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Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges

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Abstract

We performed a combination of field and laboratory experiments designed to investigate sponge-hosted microbial nitrification processes, plus a survey of the prevalence of nitrification among sponge species at patch and outer reefs, Florida Keys, USA. Results from dissolved inorganic nitrogen (DIN) measurements, chemical inhibition, and ^{15}N isotope tracer experiments with Caribbean sponges show that 9 of 12 species tested hosted nitrification. Of the species in our study that are reported to host internal microbial populations, all but one has been found to host this process. Nitrification occurred with an apparent N isotopic fractionation factor of $11 \pm 2.6‰$ (standard error) and was inhibited by nitrapyrin. Both of these characteristics are consistent with bacterially mediated ammonium oxidation; however, ammonium oxidation by crenarchaeota is also possible. The isotopic composition of nitrate expelled from sponges in situ had lower $\delta^{15}N$ values than nitrate from the ambient water column, suggesting active utilization of the nitrate product after it is released. The frequency of the association between nitrifying microbial communities and sponges that harbor large microbial communities suggests that nitrification is an integral part of the metabolic function of many sponge species. Given the abundance of these species on Conch Reef and the magnitude of their nitrification rates, the majority of benthic nitrification on the Florida Keys outer reef tract probably occurs in sponges. Sponge population size and composition could therefore strongly influence the concentration and speciation of DIN in the reef water column, and possibly other ecosystems where sponges are abundant.

1. Introduction

Nitrate fluxes as high as 12 ± 2.2 mmol m^{-2} d^{-1} have been reported from sponges on Caribbean reefs (Corredor et al., 1988). This implies a nitrification rate that far exceeds those measured in other marine benthic systems such as microbial mats (1 mmol m^{-2} d^{-1} Bonin and Michotey 2006), coral reef sediments (1.68 mmol m^{-2} d^{-1} Capone et al., 1992), and temperate continental shelf sediments (2.1 mmol m^{-2} d^{-1} Hopkinson et al., 2001). To date, weight-specific rates of nitrification have been reported for five tropical (Corredor et al., 1988; Diaz and Ward, 1997) and six temperate sponge species (Jimenez and Ribes, 2007) and benthic fluxes of nitrate have been calculated for only 3 species (Corredor et al., 1988; Diaz

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and Ward, 1997). These reported rates and the abundance of sponges on some coral reefs (up to 3.62 L m^{-2}, Southwell, 2007) suggest that sponge-hosted nitrification may strongly influence DIN speciation in coral reef waters. However, surprisingly little is known about this process, such as the frequency of occurrence among the species, environmental controls on rates, and the fractionation of stable isotopes that is expected to occur during nitrification (Mariotti et al., 1981).

It was once thought that ammonium oxidation, the first step in nitrification, was performed only by beta- or gamma-proteobacteria. However, recent studies have shown that crenarchaeota from Marine Group 1 are capable of oxidizing ammonium, and may constitute a large portion of the oceanic ammonium oxidizing community (Karner et al., 2001; Francis et al., 2005; Francis et al., 2007). Both bacterial nitrifiers and Marine Group 1 archaea have been detected in sponges, along with a wide variety of other microbial organisms (Taylor et al., 2007, and references therein). Thus, it is presently unclear whether archaea or bacteria (or both) might be responsible for the impressive rates of ammonium oxidation observed in sponges. Although both bacterial and archaeal ammonium oxidizers appear to perform the same biogeochemical function, there may be important differences in the rates, carbon fixation mechanisms, environmental controls, or competitive ability between these groups. Therefore, the identity of the microbial partner may, in fact, influence the biogeochemical processes that occur as a result of this association.

The release of nitrate from sponges has been interpreted as evidence for microbially-mediated nitrification (Corredor et al., 1988; Diaz and Ward, 1997; Jimenez and Ribes, 2007) because animals typically release inorganic metabolic waste in the form of ammonium rather than nitrate. Although nitrate release from sponges was first observed almost 20 years ago, no studies exist, to our knowledge, that directly test this empirical evidence or that determine the prevalence of nitrification among the large number of species (up to 300) found on tropical reefs (Diaz and Rutzler, 2001, and references therein). Furthermore, nothing is known about nitrogen isotopic fractionation that we expect to occur during sponge-hosted nitrification (Mariotti et al., 1981). Such investigations could provide information about microbial communities performing the process because the magnitude of isotopic fractionation partially depends on the enzymes employed in the reaction (Casciotti et al., 2003). However, these effects may be confounded by environmental factors such as oxygen depletion within the sponge tissue (Hoffmann et al., 2005). Stable isotopic analysis of sponge-produced nit-

rate may also provide clues to concurrent biogeochemical processes that have been speculated to occur within sponges, such as assimilatory or dissimilatory uptake of nitrate or nitrite (Southwell, 2007; Taylor et al., 2007).

Sponges can be a large source of DIN on coral reefs where they are abundant (Corredor et al., 1988; Diaz and Ward, 1997; Jimenez and Ribes, 2007), and the partitioning of these nutrients between benthic and pelagic primary producers could be an important aspect of nutrient recycling and benthic–pelagic coupling. Stable isotopic compositions have been used extensively to trace sources of nutrients in marine organisms (Marguillier et al., 1997; Sammarco et al., 1999; Heikoop et al., 2000) and to indicate specific transformations of inorganic nitrogen species (Sutka et al., 2004; Sigman et al., 2000). Therefore, characterizing the isotopic composition of the nitrate expelled by sponges is a logical first step for investigating the reaction. If the isotopic composition can be successfully constrained, then the relative importance of sponges as a source of nitrate for the coral reef food web could also be investigated using isotope mass balance. This study presents 1) an updated list of nitrate or nitrite production in sponges that expands the number of known species hosting this process to twenty-one, 2) multiple lines of direct evidence for microbial conversion of ammonium to nitrate within sponges, and 3) isotopic analysis of sponge nitrate from both *in situ* and laboratory incubations. These results are used to generate models of N-cycling processes based on isotope mass balance of nitrate and to provide new insights into the provenance and fate of nitrate produced in sponges.

2. Methods

2.1. Study sites

Samples of ambient and sponge excurrent water (water exiting the osculum) were collected at Conch Reef (24° 57.43′ N, 80° 26.82′ W), approximately 8.5 km east of Tavernier Key, and at Diadema Reef (24° 59.198′ N, 80° 26.096′ W), a small patch reef approximately 8 km east of Key Largo. Sampling and experiments took place in 2004–2006. Live sponges used in land-based incubation experiments were obtained at Three Sisters (25°01.76 N, 80°23.89 W), a patch reef approximately 7 km east of Key Largo, FL and approximately 4 m deep.

