High Temperature and Crab Density Reduce Atmospheric Nitrogen Fixation in Red Sea Mangrove Sediments

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Abstract

Mangrove ecosystems are highly productive and provide important ecosystem services. However, in the Red Sea mangroves are under severe nutrient-limiting conditions, reflected in dwarf plants. The nutrient limitation is especially acute for iron, as verified experimentally, although the low carbon-to-nutrient stoichiometric ratios reported for Red Sea mangrove leaves are indicative of general nutrient depletion. Therefore, atmospheric nitrogen (N₂) fixation in mangrove sediments might be particularly important considering the minimal nitrogen inputs from land. Here, we tested the effect of temperature and crab density on sediment N₂ fixation rates in mature and juvenile mangrove (*Avicennia marina*) stands in the central Red Sea. The average N₂ fixation rates (from 0.002 ± 0.002 to 0.46 ± 0.12 mg N m⁻² d⁻¹) fall in the low range of N₂ fixation rates reported in mangroves elsewhere, which is in agreement with the small size of the mangrove plants. Mature mangrove sediments hold higher N₂ fixation rates than the juvenile mangrove sediment, related to a higher sediment organic matter and carbon content. We found a detrimental effect of temperature and crab density on sediment N₂ fixation rates. Maximum N₂ fixation rates were detected at 28°C with a sharp decrease at 35°C. Similarly, high crab-density reduced N₂ fixation, likely due to the sediment oxygenation or the grazing of cyanobacteria by crabs. This is supported by i) previously reported higher oxygen concentration and redox around burrows compared to undisturbed sediment and ii) lighter sediment carbon isotopic composition in high crab-density than in low crab-density sediments, indicating a higher contribution of microphytobenthos in the mature sediments supporting low crab-density. Our data document temperature and crab density as factors affecting N₂ fixation in the Red Sea mangrove sediments.

1. Introduction

Mangroves are highly productive ecosystems occupying the upper intertidal zone in the land-sea interface of tropical and subtropical regions (Giri et al. 2011). They provide important ecosystem services, being habitat for a wide range of living organisms such as birds, crabs, and fish (Polidoro et al. 2010; Reef, Feller, and Lovelock 2010), protecting the coast from erosion due to exposure to tides, storms, and currents (Mazda et al. 1997), providing wood for construction and fuel (Walters et al. 2008), and acting as intense carbon sinks (Bouillon et al. 2008). Due to their large carbon storage capacity, mangroves rank among the most carbon-rich forests in the tropics (Donato et al. 2011).

A significant part of mangrove biomass is exported to adjacent coastal areas, as litter and particulate and dissolved organic matter, or stored in sediments. At the same time, mangroves receive organic matter from adjacent coastal areas by tides and rivers. The magnitude of these fluxes is highly variable (Lee 1995; Kristensen et al. 2008). However, the continuous export and sink of nutrients could lead to nutrient limitation for plant growth and production. As they often grow in river deltaic areas, they receive high inputs of nutrients and sediment that contribute to maintain a positive nutrient balance required to support high primary production, nutrient sequestration in sediments and export to adjacent marine habitats (Jennerjahn and Ittekkot 2002). However, nutrient supply to mangrove stands is restricted in arid and/or karstic areas, where freshwater runoff and the associated nutrient and sediment delivery is limited, often resulting in dwarf, nutrient-limited mangrove trees (Almahasheer, Duarte, and Irigoien 2016a, 2016b).

Central Red Sea mangroves are stunted and severely nutrient-limited. Experimental nutrient additions showed that they are iron-limited (Almahasheer, Duarte, and Irigoien

2016a) due to absence of inputs from land and low nutrient concentration in Red Sea seawater (Mandura 1997; Saifullah 1997). Iron is a co-factor in the nitrogenase enzyme (Howard and Rees 1996), and its deficiency limits atmospheric nitrogen (N_2) fixation, a key process for nitrogen (N) supply, particularly where terrestrial inputs are minimal. *Avicennia marina* is the most abundant mangrove species (El-Juhany 2009) in the 135 km² mangrove forest of the Red Sea (Almahasheer, Duarte, and Irigoien 2016a) and, considering the low nutrient inputs to the area, N_2 fixation could be an essential N source to support its growth. Recent studies in the Red Sea pointed out an increasing importance of N_2 fixation as a source of N in mangrove ecosystems towards the north, based on the stable N isotopic composition of mangrove leaves and sediments (Duarte et al. 2018; Garcias-Bonet et al. 2019a). However, to the best of our knowledge, actual N_2 fixation rates in Red Sea mangrove sediments have not been estimated yet.

