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High denitrification and anaerobic ammonium oxidation contributes to net nitrogen loss in a seagrass ecosystem in the central Red Sea

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Running head: Nitrogen removal by seagrasses

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Abstract

Nitrogen loads in coastal areas have increased dramatically with detrimental consequences for coastal ecosystems. Shallow sediments and seagrass meadows are hotspots for denitrification, favoring N loss. However, atmospheric dinitrogen (N_2) fixation has been reported to support seagrass growth. Therefore, the role of coastal marine systems dominated by seagrasses in the net N_2 flux remains unclear. Here, we measured denitrification, anaerobic ammonium oxidation (anammox), and N_2 fixation in tropical seagrass (*Enhalus acoroides*) meadow and the adjacent bare sediment in a coastal lagoon in the central Red Sea. We detected high annual mean rates of denitrification (34.9 ± 10.3 and 31.6 ± 8.9 mg N m^{-2} d^{-1}) and anammox (12.4 ± 3.4 and 19.8 ± 4.4 mg N m^{-2} d^{-1}) in vegetated and bare sediments. The annual mean N loss was higher (8 and 63-fold higher) than the N_2 fixed (annual mean=5.9 ± 0.2 and 0.8 ± 0.3 mg N m^{-2} d^{-1}) in the meadow and bare sediment, leading to a net flux of N_2 from sediments to the atmosphere. Despite the importance of this coastal lagoon in removing N from the system, N_2 fixation can contribute substantially to seagrass growth since N_2 fixation rates found here could contribute up to 36% of plant N requirements. In vegetated sediments, anammox rates decreased with increasing organic matter (OM) content, while N_2 fixation increased with OM content. Denitrification and anammox increased linearly with temperature, while N_2 fixation showed a maximum at intermediate temperatures. Therefore, the forecasted warming could further increase the N_2 flux from sediments to the atmosphere, potentially impacting seagrass productivity and their capacity to mitigate climate change but also enhancing their potential N removal.

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1. Introduction

Nutrient supply is an important driver of marine primary production (Field et al., 1998;Howarth, 1988), where nitrogen (N) availability is believed to exert a key role in regulating net primary production (Howarth, 1988) and driving eutrophication (Howarth and Marino, 2006) in coastal ecosystems. Anthropogenic activities have led to a doubling of the global amount of fixed N, with important changes in ecosystem productivity, diversity, air quality, and, ultimately, climate (Fowler et al., 2013;Vitousek et al., 1997). Whereas natural atmospheric dinitrogen (N_2) fixation is globally estimated in 203 Tg N yr^{-1} (from which 140 Tg N yr^{-1} occurs in marine systems), the anthropogenic contribution to new N supply has been estimated in 210 Tg N yr^{-1}, mainly produced by N_2-fixing crops, combustion of fossil fuels and the Haber-Bosch industrial reaction (Fowler et al., 2013).

Coastal areas receive high inputs of fixed N by river and groundwater discharges and atmospheric deposition (Galloway et al., 2003;Voss et al., 2013), causing severe problems related to eutrophication and, potentially, dystrophic crisis (Galloway et al., 2003;Herbert, 1999). High N inputs can be partially balanced through losses, as coastal marine sediments are hotspots of denitrification (Devol, 2015), the conversion of nitrates and nitrites to N_2 (and N_2O partially), leading to the loss of fixed N. Globally, coastal denitrification has been estimated to range from 4 to 8 Tg N yr^{-1} (Voss et al., 2013), which is modest compared to the global riverine input of 66 Tg N yr^{-1} (Seitzinger et al., 2005), N_2 fixation of about 15 Tg N yr^{-1} (Voss et al., 2013), and atmospheric deposition of 1 Tg N yr^{-1} (Voss et al., 2013) to the coastal ocean. Recently, however, anaerobic ammonium oxidation (anammox), the chemoautotrophic conversion of ammonium and nitrite to N_2, has also been regarded as an important process in marine

65 sediments. It has been estimated to account from nearly zero to up to 80% of the total N loss in
66 sediments (Dalsgaard et al., 2005;Devol, 2015).
67 Within the coastal ocean, seagrass ecosystems support high rates of N_2 fixation (McGlathery,
68 2008), particularly so in tropical and subtropical ecosystems (Welsh, 2000;Herbert, 1999).
69 Nitrogen supplied by N_2 fixation can account for up to 90% of plant nutrient requirements
70 (Hansen et al., 2000). It has been suggested that seagrasses have a close mutualistic relationship
71 with N_2-fixing bacteria inhabiting the rhizosphere (Herbert, 1999) and roots (Garcias-Bonet et
72 al., 2016), where bacteria would benefit from root exudates and plants would benefit from fixed
73 N supply. Yet, seagrass ecosystems also support high denitrification rates (Eyre et al., 2011b)
74 and have been identified as key habitats for N loss in temperate (Eyre et al., 2016) and sub-
75 tropical (Eyre et al., 2011a) estuaries. Although anammox rates have not been assessed for
76 seagrass sediments as yet, Salk et al. (Salk et al., 2017) recently reported very low anammox
77 rates (0.18 μmol N m^{-2} h^{-1}), although accounting for 74% of N loss, on bare sediment adjacent to
78 a *Zostera muelleri* meadow in a sub-tropical estuarine system. Therefore, the role of seagrass
79 ecosystems as net sinks or sources of N remains unclear. Welsh et al. (Welsh et al., 2000)
80 reported very low denitrification rates compared to N_2 fixation rates in a temperate intertidal
81 seagrass meadow, whereas denitrification seems to exceed N_2 fixation in tropical (Alongi et al.,
82 2008) and sub-tropical (Eyre et al., 2011a) seagrass ecosystems. The balance between
83 transformations of inert atmospheric N_2 and reactive N in seagrass ecosystems is an important
84 driver of their net N budget (Hemminga et al., 1991), particularly so in areas with limited N
85 inputs from land. This is the case of seagrass meadows in arid regions lacking riverine inputs,
86 such as the Red Sea, characterized by general oligotrophic conditions in surface waters (Raitsos
87 et al., 2013;Weikert, 1987).

Nitrogen cycling in seagrass ecosystems is mediated by complex microbial communities requiring specific physicochemical conditions, which may ultimately determine the balance between transformations of inert atmospheric N_2 and reactive N. Seagrass meadows offer patchy sediment conditions, affected by the release of organic exudates and oxygen by seagrass roots (Pedersen et al., 1998), as well as the activity of burrowing infauna, which facilitates the exchange and diffusion of nutrients and allows the oxygenation of deep sediment layers (Welsh, 2003). These processes contribute to high spatial heterogeneity in seagrass sediments, therefore, modifying the redox potential (Enriquez et al., 2001) and allowing for the co-occurrence of processes requiring different environmental conditions (Herbert, 1999;Hemminga et al., 1991). Here, we assess the annual balance between losses of reactive N as N_2, via denitrification and anammox, and gains of reactive N, by N_2 fixation, in a tropical seagrass (*Enhalus acoroides*) meadow and the adjacent bare sediment in a coastal lagoon located in the central Red Sea. We first describe the environmental conditions in the sediments, based on microprofiles of oxygen (O_2), sulfide (H_2S), and redox, and then evaluate denitrification, anammox, and N_2 fixation rates in seagrass sediments and adjacent bare sediments. In addition, we analyze the thermal dependence of denitrification, anammox, and N_2 fixation throughout the annual *in situ* thermal range. The Red Sea is one of the warmest seas and is warming faster than other seas (Chaidez et al., 2017), thereby offering an opportunity to assess if the balance between losses of reactive N as N_2, via denitrification and anammox, and gains of reactive N, by N_2 fixation, may be affected by warming.

