LETTER

Hiding in plain sight: The secret contribution of the solitary ascidian *Herdmania grandis* to temperate reef nitrous oxide production

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**Scientific Significance Statement**

Solitary ascidians can dominate the benthic substrates of temperate reefs, yet very little is known about their role in the cycling of nitrogen, including whether they are important sources of nitrous oxide, a potent greenhouse gas. Understanding the role ascidians play in nitrogen cycling is important as their abundance could increase as temperate reefs respond to climate change. In this study, we quantified the contribution of the solitary ascidian *Herdmania grandis* (Heller) to nitrogen cycling on a temperate reef on the east coast of Australia, finding that they are a potentially significant contributor to coastal nitrous oxide fluxes.

**Abstract**

Large solitary ascidians, like *Herdmania grandis* (Heller), can dominate the benthic substrates of subtropical and temperate reefs; however, their influence on nitrogen cycling, particularly nitrous oxide (N$_2$O) production, is unknown. Here, we incubated individual *H. grandis* and compared fluxes of dissolved inorganic and gaseous nitrogen species to fluxes from reef sediments. Nitrous oxide production rates per individual ascidian ($21\pm8$ nmol ind h$^{-1}$) are the highest reported for any marine invertebrate. An individual ascidian produced more N$_2$O than 1 m$^2$ of inter-reef sediment ($1.7\pm1.7$ nmol m$^{-2}$ h$^{-1}$). Ascidian mediated N$_2$O production was found to occur under nutrient depleted conditions. The addition of $^{15}$N labeled organic material showed that the microbiota associated with *H. grandis* is capable of both nitrification and denitrification, but the contribution of these pathways to N$_2$O production could not be ascertained. As the ecology of temperate reefs change, any range expansion of *H. grandis* will increase coastal N$_2$O production.

Invertebrate dominated temperate reefs extend from the tropics to the poles and encompass diverse habitats that can include lichen and algal encrusted boulders, sponge and ascidian gardens, and kelp forests. In Australia, temperate reefs span ~8000 km of coastline and are collectively referred to as the Great Southern Reefs (GSR) (Bennett et al. 2016). Suspension-feeding organisms are numerous on the temperate reefs of the GSR, including many species of ascidians (Phylum: Chordata, Subphylum: Tunicata). In particular, the large suspension feeding ascidian *Herdmania grandis* is found along the entire GSR (Kott 2002), and on some reefs can completely dominate the benthic substrate (see Fig. 1A,B).

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**Data Availability Statement:** Data are available in the Dryad repository at https://doi.org/10.5061/dryad.3tx95x6fp.

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Given their dominance on temperate reefs, ascidians are likely to play an important role in local nutrient cycling (López-Legentil et al. 2015); however, few studies investigating their impact on nitrogen (N) cycling exist. Early observations demonstrated that ascidians excrete ammonium (NH$_4^+$) which can fuel algal productivity (Goodbody 1957), but very little is known about how ascidians influence other N cycling pathways, for example, the capacity of their microbiota (i.e. the microbial community living in and on the animal) to produce the greenhouse gas N$_2$O (Stief et al. 2009; Heisterkamp et al. 2010).

Our current understanding about benthic invertebrate organisms and N cycling is largely based on research involving bivalves (Stief et al. 2009; Welsh et al. 2015; McCarthy et al. 2019), sponges (Hoffmann et al. 2009; Fiore et al. 2013), and corals (Middelburg et al. 2015), with only a few studies targeting ascidians (Heisterkamp et al. 2010; Evans et al. 2017). This is surprising given the abundance of ascidians across temperate reef systems, and the fact that the range of large solitary ascidians appears to be expanding as oceans warm (Gewing et al. 2019).

Here, we conducted a pulse chase stable isotope labeling experiment to quantify ascidian mediated N cycling rates on a temperate reef in the GSR, specifically addressing the role of ascidians in mobilizing organic N and their contribution to N$_2$O production. As a comparison, sediment N cycling rates were also measured.

**Materials and methods**

**Study site**

Specimens of *H. grandis* were collected from Surgeons Reef, an oligotrophic coastal temperate reef (Northwest Solitary Island) in the Solitary Islands Marine Park, NSW, Australia (30.0178°S, 153.2714°E), in April 2019 (Fig. 1). This reef is
dominated by the solitary ascidian *H. grandis* with coverage ranging from 0 to over 200 individuals per m² of reef area. Sandy sediments (10 cm² by 5 cm depth) were collected with a perspex corer from the inter-reef sediments at the same site and pooled in a shallow crate.