N_2 fixation has been detected in sediments, roots, rhizosphere and decomposing leaves in mangrove systems elsewhere and represents, therefore, a key, rate-limiting step in the N cycle (Alongi et al. 2002; Ray et al. 2014). It also occurs in large cyanobacterial mats that typically cover mangrove sediments and contributes to mangroves primary productivity (Alvarenga et al. 2015). High concentrations of calcium, carbon, magnesium, nitrogen, phosphorus, and sulphur, as well as several soluble extracellular carbohydrates, have been detected in cyanobacterial mats providing nutrients to mangroves (Lovelock et al. 2010). A substantial heterotrophic bacterial community exploits this capacity by colonizing and producing strong attachments to the sheaths and mucilage of some cyanobacteria (Simmons et al. 2008).

Mangrove ecosystems support abundant fauna (Nagelkerken et al. 2008), whose activity alter the sediment structure, chemical composition and affect biogeochemical processes (Aller 1994; Kristensen 2008; Bertics et al. 2010). Macrobenthic animals such as crabs, shrimps and mollusks bioturbate the sediment by excavating galleries, thereby increasing oxygen supply and oxidation of sediment materials (Laverock et al. 2011), and by feeding on the first layer of the sediment, which removes microbes, algae and meiobenthic fauna (Reinsel 2004).

The effect of bioturbators on N_2 fixation in mangrove soils has not been extensively studied. Available data provide evidence that sediment aeration inhibits nitrogenase enzyme activity (Goldberg, Nadler, and Hochman 1987; Bertics et al. 2010). However, oxygen inputs also oxidize iron sulfide produced in anoxic sediments, favoring the release of iron and its uptake in the presence of organic ligands (Luther et al. 1992). Indeed, bioturbators have been reported to enhance metal, including iron, release (Boto and Robertson 1990). In Brazilian mangroves iron sulfide oxidation has been reported to be enhanced under elevated temperatures, when mangrove evapotranspiration is highest, and increased crab activity (*Uca* spp.), which leads to release of iron from pyrite (Ferreira et al. 2007). As iron is a limiting nutrient in the Central Red Sea and required to synthesize nitrogenase, aeration of sediments through bioturbation may enhance iron mobilization and, therefore, nitrogenase synthesis. However, crab feeding on cyanobacterial mats may reduce N_2 fixation.

In addition to bioturbators, temperature maybe an important driver of N_2 fixation in Red Sea mangrove sediments, as it is considered the warmest of all seas, reaching up to 35°C in summer (Burkholz et al. 2019; Chaidez et al. 2017). There is growing evidence

that N₂ fixation is temperature-dependent, with maximum rates typically achieved at about 25°C across ecosystems (20 to 30°C, (Houlton et al. 2008; Zhou et al. 2016)). This is consistent with findings for seagrass in the Red Sea (Garcias-Bonet et al. 2018) and the Mediterranean Sea (Garcias-Bonet et al. 2019b), showing declining N₂ fixation rates at temperatures > 30°C. Hence, this suggests that N₂ fixation in Red Sea mangrove forests can also be suppressed at temperatures > 30°C.

Based on these premises, here we aim to determine if temperature and crab density influence N₂ fixation rates in *Avicennia marina* mangrove sediments in the central Red Sea coast. In particular, we hypothesize that: i) N₂ fixation is suppressed at high temperature, and ii) high crab-density affects N₂ fixation, although the effects may vary from negative, where crabs remove N-fixing microorganism and therefore reduce N₂ fixation in mangroves, to positive where bioturbation increases nutrient supply to support N₂ fixation, to neutral when both effects cancel each other. To test our hypothesis, we experimentally set up plots with different bioturbation levels by manipulating the density of *Cranuca inversa* and *Dotilla sulcata* crab species, in a mature and a juvenile mangrove stand and we measured N₂ fixation rates in the first 10 cm of mangrove sediments at two *in situ* temperatures (28°C and 35°C). We note that our measurements are not designed to resolve all components of N₂ fixation in the mangrove ecosystems, as we do not consider N₂ fixation associated with mangrove roots (Alfaro-Espinoza and Ullrich 2015) or leaf litter (Pelegrí, Rivera-Monroy, and Twilley 1998), as these would differ greatly between plots supporting mature trees and young seedlings.

2. Material and methods

2.1. Study site

The study site is located at the ‘Ibn Sina field research station and nature conservation area’ in KAUST (Thuwal, Saudi Arabia, 22° 20’ 25.032’’ N; 39° 5’ 17.411’’E). This site is a natural mangrove fringe entirely vegetated by *A. marina* species and supports a high density of ecosystem engineering burrowing crabs, *C. inversa* and *D. sulcata*. Two experimental sites were chosen, one colonized by natural mature trees of *A. marina* (mature stand) and the second colonized by one-year old planted propagules of *A. marina*. (juvenile stand). For establishment of the juvenile stand, propagules, collected from the same location, were grown in a nursery in autochthonous sediment.

