Impact of Crab Bioturbation on Nitrogen-Fixation Rates in Red Sea Mangrove Sediment

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EXAMINATION COMMITTEE PAGE

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ABSTRACT

Impact of Crab Bioturbation on Nitrogen-Fixation Rates in Mangrove Ecosystem in Thuwal, Saudi Arabia

Maryam Saber Qashqari

Mangrove plants are a productive ecosystem that provide several benefits for marine organisms and industry. They are considered to be a food source and habitat for many organisms. However, mangrove growth is limited by nutrient availability. According to some recent studies, the dwarfism of the mangrove plants is due to the limitation of nitrogen in the environment. Biological nitrogen fixation is the process by which atmospheric nitrogen is fixed into ammonium. Then, this fixed nitrogen can be uptaken by plants. Hence, biological nitrogen fixation increases the input of nitrogen in the mangrove ecosystem. In this project, we focus on measuring the rates of nitrogen fixation on Red Sea mangrove (*Avicennia marina*) located at Thuwal, Saudi Arabia. The nitrogen fixation rates are calculated by the acetylene reduction assay. The experimental setup will allow us to analyze the effect of crab bioturbation on nitrogen fixing rates. This study will help to better understand the nitrogen dynamics in mangrove ecosystems in Saudi Arabia. Furthermore, this study points out the importance of the sediment microbial community in mangrove trees development. Finally, the role of nitrogen fixing bacteria should be taken in account for future restoration activities.

Table of Contents

LIST OF ABBREVIATIONS

ARA	Acetylene reduction assay
GC-FID	Gas Chromatography-Flame Ionization Detector
HT	High tide
LT	Low tide
LOI	Loss on ignition

LIST OF ILLUSTRATIONS

LIST OF TABLES

Chapter 1

Introduction

1.1 Mangrove ecosystem

Mangroves are highly productive ecosystems occupying the upper intertidal zone in the land-sea interface of tropical and subtropical regions [1]. They provide important ecosystem services, including serving as habitat for a wide range of living organisms such as birds, crabs and fish [2][3], protecting the coast from erosion due to exposure to tides, storms and currents [4][5], providing wood for construction and fuel [6]; and acting as the most intense carbon sinks in the biosphere, building very large carbon stocks in their soils [7].

Mangrove ecosystem export a significant fraction of their production, also involving continuous loss of nutrients, often leading to nutrient limitation of mangrove growth and production. As they often grow in association with rivers in deltaic areas, they receive high inputs of nutrient and sediment that contribute to maintain a positive nutrient balance, allowing for the excess nutrients required to support high primary production, nutrient sequestration in soils and export to adjacent marine habitats [8][9].

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 [10][11], often resulting in dwarf mangrove trees in these regions.

This is the case of Red Sea mangroves, which are stunted and severe nutrient-limited in the Central Red Sea [10]. Experimental nutrient additions showed that Central Red Se mangroves are iron limited [10], as expected due to absence of inputs from land and low nutrient concentration in Red Sea water [12][13][14]. Iron, however, is a co-factor in the nitrogenase enzyme [15], and its deficiency may, thus, limit nitrogen fixation rates, which is a key process supplying nitrogen to mangrove ecosystem, particularly where, as is the case for the Red Sea, land inputs are minimal.

Mangrove forests cover about 135 km^2 of the Red Sea coastline [12], where *Avicenna marina* is the most abundant mangrove species [16]. Provided the low nutrient supply to the Red Sea, nitrogen fixation supported by Red Sea mangroves could be an important source of nutrients to the ecosystem.

1.2 Nitrogen and nitrogen fixation in mangroves

Atmospheric nitrogen (N_2) is the dominant gas in the atmosphere, comprising 78% of atmospheric gases, but is inert and cannot be used directly by organisms [17]. Nitrogen fixation is the process through which microorganisms are able to convert N_2 gas into ammonium (NH_4^+), which is the nitrogen source that can be taken up directly by mangroves [2][18]. Nitrogen fixation has been detected in sediments, roots, rhizosphere

and decomposing leaves in mangrove systems and represents, therefore, a key, rate-limiting step, in the nitrogen cycle [19][20].

Nitrogen fixation uses a nitrogenase enzyme to reduce the di-nitrogen [21]. The nitrogenase enzyme is produced by a group of nitrogen fixing bacteria and is composed of two metalloproteins, the iron (Fe) protein (nitrogenase) and the Molybdenum (MoFe) iron protein (nitrogenase reductase) [22].