Ascidians and sediments were transported back to the National Marine Science Centre (Coffs Harbour, NSW) for the experiments. Animals were gently brushed to remove large external epiphytes from the tunic before being placed in the holding tanks. The ascidians and sediments were preincubated for 7 d in 1000 litter flow-through tanks (3 L min⁻¹ of 20 μm filtered seawater) at the same temperature (23°C) as the collection site and under a 12 : 12 light : dark cycle. Seawater used for the preincubation and experiment was of very similar quality to the ambient seawater at the collection site. During the preincubation, the ascidians were fed 1 liter of cultured algae (*Proteomonas sulcata*) once on the fourth day of the 7-d preincubation period to ensure they remained healthy for the trial.

**Experimental design**

The ascidians and sediments were incubated in sealed chambers with ¹⁵N enriched particulate (40.1 μmol L⁻¹ ¹⁵N-PON—freeze-dried algal product, Cambridge Isotope Laboratories, lot no. CNLM-455-1), or dissolved organic N (108.2 μmol L⁻¹ ¹⁵N-DON—amino acid mixture, Cambridge Isotope Laboratories, lot no. NLM-2161-1). Nine 8 liter circular perspex chambers were filled with filtered seawater at in situ temperature. One ascidian individual was placed in each chamber. Three chambers received ¹⁵N-PON, three received ¹⁵N-DON, and three received no amendment (controls). Sediments had the same treatments, but in smaller chambers (0.25 L). An additional incubation in which ¹⁵N-PON and ¹⁵N-DON were incubated in seawater only was carried out to quantify leaching of NO₃⁻ and NH₄⁺ from the tracer itself. Chambers were incubated under fluorescent lights for 6 h and the experiment was repeated in the dark with new animals.

**Sample collection and analysis**

Dissolved oxygen (DO) was measured every hour in the chambers with a PreSens optode. Water samples for concentration and ¹⁵N determination in NH₄⁺ and NO₃⁻ were collected with a plastic syringe (2 × 60 mL) from taps in the chamber lid at 0, 3, and 6 h. For the ¹⁵N₂ samples, water samples were collected and added to duplicate 12 mL exetainers without a headspace and immediately injected with mercuric chloride solution (20 μL ~ 8% w/v). Samples for N₂O concentration were collected at each time point with a gas tight syringe (3 × 6 mL) from each chamber and added into 12 mL exetainers that had been previously flushed with helium (He). During sampling chamber water was replaced with seawater through a second tap in the chamber lid.

Following the incubations, the ascidians and sediments were flushed with filtered seawater (0.5 h) and then frozen at −20°C. Specimens were defrosted, weighed, and dissected into the tunic (external and internal surfaces) and internal organs (basket, stomach, and gonads combined), freeze-dried, cut into fine pieces, and weighed (0.6–0.8 mg) into tin capsules for bulk δ¹⁵N analysis. Sediments were dried in an oven (60°C), ground into a fine powder and weighed (100 mg) into tin capsules for bulk δ¹⁵N analysis. The %N and δ¹⁵N of the ascidian compartments were measured via elemental analysis (Thermo Finnigan Flash EA 112, coupled to a Thermo Delta V Plus IRMS via Thermo Conflo III) (precision ± 1% and 0.15‰ respectively).

Concentrations of NH₄⁺ and NO₃⁻ (i.e., NO₂⁻ + NO₃⁻) were analyzed via a Lachat QuickChem 8000 Flow Injection Analyzer. Headspace N₂O concentrations were determined by gas chromatography combined with a micro-electron capture detector (Sturm et al. 2015) (Agilent 7890A gas chromatograph [GC] fitted with a Gerstel multipurpose autosampler). The net nutrient fluxes and N₂O fluxes were determined as:

\[
\text{Net flux} = \frac{(\text{conc}_f - \text{conc}_i) \cdot V}{A \cdot t}
\]