Subsequently, one-month old seedlings were transplanted into the mangrove study site.

To evaluate the effect of crab density on sediment N₂ fixation rates, in each experimental site (mature and juvenile stands) we randomly selected 5 plots of 4 m² (2×2 m), which we enriched with *C. inversa* and *D. sulcata* crabs (high crab-density mangrove plots) and 5 plots of 4 m² (2×2 m) with natural crab densities (low crab-density mangrove plots). One year prior to performing the experiment, we created plots artificially enriched with these two species of crabs, continuously surveying crab numbers following Skov et al. (2002). Throughout the study period the crab densities in the low crab-density plots of the mature and juvenile mangrove stands were 3.3 ± 1.1 and 11.5 ± 0.6 crabs m⁻² (average $\pm$ SEM), respectively, while in the high crab-density plots of the mature and juvenile mangrove stands, i.e. enriched in crabs, crab densities were 17.6 ± 5.5 and 32.6 ± 1.6 crabs m⁻² (average $\pm$ SEM), respectively (see Fig. S1 for details). The size of crab burrows was on average 1 cm in diameter $\times$ 5 cm in depth, with a determined 10 cm diameter halo of influence resulting in an area of 78.5 cm² (Booth et al. 2019). The enrichment approach was adopted for two main reasons. Firstly, it is almost impossible to

effectively remove crabs from sediment without altering the structure, since it requires digging up at least the first 20 cm of sediment, compromising the N-fixing microbiome object of this study. Secondly, crab enrichment is effective due to the high burrow fidelity of these animals which tend to maintain the same burrow if the environmental conditions are favorable (Booth et al. 2019). Crab activity was monitored before the experiment by manually plugging the burrow with a thin layer of sediment and waiting for 30 minutes. Active crabs quickly remove this layer of sediment to exit and forage on the surrounding area. Therefore, the number of burrows opened indicates how many crabs were effectively active (Andreetta et al. 2014).

We performed two independent assessments of N₂ fixation rates in sediments collected on experimental plots: the first sampling was performed in November/December 2016 when seawater temperature was 35°C, and the second was performed in January/February 2017 when seawater temperature was 28°C. Based on prior research in the Red Sea (Garcias-Bonet et al. 2018) and elsewhere (Houlton et al. 2008; Zhou et al. 2016; Garcias-Bonet et al. 2019b), we expected N₂ fixation at 35°C, which corresponds to the maximum water temperature observed in the Central Red Sea (Chaidez et al. 2017), to be low, compared to those rates at 28°C. These temperature treatments were guided by previous results in seagrass sediments close to our study site, which showed maximum N₂ fixation rates at 28°C and minimum rates at 33°C, the maximum temperature tested (Garcias-Bonet et al. 2018). The *in situ* air and sediment temperature were measured and continuously recorded every 15 minutes by an on-site weather station (Ibimet, Florence, Italy). Sediment temperature was measured at the sediment surface and 15 cm below ground. The *in situ* seawater salinity was measured

from 13 November to 12 December 2016 (corresponding to the first sampling time) and from 28 January to 27 February 2017 (corresponding to the second sampling time) by deploying an EXO1 multiparameter probe (Xylem Inc., USA) at the closest distance from the mangrove edge where it remained completely submerged. Salinity was measured and recorded every 5 minutes for two periods of one month each.

2.2. Sediment characterization

The organic matter (OM) content in mangrove sediments was calculated by loss on ignition (Dean 1974). The sediment organic carbon (C) and nitrogen (N) contents were measured using a CHNS Elemental Analyzer (Flash 2000, KAUST analytical core laboratory, Saudi Arabia). Sediment samples were dried, ground and acidified before the analysis to remove carbonates. Sediment OM, C, and N contents were measured on five replicates per treatment. Sediment C and N isotopic composition ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) was analyzed on five replicates per treatment only for samples collected in January and February 2017, when seawater temperature was 28°C. Dried and ground sediment samples were acidified and analyzed using an isotope ratio mass spectrometer (Thermo-Finnegan Delta V IRMS, UH-Hilo Analytical laboratory, Hawaii). Results of the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic analysis are reported in parts per thousand (‰) and Vienna Pee Dee Belemnite limestone (V-PDB) was used as a standard for the stable C isotope and atmospheric N_2 for the stable N isotope.

