoffmann et al. (2009) suggested that the products might be used to fuel denitrification and anammox. Earlier studies have suggested that nitrification may be common in sponges (Jimenez and Ribes 2007; Bayer et al. 2008; Hoffmann et al. 2009; Ribes et al. 2012; Morganti et al., 2017), but the rates may be variable (Corredor et al. 1988; Diaz and Ward 1997; Bayer et al. 2007, 2008; Southwell et al. 2008; Hoffmann et al. 2009; Schlappy et al. 2010; Radax et al. 2012). The main benefit of nitrification in sponges is in the efficient removal of waste products (Hoffmann et al. 2009). The nitrification rate of *C. cavernosa* was  $0.15 \pm 0.04 \mu\text{M g}^{-1} \text{h}^{-1}$ . This value was close to the reported values ( $0.03\text{--}2.65 \mu\text{M g}^{-1} \text{h}^{-1}$ ) in the tropical sponges (Diaz and Ward 1997). However the nitrification rate of *C. cavernosa* was 2–12x higher than that reported in the studies carried out with sponge explants as well as deep water sponges from the temperate region and cold water sponges (Bayer et al. 2008; Hoffmann et al. 2009; Radax et al. 2012) and it was 2–3 orders of magnitude higher than that of the estuarine sediment (Bernhard et al. 2007). This high nitrification rate may be due to its intertidal habitat thereby exposed to nutrient rich environment (Table S3), compared to the deep water sponges or aquarium maintained explant. Further it has also been suggested that the different initial ambient concentration of dissolved inorganic nitrogen compounds alters sponge metabolism as sponge, *Dysidea avaraca* either remove or excrete ammonium depending on the initial inorganic concentration (Ribes et al. 2012; Morganti et al. 2017). Moreover, being an intertidal HMA sponge, *C. cavernosa* actively and non-selectively filters bacteria and other organic sources from ambient water, which provides a significant amount of particulate organic nitrogen (Lesser 2006; Trussel et al. 2006) and the utilisation of nitrogen-rich particulate organic matter results in the production of ammonium (Fiore et al. 2013), which fuels nitrification.

The increase in nitrite and decrease in ammonium concentration in the first 4 and 6 hours in the intracellular section and cortex and till 10 hours in the extracellular and intercellular sections indicated the consumption of ammonium by ammonium oxidisers ( $10^{3-4} \text{CFU g}^{-1}$ ). Further increase in the ammonium concentration during incubation may be due to decaying of some sponge tissues or fermentation process during anoxia, as a decline in oxygen and accumulation of excretion compounds occur when incubation progresses in closed chambers (Schlappy et al. 2010). A significant amount of ammonium release was also reported in other HMA sponges during incubation

experiments in aquaria and *in situ* experiments. For example, Mediterranean sponges *Aplysina aerophoba* and *Chondrosia reniformis*, and tropical sponges, *Chondrilla nucula*, *Pseudaxinella zeai*, *Oligoeras violacea* and *Plakortis haichondroides* produced 0.02 to 1.8  $\mu\text{M g}^{-1} \text{h}^{-1}$  ammonium and cold water sponge *Geodiabarretti* produced 0.17  $\mu\text{M cm}^{-3} \text{h}^{-1}$  ammonium during incubation experiments (Diaz and Ward 1997; Bayer et al. 2007; Hoffmann et al. 2009; Schlappy et al., 2010). Nitrite being an intermediate product was produced in very small amount (0.003–0.05  $\mu\text{M g}^{-1} \text{h}^{-1}$ ) which has also been reported in tropical, Mediterranean and cold water HMA sponges (Diaz and Ward 1998; Schlappy 2008; Hoffmann et al. 2009; Schlappy et al. 2010). Nitrate production was 10x higher than nitrite production in *C. cavernosa* which ranged from 0.02 to 0.15  $\mu\text{M g}^{-1} \text{h}^{-1}$ . Similar results have been obtained in Mediterranean HMA sponges, *A. aerophoba* and *C. reniformis* (Bayer et al. 2007, 2008; Schlappy et al. 2010), tropical HMA sponges, *C. nucula*, *P. zeai*, *O. violacea* and *P. haichondroides* (Diaz and Ward 1997), and Florida Key HMA sponges, *Aplysina archeri*, *Aplysina lacunose*, *Ircinia strobilina*, *Smenospongia aurea* and *Xestospongia muta* (Southwell et al. 2008). The *in vitro* incubation experiment in the present study may not be directly attributed to the nitrogen metabolism of the sponge under the natural condition as we used closed chambers and dark condition.

