Nitrification activity of the sponge *Chondrosia reniformis* under elevated concentrations of ammonium

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Abstract

This study examined the ability of a Mediterranean demosponge *Chondrosia reniformis* to oxidize exogenous ammonium, simulating N-rich conditions that occur near finfish farms. We hypothesized that as the concentration of ammonium increases in the surrounding seawater, nitrification mediated by microbes associated with *C. reniformis* will lead to enhancement of ammonium uptake, nitrate excretion and oxygen consumption by the sponge holobiont.

To test this hypothesis, we conducted laboratory experiments with *C. reniformis* explants exposed to ammonium enrichments (300 – 6667 nM) and to ambient seawater (45 – 1511 nM ammonium). We analyzed inhaled (IN) and exhaled (EX) water samples for dissolved oxygen, ammonium, nitrates and retention of picoplankton cells.

We observed ammonium uptake in nearly half the cases and excretion of nitrate in most experimental outcomes. Yet, ammonium and oxygen consumptions were not linked to nitrate excretion, and this suggests that nitrification activity of sponge-associated microbes is not necessarily related to the concentration of ammonium in the surrounding seawater. Further research is required to reveal the sources of nitrate released from sponges and the fate of this nitrate in natural and manipulated ecosystems.

# Introduction (527 words)

Marine sponges feed on microorganisms and host dense and diverse microbial communities in their bodies (Taylor *et al.* 2007, Webster and Taylor 2012, Fan *et al.* 2012, Pita *et al.* 2018). Owing to the importance of microbe-mediated processes in the sponge’s life, the term “holobiont”, i.e. “whole organism”, is used to emphasize that microbial consortia are integral parts of the sponge (Hentschel *et al.* 2003). The concept of “nested ecosystem” highlights the roles played by sponge-associated microorganisms in the functioning of sponges in aquatic environments (Pita *et al.* 2018). Microbial associates contribute to sponge metabolism and ecological functioning in various ways, such as the processing of dissolved inorganic nitrogen (DIN), which is the subject of the present study.

Sponges produce ammonium ($NH\_{4}^{+}$) as a metabolic waste (endogenous ammonium), which makes the sponge body an attractive niche for microorganisms in nitrogen-limited marine environments (Han *et al.* 2013). Nitrification is an exclusively prokaryotic process whereby ammonium is oxidized to nitrite ($NO\_{2}^{-}$) and subsequently to nitrate ($NO\_{3}^{-}$), and it is utilized to obtain energy by different groups of bacteria and archaea associated with sponges (Kowalchuk and Stephen 2001; Schläppy *et al.* 2010b; Ribes *et al.* 2012). Several sponge species were found to consume exogenous ammonium while excreting nitrate, thereby resembling microbe-mediated nitrification (Corredor *et al.* 1988; Diaz and Ward 1997; Jiménez and Ribes 2007; Schläppy *et al.* 2010; Ribes *et al.* 2012).

The present study examines the potential of a Mediterranean high microbial abundance (HMA) sponge *Chondrosia reniformis* (Nardo, 1847) to act as a sink for ammonium under conditions of elevated concentrations of ammonium in seawater. Such conditions are expected when sponges are cultivated near finfish farms, where the concentrations of ammonium are elevated because of fish metabolic wastes (Hargrave *et al.* 1993; Pitta *et al.* 1998; 2006; Korzen *et al.* 2016). By doing so, we tested the possible functioning of *C. reniformis* as an ammonium sink in Integrated Multi Trophic Aquaculture (IMTA), as suggested by Pronzato (1998), Chopin (2012) and Gökalp *et al.* (2019).