2.3. Sediment N_2 fixation rates

N_2 fixation rates in mangrove sediment samples were estimated using the acetylene reduction assay (ARA) (Boto and Robertson 1990; Capone 1993; Howard and Rees 1996). The sediments were sampled during high tide using cores (cylindrical Plexiglas

206 cores of 10.4 cm in diameter and 27 cm in height) to avoid disruption of the sediment
207 structure. For each sampling and sediment type, five replicated cores were collected from
208 random positions in high and low crab-density plots in mature and juvenile mangrove
209 stands and immediately transported to the laboratory and processed. The sediment
210 samples were extracted from the cores and processed by slicing 10 cm from the surface.
211 Sediment samples (200 ml of the first 10 cm of sediment) were placed in 500 ml Pyrex
212 glass incubation jars and amended with 80 ml of seawater collected from the same
213 sampling location. The Pyrex jars containing the sediment slurry were closed with lids
214 equipped with a gas-tight valve to allow sampling of headspace air. To avoid
215 underestimation of N₂ fixation rates due to poor diffusion, acetylene was added to the
216 incubation jars as acetylene-enriched seawater prepared according to Wilson et al. (2012),
217 thereby reducing equilibration time. Briefly, acetylene-enriched seawater was prepared by
218 bubbling acetylene gas (99.9%, supplied by Abdullah Hashim Industrial Gases &
219 Equipment Co. Ltd. Jeddah, Saudi Arabia) for five minutes. We added 20 ml of
220 acetylene-enriched seawater to each incubation jar through the gas-tight valve to obtain a
221 final acetylene concentration of 4 mM. After the addition of acetylene, samples were
222 incubated in Percival chambers for 24 h at the temperatures recorded *in situ* (35°C or
223 28°C) under light (200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and dark conditions following the natural
224 photoperiod (12 h light, 12 h dark). The following negative controls were run for each
225 sampling: 1) sediment samples without addition of acetylene-saturated seawater in order
226 to confirm that ethylene was not naturally produced; 2) seawater used in the preparation
227 of the sediment slurries with addition of acetylene-saturated seawater in order to measure
228 the N₂ fixation due to pelagic diazotrophs. During the 24 h incubations, we sampled the

headspace of each jar at five different time points by taking three ml of the headspace air. The headspace air samples were injected in gas-tight vacuum vials using a syringe to be analyzed later for ethylene production. The concentration of ethylene in the headspace air samples was analyzed on a gas chromatographer coupled to a flame ionization detector and a mass spectrometer (MS-FID-GC, Agilent 7890) using HP-AL/S (30 m $\times$ 0.250 mm $\times$ 5.0 μ m) and GS-CarbonPLOT (60 m $\times$ 0.320 mm $\times$ 1.50 μ m) columns (Agilent Technologies, USA). We used two different columns due to the over use of the first column. The ethylene concentration of each headspace air sample was calculated using a standard curve, which was built from the peak area of three ethylene gas standards of known concentrations (93 ppm, 9 ppm and 1.5 ppm containing Helium as balance gas (Abdullah Hashim Industrial Gases & Equipment Co. Ltd. Jeddah, Saudi Arabia)). Three replicates of each ethylene gas standard were analyzed to make the calibration curves for each column and batch of samples. The concentration of dissolved ethylene before equilibrium with the headspace was calculated from the ethylene concentration measured in the equilibrated headspace samples according to Wilson et al. (2012) and applying the solubility coefficient of ethylene extracted from Breitbarth et al. (2004) as a function of temperature and salinity. Monthly mean *in situ* seawater salinity values measured at each sampling time were used to calculate the solubility coefficient (i.e. 39.9 in Nov/Dec 2016 and 40.5 in Jan/Feb 2017). Acetylene reduction rates were converted to N₂ fixation rates following the theoretical ratio 3:1; which means that one N₂ molecule is fixed for each three acetylene molecules reduced (Alongi et al. 2000; 2002). No ethylene production was detected in the negative controls.

2.4. Statistical analysis

Differences in sediment OM, organic C and N content, and isotopic ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) composition among mature mangrove sediments with high and low crab-density, and juvenile mangrove sediments with high and low crab-density were tested by ANOVA analysis followed by Tukey's post-hoc test. To evaluate the effect of the mangrove age, temperature and crab density on sediment N_2 fixation rates, we performed an ANCOVA by considering three explanatory variables: two categorical factors as Age (two levels, mature and juvenile) and Temperature (two levels, 35°C and 28°C) both fixed and orthogonal, and crab density as our continuous explanatory variable, since crab abundance could not be tightly controlled and the replicated plots within different treatments showed variability in crab densities (Fig. S1). To test our model, we performed a generalized linear model with a Quasipoisson family of error. We updated the model to the first significant terms using the function *update()* in R. Statistical analysis and graphs were produced with JMP, PRISM, and R software (R core team, 2017) using "ggplot2" package (Wickham 2010).