2.2. In situ sampling

SCUBA divers collected triplicate samples of sponge excurrent water (water exiting the osculum) and ambient water (adjacent to the sponge) in acid-washed (10% HCl) and DI-water rinsed 60-mL syringes fitted with a stopcock and a short
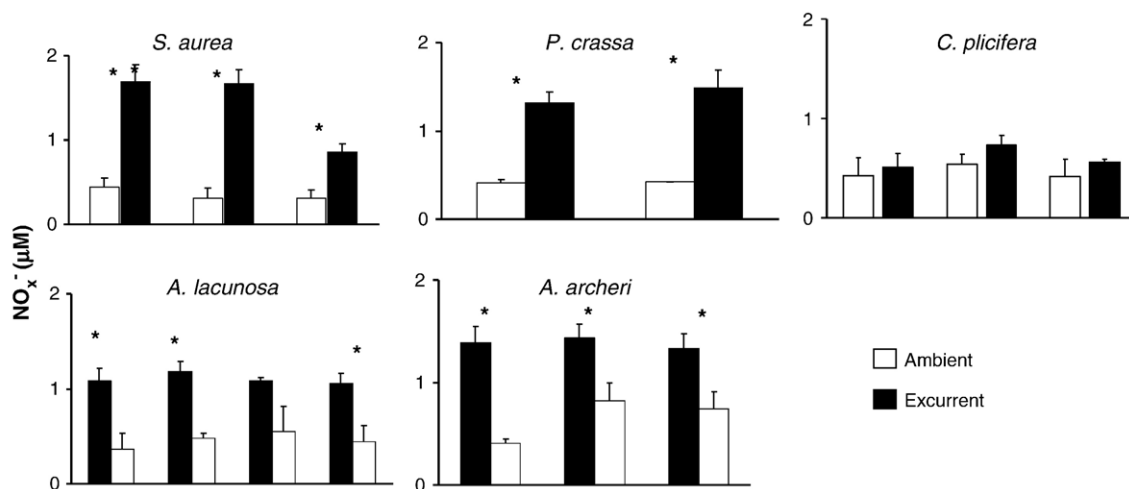
Fig. 1. NO_x^- concentrations in ambient and excurrent samples for sponges. Each set of bars represents an individual sponge, and error bars are standard deviation of triplicate samples. Asterisks (*) indicate a significant difference ($p < 0.05$).

length of narrow tubing. For each species, 2–4 individuals were sampled. Fluorescein dye was used to confirm that the sponge was pumping before samples were taken. In order to obtain water from the excurrent plume only, excurrent samples were collected slowly (~ 2 mL s^{-1}), and the syringe was flushed with sample first before the final collection. Samples were kept on ice during transportation to the shore-based laboratory, where they were filtered (Whatman GF/F, nominal porosity 0.7 μm) and frozen in acid-washed and DI-water rinsed polypropylene centrifuge tubes for nutrient analysis, which was usually performed within one month.

For the analysis of nitrate isotopic composition, we collected 300 mL samples of sponge excurrent water in five 60-mL syringes. Ambient water from approximately 2 m above the sea floor was also sampled in this way ($n=3$). Samples were kept on ice during transportation to the shore-based laboratory and filtered as described above. Aliquots of 50-mL were frozen at −20 °C (Dore et al., 1996) in acid-washed and DI-water rinsed polypropylene centrifuge tubes for analysis of nitrate plus nitrite concentration, and the rest

frozen at −20 °C in acid-washed, DI-water rinsed 250-mL HDPE bottles for analysis of nitrate isotopic composition.

2.3. Incubation experiments

SCUBA divers cut pieces of sponge from healthy adults (2–8 cm^3, depending on species), attached them to PVC plates with plastic cable ties, and then left the plates attached to weighted platforms on the reef to recover. Healed sponges for experiments were collected from the platform on a time scale of days to weeks, depending on species. Only sponges with healthy appearance (i.e., no visible wounds or decay) were selected for incubation experiments. The selected sponges were incubated in 2–4 L HDPE containers of aerated seawater for 6–8 h, except for *Verongula rigidia*, whose incubation was ended after 3 h due to a hurricane. At no time were the sponges exposed to air. Incubations with coral rubble ($n=4$) and seawater blanks ($n=3$) were also conducted to evaluate potential background nitrification rates. Two aliquots of water were taken at each time point; one was analyzed immediately for ammonium concentration, and the other
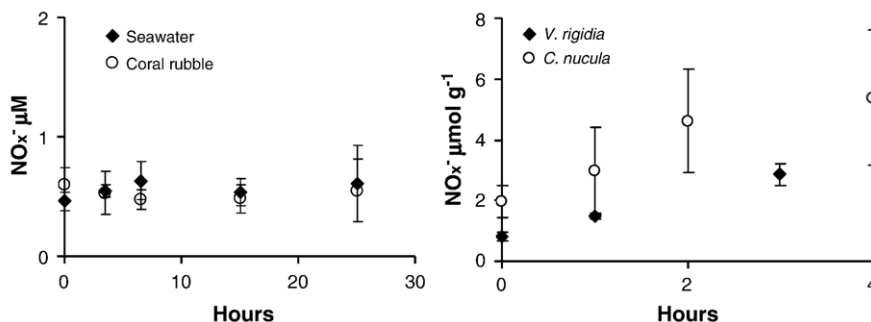

