Deep-sea sponge grounds as nutrient sinks: Denitrification is common in boreo-arctic sponges

Christine Rooks[1], James Kar-Hei Fang[2], Pål Tore Mørkved[3], Rui Zhao[1], Hans Tore Rapp[1, 4, 5], Joana R. Xavier[1, 6] and Friederike Hoffmann[1].

[1]Department of Biological Sciences, University of Bergen, Postboks 7803, 5020, Bergen, Norway.

[2]Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Hong Kong.

[3]Department of Earth Sciences, University of Bergen, Postboks 7803, 5020, Bergen, Norway.

[4]K.G. Jebsen Centre for Deep Sea Research, University of Bergen, Postboks 7803, 5020, Bergen, Norway.

[5]NORCE, Norwegian Research Centre, NORCE Environment, Nygårdsgaten 112, 5008 Bergen, Norway

[6]CIIMAR – Interdisciplinary Centre of Marine and Environmental Research of the University of Porto, 4450-208 Matosinhos, Portugal.

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Abstract

Sponges are commonly known as general nutrient providers for the marine ecosystem, recycling organic matter into various forms of bio-available nutrients such as ammonium and nitrate. In this study we challenge this view. We show that nutrient removal through microbial denitrification is a common feature in six cold-water sponge species from boreal and Arctic sponge grounds. Denitrification rates were quantified by incubating sponge tissue sections with $^{15}NO_3^-$ - amended oxygen saturated seawater, mimicking conditions in pumping sponges, and de-oxygenated seawater, mimicking non-pumping sponges. Rates of anaerobic ammonium oxidation (anammox) using incubations with $^{15}NH_4^+$ could not be detected. Denitrification rates of the different sponge species ranged from below detection to 97 nmol N cm^{-3} sponge day^{-1} under oxic conditions, and from 24 to 279 nmol N cm^{-3} sponge day^{-1} under anoxic conditions.

A positive relationship between the highest potential rates of denitrification (in the absence of oxygen) and the species-specific abundances of *nir*S and *nir*K genes encoding nitrite reductase, a key enzyme for denitrification, suggests that the denitrifying community in these sponge species is active and prepared for denitrification. The lack of a lag phase in the linear accumulation of the ^{15}N labelled N_2 gas in any of our tissue incubations is another indicator for an active community of denitrifiers in the investigated sponge species.

Low rates for coupled nitrification-denitrification indicate that also under oxic conditions, nitrate to fuel denitrification rates was derived rather from the ambient seawater than from sponge nitrification. The lack of *nif*H genes encoding nitrogenase, the key enzyme for nitrogen fixation, shows that the nitrogen cycle is not closed in the sponge grounds. The denitrified nitrogen, no matter of its origin, is then no longer available as a nutrient for the marine ecosystem.

These results suggest a high potential denitrification capacity of deep-sea sponge grounds based on typical sponge biomass on boreal and Arctic sponge grounds, with areal denitrification rates of

0.6 mmol N m^{-2} day^{-1} assuming non-pumping sponges and still 0.3 mmol N m^{-2} day^{-1} assuming pumping sponges. This is well within the range of denitrification rates of continental shelf sediments. Anthropogenic impact and global change processes affecting the sponge redox state may thus lead to that deep-sea sponge grounds change their role in the marine ecosystem from being mainly nutrient sources to becoming mainly nutrient sinks.

1 Introduction

Sponges are sessile filter feeders with an immense capacity to process large volumes of seawater (Kahn et al., 2015;Reiswig, 1974). As such, they play a critical role in benthic-pelagic coupling, recycling particulate or dissolved organic matter from the water column into various forms of bio-available nutrients (Brusca and Brusca;Reiswig, 1974;Yahel et al., 2003;Hoffmann et al., 2009;Schläppy et al., 2010a;Maldonado et al., 2012;de Goeij et al., 2013;Rix et al., 2016). Sponges show an active nitrogen metabolism, as recently reviewed by (Feng and Li, 2019;Folkers and Rombouts, 2020;Pawlik and McMurray, 2019;Zhang et al., 2019). Actively pumping sponges have been associated with the release of dissolved inorganic nitrogen (DIN), enriching ex-current waters with excess ammonium (NH_4^+) and/or nitrite and nitrate (NO_3^- and/or NO_2^-, summarized as NO_x^-) (Southwell et al., 2008;Fiore et al., 2013;Keesing et al., 2013;Leys et al., 2018;Hoer et al., 2018). Whilst NH_4^+ is excreted by sponge cells as a metabolic waste product (Yahel et al., 2003), NO_x^- is derived from the microbial oxidation of NH_4^+, through NO_2^-, to NO_3^- in aerobic nitrification (Painter, 1970;Corredor et al., 1988;Diaz and Ward, 1997;Jiménez and Ribes, 2007;Schläppy et al., 2010a;Southwell et al., 2008;Radax et al., 2012;Fiore et al., 2010).Nitrogen fixation has also been reported in shallow water sponges (Wilkinson and Fay, 1979;Wilkinson et al., 1999;Mohamed et al., 2008;Ribes et al., 2015), reducing biologically inaccessible N_2 gas to NH_4^+, which represents yet another source of DIN from sponges. DIN release has been affiliated with a number of deep-sea and shallow water sponges and varies according to species (Schläppy et al., 2010a;Radax et al., 2012;Keesing et al., 2013), as well as on temporal (Bayer et al., 2008;Radax et al., 2012) and spatial scales (Fiore et al., 2013;Archer et al., 2017). Such variations have been linked to abiotic conditions, as well as the availability of organic matter or nutrients in the water column (Bayer et al., 2008;Archer et al., 2017;Fiore et al., 2013).

In any case, since nitrification is dependent on oxygen, NO_x^- release is dependent on active filtration, delivering an excess of O_2 to sponge tissues. (Reiswig, 1974;Hoffmann et al., 2008;Southwell et al., 2008;Pfannkuchen et al., 2009;Fiore et al., 2013;Keesing et al., 2013;Leys et al., 2018). Fluctuations in pumping activity, however, disrupt the delivery of O_2 to sponge tissues, resulting in either heterogeneous oxygenation within the sponge matrix or complete anoxia (Hoffmann et al., 2005;Hoffmann et al., 2008;Schläppy et al., 2010b;Schläppy et al., 2007). Under such conditions, a paucity of oxygen would inevitably promote anaerobic microbial processes.

Anaerobic N-transformations have been quantified using ^{15}N tracer experiments in deep-sea (Hoffmann et al., 2009) and shallow water sponges (Schläppy et al., 2010a;Fiore et al., 2013). In the deep-sea sponge, *Geodia barretti*, the removal of fixed nitrogen via heterotrophic denitrification (the sequential and anaerobic reduction of NO_3^-, via NO_2^-, to N_2) was shown to exceed sedimentary denitrification rates at equivalent depths by a factor 2 to 10 (Hoffmann et al., 2009). Also anaerobic ammonium oxidation (anammox, transforming NH_4^+ and NO_2^- to N_2) was quantified in that study, as well as nitrification performed simultaneously with denitrification (coupled nitrification-denitrification). Given that marine sediments are considered the major sites of marine N-transformations (Middelburg et al., 1996;Seitzinger, 1988), sponges may thus represent a significant, yet largely overlooked sink for bioavailable nitrogen (Hoffmann et al., 2009).

The redox state of the sponge tissue as well as the rates of the different N-transforming processes could thus determine whether the sponge may act as a nutrient source or a nutrient sink. It has been observed in field studies that sponges can act as both net sources or sinks for NH_4^+ and NO_3^- (Fiore et al., 2013;Archer et al., 2017); however the

balance of the underlying processes and their controlling factors, have not as yet been quantified.