3. Results

3.1. *In situ* temperature and salinity

The air temperature at the study site ranged from 14°C in January to 40°C in July during a one-year period from July 2016 to July 2017. Similarly, the surface sediment temperature ranged annually from 11°C to 57°C , with maximum temperature recorded in July and minimum temperature in February. Surface sediment temperature oscillated from 11°C to 32°C in the colder period and from 30°C to 57°C in the hotter period. Belowground sediment temperature ranged from approximately 15°C in February to 50°C in July. Belowground sediment temperature oscillated from 15°C to 29°C in the

colder period and from 30°C to 50°C in the hotter period. Seawater temperature was 35°C in November/December 2016 and 28°C in January/February 2017. Monthly mean ($\pm$ SEM) seawater salinity was 39.9 ± 0.01 from 13 November to 12 December 2016 (first sampling time when temperature was 35°C) and 40.5 ± 0.01 from 28 January to 27 February 2017 (second sampling time when temperature was 28°C).

3.2. Sediment characteristics

The sediment OM content differed among the four experimental treatments (ANOVA, $F_{3,16} = 196$, $p < 0.0001$, Fig. S2A), with higher values (mean $\pm$ SEM) recorded in mature mangrove sediments (3.26 ± 0.04 and $2.81 \pm 0.04\%$ of dry weight in high and low crab-density sediments, respectively) compared to juvenile mangrove sediments (2.06 ± 0.05 and $2.05 \pm 0.04\%$ of dry weight in high and low crab-density sediments, respectively). Similarly, the sediment organic C content differed among sediment types (ANOVA, $F_{3,16} = 72.14$, $p < 0.0001$, Fig. S2B), with the highest organic C content measured in mature mangrove sediments with high crab-density (0.38 ± 0.01 mmol C g DW sed⁻¹), followed by mature mangrove sediments with low crab density (0.25 ± 0.01 mmol C g DW sed⁻¹) and juvenile mangrove sediments (0.21 ± 0.01 and 0.20 ± 0.01 mmol C g DW sed⁻¹ in sediments supporting high and low crab-density, respectively). The sediment N content also differed among sediment types (ANOVA, $F_{3,16} = 6.89$, $p = 0.003$, Fig. S2C). The N content in mature mangrove sediments supporting high crab-density (0.04 ± 0.0003 mmol N g DW sed⁻¹) was significantly higher than the N content in mature mangrove sediments supporting low crab-density (0.03 ± 0.002 mmol N g DW sed⁻¹), juvenile mangrove sediments supporting high crab density (0.03 ± 0.002 mmol N g DW sed⁻¹) and juvenile mangrove sediments supporting low crab-density (0.03 ± 0.002

mmol N g DW sed⁻¹) sediments (Tukey post-hoc test, $p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively).

The sediment carbon isotopic ($\delta^{13}\text{C}$) signature differed among sediment types (ANOVA, $F_{3,16} = 9.04$, $p = 0.001$, Fig. S2D). The sediment from mature mangrove with low crab-density plots showed the heaviest average $\delta^{13}\text{C}$ ($-19.20 \pm 0.25 \text{ ‰}$) compared to mature mangrove with high crab-density ($-20.72 \pm 0.09 \text{ ‰}$), juvenile mangrove with high crab-density ($-20.74 \pm 0.34 \text{ ‰}$), and juvenile mangrove with low crab-density ($-20.32 \pm 0.20 \text{ ‰}$) sediments (Tukey post-hoc test, $p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively).

The sediment N isotopic ($\delta^{15}\text{N}$) signature ranged from $1.68 \pm 0.24 \text{ ‰}$ and $1.76 \pm 0.44 \text{ ‰}$ in juvenile and mature mangrove sediments with low crab-density, to $2.16 \pm 0.12 \text{ ‰}$ and $2.18 \pm 0.86 \text{ ‰}$ in mature and juvenile mangrove sediments with high crab-density (Fig. S2E), but these differences were not statistically significant (ANOVA, $F_{3,16} = 0.27$, $p = 0.85$).

3.3. Sediment N₂ fixation rates

Sediment N₂ fixation rates ranged 200-fold from $0.002 \pm 0.002 \text{ mg N m}^{-2} \text{ d}^{-1}$ in juvenile mangrove sediments with high crab-density at 35°C, to $0.46 \pm 0.12 \text{ mg N m}^{-2} \text{ d}^{-1}$ in mature mangrove sediments with low crab-density at 28°C (Fig. 1). The age of the mangrove stand, temperature, and crab density had significant statistical effects on sediment N₂ fixation rates, as did the interaction between temperature, crab density, and age of the mangrove stand (GLM, Table 1). N₂ fixation rates were higher in mature mangrove sediments than in juvenile sediments and the rates dropped when temperature increased from 28°C to 35°C. Similarly, sediment N₂ fixation rates decreased under high crab-density (Fig. 1), with a linear decrease in rates with increasing crab-density (Fig. S3).

