Dear editor,

Please find our replies to all reviewers' comments attached. Here we summarize the most important changes to the manuscript:

1. We realized that the incubation experiments were not clearly described in the method section, in particular it was not clear which kind of 15N species was added to which experiment. This caused some fundamental misunderstanding about the method for Ref 1 and 3. This is now corrected and explained.
2. Challenged by some critical comments by Ref 1 and 3 about calculation of denitrification rates, the first author CR re-calculated all the rates from scratch, going back to integration of peak areas and data plotting. This resulted in a change of some of the final denitrification rates, which however did not affect the main story of the manuscript.
3. By going through the entire raw data set again, CR discovered a column mix-up between 29N and 30N data, which had lead to the surprisingly high rates for coupled nitrification-denitrification in our previous manuscript version. The new rates make much more sense and make a better fit with previously reported values.
4. As a result of this, we abandoned our calculations of minimum nitrification rates based on the coupled nitrification-denitrification rates, which also had been commented critically by some reviewers. We replaced by a statement that coupled rates indicate the presence of nitrification in these sponges under aerobic conditions, which is well documented for many sponge species.
5. We updated figures and tables accordingly, an updated data set was sent to PANGAEA.

Finally, we are aware that there are still some mistakes in the format of the in-text citations and the reference list. Reference format is generated automatically through EndNote each time we open the document. We will do the final formatting manually when the manuscript eventually may be accepted and no references have to be removed or added.

We would like to thank you for your fair decision to reconsider publishing after major revisions. We also appreciate your opinion that a revised version might eventually be publishable, and that it will be a very useful addition to the literature.

With best regards,
On behalf of all co-authors,
Friederike Hoffmann

Deep-sea sponge grounds as nutrient sinks: High denitrification rates in boreo-arctic sponges

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Key words: Denitrification, ~~nitrification~~, boreal, Arctic, deep-sea sponges, sponge grounds.

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 0 to ~~114~~ 97 nmol N cm^{-3} sponge day^{-1} under oxic conditions, and from ~~47~~ 24 to ~~342~~ 279 nmol N cm^{-3} sponge day^{-1} under anoxic conditions.

A positive~~n exponential~~ 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 ~~both~~ prepared ~~and optimized~~ 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~~High~~ rates for coupled nitrification-denitrification indicate that also under oxic conditions, nitrate to fuel denitrification rates was derived rather from the ambient sea-water than from sponge nitrification. ~~(up to 89% of nitrate reduction in the presence of oxygen) shows that under these conditions, the NO_3^- reduced in denitrification was primarily derived from nitrification within the sponge, directly coupling organic matter degradation and nitrification to denitrification in sponge tissues. Under anoxic condition when nitrification was not possible, nitrate to fuel the much higher denitrification rates had to be retrieved directly from the seawater.~~ 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 reveal the following scenario for the potential denitrification capacity of sponge grounds based on typical sponge biomass ~~Considering average sponge biomasses~~ on ~~typical~~ boreal and Arctic sponge grounds~~:~~. A~~our sponge denitrification rates reveal a~~real denitrification rates of 0.~~8~~ 6 mmol N m^{-2} day^{-1} assuming non-pumping sponges and still 0.3 mmol N m^{-2} day^{-1} assuming pumping sponges may be possible. This is well within the range of denitrification rates of continental shelf sediments. For the most densely populated boreal sponge grounds we calculated potential denitrification rates of up to ~~2~~ 1.7 mmol N m^{-2} day^{-1}, which is ~~comparable to rates in coastal sediments~~higher than typical rates in continental shelf sediments. Increased future impact of sponge grounds by anthropogenic stressors reducing sponge pumping activity and further stimulating sponge anaerobic processes 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.

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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., 2009b;Schläppy et al., 2010a;Maldonado et al., 2012;de Goeij et al., 2013;Rix et al., 2016). Sponges show an active nitrogen metabolism (Feng and Li 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_x^-~~ (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^+. ~~Although this pathway~~ which represents yet another source of ~~bio-available N~~DIN from sponges. ~~the presence of *nifH* (encoding the essential nitrogenase responsible for N_2 fixation) does not necessarily confer to nitrogen fixing activity, even under seasonal N-limitation (Ribes et al., 2015;Bentzon-Tilia et al., 2014).~~ 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 and the availability of N-rich particulate organic matter in the water

column (Bayer et al., 2008;Archer et al., 2017;Fiore et al., 2013)~~., although where archaea dominate the nitrifying community, NO_3^- release could potentially be sustained by nitrifying archaea scavenging for NH_4^+ under N-limiting conditions (Radax et al., 2012;Tian et al., 2016)~~

In any case, since nitrification is dependent on oxygen, NO_3^- release is dependent on active filtration, delivering an excess of O_2 to sponge tissues~~., and in turn, sustaining aerobic nitrification within the sponge~~ (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.

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Anaerobic N-transformations have been quantified using ^{15}N tracer experiments in deep-sea (Hoffmann et al., 2009b) 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). 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).

Denitrification in sponges ~~This~~thus opens for an alternative role of sponges as nutrient scavengers, and an alternative explanation for the ~~observed~~ variations in DIN~~nutrient~~ release as described

above~~by sponges~~: an interplay of both, variability~~a combination of variations~~ in remineralisation rates~~processes~~ associated with food availability **and** direct consumption of endogenous and ambient nutrients by microbial processes in sponges. Sponges can even perform competing nitrogen cycling processes such as nitrification and denitrification simultaneously(Hoffmann et al., 2009a), where the rates of the different processes determine whether the sponge acts as a nutrient source or a nutrient sink (Pita et al., 2018)~~(Pita et all 2018, and references cited therein),~~ . The balance of these processes and their controlling factors, however, 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*) (Klitgaard and Tendal, 2004; Cárdenas et al, 2013; 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* (Klitgaard and Tendal, 2004; (Cardenas et al., 2013)~~Cárdenas et al, 2013;~~ Murillo et al, 2014). ~~*Geodia barretti* is a key species on boreo-arctic sponge grounds, which cover wide expanses of the seafloor both in the Eastern and Western~~

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~~North Atlantic, as well as the sub-Arctic (Klitgaard and Tendal, 2004;Murillo et al., 2012).~~ ~~However,~~ Tto 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 ~~show 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. ~~As sponges were collected at the rocky slope of the fjord, it was not possible to collect sediment from that sampling site.~~

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.

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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, ~~s~~sponges were collected from~~at~~ the rocky slope of the fjord, ~~so~~ It was therefore~~it was~~ not possible to collect sediment from ~~that sampling~~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 ~~a~~ gas-tight ~~atmosphere~~ 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 ~~surface~~ 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

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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; ~~$^{14}NH_4^+$ Cl^-~~ or ii) $^{15}NH_4^+$ Cl^- (≥98. ^{15}N atm %) and~~,~~ $Na^{14}NO_3^-$, screening for anammox. Solutions were ~~and~~ shaken vigorously. The final concentrations of i) $^{15}NO_3^-$; ~~$^{14}NH_4^+$ (screening for denitrification and/or anammox)~~ or ii) $^{15}NH_4^+$; $^{14}NO_3^-$ were 100µM NO_3^- and 10µM NH_4^+ respectively. These values were essentially ~~90%~~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 ~~in situ temperature~~ (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.

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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) ~~a selection of samples~~ 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. We never observed 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), but some samples showed an abrupt increase in N_2 production,

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indicating 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 ~~the~~ 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 $^{15}NO_3^-$/~~$^{14}NH_4^+$~~ , 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 ~~(e.g. tissue pieces turn black, see for example (Hoffmann et al., 2003;Osinga et al., 2001;Osinga et al., 1999),~~ this ~~non-linear~~ 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. ~~Also here, no signs for tissue degradation were observed.~~

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). ~~In brief, the~~ 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).

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

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

The rate of denitrification was measured from ^{15}N isotope production (equations 1 and 2). D_{14} and D_{15} represent denitrification of labelled $^{15}NO_3^-$ and $^{14}NO_3^-$. p ($^{14}N^{15}N$) and p ($^{15}N^{15}N$) are the

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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 overlying water.

