



# Simultaneous Measurements of Dinitrogen Fixation and Denitrification Associated With Coral Reef Substrates: Advantages and Limitations of a Combined Acetylene Assay

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Nitrogen (N) cycling in coral reefs is of key importance for these oligotrophic ecosystems, but knowledge about its pathways is limited. While dinitrogen (N<sub>2</sub>) fixation is comparably well studied, the counteracting denitrification pathway is under-investigated, mainly because of expensive and relatively complex experimental techniques currently available. Here, we combined two established acetylene-based assays to one single setup to determine N<sub>2</sub>-fixation and denitrification performed by microbes associated with coral reef substrates/organisms simultaneously. Accumulating target gases (ethylene for N<sub>2</sub>-fixation, nitrous oxide for denitrification) were measured in gaseous headspace samples via gas chromatography. We measured N<sub>2</sub>-fixation and denitrification rates of two Red Sea coral reef substrates (filamentous turf algae, coral rubble), and demonstrated, for the first time, the co-occurrence of both N-cycling processes in both substrates. N<sub>2</sub>-fixation rates were up to eight times higher during the light compared to the dark, whereas denitrification rates during dark incubations were stimulated for turf algae and suppressed for coral rubble compared to light incubations. Our results highlight the importance of both substrates in fixing N, but their role in relieving N is potentially divergent. Absolute N<sub>2</sub>-fixation rates of the present study correspond with rates reported previously, even though likely underestimated due to an initial lag phase. Denitrification is also presumably underestimated due to incomplete nitrous oxide inhibition and/or substrate limitation. Besides these inherent limitations, we show that a relative comparison of N<sub>2</sub>-fixation and denitrification activity between functional groups is possible. Thus, our approach facilitates cost-efficient sample processing in studies interested in comparing relative rates of N<sub>2</sub>-fixation and denitrification.

**Keywords:** nitrogen cycling, metabolism, gas chromatography, ethylene, nitrous oxide

## INTRODUCTION

Nitrogen (N) is one of the primary nutrients critical for the survival of all living organisms. Natural N availability is limited in many ecosystems, while others suffer from eutrophication. Thus, understanding N cycling processes is of paramount interest. Two antagonistic biological processes within the N cycle are of particular importance: (a) the import of new N into the ecosystem via microbes (diazotrophs) capable of converting atmospheric dinitrogen ( $N_2$ ) into bioavailable forms of N, which is called biological  $N_2$ -fixation (hereafter  $N_2$ -fix), and (b) its counteracting process that removes N from the ecosystem via a reduction of nitrate to  $N_2$ , commonly described as denitrification (hereafter DENI; Vitousek et al., 1997; Gruber and Sarmiento, 2002; Jickells and Weston, 2011). Both processes appear in terrestrial and aquatic ecosystems, where N can act as an important factor limiting productivity (Lesser et al., 2007).

In coral reefs, N cycling is of particular importance as these ecosystems flourish in the oligotrophic waters of the tropics. However, attempts to describe microbial N cycling in coral reef environments are primarily restricted to  $N_2$ -fix to provide information about how coral reefs flourish in nutrient-poor waters and how their N demand is satisfied (Neil and Capone, 2008). Appropriate methods to quantify other pathways are largely missing to date (Groffman et al., 2006).

$N_2$ -fix is often quantified by one of two commonly applied methods: labeled isotope tracing techniques (Montoya et al., 1996; Grover et al., 2014) or acetylene reduction assays (ARA; Pogoreutz et al., 2017; Tilstra et al., 2017). Isotope based techniques can be highly specific in identifying the exact location of  $N_2$ -fix, but they are comparatively expensive and require higher effort to set up, perform and analyze samples. Whereas isotope tracer approaches track the fate of fixed N to the cellular compartments of a target organism/substrate (Wilson et al., 2012), acetylene assays measure gross  $N_2$ -fix activities on a “holobiont-wide” level of an organisms/substrate (Mulholland et al., 2004; Mohr et al., 2010; Grover et al., 2014). This property is of significance, as coral reef organisms/substrates are considered as holobionts, consisting of the host organism/substrate and all of its associated symbiotic microorganisms (Rosenberg et al., 2007). The ARA is frequently used due to its comparatively easy handling and cost-effectiveness and has received first attention in the 1960s (Hardy et al., 1968). It makes use of the fact that acetylene acts as an alternative substrate to  $N_2$ , resulting in the preferential reduction of acetylene to ethylene ( $C_2H_4$ ) instead of  $N_2$  to ammonium by the nitrogenase enzyme (**Supplementary Figure S1**). The  $C_2H_4$  evolution is then quantified, e.g., via gas chromatography (GC), as an indirect measurement of  $N_2$ -fix.