The understanding of such processes and their dynamics is particularly relevant for areas where sponges occur in high densities forming highly structured habitats as is the case of the sponge grounds found widely distributed across the deeper areas of the oceans. In such areas, sponges can represent up to 95% of the total invertebrate biomass (Murillo et al., 2012) and attain densities of up to 20 individuals m^{-2} (Hughes and Gage, 2004). In the North Atlantic boreo-arctic region the widely distributed sponge grounds have traditionally been divided into two main types. The cold-water (arctic) type, generally found along continental slopes and mid-ocean ridges at negative temperatures, or at least below 3-4°C, and comprising a multi specific assemblage of demosponges (the astrophorids *Geodia parva, G. hentscheli* and *Stelletta rhaphidiophora*) and glass sponges (the hexactinellids *Schaudinnia rosea, Trichasterina borealis, Scyphidium septentrionale* and *Asconema foliata*) (Cardenas et al., 2013;Klitgaard and Tendal, 2004;Roberts et al., 2018) . The boreal type is mainly found along continental shelves and upper slopes and at temperatures above 4 °C. These grounds are dominated by the astrophorids *Geodia barretti, G. atlantica, Stryphnus fortis* and *Stelletta normani* (Cardenas et al., 2013;Klitgaard and Tendal, 2004;Murillo et al., 2012).To make reliable estimates on the potential nitrogen sink function of these deep-sea sponge grounds, denitrification rates from more sponge ground species are needed.

In this study we quantify the potential nutrient sink function of six sponge species which characterize the two main types of boreo-arctic Tetractinellid sponge grounds. We aim to test our hypothesis that nutrient removal through microbial denitrification is a common feature in cold-water sponges, and that rates are dependent on oxygen availability in the sponge tissue. Based on these results we aim to estimate the potential nutrient sink function of boreo-arctic sponge grounds for the marine ecosystem.

2 Materials and Methods

2.1 Site description

Arctic sponge species were collected at the Schulz Bank (73° 50’ N, 7° 34’ E). This is a large seamount located at the transition between the Mohn and the Knipovich ridges, two of the main sections of the Arctic Mid-Ocean Ridge (AMOR). The seamount rises from more than 2.500 m depth and its summit and shallower areas (550-700 m depth) host a dense and diverse sponge ground composed of a multispecific assemblage of species dominated by tetractinellid desmosponges (*Geodia parva*, *G. hentscheli*, and *Stelletta rhaphidiophora*) and hexactinellid sponges (*Schaudinnia rosea*, *Trichasterina borealis*, *Scyphidium septentrionale*, and *Asconema foliata*). Exact hydrodynamic settings at the summit is not known, but conditions measured using a benthic lander at 670 m (i.e. 70-80 m below it) revealed a water temperature just below 0 °C, salinity of 34.9, and dissolved oxygen between 12.4-12.6 mg L^{-1}. Near-bed suspended particulate matter concentrations was determined to be 3.2 mg L^{-1}, considerably larger than those observed both in surface and deeper waters (where values range from less than 1 and 2 mg L^{-1}) (Roberts and J., 2018;Roberts et al., 2018).

Boreal sponge species were collected on the hard bottom slope of the fjord Korsfjord (60°09′12″N, 05°08′52″E) near the city of Bergen on the west coast of Norway. Hard bottom slopes of these fjords, which can be several hundred meters in deep, host dense assemblies of typical boreal sponges, dominated by tetractinellid demosponges such as different species of the Geodiidae. Site characteristics are described elsewhere (Hoffmann et al., 2003).

Average sponge biomass (kg/m^2) in both Arctic and boreal grounds was estimated from trawl catches and underwater imagery collected in the course of various sampling campaigns.

2.2 Sample collection and preparation

Intact individuals from each of the key Arctic species, *Geodia hentscheli* (n=3), *Geodia parva* (n=3) and *Stelletta rhaphidiophora* (n=3) were retrieved from a depth of 700m at the top of Schulz Bank. Sponges were collected with a remotely operated vehicle (ROV) on board the R/V GO Sars in June 2016

Intact individuals from each of the key boreal species, *Geodia barretti* (n=3), *Geodia atlantica* (n=3) and *Stryphnus fortis* (n=3) were collected from a depth of 200m at the slope of Korsfjorden, Norway. Sponge individuals were retrieved using a triangular dredge deployed from the R/V Hans Brattstrøm in November 2016.

Upon retrieval, samples were immediately transferred into containers holding low-nutrient seawater, directly recovered from the sampling site. Following species identification, intact individuals were either transported to the aquaria at the University of Bergen (ca. 1h; boreal species), or immediately to the lab on board the R/V G.O. SARS (Arctic species). Sponge tissue, from three intact individuals, was then dissected for use in either ^{15}N-labelled tissue incubations or preserved for subsequent DNA extraction for each species.

Whilst completely immersed in site water, the massive sponge individuals were cut into 3 sections of approximately equal size to aid dissection. Using an autoclaved stainless steel core (internal diameter = 0.74cm; length = 7cm), the choanosomal portion of the sponge was sliced from each section to produce cylindrically-shaped tissue samples. Three whole sponges (n=3) were collected for each species. The dissected tissue from a single sponge individual represented one replicate. Avoiding exposure to air, tissue samples were then transferred to 1L containers holding site water. Using a sterile scalpel, the tissue cylinders were further sectioned (under water) into pieces of equal

size (volume = 0.45cm^3). The samples were then either distributed into 12mL gas-tight vials (Exetainer, Labco, High Wycombe, UK) for incubation with ^{15}N isotopes, or into 1.5mL microcentrifuge tubes, snap frozen and stored at -80°C for subsequent DNA extraction.

Sediment was collected from the Arctic sponge grounds using a box-corer. The upper few centimeters were sampled, homogenised and packed into 10 mL sterile cut-off syringes. 1mL of sediment was then either distributed into 3mL gas tight vials (Exetainer, Labco, High Wycombe, UK) for ^{15}N isotope incubations or into 1.5ml microcentrifuge tubes (Eppendorf), snap-frozen and stored at -80°C for subsequent DNA extraction. At the boreal sponge ground, sponges were collected from the rocky slope of the fjord,.It was therefore not possible to collect sediment from this site.

2.3 Quantifying rates of N-removal processes in sponge tissues and deep-sea sediments

2.3.1 Sponge tissue incubations

For simulating conditions in pumping and non-pumping sponges, sponge tissue sections were incubated with oxygen-saturated (standard temperature and pressure) and degassed site water (oxygen free seawater, degassed with ultra high purity He). Site water was retrieved using 10L Niskin flasks mounted on a CTD rosette water sampler aboard the R/V GO Sars. This water was collected at a depth of approximately 650m, just above the summit of the seamount. It was then filtered to remove water column bacteria and or phytoplankton (0.2µm polycarbonate filters, Whatman Nucleopore) and added to all incubations with Arctic specimens. Boreal specimens were incubated with sand filtered seawater, pumped into the aquaria at the University of Bergen from a local fjord. This water was sourced from a depth of 130m.

To ensure that all labelled N_2 gas was retained, it was necessary to maintain gas-tight conditions in each of the incubations. Consequently, no oxygen could be added during the experiment. Estimating from typical respiration rates of 0.32 µmol O_2 mL sponge^{-1} h^{-1} in *G. barretti* (Leys et al., 2018), this would suggest the complete removal of oxygen (by sponge cells and associated microbes) following 26 hours of incubation (12 ml exetainer, sponge pieces 0.45 cm^3, oxygen concentration at experiment start 313 µmol/L). This means that oxygen concentrations in the aerobic incubation continuously decreased from oxygen saturation to zero throughout the course of the experiment, thus mimicking conditions where a sponge has recently ceased pumping, or where pumping occurs at a low rate (Fang et al., 2018;Hoffmann et al., 2008;Schläppy et al., 2010b). Nevertheless, we can assume that oxygen was available during the first 26 hours of incubation in the oxic experiment, in contrast to the anoxic experiment where oxygen was absent from the beginning of the incubation, thus mimicking non-pumping conditions (Hoffmann et al., 2008;Schläppy et al., 2010b).

For the oxic incubations, 12 mL of air-saturated (standard temperature and pressure) seawater was transferred into 12 mL gas tight vials. Using autoclaved forceps, one piece of freshly dissected tissue was then placed into each gas tight vial, until a sufficient number of samples were prepared for the incubations. The caps were then replaced and the vial was carefully sealed to exclude any air bubbles.

For the anoxic incubations, 2L of site water was de-gassed with ultra high purity He for 2h. To verify the absence of oxygen in the de-gassed water, an anaerob strip test (colour change from pink to white under anaerobic conditions; Sigma Aldrich) was performed prior to transfer into 12mL exetainers. The caps were then replaced and the gas tight vials were carefully sealed to exclude any

air bubbles. An anaerob strip was added to control exetainers (seawater only) to verify the absence of oxygen during anaerobic incubations.