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2. Materials and methods

2.1. Study site

The study was conducted on an *Enhalus acoroides* seagrass meadow in Khor Almesena'a, a shallow enclosed coastal lagoon located in the central Red Sea (22º23'23.2" N, 39º08'08" E). The study site was on a monospecific seagrass meadow at 2-m depth and the adjacent (about 5 m from the edge of the seagrass meadow) bare sediment. The study was conducted between June 2016 and April 2017. We assessed sediment microprofiles (O_2, H_2S, and redox) and denitrification, anammox, and N_2 fixation rates along five sampling events, two in summer (June and August 2016), one in the fall (November 2016), one in winter (February 2017), and one in spring (April 2017). At each sampling time, we also monitored and recorded salinity and seawater temperature *in situ* every 10 min for 24 hours using an EXO1 Multiparameter Sonde (YSI, USA).

2.2. Sediment microprofiles

At each sampling event, we performed O_2, H_2S, and redox microprofiles on vegetated sediments and adjacent bare sediment reaching an average depth of 7 cm below the sediment surface, using the Field Microprofiling system by Unisense (Aarhus, Denmark). At each sampling event, we collected four sediment cores (40 cm length and 10 cm in diameter, two replicate cores per each sediment type) containing at least 15 cm of undisturbed sediment. The cores were transported immediately to the laboratory and the microprofile analysis started within the next 3 h. Oxygen microsensors (Ox-200), with a tip diameter of 200 µm, were calibrated in sterile water at oxygen partial pressures of 0 and 21 kPa. For H_2S measurements, we used H_2S-200 microelectrodes (200 µm tip diameter). Calibration of the microsensors was performed following manufacturer

specifications and following Seitaj et al. (Seitaj et al., 2015). Redox measurements were performed using Redox-200 microelectrodes (200 µm tip diameter). The electrode potentials in the sediment were measured against the Unisense Ag-AgCl reference electrode and the calibration was performed using two quinidrone solutions (10 mg ml^{-1}) buffered at pH 4 and 7, respectively. Microsensors were positioned using a manual micromanipulator (Märzhäuser, Wetzlar, Germany), and the tip position was visually controlled with a horizontally mounted USB stereomicroscope (Veho VMS-004). Oxygen, H_2S, and redox microprofiling measurements started 10 min after embedding and lasted about 4 hours per sediment core. The sediment surface position was adjusted using the software Sensor Trace Suite v2.7.100 (Unisense, Aarhus, Denmark). The microprofiles were performed under light and dark conditions per triplicate. The cores were left for 12 h under dark conditions before starting the microprofiles under dark conditions.

2.3. Denitrification and anammox rates

We measured denitrification and anammox rates by a batch incubation experiment with ^{15}N-labeled nitrogen compounds in vegetated sediment and the adjacent bare sediment. At each sampling event, we collected 6 cylindrical plastic cores (40 cm length and 5 cm in diameter, 3 replicate cores per each sediment type) containing at least 15 cm of undisturbed sediment. The cores were transported immediately to the laboratory. Denitrification and anammox rates were measured at three sediment horizons: from sediment surface to 1 cm deep, from 1 to 2 cm deep and from 2 to 3 cm deep. ^{15}N isotope pairing technique was applied for measurement of N-related activities. The principle and procedure for measuring N_2 production via anammox were essentially based on a ^{15}N-tracer incubation method reported elsewhere (Thamdrup and

157 Dalsgaard, 2002). However, in this study, we used a simpler method developed previously
158 (Amano et al., 2011;Yoshinaga et al., 2011). Briefly, about two grams of sediments was
159 dispensed into 10-ml glass serum vials, which were sealed with butyl rubber stoppers and
160 aluminum caps. All these procedures were performed in an anaerobic chamber (Coy Laboratory
161 Products, Grass Lake Charter Township, MI) where O_2 concentration was <1 ppm. Headspace in
162 the vials was exchanged with highly pure He gas (>99.9999%) by vacuuming and purging.
163 Positive pressure (50-75 kPa) was added to the headspace to prevent unintentional contamination
164 with ambient air during the incubation and gas sampling. The vials containing oxygen-free
165 sediment suspensions were pre-incubated overnight in the dark to eliminate the remaining
166 substrates. Four different combinations of ^{15}N labeled and/or unlabeled substrates were
167 supplemented from anoxic stock solutions to these pre-incubated vials: 1) 0.5 mM $^{15}NH_4Cl$ (≥98
168 atom % ^{15}N, Sigma-Aldrich, Inc.); 2) 0.5 mM $^{15}NH_4Cl$ and 0.5 mM $Na^{14}NO_2$ (Sigma-Aldrich,
169 Inc.); 3) 0.5 mM $Na^{15}NO_2$ (98 atom % ^{15}N, Sigma-Aldrich, Inc.); and 4) 0.5 mM $K^{15}NO_3$ (98
170 atom % ^{15}N, Sigma-Aldrich, Inc.). The concentration of the ^{15}N-labeled and unlabeled substrate
171 was chosen as suggested previously (Amano et al., 2007). The vials were incubated under anoxic
172 conditions at *in situ* temperature. The concentrations of $^{29}N_2$ and $^{30}N_2$ gas were determined by
173 gas chromatography-mass spectrometry (GC-MS) analysis at different intervals as described
174 previously (Isobe et al., 2011a;Isobe et al., 2011b). In combination with GC inlet and peak
175 integration software, a quadrupole MS system can be used to determine N_2 production rates as
176 low as a few nmol N l^{-1} d^{-1} (Holtappels et al., 2011). Fifty microliters of headspace gas were
177 collected using a gas-tight syringe (VICI; Baton Rouge, LA, USA), immediately injected into a
178 GC (Agilent 7890A system equipped with a CP-7348 PoraBond Q column) combined with
179 5975C quadrupole inert MS (Agilent Technologies; Santa Clara, CA, USA). The m/z = 29 and