Nitrogenase can also reduce other substrates, such as azide, acetylene and cyanide [15], which allows biological nitrogen fixation rates to be resolved indirectly through the acetylene reduction assay (ARA). This method is based on the experimental evaluation of the rates of reduction of acetylene gas (C_2H_2) to ethylene gas (C_2H_4) using nitrogenase enzyme [23]. The rates can then be converted to nitrogen-fixing rates according to the theoretical ratio 3 (C_2H_4): 1 (NH_4^+) [24]. This assay is sensitive and inexpensive and can be performed in short experiments. It requires the addition of acetylene to the field sample and measuring ethylene production along an incubation period. Ethylene (C_2H_4) can then be quantified using Gas Chromatography-Flame Ionization Detector (GC-FID), which separates gases in the sample and detect the gas of interest [25][23]. ARA is the method most commonly employed to resolve nitrogen fixation rates in mangrove ecosystem [26].

In addition to iron to synthesize the nitrogenase enzyme, nitrogen fixation requires anaerobic conditions, and is believed to be enhanced in anaerobic, organic-rich sediments [2]. Whereas mangrove sediments are organic rich and often anaerobic, mangrove ecosystems support abundant fauna, whose activity alter the sediment structure, chemical composition and affect biogeochemical process [27][28]. Burrowing animals such as crabs and shrimps, excavate galleries, which the actively ventilate, thereby increasing oxygen supply and oxidation of sediment materials [29]. The effect of bioturbation on nitrogen fixation has not been extensively studied, a number of reports provide evidence that burrowing affects nitrogen fixation process [27][30]. The effect reported is positively affect nitrogenase, because bioturbation leads to sediment aeration, which involves an increased oxygen penetration through the sediment, possibly inhibiting the nitrogenase enzyme [27][30][31]. However, oxygen inputs may oxidize sulfur pyrites, formed by the binding of iron with sulfide produced in anoxic sediments, which may release iron and allow its uptake in the presence of organic ligands [32]. Indeed, bioturbators have been reported to enhance metal, including iron, release [33]. Experimental assessments in Brazilian mangroves have shown that oxidation of iron sulfide is enhanced under elevated temperatures, when mangrove activity is highest, and that increased crab activity (*Uca* spp) leads to release of iron from pyrite [34]. As iron is limiting nutrient in the Central Red Sea sediments and required to synthesize nitrogenase, oxidation of sediments through the activity of bioturbators may enhance iron mobilization and, therefore, synthesis of nitrogenase.

Here we quantify, for the first time, nitrogen fixation rates in Red Sea mangrove (*Avicenna marina*) stand and test experimentally the effect of mangrove plantation and crab (*Uca inversa*) with the invasion of (*Dotilla sulcata*) bioturbation on nitrogen fixation rates. Furthermore, it will help to better understand the nitrogen dynamics of those mangrove ecosystems.

Chapter 2

Materials and Methods

2.1 Study site

The study site is a Central Red Sea mangrove stand located next to King Abdullah monument at KAUST (Thuwal, Saudi Arabia, 22° 20’ 25.032’’ N, 39° 5’ 17.411’’), (Fig. 1). This site is occupied by mature and juvenile *Avicennia marina* mangrove trees species and supports a high density of crab (*Uca inversa* and *Dotilla sulcata*) bioturbators, as reflected in high density of burrows. Air temperature in Thuwal ranged from 20 to 39°C during the year of 2016 and the salinity of Red Sea at this location is (average ± SE) 42.0 ± 1.3 ‰.