DENI can be detected using the same methods as described above (Koop et al., 2001; Groffman et al., 2006; Hoffmann et al., 2009; Myrsten et al., 2016). Acetylene assays are carried out as acetylene inhibits the nitrous oxide ( $N_2O$ ) reductase of denitrifying bacteria, which subsequently results in an accumulation of  $N_2O$  (**Supplementary Figure S1**; Fedorova et al., 1973; Balderston et al., 1976; Yoshinari and Knowles, 1976). Accumulated  $N_2O$  can then be quantified via GC and used as an approximation of DENI activity.

DENI received attention as a mechanism to relieve coral reefs from excessive N only recently, as coral reefs experience and suffer more frequently from anthropogenically N inputs (Halpern et al., 2008; Smith and Schindler, 2009); upcoming studies should, thus, focus on determining the capacities of coral reef environments to cope both with limited and excessive N by quantifying  $N_2$ -fix and DENI activities simultaneously (Rädecker et al., 2015).

In this context, acetylene assays have the potential for detecting both  $N_2$ -fix and DENI simultaneously. Indeed, both methods have been applied in terrestrial (Yoshinari et al., 1977) and aquatic (Bertics et al., 2012) systems. Capone and Montoya (2001) already hypothesized a potential simultaneous usage in coral reef environments, but applications on various coral reef organisms and substrates are still missing to date. Comparing multiple functional groups of coral reefs, however, is of key importance, as coral reefs experience regime-shifts that in return alter N cycling patterns that potentially exacerbate or alleviate anthropogenic impacts.

We acknowledge the ongoing scientific debate whether isotope-based approaches or acetylene assays are the methods of choice in order to investigate N cycling pathways. We, therefore, do not compare or discuss both approaches in the present study. Instead, we highlight the application of a combined acetylene-based assay to two common coral reef substrates. We were able to evaluate both process measurements in one single experimental setup and analysis, which we termed COBRA (= combined blockage/reduction acetylene assay). Based on the results of the application, we discuss advantages and limitations as well as its potential use in coral reef science.

## MATERIALS AND PROCEDURES

A complete list of all materials and the equipment used in this protocol as well as a full description of how the acetylene assay was performed can be found in **Supplementary Material**.

### Collection and Maintenance

Specimens were collected randomly from a semi-exposed area of Abu Shosha reef in the Jeddah region ( $22^{\circ}18'15''$  N,  $39^{\circ}02'56''$  E) on the west coast of Saudi Arabia in the central Red Sea in March 2018. Turf algae ( $n = 5$ ) and coral rubble fragments ( $n = 4$ ) were selected in order to cover prevailing benthic substrates of the region. Turf algae were defined as dense and flat (<2 cm in height) assemblages of filamentous algae of different species, including small individuals of macroalgae and cyanobacteria. Coral rubble was defined as dislodged parts of framework builders or loose reef rock larger than the sand fraction with its associated microbial community according to Rasser and Riegl (2002). All fragments were ~10 cm long and were collected with hammer and chisel from 5 to 6 m water depth. They were immediately transferred to recirculation aquaria on the boat (each filled with 10 L of ambient seawater) and kept until experiments at ambient water temperature and light conditions.