Sediment N₂ fixation rates measured in January/February at 28°C increased with increasing sediment OM content in high crab-density (lm, $F_{1,8} = 7.63$, $p = 0.02$) and in low crab-density (lm, $F_{1,8} = 12.17$, $p = 0.008$) mangrove sediments (Fig. 2A, B). Similarly, sediment N₂ fixation rates increased with increasing sediment organic C content in high (lm, $F_{1,8} = 7.68$, $p = 0.02$) and low crab-density (lm, $F_{1,8} = 12.45$, $p = 0.008$) mangrove sediments (Fig. 2C, D). However, the sediment N content and sediment N₂ fixation rates at 28°C were not linearly related in high crab-density (lm, $F_{1,8} = 2.82$, $p = 0.13$) and in low crab-density (lm, $F_{1,8} = 4.33$, $p = 0.07$) mangrove sediments (Fig. 2E, F). Similarly, sediment N₂ fixation rates measured in January/February at 28°C increased with heavier sediment C isotopic ($\delta^{13}\text{C}$) composition of organic matter pools (lm, $F_{1,18} = 42.05$, $p < 0.0001$, Fig. 3A). However, sediment N₂ fixation rates measured in January/February at 28°C were not linearly related to the sediment N isotopic ($\delta^{15}\text{N}$) composition (lm, $F_{1,18} = 3.3$, $p = 0.09$, Fig. 3B).

4. Discussion

This study provides evidence that high temperature and crab density can reduce N₂ fixation rates in mangrove sediments from arid regions. Sediment N₂ fixation rates detected in *A. marina* mangrove stands in the Central Red Sea (from 0.0007 to 0.168 g N m⁻² yr⁻¹) were lower than the average N₂ fixation rates previously reported in mangroves (from 0.03 to 2.6 g N m⁻² yr⁻¹, Zuberer and Silver 1978; Howarth et al. 1988). The comparatively low N₂ fixation rates measured here could be explained by the high salinity values in our study site, in agreement with Vovides et al. (2011) that found a strong negative relation between N₂ fixation rates and salinity in an impacted black mangrove in the Gulf of Mexico. Central Red Sea mangrove stands are stunted, with low productivity

and with organic-poor sediments when compared to mangrove stands elsewhere (Almahasheer, Duarte, and Irigoien 2016b; Almahasheer et al. 2017; Garcias-Bonet et al. 2019a), due to a critical nutrient limitation, including iron-limitation (Almahasheer, Duarte, and Irigoien 2016a). The low N₂ fixation detected here is consistent with the iron-limiting condition of these mangrove stands as the nitrogenase enzyme requires iron as a cofactor (Howard and Rees 1996). Despite the low rates of sediment N₂ fixation, we revealed a number of drivers eliciting a 200-fold range in N₂ fixation rates in central Red Sea mangroves.

N₂ fixation was strongly temperature-dependent, with 10-fold higher rates at 28°C than at 35°C, where higher temperatures resulted in undetectable N₂ fixation rates. This is consistent with the thermal optima for N₂ fixation, ranging between 15°C and 30°C, described in terrestrial ecosystems elsewhere (Houlton et al. 2008), and reports of reduced N₂ fixation rates at > 30°C in desert soils (Barger, Castle, and Dean 2013; Zhou et al. 2016), and seagrass meadows (Garcias-Bonet et al. 2018; Garcias-Bonet et al. 2019b). The temperature dependence of N₂ fixation can be explained by a thermal suppression of nitrogenase synthesis (Brooks, Collins, and Brill 1984). In addition, temperature can control the microbial community structure (Wang et al. 2013), including microorganisms responsible for N₂ fixation. Although thermophilic N₂-fixing microorganisms have been reported in anoxic environments and hydrothermal vents (Wahlund and Madigan 1993; Mehta and Baross 2006), high temperature conditions clearly reduce N₂-fixing activity in mangrove sediments in the Central Red Sea.