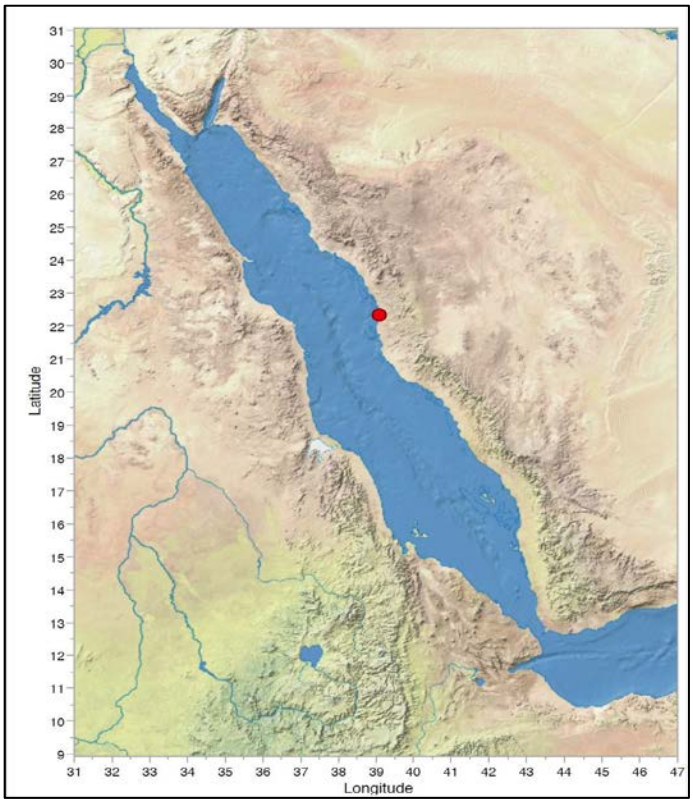


Figure 1: Location of the study site, Thuwal, Saudi Arabia.

2.2 Experimental design

The experimental design consists on naturally growing mature mangrove trees and planted juvenile mangrove stands.

The juvenile mangrove plants were transplanted in the field after growing the propagules in a nursery. They were collected from the mangrove stand next to King Abdullah monument in January, 2016. The propagules were grown in a nursery during February, 2016, and planted in the same site by digging a hole big enough to contain the roots at the first week of March, 2016. Sediment temperature during that period was

around 39°C and the water temperature was around 28°C, provided by a logger located at the site to retrieve the data.

Both mature and juvenile mangrove plants were exposed to different crab densities and therefore, different bioturbation pressure. The main crab species found in this system are *Uca inversa* and *Dotilla sulcata*. First, the non-bioturbated (not enriched) mature and juvenile stands hold a crab density of 2.3 ± 0.5 per square meter and 23.4 ± 2.6 per square meter (average ± SE), respectively. Then for the bioturbated (enriched) mature and juvenile stands, the crab density recorded was 74.5 ± 7.4 per square meter and 27 ± 2.6 per square meter (average ± SE), respectively. The crab density in the enriched plots was higher than the density of crabs occurring naturally, where it was artificially increased.

2.3 Sample collection and preparation

All sediment samples were obtained from bioturbated and non-bioturbated areas of mature and juvenile mangrove trees. They were collected at two different water conditions, either at high-tide (HT) or low-tide (LT). Assessment of nitrogen fixation rates were conducted in two period, November to December 2016 when water temperature is very high (35°C) and January to February 2017 when seawater temperature was much lower (28°C). Plexiglas cores (10.4 cm in diameter and 27 cm in

height) with open ends and rubber stoppers were used to extract sediment samples at the different plots. Three replicated cores were retrieved from bioturbated plots and an additional three for the non-bioturbated plots. In laboratory, samples were processed differently according to the tide level during sampling.

For high-tide samples, the water filling the cores was suctioned out without disturbing sediment surface, using a silicon tube and a syringe. About 10 cm from the surface of two cores from the same plot (bioturbated or non-bioturbated) were sliced using a ceramic knife and a plastic holder. From each treatment (bioturbated or non-bioturbated), 200 ml of the sediment was placed in a 500 ml Pyrex glass incubation jar, and amended with 80 ml of sea water collected from the sampling location and stirred to produce a slurry. Seawater was added to resemble the high-tide condition that covers the sediment in the field. The Pyrex jar containing the slurry was closed with a lid containing a gas inlet closed with a silicone septum to allow sampling of headspace gases. The low-tide samples followed the same process of the high-tide samples except the addition of water.

2.4 Acetylene reduction assay (ARA)

Nitrogen fixation activity in sediment samples of the mangrove ecosystem was indirectly estimated using the acetylene reduction assay (ARA). ARA assay measures the nitrogenase enzyme activity, which reduces acetylene gas (C_2H_2) into ethylene gas (C_2H_4) [25][33][15]. Acetylene reduction rates are converted to nitrogen fixing rates following the theoretical ratio 3:1; which means for each three acetylene molecules reduced one

nitrogen molecule is fixed [34][19]. Acetylene was added to the incubation bottles in two different forms depending on the tide condition. For high-tide samples 20 ml of acetylene-enriched seawater was added to the incubation bottle [35]. However for the low-tide samples 30 ml was added in a gas form which equals 10% of the headspace [25][23].