Fig. 2. NO_x^- accumulation in incubation experiments; $N=3$ individuals for each species, $N=4$ for coral rubble, and $N=2$ for seawater blanks, error bars are standard deviation.
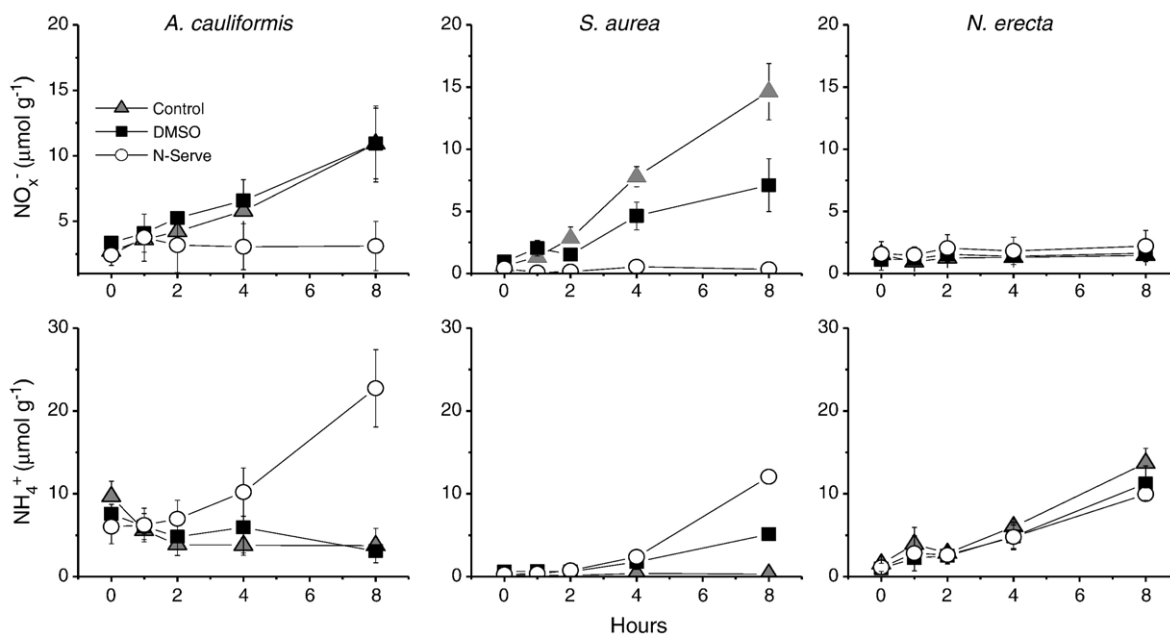
Fig. 3. N-Serve inhibition experiments with *A. cauliformis*, *S. aurea,* and *N. erecta*. NO_x^- and NH_4^+ concentrations over time during incubations of the sponge alone (control), with DMSO, and with DMSO plus N-Serve. Error bars are standard deviation ($n=2$ for control and DMSO treatments, $n=3$ for N-Serve).

was frozen in an acid-washed, DI-water rinsed 50-mL polypropylene centrifuge tube for nitrate and nitrite analysis, usually within one month.

2.4. Nutrient analysis

Ammonium was measured by fluorescence following the method of Holmes et al. (1999). Briefly, 3 mL of o-phthalaldehyde working reagent was added to 12 mL of sample in an acid-washed

Fig. 4. [15]N tracer addition experiment. Black bars indicate N isotopic composition of nitrate (in atom%) after an 8 h incubation of a single sponge individual with [15]NH$_4^+$ tracer added at a concentration of 0.2 μM. White bars indicate the amount of ^{15}N tracer recovered as [15]NO$_3^-$.

15-mL centrifuge tube. Samples were incubated in the dark for 2 h, and then analyzed using a Turner Designs fluorometer, model TD-700, fitted with an ammonium optical kit. Ammonium standards were made fresh each day, and measured along with samples. The limit of detection for ammonium was determined to be 0.2 μM by repeated measurement of standards. Nitrate plus nitrite (NO_x^-) was measured using standard colorimetric methods on a QuickChem flow-through autoanalyzer (Strickland and Parsons, 1972). A subset of samples was analyzed for nitrite only, and the contribution of nitrite was found to be negligible. The limit of detection for NO_x^- was determined to be 0.25 μM by repeated measurement of standards, and standard deviation of triplicate samples was 0.17 μM for both ammonium and nitrate.

2.5. [15]N tracer experiment

Sponges were screened for visible signs of necrosis, and visually healthy individuals were incubated in natural seawater amended with [15]NH$_4^+$ to a final concentration of 0.2 μM, which was approximately 20% of starting ammonium concentrations (determined in the field). After 8 h, water samples of 50 mL each were collected and frozen in acid-washed centrifuge tubes for analysis of the concentration and nitrogen isotopic composition of NO_3^-.

2.6. Inhibition experiment

Nitrapyrin (commercial name N-Serve) blocks oxidation of ammonium (in bacteria), the first of two steps in its conversion to nitrate. Therefore the difference in ammonium flux with and without N-Serve is taken as a measure of gross nitrification
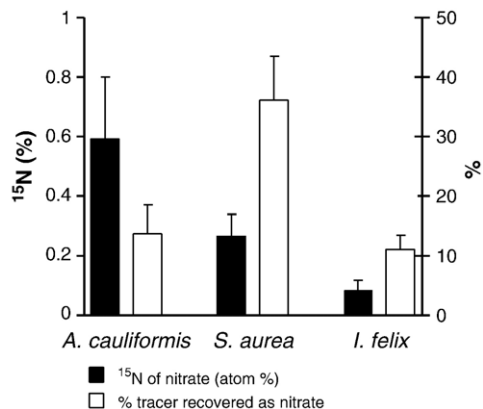
Table 1
Sponges that release NO_x^- from our survey and from the literature

Species	Method	NO_x^-	References
Agelas conifera	Inc/In situ	+	2
Agelas oroides	Incubation	+	5
Amphimedon compressa	Incubation	−	2
Anthosigmella varians	Incubation	+	3
Aplysina aerophoba	Incubation	+	5
Aplysina archeri	In situ	+	1,2
Aplysina cauliforms	Incubation	+	1,2
Aplysina fistularis	Incubation	+	2
Aplysina lacunose	In situ	+	1,2
Axinella polypoides	Incubation	+	5
Callyspongia plicifera	In situ	−	1
Callyspongia vaginalis	Incubation	−	2
Chondrilla nucula	Incubation	+	3,4
Chondrosia reniformis	Incubation	+	5
Dysidea avara	Incubation	−	5
Ircinia campana	Incubation	+	2
Ircinia felix	Incubation	+	1,2
Ircinia oros	Incubation	+	5
Ircinia strobilina	In situ	+	1,2
Niphates digitalis	In situ	−	2
Niphates erecta	Incubation	−	1,2
Oligoceras violacea	Incubation	+	4
Plakortis halichondroides	Incubation	−	4
Pseudaxinella zeai	Incubation	+	4
Pseudoceratina crassa	In situ	+	1
Smenospongia aurea	Inc/In situ	+	1,2
Spheciospongia vesparium	In situ	−	1
Verongia rigidia	Incubation	+	1
Xestospongia muta	Inc/In situ	+	1,2

Inc = incubation method. Plus (+) indicates NO_x^- production; minus (−) indicates no NO_x^- production. Reference numbers indicate 1: this study, 2: Southwell, 2007, 3: Corredor et al., 1988, 4: Diaz and Ward, 1997, 5: Jimenez and Ribes, 2007.

rates, and the difference between net nitrate release rates from controls and gross nitrification is assumed to be nitrate uptake. Nitrapyrin is insoluble in water, so it was first dissolved in dimethyl sulfoxide (DMSO) before it was added to the incubation chambers at a final concentration of 150 μM. *Aplysina cauliformis* and *Smenospongia aurea,* two sponges that release nitrate, plus *Niphates erecta*, which releases only ammonium, were incubated in three parallel treatments: DMSO plus N-Serve, DMSO only, and no additions ($n=2$ for control and DMSO treatments and $n=3$ for N-Serve treatments). A blank experiment (seawater alone) was included to test for experimental artifacts and changes in nutrient concentration due to the phytoplankton community. Otherwise, experimental procedures were as described above for incubation experiments.