The rate of nitrification was different in different sections and also varied in each section during the incubation time, with cortex showing highest nitrification rate. One of the reasons attributed for this variability in nitrification rate is the loss of nitrate under anaerobic conditions through denitrification. However, in this sponge, denitrification rate (5.25–14.4  $\text{nM g}^{-1} \text{h}^{-1}$ , data not shown) was much lower compared to nitrification rate. Other possibility was the involvement of photosynthetic community for ammonium removal. However, it was not considered as the experiment was carried out in the dark condition. Thus, the difference in the nitrification rate may be due to the difference in their internal environment such as availability of nutrients, oxygen and space (Yang and Li 2012) in different sections of the sponge.

The discovery of genes responsible for ammonium oxidation in sponge and evidence of vertical transmission of these symbionts suggest that ammonium oxidisers are essential for the metabolism of sponge (Steger et al. 2008). The nitrifying community probably plays an important role in the ammonia detoxification within marine sponges (Steger et al. 2008). A total of 51 OTUs (1431 bacterial reads and 3815 archaeal reads) of nitrifiers were detected in different sections of sponge. These included ammonium oxidisers like *Nitrosomonas*, *Nitrosopumilus*, nitrite oxidisers like *Nitrospina* and *Nitrobacter* and *Nitrospira*, which carry out both ammonium and nitrite oxidation.



Different sections of the sponge had a significantly different number of ammonium and nitrite oxidisers pointing to the existence of different microbial niches within the sponge body. Apart from nitrifying bacteria, ammonium oxidising archaea were present in all the sections, contributing to the nitrification activity. Liu et al. (2011) also reported the presence of ammonia oxidising archaea in different sections of the sponge, with higher number in intercellular section, whereas in *C. cavernosa* higher number of ammonium oxidising archaea was observed in the intracellular section than in other sections suggests that ammonium oxidising archaea may be the predominant nitrifiers in the intracellular section of *C. cavernosa*. Besides, Radax et al. (2012) have reported that ammonium oxidising archaea are the major drivers of nitrification in cold water sponges as they are adapted to a broad range of growth conditions and might have a more versatile metabolism than ammonium oxidising bacteria (Leininger et al. 2006).

Cluster analysis based on the abundance of nitrifiers and their activity showed that the extracellular and intercellular sections clustered together. Cortex was differed from the other sections due to high bacterial abundance (39 OTUs of 1078 reads) and high nitrification rate. Similarly, the intracellular section differed from the extracellular and intercellular sections due to its low bacterial abundance (one OTU of 14 reads), high archaeal abundance (5 OTUs of 3798 reads) and relatively high nitrification rate. CCA ordination plot also showed the distinct nitrifying community in different sections with respect to the inorganic nutrient production. Correlation analysis and CCA showed a strong relation between nitrifiers and their activity. The order of nitrifying bacterial reads in different sections was cortex > extracellular > intercellular > intracellular and it was the vice versa for archaeal reads. This might be related to the availability of oxygen as there are oxic and anoxic zones inside the sponge body which was achieved by controlling the pumping activity of sponge (Schlappy et al. 2010). The negative correlation of *Nitrospira* with ammonium concentration ( $r = -0.596$ ,  $p < 0.05$ ) might be due to their higher number in cortex, intercellular and extracellular regions (836, 160 and 154 reads, respectively), which might have utilized ammonium produced by the sponge and thus resulted in low ammonium concentration in these sections. The abundance of *Nitrosopumilus* increased with ammonium concentration ( $r = 0.949$ ,  $p < 0.005$ ), which, however may not be a direct correlation. All nitrifying bacteria showed a positive correlation with nitrate and negative correlation with nitrite concentration, due to the conversion of nitrite to nitrate by these bacteria. In the cortex, the nitrification was mainly controlled by *Nitrosomonas* and *Nitrobacter* whereas *Nitrospira* and *Nitrospina* controlled nitrification in the extracellular and intercellular section of the sponge.