Acetylene enriched seawater was prepared on a 1000 ml bottle filled with 300 ml seawater taken at the same date of sampling. The bottle lid had two gas inlets with controllable valves to allow the diffusion of acetylene gas into the bottle. A commercial acetylene gas cylinder was used to enrich the seawater by bubbling it for 5 minutes [35]. After that, the valves were closed immediately to prevent the gas flow.

2.5 Samples incubation and readings

After the addition of acetylene, samples were mixed well to ensure the diffusion of acetylene through the sample. Then, 3 ml of the headspace gas was taken from each incubation bottle and placed in a gas tight vacuum vials using a syringe (T_0). Samples were incubated in a Percival for 24 hours at *in situ* temperatures (35°C or 28°C) under light and dark conditions. During different time points of the incubation period, four

more gas samples of each sample were taken (T_1-T_4) to be analyzed later for ethylene production.

2.6 Quantification of Ethylene using Gas Chromatography-Flame Ionization Detector (GC-FID)

Samples were analyzed for ethylene production using gas chromatography-flame ionization detector (GC-FID) after the incubation period. Two different columns used for the gas separation in the instrument. The GS-CARBON PLOT column (60 m in length, 0.320 mm in diameter and 1.50 µm film) used with the samples incubated at 28°C. Whereas the HP-AL/S column (30 m in length, 0.250 mm in diameter and 5.0 µm film) used with the samples incubated at 35°C. The column allow the passage of the gas sample via a carrier gas from the injector into the FID-detector and gets recognized. Concentrations of ethylene from each gas were calculated using a standard curve. The standard curve was built from the peak area of three ethylene standards of known concentrations (93 ppm, 9 ppm and 1.5 ppm). Three gas samples replicates of each ethylene standard were analyzed to make the calibration curve. A gas tight syringe (1 ml) was used to inject gas samples from the gas tight vials to the inlet of the instrument to be analyzed. The peak area of ethylene peak in each sample was measured.

2.6.1 Calculation of nitrogen fixing rates in high-tide samples

First, the equilibrated ethylene concentration in the headspace (ppm) was calculated using the calibration curve:

$$y = ax + b,$$

where the $concentration\ (x)\ =\ peak\ area\ (y)$ – (b/a).

The ethylene production rates are calculated as described previously by Wilson et al. [35]. Briefly, the total dissolved ethylene concentration in the water after equilibration (ml of C_2H_4/ ml of H_2O) was calculated by:

$$\{C_2H_4\}_w\ = 10^{-6}\ \beta\ m_a\ p$$

where β is the Bunsen solubility coefficient of ethylene at given temperature and salinity, m_a is the ethylene concentration measured in the equilibrated headspace and *p* is the atmospheric pressure of dry air.

Then, the initial concentration of ethylene in seawater before equilibration was calculated following:

$$\{C_2H_4\}_{aq}\ =\ (\{C_2H_4\}_w\ V_w\ +\ 10^{-6}\ m_a\ V_a)\ /\ V_w$$

$$= 10^{-6}\ m_a\ (\beta p\ V_w +\ V_a)/\ \ V_w$$

where *V*w is the water volume (ml) in the incubation bottle and *V*a is the headspace volume (ml).

After that, ethylene concentration in initial seawater was converted into nmol L^{-1} by:

$$[C_2H_4]_{aq} = 10^9 \times p\{C_2H_4\}_{aq} / (RT)$$

where *R* is the gas constant (0.08206 atm liter mol^{-1} K^{-1}) and *T* is the temperature in Kelvin (K).

We calculated the ethylene production rate (nmol L^{-1} h^{-1}) based on the increase in ethylene concentration through the incubation time. Finally, ethylene production rates were converted to nitrogen fixing rates (nmol N_2 L^{-1} h^{-1}) by using a ratio of 3:1. Rates of nitrogen fixation (nmol N_2 L^{-1} h^{-1}) were then converted into rates measured per area (mg N m-2 d-1) by:

$$mg\ N\ m^{-2}\ d^{-1} =$$

$$\frac{N - Fix\ rate\ (nmol\ N2\ cm^{-3}\ h^{-1}) \times MW \times Sediment\ volume\ in\ the\ bottle\ (ml) \times 10000}{1000000 \times Area\ of\ the\ core\ sediment\ (cm^2)}$$

2.6.2 Calculation of nitrogen fixation rates in low-tide samples

Ethylene concentrations in the headspace samples were calculated using the calibration curve equation. Then, the ethylene production rates (ppm h^{-1}) were calculated based on the increase in ethylene concentration (ppm) through the incubation time. Thereafter, rates were converted to mol C_2H_4 m^{-3} h^{-1} using the formula:

$$PV = nRT$$

where P equals pressure (101325 pascal), V is the volume of gas (1 ppm =10^{-6}), n is number of moles, R is the gas constant (8.31441 J mol^{-1} K^{-1}) and T is temperature in (kelvin).