The working hypothesis of this study, hereafter referred to as the “induced nitrification” hypothesis, predicted elevated uptake of ammonium, elevated excretion of nitrate and elevated consumption of oxygen by *C. reniformis* as the concentration of ammonium increases in seawater, due to the oxidation of ammonium to nitrate (nitrification) by the sponge-associated microbes. The “induced nitrification” hypothesis stems from the evidence of nitrification in *C. reniformis* obtained by Jiménez and Ribes (2007) and Schläppy *et al.* (2010b). Ammonia-oxidizing prokaryotes (bacteria and archaea) and functional genes for nitrification (ammonia monooxygenase, *amoA/amoB*) are usually found in HMA sponges including *C. reniformis* (Han *et al.* 2013, Bayer *et al.* 2014, Gantt *et al.* 2019). Hence, we chose to perform a practical analysis of nitrification in *C. reniformis*, especially regarding its possible role as an ammonium sink in human-impacted marine ecosystems such as aquaculture facilities.

Here we present simultaneous measurements of all nitrification components: ammonium, nitrite, nitrate and oxygen by non-destructive methods which require no physical contact with the sponge. We tested the “induced nitrification” hypothesis in laboratory aquaria enriched with ammonium, and the results were used to predict if *C. reniformis* would act as a sink for ammonium in integrated aquaculture.

# Materials and Methods (1352 words)

## Sponge collection and handling

Live sponges were collected by SCUBA diving from 2 to 4 m depths at a rocky reef in the Eastern Mediterranean Sea (32°29'24.71"N, 34°53'9.29"E) in July 2014. Specimens covering roughly 10 cm2 each were cut from different individuals with a sharp blade, such that the larger part of the sponge was not removed to allow for its regeneration. The excised specimens, from here on referred to as ‘explants’, were inserted underwater into sealed plastic bags (Whirl-Pak, USA) and brought to the laboratory within two hours after collection while constantly immersed in seawater to prevent possible damage to sponges from exposure to air.

In the laboratory, the explants were placed on porcelain tiles to provide them with substrate for attachment and kept in individual 6 L aquaria immersed in a sealed wooden table filled with seawater to maintain equal temperature between the aquaria. The aquaria were supplied with a constant flow of sand-filtered seawater that was pumped from 150 m off the coast at 2 m depth. Prior to initiation of experiments the sponges were checked for: 1) attachment to the substrate; 2) opening of osculae; 3) natural color; and 4) pumping of water by spreading fluorescein near the sponge and observing the efflux of the dye through the osculae.

The experiments were conducted in two seasons during 2014: summer (July – August) and autumn (November – December). The water temperature in experimental aquaria ranged between 27 – 28 ºC in the summer and between 18 – 21 ºC in the autumn.

##  Experimental design

To evaluate the influence of sponge filtration on the concentrations of ammonium, nitrite and nitrate, we used direct sampling of water that is inhaled and exhaled by the sponge, IN-EX, in a method modified from Yahel *et al.* (2005), and calculated the differences in concentrations of ammonium (ΔNH4), nitrite (ΔNO3-) and nitrate (ΔNO2-) combined as ΔNOx, between the inhaled (IN) and the exhaled (EX) water samples. To evaluate the oxygen consumption by sponge explants during the IN-EX sampling, we measured the concentrations of dissolved oxygen at IN and EX sites by needle-type optode sensors (Fire-Sting®, Pyro-Science GmbH Germany) as depicted in Fig. S1 and calculated the differences between the IN and EX oxygen concentrations as ΔO2. The working hypothesis predicted that an increase in concentration of ammonium inhaled by the sponge (NH4IN) would result in elevated uptake of ammonium (ΔNH4), elevated excretion of nitrate (ΔNOx) and elevated oxygen consumption (ΔO2).

We used a split-plot design to test the effect of inhaled ammonium concentration (NH4IN) on three response variables: ΔNH4, ΔNOx and ΔO2. We applied two treatments, “ambient” and “enrichment”, to all explants as a whole-plot factor. For the ambient treatment, the concentration of ammonium in aquaria was not augmented artificially and thus represented natural concentrations. For the enrichment treatment, we added ammonium chloride (Sigma-Aldrich PN 254134) into a 10 L container with seawater, and from that container to an aquarium with a sponge in a flow-through manner. The ranges of ammonium concentrations were 0.045 – 1.511 µM and 0.344 – 6.667 µM respectively for the ambient and enrichment treatments. To account for the variance in responses to elevated ammonium concentration between the explants, we used explant as a split-plot factor and applied both treatments alternately to each explant.