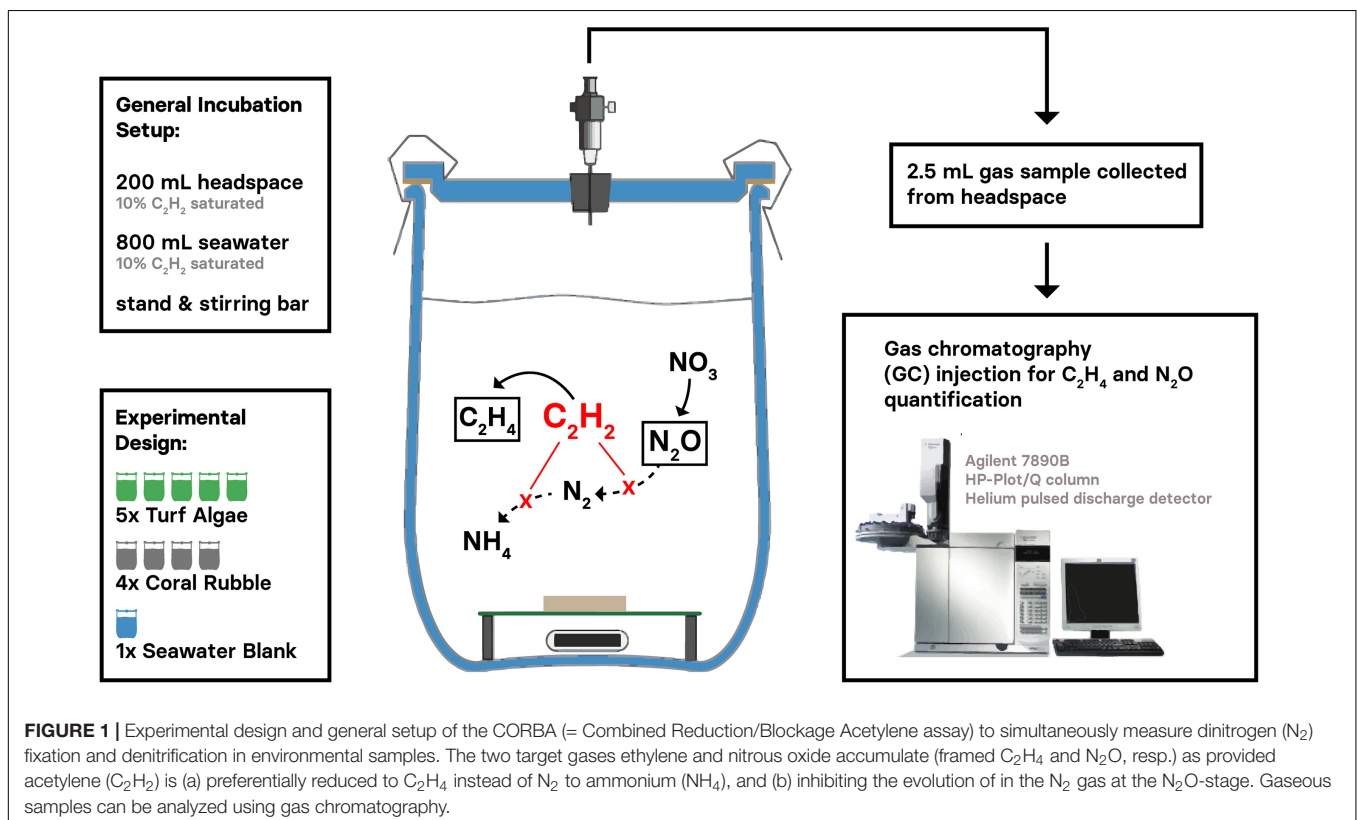
## Dinitrogen Fixation and Denitrification Measurements

Incubations were conducted *ex situ* and directly after sample collection (<3 h after collection).  $N_2$ -fix and DENI incubations were conducted using COBRA according to the steps outlined in detail in **Supplementary Material S1**. Briefly, all COBRA incubations were conducted in gas-tight 1 L glass chambers, each filled with 800 mL of nutrient-enriched seawater ( $5 \mu\text{M NO}_3^-$ ;  $\text{NO}_3^-$  enrichment consisted of previously prepared  $\text{NaNO}_3$  stock solution, prepared with MilliQ water and  $\text{NaNO}_3$ ,  $\geq 99.0\%$ , Sigma-Aldrich) and 200 mL headspace (**Figure 1**). Both incubation water and headspace were 10% acetylene enriched, as a 10% saturation has been successfully applied in acetylene-based assays for the quantification of  $N_2$ -fix (Mulholland et al., 2004; Pogoreutz et al., 2017) and DENI (Yoshinari and Knowles, 1976; Shrewsbury et al., 2016) previously. Nutrient enriched seawater was used, as acetylene inhibits the production of  $\text{NO}_3^-$  in the nitrification pathway (Hynes and Knowles, 1978; Oremland and Capone, 1988). Thus, added  $\text{NO}_3^-$  served as a substrate for DENI (see section “Limitations” for detailed information). Replicates were arranged in individual incubation chambers, and an additional chamber that was filled exclusively with  $5 \mu\text{M NO}_3^-$  enriched seawater as “seawater blank” (**Figure 1**). Solely one seawater blank was chosen because negligible microbial activity (diazotrophs and hypothetically denitrifiers) in seawater communities was expected according to previous studies (Foster et al., 2009; Bednarz et al., 2015; Cardini et al., 2016). All incubation chambers were placed in a tempered water bath and

stirred continuously (500 rpm) to ensure stable measurement conditions for 12 h at *in situ* temperatures ( $25^\circ\text{C}$ ). Dark and light incubations were conducted separately; dark incubations were performed at night in complete darkness. Light incubations were performed with a photon flux of  $\sim 200 \mu\text{M quanta m}^{-2} \text{ s}^{-1}$ . Gaseous samples were taken immediately after starting the incubations ( $t_0$ ), after 2 h ( $t_2$ ), 4 h ( $t_4$ ), 8 h ( $t_8$ ), and 12 h ( $t_{12}$ ) and analyzed using GC (Agilent 7890A, HP-Plot/Q column, helium pulsed discharge detector) via manual injection.  $N_2$ -fix and DENI were quantified by changes in gas concentrations according to equations 2–6 outlined in **Supplementary Material S1**. Besides start and end concentrations, two different intervals were selected as basis for the rate calculations, particularly  $t_4$ – $t_{12}$  for  $N_2$ -fix and  $t_0$ – $t_4$  for DENI activity. Finally, concentrations were corrected for the seawater blank signal, related to incubation volume and normalized to the surface area of the substrates (**Supplementary Tables S2, S3**). Surface areas for turf algae and coral rubble fragments were calculated using cloud-based 3D models of samples (Autodesk Remake v19.1.1.2; Lavy et al., 2015; Gutierrez-Heredia et al., 2016).