Incubations were prepared in four sets of 1 un-amended reference (no isotope added) and 5 amended (^{15}N labelled) samples per in-tact sponge (x3 in-tact sponge individuals/ species). Each set was then either injected (gas tight luer lock syringes, VICI, USA) with air saturated (at standard temperature and pressure, for oxic incubations) or oxygen free (de-gassed; for anoxic incubations) concentrated stock solutions of i) $Na^{15}NO_3^-$ (99.2 ^{15}N atm. %), screening for denitrification; $^-$ or ii) $^{15}NH_4^+$ Cl^- (≥98. ^{15}N atm %) and $Na^{14}NO_3^-$, screening for anammox. Solutions were shaken vigorously. The final concentrations of i) $^{15}NO_3^-$; or ii) $^{15}NH_4^+$; $^{14}NO_3^-$ were 100µM NO_3^- and 10µM NH_4^+ respectively. These values were essentially 10 times above ambient NO_3^- (10 µM NO_3^-) and NH_4^+ concentrations (<1µM NH_4^+) present in the seawater. Prior to the incubations, however, background nutrient concentrations were unknown. In this regard, to ensure that the availability of ^{15}N was sufficient for the measurement of denitrification and or anammox (e.g. at least 50% above the ambient pool of ^{14}N), we selected high concentrations of stock solutions (Holtappels et al., 2011). To enable continuous homogenisation of the isotopic label with sponge tissue, exetainers were placed on rollers (Spiromix, Denley) and incubated at (6°C) in the dark. At zero hours, and at subsequent 3-6 hour intervals, a selection of samples were injected with 2mL of ultra high purity helium to create an oxygen free headspace using a gas-tight syringe. The vials were then injected with 200µL of formaldehyde, and shaken vigorously to inhibit further microbial activity. This was repeated over a period of 48 hours.

2.3.2 Sediment slurry incubations

One mL of the homogenised sediment was distributed into 3mL gas-tight vials (Exetainer, Labco, High Wycombe, UK) with 1mL of de-gassed site water (as above). The cap was replaced, the headspace (1mL) flushed with ultra-high purity helium and each vial was shaken vigorously to produce an anaerobic sediment slurry. Anaerobic slurries were prepared as 2 sets of un-amended references (no isotopic mixture added) and 5 amended samples in incubations screening for either anammox and or denitrification. Amended samples were injected with oxygen free isotopic mixtures (as above) and placed on rotating rollers (Spiromix, Denley) in a constant temperature room (6°C) in the dark. At zero hours, and every subsequent 3-6 hours, 3 samples per sponge species (one from each replicate specimen) was injected with 200µL of formaldehyde, and shaken vigorously to inhibit further microbial activity. This was repeated over a period of 48 hours. Concentrations of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ were measured as above and calculations for denitrification and or anammox were performed as per (Thamdrup and Dalsgaard, 2002) and (Risgaard-Petersen et al., 2003).

2.3.3 Calculation of denitrification and anammox rates

Concentrations of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ were measured by directly sub-sampling 70µL from the gas headspace on a GC (Trace GC, Thermo Fisher Scientific, Bremen) connected to a continuous flow isotope ratio mass spectrometer (Delta V plus, Thermo Fisher Scientific, Bremen) calibrated with in house reference gas and air. Though we never observed visual signs for tissue degradation (see for example (Hoffmann et al., 2003;Osinga et al., 2001;Osinga et al., 1999) for description of how to spot signs of sponge tissue degradation), some samples showed an abrupt increase in N_2 production, indicating the onset of tissue degradation. These were not included in the analyses and

rate calculations. Calculations for rates of both anammox and denitrification were based on established methods for measuring these processes in sediments (Thamdrup and Dalsgaard, 2002;Risgaard-Petersen et al., 2003). Rates were calculated from the linear increase in excess N_2-accumulation over time as measured from the isotope ratio mass spectrometer.

The accumulation of excess $^{29}N_2$ and $^{30}N_2$, from incubations with $^{15}NO_3^-$, was linear over a 24h period (p<0.05) and precluded an initial lag phase (Figures 1a and 1b). This was the case for all species. In the oxic incubations, after 24 hours a sharp non-linear increase in labelled N_2 was detected. This is in good agreement with our calculations for oxygen depletion (26 hours, see above). Since we observed no signs of tissue degradation in these during the 48 hours of incubation this increase was taken to indicate a switch of metabolic processes within the sponge towards predominantly anaerobic pathways, and thus, a different denitrification rate. For the anoxic incubations, N_2-production was also linear during the first 24 hours of incubations, although the data were more scattered when compared with oxic incubations. The scatter increased after 24 hours, though most incubations still followed a similar linear trend.

For best comparability of denitrification rates from oxic and anoxic incubations, only the first 24 hours, where N_2 production was linear in all experiments, and where oxygen was assumed to be present in the exetainers of the oxic incubation, were used to calculate denitrification rates.

No $^{29}N_2$ production was detected following labelling with $^{15}NH_4^+$ and $^{14}NO_3^-$, suggesting an absence of anammox activity. Therefore, no anammox rates could be calculated. The N_2 produced during the $^{15}NO_3^-/^{14}NH_4^+$ experiments is assumed to originate entirely from denitrification.

2.3.4 Calculation of coupled nitrification-denitrification and the denitrification of NO_3^- derived from ambient seawater

To determine the predominant source of NO_3^- fueling denitrification, rates of coupled nitrification-denitrification and the denitrification of NO_3^- supplied by the ambient seawater, were calculated according to the methods of Nielsen (1992) (Nielsen, 1992). Production of NO_3^- can occur endogenously via the aerobic oxidation of NH_4^+ to NO_3^- within the sponge tissues. In turn, this represents a source of NO_3^- for denitrification which 'couples' nitrification to denitrification. Alternatively, denitrification can simply be fueled by NO_3^- diffusing from the ambient seawater. By taking into consideration the frequency of ^{14} and $^{15}NO_3^-$ availability, in addition to random isotope pairing, it is possible to calculate the source of denitrified NO_3^- from the abundance of ^{28}, ^{29} and $^{30}N_2$ in all oxic incubations.

Denitrification rates were calculated from the production of ^{15}N isotopes (see below) according to the method described by Nielsen (1992).

$$D_{15} = p\,(^{14}N\ ^{15}N) + 2p\,(^{15}N\ ^{15}N) \quad (1)$$

$$D_{14} = \frac{p\,(^{14}N\ ^{15}N)}{2p\,(^{15}N\ ^{15}N)}\,D_{15} \quad (2)$$

The rate of denitrification was measured from ^{15}N isotope production (equations 1 and 2). D_{15} and D_{14} represent denitrification of labelled $^{15}NO_3^-$ and $^{14}NO_3^-$, respectively. p ($^{14}N^{15}N$) and p ($^{15}N^{15}N$) are the production rates of the 2 labelled N_2 species $^{14}N^{15}N$ and $^{15}N^{15}N$ (Rysgaard et al., 1995). Essentially, D_{15} is indicative of denitrification of labelled $^{15}NO_3^-$ and D_{14} represents *in situ* denitrification of $^{14}NO_3^-$.

To estimate denitrification of NO_3^- from the ambient water (D_w), in terms of D_{14}, the following calculation was applied (equation 3):

$$D_w = D_{15} [^{14}NO_3]_w / [^{15}NO_3]_w \quad (3)$$

where $[^{14}NO_3]_w$ and $[^{15}NO_3]_w$ represent the concentration of unlabeled and labelled NO_3^- in the ambient water. Dw thus represents an estimate of denitrification of NO_3^- at ambient NO3- concentrations (approximately 10uM) and we are in the rest of the publication referring to this as denitrification rates if nothing else is stated.

