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High plasticity of nitrogen fixation and denitrification of common coral reef substrates in response to nitrate availability

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ABSTRACT

Nitrogen cycling in coral reefs may be affected by nutrient availability, but knowledge about concentration-dependent thresholds that modulate dinitrogen fixation and denitrification is missing. We determined the effects of different nitrate concentrations (ambient, 1, 5, 10 μM nitrate addition) on both processes under two light scenarios (i.e., light and dark) using a combined acetylene assay for two common benthic reef substrates, i.e., turf algae and coral rubble. For both substrates, dinitrogen fixation rates peaked at 5 μM nitrate addition in light, whereas denitrification was highest at 10 μM nitrate addition in the dark. At 10 μm nitrate addition in the dark, a near-complete collapse of dinitrogen fixation concurrent with a 76-fold increase in denitrification observed for coral rubble, suggesting potential threshold responses linked to the nutritional state of the community. We conclude that dynamic nitrogen cycling activity may help stabilise nitrogen availability in microbial communities associated with coral reef substrates.

1. Introduction

In the Anthropocene, almost all marine ecosystems worldwide have experienced a range of threats that are relatable to human activities (Halpern et al., 2007), and those anthropogenic impacts are increasing (Halpern et al., 2015). Consequences of anthropogenic stressors are well-documented, ranging from ecosystem to individual and cellular levels. Eutrophication, as a direct consequence of increasing coastal populations (Burke et al., 2011), is among the most impactful local stressors. This is particularly true for ecosystems adapted to oligotrophic, i.e., nutrient-poor environmental conditions, such as tropical coral reefs, where nitrogen (N) is a key factor limiting primary production (Webb et al., 1975; Vitousek and Howarth, 1991; Lesser et al., 2007).

The acquisition, retention and disposal of N are of particular importance in coral reefs, which are partly moderated by microbial N cycling (Rädecker et al., 2015). More specifically, the interplay between two counteracting N cycling pathways, i.e., dinitrogen (N_2) fixation and denitrification contribute to maintaining a stable N availability, and ultimately, ecosystem functioning. N_2 fixation is the conversion of atmospheric N_2 into bioavailable ammonium by prokaryotic microbes (diazotrophs) serving as a potential alternative nutrient source in times of N scarcity that fluctuates seasonally and in response to varying environmental conditions (Cardini et al., 2016a). In contrast, the microbially performed conversion of nitrate to atmospheric N_2, referred to as denitrification, effectively removes excess N from the reef system. The relief of N could be vital to coral reef functioning during excess N availability, particularly under eutrophic conditions (Rädecker et al., 2015). For example, the main framework builders, scleractinian corals, rely on low N levels, as elevated N concentrations can destabilise the host-symbiont symbiosis (Wiedenmann et al., 2012; Morris et al., 2019), which in combination with limited phosphate availability results in an increased coral bleaching susceptibility (Wiedenmann et al., 2012).

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Eutrophication divergently affects N fluxes in coral reef environments at the community (Koop et al., 2001, Roth et al., 2021) and organism level (El-Khaled et al., 2020a; Karcher et al., 2020). For example, Koop et al. (2001) reported consistently lower N_2 fixation activities and concurrent higher denitrification rates in reef patches under elevated nutrient availability (up to 20 μM N), Roth et al. (2021) reported a shift in small, distinct coral reef communities from a sink to a source of N due to nutrient pollution. A recent study, further] demonstrated that moderately (1–2 μM N) elevated nutrient concentrations may stimulate both N_2 fixation and denitrification in turf algae, reef sediments and hard corals (El-Khaled et al., 2020a), suggesting ambivalent responses of N fluxes to nutrient pollution. Consequentially, certain nutrient concentration thresholds that regulate the suppression or stimulation of both N cycling pathways might exist but have not been determined in coral reef environments yet. Additionally, all aforementioned studies have performed their experimental eutrophication experiments on a relatively large time-scale ranging from months (El-Khaled et al., 2020a, Roth et al., 2021) to years (Koop et al., 2001). Due to these timescales, these studies do not allow us to disentangle the indirect consequences of potential community shifts from the direct consequences of altered N availability on the N cycling pathways themselves. Studies investigating responses on a shorter time scale in coral reefs are, however, non-existent. Rapid responses of both N cycling pathways to runoff events (and a subsequent increase in inorganic N) were previously observed in intertidal mudflats covered by microbial mats (Joye and Paerl, 1993). Hence, we hypothesised decreasing N_2 fixation and increasing denitrification rates with increasing nitrate availability.