where the initial concentration in each incubation vessel (μmol L⁻¹) is concᵢ, the final nutrient concentration is concᵢ (μmol L⁻¹), the incubation volume (L) is V, A is the dry weight of the ascidian or the sediment surface area (m²), and t is time (hours). The net flux of nutrient from the ¹⁵N-PON and ¹⁵N-DON amendments in water only (i.e., no ascidians) was subtracted off the rates for the ¹⁵N-PON and ¹⁵N-DON treatments to account for water column processing and/or leaching of nutrients from the isotopic label. The sediment rates are given in μmol m⁻² h⁻¹, and the rates in the ascidian treatments are in units of μmol g⁻¹ h⁻¹ (rates for N₂O are in nmol). To aid comparison between the ascidian and sediment rates, we converted the ascidian rate to an areal rate. To do this, the rate measured in each chamber was multiplied by the total average dry weight of all the ascidians used in the experiment to give units of nmol animal⁻¹ h⁻¹. The abundance of ascidians on Surgeons Reef (Northwest Solitary Island) can vary from 1 to 200 per m², so a conservative estimate of 10 m⁻² was used. The μmol animal⁻¹ h⁻¹ was multiplied by 10 to give units of μmol m⁻² h⁻¹. Rates were averaged over the light and dark incubations.

For ¹⁵N-N₂₂, 2 mL of pure He was added to the exetainer vials containing the sample, 10 μL of this was subsequently analyzed for ²⁹N₂ and ³⁰N₂ on a Thermo Trace GC Ultra with a 25 m × 0.32 mm PoraPLOT Q column interfaced to a Thermo Delta V Plus IRMS (precision ± 0.15‰). The instrument precision for ³⁰N₂ was poor due to interference from NO in the IRMS ion source (even following correction with standard gases of known isotopic composition). As such, the rate of total ¹⁵N-N₂ production was calculated via the production of ²⁸N₂ only (Lewicka-Szczebak et al. 2013). The labeling of the N pool for denitrification, which is required for the calculation, was directly determined by measuring the ratio of ¹⁵N/¹⁴N in NO₃⁻ in chamber water at the end of the trial. The production of
Fig. 2. Areal fluxes of dissolved oxygen (A, B), NH₄⁺ (C, D), NO₃⁻ (E, F) (all μmol m⁻² h⁻¹), and N₂O (G, H) (nmol m⁻² h⁻¹) in the ascidian (A, C, E, G) and sediment (B, D, F, H) incubations with different amendments (control, ¹⁵N-PON, ¹⁵N-DON). For the ascidians, the areal flux is based on a density of 10 individuals per m² (this density is used to provide a useful areal comparison but may differ between reefs and therefore requires further validation). Note the differences in the scale for the dissolved oxygen and N₂O comparisons. Values are mean ± SD.

Table 1. Areal rates of denitrification and nitrification (in units of μmol m⁻² h⁻¹) in the ascidian and sediments receiving ¹⁵N-DON and ¹⁵N-PON. For the ascidians, the areal flux is based on a density of 10 individuals per m². Errors are standard deviation.

|        | Ascidians |          | Sediment |          |
|--------|-----------|-----------|----------|----------|
|        | Nitrification | Denitriication | Nitrification | Denitriication |
| ¹⁵N-DON| 86.1±24.4  | 0.39±0.15  | 4.2±0.5   | 0.55±0.21 |
| ¹⁵N-PON| 8.5±0.7    | 0.11±0.04  | 0.2±0.1   | 0.25±0.17 |
Table 2. Percent of recovered $^{15}$N tracer found in the ascidian or sediment, and water column pools. Errors are standard deviation.

| Ascidian | Tunic | Organs | NO$_3^-$ | NH$_4^+$ | N$_2$ |
|----------|-------|--------|----------|----------|-------|
| $^{15}$N-DON | 22.7±4.8 | 3.6±1.3 | 7.8±4 | 65.4±22.8 | 0.6±0.3 |
| $^{15}$N-PON | 3.9±2.3 | 4.2±1.5 | 2.3±1.9 | 88.9±41.5 | 0.3±0.2 |

| Sediment | Sediment organics | NO$_3^-$ | NH$_4^+$ | N$_2$ |
|----------|------------------|----------|----------|-------|
| $^{15}$N-DON | 30.4±4.8 | 1.9±3.1 | 1.3±1.7 | 0.3±0.1 |
| $^{15}$N-PON | 97.6±22 | 67.4±28.1 | 0.1±0.1 |

$^{14}$N-N$_2$ was calculated by dividing the $^{15}$N-N$_2$ production by the ratio of $^{15}$N/$^{14}$N in NO$_3^-$, and total N$_2$ production was the sum of $^{15}$N-N$_2$ and $^{14}$N-N$_2$ production. This approach will over-estimate denitrification rates if anammox is active.