180 30 values monitored at the same retention time for each measurement. The amounts of $^{29}N_2$ and
181 $^{30}N_2$ gas were determined using a standard curve prepared with $^{30}N_2$ standard gas (> 98% purity)
182 (Cambridge Isotope Laboratories; Tewksbury, MA, USA). The potential denitrification and
183 anammox rates were estimated from the production of $^{29}N_2$ and $^{30}N_2$ using the equations
184 described elsewhere (Holtappels et al., 2011). All the batch tests were performed in triplicate.
185 Finally, the denitrification and anammox rates were standardized to surface area integrating 3 cm
186 sediment depth by averaging the rates measured at different horizons and taking into account the
187 sediment bulk density.
188
189 **2.4. Atmospheric N_2 fixation rates**
190 We measured N_2 fixation by Acetylene Reduction Assay (Capone and Taylor, 1980) in seagrass
191 roots, seagrass shoot epiphytes, vegetated sediment, and adjacent bare sediment. At each
192 sampling time, we carefully collected shoots containing roots and placed them in zip-lock plastic
193 bags. The shoots were immediately transported to the laboratory in a cooler box protected from
194 sunlight. We also collected six cylindrical plastic cores (40 cm length and 10 cm in diameter,
195 three replicate cores per each sediment type) containing at least 15 cm of undisturbed sediment.
196 The sediment cores were transported immediately to the laboratory. Once in the laboratory the
197 roots were carefully separated from shoots and rinsed with sterilized seawater. Then, we placed
198 10 g (fresh weight) of healthy roots in a 250 ml glass bottle. Similarly, we placed one shoot
199 (without roots) in a 500 ml glass bottle. We added 100 and 250 ml of fresh seawater collected
200 from the same location to the root and shoot samples, respectively, and the bottles were closed
201 with lids fitted with gas-tight valves. In parallel, the sediment from the cores was extruded
202 carefully using a plunger, and the sediment was cut in four different horizons: from sediment

surface to 1 cm deep, from 1 to 2 cm deep, from 2 to 3 cm deep, and from 9 to 10 cm deep. We added the additional deeper sediment layer (9 to 10 cm), matching the maximum depth at which seagrass roots were detected. For each horizon, 80 ml of sediment was placed in a 500 ml glass bottle. Then, we added 200 ml of fresh seawater collected from the same location and the bottles were closed with a lid fitted with a gas-tight valve. Finally, we added acetylene-saturated seawater, prepared according to Wilson et al. (Wilson et al., 2012), through the gas-tight valve in order to achieve a final acetylene concentration of 4 mM (10 ml to roots and 20 ml to shoots and sediment incubations). We run the root and shoot incubations in triplicate. Similarly, we run the sediment incubation in triplicate for each horizon and sediment type. The roots and sediment slurries were incubated under dark conditions, and the shoots were incubated mimicking the natural photoperiod (12 h light at 200 µmol photons m^{-2} s^{-1}: 12 h dark) at *in situ* temperature. We sampled the headspace five times, distributed along the 24 h incubations. Specifically, we withdrew 3 ml of air from the headspace with a gas-tight syringe. The headspace air sample was immediately injected into a 3 ml vacuum vial for further analysis of ethylene concentration on a gas chromatographer equipped with a flame ionization detector and coupled to a mass spectrometer (MS-FID-GC, Agilent 7890) using a GS-CarbonPLOT column (60 m × 320 µm × 1.5 µm, Agilent Technologies, USA). We built a calibration curve using three ethylene standards of known concentration (1.5, 9 and 93 ppm) and Helium as a balance gas, supplied by Abdullah Hashim Industrial Gases & Equipment Co. Ltd. (Jeddah, Saudi Arabia). We estimated the concentration of dissolved ethylene before equilibrium with the headspace, from the ethylene concentration in the equilibrated air according to Wilson et al. (Wilson et al., 2012) and applying the solubility coefficient of ethylene extracted from Breitbarth et al. (Breitbarth et al., 2004) as a function of temperature and salinity.

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We run the following negative controls at each sampling event: i) roots, shoots, and sediment without addition of acetylene-saturated seawater in order to confirm that ethylene was not naturally produced by our samples, and ii) seawater collected from the study site and used in the preparation of the incubations with addition of acetylene-saturated seawater in order to measure the N_2 fixation due to pelagic diazotrophs. The ethylene production rate measured in the seawater control was subtracted from the ethylene production rates detected in our samples. The net ethylene rates (after subtracting the background seawater rate) were converted into N_2 fixation rates by applying the common ratio of 3 mol of acetylene:1 mol of N_2 (Welsh, 2000). At the end of the incubation, we dried the roots, shoots, and sediment samples at 60ºC and recorded the dry weight for further calculations. Moreover, we calculated the sediment organic matter (OM) content of each replicate sediment horizon by loss on ignition (Dean Jr, 1974). Then, the sediment N_2 fixation rates were standardized to surface area integrated over 3 cm sediment depth by averaging the rates measured at the first 3 sediment horizons and taking into account the sediment bulk density in order to compare N_2 fixation to denitrification and anammox rates. The N_2 fixation rates of roots and shoot epiphytes were standardized to surface area taking into account the biomass density.

2.5. Statistical analysis

Differences in OM content (our continuous response variable) were tested considering the categorical explanatory variables ‘sediment type’ (2 levels: vegetated and bare sediments) with the non-parametric Wilcoxon test. Furthermore, we analyzed OM content considering also as explanatory variable ‘sediment horizons’ (4 levels: from sediment surface to 1 cm deep, from 1 to 2 cm deep, from 2 to 3 cm deep, and from 9 to 10 cm deep), and ‘sampling events’ (5 levels:

249 June, August, November, February and April) by performing a Generalized Linear Model
250 (GLM) and considering their interaction. All the factors were fixed and orthogonal.
251 Differences in O_2 concentration and H_2S concentration between vegetated and bare sediments
252 and between light and dark were tested by nonparametric Wilcoxon test.
253 Differences in denitrification, anammox, and N_2 fixation rates per gram of sediment along the
254 sediment horizons between vegetated and bare sediments were tested by Wilcoxon matched-
255 pairs signed rank test. Similarly, we test for differences between denitrification, anammox, and
256 N_2 fixation rates by Wilcoxon matched-pairs signed rank test. Moreover, we analyzed the
257 difference in denitrification, anammox, and N_2 fixation rates per gram of sediment considering
258 the type of sediment and the sediment OM content as our categorical and continuous explanatory
259 variables, respectively. Since the distribution of denitrification, anammox, and N_2 fixation rates
260 per gram of sediment was not normal, we used a GLM to test for differences.
261 Finally, we analyzed the difference in depth-integrated denitrification, anammox, and N_2 fixation
262 rates, our continuous response variables, considering the type of sediment and sampling event as
263 our categorical explanatory variables. We furthermore analyzed the difference in depth-
264 integrated denitrification, anammox, and N_2 fixation rates, considering the type of sediment and
265 temperature as our categorical and continuous explanatory variables, respectively. Since the
266 distribution of the depth-integrated denitrification and N_2 fixation rates was not normal, we used
267 a GLM to test for differences, while we used a linear model test to analyze the depth-integrated
268 anammox rates as its distribution was normal. The effect of temperature and sediment type on
269 the net N_2 flux was tested by using a linear model. All statistical analyses were performed using
270 JMP (SAS Institute Inc., USA) and PRISM (GraphPad Software Inc., USA) statistical software.
271

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3. Results

3.1. Water and sediment properties

The *in situ* daily average seawater temperature ranged from 22.3°C in February to 32.5°C in June (Table 1), while annual mean salinity was 41.2 ± 0.4 PSU. The OM content was consistently higher (about 40% higher) in the vegetated sediments compared to the bare sediment (nonparametric Wilcoxon test, $p < 0.0001$), with annual mean (± SEM) OM content of 13.5 ± 0.1 and 8.5 ± 0.1% of sediment dry weight, respectively, and decreased with increasing depth (Fig. 1a). The sediment OM content significantly differed among sediment type, sampling event, and sediment horizon (GLM; sediment type*sampling event*sediment horizon $\chi^2_{12,80} = 28.7$; $p = 0.004$). The maximum depth-integrated mean OM content in vegetated sediments (15% of sediment dry weight) and bare sediments (9.5% of sediment dry weight) was found in November and June, respectively.