For this purpose, we investigated the plasticity of coral reef-associated N_2 fixation and denitrification in response to inorganic N availability of two coral reef substrates (i.e., heterogeneous algal assemblages and coral rubble) that commonly increase in their abundance when coral-algae phase shifts occur (Holmes et al., 2000; McManus and Polsenberg, 2004; Norström et al., 2009) and, thus, may have an impact on 'post phase shift' reef-wide N budgets (El-Khaled et al. accepted). The selection and investigation of both substrates will, thus, contribute to the understanding of rapid responses of contemporaneous N_2 fixation and denitrification to eutrophication of post phase shift coral reefs. We aimed i) to quantify the relative activity of N_2 fixation and denitrification in the two coral reef substrates, ii) to assess the direct responses of both N fluxes quantified for both substrates to nutrient enrichment, and iii) to identify potential thresholds that mediate both N cycling pathways. For this purpose, we carried out acetylene-based short-term incubations, which allowed us to quantify the effect of four different nitrate concentrations on N_2 fixation and denitrification simultaneously.

2. Material and methods

2.1. Study site, sampling and experimental design

The experiment was conducted at the laboratory facilities of the King Abdullah University of Science and Technology (KAUST), Saudi Arabia, in May 2018. Sampling of substrates took place at a semi-exposed area of the Abu Shosha reef in the Jeddah Region (22° 18′ 15" N, 39° 02′ 56″ E) on the west coast of Saudi Arabia in the central Red Sea. Turf algae and coral rubble as predominant coral reef substrates were chosen a) for their dominant spatial benthic distribution (26.2 to 47.5% and 3.3 to 12.1%, resp., benthic cover; Roth et al. (2018)), particularly when phase shifts occur (Roth et al., 2018), and b) their evidently active role as important N fixers and denitrifiers (Davey et al., 2008; Rix et al., 2015; El-Khaled et al., 2020b). 'Turf algae' were defined as dead coral substrates of approx. 10 cm length that were overgrown (1–2 cm in height) with heterogeneous assemblages of different filamentous algae and cyanobacteria (see Fig. S1). 'Coral rubble' was defined as dislodged parts of dead coral skeletons of approx. 5–10 cm length that were partly covered with crustose coralline algae and microbial biofilms (see Fig. S2; Rasser and Riegl, 2002). Both substrates were sampled for the present study to allow for comparisons between fragile filamentous algal assemblages and cyanobacterial structures and coral rubble fragments without turf algal cover. A total of 40 turf algae fragments and 32 coral rubble fragments were sampled from 5 m water depth using hammer and chisel. All samples were stored in recirculation aquaria equipped with ambient reef water on the research vessel. Aquaria were kept at ambient temperature and light levels. Experimental incubations (see below) started < 3 h after sampling.

2.2. Nitrogen fluxes

Four different N concentrations were used for the present study: ambient concentration typical for the central Red Sea (0.1–0.3 μM nitrate) as measured previously (Roth et al., 2018), 1 and 5 μM nitrate addition covering ranges of natural (Cardini et al., 2016a) as well as anthropogenic (Loya et al., 2004) nutrient inputs to local waters and as previously utilised in nutrient-enrichment studies (Wiedenmann et al., 2013; El-Khaled et al., 2020a), and 10 μM nitrate addition being well above observed scenarios in the Red Sea. Nitrate enrichment consisted of previously prepared nitrate stock solution, prepared with MilliQ water and sodium nitrate (≥ 99.0%, Sigma-Aldrich). Nitrate was added immediately prior to incubations (< 5 min). To determine N_2 fixation and denitrification rates, a combined blockage/reduction acetylene assay (COBRA) was performed as previously described in (El-Khaled et al., 2020b). Briefly, all COBRA incubations were conducted in 1 L gastight glass chambers, each filled with 800 mL of either ambient or nutrient-amended seawater. Acetylene gas was then added to both incubation water and remaining headspace at a concentration of 10%. Acetylene in the gastight chambers leads to the preferential reduction of acetylene to ethylene (C_2H_4), instead of N_2 to ammonia by the key enzyme nitrogenase (Balderston et al., 1976). Further, acetylene blocks the nitrous oxide (N_2O) activity in the denitrification pathway leading to an accumulation of N_2O (Yoshinari and Knowles, 1976). Replicate samples were incubated and two additional chambers per respective nutrient concentration and light condition without specimens served as controls to correct for planktonic background activity (Fig. 1). All incubations lasted 12 h, with dark and light incubations being performed separately with differing specimens (Fig. 1). Light was set at a photon flux of ~200 μM quanta m^{-2} s^{-1}, representing the daytime average photon flux of the studied reef and water depth at this time of the year. All incubation chambers were submerged in a temperature-controlled water bath at 27 °C, resembling the ambient water temperature measured at the reef in 5 m water depth during sampling. Incubation chambers were constantly stirred (500 rpm) to ensure sufficient exchange between the water body and headspace. Gas samples were taken at the start (t_0), after 4 h (t_4), and after 12 h (t_{12}, i.e., at the end of the incubation), to consider potential initial lag phases (El-Khaled et al., 2020b) and to account for incomplete inhibition of the denitrification pathway (Yu et al., 2010; El-Khaled et al., 2020b). Both target gases (i.e., C_2H_4 and N_2O) were quantified using gas chromatography (Agilent 7890B GC system, Agilent Technologies) and helium pulsed discharge detection with an HP-Plot/Q 19091P-QO4 column (30 m length, I. D. 0.320 mm, film 20.0 μM; Agilent J&W GC Columns, Agilent Technologies). The lower detection limit for both target gases was 0.3 ppm. Gas concentrations were normalised to incubation time and surface area. The latter was calculated using cloud-based 3D models (Lavy et al., 2015; Gutierrez-Heredia et al., 2016) for all specimens (Autodesk Remake v19.1.1.2). We refrained from converting C_2H_4 into actual N_2 fixation rates as we acknowledge the ongoing discussion regarding an appropriate conversion factor (Hardy et al., 1968; Wilson et al., 2012). We used sampling point t_4 and t_{12} as a basis for N_2 fixation rate calculation, therefore omitting an initial lag phase; and t_0 and t_4 for denitrification to avoid an underestimation due to a potential incomplete inhibition of the denitrification pathway (see El-Khaled et al. (2020b) and references therein).