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

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

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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°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: 94°C for 15 min, and 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C 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: 95°C for 15 min, 45 cycles of denaturing at 95°C for 15 s, annealing at 51°C for 30 s, and elongation at 72°C for 45 s. The *nirK* gene was amplified using the primer pair nirK_F1aCu/ nirK_R3Cu, with the following thermal conditions: 95°C for 15 min, 45 cycles of denaturing at 95°C for 30 s, annealing at 51°C for 45 s, and elongation at 72°C 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. ~~Standard of~~ 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_3~~NO_2^- (which is not labelled) and 1 N from NH_4^+ (which is ^{15}N labelled). ~~The lack of $^{29}N_2$ production following labelling with $^{15}NH_4^+$ as observed in our study suggests an absence of anammox~~. 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 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~~39.339~~, p<0.01) and in the presence or absence of dissolved oxygen (two-way ANOVA, $F_{1,5}$=141,235~~50.260~~, 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~~2.847~~, 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 0 nmol N cm^{-3} sponge day^{-1} in *Stryphnus fortis* to a maximum of ~~114~~ 96 nmol N cm^{-3} sponge day^{-1} in ~~*Stelletta rhaphidiophora*~~*Geodia barretti*. However, under anoxic conditions, rates of denitrification ranged from ~~47~~ 24 nmol N cm^{-3} sponge day^{-1} in ~~*Stryphnus fortis*~~*Geodia atlantica* to ~~342~~ 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=2.075~~6.591~~, p<0.05), *Geodia barretti* (t=2.197~~3.277~~, p<0.05), *Geodia hentscheli* (t=3.495~~4.577~~, p<0.05) ~~and~~ *Geodia parva* (t=8.788~~5.789~~, 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.

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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 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. ~~For these species 67 – 89% of the nitrate reduced in denitrification was coupled to nitrification within the sponge tissue. This indicates a minimum nitrification rate of 29 – 102 nmol N cm^{-3} sponge day^{-1} and the predominance of this process as a source of nitrate for denitrification under oxic conditions (Table 1).~~

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 ~~A~~ positive ~~exponential~~ relationship between denitrification rates under anoxic conditions, and total *nir* copy number, for all species except *G. atlantica*, the species with the

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lowest denitrification rate. ~~were associated with 4 of the sponge species (*Stelletta rhaphidophora*, *Geodia barretti*, *G. hentscheli and G. parva*) with the highest anaerobic denitrification rates (>140 nmol N cm^{-3} sponge day^{-1}, Fig. 3.). No correlation, however, was detected for species with low denitrification rates under anoxic conditions (<60 nmol N cm^{-3} sponge day^{-1}),~~ No correlation to *nir* copy number was detected~~nor~~ for denitrification rates under oxic conditions~~, where the rates were quite similar for the 4 most active species despite variations in *nir* gene copy number~~ (Fig. 3.).

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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 hypothesesshow 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 86 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 (with 342 N cm^{-3} sponge day^{-1} for *G. parva* as our highest rate). Considering generally Hhigher metabolic ratessms in warm and shallow water spongess compared to cold deep water sponges is not surprising.s, this is as expected. In addition to these rather few direct quantifications of denitrification rates in sponges, the presence of denitrification activity has been indicatedshown 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 demonstratingproving 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 ~~With~~ denitrification is~~being~~ an anaerobic process, this is not surprising. More surprising is our detection of considerable denitrification rates (up to ~~114~~ 96 nmol N cm^{-3} sponge day^{-1}) when sponge tissue sections were incubated in oxygenated seawater. Furthermore, evidence for ~~the high percentage of~~ coupled nitrification/denitrification ~~(up to 89.5% for *Stelletta rhaphidiophora*, the sponge with the highest oxic denitrification rate!)~~, proves that both aerobic and anaerobic processes can happen~~ed~~ 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~~(some)~~ sponge species, ~~with two of the three Arctic species (*G. hentscheli* and *G. parva*) displaying the highest rates. Sampling coincided with a seasonal pulse of organic matter in the water column above the Schulz Bank (this was measured on the 2016 cruise at the time of sampling). An increase in the availability of organic matter is known to stimulate denitrification (Devol, 2015) and could explain, to some extent, why rates are higher among two of the Arctic samples retrieved in June 2016, relative to boreal samples retrieved in November 2017. Another explanation is that there are simply~~ indicating species-specific differences in maximum potential denitrification rates~~, independent of sampling site and time~~. 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 (O °C), wich may have led to an overestimation of the potential rates for the Arctic species.

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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 ~~High rates for coupled nitrification-denitrification shows that under these conditions, the NO_3^- reduced in denitrification was primarily derived from nitrification, coupling aerobic ammonium oxidation to NO_3^- reduction in sponge tissues. Mean values of coupled nitrification-denitrification indicate that nitrification fuels as much as 67-89% of NO_3^- reduction (Table 1). This exceeds values previously reported for explants of *Geodia barretti*, which showed similar denitrification rates as *G. barretti* sections incubated in oxic seawater in this study (see above,), but where only 26% of N-loss was attributed to coupled nitrification-denitrification~~ (Hoffmann et al., 2009). ~~Those measurements were derived from sponge explants, which inherently lack an aquiferous system (Hoffmann et al., 2003, 2005, 2009). As such, the delivery of oxygen is entirely dependent on diffusion, which renders a large portion of the explant matrix permanently anoxic (<0.5-1mm below the surface; Hoffmann et al., 2005). Since nitrification requires oxygen, the high rates of coupled nitrification-denitrification for *G. barretti* and the majority of sponges investigated in the present study proves that (1) for incubations in oxygen-saturated seawater, more oxygen was~~

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~~present in the sponge tissue sections compared to sponge explants incubated under the same conditions and (2) nitrate derived from nitrification within the sponge tissue fueled most of the denitrification under oxic conditions.~~ Evidence for coupled nitrification-denitrification in most sponge species of this study indicates that nitrification was present in these species.

~~The calculated rates of coupled nitrification-denitrification represent the minimum nitrification rates. Since we did not measure nitrate production in the current study, true nitrification rates may be even higher. Nevertheless, minimum nitrification rates calculated in this study of up to 102 nmol N cm^{-3} sponge day^{-1}, are just below~~ N~~n~~itrification 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 ~~our calculated (and probably even underestimated)~~ 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). They occur as single copies within ~~the~~ denitrifying bacterial genomes, which generally indicates that a single copy

of nitrite reductase (*nir*S or *nir*K) corresponds to one cell with the potential for denitrification. Copies of *nir*S and *nir*K were ~~detected~~ 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~~n exponential~~ relationship between ~~the highest~~ 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 ~~*G. parva, G. hentscheli, G. barretti* og *S. rhaphidophora* i~~these species~~ both prepared and optimized for denitrification.~~ .

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~~optimized~~ for the denitrification rates observed in our experiments. This again means that the measured maximum denitrification rates are ~~realistic and will~~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.~~itrifying apparatus turned on.~~

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 under idealized conditions, and therefore show potential rates of these species under certain conditions, not real rates under current in-situ conditions. Keeping this in mind, our results still allow scenarios about the potential denitrification capacity of sponge grounds. ~~Considering denitrification rates of the three arctic and boreal sponge species investigated in this study as representative for boreal and arctic sponge grounds, we can calculate~~ Our results reveal average nitrogen removal rates for boreal sponge grounds of ~~91~~ 70 nmol N cm^{-3} sponge day^{-1} assuming all sponges are not pumping (results from the anoxic experiment), and ~~42~~ 38 nmol cm^{-3} day^{-1} when all sponges are pumping (results from the oxic experiment). For Arctic sponge grounds the rates will be ~~233~~ 167 and ~~94~~ 55 for non-pumping and pumping sponges, respectively. Based on our own observations from trawl catches and underwater imagery from several cruises, we estimate~~conclude~~ 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} ~~based on our own observations~~.

These estimates reveal a scenario of areal denitrification rates for the boreal sponge grounds of up to 0.764 587 mmol N m^{-2} sponge ground assuming non-pumping and still 0.321354 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): 0.847 608 mmol N m^{-2} day^{-1} for pumping and 0.343 201 for non-pumping sponges. These rates are well within the range - or, for the non-pumping situation, 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 1.72.3 mmol N m^{-2} day^{-1}; well above typical rates for continental shelf sediments. 2-10 times higher than in continental shelf sediments, and rather comparable to rates measured in coastal sediments (e.g. (Asmala et al., 2017).

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 do 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 ~~represent careful estimations~~point out a potential scenario, 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. Increased future impact through anthropogenic stressors reducing sponge pumping activity ~~Increased future impact through multiple stressors affecting the ocean ecosystems (Bopp et al., 2013)~~ 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~~nutrient~~ 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 denitrifiyers which show highest denitrification rates under anoxic conditions. Anoxic conditions occur when sponges are not pumping. Increased future impact of sponge grounds by anthropogenic stressors which reduce sponge pumping activity and by this increase anoxic conditions in sponges 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.