In situ coupled denitrification (D_n), in terms of D_{14}, was calculated using equation 4 (see below).

$$D_n = D_{14} - D_w \quad (4)$$

2.4 Screening and quantifying the abundance of *nir*S, *nir*K and *nif*H genes

Total DNA was extracted from dissected sponge pieces (0.45cm^{3}of sponge tissue) using a FastDNA Spin Kit for Soil (mpbio, Santa Ana, CA, USA) following the manufacturer's instructions. In total, DNA was extracted from 3 tissue samples retrieved from each of the intact sponges (3 intact individuals sampled/ key species) as well sediment samples (1mL, ~2g sediment slurry) and sample blanks (RNAse free water). DNA extracts were eluted into 100 μL of PCR grade double distilled H_2O and stored at -20^{o}C until further analysis.

The functional genes diagnostic of nitrogen fixation (*nif*H encoding nitrogenase) and denitrification (*nir*S/K encoding nitrite reductase) in sponges were screened using conventional PCR of 40 cycles. *nif*H gene was amplified using the primer pair nifHfw/nifHrv (Mehta et al., 2003) with the following thermal conditions: 94ºC for 15 min, and 40 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 60 s. nirS/K genes were amplified using the primers and thermal conditions as described below. Each reaction mixture (25µl total volume) contained the following: 1× HotStar Taq® Master Mix (Qiagen, Hilden, Germany), 1.2 µM of each primer and 1 µl template DNA. PCR products were evaluated by visual inspection on 1% agarose gels.

The abundance of *nir*S or *nir*K genes of denitrifying bacteria were quantified using quantitative PCR (qPCR) on a StepOne Real-Time PCR system (Applied Biosystems). *nir*S genes were amplified using the primer pair nirS_cd3aF/nirS_R3cd (Throback et al., 2004), with thermal conditions as follows: 95ºC for 15 min, 45 cycles of denaturing at 95ºC for 15 s, annealing at 51ºC for 30 s, and elongation at 72ºC for 45 s. The *nirK* gene was amplified using the primer pair nirK_F1aCu/ nirK_R3Cu, with the following thermal conditions: 95ºC for 15 min, 45 cycles of denaturing at 95ºC for 30 s, annealing at 51ºC for 45 s, and elongation at 72ºC for 45 s. All qPCR reactions were run in triplicate and each reaction mixture contained 1× QuantiTech SybrGreen PCR master mixture (QIAgen, Germany), 0.5 µM forward and reverse primer and 1 µl of DNA template in a final volume of 20 µL. qPCR standard of each gene was linear DNA containing respective genes from an uncultured denitrifying bacterium in an Arctic permafrost soil. For each gene, the DNA concentration of the standard was measured using BIO-analyzer (DNA 1000 chips, Agilent Technologies) and a DNA abundance gradient of 10-10^5 copies μL^{-1} were prepared by 10x serial dilution.

2.5 Statistical analyses

Statistical analyses were performed to test for significant differences in (i) species-specific rates of denitrification or (ii) variations in the rates of denitrification according to oxygen availability. The data set failed to meet the assumptions of normality or equal variance. As a result, the data set was transformed by rank prior to two-way ANOVA. All pairwise multiple comparisons were performed using the Holm-Sidak method at species level. In all cases, the level of significance was set to at least $p < 0.05$. Statistical analyses were performed using the software SigmaPlot 13.0 (Systat Software, CA, USA).

3 Results

3.1 Denitrification activity in sponge tissues

The lack of $^{29}N_2$ production following labelling with $^{15}NH_4^+$ as observed in our study suggests an absence of anammox, since N_2 production via anammox requires 1 N from NO_2^- (which is not labelled) and 1 N from NH_4^+ (which is ^{15}N labelled). Therefore, no anammox rates could be calculated and the labelled N_2 produced during the $^{15}NO_3^-$ incubations is assumed to originate entirely from denitrification. Denitrification rates at ambient NO_3^- concentrations as calculated from this linear N_2-release (Eq 1- 4 in ch 2.3) were quantified in all 6 sponge species and are shown in figure 2. Mean rates of denitrification varied significantly between species (two-way ANOVA, $F_{1,5}$=117,337, p<0.01) and in the presence or absence of dissolved oxygen (two-way ANOVA, $F_{1,5}$=141,235, p<0.01). A significant interaction between species and the availability of dissolved oxygen was also identified by two-way ANOVA ($F_{1,5}$=9,315, p=0.037). Mean rates of denitrification were always greater in incubations with de-gassed seawater relative to incubations with fully air saturated seawater (Fig. 2). Under oxic conditions, mean rates varied from below detection in *Stryphnus fortis* to a maximum of 96 nmol N cm^{-3} sponge day^{-1} in *Geodia barretti*. However, under anoxic conditions, rates of denitrification ranged from 24 nmol N cm^{-3} sponge day^{-1} in *Geodia atlantica* to 280 nmol N cm^{-3} sponge day^{-1} in *Geodia parva* (Fig.2.). Differences in the rates of denitrification under either aerobic or anaerobic conditions were significant in *Stryphnus fortis* (t=6.591, p<0.05), *Geodia barretti* (t=2.197, p<0.05), *Geodia hentscheli* (t=4.577, p<0.05) *Geodia parva* (t=8.788, p<0.05) and *Stelletta rhaphidiophora* (t=6.408, p<0.05). Notably, the Arctic sponge ground species *G. hentscheli* and *G. parva* showed the highest anaerobic denitrification rates, with the boreal species *G. barretti* only slightly below.

No labelled N_2 production was detected in the surface sediment slurries screening for denitrification or anammox.

3.2 Coupled nitrification-denitrification and the absence of nitrogen fixation

In incubations with air saturated seawater, denitrifying activity was detected in all sponges with the exception of *Stryphnus fortis* (Fig. 2). The rates for coupled nitrification-denitrification (Dn, Eq4) were generally low, with 16% for *G. barretti* and 30% for *G. atlantica* as the highest values (Table 1). This shows that sea-water nitrate was the predominant source of nitrate for denitrification also under oxic conditions.

Functional genes for nitrogen fixation were not detected in any of the six sponge species, pointing towards the absence of nitrogen fixing microorganisms in these species.

3.3 Correlation between denitrification rates and the abundance of nitrite reductase

Copies of the nitrite reductase genes, *nir*S and *nir*K, were detected in all six sponges, though in different quantities (Table 2). The total nitrite reductase copy number (the sum of mean *nir*S and *nir*K gene copies per cm^{-3} sponge tissue) ranged from 2.19E+03 copies cm^{-3} sponge in *Stryphnus fortis* to 1.03E+09 copies cm^{-3} sponge in *Geodia parva* (Table 2). Although no denitrification activity was measured in the sediment slurry incubations, nitrite reductase was present at an abundance of 2.77E+04 copies cm^{-3} sediment.

We observed a positive relationship between denitrification rates under anoxic conditions, and total *nir* copy number, for all species except *G. atlantica*, the species with the lowest denitrification rate. No correlation to *nir* copy number was detected for denitrification rates under oxic conditions (Fig. 3.).

4 Discussion

4.1 Denitrification as a common feature of cold-water sponges

The purpose of this study was to quantify the potential nutrient sink function of six sponge key species from boreal and arctic sponge grounds. **We aimed to test our hypotheses that (1) nutrient removal through microbial denitrification is a common feature in cold-water sponge species, and that (2) rates are dependent on oxygen availability in the sponge tissue.**

All six species investigated in this study showed denitrification rates under anoxic conditions, five of them even under oxic conditions. Rates were always higher in the absence compared to in the presence of oxygen. All our denitrification rates are within the same range as rates previously reported for cold- and warm-water sponges: Hoffmann et al. (2009) reported 92 nmol N cm^{-3} sponge day^{-1} for explants of *G. barretti* incubated under oxic conditions, which is very close to our average rate of 97 nmol N cm^{-3} sponge day^{-1} for *G. barretti* sections incubated under oxic conditions. Rates reported by Schläppy et al. (2010a) for the two Mediterranean shallow water sponges *Chondrosia reniformis* and *Dysidea avara,* also measured on tissue sections incubated under oxic conditions, were 240 and 357 nmol N cm^{-3} sponge day^{-1}, respectively – well above our maximum rates measured under oxic conditions, but close to our maximum rates measured under anoxic conditions. Higher metabolic rates in warm and shallow water sponges compared to cold deep water sponges is not surprising. In addition to these rather few direct quantifications of denitrification rates in sponges, the presence of denitrification activity has been indicated by isotopic tracer experiments in a tropical sponge (Fiore et al., 2013), as well as by numerous reports on the presence of functional genes for denitrification in sponge microbes, or by demonstrating the ability for denitrification in sponge-derived microbial isolates from a variety of marine habitats (Bayer et al., 2014;Cleary et al., 2015;Fiore et al., 2010;Fiore et al., 2015;Han et al., 2013;Li et al.,

2014;Liu et al., 2016;Liu et al., 2012;Webster and Taylor, 2012;Zhang et al., 2013;Zhuang et al., 2018).