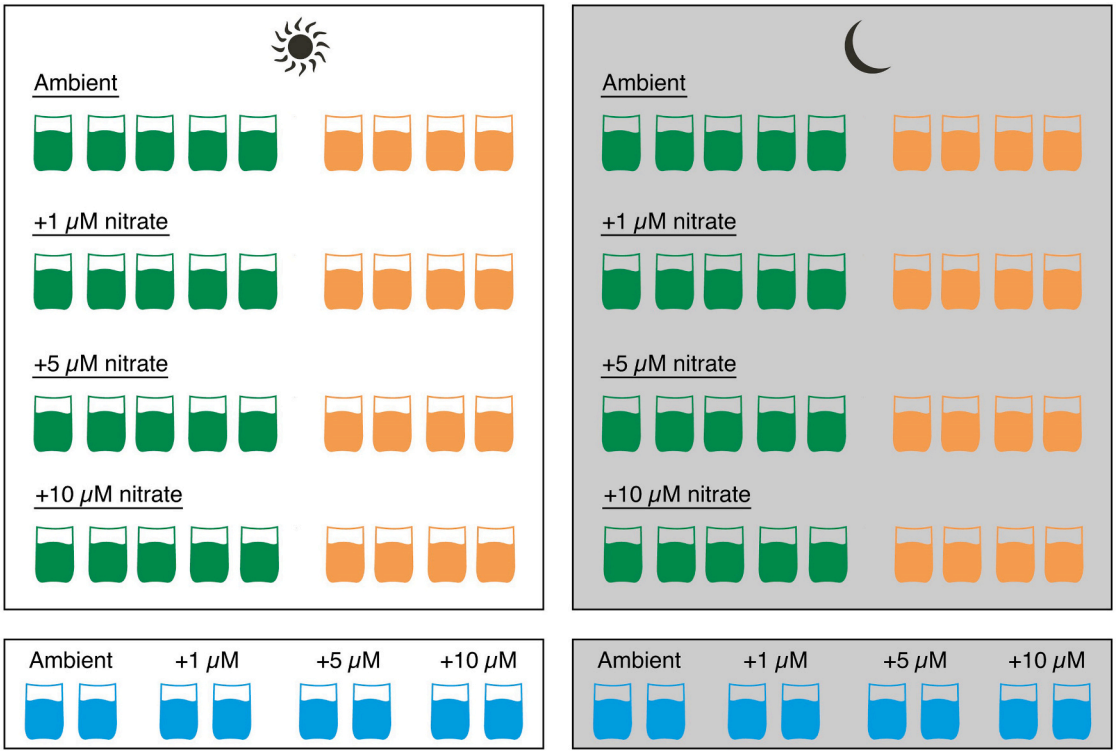

Fig. 1. Replication of turf algae (green), coral rubble (orange) and control (blue) incubations in various nitrate treatments and light and dark (grey) conditions for N_2 fixation and denitrification quantification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Statistical analysis