## Calibration

A concentration series of  $\text{C}_2\text{H}_4$  (Abdullah Hashim Industrial Gases & Equipment Co. Ltd. Specialty Gases Center, 19.6 ppm in balanced air; 210 ppm in balanced air) and  $\text{N}_2\text{O}$  (Air Liquide, 199.6 ppm in balanced Helium) was prepared by diluting commercial standards to desired standard concentrations. Standards covered the expected ranges of 0–210 ppm for  $\text{C}_2\text{H}_4$



and 0–2 ppm for  $N_2O$ , respectively, and were injected manually into the GC. For the  $C_2H_4$  calibration curve, standards with known concentrations of 210, 105, 52.5, 21, 20, 10, 5, 2, 1, and 0 ppm were measured in duplicates. The same was done for the  $N_2O$  calibration curve, where standards with known concentrations of 2, 1, 0.5, 0.2 ppm, and 0 ppm were injected.

## Statistical Analysis

The statistical analysis was performed using Sigmaplot 12 (Systat software, v12.0). A two-way analysis of variance (ANOVA) with factors “substrates” and “sampling time” was performed. In case of  $p < 0.05$ , a *post hoc* test (Tukey HSD) was performed to check for significant differences between substrates and sampling times. Significance level was set at  $\alpha = 0.05$ .

## RESULTS

Limits of detection (LOD), obtained from duplicate measurements of prepared standard samples, were 0.3 ppm for both target gases (**Supplementary Figure S2**). Standard curves showed high linearity for both target gases (linear regressions;  $R^2 > 0.99$ ;  $p < 0.0001$ ,  $F > 800$ ; **Supplementary Figure S2**).

Concentrations for both  $C_2H_4$  and  $N_2O$  in seawater blank incubations over the incubation time of 12 h were stable (**Supplementary Figure S3**). Production of  $C_2H_4$  (as a proxy for  $N_2$ -fix) and  $N_2O$  (as a proxy for DENI) were measured in both dark and light conditions (**Figure 2**, **Supplementary Tables S2, S3**). Statistical analysis identified “time” as a significant factor in both processes and regardless of light availability (**Supplementary Table S4**).  $C_2H_4$  evolution was measured in both substrates and was higher in light compared to dark incubations. Overall,  $C_2H_4$  increased over time, with an initial lag phase in the first 4 h of incubation. Consequently,  $N_2$ -fix rates calculated discounting the initial lag phase were one third higher than rates resulting from start-end concentrations (**Table 1**).

$N_2O$  evolution was detected for turf algae and coral rubble, with turf algae showing significantly higher amounts of  $N_2O$  after 2, 8, and 12 h incubation time regardless of light availability (**Supplementary Table S5**). For both substrates, the highest amounts of  $N_2O$  were measured after 4 h of incubation. Unlike to  $C_2H_4$  evolution, no initial lag phase was observed. In contrast,  $N_2O$  only increased in the first 4 h and remained stable or decreased afterward. Turf algae DENI rates were higher in dark than those for coral rubble, but similar when incubated in light (**Table 1**). DENI rates obtained from the first 4 h of incubation were up to 8 times higher than those resulting from start-end concentrations (**Table 1**).