Sediment O_2 microprofiles significantly differed between vegetated and bare sediments during light and dark measurements (nonparametric Wilcoxon test, $p = 0.0002$ and $p < 0.0001$, respectively) and between light and dark conditions in both vegetated and bare sediments (nonparametric Wilcoxon test, $p < 0.0001$ and $p < 0.0001$, respectively). The vegetated and bare sediments were anoxic below the sediment surface but the sediment depth at which anoxic conditions prevailed varied depending on sediment type, light or dark conditions, and the time of the year (Fig. 1b, c). In vegetated sediments, the anoxic conditions appeared at deeper layers than in the bare sediments. Similarly, O_2 diffused into deeper sediment layers during light incubations than during dark incubations for both vegetated and bare sediments. On average, the vegetated sediments were anoxic at 1 ± 0.3 cm and 0.7 ± 0.1 cm below the surface under light and dark conditions, respectively, while bare sediments were anoxic at 0.6 ± 0.2 cm and 0.4 ± 0.1 cm

295 below the surface under light and dark conditions, respectively. In some vegetated sediments
296 under light conditions (Fig. 1b1, b3), the O_2 concentration increased again at deep layers, likely
297 indicating O_2 release by seagrass roots into the sediment or O_2 diffusion through animal burrows.
298 Sediment H_2S microprofiles were highly variable along the year (Fig. 1b, c). Under light
299 conditions, the H_2S concentration in bare sediments (median = 1.28 µmol H_2S L^{-1}) was
300 significantly higher than in vegetated sediments (median = 0 µmol H_2S L^{-1}) (nonparametric
301 Wilcoxon test, $p < 0.0001$). Similarly, under dark conditions, the H_2S concentration in bare
302 sediments (median = 1.17 µmol H_2S L^{-1}) was significantly higher than in vegetated sediments
303 (median = 0.008 µmol H_2S L^{-1}) (nonparametric Wilcoxon test, $p < 0.0001$). In vegetated
304 sediments, the H_2S concentration was very low (< 0.5 µM) during the summer months (June and
305 August, Fig. 1b1-2) and the maximum H_2S concentration (10.4 µM) was detected in November
306 under dark conditions (Fig. 1b3) at 2.2 cm below the sediment surface. Bare sediments showed
307 similar H_2S profiles under light and dark conditions, except for the dark measurement in
308 November. The maximum H_2S concentration in bare sediments (15.2 µM) was also detected in
309 November under light conditions, but it was higher than that in vegetated ones and at deeper
310 sediment layers (Fig. 1c3), about 6 cm below the surface. The redox potential ranged from about
311 550 mV to -450 mV (Fig. S1) and decreased abruptly with increasing sediment depth.
312
313 **3.2. Denitrification, anammox and N_2 fixation rates**
314 Sediment denitrification rates per gram of sediment were consistently higher in vegetated
315 sediments compared to bare sediments (Wilcoxon matched-pairs signed rank test, $p = 0.0015$,
316 Fig. 2a). The highest denitrification rates were detected in summer (June and August, Fig. 2a1-2)
317 for both vegetated and bare sediments. In vegetated sediments, the maximum denitrification rate

318 (20.52 ± 0.6 nmol N g DW^{-1} h^{-1}) was found in June and was almost twice the maximum rate
319 measured in bare sediments (11.5 ± 4.2 nmol N g DW^{-1} h^{-1}), which was found in August.
320 Although the rates varied throughout the year, the maximum denitrification rates took place
321 between 1 and 2 cm below surface, with minimum rates detected in the sediment surface.
322 Anammox rates per gram of sediment were large but consistently lower than denitrification rates
323 (Wilcoxon matched-pairs signed rank test, $p < 0.0001$) and without significant difference
324 between vegetated and bare sediments (Wilcoxon matched-pairs signed rank test, $p = 0.6788$,
325 Fig. 2b). In vegetated sediments, the maximum anammox rate (6.88 ± 0.5 nmol N g DW^{-1} h^{-1})
326 was detected in August and was similar to the maximum rate in bare sediments (6.89 ± 0.4 nmol
327 N g DW^{-1} h^{-1}), measured in April. The minimum denitrification and anammox rates were
328 measured in November. Sediment N_2 fixation rates per gram of sediment (Fig. 2c) were
329 significantly lower than denitrification and anammox rates (Wilcoxon matched-pairs signed rank
330 test, $p < 0.0001$ and $p < 0.0001$, respectively), with maximum N_2 fixation rates (1.25 ± 0.1 nmol
331 N g DW^{-1} h^{-1}) detected in November, in contrast to the denitrification and anammox patterns.
332 The N_2 fixation rates were significantly higher in vegetated sediments than those rates measured
333 in bare sediments (Wilcoxon matched-pairs signed rank test, $p < 0.0001$, Fig. 2c). Denitrification
334 rates per gram of sediment differed between vegetated and bare sediments at different sediment
335 horizons, however sediment OM content did not have a significant effect (GLM; sediment type
336 $\chi^2_{1,28} = 5.6$, $p < 0.05$; OM content $\chi^2_{1,28} = 3.1$, $p = 0.08$) (Fig. 3a). The sediment OM content and
337 the type of sediment had a significant effect on anammox rates (GLM; sediment type $\chi^2_{1,28} = 4.5$,
338 $p < 0.05$; OM content $\chi^2_{1,28} = 5.1$, $p < 0.05$) and N_2 fixation rates (GLM; sediment type*OM
339 content $\chi^2_{1,36} = 14.2$, $p < 0.001$). Anammox rates decreased with increasing OM content in

vegetated sediments ($Y = -1.04X + 17.8$, $p < 0.05$, Fig. 3b), while N_2 fixation rates increased with increasing OM content in vegetated sediment ($Y = 0.24X - 2.9$, $p < 0.0001$, Fig. 3c). The differences in denitrification rates between vegetated and bare sediment rates became smaller when depth-integrated (0 – 3 cm) rates were compared (Fig. 4a), largely due to the higher (1.5-fold) bulk density in bare sediments compared to vegetated sediments. Depth-integrated denitrification rates significantly differed among sampling events but not between vegetated and bare sediments (GLM; sampling event $\chi^2_{4,24} = 70.6$, $p < 0.0001$; sediment type $\chi^2_{1,24} = 3.1$, $p = 0.08$). Depth-integrated anammox rates (Fig. 4b) significantly differed among sampling events and between vegetated and bare sediments (lm, sampling event*sediment type; $F_{4,29} = 30.05$, $p < 0.0001$). Similarly, depth-integrated N_2 fixation rates (Fig. 4c) significantly differed among sampling events and between vegetated and bare sediments (GLM, sampling event*sediment type $\chi^2_{4,20} = 73.31$, $p < 0.0001$).