2.7. Nitrate isotopic composition

Samples for determination of the $\delta^{15}N$ value of nitrate ($\delta^{15}NO_3^-$) were collected at the end of incubation experiments (with and without $^{15}NH_4^+$ added), and also *in situ* from the excurrent plumes of sponges as described above. $\delta^{15}NO_3^-$ values were determined using the denitrifier method of Sigman et al. (2001) in the Stable Isotope Biogeochemistry Laboratories at the University of Hawaii at Manoa. This method uses the denitrifying bacteria *Pseudomonas chlororaphis*, which lack the enzyme to convert N_2O to N_2. Briefly, 20-mL headspace vials containing pure cultures of *P. chlororaphis* were capped with a Viton stopper and flushed with N_2 or He for 2 h. An aliquot of the sample was then injected into the vial (2–8 ml, depending on NO_3^- concentration, previously determined) and the culture was allowed to incubate in the dark overnight. The culture was then killed by addition of 0.2 mL of 10 N NaOH. The isotopic composition of the N_2O in the headspace was then analyzed by isotope-ratio-monitoring gas chromatography-mass spectrometry using methods described by Popp et al. (1995) and Dore et al. (1998). Repeated injections were performed on 13% of samples to evaluate instrumental precision, and analysis of duplicate samples was performed on 19% of samples to evaluate overall sampling and analytical error. The standard deviation of duplicate injections of samples was 0.15‰, and the standard deviation of duplicate samples was 0.33‰. For ^{15}N-labeled nitrate samples, replicate analysis of samples had a standard deviation of 0.04 atom% ^{15}N.

2.8. Statistical treatment

Sponges were considered to host nitrification if *in situ* samples of excurrent water had significantly higher concentrations of nitrate compared to ambient water adjacent to the sponge individual ($n=3$) or if nitrate concentrations increased significantly during laboratory incubation experiments with live sponges compared to controls. At least two individuals were tested for each species. Statistical significance of differences between ambient and excurrent samples was determined by using a 2-tailed *t*-test assuming unequal variance and for incubation experiments by linear regression of nitrate

Table 2
Average flux rates from N-Serve inhibition experiment

Species	Treatment	NO_x^- μmol $g^{-1} h^{-1}$	NH_4^+ μmol $g^{-1} h^{-1}$	Total DIN μmol $g^{-1} hs^{-1}$
A. cauliformis	Control	1.7 (0.5)	−0.5 (0.5)	1.2 (0.7)
A. cauliformis	DMSO	1.5 (0.5)	0.4 (1.0)	1.9 (1.1)
A. cauliformis	N-Serve+ DMSO	−0.1 (0.1)	2.2 (0.4)	2.1 (0.4)
S. aurea	Control	1.8 (0.3)	0.0 (0.0)	1.8 (0.3)
S. aurea	DMSO	0.8 (0.3)	0.6 (0.1)	1.4 (0.3)
S. aurea	N-Serve+ DMSO	0.0 (0.0)	1.5 (0.1)	1.5 (0.1)
N. erecta	Control	0.0 (0.0)	1.5 (0.3)	1.5 (0.3)
N. erecta	DMSO	0.1 (0.1)	1.3 (0.2)	1.4 (0.2)
N. erecta	N-Serve+ DMSO	0.1 (0.1)	1.1 (0.2)	1.2 (0.2)

Numbers in parentheses are standard deviation.

concentration versus time. This test was also used to evaluate differences between fluxes of different treatments in the chemical inhibition experiments. Unless otherwise noted, uncertainties are ±1 standard deviation.

3. Results

3.1. Nitrification survey

Results from *in situ* samples (Fig. 1), incubation experiments (Fig. 2), chemical inhibition (Fig. 3) and ^{15}N tracer experiments (Fig. 4) demonstrated active nitrification in 9 of the 12 species studied. Nitrification was detected in the genera *Chondrilla, Ircinia, Aplysina, Xestospongia, Smenospongia, Pseudoceratina,* and *Verongula.* Species in the genera *Callyspongia, Niphates,* and *Spheciospongia* produced solely ammonium (Table 1). During the incubation experiments, there was no significant increase in NH_4^+ or NO_x^- in either the seawater blanks or the coral rubble incubations over 24 h (Fig. 2).

3.2. Chemical inhibition

Only NH_4^+ (and not NO_x^-) accumulated in incubations with *Niphates erecta*, regardless of treatment (Fig. 3 and Table 2). The total DIN fluxes for *N. erecta* were not significantly different for control, DMSO, or N-Serve treatments. In contrast, N-Serve clearly inhibited nitrification in *Aplysina cauliformis* and *Smenospongia aurea*, as ammonium accumulated rather than NO_x^- (Fig. 3). DMSO alone had no apparent effect on the production of NO_x^- in *A. cauliformis*, but had a significant effect on NO_x^- production in *S. aurea*. The partial inhibition in the DMSO-treated *S. aurea* may have been caused by the DMSO itself or by cross-contamination with the N-Serve inhibitor, as ammonium-oxidizing organisms can be sensitive to this chemical even at low concentration (Popp, B. pers. obs.). For *S. aurea* and *A. cauliformis*, the rates of total DIN release for the N-Serve, DMSO only, and controls were not statistically different.
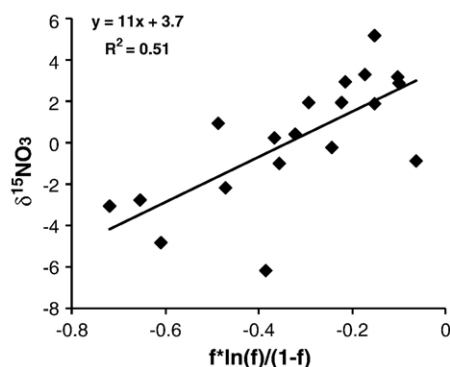

Fig. 5. Natural abundance N-isotopic composition of nitrate produced by sponges during incubation experiments (with no ^{15}N tracer added) fitted with the Rayleigh distillation model for a closed system.