Then, the rates are transformed on aerial base (mol C_2H_4 m^{-2} h^{-1}) by:

$$\frac{Ethylene\ production\ rate\ (mol\ m^{-3}\ h^{-1}) \times Air\ volume\ (m^3)}{Core\ surface\ (m^2)}$$

Finally, those rates were calculated on a daily base and converted to nitrogen fixing rates (mg N_2 m^{-2} d^{-1}) by using the ratio 3:1.

2.7 Sediment analysis

The percentage of organic matter (%OM) in mangrove sediment was calculated from loss on ignition (LOI). First, sediment samples were dried at 60°C for about two days and grinded to a size of approximately 2mm particles. A subsample of the dried sediment (approximately 3 grams) were weight in the crucibles using the high precision balance. This step was performed quickly to prevent moisture absorption. Samples were heated in the furnace oven for 5 hours at 450°C and dried and burnt sediment weight were used in the below formula to calculate the organic matter content in the samples:

$$\%OM = \frac{(pre - ignition\ weight\ (g)) - (post - ignition\ weight\ (g))}{(pre - ignition\ weight\ (g))} \times 100$$

Carbon and nitrogen in the sediments was evaluated using a CHN analyzer. Sediment samples were dried, grinded and acidified before analysis, to remove carbonates. About 10-11 mg of the sediment sample was analyzed using a CHNSO-2 instrument at the analytical core lab in KAUST.

2.8 Statistical analysis

The general linear model was used to assess the significance of each factor, in accounting for variability in nitrogen fixation rates, and also assesses the interaction between them.

Chapter 3

Results

3.1 Sediment characteristics

The sediment organic matter content varied between 1.7 and 3.3 % of dry weight with the organic carbon concentration ranging between 0.2% to 0.4% C (% of dry weight), and tended to be higher in the sediments supported mature, compared to those

supporting juvenile, mangroves (Fig. 2 and 3). Nitrogen content ranged from 0.02% to 0.05% N (% of dry weight) between the different treatments, and tended to be lower in sediments supporting juvenile compared to mature mangroves as well as lower under high compared to low temperatures (table 2).

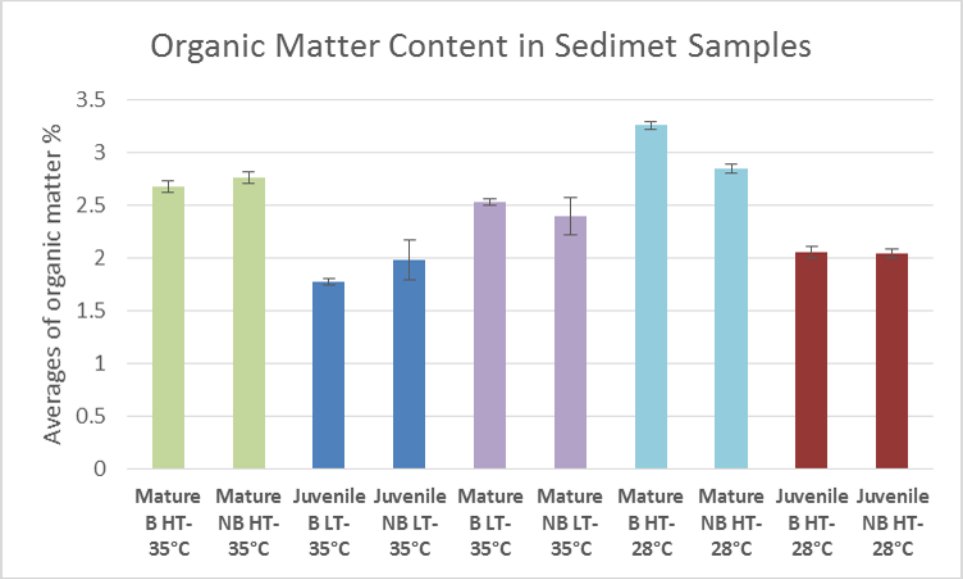


Figure 2: Variation of organic matter content in sediment samples of mature and juvenile stands. Bioturbated (B), non-bioturbated (NB), high-tide (HT), low-tide (LT).