Competing Interests. The authors declare that they have no conflict of interest.

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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 (~~S.T.P.,~~ 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.

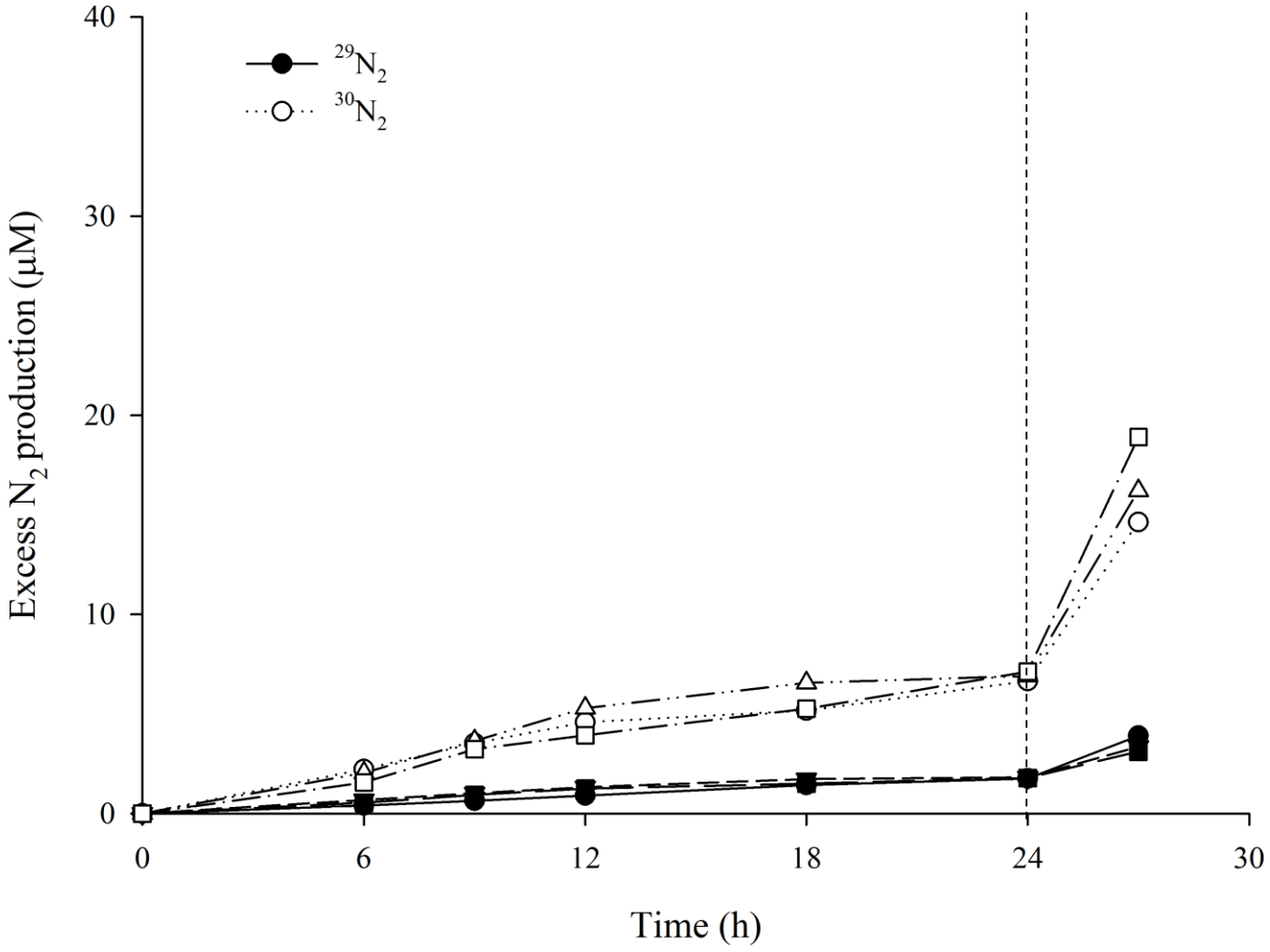
Fig. 3. Mean species-specific denitrification rates in incubations with air-saturated site water (with O_2, open circles) and de-gassed site water (without O_2, closed circles) as a function of nitrite reductase copy number. The nitrite reductase gene copy number is the sum of the mean number of *nir*S 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. ~~A positive exponential relationship, based on the mean rates of denitrification and *nir* copy number in *S. rhaphidiophora (Sr), G. barretti (Gb), G. hentscheli (Gh), G. phlegraei (Gp),* is shown.~~

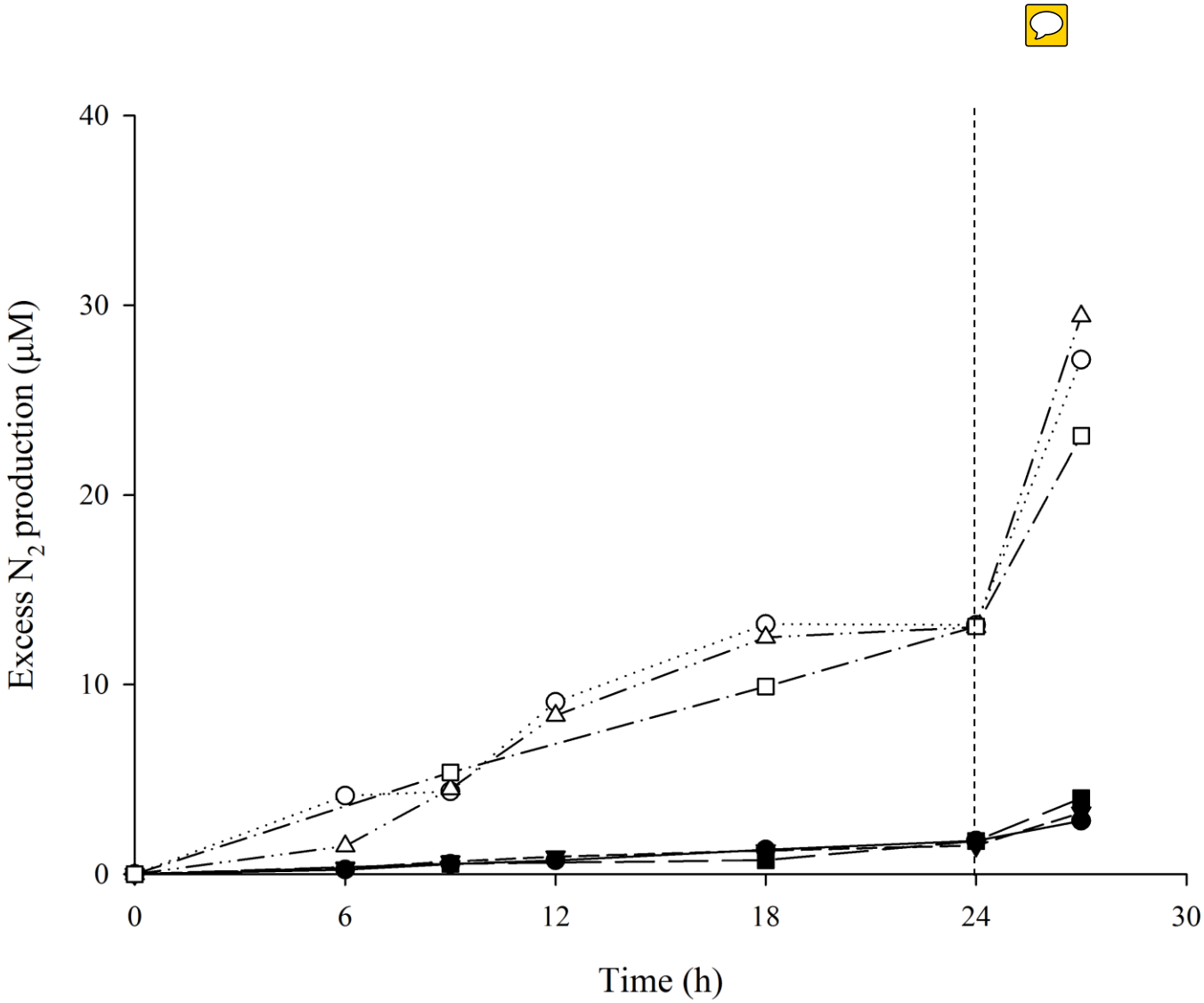
Table 1. Nitrate sources for denitrification in the presence of dissolved oxygen. ~~A large portion~~Most of nitrate removed by sponge denitrification in incubations with air-saturated seawater originates from seawater, while some originates from sponge nitrification (coupled nitrification-denitrification).