## DISCUSSION

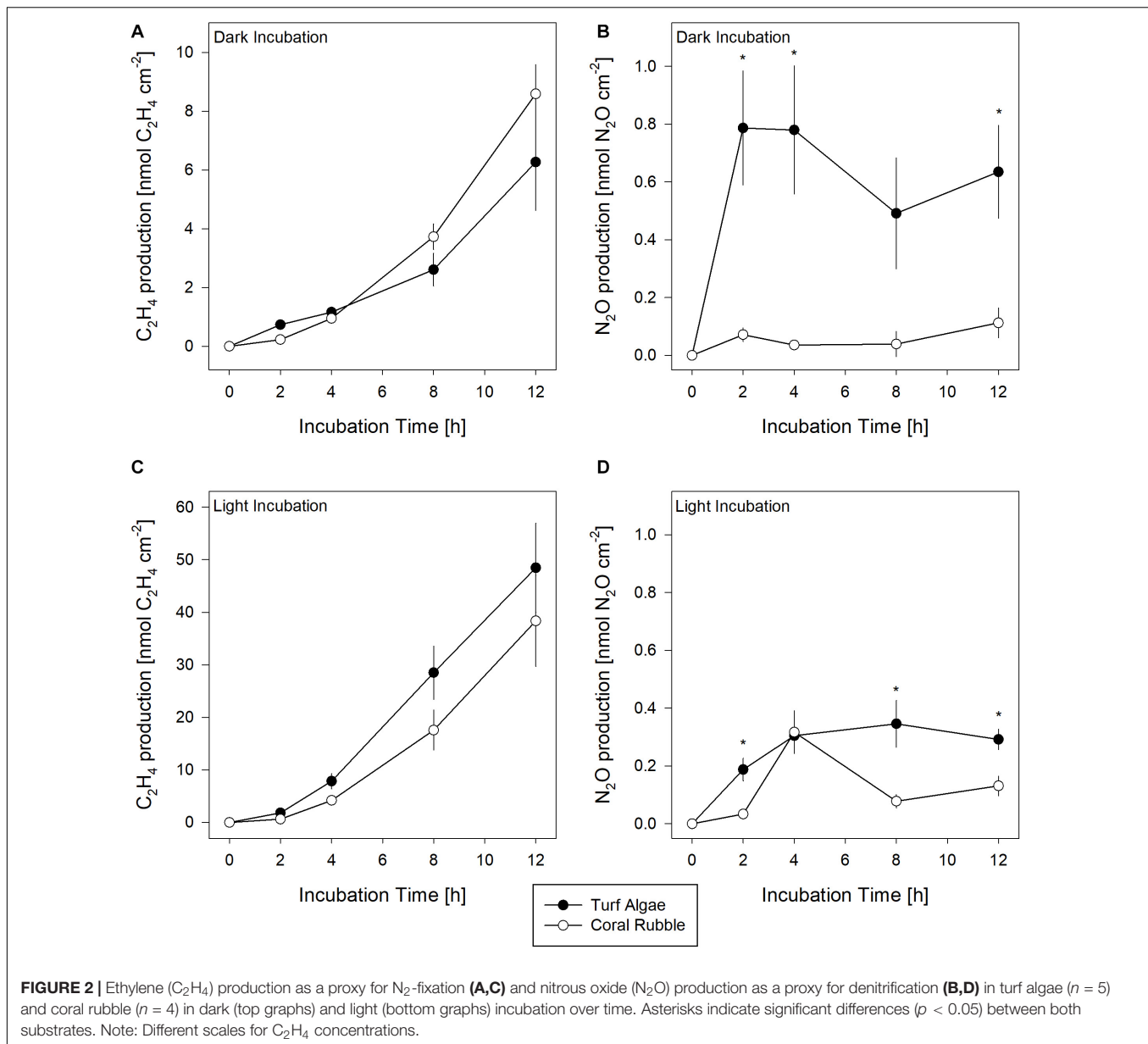
### Dinitrogen Fixation and Denitrification Measurements

In the present study, we were able to demonstrate the co-occurrence of  $N_2$ -fix and DENI in turf algae and coral rubble for the first time. The simultaneous presence of these antinomic

processes has been detected in coral reef associated sediments (Koop et al., 2001) and microbial mats (Joye and Paerl, 1993) before. Potentially, both pathways can be carried out by the same microbes (Bothe et al., 1981) and are indirectly linked by their similar environmental requirements (Tilstra et al., 2019). Absolute  $N_2$ -fix rates (i.e., calculated based on sampling points  $t_0$  and  $t_{12}$ ) measured in the present study were in the same range for turf algae (Rix et al., 2015) and coral rubble (Cardini et al., 2016), who also obtained  $N_2$ -fix rates via acetylene-based assays (**Table 2**). Potentially, the similarity of rates of both substrates was due to a similar associated microbial composition on the substrates in terms of ecological niche and thus  $N_2$ -fix activity. To the best of our knowledge, this was the first study to quantify rates of DENI in coral rubble and turf algae, and, thus, no comparison to previous reports is possible.