We therefore, conclude that intertidal HMA sponge, *C. cavernosa* harboured nitrifiers in different sections of its body which partake in nitrification. The rate of nitrification varied among different sections. These differential activities of nitrifying bacteria in different sections of sponge may be due to the temporal and spatial occurrence of oxic and anoxic zones, achieved by controlling the pumping activity of sponge and also due to the difference in the number of nitrifiers. This study for the first time reports the nitrification process inside the sponge cells. The high number of nitrifiers associated with the sponge and their activity may help in getting rid of the ammonium and nitrite and may also inhibit the sulphate reducing and denitrifying bacteria.

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### **Figure legends**

Figure 1. Production or consumption rate of (a) Ammonium, (b) nitrite and (c) nitrate in different sections of sponge. Negative values show consumption of nutrient.

Figure 2. Abundance of ammonium oxidisers (AOB) and nitrite oxidisers (NOB) in different sections of sponge.

Figure 3. Relative abundance of nitrifiers in different sections of sponge.

Figure 4. CCA ordination plot showing the relationship of nutrient production and nitrifiers.

Figure S1. Clustering of different sections of sponge based on the production of ammonium, nitrite and nitrate.

Figure S2. Clustering of different sections of sponge based on the abundance of nitrifiers. Figures

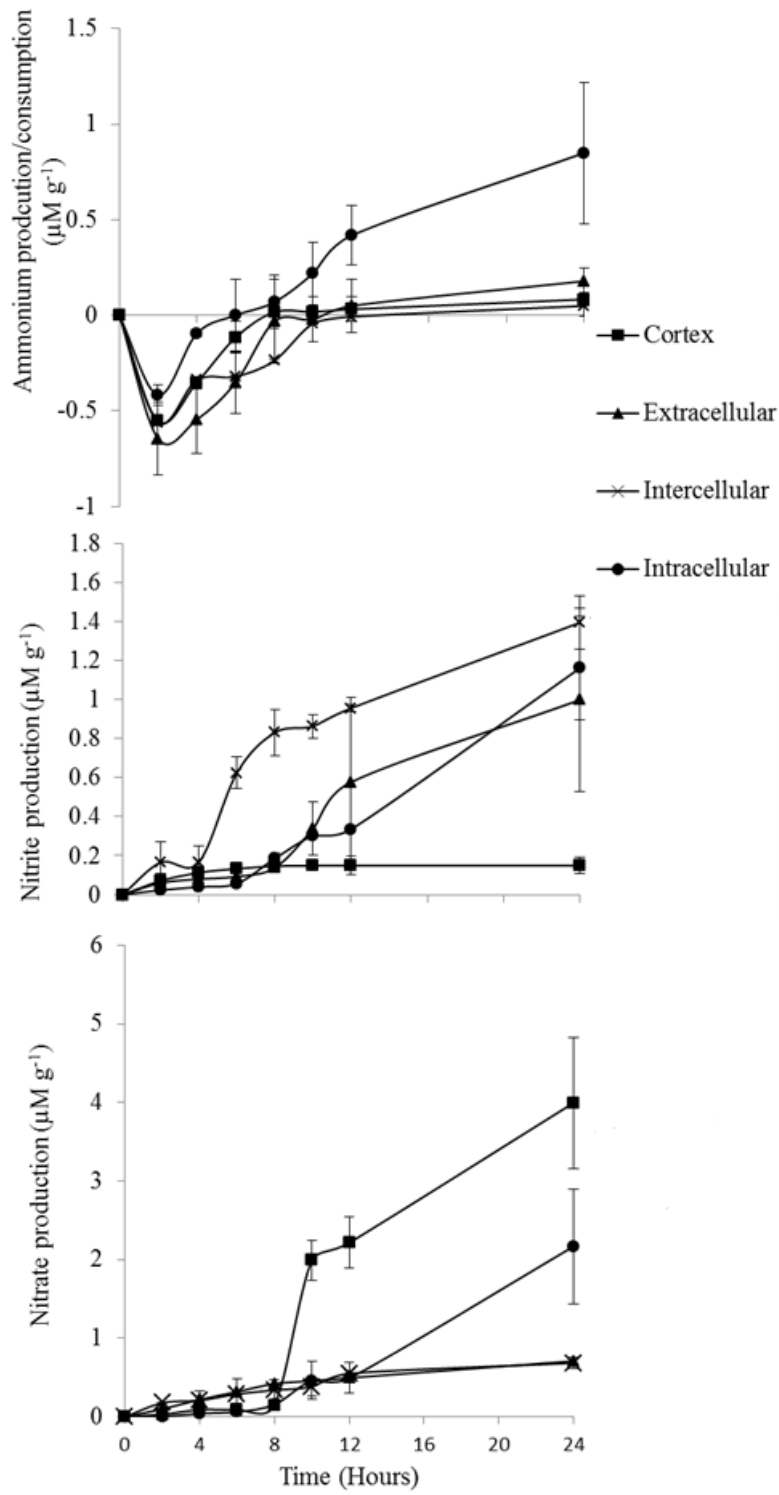


Figure 1

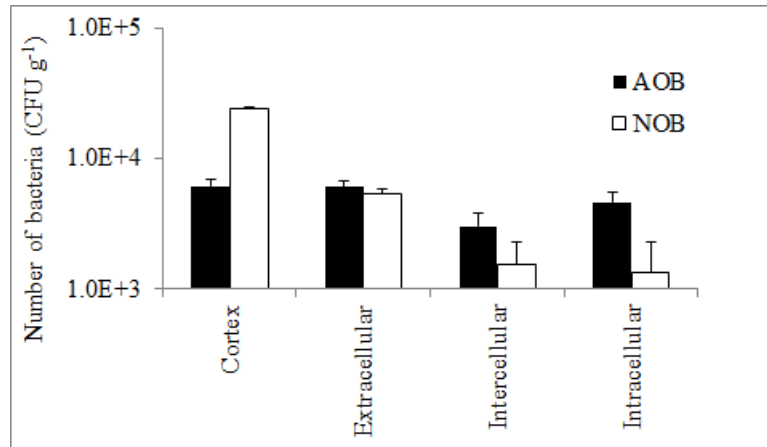


Figure 2

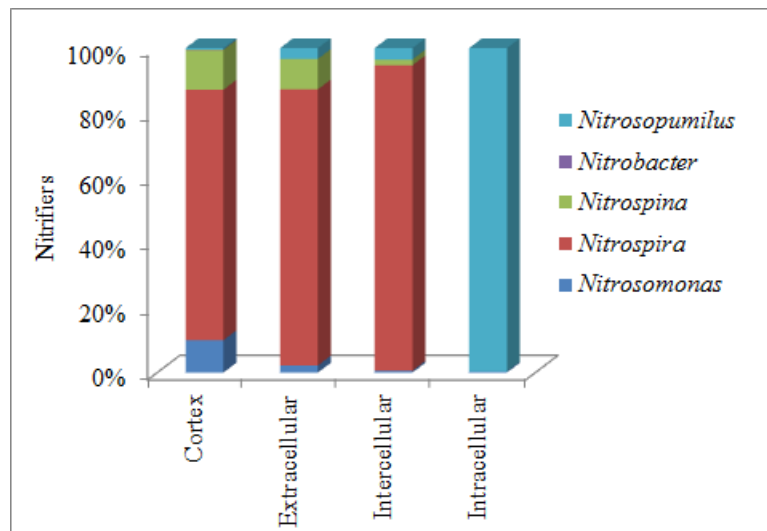


Figure 3





Table 1. Net nitrification and average rate of production of nutrients in different sections of sponge

Parameters	Cortex	Extracellular	Intercellular	Intracellular
( $\mu\text{M g}^{-1} \text{h}^{-1}$ )				
Ammonium	0.28±0.45	0.29±0.44	0.28±0.45	0.29±0.44
Nitrite	0.003±0.00	0.04±0.02	0.05±0.00	0.04±0.01
Nitrate	0.15±0.03	0.02±0.01	0.02±0.00	0.08±0.03
Net nitrification	0.15±0.04	0.06±0.03	0.07±0.00	0.13±0.04

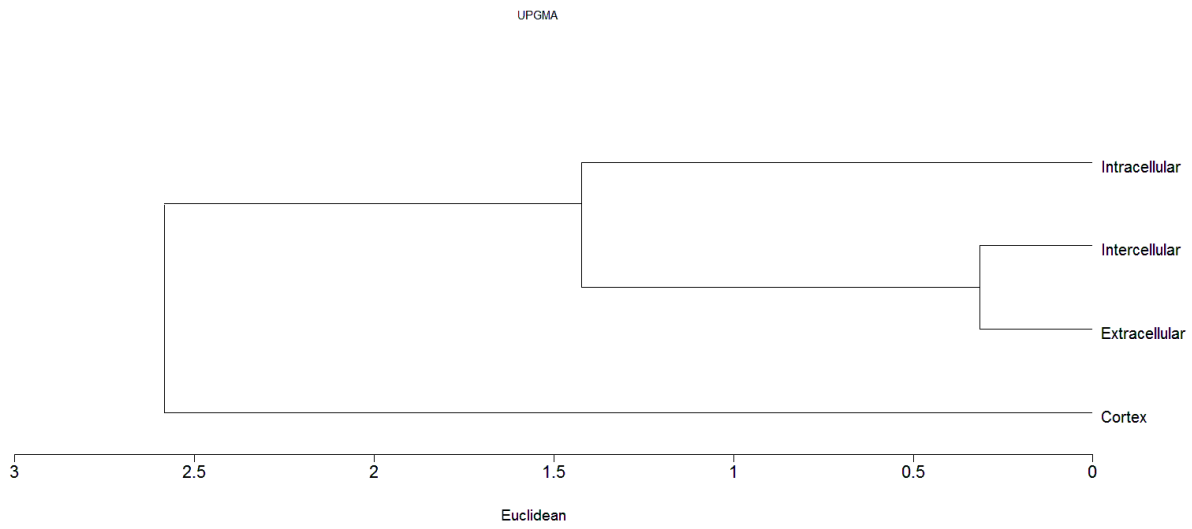
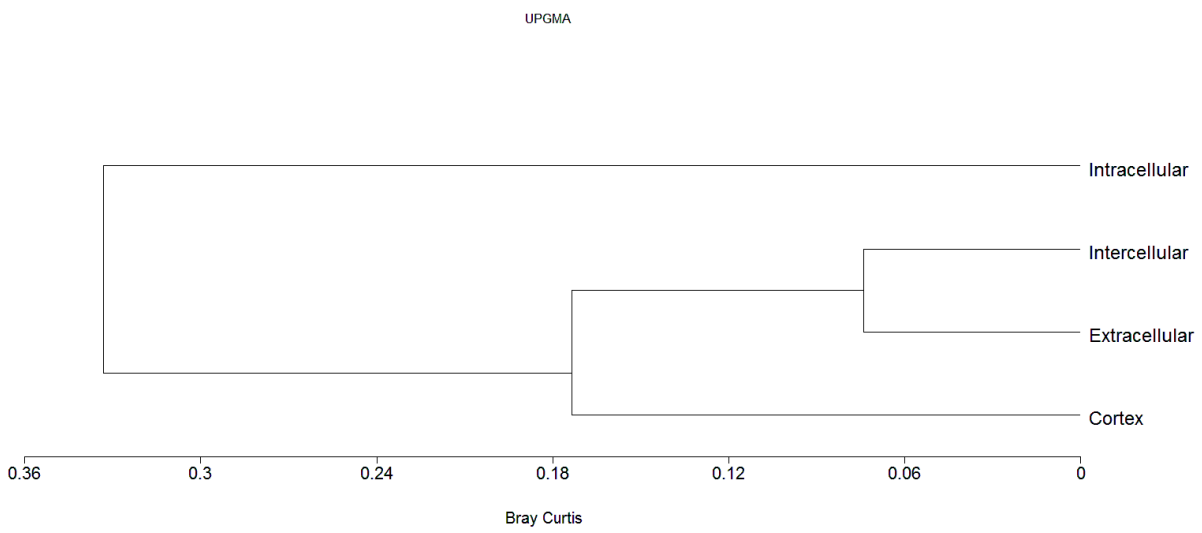