Data were analysed using PRIMER-E version 6 software (Clarke and Gorley, 2006) with the PERMANOVA+ add-on (Anderson, 2001). Since the assumptions for the parametric analyses were not met, data were analysed using a non-parametric permutational analysis of variance (PERMANOVA). Analyses were based on Bray-Curtis similarities of measured physiological parameters (square-root transformed data). To test for differences between N fluxes and light conditions between the substrates under various nutrient treatments, 3-factorial PERMANOVAs (factor: N flux, light condition, nutrient treatment) with type III (partial) sums of squares and unrestricted permutation of raw data (999 permutations) with Monte Carlo tests were used. Subsequent pairwise tests were performed when significant differences occurred ($p \leq 0.05$).

3. Results

3.1. N_2 fixation

Both substrates showed detectable rates of N_2 fixation under all scenarios (Fig. 2, Table S1). The detected N_2 fixation throughout all treatments ranged from 0.04 ± 0.00 nmol C_2H_4 cm^{-2} h^{-1} (coral rubble dark 10 μM) to 5.08 ± 0.88 nmol C_2H_4 cm^{-2} h^{-1} (turf light 5 μM). The highest N_2 fixation rates for turf algae were observed in light conditions showing an optimum curve response peaking at 5 μM nitrate addition (Fig. 2A). A similar pattern was observed for coral rubble during light with an optimum being at 3 to 4 μM nitrate addition (Fig. 2A). During dark incubations, turf algae N_2 fixation rates were highest in 5 and 10 μM and did not decrease under the highest nitrate treatment. In contrast to this, for coral rubble in the dark, a significant decrease of N_2 fixation rates at 10 μM compared to all other nutrient treatments was observed.

3.2. Denitrification

Both substrates also exhibited denitrification in all scenarios, except for coral rubble during dark incubations under ambient conditions, when denitrification remained below the detection limit (Fig. 2B, Table S1). Overall, a trend of increasing denitrification with increasing nitrate addition was apparent. Under light conditions, this trend was less pronounced, and coral rubble and turf algae performed comparable denitrification rates. For both substrates, the peaks of denitrification rates were observed at 10 μM under dark conditions. The observed denitrification rate for coral rubble at 10 μM in the dark was significantly higher than all other denitrification rates quantified for turf algae or coral rubble. This significant increase in denitrification corresponds with the sharp decrease in N_2 fixation for coral rubble in the dark (described above; Fig. 2, Table 1). A similar increase in denitrification activities at 10 μM compared to other nutrient treatments was observed for turf algae (Fig. 2B). The aforementioned peaks of denitrification for both turf algae and coral rubble in the dark were about four- to 800-fold higher, respectively, compared to all other quantified denitrification rates (Fig. 2, Table 1).

4. Discussion

The interplay of N_2 fixation and denitrification is key for moderating N availability to either supply N or relieve excess N depending on environmental conditions. We here demonstrate that the (in-)activity of N cycling pathways appears to be nitrate-dependent with substrate-specific thresholds stimulating or suppressing N_2 fixation and denitrification in coral reef environments.

4.1. Nitrogen fluxes of turf algae and coral rubble

Both substrates showed detectable levels of N_2 fixation and denitrification simultaneously under all given scenarios except for the absence of detectable denitrification in coral rubble substrates in the dark with no additional nitrate (i.e., under ambient conditions). Potentially, rates for coral rubble that were below the detection limit can be explained as follows: Nitrification, the oxidation of ammonium to nitrite and nitrate,