Sample	Location	Denitrification in oxic incubations nmol N cm^{-3} sponge day^{-1}	Nitrate from nitrification	Nitrate from seawater	% coupled nitrification-denitrification
S. fortis	Boreal	0	0	0	0
G. atlantica	Boreal	~~40.96~~18.87	~~29.52~~5.68	~~11.44~~13.19	~~72.06~~30.1
G. barretti	Boreal	~~86.46~~96.54	~~71.28~~15.87	~~15.18~~80.67	~~82.44~~16.4
S. rhaphidiophora	Arctic	~~113.84~~8.42	~~101.52~~0	~~12.32~~8.42	~~89.18~~0
G. parva~~hentscheli~~	Arctic	~~86.73~~64.46	~~72.2~~1.1	~~14.53~~65.28	~~83.25~~1.72
G. parva~~hentschelii~~	Arctic	~~82.09~~93.15	~~55.15~~0	93.15~~26.94~~	~~67.18~~0
Sediment	Arctic	0	0	0	0

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.
S. fortis	Boreal	ND	2.19E+03	2.19E+03
G. atlantica	Boreal	2.67E+02	6.00E+07	6.00E+07
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
G. hentscheli	Arctic	1.25E +03	1.82E+08	1.82E+08
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)


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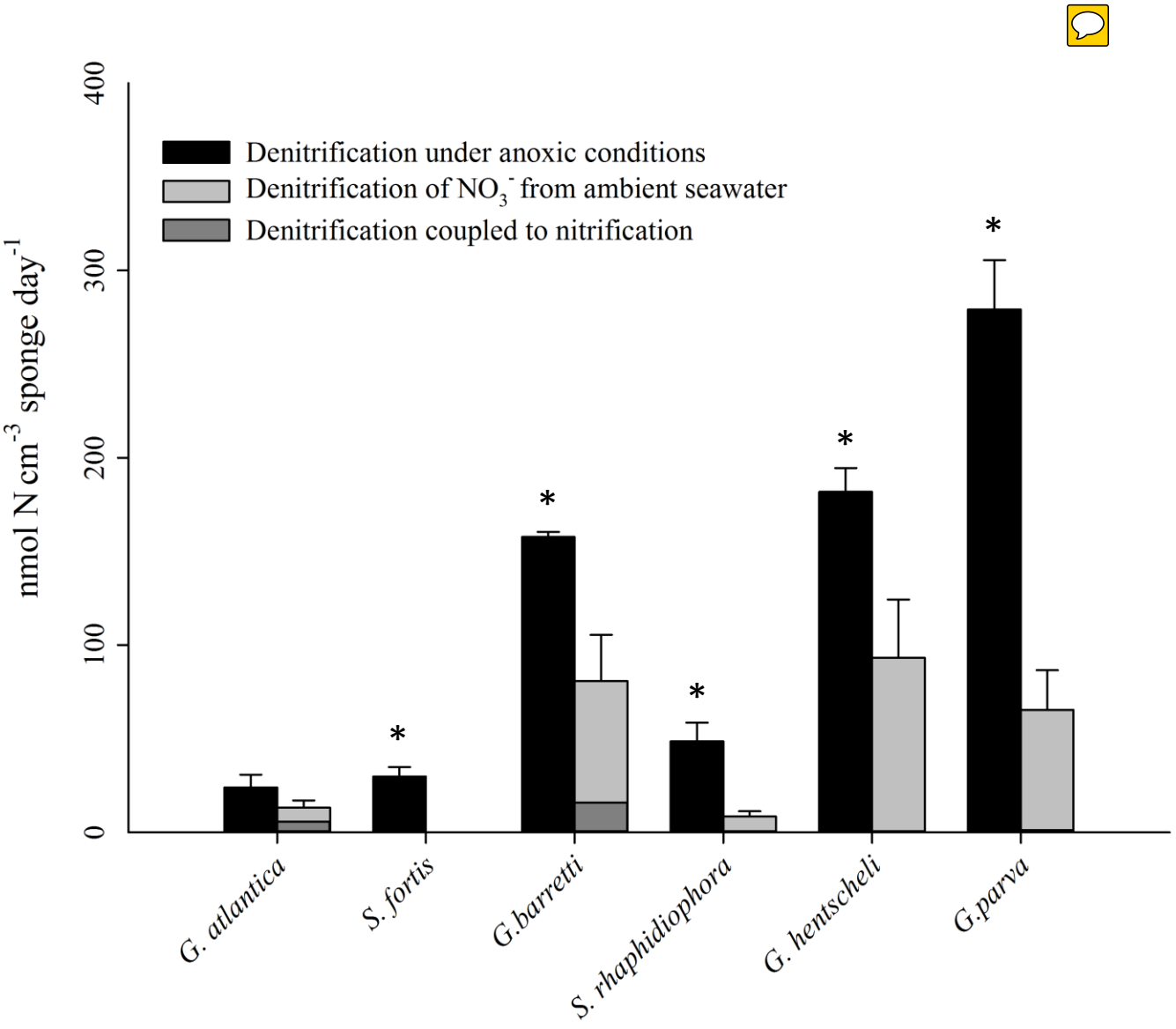
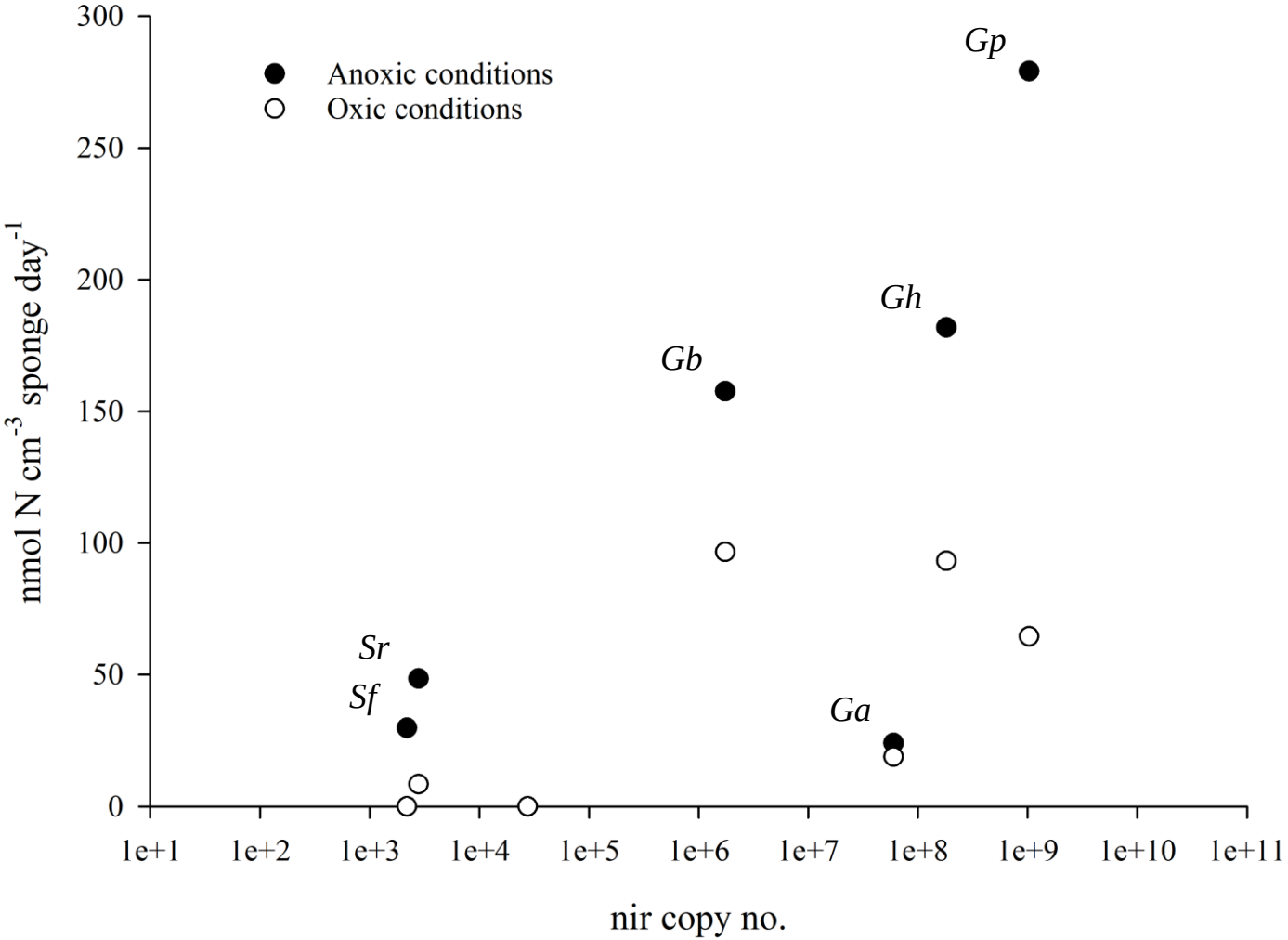
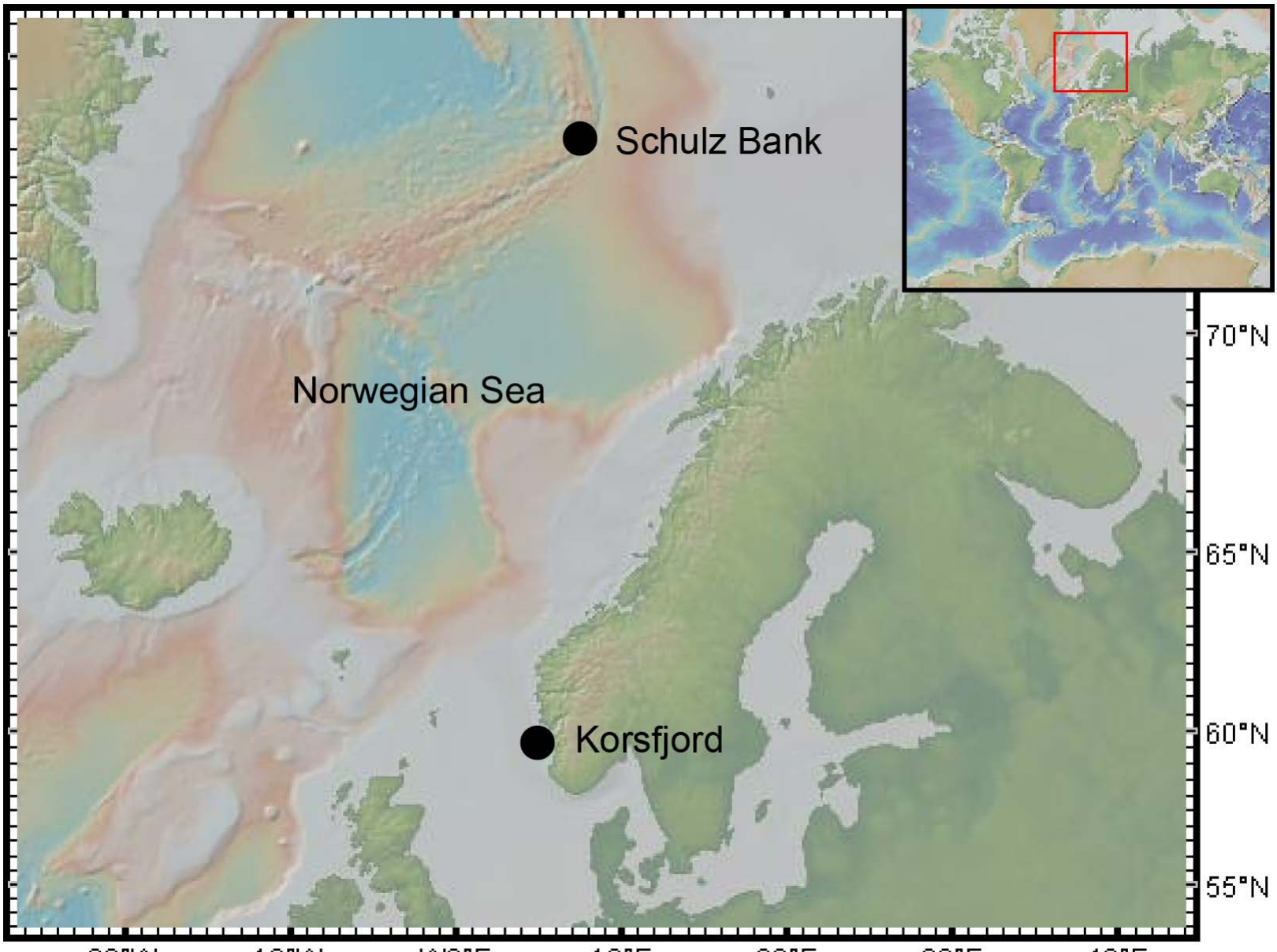
Time (h)