Interestingly, both substrates showed stimulated  $N_2$ -fix during light compared to dark incubations. DENI activity was similar in both substrates during light incubations, whereas dark incubations showed suppressed DENI activity in coral rubble but stimulated DENI in turf algae (**Table 1**). Nitrogenase, the key enzyme performing  $N_2$ -fix, is extremely sensitive to oxygen (Robson and Postgate, 1980), and  $N_2$ -fix is generally considered as an anaerobic process (Compaoré and Stal, 2010). Likewise, DENI is also an anaerobic process (Zumft, 1997). Both incubations (dark and light) were started with freshly collected seawater containing natural, ambient oxygen concentrations, which likely increased during light and decreased during dark incubations due to photosynthesis or respiration (Mague et al., 1974; Rix et al., 2015; Cardini et al., 2016). The occurrence of both anaerobic processes despite the presence of oxygen (i.e., during light) indicates that both  $N_2$ -fix and DENI may be spatially separated from oxygen evolution in both substrates, or that the involved diazotrophs and denitrifiers are capable of performing  $N_2$ -fix and DENI, resp., in the presence of oxygen (Lloyd et al., 1987; Berman-Frank et al., 2001; Silvennoinen et al., 2008; Bednarz et al., 2018). Furthermore, aerobic conditions promote nitrification (Rysgaard et al., 1994), i.e., the oxidation from ammonium to nitrite and  $NO_3^-$ , which serves as a substrate for DENI (Devol, 2008). However, in how far photosynthesis and respiration affected  $N_2$ -fix and DENI activities in both substrates in the present study remains speculative. Synoptically, the method aids to reveal interesting N cycling patterns. This emphasizes the general applicability of the combined technique for the quantification of  $N_2$ -fix rates, yet revealing interesting patterns for both N cycling pathways.

Our findings revealed increasing  $C_2H_4$  concentrations over the total incubation time of 12 h with an initial lag phase in the first 4 h, which is shorter than reported before (Patriquin and McClung, 1978; Williams et al., 1987; Shieh and Lin, 1992). Considering start ( $t_0$ ) and end ( $t_{12}$ ) concentrations will thus result in an underestimation of  $N_2$ -fix rates (**Table 1**). However,  $N_2$ -fix rates based on start/end concentrations are comparable to previous studies that have applied standard acetylene reduction assays (**Table 2**), indicating that COBRA still provides sufficient information to go beyond relative comparisons between functional groups but also allowing comparative analysis with other studies. Nevertheless, the most accurate rates are derived



**FIGURE 2** | Ethylene ( $C_2H_4$ ) production as a proxy for  $N_2$ -fixation (A,C) and nitrous oxide ( $N_2O$ ) production as a proxy for denitrification (B,D) in turf algae ( $n = 5$ ) and coral rubble ( $n = 4$ ) in dark (top graphs) and light (bottom graphs) incubation over time. Asterisks indicate significant differences ( $p < 0.05$ ) between both substrates. Note: Different scales for  $C_2H_4$  concentrations.

by omitting the initial lag phase when calculating rates and considering sampling points  $t_4$  and  $t_{12}$  instead.

In the case of  $N_2O$  rates, we revealed higher  $N_2O$  rates obtained from the first 4 h incubation time (Table 1). This leads to the suggestion of a short sampling interval to identify DENI potentials (i.e.,  $t_0$  and  $t_4$ ). The detected maximum after 4 h likely reflects an incomplete blockage of the DENI pathway (Yu et al., 2010). A depletion of accumulated  $N_2O$  gas concentrations via denitrifying bacteria is likely due to reduced DENI inactivity as  $NO_3^-$  substrate has been consumed (see next section). However, our findings reveal a significant difference in accumulated  $N_2O$  after 12 h between turf algae and coral rubble fragments, despite a decrease in  $N_2O$  concentrations after 4 h. As a result, we recommend short sampling intervals but also conclude that even over 12 h incubation time, a

relative comparison between different functional groups may be possible as long as methodological limitations (e.g., substrate availability) are considered.

Altogether, we recommend performing incubations for 12 h, with sampling points at  $t_0$ ,  $t_4$ , and  $t_{12}$ . If feasible with regard to costs and workload, we suggest a higher temporal resolution (i.e., more sampling points) to achieve rates that are potentially more precise. By all means, the temporal resolution chosen in the present study enables calculations for most reliable  $N_2$ -fix (from  $t_4$  until  $t_{12}$ ) rates and DENI (from  $t_0$  until  $t_4$ ) potentials. In case that solely start- and end-measurements can be performed (from  $t_0$  until  $t_{12}$ ), we conclude that a) the rates measured with the COBRA still allow comparisons with other studies in case of  $N_2$ -fix (Table 2); and b) COBRA provides sufficient information about the relative importance by accounting for relative changes

**TABLE 1** |  $N_2$ -fixation ( $\text{nmol } N_2 \text{ cm}^{-2} \text{ h}^{-1}$ ) and denitrification ( $\text{nmol } N_2O \text{ cm}^{-2} \text{ h}^{-1}$ ) rates/potentials in turf algae and coral rubble.