To verify that sponges were filtering the water, all IN-EX samples were examined by flow cytometry for retention of picoplankton. To control for the variability in analyte concentrations which are not caused by sponge activity, three pairs of samples from an aquarium without a sponge were collected under two ambient and two enrichment treatments and analyzed in the same way as the samples obtained from sponges.

## Sampling procedure

The IN-EX samples were collected by siphoning through two identical Teflon hoses of 1 m length and internal diameter of 0.5 mm, equipped with glass capillary tips of 300 μm internal diameter (Fig. S1). The samples of inhaled water (IN) were collected at a distance of ~ 5 mm from the sponge’s surface and at least 1 cm away from an osculum, and the samples of exhaled water (EX) were collected in front of the sponge’s osculum, by placing the sampling gear as close as possible to the osculum while not touching the sponge. The samples were collected into sterile test tubes and kept in a dark styrofoam box filled with ice in order to slow down the microbial transformations of DIN during the collection and handling. All sampling equipment was cleaned with a 10 % HCl solution and rinsed with double-distilled water between the experiments.

## Analytical procedures

From each sample (30 ml) of an IN-EX pair, 2 ml were analyzed for ammonium immediately after collection, 10 ml were filtered through 0.45 μm membranes (Millex PVDF, Millipore) and stored at 4°C until the analyses of nitrite and nitrate, and 1.5 ml were preserved with 10% glutaraldehyde (Sigma-Aldrich PN G7651) and stored at -80°C for the flow cytometry analysis.

### Ammonium

The concentration of ammonium (NH4+) was determined by the fluorometric method amended from Holmes *et al.* (1999) (Appendix 1, Protocol for Determination of Ammonium in Seawater). Fluorescence was measured by a Trilogy fluorometer (Turner Designs, USA) with excitation at 350–380 nm and emission at 410-450 nm (ammonium kit P/N 7200-041). The accuracy of the ammonium assay was tested against a certified reference material (Fluka QC3179) and the precision between duplicate analyses of sample splits was <50 nM.

### Nitrite and nitrate

The analyses of nitrite (NO3-) and nitrate (NO2-) were conducted using a flow injection autoanalyzer (Lachat Instruments, QuikChem 8000) following the method of Grasshoff *et al.* (1999). The analyses were fully automated and peak areas were calibrated using 0 to 5 μM standards prepared in nutrient-depleted filtered seawater (Meeder *et al.* 2012). The precision between duplicate analyses of sample splits was ±0.02 and ±0.05 μM for nitrite and nitrate, respectively.

### Oxygen

Oxygen optodes underwent two-point calibration using 100% oxygen-saturated seawater and oxygen-depleted seawater made anoxic by addition of sodium sulfite. Dissolved oxygen readings were automatically adjusted to the water temperature that was recorded *in situ* by the instrument’s temperature sensor.

### Flow cytometry

Concentrations of picoplankton cells in the IN-EX samples were measured using an LSR II flow cytometer (Becton, Dickinson and Co., USA) and using the FCS Express v.5 software for data analysis. The specifications of the flow cytometer were adjusted to fit the concentrations of cells in the Eastern Mediterranean water (Supporting Information 2) and 1 μm beads (Invitrogen) were used for size reference.

Phytoplankton cells with red and orange fluorescence were classified as picoeukaryotes (Euk), prokaryotic *Prochlorococcus*-like (Pro) or *Synechococcus*-like (Syn) populations, and detected using the auto fluorescence, size and shape characteristics, according to Marie *et al.* (2001). The non-photosynthetic heterotrophic bacteria (Het) were detected using SYBR Green (SG) staining for DNA as in Perea-Blázquez *et al.* (2012).