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


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


Schulz Bank
Norwegian Sea
Korsfjord
70°N
65°N
60°N
55°N
20°W
10°W
W0°E
10°E
20°E
30°E
40°E

Referee #1 Reply

Major issues

1) Experiments were conducted with nitrate and ammonium added at at least 10 fold higher concentrations than in situ values (100 μM vs. 10 μM and 10 μM vs. 1 μM, respectively, i.e., 1000% above ambient, and not 90% as stated in the text p. 11 l. 6).

We rephrased the text to avoid misunderstanding (p 12 line 8)

This means that the measured rates must be treated as potential rates unless the authors can establish an argument for 0th-order kinetics for both denitrification and nitrification. In turn, this implies that the estimated sponge-ground rates may be vastly (10-fold) overestimated. This issue should be discussed and the conclusions modified accordingly.

We agree that these are potential rates. We make this now more clear in the discussion, beginning of chapter 4.4.

^{15}N incubations were based on standard methods (Dalsgaard et al., 2003 and Hannig et al., 2007) with minor modifications as per Hoffmann et al. 2009. These methods allow us to estimate the rates at ambient NO3 concentrations based on 15N incubations. They are not the rates measured directly by the 15N labelled N2 production. This is clarified in Section 2.3. Although concentrations of labelled $^{15}N^-$ exceeded background ^{14}N 10-fold, potential denitrification rates were well within the range of those previously reported for cold and warm water sponges, where ^{15}N amendments were more reflective of ambient NO_3^- concentrations (Hoffmann et al., 2009;Schläppy et al 2010a.

In the oxic experiments, denitrification rates could, in principle, be calculated using the classic isotope pairing calculations for sediment cores (D14 sensu Nielsen 1992), but then the incubations should have been performed without addition of unlabelled ammonium and with maintenance of steady state

Labelled ammonium was added to the annamox incubations. No ammonium was added to the denitrification experiment. We now discovered that this was not clearly explained in the method section, which have led to confusion. The text is now corrected.

Calculations of coupled nitrification-denitrification were based on the approach of Hoffmann et al., 2009, where a similar experimental set-up was employed. The potential rates of denitrification and proportion of coupled nitrification-denitrification are comparable for Geodia barretti – 92 nmol N cm-3 sponge day-1 ; 26% coupled nitrification-denitrification (Hoffmann et al., 2009) relative to 96 nmol N cm-3 sponge day-1; 16% coupled nitrification-denitrification (this study).

2) Nitrification-based denitrification rates are calculated from the accumulation of single labelled 29N2. Firstly, it is not entirely clear how these rates and relative contributions were calculated, and I suggest to include the essential equations in Methods.

The abundance of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ were analysed from gas samples using a continuous flow isotope ratio mass spectrometer (CF/IRMS). Calibrations were achieved by injecting lab air and additional in house reference gas samples. Denitrification rates were calculated from the production of ^{15}N isotopes (see below) according to the method described by Nielsen (1992).

The rate of denitrification was measured from ^{15}N isotope production (equations 1 and 2). D_{14} and D_{15} represent denitrification of labelled $^{15}NO_3^-$ and $^{14}NO_3^-$. 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). D_{15}

is indicative of denitrification of labelled $^{15}NO_3^-$ and D_{14} represents in situ denitrification of $^{14}NO_3^-$.

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

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

To estimate denitrification of NO_3^- from ambient sea 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 unlabelled and labelled NO_3^- in ambient seawater.

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

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

These equations are now given in the method section, page 15.