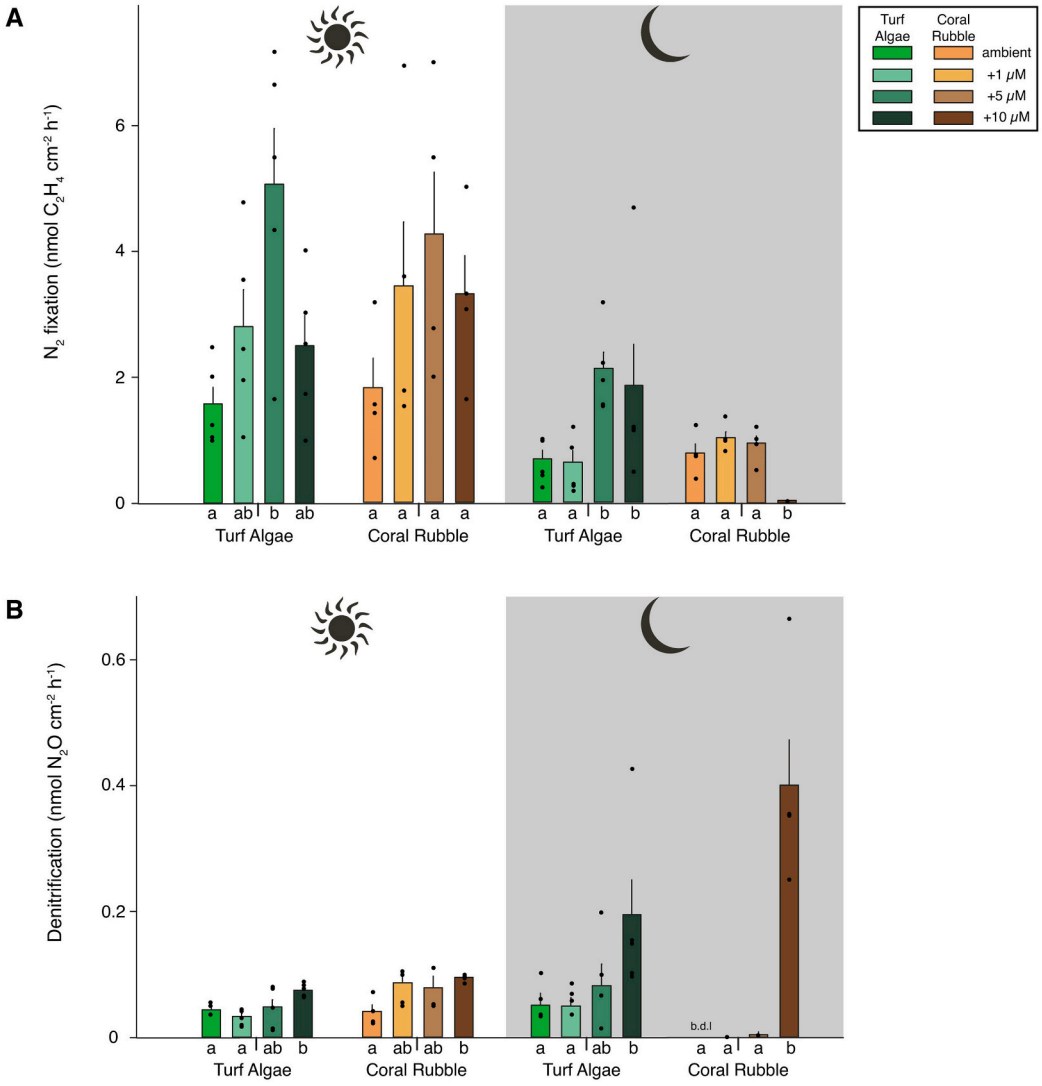

Fig. 2. N_2 fixation (A) and denitrification (B) activities for turf algae (green) and coral rubble (earth coloured) measured under four different nutrient treatments (ambient, 1, 5, and 10 μM nitrate addition) in light and dark (grey) incubations. Means are shown ± SE. Different letters on x-axis indicate significant differences between groups from the same substrate and light/dark condition. Black dots represent quantified N_2 fixation/denitrification rates. Note different scales for N_2 fixation and denitrification. b.d.l. = below detection limit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

functions as a recycling mechanism within the N cycle (Webb et al., 1975), with its final product serving as a substrate for denitrification. Nitrification is an aerobic pathway (O'Neil and Capone, 2008) that potentially redeems substrate limitation for the denitrification pathway during light incubations (Wiebe et al., 1975; Wafar et al., 1990). This hypothesis is supported through the quantification of denitrification rates for coral rubble during light incubations (Fig. 2B) and for incubations where nitrate was provided. In return, denitrification remained below the detection limit under ambient conditions and during dark incubations where nitrate is limited. Similarly, denitrification rates quantified for coral rubble in dark were consistently lower than under light conditions (except for 10 μM nitrate addition, see below). The denitrification rates of turf algae in all scenarios (Fig. 2B) indicate that these algal assemblages differ in their N cycling properties compared to coral rubble and may be able to persist higher levels of nitrate concentrations even at night. Potentially, another N-relieving mechanism, i.e., anaerobic ammonium oxidation (ANAMMOX) may remove bioavailable N from coral reefs. ANAMMOX can occur in coral reef sponges (Hoffmann et al., 2009), direct evidence for its reef-wide occurrence is, however, missing to date. In how far ANAMMOX is a) an active pathway, and b) potentially varying among coral reef-associated functional groups, hence, needs to be determined in future studies.