Temperature had a significant effect on depth-integrated denitrification rates regardless of the type of sediment (GLM; temperature $\chi^2_{1,27} = 16.67$, $p < 0.0001$; sediment type $\chi^2_{1,27} = 0.53$, $p = 0.46$;). Depth-integrated denitrification rates increased linearly with temperature ($Y = 3.569X - 65$, Fig. 5a). Temperature and sediment type had a significant effect on depth-integrated anammox rates (lm; temperature $F_{1,29} = 14.8$, $p = 0.0007$; sediment type, $F_{1,29} = 7.7$, $p = 0.01$), with rates increasing linearly in vegetated ($Y = 1.3X - 20.36$) and bare ($Y = 1.3X - 16.94$) sediments (Fig. 5b). However, depth-integrated N_2 fixation rates did not increase linearly with temperature and the differences in rates were explained by sediment type (GLM; sediment type $\chi^2_{1,27} = 4.93$, $p = 0.03$). Sediment N_2 fixation rates in vegetated and bare sediments showed a different thermal response than denitrification and anammox processes, with maximum rates reported at 28.5°C and decreasing rates at either lower and higher temperatures (Fig. 5c). N_2

fixation rates followed a second-degree polynomial curve ($Y = 16.94 - 0.45X - 0.13X^2$, r^2 = 0.40, $p < 0.05$) in vegetated sediments. N_2 fixation rates in seagrass roots and epiphytes showed the same annual pattern that the rates reported for the rhizosphere. The maximum rates in seagrass roots (21.9 ± 210.7 µg N g DW^{-1} d^{-1}) and epiphytes (10.4 ± 1.5 µg N g DW^{-1} d^{-1}) were also recorded in November when *in situ* seawater temperature was 28.5ºC (Fig. 5c).

The net N_2 fluxes ranged from 3.6 ± 0.8 and 19.73 ± 0.9 mg N m^{-2} d^{-1} in November, to 85.1 ± 3.7 and 85.1 ± 2.6 mg N m^{-2} d^{-1} in summer months for the seagrass meadow and bare sediments, respectively (Fig. 6). The net N_2 flux significantly differed among sampling events but not between sediment type (lm; sampling event $F_{4,9}$ = 24.76, p = 0.004; sediment type, $F_{1,9}$ = 1.83, p = 0.25). Net N_2 flux increased linearly with temperature ($Y = 4.99X - 91.86$, r^2 = 0.43, $p <$ 0.05, Fig. 7).

4. Discussion

The sediment organic matter content in the Red Sea lagoon system studied here was extremely high, especially in the vegetated sediments (mean = 13.5%), compared to the values reported for other seagrass sediments (mean = 4.1%, (Kennedy et al., 2010)). The higher sediment organic matter content in vegetated sediments, compared to bare sediments, corroborates the evidence that seagrasses accumulate and store large amounts of organic carbon in their sediments (Duarte et al., 2005). In addition, seagrasses modified the physicochemical conditions of the sediment (Enriquez et al., 2001), as reflected in higher O_2 and lower sulfide sediment concentrations than those in the adjacent bare sediment. Moreover, O_2 profiles showed higher variability in vegetated sediments. This can be directly related to bioturbation (Kristensen et al., 2012) and to the radial

oxygen loss by roots leading to oxic layers at depth (Pedersen et al., 1998), enhancing the complexity and heterogeneity of seagrass sediments.

The denitrification rate in *E. acoroides* sediments reported here (annual mean = 34.9 ± 10.3 mg N m^{-2} d^{-1}) is 6-fold higher than the rate reported for a restored *Zostera marina* meadow in Virginia using an *in situ* push-pull incubation method (Aoki and McGlathery, 2017), 1.3 to 2.5-fold higher than the rate previously reported for tropical meadows dominated by *E. acoroides* on slurries from the top 5 cm sediment (Alongi et al., 2008), comparable to the rates reported for temperate seagrasses (Eyre et al., 2016), and 8-fold lower than the rates reported for sub-tropical estuarine seagrasses (Eyre et al., 2011a) using *in situ* benthic chambers. Denitrification rates were higher in vegetated sediments than bare sediments when expressed per gram of dried sediment. However, we did not find differences between depth-integrated denitrification rates in vegetated and bare sediments (annual mean = 34.9 ± 10.3 and 31.6 ± 8.9 mg N m^{-2} d^{-1}, respectively) contrary to previous findings (Eyre et al., 2011b).

The potential sediment anammox rates reported here, ranging from 0.5 to 6.9 nmol N g DW^{-1} h^{-1}, are comparable to potential anammox rates reported elsewhere (i.e. from 0 to 8 nmol N cm^{-3} h^{-1} in subtropical mangrove sediments (Meyer et al., 2005); from <0.5 to 7.6 nmol N g DW^{-1} h^{-1} in marine sediments from a Fjord in Sweden (Brandsma et al., 2011) and from 0 to 3 nmol N cm^{-3} h^{-1} in sandy sediments in the southern North Sea (Bale et al., 2014)). However, the potential sediment anammox rates detected here (annual mean depth-integrated anammox rates = 12.4 ± 3.4 and 19.8 ± 4.4 mg N m^{-2} d^{-1} in vegetated and bare sediments, respectively) are higher than the only estimate available, to the best of our knowledge, for a seagrass ecosystem (0.06 mg N m^{-2} d^{-1} in bare sediment adjacent to a sub-tropical seagrass meadow in Australia on intact cores (Salk et al., 2017)). The contribution of anammox to the total loss of N reported here (27 and

38% in vegetated and bare sediments, respectively, Fig. S2) is smaller than the contribution reported by Salk et al. (Salk et al., 2017), with anammox accounting for 64 to 86% of the total N loss, but still within the range of anammox-supported N losses reported for other marine sediments (Devol, 2015;Bale et al., 2014).

The maximum N_2 fixation rates reported for *E. acoroides* sediments here (6.3 ± 0.5 mg N m^{-2} d^{-1}) are lower than the previously reported maximum N_2 fixation rates in sediments of a tropical mixed meadow dominated by *E. acoroides* (19.4 ± 3.2 mg N m^{-2} d^{-1}, (Alongi et al., 2008)). Similarly, Moriarty and O'Donohue (Moriarty and O'Donohue, 1993) reported higher N_2 fixation rates for a mixed meadow dominated by *E. acoroides* (25 ± 2.6 mg N m^{-2} d^{-1}) than those reported here during the same time of the year (16.4 ± 0.4 mg N m^{-2} d^{-1},) although with a smaller contribution from leaf epiphytes (4.2 ± 0.1 mg N m^{-2} d^{-1}) compared with our N_2 fixation rates from epiphytes (7.9 ± 1.1 mg N m^{-2} d^{-1}). The N_2 fixation rates supported by roots are in agreement with previous findings of N_2-fixing bacteria in association with seagrass roots (Garcias-Bonet et al., 2012;Garcias-Bonet et al., 2016). Moreover, the N_2 fixation rates previously reported for surface-sterilized *E. acoroides* roots (0.13 mg N m^{-2} d^{-1} (Raja et al., 2012)) are 17-fold lower than the rates reported here (2.3 ±1.5 mg N m^{-2} d^{-1}) for the same seawater temperature (29°C), pointing out at the role of bacteria inhabiting the rhizoplane of *E. acoroides* roots in nutrient supply.

The highest N_2 fixation rates in vegetated and bare sediments coincided with the highest sediment sulfide concentrations (10.4 and 15.2 µmol H_2S L^{-1} in vegetated and bare sediments, respectively), suggesting a contribution of sulfate-reducing bacteria to the pool of N_2-fixing bacteria, as reported in other seagrass rhizospheres (Welsh et al., 1996). Although sulfide has detrimental effects on seagrasses (Garcias-Bonet et al., 2008), the sulfide concentrations in the

vegetated sediments were generally below the threshold limit of 10 µmol H_2S L^{-1} for seagrass decline (Calleja et al., 2007).