Secondly,
the concept of water-based and nitrification-based denitrification was developed by Nielsen for sediment cores with steady state distributions of oxygen and nitrate (and it was challenged by Middelburg in L&O 41:1839). In the present study, oxygen was clearly not at steady state during the oxic incubations, and it also seems likely that new formed nitrate may have leaked from the sponge tissue thus gradually decreasing the C2 labelling of the ambient nitrate pool, and increasing 29N2 production from the ambient water. Moreover, the data presented in Fig. 1, for one of the six sponges, suggests that there is an issue with the mass balance of unlabelled N in the incubations. Thus, at the end of the anoxic incubations, excess 29N2 dominated over 30N2 in two of three incubations despite the stated _90% labelling of the nitrate pool, and the accumulated 29N2, reaching up to _23 μM, exceeds the amount of unlabelled nitrate initially available (10 μM in situ + 1 μM from the 99% 15N tracer). Also during the first 24 h, 29N2 production in the anoxic incubations seems higher than predicted by nitrate labelling in the absence of nitrification. Altogether, these uncertainties and discrepancies undermine the conclusion concerning the role of nitrification. Plots of excess 29N2 vs. excess 30N2 could potentially help the authors to evaluate and constrain some of these issues.

Ambient seawater was filtered to remove water column bacteria and or phytoplankton, thus reducing the potential for background nitrification. Although conditions were not at steady state, previous application of this method was considered suitable for dissected sponge explants (Hoffmann et al., 2009). The data has been re-analysed as suggested and errors in the calculation have been corrected. Issues with mass balance and $^{29}N_2$ production have now been resolved.

Specific comments 3, 8-12: The final statement is highly speculative and does not belong in an abstract.
We agree that this statement sounds provocative, but we still consider this a valid interpretation of our data, see justification below for comment on 25,11
4, 16-7: The statement about nif genes seems out of context. *Agree, removed*
6, 14: Science should never aim to show specific results but rather test hypotheses! *Rephrased*
7, 11
9, 4-5: "Upper few centimetres" is vague – considering the negative result, the question is whether only the oxic surface layer was sampled.
As the reviewer pointed out, the "upper few centimetres" sediments are very likely oxic (our microsensor measurements indicate that oxygen can penetrate to ~75 cm below seafloor). However, denitrification (at also other anaerobic processes) is most active in the upper most sediments, due to the widespread of bioturbation and bioirrigation in marine surface sediments (e.g. see the recent paper regarding sulfate reduction rates in the Aarhus Bay sediments (Andrew Dale et al, 2019, GCA)). Therefore, measurements made using the most surface sediments are believed to represent the majority of denitrification activities in a marine sediment column. (Of course, this is supported by our reaction-transport model!)
9, 20: There was no "atmosphere" in the vials? However, incubation with a helium/ oxygen headspace would have kept the incubations oxic throughout.
No atmosphere in the exetainers as described on p11. We followed a standard protocol here.
10, 7-8: This seems a very shaky assumption. Respiration rates must vary with species, temperature, and trophic state.
They certainly do, but we do not have these details for all investigated species and needed to make the best possible estimation.
10, 18-9: Some oxygen is likely introduced during transfer – did you test the water in the Exetainers?
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 in anaerobic incubations. See p 11, line 19
11, 6: The values are _1000% above ambient.
10 times above, corrected.
11, 12: According to 7, 11 the in situ temperature was below 0 _C! How would the higher incubation temperature affect the rates?
Lab experiments can never perfectly mimmick in situ conditions, and in our case, there were no cool room available at 0 C. We are aware that this may have led to over-estimation of the Arctic rates and made a comment in the discussion.
12, 18: The accumulations in Fig. 1 look only approximately linear – which test gave p < 0.05? Did the same apply to the linearity of the anoxic rates (13, 4)? *Figures modified after re-calculation of rates*
13, 15: Please specify the equations used here (see major issue #2).
See above, our reply to major issue #2.

16, 3-5: The opening of the Results is very confusing with the first two sentences referring to two different treatments. Delete the first sentence.
The first two sentences explain why our results show the absence of anammox, they refer to the same treatment. We rephrased for more clarity.

16, 22-3: The sediment experiment has little value. The origin of the sediment is unclear, and it does not seem representative of Arctic sediments.
The origin of the sediment samples (next to Arctic sponge ground at Schulz Massiv) is clearly stated in chapter 2.2. The sediment itself is of pelagic origin (ultimately from the primary production in the surface ocean rather than terrestrial origin), this is obvious from the geographic position of the sampling site and does not need to be mentioned.
18, 5: See 6, 14.
Rephrased
18, 18-9: Metabolisms in sponges or what? Please clarify/reference.
Rephrased for clarification
18, 20-5: The presence of denitrification genes and isolation of denitrifiers cannot prove "the presence of denitrification activity".
Rephrased

20, 11: How would the "pulse of organic matter in the water column" (where in the water column?) affect potential denitrification in the sponges' tissue?
Section rephrased

21, 16: "proves" is an overstatement.
Changed to "shows"
22, 1-2: It is not the in situ concentration but the 10 μM ammonium added, that is of relevance here.
Disagree. Ammonium was only added to the annamox experiments, not to the denitrification experiments. So we have a point here.
22, 13-5: Please provide a reference for the single copies.
This is text book knowledge, we do not see a need to provide a reference
22, 16-20: The curve in Fig. 3 does not look like an exponential function. It there statistical support for this relationship?
The figure changed after re-calculating the data. The point is that there is a clear positive correlation between anaerobic denitrification rates and nir genes for most species, we made this more clear now.
22, 20: What is meant by "optimized"?
Rephrased

23, 9-10: With 6 orders of magnitude variation, this is not very telling.
Our point is that there were not less nir genes in the sediment than in some of the sponges, but no denitrification.
23, 19 on: The calculations of sponge ground rates need explanation, but see Major issue #1.
See text added and rephrased at beginning of chapter 4.4
Furthermore, it seems that results of population density surveys are presented here for the first time. If this is the case, the methods and results should be specified i the appropriate sections. Otherwise, a reference should be included.
This is described in the methods, p 8 l 21-22. We rephrased also in section 4.4 to make clear that these are careful estimates.

24, 24: What was the frequency of non-pumping?
Not possible to say something in general because this varies between species, environmental conditions etc. In the particular study quoted here, there was one non-pumping period of 1-2

hours during the experiments of 12-20 hours, this can easily be looked up in the cited reference.
25, 11-2: Is this a short-term or permanent effect? Would reduced pumping rates/increased anoxia not result in reduced growth, reduced biomass, and thereby reduced nitrogen removal in a longer perspective?
Reduced growth would first lead to reduced remineralisation activity – so it would first of all weaken the classical DIN source function of the sponge. We know for sure that reduced pumping leads to reduced oxygen in sponge tissue, but we do not know if reduced pumping leads to reduced growth, so there is no point speculating about it here.
The system effect of the stressors seems speculative.
We rephrased to make clear that this is not speculation, but a potential scenario based on valid data interpretation.

Table 1: The number of significant digits should be adjusted.
Adjusted to what? There are two digits per value, what is wrong?

Fig. 1: Different triangles are used for 29N2 and 30N2.
Resolved

Ref #2

Answers

Biogeosciences review
General Comments:
This is an interesting and well-planned study. It is nicely focused and well-suited to address the question outlined by the authors. I do not see any major flaw with the experimental design or the interpretation, however, I think there are several places where some more clarity and/or improved organization would be helpful to the readers.

My “major” comments are that the
authors could setup arctic vs boral comparison a little more purposefully and clearly in the introduction.
Done, p 6.
Also, a map of the sampling are would be really helpful as I (and I think most
people) do not have a good image of this region in my head, it could be a supplemental file if need be.
We now provide a simple map, which we suggest to make available as supplementary information (Fig suppl). Geographical position of the sampling sites can also be visualized through the map tool in the Pangaea database.

There are also a number of typos and grammatical errors that need to be addressed.
Aside from those, I have also outlined below some minor comments that may help improve clarity and a few places where some more context or broader discussion is warranted.
Overall, I enjoyed reading this manuscript and the methods, statistics, and interpretation are sound.

Specific Comments:
Pg 4
L8: I would think that each compound should be defined first with the name and formula in parentheses, then you can use the formula after that
OK, corrected
L10-13: This sentence and really the whole first pp is on one hand a logical introduction, yet it still leaves me with the thought “what is the point of this pp”? Can the authors make it a more cohesive and setup the transition to N fixation (in the next pp) a little better? (and in the second pp it starts with N fix then goes back to DIN)
We rephrased according to suggestion
L22-26: This last sentence in the paragraph could be clearer. I follow the first half ok, but the second half starting on L24 is not clear to me.
removed

P5
L21: This goes back to a topic discussed in the first pp so I think some more context is needed here because it is a sudden transition to an earlier topic. Also what “observed variations” do the authors refer to here on L22?