The N_2 fixation and denitrification rates in the present study are in line with rates reported for the Red Sea using acetylene-based measuring techniques (Rix et al., 2015; Cardini et al., 2016a; El-Khaled et al., 2020a). N_2 fixation rates for turf algae and coral rubble quantified in the present study are higher than those of other coral reef organisms and substrates such as scleractinian corals (Cardini et al., 2016b; Tilstra et al., 2019), soft corals (Bednarz et al., 2015), or top-layers of coral reef-associated reef sediments (El-Khaled et al., 2020a). Comparatively high N_2 fixation rates associated with both substrates confirm their role as important N_2-fixers on coral reefs (Cardini et al., 2016a). Turf algae, in particular, have been described as opportunists (Rosenberg and Ramus, 1984; Littler and Littler, 2013), showing a high N demand to build up biomass and to fuel their metabolism. Previous findings suggest that this high N demand can be satisfied, at least in part, by N_2 fixation (Yamamuro et al., 1995; Rix et al., 2015; Roth et al., 2020), which in return could explain the findings of the present study.

Both substrates showed more N_2 fixation during light than during dark incubations (Fig. 2A). For turf algae, these findings are in line with previous studies (Williams and Carpenter, 1997; den Haan et al., 2014), but contradict those of Rix et al. (2015) who showed substantially higher night-time N_2 fixation. Varying diel N_2 fixation rates for turf algae with high rates depending on the presence of light hypothetically reflect the presence of rather oxygen-tolerant diazotroph communities. Potentially, heterocystous cyanobacteria dominated in turf algae fragments used for the present study. They prevalently perform N_2 fixation despite the

Table 1
Results of pairwise permutational analysis of variance (PERMANOVA) for N_2 fixation (A, B) and denitrification (C, D) measured under four nutrient treatments (ambient, 1, 5, and 10 μM nitrate addition) under light and dark (grey) incubations. Significant values in bold. Top: t-values; bottom: *p*-values.

A - N_2 Fixation		Turf Algae			
		Ambient	+1 μM	+5 μM	+10 μM
Turf Algae	Ambient		0.0495 0.639	4.437 **0.001**	2.011 0.061
	+1 μM	1.636 0.144		3.944 **0.006**	2.126 0.053
	+5 μM	3.380 **0.011**	1.600 0.139		1.065 0.324
	+10 μM	1.336 0.204	0.277 0.826	1.874 0.102	

B - N_2 Fixation		Coral Rubble			
		Ambient	+1 μM	+5 μM	+10 μM
Coral Rubble	Ambient		1.349 0.233	0.8377 0.411	6.155 **0.001**
	1 μM	1.285 0.253		0.567 0.583	7.603 **0.001**
	5 μM	1.993 0.075	0.605 0.556		7.098 **0.002**
	10 μM	1.691 0.109	0.209 0.902	0.568 0.592	

C - Denitrification		Turf Algae			
		Ambient	+1 μM	+5 μM	+10 μM
Turf Algae	Ambient		0.004 0.971	0.685 0.519	2.345 **0.032**
	+1 μM	0.826 0.406		0.757 0.468	2.451 **0.043**
	+5 μM	0.236 0.801	1.059 0.321		1.612 0.154
	+10 μM	2.448 **0.049**	6.89 **0.001**	1.948 0.098	

D - Denitrification		Coral Rubble			
		Ambient	+1 μM	+5 μM	+10 μM
Coral Rubble	Ambient		1.000 0.358	1.000 0.356	5.7759 **0.002**
	+1 μM	2.302 0.069		0.885 0.432	5.767 **0.001**
	+5 μM	1.571 0.164	0.298 0.783		5.672 **0.002**
	+10 μM	4.160 **0.006**	0.570 0.604	0.794 0.471	

presence of oxygen generated via photosynthesis (Bergman et al., 1997; Staal et al., 2002), even though being capable of fixing N in the dark under oxygen-depleted conditions (Millineaux et al., 1981; Staal et al., 2002). Heterogeneous cyanobacterial community compositions between replicates could also explain the relatively large variation in the present data (Fig. 2A). We, hence, recommend simultaneous molecular analyses in future studies to define community compositions (Bauer et al., 2008), and to ultimately identify the role of (non-)heterocystous cyanobacteria in the respective specimens that potentially drive N_2 fixation. For coral rubble, we hypothesise the abundance of a diazotroph community consisting of heterotrophic bacteria equally adapted to both dark and light conditions (Staal et al., 2002), as N_2 fixation was observed during light and dark (Fig. 2A).