	$N_2$ -fixation [ $t_0$ - $t_{12}$ ]	$N_2$ -fixation [ $t_4$ - $t_{12}$ ]	Denitrification [ $t_0$ - $t_{12}$ ]	Denitrification [ $t_0$ - $t_4$ ]
Turf algae	0.12 ± 0.03	0.16 ± 0.05	0.05 ± 0.01	0.19 ± 0.06
Coral rubble	0.18 ± 0.02	0.24 ± 0.03	0.01 ± 0.00	0.01 ± 0.00
Turf algae	1.00 ± 0.17	1.27 ± 0.22	0.02 ± 0.00	0.08 ± 0.00
Coral rubble	0.80 ± 0.18	1.07 ± 0.24	0.01 ± 0.00	0.08 ± 0.02

Rates are obtained from varying sampling times (in squared brackets) to highlight underestimations when solely start/end values are used to generate  $N_2$ -fixation and denitrification rates. Dark incubation rates are highlighted in gray color, light incubation rates are highlighted in white color. All  $N_2$ -fixation rates were converted with a conservative conversion factor of 4:1 ( $C_2H_4:N_2$ ), according to Mulholland et al. (2004). Values are presented in mean ± SE.

**TABLE 2** |  $N_2$ -fixation ( $\text{nmol } N_2 \text{ cm}^{-2} \text{ h}^{-1}$ ) and denitrification ( $\text{nmol } N_2O \text{ cm}^{-2} \text{ h}^{-1}$ ) rates of turf algae and coral rubble in comparison with values reported from other coral reef areas worldwide acquired via acetylene assays.

$N_2$ -fixation	Denitrification	Location	References
<b>Turf algae</b>			
0.56 ± 0.10	0.04 ± 0.01	Central Red Sea	Present study
0.44 ± 0.04*	n.d.a.	Northern Red Sea	Rix et al., 2015
<b>Coral rubble</b>			
0.49 ± 0.10	0.01 ± 0.00	Central Red Sea	Present study
1.00 ± 0.25	n.d.a.	GBR	Davey et al., 2008
0.58 ± 0.20*	n.d.a.	Northern Red Sea	Cardini et al., 2016
0.90–4.00	n.d.a.	GBR	Larkum, 1988
0.74–5.70	n.d.a.	GBR	Larkum et al., 1988

\*Winter season. Rates of the presented study were obtained from mean values from  $t_0$  and  $t_{12}$  sampling times of both dark and light incubations to demonstrate comparability though being ultimately underestimated. All  $N_2$ -fixation rates were converted with a conservative conversion factor of 4:1 ( $C_2H_4:N_2$ ), according to Mulholland et al. (2004). Values are presented in mean ± SE. GBR, Great Barrier Reef, Australia. n.d.a., no data available.

in rates across substrates in case of DENI activity, even though both N cycling pathways may be underestimated.

## Limitations

Acetylene assays have faced criticism in the last decades. Limitations of acetylene assays have been reviewed extensively in Giller (1987), Groffman et al. (2006), and Wilson et al. (2012). Reported restraints are of general nature and cannot be directly related to a combination of both acetylene reduction and inhibition as in the presented setup here. Thus, we focus on methodological limitations that are important to consider for the interpretation of relative rather than absolute rates (Groffman et al., 2006).

Firstly, Oremland and Capone (1988) observed an unspecific inhibitory effect of acetylene on other N cycling pathways, as acetylene inhibits the formation of  $NO_3^-$  via the nitrification pathway. This can ultimately result in an underestimation of DENI (Hynes and Knowles, 1978; Jenkins and Kemp, 1984; Seitzinger, 1993; Groffman et al., 2006), as there is a close coupling of both nitrification and DENI (i.e., the production of  $NO_3^-$  via nitrification serves as a substrate for DENI). This

is particularly the case in oligotrophic systems, where  $NO_3^-$  may be a limiting factor for DENI (Miyajima et al., 2001). Hence, to preclude substrate limitation, incubations with  $NO_3^-$  addition, as used in this study, can aid maintaining DENI activity in the absence of nitrification (Haines et al., 1981; Joye and Paerl, 1993; Miyajima et al., 2001). However, the results obtained with COBRA and nutrient addition reflect, thus, a DENI potential rather than actual DENI rates, as artificially increased substrate availability drives DENI above natural occurring rates. A potential side effect of added  $NO_3^-$  on  $N_2$ -fix activity should be addressed in future studies. Theoretically, with the addition of  $NO_3^-$ , an energetically more cost-efficient alternative N source (i.e., through assimilation) is provided, as compared to  $N_2$ -fix (Falkowski, 1983). The preferential  $NO_3^-$  assimilation potentially results in lower  $N_2$ -fix rates. Yet, there is both evidence for the inhibition of  $N_2$ -fix by the availability of  $NO_3^-$  and counterproof that  $N_2$ -fix occurs with substantial rates in the presence of up to 30  $\mu\text{M } NO_3^-$  (Knapp, 2012).