Table 3
Concentrations and isotopic compositions from nitrate in sponge excurrent plumes sampled *in situ* and calculated δ^{15}N values for sponge-produced nitrate

Species	Excurrent plume		Sponge-produced nitrate	
	$[NO_3^-]$ μM	δ^{15}N ‰	δ^{15}N ‰	SD ‰
A. archerii	1.33	1.0	−4.5	3.5
A. lacunose	1.15	0.7	−8.7	8.8
A. lacunose	1.81	−0.3	−4.3	1.8
I. strobilina	1.14	0.8	−8.6	8.9
I. strobilina	1.27	3.0	0.8	3.1
I. strobilina	1.36	1.7	−2.3	2.6
S. aurea	1.18	−0.7	−12.6	11.0
S. aurea	1.02	0.1	−19.4	31.2
X. muta	1.75	2.5	0.9	1.5
X. muta	1.81	2.9	1.8	1.5
X. muta	1.80	2.2	0.4	1.4
Ambient water	0.84	4.2		

Sponge-produced nitrate δ^{15}N values were calculated by isotopic mass balance of excurrent nitrate minus the contribution from ambient nitrate. SD=standard deviation. For propagation of error calculations, uncertainties were based on standard deviations of triplicate samples from the same sponge individual.

3.3. ^{15}N tracer experiment

Nitrate from $^{15}NH_4Cl$ amended incubation experiments was highly enriched in ^{15}N for both *A. cauliformis* and *S. aurea* (Fig. 4). The nitrate recovered from the *A. cauliformis* incubation ranged from 0.46–0.83 atom% ^{15}N, and nitrate from the *S. aurea* incubation ranged from 0.18–0.32 atom% ^{15}N. *Ircinia felix* produced the least ^{15}N-enriched nitrate, ranging from 0.05 to 0.11 atom%. With *A. cauliformis*, an average of 14% of the ^{15}N labeled NH_4^+ was recovered as nitrate, whereas an average of 37% was recovered as nitrate in experiments with *S. aurea*. Recovery of ^{15}N tracer as nitrate from *I. felix* could not be calculated directly because the concentration of nitrate was not measured (due to sample loss). However, the nitrate concentration was estimated from the peak area measured during analysis of $\delta^{15}NO_3^-$ (described above). The nitrate concentrations estimated using this method agreed with previously measured nitrate release rates for this species under similar experimental conditions (Southwell, 2007). The estimated recovery of tracer from the *I. felix* incubation was 8.4–13.2%.

3.4. Natural abundance δ^{15}N of sponge nitrate

The δ^{15}N values of nitrate collected from incubation experiments in which no isotopic tracer was added (i.e. natural abundance) ranged from −6.2 to 5.2‰ (Fig. 5). Nitrate from *in situ* sampling of the sponge excurrent plumes ranged from −0.7 to 3.4‰, with an average of 1.5‰±1.4 (Table 3). Nitrate concentration in the ambient water samples obtained for isotope analysis averaged 0.84±0.1 μM (n=3), and the δ^{15}N value was 4.2±0.8‰ (n=3).

4. Discussion

4.1. Nitrification survey

Previous research on sponges that host large microbial communities, or High Microbial Abundance (HMA) sponges (*sensu* Hentschel et al., 2006), shows that microbial communities in HMA sponges can occupy up to 50% of the sponge volume (Rutzler, 1985), and generally have community compositions that are different from that found in the surrounding water column (Hentschel et al., 2006). The large body of phylogenetic research on sponge microbial communities shows that community composition appears to be stable over time and space, even under different environmental conditions and in different oceans (Taylor et al., 2007, and references therein). Thus, it is unsurprising that, in our study and in previous sponge nitrification studies, the presence or absence of nitrification was generally consistent among sponges of the same species and also within a genus. In contrast, Welsh and Castadelli (2004) detected nitrification potential in many marine invertebrates, but the rates were highly variable and there was no apparent correlation with taxonomic group. Overall, we found that nitrification was extremely common among HMA sponges; *Spheciospongia vesparium* was the only sponge in our study reported to host a significant microbial population (Weisz et al., in press, and references therein) that did not show evidence for nitrification (NO_x^- was below the limit of detection in both excurrent and ambient samples, data not shown). No low microbial abundance (LMA) sponges were found to host nitrification. Given the frequency and consistency with which nitrification occurs, and the known stability of sponge microbial communities, it is reasonable to extrapolate our findings to an extent, both temporally and geographically. That is, those species that exhibited nitrification during our study probably also host this process in other locales that they inhabit.

4.2. Chemical inhibition

The N-Serve experiments indicate that ammonium-oxidizers in sponges are inhibited by this chemical, as are other known ammonium oxidizers (Fig. 3). For all three species, there were no significant differences in DIN fluxes between controls and DMSO treatments (Table 2). Furthermore, DIN fluxes from *N. erecta* (which based on our evaluation does not host nitrification) were unaffected by the N-Serve treatment as well. These results indicate that exposure to the chemicals used in this experiment did not appear to affect sponge function or DIN flux. Therefore, the differences in fluxes among treatment types and species are likely caused by the effect of the treatment on the sponge-hosted microbial community, rather than by its effects on the sponge itself.

Overall, the results of the N-Serve inhibition experiment are consistent with the presence of microbially-mediated nitrification in sponges. It is possible that some of the nitrate produced in *A. cauliformis* or *S. aurea* might by removed by microbial associates, either via assimilation or conversion to N_2 or N_2O. Both these species are HMA sponges, but *A. cauliformis* hosts a dense layer of cyanobacteria in its outer tissues (Southwell, 2007), whereas *S. aurea* hosts only non-photosynthetic microbes (Fieseler et al., 2004; Southwell, 2007). The third species in the inhibition experiment, *N. erecta*, is nearly devoid of microbial cells (Weisz et al., 2007). However, the total DIN fluxes from DMSO-treated and N-Serve-treated sponges were not statistically different for any of the three species. Although the number of replicates in each treatment (and thus the statistical power) was limited by the availability of appropriate sponge individuals, these data suggest that that assimilatory and/or dissimilatory uptake of metabolically-produced NO_x^- by the internal microbial communities of the sponges is small relative to rates of NO_x^- release. Therefore, the inhibition data suggest that, if the microbial communities of *A. cauliformis* and *S. aurea* represent a sink for DIN, then ammonium is the preferred substrate rather than nitrate or nitrite. Because both denitrification and anammox require either nitrite or nitrate, any DIN removal by sponge microbes must be via ammonium assimilation and would therefore represent only temporary removal of nutrients from the water column.