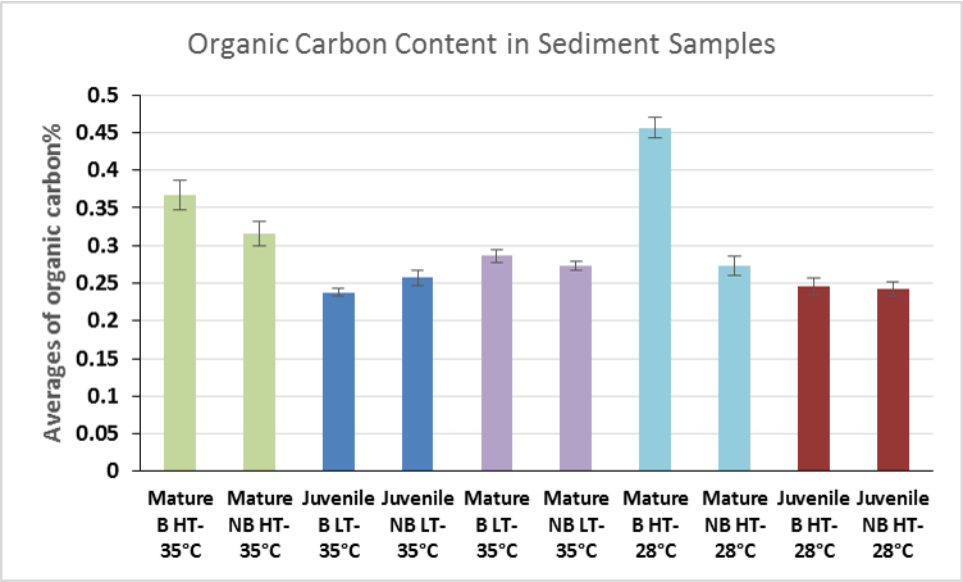


Figure 3: Variation of organic carbon content in sediment samples of mature and juvenile stands. Bioturbated (B), non-bioturbated (NB), high-tide (HT), low-tide (LT).

3.2 Nitrogen fixing rates at 35°C

Nitrogen fixation rates resulted from sediment samples at 35°C were generally low. Nitrogen fixation rates detected in sediments of established mangrove stand resulted in an average ± SE of 0.036 ± 0.010 mg N m^{-2} day^{-1} for bioturbated sediment and were significantly greater, at 0.039 ± 0.006 mg N m^{-2} day^{-1}, for the non-bioturbated sediment. On the other hand, N_2 fixation rates in stands planted with mangrove saplings approached detection limit, yielding an average ± SE rate of 0 ± 0.004 mg N m^{-2} day^{-1} for both bioturbated and non-bioturbated sediments (Fig. 4). Negative control sample of sediment with seawater without acetylene addition yielded no detected ethylene production. However, seawater (SW) incubated with acetylene (C_2H_2) had low ethylene

production rates that were subtracted from the fixation values of sediment samples. Thus, only the fixation rates occurring in the sediment are presented here.

The low tide set of sediment samples had slightly lower fixation rate than the high tide set (Fig. 7). Whereas the mature mangrove stand presented similar average ± SE nitrogen fixation rates of 0.020 ± 0.005 mg N m^{-2} day^{-1} for bioturbated sediment and non-bioturbated sediments, respectively. As in the case of high tide sediments planted with mangrove saplings had no detectable nitrogen fixation rates for either bioturbated or non-bioturbated sediments, at 0 ± 0.001 mg N m^{-2} day^{-1} (Fig. 5).