A time-dependent incomplete inhibition of  $N_2O$  through the presence of acetylene has been discussed before (Groffman et al., 2006; Yu et al., 2010). An incomplete inhibition and a subsequent  $N_2O$  reduction in the presence of acetylene can, thus, result in an incorrect estimation of total DENI. Yu et al. (2010) have reported incomplete inhibitions after  $\geq 24$  h. Our results indicate that observed patterns of decreasing  $N_2O$  concentrations after  $> 4$  h incubation time potentially occur due to an incomplete blockage. Possibly, substrate depletion during the incubation time also lead to decreasing  $N_2O$  concentrations after  $> 4$  h of incubation time, as  $NO_3^-$  a) is potentially used as a substrate for DENI (Devol, 2008) or) may be assimilated immediately by turf algae (den Haan et al., 2016) and potentially also by coral rubble. It remains unclear if saturating the incubation chamber with  $> 10\%$  acetylene will result in a complete inhibition.

## Advantages and Use in Research

Generally, acetylene assays are applied as indirect approaches to identify  $N_2$ -fix as well as DENI. Despite their limitations (see above), they have provided  $N_2$ -fix and DENI rate quantifications and estimates for many aquatic systems.

We could demonstrate that a combination of both approaches (i.e., acetylene reduction and acetylene blockage assay) can be performed simultaneously. Both assays have been performed separately over a wide range of aquatic substrates and organisms before (Olson et al., 1998; Cardini et al., 2016). As no changes in the basic setup have been carried out here, we hypothesize that the here presented approach (similar to commonly applied acetylene assays) can be used as a versatile method for organisms and substrates of various aquatic environments.

By using COBRA, the number of samples and measuring time is halved, as only one gaseous sample and consequently, only one sample run is required to detect both target gases. In addition, we were able to measure four gaseous headspace samples per hour using either manual or autosampler injection (see **Supplementary Material**), whereby the autosampler has the capacity to measure 71 samples within  $< 18$  h. Overall,

the high sample throughput enables the generation of profound datasets in a minimum of time, with the possibility to receive a high temporal resolution with multiple functional groups of a targeted coral environment. This opens the door to a deeper understanding of temporal and spatial patterns of N<sub>2</sub>-fix and DENI across various substrates and organisms and their associated microbial community.

Previous acetylene-based investigations mostly used flame ionization detectors for N<sub>2</sub>-fix quantification and either electron capture or thermal conductivity detectors for DENI detection (Haines et al., 1981; Capone et al., 1992; Joye and Paerl, 1993; Capone and Montoya, 2001). The latter have a high sensitivity for N<sub>2</sub>O but are also prone to interference from other compounds (Capone and Montoya, 2001; Roberge et al., 2004). In the present study, we used a helium pulsed discharge ionization detector. This detector is up to 500 times more sensitive than the thermal conductivity sensor and up to 50 times more sensitive than the flame ionization detector (Woo et al., 1996; Hunter et al., 1998). Owing to the use of only one single and very sensitive detector (Roberge et al., 2004) only one type of carrier gas was required for one sample run. This leads to a reduction in costs, time, storage, and usage of further gases.

In the future, unprecedented dramatic environmental changes will be a major challenge for life in the earth's oceans (Harnik et al., 2012). Thus, research will undoubtedly focus on understanding and predicting the effects of climate change and other anthropogenic pressures on marine ecosystems and their organisms (Bijma et al., 2013; Pandolfi et al., 2011; Runge et al., 2016). Special emphasis will be laid on the effects of environmental change on marine microbial communities, which act as the major drivers of elemental transformations of terrestrial and marine biogeochemical cycles (Gruber and Galloway, 2008; Gruber, 2011). N cycling is one of the most important biogeochemical cycles, and, as such, changes in N availability (through eutrophication) will likely evoke physiological and metabolic responses in coral reef organisms/substrates. Their study is paramount for understanding N cycling from a species to ecosystem level with knowledge of a changing environment (Cardini et al., 2014). Eutrophication, as well as ocean warming and acidification, belong to the mainly discussed and assessed threats. These can easily be implemented in the COBRA by adjusting incubation parameters, e.g., temperature, nutrient availability and pH, or a combination of such. Hence, the described N cycling pathways can be investigated in an

anthropogenically influenced system that simulates conditions comparable to common anthropogenic stressor scenarios. As investigations on the effects of climate change have become a major scientific interest, the opportunities to include them in the here presented technique underline its potential.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

YE-K, CW, FR, BJ, and CV conceptualized and designed the research. YE-K and NK performed the research. YE-K, FR, and NR analyzed the data. YE-K, FR, NR, and CW wrote the manuscript. All authors read and approved the final version of this manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00411/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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