The $\delta^{15}$N of NO$_3^-$ was determined by the denitrifier protocol (Sigman et al. 2001), and the $\delta^{15}$N of NH$_4^+$ was determined using the hypobromite/azide protocol (Zhang et al. 2007). The net rate of nitrification was calculated by dividing the production rate of $^{15}$N-NO$_3^-$ by the $^{15}$N/$^{14}$N of the NH$_4^+$ pool. This is net nitrification as it does not account for any NO$_3^-$ uptake following nitrification.

For the isotope mass balance, the $^{15}$N content of each ascidian pool or sediment sample was calculated by multiplying the $^{15}$N abundance (corrected for natural abundance) by the amount of N present. The $\delta^{15}$N was converted to Atom % for this calculation. Similarly, the $^{15}$N abundance of NO$_3^-$ and NH$_4^+$ was multiplied by their change in concentration over the incubation period to give a mass of $^{15}$N in each pool. Statistical comparisons (ANOVA and t-tests) were made with SPSS Statistics 25 after testing for normality and equality of variances.

All data are available online (Erler and Kelly 2020).

Table 3. Marine organism N$_2$O fluxes normalized per g of dry weight and per individual (mean ± SD).

| Species | Location (ref) | N$_2$O flux (nmol g h$^{-1}$) | N$_2$O flux (nmol ind h$^{-1}$) |
|---------|----------------|-------------------------------|--------------------------------|
| Ascidian* – Herdmania grandis | Solitary Islands (this study) | 0.9±0.4 | 21±8 |
| Blue mussel† – Mytilus edulis | Ârhus Harbor (Stief et al. 2009) | 12.2±5.3 | 0.7±0.2 |
| Ascidian† – Ascidia sp. | Ærhus Bay (Heisterkamp et al. 2010) | 0.04±0.024 | 0.3 |
| Green Crab† – Carcinus maenas | Ærhus Bay (Heisterkamp et al. 2010) | 0.369±0.137 | 1.1 |
| Amphipod† – Corophium volutator | Wadden Sea (Heisterkamp et al. 2010) | 0.955±0.664 | 0.01 |
| Bivalve mollusk† – Scrobicularia plana | Wadden Sea (Heisterkamp et al. 2010) | 0.302±0.083 | 1.39 |
| Mud snail† – Hydrobia ulvae | Wadden Sea (Heisterkamp et al. 2010) | 5.449±1.822 | 0.01 |
| Polychaete worm† – Nereis diversicolor | Wadden Sea (Heisterkamp et al. 2010) | 0.398±0.319 | 0.05 |
| White Shrimp† – Litopenaeus vannamei | Aquacultured (Heisterkamp et al. 2010) | 0.18±0.06 | 3.95 |
| Blue mussel – Mytilus edulis | Rhode Island (Middelburg et al. 2015) | 11.5±2.9 | 1.9±0.4 |
| Manila clam – Ruditapes philippinarum | Sacca di Goro lagoon (Welsh et al. 2015) | 11.5±2.9 | 1.9±0.4 |
| White Shrimp – Litopenaeus vannamei | Aquacultured (Heisterkamp et al. 2016) | 0.20±0.07 | 4.3±1.5 |
| Oyster – Crassostrea glomerate | Walls Lake (Erler et al. 2017) | 0.39±0.16 | 1.40±0.57 |
| Oyster – Crassostrea virginica | Duxbury Bay (Carthy et al. 2019) | 0.16±0.06 | 0.36±0.13 |
| Blue mussel – Mytilus edulis | Rhode Island (Garate et al. 2019) | 0.6±0.2 | 5±1 |
| Blue mussel – Mytilus mercenaria | Rhode Island (Garate et al. 2019) | 0.04±0.06 | 2±1 |
| Oyster‡ – Crassostrea virginica | Rhode Island (Garate et al. 2019) | −0.04±0.06 | −5.5±1.5 |

*From the control incubation in this study.
†The rate per individual is based on wet weight which means the rate based on dry weight would be lower.
‡Values are for the short-term control incubation in this reference.
§Denotes the rate normalized to wet weight of the ascidians.
Results

The average dry weight of the animals was 23.8 ± 8.1 g and there was no significant difference (p > 0.05) between the weights of animals used in the difference treatments of the controls. Day/night differences in fluxes were insignificant (p > 0.05) for most parameters with the exception of N2O. As such, we combined the day/night treatments so that there were six rather than three replicates. The ascidians consumed significantly more DO (p < 0.05, n = 18, df = 1, F = 162) than the sediments, but the amendment type (i.e., control, 15N-PON and 15N-DON) (Fig. 2) did not influence DO flux. Ammonium flux was similar between sediments and ascidians. For the ascidians, the flux of NH4+ was significantly higher when 15N-PON and 15N-DON were present relative to the control (p < 0.05, n = 6, df = 2, F = 13.4). Sediment NH4+ flux in the 15N-DON amended sediments was also positive and significantly higher (p < 0.05, n = 6, df = 2, F = 19.8) than the control or 15N-PON amended sediments (Fig. 2C, D).