The contrasting annual patterns in denitrification and anammox compared to those of N_2 fixation, with highest rates of denitrification and anammox in summer and spring while maximum N_2 fixation in autumn (Fig. 3), suggest differential specific thermal responses. The linear increase of denitrification and anammox with temperature found here was already described for net sediment N_2 fluxes in estuaries (Nowicki, 1994). Similarly, the thermal response of N_2 fixation found here, with maximum rates at intermediate temperatures (29ºC) and a decrease in rates at lower and higher temperatures, is in agreement with the notable decrease in N_2 fixation rates at 35ºC reported for cyanobacteria in soil crusts (Zhou et al. 2016). Moreover, the sediment microbial activity is modulated, as well, by the quantity and quality of the OM. Decomposition and remineralization rates of OM depends on its lability (Herbert, 1999) which is indicated by the C:N:P ratio and differs among sources (Enríquez et al., 1993). OM from phytoplankton decomposes faster than OM from seagrasses, due to their higher N content and therefore lower C:N:P ratios. Eyre et al. (Eyre et al., 2013) demonstrated that the source of the OM, and therefore, its C:N ratio controls denitrification rates in coastal sediments. Tibbles et al. (Tibbles et al., 1994) showed an increase in sediment N_2 fixation following the addition of complex plant polysaccharides and Fulweiler et al. (Fulweiler et al., 2013) argued that an increase in the C:N ratio of OM was responsible for the decrease in denitrification and the increase in N_2 fixation, in agreement with the effect of OM reported here.

The net N_2 fluxes in the Red Sea lagoon ecosystem indicates this ecosystem supports a net loss of reactive N as N_2 gas throughout the year, as sediment denitrification and anammox consistently exceeded N_2 fixation in sediment and seagrass tissues. Alongi et al. (Alongi et al.,

454 2008) also reported higher denitrification than N_2 fixation rates in an *E. acoroides* meadow.
455 Integrating the average seasonal rates, we estimate the annual N loss in 14.9 g N m^{-2} yr^{-1} in the
456 seagrass meadow and 18.2 g N m^{-2} yr^{-1} in bare sediments. Despite the lack of rivers discharging
457 into the Red Sea, the occasional heavy rains, groundwater discharge, and atmospheric deposition
458 might lead to high reactive N loads reaching coastal systems (Voss et al., 2013). Therefore, the
459 high denitrification and anammox rates in this coastal lagoon provide a mechanism adding
460 resistance to excess N inputs (Zarnoch et al., 2017;Galloway et al., 2003), which might lead to
461 permanent seagrass losses due to hypoxia after algal blooms produced by severe eutrophication
462 (Herbert, 1999;Duarte, 1995). Nevertheless, N_2 fixation plays an important role in supporting
463 seagrass meadows in the Red Sea, as the maximum N_2 fixation rate reported here could
464 contribute from 7 to 36.4% of the N requirements to support *E. acoroides* growth, calculated
465 using previous estimates of biomass productivity (Alongi et al., 2008;Pedersen et al., 2016) and
466 measured N content of leaf tissues in this central Red Sea lagoon (Almahasheer et al., 2017).
467 Furthermore, the N isotopic composition reported for *E. acoroides* tissues in this location
468 ($\delta^{15}N_{leaves}$ = 0.17‰ and $\delta^{15}N_{rhizomes}$ = -1.56‰ (Almahasheer et al., 2017)), provides evidence for
469 the atmospheric origin of the assimilated N.
470 The differential apparent thermal response of denitrification and anammox, which increased with
471 increasing temperature, and N_2 fixation, which showed a maximum at about 28°C, leads to an
472 increase in the net N_2 flux with temperature (fig. 6). This has important implications in a context
473 of rapid warming of the Red Sea (Chaidez et al. 2017). In particular, a further increase in
474 temperature, with maximum temperatures in excess of 33°C at present (Chaidez et al. 2017),
475 might lead to a further imbalance in N cycling in tropical seagrass ecosystems, similar to that
476 predicted for soil crusts (Zhou et al., 2016). Therefore, the forecasted warming might have an

important impact on N availability and therefore on seagrass productivity and their capacity to mitigate climate change.

Conclusion

The studied coastal lagoon ecosystem supported a net loss of reactive N as N_2, with anammox accounting for about one-third of N_2 production. However, N_2 fixation supported part of seagrass growth. The results presented suggest that, as a consequence of the differential thermal responses of processes supporting losses and gains of reactive N, future warming can enhance the role of seagrass meadows as sites of reactive N loss in an already warm Red Sea.

Author contribution

NG-B and CMD designed the study. NG-B and MF performed the fieldwork. NG-B performed the N_2 fixation measurements. MF performed the sediment microprofiles. MA and DRS performed the denitrification and anammox activity measurements. NG-B, MF and CMD interpreted the results. NG-B wrote the first draft of the manuscript. All authors contributed substantially to the final manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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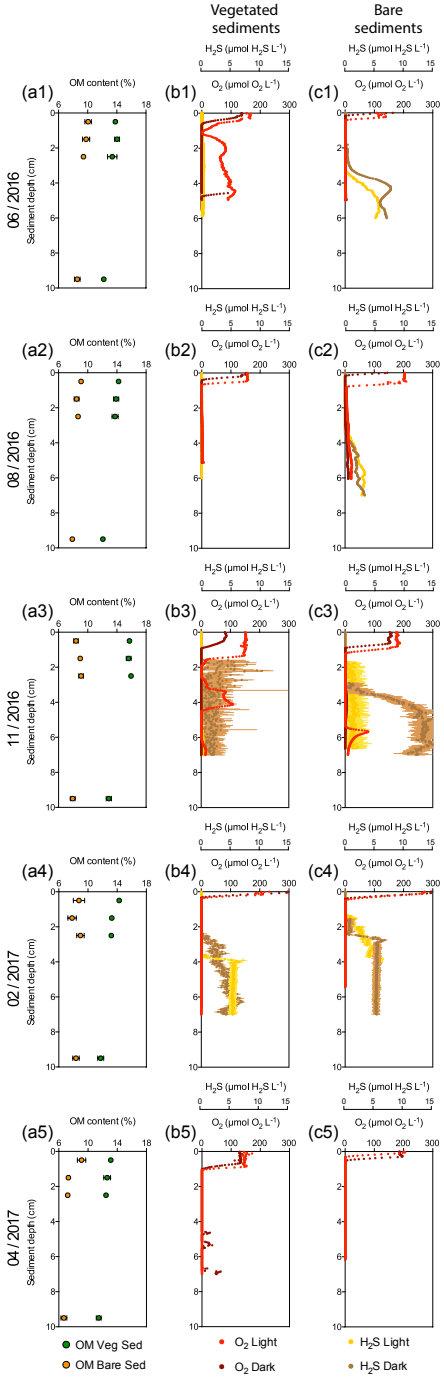
694 **Figure 1**