We could not detect any anammox rates in any of the sponges investigated in this study. The only literature report for anammox rates quantified in a sponge was a very low rate of 3 nmol cm^{-3} sponge day^{-1} in explants of *G. barretti* (Hoffmann et al 2009). In the present study, we could not reproduce these rates in the tissue sections of *G. barretti* nor detect the functional genes associated with this process. There are no other quantifications of anammox rates in sponges, and only few studies on the presence of anammox bacteria and genes in some sponge species (Han et al., 2012;Mohamed et al., 2010;Webster and Taylor, 2012).

Our study further clearly shows that denitrification rates are generally higher under anoxic conditions. As denitrification is an anaerobic process, this is not surprising. More surprising is our detection of considerable denitrification rates (up to 96 nmol N cm^{-3} sponge day^{-1}) when sponge tissue sections were incubated in oxygenated seawater. Furthermore, evidence for coupled nitrification-denitrification, proves that both aerobic and anaerobic processes can happen in the sponge sections at the same time. Oxygen was assumed to be present in the experimental vial at least during the first 26 hours of the experiment, though continuously decreasing due to sponge respiration (see calculation in method section), but we do not have control over oxygen concentration in the sponge tissue pieces during the experiment. From marine sediments, there are numerous studies reporting denitrification in bulk oxic conditions, either in anoxic microniches or under complete oxygenated conditions. e.g. (Wilson, 1978); (Marchant et al., 2017;Robertson et al., 1995;Chen and Strous, 2013). For the present study, we do not know if denitrification actually happened in the presence of oxygen, in anoxic microniches, which were present in the sponge tissue already at experiment start, or in tissue sections rapidly becoming

anoxic while not continuously flushed with oxygen. Nevertheless since all these scenarios reflect the situation in a sponge which is pumping on a low rate or occasionally stops pumping (Hoffmann et al., 2008;Schläppy et al., 2010b;Schläppy et al., 2007), which are typical features in sponges, we assume that our results are representative for sponges under normal conditions.

Our study further indicates significant differences in anaerobic denitrification rates between most sponge species, indicating species-specific differences in maximum potential denitrification rates. Two of the Arctic sponges (*G. hentscheli* and *G. parva*) showed the highest denitrification rates. It is worth noticing that due to technical reasons the Arctic incubations had to be performed at a higher temperature (6 °C) compared to current in-situ conditions (0 °C), wich may have led to an overestimation of the potential rates for the Arctic species.

Our systematic screening of 6 cold-water sponge species, together with reports of denitrification activity from other sponge species all over the world and from different habitats (see above), **strengthens the view that denitrification is a common feature in many sponge species – both under oxygenated (pumping) and deoxygenated (non-pumping) tissue conditions, with rates being highest when oxygen is absent.** Anammox in contrast seems to be a more rare and occasional feature in sponges, which may not have quantitative importance for sponge-mediated nitrogen cycling.

4.2 The fate of nitrogen in sponges

With the exception of *Stryphnus fortis*, denitrification was verified in the presence of dissolved oxygen across all species. For most species, denitrification was partly coupled to nitrification. For *G. barretti*, 16% of nitrate used for denitrification under oxic conditions was derived from nitrification, which is very close to previously reported values of 26% as reported for the same

species (Hoffmann et al., 2009). Evidence for coupled nitrification-denitrification in most sponge species of this study indicates that nitrification was present in these species. Nitrification rates have been quantified in the cold-water species *Phakellia ventilabrum, Antho dichotoma, Geodia barretti and Stryphnus fortis* (120-1880 nmol N cm^{-3} sponge day^{-1}; Radax et al., 2012; Fang et al., 2018; Hoffmann et al., 2009), and we may assume similar rates for the species in this study. Since the ammonium concentration in bottom seawater at our sampling sites is far too low (under detection limit of 1 µM NH_4^+) to fuel these nitrification rates, ammonium needs to originate from organic nitrogen remineralized from organic matter by the sponge cells or by heterotrophic sponge microbes. Under anoxic conditions, there is no nitrification, and nitrate to fuel the much higher denitrification rates has to be retrieved directly from the seawater. We did not detect any genes for nitrogen fixation; the N-cycle is not closed in the cold-water sponges. **The denitrified nitrogen, no matter of its origin, is no longer available as a nutrient and thus inevitably lost as a good and service for the marine ecosystem**.

4.3 The sponge microbial community is ready for denitrification

*Nir*S and *nir*K are functionally equivalent genes that code for the reduction of nitrite to nitric oxide, the first step towards the production of a gas in denitrification (Shapleigh, 2013). Copies of *nir*S and *nir*K were quantified in all six sponge species, and also in the sediment (Table 2). Scattering denitrification rates against nitrite reductase copy numbers, revealed a clear positive relationship between denitrification rates (in the absence of oxygen) and the species-specific abundance of *nir*S and *nir*K (Fig. 3) for 5 of the 6 sponge species. This relationship suggests that there is an active denitrifying community present in these species.

This is further corroborated by our observation of a linear accumulation of ^{15}N labelled N_2 gas already from incubation start for our 15N incubation experiments as shown in Fig.1. The lack of a

lag phase is frequently associated with 'active' denitrification (Bulow et al., 2010;Ward et al., 2009). Conversely, denitrifiers in pure culture require a 24-48h reactivation period to recover from dormancy (Baumann et al., 1997;Baumann et al., 1996). There was no lag phase in any of our sponge tissue incubations, which strengthens our conclusion that the denitrifying community is active and prepared for the denitrification rates observed in our experiments. This again means that the measured maximum denitrification rates are likely to occur *in situ* in situations where the sponge tissue becomes completely anoxic. This also suggests that the heterotrophic microflora in these sponges regularly find themselves in an anoxic or microoxic environment where it is beneficial to have the denitrification genes readily expressed.

In the slurries of surface sediments from the Schulz Massive, *nir*K and *nir*S copy numbers were comparable to those in the sponges (Table 2); however, in these samples we did not detect any labelled N_2 production within 48h of incubation. This would suggest that although a microbial community capable of denitrification is present in the surface sediments of the Schulz Bank, its activity was under detection limit. Low availability of reactive carbon in these Arctic sediments (Baumberger et al., 2016) may be the reason for this lack of detectable denitrification activity, in contrast to a high availability of reactive carbon within a living sponge. Our results indicate that in the Arctic deep sea, sponge grounds play a much more important role for nitrogen cycling and benthic-pelagic coupling than the surrounding sediment.

4.4 Sponge grounds as nutrient sinks

Denitrification rates in this study were quantified in lab experiments, and therefore show potential rates of these species under certain conditions, not real rates under current in-situ conditions. Keeping this in mind, and also considering that denitrification rates are calculated to represent that of the ambient NO_3^-, no carbon source was added and the incubation temperature was realistic for

natural conditions, our results allow estimates of the potential denitrification capacity of sponge grounds. Our results reveal average nitrogen removal rates for boreal sponge grounds of 70 nmol N cm^{-3} sponge day^{-1} assuming all sponges are not pumping (results from the anoxic experiment), and 38 nmol cm^{-3} day^{-1} when all sponges are pumping (results from the oxic experiment). For Arctic sponge grounds the rates will be 167 and 55 for non-pumping and pumping sponges, respectively. Based on our own observations from trawl catches and underwater imagery from several cruises, we estimate that masses of 10 kg m^{-2} are common in boreal sponge grounds, while smaller areas both in shelfs and fjords may even come up to densities of 30 kg m^{-2}. In other areas masses can be considerably lower and more patchy, e.g. 3.5 kg in the Traena area, as reported by (Kutti et al., 2013). In the Arctic sponge grounds investigated in this study we estimate the sponge biomass to be approximately 4 kg m^{-2}.