4.3. ^{15}N tracer

The ^{15}N-labeled ammonium added to the incubations with nitrifying sponges was converted into ^{15}N-labeled nitrate within at least 8 h (Fig. 4), demonstrating that sponge-associated microbes oxidized ammonium from the ambient water as well as metabolically produced ammonium. The differences in the level of ^{15}N enrichment of nitrate produced by the three species were probably due to different rates of metabolic production of ammonium by the sponges that diluted the labeled tracer. *S. aurea* and *I. felix* are larger sponges with high respiration rates (C.S. Martens, M.W. Southwell, unpub. data) and therefore probably produced more ammonium, which diluted the tracer to a greater extent.

However, as the $\delta^{15}N$ value of NH_4^+ was not measured, this hypothesis cannot be directly tested.

The relatively low recovery of the added ^{15}N as nitrate for *A. cauliformis* (10.6–19.1%) and *I. felix* (8.4–13.2%) compared to *S. aurea* (28.9–43.6%) could be due to less efficient nitrification in these sponges, or assimilation of ammonium by the microbial community (Southwell, 2007). Previous work has shown that nitrate constitutes 78%, 87%, and 98% of the net DIN flux for *A. cauliformis, I. felix*, and *S. aurea*, respectively (Southwell et al. submitted). Therefore, although *S. aurea* exhibits more complete oxidation of ammonium compared to the other species, the magnitude of the difference is not sufficient to explain the 2-fold difference in ^{15}N recovery. All three sponges are HMA species, yet only *A. cauliformis* and *I. felix* host significant photosynthetic microbial communities (Southwell, 2007). The differences in $^{15}NO_3^-$ recovery may therefore be due to uptake of ^{15}N-labeled ammonium by these phototrophic cells. If ammonium substrate is a limiting resource for nitrifiers, such uptake by photosynthetic cells could reduce nitrification rates in the sponge by consuming part of the available ammonium substrate (Risgaard-Petersen et al., 2004). However, oxygen may also be a limiting factor, as many sponges have extremely hypoxic (Southwell, 2007) or anoxic tissues (Hoffmann et al., 2005). If oxygen limits nitrification rates, then oxygen generated by photosynthesis in the surface layer of the sponge could increase nitrification rates, as has been reported in some sediments (An and Joye, 2001, and references therein). Furthermore, because of the heterogeneous nature of sponge tissue, the potential limitation of nitrification by oxygen and/or ammonium may depend on the location of the nitrifying community within the sponge. Nevertheless, the reduced recovery of ^{15}N as nitrate from the two species with photosynthetic communities does suggest significant uptake by these cells.

4.4. Natural abundance $\delta^{15}N$

4.4.1. Incubation experiments

The natural abundance isotopic composition of nitrate produced by sponges during incubation experiments was influenced by the fraction of ammonium oxidized (Fig. 5). Although the known mechanism for oxidation of ammonium to nitrate is a two-step process, all isotopic selectivity should occur in the first step if no nitrite intermediate accumulates (Casciotti et al., 2003), therefore the data may be modeled as a single reaction. Both the residual ammonium reactant and the nitrate product were retained in the incubation vessel, so the reaction

might be expected to behave as a closed system and thus modeled as a Rayleigh distillation:

$$\delta_{nitrate} = \delta_{ammonium} - \varepsilon \frac{f(\ln f)}{1-f} \qquad (1)$$

where the fraction of ammonium substrate remaining at the end of the experiment (f)

$$f = \frac{[NH_4^+]}{[NO_x^- + NH_4^+]} \qquad (2)$$

and ε = the fractionation factor for the reaction (Mariotti et al., 1981). However, the ammonium substrate was produced continuously by the remineralization of organic matter, which violates the assumption of a closed system. One could therefore argue that the reaction has characteristics of an open system at steady state, (e.g. see Hayes, 2001), which can be modeled using Eq. (3):

$$\delta_{nitrate} = \delta_{org} + \varepsilon f \qquad (3)$$

However, this model describes a system in which the residual ammonium and the nitrate product do not accumulate over time, but are continually removed. This is not true of our closed-container incubations. In reality, the reaction probably represents an intermediate between these two situations, but we can constrain the result by considering both models. Applying the closed system Rayleigh model (Eq. (1)) produced a slope (ε) of approximately 11±2.6‰ standard error (S.E.) and a y-intercept of 3.7±1.0‰ (S.E.), with an r^2 of 0.51 (Fig. 5). The open system, steady state model (Eq. (3)) produced a slope of 14±3.5‰ (S.E.), an intercept of 2.5±0.8‰ (S.E.), and an r^2 value of 0.48 (data not shown). Therefore, whether modeled as a closed or steady state system, only approximately half of the variability in the isotopic composition of the nitrate produced by sponges during incubation experiments (with no ^{15}N addition) can be explained by N isotope fractionation during oxidation of ammonium. However, this experiment did not employ the pure cultures or tightly controlled environmental conditions typical of other experiments in which ε values were determined (Mariotti et al., 1981; Casciotti et al., 2003). Rather, the results are based on data from 4 different sponge species, which may contain different microbial communities and have different metabolic rates. Some of the unexplained variability in the regression may therefore be due to differences in microbial communities and to fluctuations in community metabolism.

Because of the variability in the data, the fractionation factors and y-intercepts produced by the two models are not statistically different. For the purposes of further discussion, however, we use the values produced by the closed system model because it more closely resembles our actual experimental conditions. The apparent fractionation factor produced by the closed system Rayleigh distillation model (11 ± 2.6‰) is lower than those previously reported for oxidation of ammonium (14–38‰, Casciotti et al., 2003). However, given the large uncertainty associated with the slope of the regression (95% confidence intervals are ± 5.1‰), the potential for intermediate behavior, and the non-ideal conditions of the incubation, this difference is not statistically significant. It is, however, in the lower range of values, which could indicate that the reaction may have been diffusion limited with respect to the ammonium substrate. As discussed above, ammonium limitation of nitrifiers could be exacerbated by assimilation of ammonium by photosynthetic cells (or other microbes). The y-intercept corresponds to a $\delta^{15}N$ value of 3.7 ± 1.0‰ (S.E.) for the starting pool of ammonium, which is a reasonable isotopic composition for ammonium derived from the catabolism of marine organic matter (Fogel and Cifuentes, 1993). It is also similar to the mean $\delta^{15}N$ value of tissue from eight sponge species sampled at Conch Reef (3.4 ± 1.2‰, Southwell, 2007).