Figure S1.



. Figure S2.

Table S1. Media composition of nitrifying bacteria

Media component	Quantity (g l <sup>-1</sup> )
<i>Nitrifying bacteria (Ammonia and nitrite oxidizing bacteria)</i>	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.5
K <sub>2</sub> SO <sub>4</sub>	1.0
CaCl <sub>2</sub>	7.5
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>3</sub>	0.5
FeSO <sub>4</sub> ·7 H <sub>2</sub> O	0.03
NaCl	0.3
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	0.3
<i>Nitrite oxidizing bacteria</i>	
NaNO <sub>2</sub>	0.01
K <sub>2</sub> SO <sub>4</sub>	1.0
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>3</sub>	0.5
NaCl	0.3
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	0.1
FeSO <sub>4</sub> ·7 H <sub>2</sub> O	0.03
CaCl <sub>2</sub>	1.3

Table S2. Pearson correlation matrix showing the relationship between nitrifiers and nutrient production rate.

The values indicate correlation coefficient (r-value).

	<i>Nitrosomonas</i>	<i>Nitrospira</i>	<i>Nitrospina</i>	<i>Nitrobacter</i>	<i>Nitrosopumilus</i>	AOB	NOB
Net							
nitrification	0.8242***	0.6193*	0.7033**	0.7619***	0.3535	-0.0949	0.6847**
Nitrite	-0.9674***	-0.9619***	-0.9903***	-0.9730***	0.3706	-0.6490**	-0.9956***
Nitrate	0.9293***	0.7745***	0.8464***	0.8846**	0.1439	0.1395	0.8342***
Ammonium	-0.3333	-0.5959*	-0.4676	-0.4332	0.9492***	-0.7559**	-0.4623

\*\*\* p<0.001, \*\* p<0.01, \* p<0.05

Table S3. Characteristics of ambient water around the sponge

Parameters	Values (Average $\pm$ SD )
Temperature ( $^{\circ}$ C)	28.1 $\pm$ 2.1
Salinity	34.6 $\pm$ 1.7
pH	8.1 $\pm$ 0.2
DO ( $\text{mg l}^{-1}$ )	5.1 $\pm$ 1.2
Nitrite ( $\mu\text{M}$ )	0.8 $\pm$ 0.6
Nitrate ( $\mu\text{M}$ )	2.7 $\pm$ 0.9
Ammonia ( $\mu\text{M}$ )	2.3 $\pm$ 0.9