4.2. Rapid N_2 fixation and denitrification responses to nutrient enrichment

Both substrates followed a pattern of increasing N_2 fixation activities with increasing nutrient concentrations up to a treatment of 5 μM nitrate, despite lacking statistical significance. Interestingly, N_2 fixation rates for both substrates tend to diminish with higher nitrate treatments (i.e., 10 μM), except for turf algae during dark incubations, where rates quantified in 5 and 10 μM nitrate treatments remained higher than those at ambient and 1 μM nitrate treatments. Congruent to decreasing N_2 fixation rates under 10 μM nitrate treatments, we observed significant increases in denitrification rates for both substrates in both dark and light incubations compared to ambient, and to a certain extend to 1 or 5 μM nitrate amended incubations (Fig. 2B).

Interestingly, turf algae continued to fix N despite the presence of nitrate well above ambient concentrations, even though acquiring N via N_2 fixation is an energetically costly pathway (Holl and Montoya, 2005; Knapp, 2012). Turf algae are also capable of rapidly taking up environmentally available N (den Haan et al., 2016; Karcher et al., 2020), which we assume to be the case in the present study as well. Further, turf algal morphology (high surface area to volume ratio) is a key parameter shaping the uptake of environmentally offered nutrients (Littler and Littler, 1980; Rosenberg and Ramus, 1984). We, thus, conclude that the large N demand of turf algae may effectively limit N availability within the substrate even under high levels of seawater nitrate concentrations. Hence, efficient N uptake (via assimilation) and N_2 fixation contribute to the algae's capacity to proliferate rapidly, particularly in continuously eutrophied environments (Kuffner and Paul, 2001; Smith et al., 2005). Following this pattern, N-stimulated growth of the algae is likely closely linked to an enhanced growth of the overall microbial community (Kopp et al., 2013). Thereby, the abundance of diazotrophs (Muscatine et al., 1989) and potentially denitrifiers may increase, which in return could explain the increased N_2 fixation and denitrification rates under 5 and 10 μM nitrate concentrations during incubations.

Coral rubble-associated microbes continued to fix N in all scenarios except for dark incubations at 10 μM nitrate enriched conditions. Coral rubble can be colonised by epilithic and endolithic microbial communities (Tribollet et al., 2006) enabling the substrate 'holobiont' to fix N (Charpy et al., 2010). We conclude that microbial communities associated with coral rubble utilise the available N to stimulate N_2 fixation and to promote growth. The latter, even though not quantified in the present study, could lead to an increased abundance of diazotrophs, thus, explaining increased N_2 fixation rates.

Additionally, Mueller et al. (2016) demonstrated that both light and nutrient availability together determine dissolved organic carbon (DOC) release by turf algae. Most diazotrophs and denitrifiers rely on DOC as part of the organic carbon pool as their main energy source (Her and Huang, 1995; Lema et al., 2012; Chen et al., 2018). Increased N_2 fixation and denitrification rates with increasing nitrate availability could, thus, be explained by an increased DOC availability, that could even be translocated to neighbouring functional groups (Haas et al., 2011).

4.3. Threshold identification

Interestingly, the short time scale, which was intentionally set for the present experimental design, suggests a rapid, definite switch from active N_2 fixation to stimulation of denitrification to relieve N depending on nitrate addition. In the present study, we were able to observe the stimulation or suppression of N_2 fixation and denitrification (Fig. 2). This ambiguity has been recognised in coral reef environments in previous studies. For example, reef patch-wide denitrification increased with a congruent decrease of N_2 fixation under high (i.e., 10–30 μM) N addition (Koop et al., 2001), Roth et al. (2020) modelled decreasing N_2 fixation with increasing nitrate availability for benthic pioneer communities, whereas a nitrate-depending stimulation (10–500 μM) of denitrification was observed for coral reef sediments (Capone et al., 1992). Opposite effects on N_2 fixation and denitrification, however, were reported for turf algae, reef sediments and a hard coral under moderate eutrophication (El-Khaled et al., 2020a). We conclude that divergent effects of nutrient enrichment on both N_2 fixation and denitrification observed here and in previous studies (Capone et al., 1992; Joye and Paerl, 1993; Koop et al., 2001; El-Khaled et al., 2020a) could be related to the amount of nutrients added, either stimulating or suppressing N fluxes depending on nutrient availability. In how far divergent effects can be observed in other substrates, with potentially different microbial communities or life histories, e.g., through the exposure to other environmental conditions, remains to be targeted in future studies. Furthermore, the role of P and other micronutrients (Ferrier-Pagès et al., 2016; Luo et al., 2019) and potential shift from N to P-limitation (Wiedenmann et al., 2013) needs to be considered, and should be targeted in future studies. Based on the data of the present study, we hypothesise an identification of a threshold for a common benthic coral reef substrate (i.e., coral rubble), at which N_2 fixation is largely inactive and denitrification is initiated. Our data suggest that thresholds are substrate-specific and that thresholds for N_2 fixation and denitrification may (i.e., as identified for coral rubble) but do not have to be (i.e., as identified for turf algae) linked.