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696 **Fig. 1.** Characterization of *Enhalus acoroides* seagrass vegetated sediments and adjacent bare
697 sediments at five samplings times along the year. **a1-5**. Sediment organic matter content in
698 vegetated (green dots) and bare (orange dots) sediment horizons. **b1-5**. Vegetated sediment O_2
699 microprofiles under light (light red) and dark (dark red) incubations and H_2S microprofiles
700 during light (yellow) and dark (brown) incubations (no data available for H_2S profiles on the last
701 sampling). **c1-5**. Bare sediment O_2 microprofiles under light (light red) and dark (dark red)
702 incubations and H_2S microprofiles under light (yellow) and dark (brown) incubations (no data
703 available for H_2S profiles on the last sampling).
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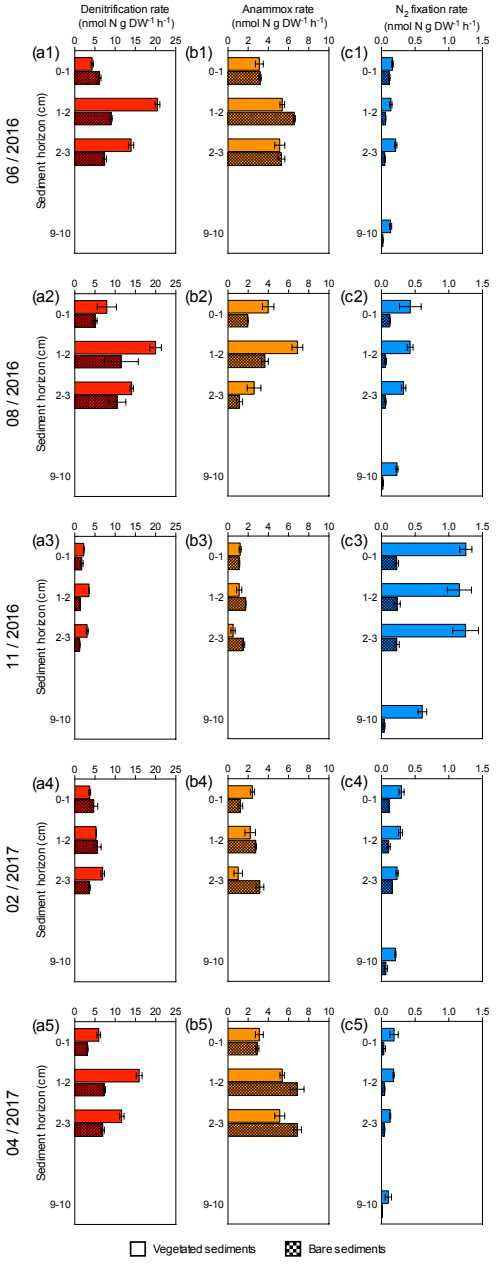
706 **Fig. 2**



707
708 **Fig. 2.** Sediment profiles of denitrification, anammox and N_2 fixation rates at five samplings
709 times. **a1-5**. Sediment denitrification rates in vegetated (red) and bare (red square pattern)
710 sediment horizons. **b1-5**. Sediment anammox rates in vegetated (orange) and bare (orange square
711 pattern) sediment horizons. **c1-5**. Sediment N_2 fixation rates in vegetated (blue) and bare (blue
712 square pattern) sediment horizons. Error bars indicate SEM.

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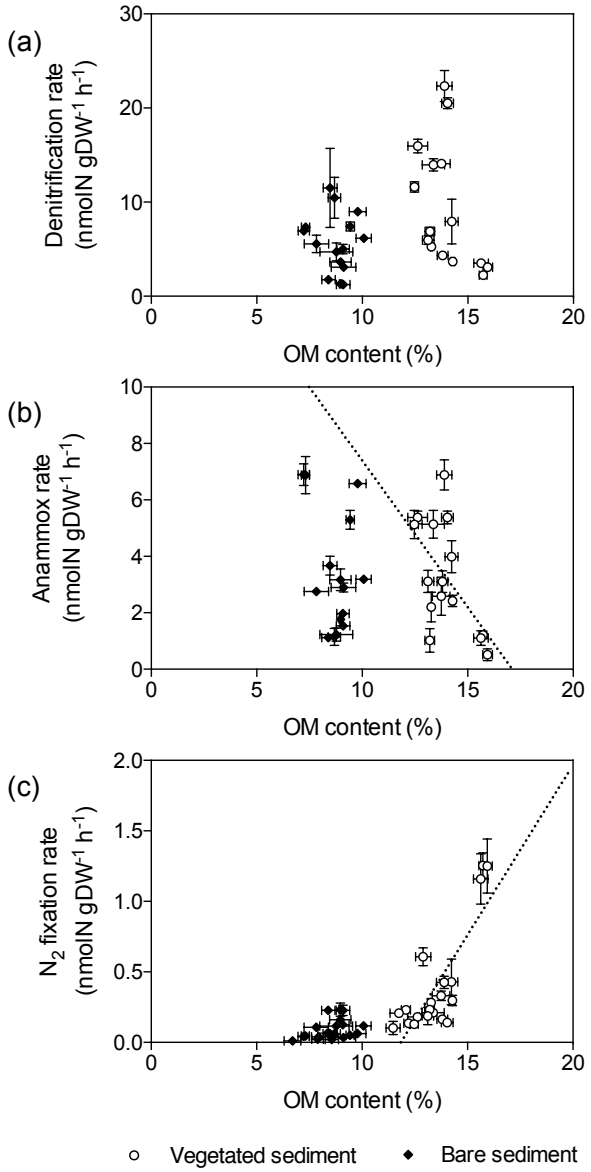
713 **Fig. 3**



714
715 **Fig. 3.** Relation of denitrification, anammox, and N_2 fixation rates with sediment OM content. **a**.
716 Denitrification rates in vegetated sediments (white dots) and bare sediments (black diamonds). **b**.
717 Anammox rates in vegetated (white dots) and bare (black diamonds) sediments, showing the
718 linear decrease of anammox rates in vegetated sediments with increasing OM content (dotted
719 line) **c**. N_2 fixation rates in vegetated sediments (white dots) and bare sediments (black
720 diamonds), showing the linear increase of N_2 fixation rates in vegetated sediments with
721 increasing OM content (dotted line). Error bars indicate SEM.

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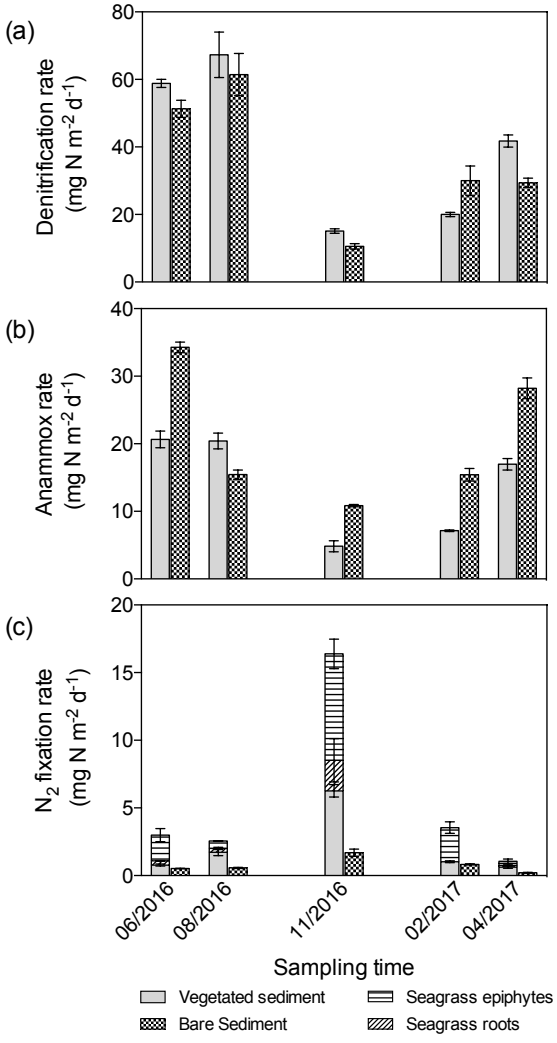
722 **Fig. 4**