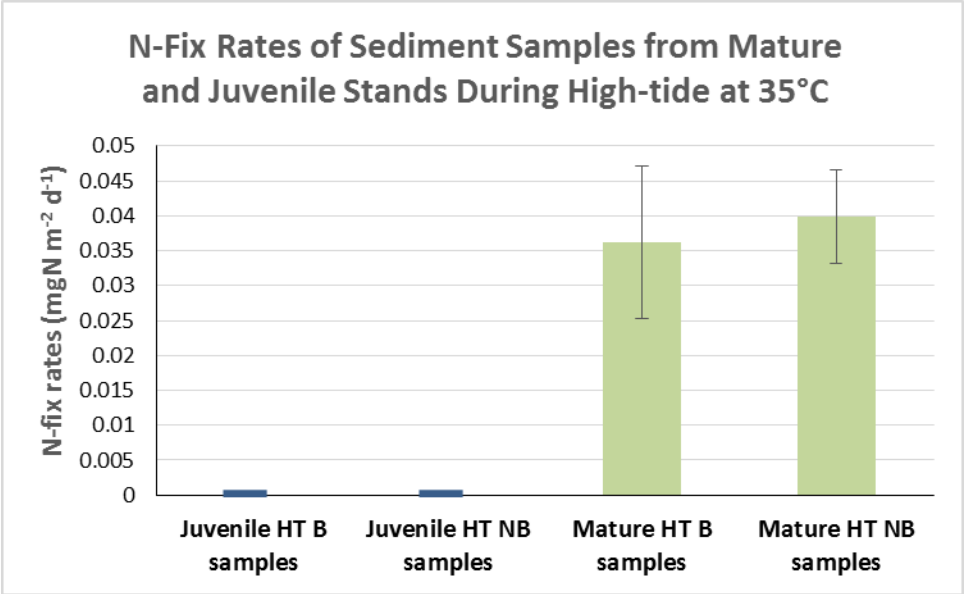


Figure 4: Average nitrogen fixation rates in sediment samples of mature and juvenile mangrove stands sampled at high-tide (HT) and incubated at 35°C, for, bioturbated sediment (B) and non-bioturbated sediment (NB).

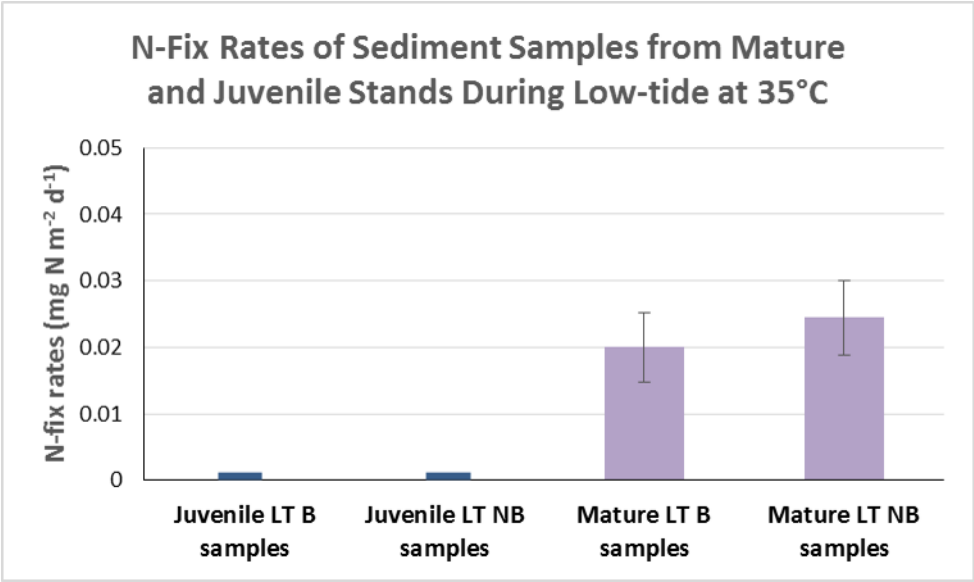


Figure 5: Averages of nitrogen fixing rates in sediment samples of mature and juvenile mangrove stands during the low-tide (LT) and incubated at 35°C for bioturbated sediment (B) and non-bioturbated sediment (NB).

3.3 Nitrogen fixing rates at 28°C

The nitrogen fixation rates derived for mangrove sediments in winter, at high tide, when in situ temperature was 28°C were much higher than those derived at 35°C. Nitrogen fixation rates detected in the bioturbated sediment of mature mangrove stands were similar to those detected at high tide conditions with an average ± SE rate of 0.030 ± 0.007 mg N m^{-2} day^{-1}. However, fixation rates detected in the non bioturbated sediment of mature mangrove stands was 15 times greater, with an average ± SE rate of 0.463 ± 0.107 mg N m^{-2} day^{-1} for the non-bioturbated sediment (Fig. 6). Nitrogen fixation rates of sediments supporting planted mangrove saplings were detectable, but significantly lower than those in mature mangrove stands, with an average rate of 0.018 ± 0.003 mg

N m^{-2} day^{-1} for bioturbated samples and 0.016 ± 0.006 mg N m^{-2} day^{-1} for non-bioturbated sediment (Fig. 6).