Production of NO3− was significantly higher when ascidians were present relative to the sediments (p < 0.05, n = 18, df = 1, F = 26.5). Nitrate fluxes were positive in the ascidian treatments, but unlike NH4+ did not differ between the amendments or control. The areal production of N2O was significantly higher in the ascidian incubations compared to the sediments (p < 0.05, n = 18, df = 1, F = 73.3) (Fig. 2G, H). In the ascidian incubations, the presence of 15N-PON and 15N-DON did not significantly increase N2O production (p > 0.05) relative to the control (Fig. 2G). In the ascidian control incubation, that is, without organic N addition, the average daily flux of N2O was 0.9 ± 0.4 nmol g−1 h−1 or 21 ± 8 nmol ind−1 h−1. At 10 individuals per m², this equates to 209 ± 77 nmol m−2 h−1. For the sediments, the average daily flux of N2O was 1.7 ± 1.7 nmol m⁻² h⁻¹. The flux of N2O was significantly higher in the light than in the dark (p < 0.05, n = 9, df = 1, F = 15.5) (data not shown).

Labeled 2H2O was detected in both the ascidian and sediment incubations receiving 15N amendments, indicative of denitrification. We cannot rule out that ammonia was active, in which case the calculated rate of denitrification would be overestimated. The rates of total N2 production were low and statistically similar between ascidian and sediment treatments (Table 1). In the treatments receiving 15N-DON and 15N-PON, both 15N-NH4+ and 15N-NO3− were detected, indicating that mineralization and nitrification, respectively, were occurring. The rate of nitrification was significantly higher when DON was added (Table 1).

Combining all the different N pools, the recovery of added 15N was 27% and 37% in the ascidian treatments receiving 15N-DON and 15N-PON, respectively. In the treatment receiving 15N-DON, most of the recovered 15N was in the NH4+ pool (65% of recovered 15N), followed by the tunic (23%) (Table 2). Similarly, most of the recovered 15N was in the NH4+ pool of the 15N-PON treatment (89%). In both the 15N-DON and 15N-PON treatments, 15N-NO3− was detected indicating the presence of active nitrification. When 15N-PON was added to sediment incubations the largest proportion of 15N was found in the sediments, reflecting the recovery of the added particulate material. The recovery of 15N in NO3− was < 2% of the added label in either of the sediment treatments (Table 1) indicating low rates of nitrification. Isotopically labeled 15N-NH4+ was only detected in the sediments receiving 15N-DON. Overall, 27% and 78% of the added 15N was recovered in the 15N-DON and 15N-PON sediment treatments.

Discussion

In this study, we have identified that the Ascidian H. grandis is capable of producing significant quantities of N2O. There are two main sites for N2O production in marine invertebrates; these are on the organism’s surfaces and within their stomach (Stief et al. 2009; Heisterkamp et al. 2013). Nitrification and denitrification can occur in either location (Svenningsen et al. 2012) although denitrification is more likely to occur in the anoxic stomach. Another possible pathway is nitrifier denitrification, that is, the reduction of nitrite (NO3−) to N2O carried out by ammonium oxidizing bacteria under oxygen limitation (Poth and Focht 1985; Wrage et al. 2001). Denitrification was confirmed in the ascidian treatments receiving 15N by the production of 15N-N2 (Table 1), but rates were much lower than reported for other benthic invertebrates (Smyth et al. 2013; Welsh et al. 2015; Erler et al. 2017). Consequently the specific N2O yield (i.e., N2O/(N2O + N2) was large (44% and 21%) in the 15N-PON and 15N-DON amendments, respectively) compared to other studies (Heisterkamp et al. 2013; Welsh et al. 2015; Erler et al. 2017). The specific N2O yield for the sediment was < 1% for either amendment.