The efficiency of cell retention by the sponge (RE) was calculated as:

RE [%] = (([IN] – [EX])/[IN]))\*100%, where [IN], [EX] – are the concentrations of a given cell type [cells/ml] in the inhaled and the exhaled samples, respectively.

Negative retention rates resulting from cell concentrations in exhaled samples being higher than the concentrations in inhaled samples were interpreted as no retention of picoplankton and consequently changed to zero.

## Statistical analyses

We collected 87 IN-EX pairs of water samples from 10 *C. reniformis* explants. The results of nutrient analyses were combined with the data on oxygen consumption and picoplankton retention rates, and 19 IN-EX pairs were excluded from statistical analyses due to missing results, low retention rates (<50% for all picoplankton groups) or suspicion of artifacts. The final database consisted of 68 IN-EX results for nutrients combined with average oxygen consumption for each IN-EX pair.

The effects of inhaled ammonium concentration (NH4IN) on ΔNH4, ΔNOx and ΔO2 were analyzed by the Generalized Linear Mixed Model (GLIMMIX) procedure of SAS version 9.4 with *explant* as a random blocking variable and *treatment* (ambient *vs* enrichment), *season* (summer *vs* autumn) and NH4IN as fixed effects. For each of the response variables (ΔNH4, ΔNOx and ΔO2) a full-factorial model of fixed effects and their interactions was tested first, and after removing the non-significant interactions (p≥0.05) by backward elimination, the model was re-run.

# Results (443 words)

During the experiments, the explants maintained natural color, opened osculae and pumped water. The changes in DIN and oxygen concentrations between the inhaled (IN) and the exhaled (EX) water samples collected from sponge explants were considerably larger than the changes obtained from the control aquarium without a sponge (Fig. 1)



Figure 1: Changes in dissolved inorganic nitrogen and dissolved oxygen concentrations between IN-EX samples collected from sponge and control (no sponge) aquaria. a) Dissolved inorganic nitrogen (DIN) ammonium and nitrates; b) oxygen. Averages ± SD. n = 39, 32 and 6 for ambient, enrichment and control treatments, respectively.

The retention of picoplankton by *C. reniformis* was similar under both treatments, with highest retention for *Synechococcus*-like particles (Table 1).

**Table 1: Retention of picoplankton by *Chondrosia reniformis* explants.**

|  |  |
| --- | --- |
| **Treatment** | **Retention Efficiency (%) for different picoplankton groups**(average, **median**, SD) |
| *Synechococcus*-like (*Syn*) | *Prochlorococcus*-like (*Pro*) | Pico-Eukaryotes | Heterotrophic Bacteria (*Het*) |
| **Ambient**  | 69, **95**, 35 | 16, **13**, 17 | 57, **69**, 32 | 41, **39**, 32 |
| **Enrichment** | 71, **97**, 38 | 14, **0**, 21 | 57, **71**, 31 | 49, **61**, 32 |

Positive differences between the inhaled and the exhaled concentrations of ammonium (ammonium uptake) were detected in 36 out of 68 IN-EX pairs. The differences in NOx concentrations between the inhaled and the exhaled samples were negative in most cases (63 out of 68 IN-EX pairs), i.e. nitrates were excreted by the sponge (Appendix 2, Full Data Set).

The effect of ammonium concentration (NH4IN) alone was not statistically significant on any of the response variables (ΔNH4, ΔNOx and ΔO2). The effect of treatment was of borderline statistical significance on ΔNOx; and the effects of season, NH4IN and of the interaction NH4IN\*season on ΔO2 were statistically significant (Table 2).