These estimates reveal a potential areal denitrification rate for the boreal sponge grounds of up to 0.587 mmol N m^{-2} sponge ground assuming non-pumping and still 0.321 mmol N m^{-2} day^{-1} assuming pumping sponges. For Arctic sponge grounds the numbers are quite similar (sponge biomass is lower but sponge denitrification rates are higher): 0.608 mmol N m^{-2} day^{-1} for non-pumping and 0.201 for pumping sponges. These rates are well within the range - or, for the non-pumping situation with anoxic tissue, on the upper end – of denitrification rates from continental shelf sediments, which are 0.1-1 mmol N m^{-2} day^{-1} (Middelburg et al., 1996;Seitzinger and Giblin, 1996). For the most dense boreal sponge grounds with sponge densities up to 30 kg m^{-2}, rates will be up to 1.7 mmol N m^{-2} day^{-1}; well above typical rates for continental shelf sediments.

While our denitrification rates in sponges incubated under oxic conditions may reflect normal *in-situ* conditions for pumping sponges, our numbers on denitrification rates in sponges incubated under anoxic conditions are theoretical extremes, since we know little about the *in-situ* pumping patterns of deep-sea sponges, and the environmental factors influencing them. Seawater nitrate

which fuels most of the denitrification under anoxic conditions enters the sponge through pumping. The maximum denitrification rates in non-pumping sponges can therefore only be maintained until the nitrate in the sponge pore water is used up. The length and frequency of these anoxic spells will thus determine the variability of *in situ* sponge denitrification rates. Observations by Schläppy et al. (2010b) showed non-pumping periods of sponges *in situ* of up to two hours, leading to complete tissue anoxia, followed by several hours of high pumping activity. Sponges with dense tissue and high loads of associated microbes (high-microbial abundance (HMA) sponges, such as most sponges in our study) generally show slower volume pumping rates than sponges with low microbial numbers and loose tissue structure (Weisz et al., 2008). Slow pumping rates lead to reduced and heterogeneous oxygen concentrations in sponges (e.g. (Schläppy et al., 2010b;Schläppy et al., 2007)) while they still may supply sufficient nitrate from ambient seawater to fuel denitrification. Even though our calculated areal denitrification rates of sponge grounds so far only point out a potential capacity, our study clearly shows that both boreal and arctic sponge grounds can function as efficient nutrient sinks, especially when they reduce or stop pumping and the tissue becomes anoxic. Environmental and anthropogenic stressors such as increased sediment loads (Bell et al., 2015) reduce pumping activity and increase anoxic conditions in sponges (Fang et al., 2018; (Kutti et al., 2015;Tjensvoll et al., 2013), and thus stimulate nutrient removal through denitrification. Elevated ambient nitrate concentrations have been linked to increased nitrate removal by sponges (Archer et al., 2017). Global change processes affecting sponge redox state will impact the sponge holobiont (Pita et al., 2018), and may thus lead to that deep-sea sponge grounds change their role in the marine ecosystem from functioning mainly as nutrient sources to functioning mainly as nutrient sinks.

Conclusions

In this study we have shown that several sponge species actively remove the bioavailable nutrients ammonium and nitrate from the marine ecosystem by denitrification and coupled nitrification-denitrification, which challenges the common view of sponges as main DIN providers through mineralisation of organic matter and nitrification. While variations in sponge remineralisation activity only postpone the delivery of nutrients, denitrification inevitably removes these nutrients from the marine ecosystem. The nitrogen cycle is not closed in the sponge grounds, the denitrified nitrogen, no matter of its origin, is no longer available as a nutrient and efficiently removed from the marine ecosystem. We further showed that the investigated sponges host an active community of denitrifiers which show highest denitrification rates under anoxic conditions. Anthropogenic impact and global change processes affecting the sponge redox state may thus lead to that deep-sea sponge grounds change their role in the marine ecosystem from functioning mainly as nutrient sources to functioning mainly as nutrient sinks.

Data availability. The data is available in the data publisher PANGAEA, https://doi.pangaea.de/10.1594/PANGAEA.899821

Author contribution. F Hoffmann and C Rooks designed the study. C Rooks and J K-H Fang performed the sponge experiments. C Rooks and PT Mørkved performed the stable isotope analyses. C Rooks and R Zhao quantified the functional genes. C Rooks analyzed all the data. HT Rapp organized the cruises, quantified sponge biomass at key sites and determined the sponge species. C. Rooks wrote the first draft of the manuscript, and all authors contributed substantially

with writing and revision. F Hoffmann supervised and coordinated the writing process, and finalized the manuscript.

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LIST OF FIGURE LEGENDS

Fig. 1. Production of $^{29}N_2$ (filled symbols) and $^{30}N_2$ (open symbols) as a function of time after the addition of $^{15}NO_3^-$ in incubations with **(A)** air-saturated (simulating pumping conditions) and **(B)** de-gassed site water (simulating non-pumping conditions) with tissue from *Geodia barretti* (n=3 individuals). Data associated with an individual sponge is represented by a set of symbols. Linear regressions of N_2 production within the first 24 hours of the experiments were used to calculate denitrification rates.

Fig. 2. Sponge species-specific rates of denitrification in incubations with de-gassed site water (anoxic conditions, black bars) and air-saturated site water (oxic conditions, grey bars) for 6 key species from boreal and arctic sponge grounds. Statistically significant differences between denitrification rates in the presence and absence of dissolved oxygen are indicated by an asterisk for each species. Error bars indicate SE (n=3 individuals). Coupled nitrification-denitrification under oxic conditions is visualised with dark grey colour in the grey bars. Compare also Table 1.

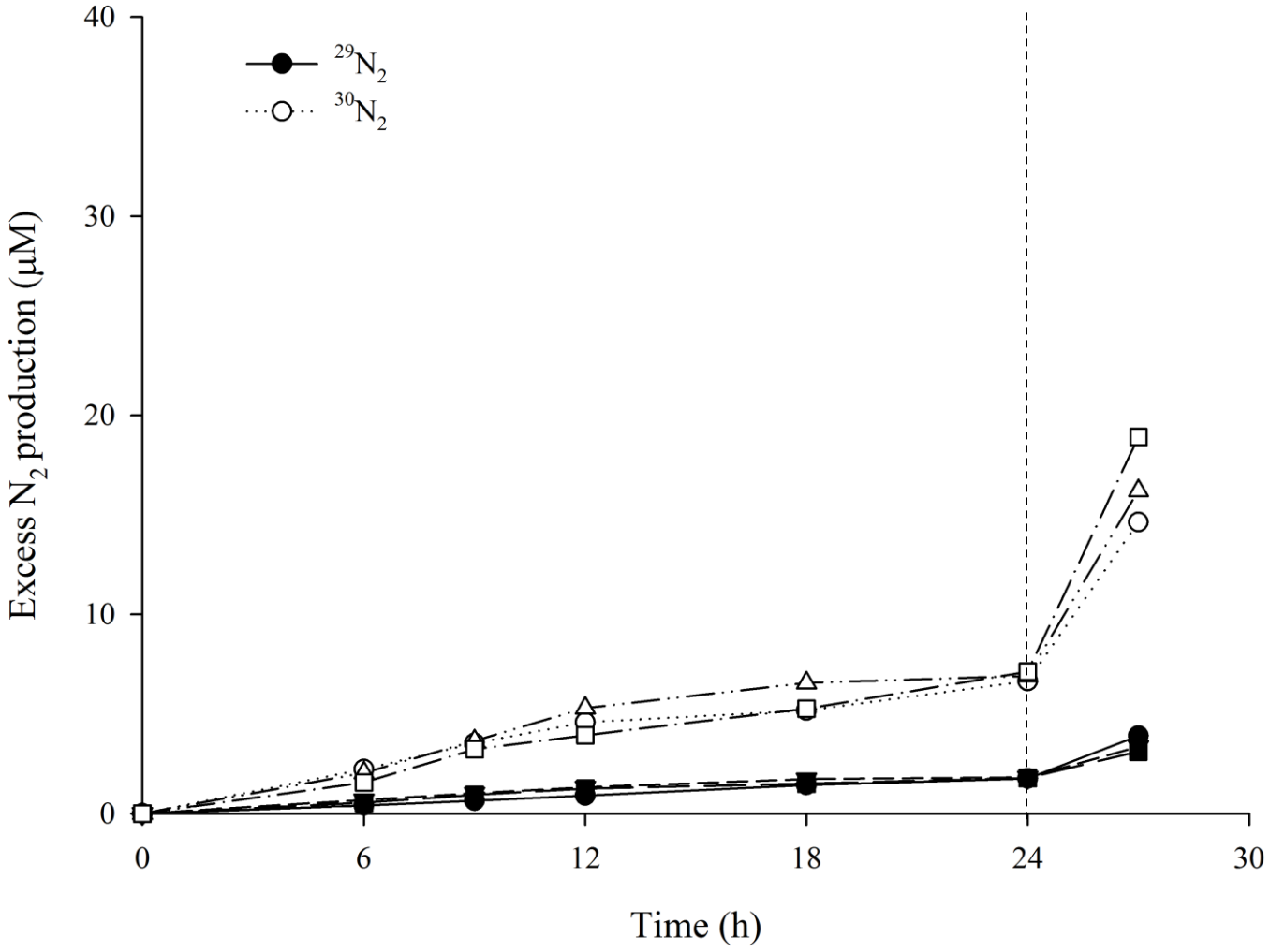
Fig. 3. Mean species-specific denitrification rates in incubations with air-saturated site water (with O_2, open circles) and de-gassed site water (without O_2, closed circles) as a function of nitrite reductase copy number. The nitrite reductase gene copy number is the sum of the mean number of *nir*S and *nir*K copies per cm^{-3} of sponge tissue (n=3). There is a positive relationship between denitrification rates (in the absence of oxygen) and the species-specific abundance of *nir*S and *nir*K for 5 of the 6 sponge species.