A high specific N2O yield suggests that denitrification is inefficient, or that nitrification and/or nitrifier denitrification are the dominant N2O production pathways. Nitrification (i.e., the production of 15N-NO3−) was confirmed when 15N-DON and 15N-PON were added to the ascidians. In fact, 15N-NO3− accounted for almost 8% of the recovered 15N in the 15N-DON treatment and was thus orders of magnitude higher than denitrification, indicating that it is more likely to be a pathway of N2O production than denitrification. We also cannot rule out nitrifier denitrification as a contributor to N2O, as it is carried out by the same microbes performing NH4+ oxidation.

We have found that H. grandis is a significant source of N2O relative to reef sediments. The average rate of N2O production in the ascidian control without organic N addition, on a dry weight basis, was 0.92 ± 0.34 nmol g h−1. This N2O production rate is within the range of other invertebrates measured in the literature (Table 3); however, when we factor in their large size, the N2O production per individual H. grandis is the highest recorded for any marine invertebrate to date. The average areal N2O flux for the ascidians in the control treatment was 209 ± 77 nmol m⁻² h⁻¹, two orders of magnitude higher than the next closest species.
magnitude higher than the sediment N₂O flux (based on 10 animals per m²). Extrapolation of the chamber incubation results to areal fluxes is problematic as we do not know the distribution of animals on different section of the reef or on other reefs. However, an important point is that based on the experimental incubations, a single ascidian of average weight produces more N₂O than 1 m² of inter-reef sediment. Therefore, the presence of ascidians is likely to have a major influence on ecosystem N₂O dynamics.

Importantly, N₂O production was active in the ascidian controls (i.e. not receiving added nitrogen). The similarity in N₂O production between the control animals and those receiving organic N may simply be a function of the short experimental duration. Other studies have clearly demonstrated that increased N availability stimulates N₂O production, but the response can take days rather than hours (Garate et al. 2019). All ascidians were acclimated in oligotrophic flow-through seawater and were only fed once during the 7-d preincubation period. Therefore, the ascidians in the control were producing N₂O with N assimilated during the preincubation period. In other words, H. grandis can produce appreciable quantities of N₂O under relatively nutrient limited conditions.

The ability of the ascidians in the control treatment to produce N₂O may be partly related to their ability to assimilate and process dissolved organic N, which was clearly demonstrated in the ¹⁵N-DON amendment where ¹⁵N was recovered in NH₄⁺, NO₃⁻, and on the tunic (Table 2). The ability of ascidians to produce N₂O under nutrient limited conditions is also important from an ecological perspective. The rate of N₂O production by H. grandis (0.21 μmol m⁻² h⁻¹) is not that much less than the average N₂O flux from subtropical estuarine sediments (0.59 μmol m⁻² h⁻¹) (Murray et al. 2015). Note that this comparison does not account for the three-dimensional structure of reefs, so a m² of reef viewed from above could actually contain many m² of effective surface area of ascidians. The implication is that temperate reefs on Australia’s continental shelf are potentially as important to N₂O production as the estuaries; however, we do not know how much area temperate reefs cover, or the densities of H. grandis on these reefs.

Another consideration in the comparison between estuarine and ascidian mediated N₂O production is that the average rate derived from Murray et al. (2015) includes estuarine systems both with and without inorganic N inputs, whereas the comparable rate measured in the H. grandis incubations occurred without additional N supply. Assuming that both estuarine sediments and ascidian dominated reefs increase N₂O production if N is supplied, we would expect the ascidian system to generate more N₂O than sediment dominated systems (as shown in our comparison between reef sediments and ascidians). If N₂O produced by benthic organisms is not consumed by denitrification in sediments then it will eventually be released to the atmosphere. The sediment incubations showed production of N₂O and therefore are not going to consume the N₂O produced by the ascidians. Our data suggest that current coastal estimates of N₂O production will be underestimated if temperate reef habitat is not included.

This experiment was not carried out for long enough to see the added organic material significantly increase N₂O production. However, NH₄⁺ production and nitrification were observed following organic amendment addition, implying that given enough time, increased organic N availability will increase N₂O production. Any increases in N availability on temperate reefs (inorganic or organic) are therefore likely to cause further increases in N₂O production. Further work is needed to quantify the abundance of H. grandis on temperate reefs, determine if its range is expanding as a result of ocean warming, quantify how temperature and food availability will affect N₂O fluxes, and better understand the processes that are responsible for N₂O production.

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