The age of mangrove trees, with the associated difference in stand and rhizosphere development and biomass, had a significant effect on sediment N₂ fixation rates, with

higher rates in the mature mangrove stand than in the juvenile mangrove stand. This suggests that mature mangrove stands, which also support increased sediment OM content and organic C stocks, provide more suitable biogeochemical conditions favoring N₂-fixing microbial communities (Holguin, Vazquez, and Bashan 2001; Inoue et al. 2019), as supported by the positive linear relationship of sediment OM and organic C content and sediment N₂ fixation in both low and high crab-density plots. Sediment N₂ fixation was independent of sediment N content. However, since N₂ fixation is known to be suppressed under high inorganic N concentrations (Knapp 2012), some of the variability detected here could be also due to differences in ambient nitrate and ammonium concentrations.

The difference in C isotopic ($\delta^{13}\text{C}$) composition of mangrove sediments, ranging from -20.74 ± 0.34 to $-19.20 \pm 0.25\text{‰}$, and mangrove tissues in the central Red Sea, $-26.08 \pm 0.11\text{‰}$ (average $\pm$ SEM, N=117, Almahasheer et al. 2017), suggests a potential additional source other than mangrove tissues to their accumulated sediment OM. Garcias-Bonet et al. (2019a) recently reported that mangrove leaves were the major contributors ($56 \pm 8\%$) to the accumulated sediment OM in Red Sea mangroves, with additional, but minor, contributions from other primary producers, such as macroalgae, seagrass, and halophytes, based on stable isotope mixing models. Similarly, the $\delta^{13}\text{C}$ of mature low crab-density sediments ($-19.20 \pm 0.25\text{‰}$) reported here likely indicates the contribution of microphytobenthos, for which $\delta^{13}\text{C}$ has been reported between -14.4‰ and -13‰ (Oakes and Eyre 2014) and we recently measured $\delta^{13}\text{C}$ values of $-15.07 \pm 0.07\text{‰}$ in microbial mats in Central Red Sea mangroves (Garcias-Bonet et al. 2019a). This highlights the role of cyanobacteria as both a source of C and N, through N₂ fixation in

mature low crab-density mangrove sediments, holding the highest N₂ fixation rates. However, N₂ fixation rates were independent of sediment N isotopic ($\delta^{15}\text{N}$) composition, possibly indicating N inputs from additional sources to N₂ fixation (Kuramoto and Minagawa 2001), as the rates measured were low, insufficient to satisfy the N demands of the ecosystem.

Sediment N₂ fixation rates were negatively affected by crab density. Low crab-density sediments consistently showed higher N₂ fixation rates than the high crab-density sediments. Crab activity can affect N₂ fixation activity in several ways. First, crab burrowing activity allows the transport of oxygen to deeper, anoxic sediment layers, increasing oxygen concentration and redox potential in sediment around the burrow (Booth et al. 2019) and making the conditions less favorable for N₂ fixation due to potential inhibition of nitrogenase enzyme activity, which requires anaerobic conditions (Goldberg, Nadler, and Hochman 1987; Reef, Feller, and Lovelock 2010). However, burrowing activity could have a positive effect on mangrove growth by favoring oxidation and release of iron from the sediment, which is limiting mangrove growth (Mokhtari et al. 2016; Almahasheer, Duarte, and Irigoien 2016a). In addition, crabs can limit N₂ fixation through their feeding behavior, which alters the microbial community in the sediments. Cyanobacteria have an important role in N₂ fixation in mangrove ecosystems (Alvarenga et al. 2015). Hence, crab grazing on cyanobacteria might reduce N₂ fixation in the system, as supported by the differences in $\delta^{13}\text{C}$ values between low and high crab-density in mature sediments, which suggest a somewhat lower contribution of microbial mats to organic C pools in sediments affected by crab activity. We acknowledge here that some crab species belonging to sesarmid family, which have a

herbivorous diet, can increase the C:N by storing organic matter such as leaves and propagules in their burrows (Andreetta et al. 2014), and therefore creating the redox conditions that favor the N₂ fixation (Booth et al. in press). However, this is not the case in our study since central Red Sea mangroves are mainly colonized by fiddler and sand bubble crabs with relatively few occurrences of sesamid crabs, which are mainly located in the south (Price et al. 1987). However, further research is needed to corroborate the suggestion that the feeding behavior of crabs may contribute to reduce N₂ fixation, for instance by microscopy or compound specific analysis.

Our experimental assessment does not capture ecosystem N₂ fixation, as we disturbed the sediments to ensure a good distribution of acetylene, and some components of N₂ fixation in the mangrove system, such as activity associated with mangrove roots (Alfaro-Espinoza and Ullrich 2015) or leaf litter (Pelegri, Rivera-Monroy, and Twilley 1998), were not included as it would have biased the comparison between juvenile and mature stands. Moreover, ARA has some methodological limitations to be considered, although it has been extensively used to measure N₂ fixation rates in coastal vegetated sediments, including mangrove (e.g. Romero et al. 2012), seagrass (e.g. Welsh 2000; Garcias-Bonet et al. 2018; Garcias-Bonet et al. 2019b) and salt marsh (Murphy, Boyer, and Carpenter 2017) sediments. Specifically, addition of acetylene has been shown to affect the microbial community composition (Fulweiler et al. 2015). Therefore, the N₂ fixation rates reported here need to be carefully interpreted.