4.4.2. $\delta^{15}NO_3^-$ of nitrate from sponges in situ

Because the incubation experiments were performed in a closed vessel, the $\delta^{15}N$ values of the nitrate probably did not reflect that which is produced in their natural environment. The $\delta^{15}N$ values of the nitrate in the sponge excurrent plumes (samples obtained *in situ* without physical contact with the sponge) averaged 1.3 ± 1.3‰ ($n = 11$), lower than those of nitrate in the ambient water. The mean $\delta^{15}N$ value of nitrate in the water column was 4.2 ± 0.8‰, similar to other N isotopic measurements of nitrate in deeper waters (>50 m depth) adjacent to this region (Leichter et al., 2007). Sponge excurrent water likely contains both nitrate produced by nitrification in the sponge and nitrate from the ambient water column that was transported passively through the animal. If no ambient nitrate is assimilated as it passes through the sponge tissue, then an isotopic mass balance can be calculated to subtract the contribution of ambient nitrate from the excurrent plume:

$$C_{sponge}\delta^{15}N_{sponge} + C_{ambient}\delta^{15}N_{ambient}$$
$$= C_{excurrent}\delta^{15}N_{excurrent} \qquad (4)$$

where C_i is the concentration of nitrate and $\delta^{15}N_i$ is the isotopic composition of nitrate. The assumption that am-

bient nitrate is not removed by sponge microbes is supported by the results of the N-Serve inhibition experiment, which indicate that no significant sink for metabolically-produced nitrate in the two nitrifying species tested (*A. cauliformis* and *S. aurea*) and by preliminary data from $^{15}NO_3^-$ tracer experiments with *A. cauliformis* (Southwell, 2007). The values for the concentration and isotopic composition of the ambient nitrate were based on samples taken on 3 different days during the study (0.8 μM$+0.1$, 4.2‰$+0.8$, $n = 3$). Uncertainties in measurements of nitrate concentration and isotopic composition were based on the standard deviation of triplicate samples (0.2 μM for [NO_3^-] and 0.3‰ for $\delta^{15}NO_3^-$). Results of this isotope mass balance calculation yield $\delta^{15}N$ values for pure sponge-produced nitrate ranging widely from -19.4 to 1.8‰, averaging $-5.1 + 6.4$‰ (Table 3). If a portion of the ambient nitrate is assimilated, then the true $\delta^{15}N$ value of sponge-produced nitrate would be an intermediate value between the measured $\delta^{15}N$ values of the excurrent plume and the calculated values for sponge-produced nitrate. Because of the small difference in the concentration of nitrate of excurrent water compared to ambient water in some sponges, the uncertainty associated with some of the calculated values is very large (Table 3). Despite this large uncertainty, these data suggest that sponge-produced nitrate generally has low $\delta^{15}N$ values, which would be consistent with the known partitioning of ^{15}N during ammonium oxidation. Previously measured fractionation factors are as large as 38‰ (Casciotti et al., 2003), and data from this study suggest that ammonium oxidation occurs in sponges with an effective fractionation factor of approximately 11‰. Therefore, catabolism of particulate organic matter followed by nitrification of a fraction of the resulting ammonium could reasonably produce $\delta^{15}N$ values in the range of the adjusted values (-19.4 to 1.8‰), depending on the fraction of ammonium that is oxidized. Furthermore, even without the adjustment for ambient nitrate (and its associated uncertainty), the lower $\delta^{15}N$ values of nitrate in sponge excurrent plumes demonstrate that sponge produced nitrate was depleted in ^{15}N compared to nitrate in the ambient water column.

The variability in $\delta^{15}N$ values of the *in situ* nitrate samples may be controlled primarily by the fraction of ammonium that is consumed by ammonium oxidizers (as in an open, steady state system, Eq. (3)). However, the isotopic composition of the nitrate could also be influenced by other microbially-mediated N processes such as DIN uptake. Significant nitrate uptake is not supported by the results of the N-Serve inhibition experiment; however, uptake of ammonium is likely, especially in species hosting autotrophic microbial

communities. Fractionation of ammonium during uptake in the sponge interior, where ammonium may be more concentrated, could thus increase the $\delta^{15}N$ value of the residual ammonium pool before nitrification occurs (Hoch et al., 1994). The internal pool of nitrate could become further enriched in ^{15}N if denitrification or anammox occurs in the low-oxygen zones of sponge tissue (Hoffmann et al., 2005). However, the highest $\delta^{15}N$ value for sponge nitrate measured was still lower than the ammonium substrate predicted by the closed-system Rayleigh distillation model from the incubation experiments (1.8‰ in *Xestospongia muta* versus 3.7‰ predicted for ammonium). Therefore, in the absence of additional data supporting denitrification or DIN uptake, the range of values measured for nitrate isotopic composition is most reasonably explained by the partitioning of isotopes during oxidation of ammonium.

4.5. Fate of sponge nitrate

The remineralization and oxidation of N by sponges likely represents a transformation of organic N compounds rather than inputs of "new" N. However, given the magnitude of the sponge nitrate flux, this recycling of nutrients may increase nitrate concentrations near the benthos, which may have implications for coral reef community structure. Certain sponge species have been shown to be a large benthic source of nitrate on Caribbean coral reefs (up to 12 mmol m^{-2} d^{-1} Corredor et al., 1988; Diaz and Ward, 1997). A recent study at our field site on Conch Reef showed that the combined nitrate flux from 14 sponge species was similarly high (13 mmol m^{-2} d^{-1} of NO_x^- Southwell, 2007). Based on species-specific nitrate release rates and biomass estimates, approximately 85% of the sponge nitrate flux on Conch Reef was produced by *X. muta*, a massive barrel sponge (Southwell, 2007). Yet, despite this large flux, the ambient nitrate in the water column over Conch Reef did not reflect the isotopic composition of sponge nitrate. The mean $\delta^{15}N$ value of nitrate produced by three *X. muta* individuals (adjusted for ambient nitrate) in this study was approximately 1‰, and all other species produced nitrate that was even more depleted in ^{15}N (Table 3), whereas nitrate in the ambient water had a mean $\delta^{15}N$ value of 4.2±0.8‰. There are two possible explanations for this difference: 1) the sponge nitrate was diluted by a flux of nitrate from an additional source with a high $\delta^{15}N$ value or 2) sponge nitrate was removed from the water column via processes that selectively use ^{14}N, leaving the remaining nitrate pool relatively enriched in ^{15}N.