4.4. Ecological implications

It is of paramount importance to understand the effects of anthropogenically-induced nutrient enrichment, such as sewage outfalls (McManus and Polsenberg, 2004), terrestrial run-offs (den Haan et al., 2016), or aquaculture (Loya et al., 2004), on nutrient cycles that contribute to the functioning of coral reef ecosystems.

We identified turf algae and coral rubble as important sources of de novo N under ambient and elevated nutrient concentrations (Fig. 2A). Algae are likely to increase in dominance in future coral reefs (Pandolfi et al., 2011; Hughes et al., 2017) due to their aforementioned opportunistic behaviour and their competitive advantage compared to classical reef framework builders (i.e., hard corals) under stressed environmental parameters. Shifts from coral to algae dominance have been recognised worldwide (Hughes, 1994; McManus and Polsenberg, 2004; Bruno et al., 2009; Norström et al., 2009), potentially resulting in an increase of bioavailable N as i) a direct consequence of increases in turf algae abundance (El-Khaled et al. accepted) or ii) due to the stimulation of N_2 fixation under moderate eutrophied conditions (El-Khaled et al., 2020a). In this context, a rapid response to short-term nutrient pollution by increasing N_2 fixation for turf algae might have several implications on a shorter time scale. The fast on-set and increase of N_2 fixation within minutes to hours after nitrate addition, for example in turf algae, might add to explaining their competitive advantages. Given turf algae's opportunistic use of N for growth, this might provide an additional 'head start' under eutrophication; however more functional groups would need to be analysed to investigate the first hours of competition under pulse N availability. Besides that, a rapid accumulation of biomass based on the availability of N may eventually result in an energy transfer to higher trophic levels (Roth et al., 2020) by either herbivorous grazing (Fong and Paul, 2011) or a decay of biomass (Duarte and Cebrián, 1996). The latter potentially causes hypoxia (oxygen deficiency) or anoxia (no oxygen) through the proliferation of oxygen-consuming microbes (Hughes et al., 2020), which eventually have adverse effects for the health of coral reefs (Dubinsky and Stambler, 1996).

Turf algae and coral rubble have been identified as major N fixers before (Cardini et al., 2016a), their role as denitrifiers under ambient conditions is comparatively small (El-Khaled et al. accepted). In that study, turf algae and coral rubble were among the least active denitrifiers under ambient conditions compared to hard and soft corals, reef sediments, and biogenic rock. However, particularly under eutrophied scenarios, coral rubble, for which we were able to identify a threshold that suppresses N_2 fixation and stimulates denitrification

(Fig. 2), seems to play a crucial role in rapidly relieving substrates from N. Hence, the fast response to short-term eutrophication, e.g., from nutrient pulses (den Haan et al., 2016) might be key for alleviating excess N via denitrification. In how far previously identified key denitrifiers such as corals (shown to perform highest denitrification rates among the aforementioned functional groups, El-Khaled et al. accepted) or reef sediments (Capone et al., 1992) respond to short-term eutrophication remains to be determined.

Given the observed threshold under high nitrate addition, it needs to be considered that such high N availability (i.e. 10 μM nitrate) is far above the environmental conditions, even under anthropogenic impact. Under lower, more realistic N availability, N_2 fixation appeared to increase more strongly and steadily with increasing nitrate additions than denitrification. In return, this means that the 'threshold' for a drastic N relief via denitrification (vs. N_2 fixation) is likely not 'hit' soon enough to counter eutrophication. Therefore, the role of microbial nutrient cycling in enhancing the effects of moderate, even short-term, eutrophication should be considered in coastal development and planning (e.g., runoff events, drainage, sewage outfalls, etc.).

Our study, concludes that short-term eutrophication has strong implications on the reef N-cycling, that substrate specific thresholds (N_2 fixation vs. denitrification) seem to exist, that those substrate- and context-specific thresholds may help explaining varying outcomes of eutrophication studies, and that the effects of moderate eutrophication may physiologically alter the overall N budget in the reef.

CRediT authorship contribution statement

YEK, FR, CRV and CW conceptualised and designed research. YEK, FR, NR and DBK performed research. YEK, RN, and DBK analysed data. BHJ, CRV and CW contributed to research materials, logistics and interpreting data. YEK wrote original draft of the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112430.

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