L22: Maybe use “DIN” instead of “nutrient” to be more specific
L22: Instead of “a combination of variations” maybe just use “variability”
Entire sentence (L21-24) rephrased

P6
L4: This pp might be a good place to clearly introduce arctic vs boreal
Done

Pg5
L4: This might be a good place to briefly discuss the dependence of nitrification on oxygen, which is not really stated anywhere upfront but that information is relevant think.
The information is given in the same sentence but we rephrased to make it more clear

L14: There is no anaerobic process quantified in Fiore et al. 2013
OK, reference removed here

Pg7
L21: average biomass for boreal sponges is given, is this true for arctic sponges too?
Yes, estimates for average biomass for both boreal and arctic sponge grounds is given in the discussion (chapter 4.4) and in the data sheet published in Pangaea

Pg3 – *you mean Pg8?*
L2 and 6: what is meant by “key” here, why were these species chosen? *We chose species that are typical and representative for this type of sponge ground, see added text about sponge ground characterisation*
L9-10: says that sediment was not collected here, so it seems like something about sediment should be mentioned in the paragraph before this one.
Moved this sentence to the section on sediment sampling
L17-22: I can tell the authors tried to make the sampling and setup of experiments clear but I am still a little confused. For one, it would be helpful to describe the shape of the sponges, presumably massive/round? Second, why were these cut into three pieces- for each isotope tracer, is that right? *Details on sponge shape added in text. The sponges were cut into three sections to aid dissection. To ensure that we used only the choanosomal portion of the tissue, the most practical way to dissect this from a large individual was to cut the sponge into three pieces. Three whole sponges (n=3) were collected for each species. The dissected tissue from a single sponge represents one replicate. Also these details were now added to the text.*

Pg 9
L17: This setup was on the ship yes? Might be good to remind the reader of that
On the ship for arctic species, in the lab for boreal species – clearly stated in line 13-15
L18: Why only sand filter for the boreal specimens?
It was the only option available in that lab facility.
Pg11 L13: Can the authors say how many samples were sampled at each time point rather than “a selection” – or am I missing something here?
Corrected: 3 samples per species (one for each replicate specimen)
Pg13 L12: This seems redundant because of pg 12, which would be ok, but it makes it a little Confusing

As we did not detect any anammox, we could not calculate the rates or contribution of anammox to total N2 production according to this method. Is this what the referee means by redundan?.
Pg13 L23: Is it possible to give a little more guidance on the calculation of nitrification derived nitrate? It would be helpful for the reader and worth the word count since this becomes an important piece of information in the discussion.
Section is now extended including equations

Pg 14: Out of curiosity, why was amoA not quantified? Since the authors are interested in nitrification rates as well
This work focusses on denitrification. Coupled nitrification/denitrification was of interest in this context, but it was never our intention to quantify total nitrification rates or the genes involved. Nitrification in sponges is well explored already, while denitrification is not.

Pg 17 L3 – I suggest adding some of these calculations (even brief) in a supplemental document
Done

Pg18 L23: "proving" is a strong word… maybe "demonstrating" works better? (when possible in
other instances for the use of "prove" is there another word that could work?)
Rephrased

Pg18 L24: Fiore et al. 2010 is a review paper so I don't know that that fits in here with this sentence
OK we took it out

Pg19
L3-9: The information in this pp is fine, but I don't really see the point of the pp. It could use some language to tie it into the paper more.
The aim of this paragraph is to explain why we were not able to reproduce the (very low) anammox rates that we had quantified in one of the species previously. If the reviewer and the editor advice to delete this paragraph, we will do so.

L11: This sentence is awkward, try to reword without using "being"- just using "As denitrification is an…" would be much more straightforward.
OK, rephrased

L15-16: certainly interesting, but this sentence also leaves me wondering, what is the point? It would be nice to have it tied in more clearly for the reader.
The point is that an aerobic process and an anaerobic process happen at the same time. Even though this has been described before, it is unusual since aerobic processes usually take over as soon as oxygen is present. We think we have a point here?

Pg20
L1-6: It would be helpful to have more context here on any work that has been done to measure pumping activity – has anyone done this? Do we have any idea of these deep sea

sponges behave the same as others? The authors do get to this type of info later in the discussion but this pp seems lacking a bit as is.
We think that the references we give here and the information we provide later is sufficient.
L7-8: The use of "(anaerobic)" and "(some)" is confusing to me
Agree, clarified

Pg21 L16-17: The first point here about oxygen in the specimens here vs explants is confusing as to what the point is. I think I get what the authors want to say but it is not all that clear here and could be tied in better.
Section entirely rephrased, as re-calculation of the data gave a much better fit with literature values, so there is no need any more to explain differences.

Pg22 L20: "prepared and optimized" – I get hung up on "optimized" here, is there better way to say this? I think the authors mean the community is well suited to this environment or adapted to this environment, but optimized sounds odd to me
Agree, rephrased

Pg23 L8: instead of "apparatus turned on" maybe say "genes expressed"
Agree, rephrased

Pg24 L14: would be helpful to give some of this info briefly (sediment denitrification rates)
I think there is no need to go into detail, this information can be looked up in the cited references.

Pg25 L19-22: seems more to the point here to contrast with nitrification studies showing release of nitrate, rather than discussing "nutrients" as a whole which is vague
No, it is the contrast with both nitrification studies showing release of nitrate, and mineralisation studies showing release of ammonium. Good point, we made this more clear now.

Pg26 L4-5: This sentence is not tied in well, so it reads a bit awkward
Rephrased

Pg26 L6-7: This last sentence is a bit awkward and unclear at the end.
Rephrased

Technical Comments:
L10 abstract: "thus lead to that"
We were not able to find this sentence
pg 5 L21: "This opens for"
Rephrased
Pg 7 L15: check reference format
Reference format is generated automatically in EndNote. We will do the formatting when the manuscript is accepted and no references have to be removed or added.
Pg 15 L 6: "Standard of qPCR"
Rephrased to "qPCR standard".
Pg19 L21: parentheses for references- it looks like it should be: (e.g., Wilson….).

Reference format is generated automatically in EndNote. We will do the formatting when the manuscript is accepted and no references have to be removed or added.

Pg22 L4: “origin” should be “originate” I think

OK

Pg24 L10-11: are the “-“ supposed to be there? Yes

Pg24 L18: “since we do know” – maybe just remove “do”

ok

Pg25 L5 and 12: extra parenthesis

Reference format is generated automatically in EndNote. We will do the formatting when the manuscript is accepted and no references have to be removed or added.

Referee #3 reply

General comments

This manuscript provides novel information on potential (de)nitrification and anammox rates combined with genomics in 6 abundant cold-water sponges, from which 5 have not been analyzed previously. The data show that denitrification is a common process in deepsea sponges and is relevant for understanding the role of sponges in nutrient cycling. The study seems well planned and conducted.

My main concern is that the potential denitrification rates measured in this study in tissue sections are upscaled to whole sponges and ecosystem level and are even used for future predictions under anthropogenic stress. The rates here should be treated as maximum or potential rates, since they were conducted with 10 times ambient concentrations, on small tissue sections in closed exetainers, under no oxygen and decreasing oxygen concentrations.
We agree that these are maximum potential rates. We make this now more clear in the discussion, beginning of chapter 4.4. Compare also comment from Referee #1, and our reply.

My second major comment is that the MS is focused on and biased towards denitrification, with limited attention for other nitrogen transforming processes, such as nitrification, anammox and perhaps DNRA. I suggest to present all labelling incubations, carefully evaluate the results and present and discuss in a more balanced overview of the different nitrogen transforming processes and also include all data in the published Pangea dataset.
This work focusses on processes which transfer DIN into N2 – denitrification and anammox. The anammox rates were under detection limit. Coupled nitrification-denitrification is of interest in this context, but it was never our intention to provide "a balanced overview of the different nitrogen transforming processes" in sponges. Nitrification and also DNRA in sponges is well explored, while denitrification (and anammox) is not. Our intention was to close this knowledge gap.