723
724
725 **Fig. 4.** Area integrated sediment rates along the year. **a**. Denitrification rates in vegetated (gray)
726 and bare (square pattern) sediments. **b**. Anammox rates in vegetated (gray) and bare (square
727 pattern) sediments. **c**. N_2 fixation rates in vegetated (gray) and bare (square pattern) sediments
728 and in seagrass roots (angled stripes) and epiphytes (horizontal stripes). Error bars indicate SEM.
729

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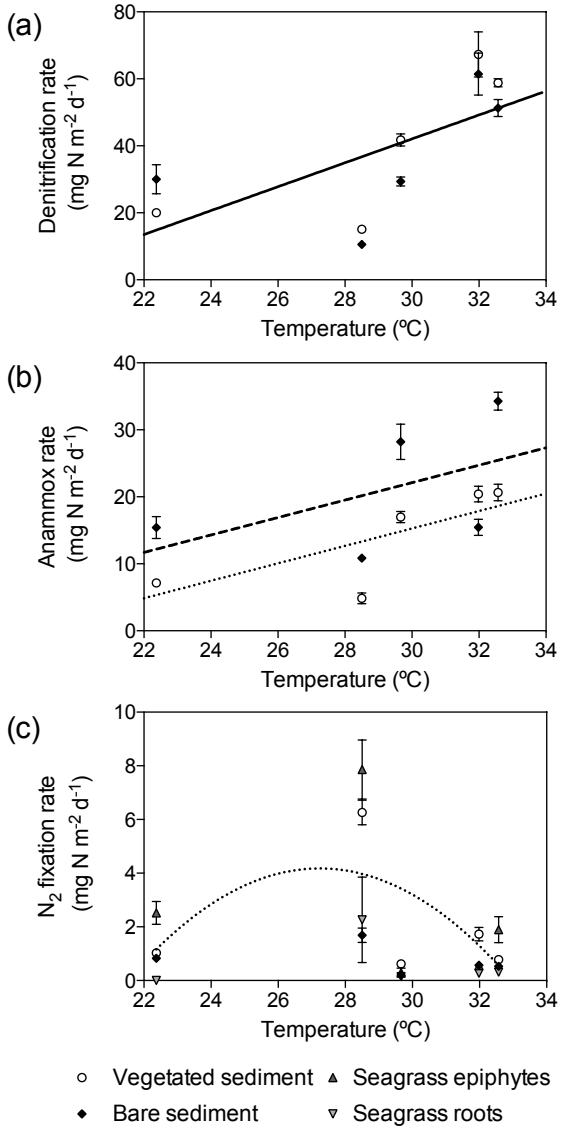
730 **Fig. 5**



731
732
733 **Fig. 5.** Relation of denitrification, anammox, and N_2 fixation rates with *in situ* seawater
734 temperature. **a**. Linear increase of denitrification rates (solid line) with temperature, showing
735 denitrification rates in vegetated sediments (white dots) and bare sediments (black diamonds). **b**.
736 Linear increase of anammox rates in vegetated (dotted line and white dots) and bare (dashed line
737 and black diamonds) sediments. **c**. Thermal response of N_2 fixation rates in vegetated sediments
738 (white dots), bare sediments (black diamonds), seagrass epiphytes (triangles) and roots (upside
739 down triangles), showing the fitted second-degree polynomial curve in vegetated sediment
740 (dotted line). Error bars indicate SEM.

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741 **Fig. 6**
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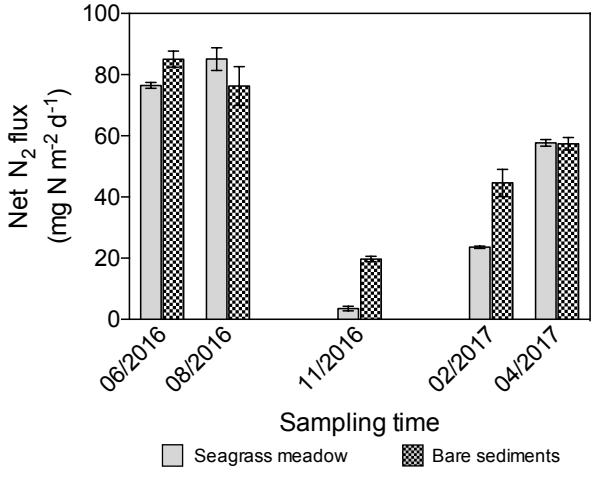

743
744
745 **Fig. 6.** Net N_2 flux in seagrass meadow (gray) and bare sediment (square pattern gray) along the
746 year, considering sediment denitrification and anammox as N losses and sediment and seagrass
747 roots and epiphytes N_2 fixation as new N inputs. Error bars indicate SEM.

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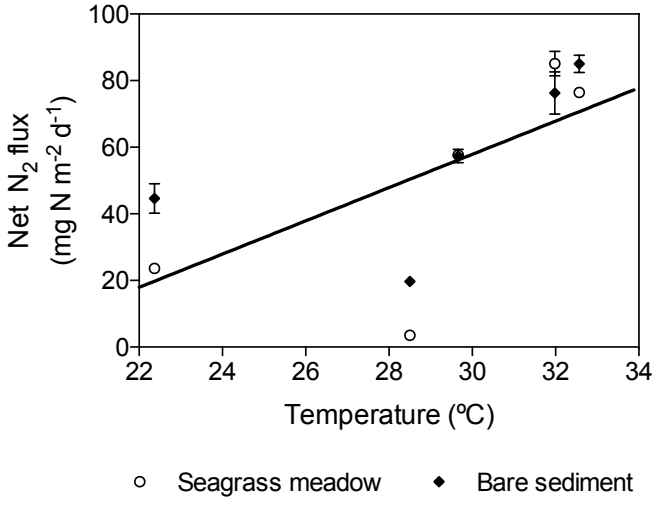
748 **Fig. 7**



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750
751 **Fig. 7.** Linear increase (solid line) of net N_2 fluxes in vegetated (white dots) and bare (black
752 diamonds) sediments. Error bars indicate SEM.
753

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754 **Table 1.** Annual variation of *in situ* seawater temperature. Mean seawater temperature values are
755 daily averages of *in situ* seawater temperature and temperature range indicate daily oscillations
756 (minimum – maximum). Seawater temperature was recorded every 10 min during 24 h for each
757 sampling event.
758

Sampling time	Mean Seawater Temperature (°C)	Seawater Temperature Range (°C)
06/2016	32.5	31.6 – 33.6
08/2016	31.9	31.1 – 32.8
11/2016	28.4	27.7 – 29.2
02/2017	22.3	21.3 – 22.8
04/2017	29.5	28.7 – 30.0