**Table 2:** **GLIMMIX results on the effects of inhaled ammonium, treatment and season on the nitrification outcomes.**

|  |
| --- |
| Type III Tests of fixed effects |
| Responsevariable | Effect | df | F-value | P-value | run |
| dNH4 | NH4IN | 52 | 0.39 | 0.5333 | 1 |
|  | treatment | 52 | 0.38 | 0.5396 | 1 |
|  | season | 52 | 0.54 | 0.4661 | 1 |
|  | NH4IN\*treatment | 52 | 0.08 | 0.7768 | 1 |
|  | NH4IN\*season | 52 | 0.34 | 0.5640 | 1 |
|  | treatment\*season | 52 | 0.86 | 0.3581 | 1 |
|  | NH4IN\*treatment\*season | 52 | 0.00 | 0.9706 | 1 |
|  | NH4IN | 56 | 0.07 | 0.7931 | 2 |
|  | treatment | 56 | 0.24 | 0.6258 | 2 |
|  | season | 56 | 0.12 | 0.7299 | 2 |
| dNOx | NH4IN | 52 | 0.07 | 0.7936 | 1 |
|  | treatment | 52 | 0.02 | 0.8875 | 1 |
|  | season | 52 | 0.93 | 0.3384 | 1 |
|  | NH4IN\*treatment | 52 | 0.06 | 0.8148 | 1 |
|  | NH4IN\*season | 52 | 0.43 | 0.5132 | 1 |
|  | treatment\*season | 52 | 0.15 | 0.6994 | 1 |
|  | NH4IN\*treatment\*season | 52 | 1.69 | 0.1999 | 1 |
|  | NH4IN | 57 | 3.23 | 0.0778 | 2 |
|  | **treatment** | **57** | **3.76** | **0.0575** | **2** |
|  | season | 58 | 0.91 | 0.3434 | 2 |
| dO2 | NH4IN | 52 | 0.48 | 0.4900 | 1 |
|  | treatment | 52 | 0.24 | 0.6265 | 1 |
|  | **season** | **52** | **12.39** | **0.0009** | **1** |
|  | NH4IN\*treatment | 52 | 0.00 | 0.9929 | 1 |
|  | **NH4IN\*season** | **52** | **11.20** | **0.0015** | **1** |
|  | treatment\*season | 52 | 1.58 | 0.2140 | 1 |
|  | NH4IN\*treatment\*season | 52 | 2.90 | 0.0947 | 1 |
|  | NH4IN | 54 | 0.85 | 0.3608 | 2 |
|  | treatment | 54 | 0.84 | 0.3630 | 2 |
|  | **season** | **54** | **12.60** | **0.0008** | **2** |
|  | **NH4IN\*season** | **54** | **8.94** | **0.0042** | **2** |

df = degree of freedom; significant effects at p ≤0.05 are highlighted in bold; run = model iteration after backward elimination of non-significant effects;

# Discussion (word count: 922)

The present study examined the effect of ammonium enrichment on microbe-mediated nitrification in the HMA sponge *Chondrosia reniformis*. The augmentation of water with ammonium simulated the cultivation of *C. reniformis* next to fish farms where the water is enriched with ammonium. Based on the previous findings of Bayer *et al.* (2008), Jiménez and Ribes (2007), Schläppy *et al.* (2010b) and Ribes *et al.* (2012), we assumed that microbe-mediated nitrification in *C. reniformis* would be enhanced by elevating the concentrations of ammonium in the water, the “induced nitrification” hypothesis. Although we observed ammonium uptake in nearly half of experimental outcomes and excretion of nitrate in most IN-EX pairs, the oxidation of exogenous ammonium (nitrification) seems not to be the source for nitrate excretion, since ammonium and oxygen consumptions were not linked to nitrate excretion, as discussed below.

## Feeding and nitrification

Sponges feed on picoplankton cells in the size range of 0.2 – 2 μm with almost 100% efficiency (Pile *et al.* 1996, Ribes *et al.* 1999, Topçu *et al.* 2010, Jiménez 2011). In the present work, the efficiencies of picoplankton retention by *C. reniformis* (Table 1) were lower than those reported by Jiménez (2011) for the same species from the Western Mediterranean Sea. We observed 16% retention of *Prochlorococcus*-like population and 45% retention of heterotrophic bacteria, whereas Jimenez (2011) observed 93% and 98% retention of the corresponding populations. *Synechococcus*-like cells were retained with similar efficiency (96%) as in the work of Jimenez (2011). Topçu *et al.* (2010) and Perea-Blázquez *et al.* (2012) also found that *Synechococcus*-like cells are the preferred food source for sponges. This indicates that in our study *C. reniformis* explants were feeding normally during the experiments.