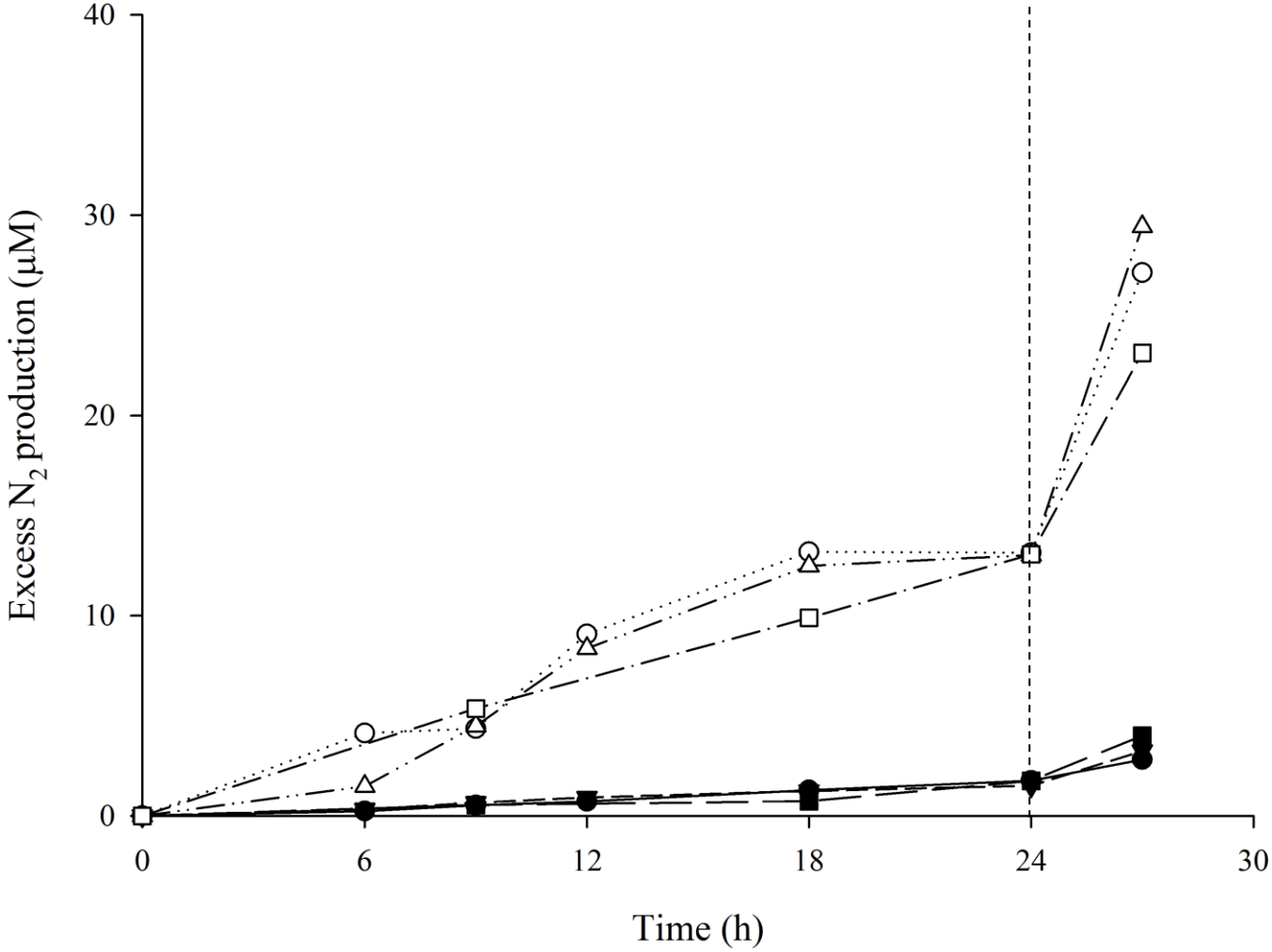
Table 1. Nitrate sources for denitrification in the presence of dissolved oxygen. Most of nitrate removed by sponge denitrification in oxic incubations originates from seawater, while some originates from sponge nitrification (coupled nitrification-denitrification). Denitrification rates in anoxic incubations (no coupled nitrification-denitrification) are also shown. Data are also presented in Fig 2.