Specific comments

- Title: The term "nutrient sink" in the title is confusing and questionable
We disagree and would like to keep it
- P3, L3-11: this is too speculative, see major comment 1.
We agree that this statement sounds provocative, but we still consider this a valid interpretation of our data. The interpretation is sufficiently justified in chapter 4.4.
- P4, L16-19: This sentence doesn't really fit and perhaps the whole part of N fixation can be moved to the discussion, since it disrupts the introduction on DIN release.
Section rephrased
- P7, L20: Add some of the relevant characteristics for this site.
The most relevant characteristic (hard bottom slope of a fjord) is given, details can be looked up in the quoted reference. Community structure of boreal vs arctic sponge grounds is now also described.
- P2.2 and p2.3: A table or flow chart with the experimental incubations would be very useful.
We consider the description in the text to be sufficient
- P10, L:16: On the previous page it is mentioned that all incubations were done with water sampled from the deep, but here surface site water is mentioned for anoxic incubations. This needs to be clarified.
Corrected for clarification

- P11, L4: Can you be sure that 15-NO3 is reduced to 15-NO2, the preferred substrate for anammox? Please elaborate
For the incubation experiment screening for anammox, 15-NH4 was added as substrate. 15-NO3 was added for the denitrification experiment. We realized that this was not clearly written in the method, this is now corrected.
- P 11, L6-7: This is 1000% above ambient concentrations, and ambient concentrations from which site, arctic or boreal grounds or both?
Section rephrased to avoid misunderstanding, see also Ref # 1
- P11, L12: This is not *in situ* temperature for the Arctic species, the temperature increase might increase your potential rates.
Lab experiments can never perfectly mimmick in situ conditions, we chose the best possible solution. We made a comment in the discussion that Arctic rates may be overestimated because incubation temperature was above in situ. See also comment by Ref #1.
- Paragraph 2.3.2: There were no oxic sediment incubations?
No. Since the denitrification was zero under anoxic conditions, there was not need to check for oxic conditions.
- Paragraph 2.3.3: Including the calculations is informative for the reader.
Some equations and calculations are now included.
- P12,L22-P13, L1: The published dataset contains individuals with tissue degradation
We now explained more clearly in chapter 2.3 that these samples were not considered for 15N analyses and rate quantification.
- P13, L:11-13: This needs more explanation. I guess you mean no 29-N2 was detected in the anoxic incubations? What about 29-N2 and 30-N2 production in oxic incubations with labeled ammonium? Some production can be expected from coupled nitrification and denitrification.
The lack of 29N2 production from 15NH4+ (anoxic incubations) suggests an absence of anammox, since N2 production via anammox requires 1 N from NO2- and 1 N from NH4+. It is also important to note that although labelled N2 production can be expected from coupled nitrification-denitrication of 15NH4+ in the oxic incubations, we did not detect labelled N2 in these oxic incubations.

- Paragraph 2.3.4: Also here, the equations would be useful. And following my comment above, can you estimate coupled nitrification-denitrification from your oxic incubations with 15N-NH4?
Equations added
- Paragraph 2.4: In the results and discussion is mentioned that the sponges were also screened for anammox and N2 fixation functional genes. The screening and description of the functional genes should be described here. Was there also screening for other genes relevant for the nitrogen cycle (e.g. nitrification, DNRA)?
Only screening for N2 fixation functional genes. This was relevant for our "nutrient sink" story, since nitrogen fixation would have closed the nitrogen cycle in the sponge. The other processes the reviewer mentions were of less importance for this particular story, and so we did not screen for genes.
- P16, L1-6: Add graphs or tables with the results under oxic and anoxic conditions.
These results are presented in Fig 2.
- P16,L22-23: What about unlabeled N2 production?
Unlabelled N2 production cannot be detected in the IR-MS.
- P18, L5: I would remove "nutrient removal"
This needs to stay, it is the main conclusion of the manuscript, and it is well justified.
- P18, L22: Denitrification has not been directly shown in Fiore et al. 2013, but is given as a potential pathway, together with anammox or DNRA, to explain net consumption of nitrate in some of the sponges.

Changed to "…has been indicated…"
- P20, L6: I won't state that results are representative for normal conditions, but state that these conditions are not atypical (or something similar).
We think this statement is well justified: the quoted literature proves that undersaturation of oxygen in the tissue is a common feature in sponges.
- P20, L9-L15: I would expect year-round higher (dissolved) organic matter concentrations at the Boreal compared to Arctic grounds. Another explanation might be related to the higher incubation temperature (if it was 6°C) compared to the *in situ* temperature.
Good point, text rephrased
- Paragraph 4.2: The relevance of your denitrification rates in view of other nitrogen transforming processes should be discussed in a balanced way (see major comment 2). This paragraph gives the impression that denitrification is more important than nitrification in sponges, even though the majority of sponge studies reveal that sponges are net sources of nitrate, with denitrification being only a fraction of nitrification. Also the possibility of DNRA as competitive process for denitrification should be discussed somewhere.
We removed the calculation of minimum nitrification rates as we see that this was confusing, and keep the statement that nitrification was present. We extensively quote publications which focus on the nitrogen source function of sponges. We do not believe that any reader will get the impression that denitrification is generally more important than nitrification in sponges. DNRA is not relevant for our publication as it conserves bioavailable nitrogen in the system. We focused on processes which remove bioavailable nitrogen from the system. As mentioned above, the aim of our study is not to give a balanced overview of the different nitrogen transforming processes in sponges, but to put the focus on sponges as potential nutrient sinks, and to point out potential scenarios based on calculated potential rates.
- P21, L11-14: What is so different between explants and tissue sections? The tissue sections will also dependent on diffusion? There are more differences between Hoffmann et al. 2009, i.e. in your study you added NH4, which will stimulate nitrification, while in Hoffmann et al. 2009, no NH4 was added. You could discuss the reliability of nitrification measurements.
Section removed and rephrased
- P21, L21-25: "May be higher" should be "are likely higher" and the reported rates are really at the low end of other reported rates.
Section rephrased
- P22, L1, yes, but you added 10 μM (unlabeled) NH4, which can result in 10 μM (unlabeled) NO3 in oxic conditions.
Labelled ammonium was added to the annomox incubations. No ammonium was added to the denitrification experiment. We now discovered that we did not write this clearly in the method section, which has led to confusion. The text is now corrected
P22,L6,9: These last two sentences are not connected to the rest of the paragraph.
Yes they are. This paragraphs lines up several facts that lead to the main conclusion that the nitrogen cycle is not closed in these sponges, so bioavailable nitrogen leaves the system.
- P22, L16-21: I won't use optimized, I guess you want to say there is an active denitrifying community. Perhaps add some statistics to the relationship, this is a nice result.
Rephrased.
- P23, L4-6: I disagree that they are realistic, see major comment one.
Rephrased, but see our reply to major comment one.
- P23, 15-17: Are there reported denitrification measurements of Arctic sediments? What about the other nitrogen transforming processes? A comparison to literature should be added with to this statement.

There is a recent publication about anammox in these Arctic sediments: https://www.biorxiv.org/content/10.1101/729350v1 *, where profiles of nitrate, ammonium and oxygen are included. The group did also predict denitrification rates based on their model but these data are not yet published. Apart from this, we are not aware of any literature on denitrification rates in deep Arctic sediments, only from the continental shelf areas.*

- Paragraph 4.4: The results are not representative for a natural situation, but rather show a potential, so I would be extremely cautious to upscale these numbers and refer to these sponges as efficient nutrient sinks (see major comment 1).

We rephrased the first paragraph to make more clear that we talk about maximum potential rates and possible scenarios. We still think that a presentation of (potential) areal rates is valid and useful to compare denitrification rates of sponge grounds to other ecosystems.

- P24, L6-10: The calculations and conversion factors going from volume to surface integrated measurements are lacking (but see major comment 1).

No, both calculations and conversion factors are given in the data publication https://doi.pangaea.de/10.1594/PANGAEA.899821 which will be publicly available when the manuscript is accepted.

- P25, L13-15: Combined anthropogenic stressors can also lead to changes in nutrient and organic matter availability which might affect microbial composition and biogeochemical processes. It is too speculative to state that sponges will become nutrient sinks in the future if they reduce pumping.

We say "may", not "will". We think it is necessary to point out this potential and so-far overlooked scenario.

- P26, L10: Please expand the dataset in Pangaea with all incubation data and results.

All results of the incubation experiments are completely presented in terms of 29N and 30N accumulations. What is lacking?

- P35, L2: STP is used for the first time, does it stand for standard temperature and pressure?

Removed, as this explanation belongs to the methods and not the figure legend

- Figure 1 and figure 2 should be swapped, based on their reference order in the text.

No, Figure 1 is mentioned for the first time on page 12, while Figure 2 is mentioned for the first time on page 16.

- The order of references in the citations and full references need to be checked.

Reference format is generated automatically in EndNote. We will do the formatting when the manuscript is accepted and no references have to be removed or added.