Intrusion of nutrient-rich deep water is known to deliver a significant amount of nitrate to the Florida Keys reef tract (Leichter et al., 2003). Leichter et al. (2007) found that the $\delta^{15}NO_3^-$ value of sub-thermocline (>50 m depth) nitrate along the FL Keys reef tract was 5.26±0.05‰; therefore, if vertical mixing and/or upwelling was occurring during our sampling, this deep water nitrate could contribute ^{15}N-enriched nitrate to the ambient pool. Assuming a sponge $\delta^{15}NO_3^-$ value of 1‰, the nitrate flux from upwelling would have to be three times that of sponges in order to maintain ambient nitrate with a $\delta^{15}N$ value averaging 4.2‰. While this is theoretically possible, our ambient samples (obtained on three different days in winter at a depth of about 15 m) showed little variation in concentration or isotopic composition (0.84±0.1 µM, and 4.2±0.8‰). Furthermore, it is unlikely that deep water intrusions would reach this depth with such frequency during the winter months (Leichter and Miller, 1999). A dual isotope approach might better elucidate the potential contribution of deep water nitrate, as coupled $\delta^{15}N$ and $\delta^{18}O$ values can provide information on nitrate processing where multiple sources and processes are possible (Wankel et al., 2006). Unfortunately, *P. chlororaphis*, which were used to prepare samples for nitrate stable isotope analysis in this study, are not appropriate for $\delta^{18}O$ analysis (Casciotti et al., 2002). Comparisons of sponge nitrate with ambient nitrate from sites that are not exposed to deep water intrusions (e.g., back reefs) could also be useful in differentiating between mixing and assimilation effects.

Large contributions from deep water nitrate are unlikely given the season and depth of our site. Therefore, the more probable explanation for the higher $\delta^{15}NO_3^-$ value of ambient nitrate is that sponge-produced nitrate is actively utilized by primary producers, and that these processes enrich the remaining ambient nitrate pool in ^{15}N. If we assume that the system is in steady state and that other inputs of nitrate are negligible, then we may apply Eq. (3) to this situation in order to estimate the fraction of sponge-produced nitrate consumed:

$$\delta_{amb} = \delta_{sponge} + f\varepsilon_{assimilation} \qquad (5)$$

(Hayes, 2001). Here δ_{amb} is the $\delta^{15}N$ of nitrate in the ambient water, δ_{sponge} is the $\delta^{15}N$ value of sponge-produced nitrate, f is the fraction assimilated, and $\varepsilon_{assimilation}$ is the fractionation factor. Assuming a fractionation factor of 5‰ for nitrate assimilation (Fogel and Cifuentes, 1993) and a δ_{sponge} value equal to that measured for *X. muta* (1‰), the model estimates that 64% of sponge-produced nitrate is assimilated on the reef. Hayes (2001) showed that, by extension, the

δ^{15}N value of the assimilated nitrate can be calculated according to:

$$\delta_{\text{biomass}} = \delta_{\text{sponge}} - (1-f)\varepsilon_{\text{assmilation}} \qquad (6)$$

Using Eq. (6), the δ^{15}N of assimilated nitrate is approximately $-0.8‰$, which suggests that organisms using sponge-produced nitrate should have low δ^{15}N values. However, δ^{15}N values near zero may also be interpreted as evidence for N_2 fixation (Fogel and Cifuentes, 1993), possibly confounding our ability to separate these two N sources. In a separate study, the isotopic composition of suspended particulate organic matter from this region had a mean δ^{15}N of $2.2\pm0.4‰$ (standard deviation, $n=5$) (Southwell, 2007). However, it is difficult to interpret sources based on this value because the potential input from N_2 fixation may contribute to low δ^{15}N values, and also because the samples likely represent a mixture of autochthonous and allochthonous particles, including autotrophic and heterotrophic cells and detritus.

4.6. Benefits of nitrification for the host sponge

The frequency of occurrence in HMA species and the apparent dominance of species hosting nitrification on some reefs (Southwell, 2007) suggest that this association is stable and beneficial. The exact nature of the metabolic exchange or other potential benefits for the respective partners is not well understood, however, autotrophic partners such as nitrifying bacteria may provide an additional source of carbon for the sponge. The ratio of carbon fixation to nitrification by bacterial ammonium oxidizers and nitrite oxidizers is approximately 1 mole of CO_2 for every 9 mol of NH_4^+ oxidized to NO_3^- (Feliatra and Bianchi, 1993). Thus, a typical nitrification rate in sponges (~ 1 µmol g^{-1} h^{-1} (dry weight), Southwell, 2007) should allow the nitrifying community in sponges to fix up to 0.110 µmol CO_2 g^{-1} h^{-1}. However, differences in carbon fixation mechanisms of archaeal ammonium oxidizers (Hallam et al., 2006) could alter these ratios. Nevertheless, the projected carbon fixation rates are small relative to expected sponge respiration rates (Reiswig, 1974) and to rates of carbon fixation by photoautotrophs that populate the outer layer of tissue in many species (Wilkinson, 1983).

5. Conclusions

The results of this study indicate high rates of nitrification within HMA sponges, in agreement with previous work on a limited number of Caribbean species

(Corredor et al., 1988; Diaz and Ward, 1997). Chemical inhibition experiments confirm the occurrence of microbially-mediated nitrification, and isotope tracer experiments indicate that the reactant substrate is derived from a combination of metabolically-produced and ambient ammonium. The apparent fractionation factor expressed during incubation experiments and the sensitivity to N-Serve inhibitor are consistent with bacterially mediated nitrification. However, nitrification by crenarchaeota is a relatively recent discovery, and, to our knowledge, little is currently known about associated isotopic fractionation or sensitivity to chemical inhibition.

Factors that could affect the overall rates of sponge-hosted nitrification and the fate of the nitrate produced include the metabolic interactions within the microbial community, oxygen concentrations within the sponge tissue (Muller et al., 2004), and respiration rates. For example, it has been hypothesized that the production of nitrate and nitrite could facilitate anaerobic processes such as denitrification or anammox in the interior hypoxic zones of the sponge (Southwell, 2007; Taylor et al., 2007). The results of this study do not provide support for this hypothesis, but are consistent with the retention of ammonium by the microbial communities of some species. The factors governing nitrate fluxes from the sponge community may therefore be quite complex. However, it is clear from the results of this study that sponge abundance and community composition could strongly influence the concentration and speciation of DIN in the reef water column. Given the magnitude of the nitrate flux from the sponge community and its likely impact on reef primary productivity and health, it is important to examine this process in order to achieve a quantitative understanding of nutrient transformations in the coral reef environment.

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