The analysis of IN-EX results (Table 2) suggests that the nitrification activity of *C. reniformis* is not related to the concentration of ammonium in the surrounding seawater. In a field study, Fiore *et al.* (2013) observed both positive and negative fluxes of ammonium and NOx through the tropical HMA sponge *Xestospongia muta*. In a laboratory study of Bayer *et al.* (2008), the Mediterranean HMA sponge *Aplysina aerophoba* acted as a sink or a source of ammonium alternately, depending on the water temperature. In the present work, the flux of ammonium through the sponge was not affected by temperature: uptake and release of ammonium were observed at similar frequencies in summer and in autumn. Taken together, the findings of Bayer *et al.* (2008), Fiore *et al.* (2013) and the present work suggest that *C. reniformis* does not always act as an ammonium sink and that we cannot generalize about HMA sponges serving as a source or a sink of ammonium.

It is noteworthy that while HMA sponges such as *C. reniformis* are usually positive for nitrate excretion (Jiménez and Ribes 2007; Bayer *et al.* 2008; Hoffman *et al.* 2009; Ribes *et al.* 2012, present study), sponges of the LMA group do not excrete nitrate or do so at much reduced rates (Jiménez and Ribes 2007; Yahel *et al.* 2007; Ribes *et al.* 2012). There is evidence for aerobic nitrification and for anaerobic processes such as denitrification and anammox in both HMA and LMA sponges (Hoffman *et al.* 2009, Mohamed *et al.* 2010, Schläppy *et al.* 2010b). It is reasonable to assume that in the present study we observed the net outcome of coexisting or even competing aerobic and anaerobic microbial processes co-occurring in the same sponge (*C. reniformis*), which might explain the variations in the excretion of nitrate. Further research is thus needed to decipher the specific contributions of co-occurring microbial process to the net outcome of nitrate excretion.

## Oxygen consumption

The changes in oxygen consumption by *C. reniformis* were not associated with the increase in ammonium concentrations in seawater (Table 2). These results suggest that the contribution of nitrification to the consumption of oxygen by *C. reniformis* holobiont is minor compared to other oxygen-consuming functions, such as water pumping, which was not accounted for in the present study. Hadas *et al.* (2008) found that “roughly 75% of sponge oxygen consumption is used for ‘maintenance’ and water propulsion (pumping)”. Leys *et al.* (2011) showed that “at least 28% of total respiration is required for maintaining even modest pumping rates”. Although we verified that sponges were feeding during the experiments (Table 1), we did not account for the possible changes in sponge pumping during the experiments, and in further studies we should perform a quantitative assessment of sponge pumping when dealing with oxygen consumption and nutrient processing by sponge holobionts.

## Conclusions and suggestions for further research

Our findings suggest that nitrification may not be the prevalent microbe-mediated process in sponges under conditions of nitrogenous enrichment and that we cannot generalize about HMA sponges serving as a source or a sink of ammonium. Particularly, this study suggests that *C. reniformis* should not be considered as a sink of ammonium in models of integrated aquaculture.

In a recent study, Gantt *et al.* (2019) showed that relative abundances of ammonia-oxidizing microbial taxa increase when sponges exhibit higher levels of ammonium consumption. In order to elucidate the mechanisms underlying the variability in sponge-mediated DIN fluxes (ammonium uptake and nitrate excretion), we suggest combining physiological experiments as performed in the present work with sampling for microbial community composition as performed by Gantt *et al.* (2019). Thus, by knowing what the sponge-associated microbes are and what these microbes do at different conditions, we should be able to better predict the contribution of sponge-mediated DIN fluxes in natural and in human-impacted ecosystems.

# Conflict of Interest

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# References

# Supplementary Material

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