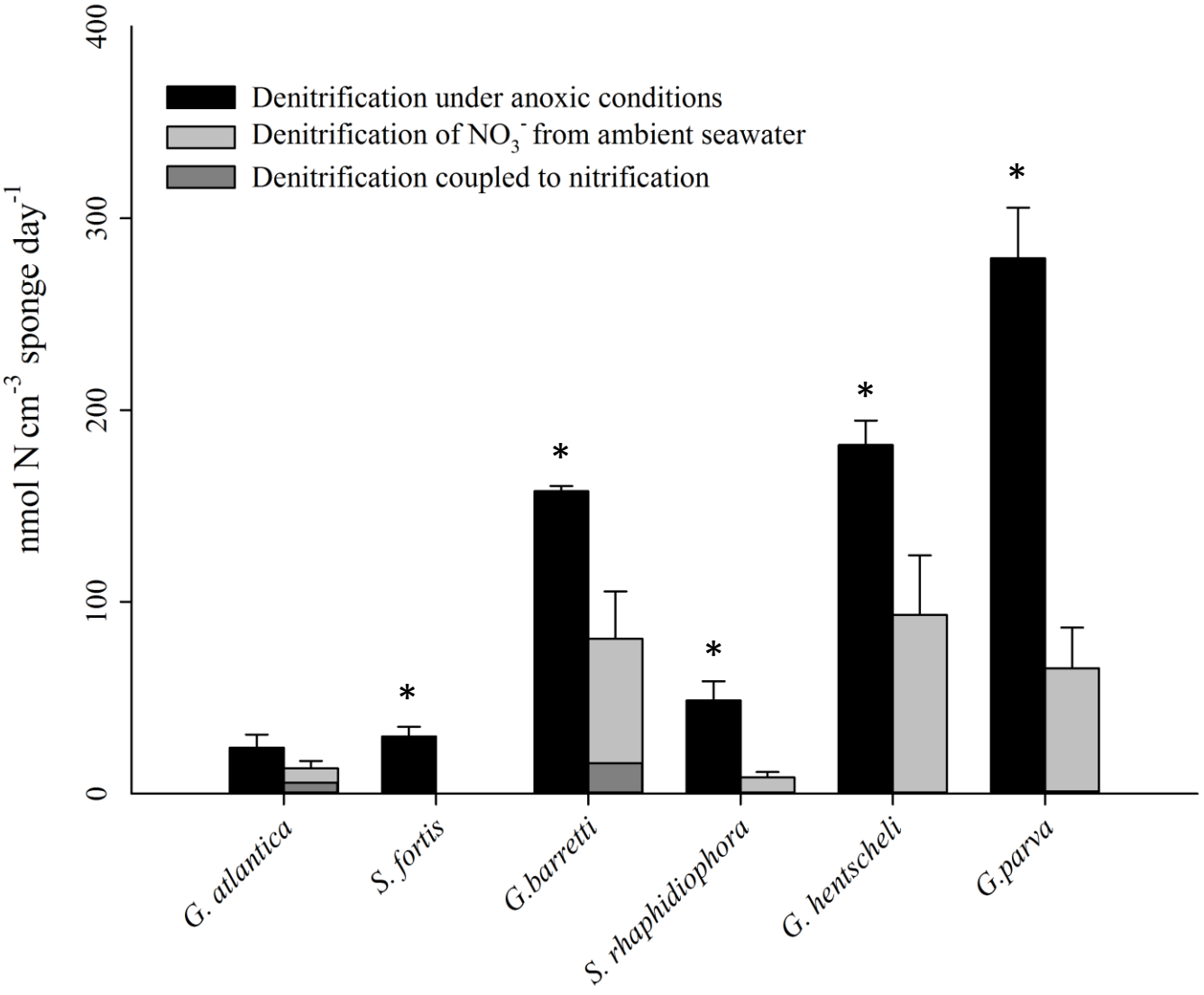
Sample	Location	Denitrification anoxic incubations	Denitrification oxic incubations	Nitrate from nitrification	Nitrate from seawater	% coupled nitrification-denitrification
		nmol N cm^{-3} sponge day^{-1}				
G. atlantica	Boreal	23.98	18.87	5.68	13.19	30.1
S. fortis	Boreal	29.80	ND	ND	ND	ND
G. barretti	Boreal	157.64	96.54	15.87	80.67	16.4
S. rhaphidiophora	Arctic	40.92	8.42	ND	8.42	ND
G. hentschelii	Arctic	181.87	93.15	ND	93.15	ND
G. parva	Arctic	279.12	64.46	1.1	65.28	1.72
Sediment	Arctic	ND	ND	ND	ND	ND

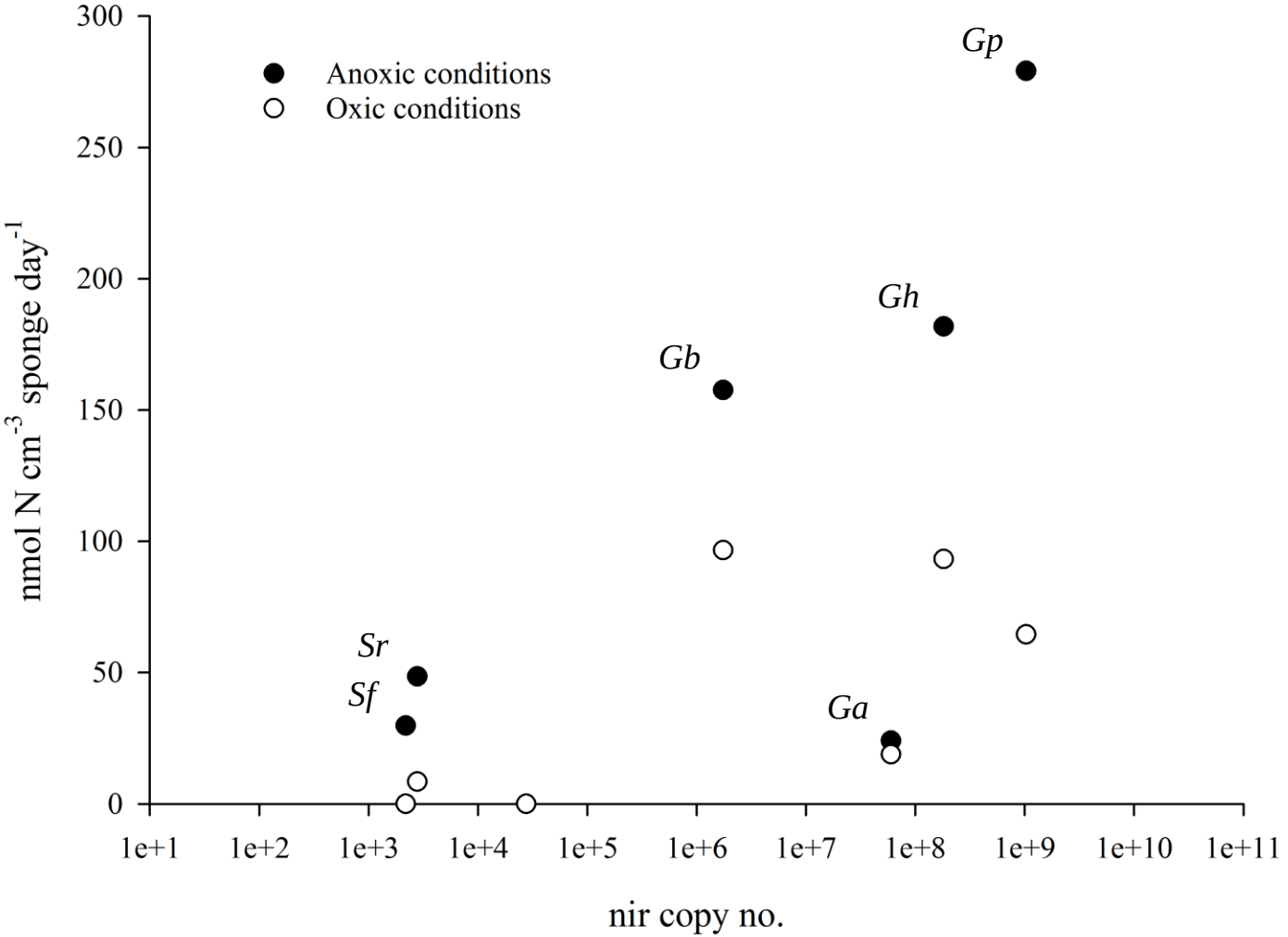
Table 2. Abundance of the nitrite reductase genes *nir*S and *nir*K in sponge and sediment samples. The nitrite reductase copy number is the sum of the mean number of *nir*S and *nir*K copies per cm^{-3} of sponge tissue (n=3). *ND = not detectable.

Sample	Location	*nir*S copy no.	*nir*K copy no.	Nitrite reductase copy no.
G. atlantica	Boreal	2.67E+02	6.00E+07	6.00E+07
S. fortis	Boreal	ND	2.19E+03	2.19E+03
G. barretti	Boreal	7.04E+02	1.75E+06	1.75E+06
S. rhaphidiophora	Arctic	4.02E+02	2.39E+03	2.80E+03
G. hentscheli	Arctic	1.25E +03	1.82E+08	1.82E+08
G. parva	Arctic	3.81E+02	1.03E+09	1.03E+09
Sediment	Arctic	ND	2.77E+04	2.77E+04


40
30
20
10
0
$^{29}N_2$
$^{30}N_2$
Excess N_2 production (μM)
0
6
12
18
24
30
Time (h)


40
30
20
10
0
Excess N_2 production (μM)
0
6
12
18
24
30
Time (h)


Denitrification under anoxic conditions
Denitrification of NO_3^- from ambient seawater
Denitrification coupled to nitrification
nmol N cm^{-3} sponge day^{-1}
0
100
200
300
400
*
*
*
*
*
G. atlantica
S. fortis
G.barretti
S. rhaphidiophora
G. hentscheli
G.parva


300
250
200
150
100
50
0
nmol N cm-3 sponge day-1
Anoxic conditions
Oxic conditions
Gp
Gh
Gb
Sr
Sf
Ga
1e+1
1e+2
1e+3
1e+4
1e+5
1e+6
1e+7
1e+8
1e+9
1e+10
1e+11
nir copy no.