5. Conclusions

Sediment N₂ fixation rates in central Red Sea mangrove ecosystems are within the low range among those reported for mangrove ecosystems, with almost undetectable N₂

fixation in sediments of juvenile mangrove stand. We identified temperature and crab density as important interacting drivers of N₂ fixation, pointing out a complex regulation of N₂ fixation in mangrove ecosystems. Rapid warming of the Red Sea (Chaidez et al. 2017) may, thus, further reduce N₂ fixation, thereby aggravating the nutrient-limiting conditions of Red Sea mangroves.

Author contributions

NG-B, MF, DD and CMD designed this study. MF, JMB and DD design and set up the experimental mangrove plots. MSQ, MF and NG-B performed the fieldwork. MSQ and NG-B performed the atmospheric N₂ fixation measurements. NG-B, MF, MSQ and CMD interpreted the results. MF, CMD and NG-B performed the statistical analysis. NG-B, MF and MSQ wrote the first draft of the manuscript. All authors contributed substantially to improving the manuscript.

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Table 1. Generalized linear model adopted to assess the effect of each factor and their interaction on sediment N₂ fixation rates. Df: degree of freedom, Rs.Df: residual degree of freedom, Rs.Dev: residual deviance; *p*: p statistic. The statistically significant results are shown in bold.

Terms	Df	Deviance	Rs.Df.	Rs.Dev	<i>p</i>
Temperature	1	1.75094	38	6.1633	< 0.0001
Age	1	3.10967	37	3.0537	< 0.0001
Crab	1	2.10009	36	0.9536	< 0.0001
Temperature × Age	1	0.04009	35	0.9135	0.162441
Temperature × Crab	1	0.09976	34	0.8137	0.027556
Age × Crab	1	0.20814	33	0.6056	0.01458

Figures

Fig. 1. N₂ fixation rates in juvenile (a, b) and mature (c, d) mangrove sediments with high crab-density (black bars) and low crab-density (gray bars) at 28°C and 35°C.

Fig. 2. Relation of N_2 fixation rates at 28°C in mangrove sediments with high and low-crab density with sediment organic matter (a, b), organic carbon (c, d) and nitrogen (e, f) content, showing linear regressions (dashed line). N_2 fixation rates in mature mangrove sediments are represented as black dots and in juvenile mangrove sediments are represented as white dots.

- Mature mangrove stands
- Juvenile mangrove stands

Fig. 3. Relation of sediment N₂ fixation rates at 28°C with sediment C isotopic ($\delta^{13}\text{C}$) signature (a) and sediment N isotopic ($\delta^{15}\text{N}$) signature (b). Mature mangrove sediments with high-crab density are represented as black dots, mature mangrove sediments with low-crab density are represented as black squares, juvenile mangrove sediments with high-crab density are represented as white dots, and juvenile mangrove sediments with low-crab density are represented as white squares. Error bars indicate SEM.

- Mature high crab-density ○ Juvenile high crab-density
- Mature low crab-density □ Juvenile low crab-density

Supplementary figures

Figure S1. Box plot showing crab density in high crab-density (High) and low crab-density (Low) plots in juvenile and mature mangrove stands at two different sampling times: in November/December 2016 when seawater temperature was 35°C and in January/February 2017 when seawater temperature was 28°C. Boxes extend from the 25th to 75th percentiles and lines inside boxes represent median values.

Figure S2. Sediment OM content (a), organic C content (b), N content (c), isotopic C composition (d) and isotopic N composition (e) in mature with high-crab density (M High), mature with low crab-density (M Low), juvenile with high crab-density (J High) and juvenile low crab density (J Low) mangrove sediments in January/February 2017 when seawater temperature was 28°C. Boxes extend from the 25th to 75th percentiles, lines inside boxes represent median values, and whiskers span from minimum to maximum values. Letters indicate significant differences among groups based on ANOVA analysis and Tuckey's post-hoc tests.

Figure S3. N₂ fixation rates in juvenile (upper panels) and mature (bottom panels) mangrove sediments versus crab density at 28°C and 35°C. Black line is the regression obtained with the function *geom_smooth()* of the package *ggplot2* in R and the gray area is the 95% interval of confidence.

759 **Supplementary Table**

760 Raw data available in Supplementary Table S1

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