3.4 Effects of the experimental factors on nitrogen fixation rates

The statistical analysis showed significant difference in nitrogen fixation rates between the treatments (temperature, mangrove state, tidal stage and bioturbation), (Fig. 7). Where the juvenile versus mature mangroves accounts for the most variability, followed by temperature, then, the interaction between the mangrove and the different factors (R^2 = 079, $p < 0.001$*, Table 1).

The differences in nitrogen fixing rates were related to sediment characteristics. Nitrogen fixation rates at high temperatures (35°C) increased significantly with increasing organic matter concentration in the sediments (R^2 =0.31, $P < 0.001$*), organic carbon concentration (R^2 = 0.31, P = 0.002) and nitrogen concentration (R^2 = 0.26, P = 0.004), (Fig. 8).

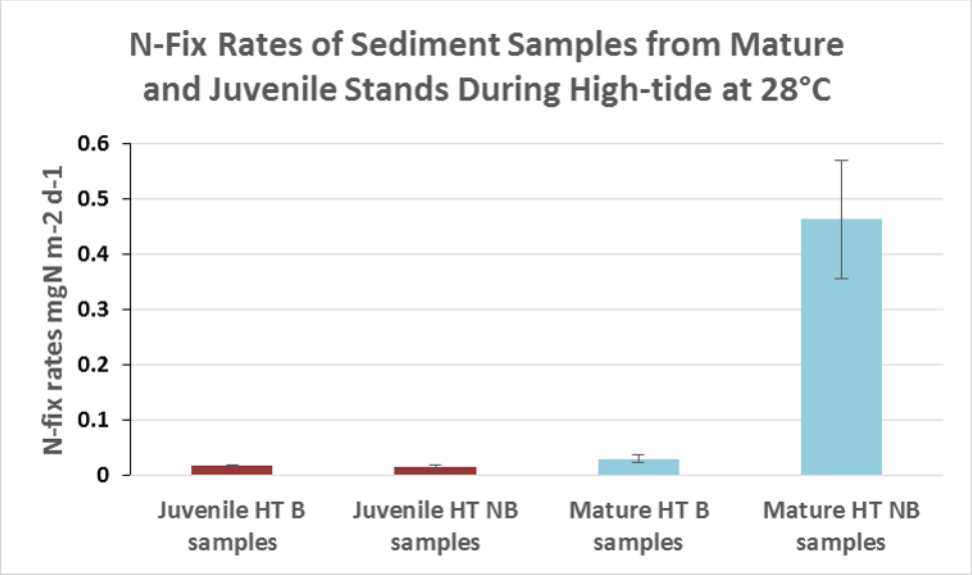


Figure 6: Averages of nitrogen fixing rates in sediment samples of mature and juvenile mangrove stands during the high-tide (HT) and incubated at 28°C, for, bioturbated sediment (B) and non-bioturbated sediment (NB).

Table 1: Analysis of variance (ANOVA) test using general linear model (GLM), to assess the significance of each treatment on the variability of nitrogen fixation rates and the interaction between them.

Summary of Fit	
RSquare	0.791488

RSquare Adj	0.756736
Root Mean Square Error	0.075049
Mean of Response	0.063371
Observations (or Sum Wgts)	50

Source	DF	Sum of Squares	Mean Square	F ratio
Model	7	0.8979545	0.128279	22.7753
Error	42	0.2365596	0.005632	**Prob > F**
C. total	49	1.1345142		<0.0001*

Parameters Estimates

Term	Estimate	Std Error	t Ratio	Prob >|t|
Intercept	0.071679	0.0111	6.46	<.0001*
Mangrove [Juvenile]	-0.06696	0.0111	-6.03	<.0001*
Bioturbation [No]	0.054445	0.0111	4.91	<.0001*
Temperature [28°C]	0.056611	0.0111	5.1	<.0001*
Temperature [28°C]*Mangrove [Juvenile]	-0.05189	0.0111	-4.67	<.0001*
Bioturbation [No]*Mangrove [Juvenile]	-0.05482	0.0111	-4.94	<.0001*
Bioturbation [No]*Mangrove [Juvenile]*Temperature [28°C]	-0.05382	0.0111	-4.858	<.0001*
Bioturbation [No]*Temperature [28°C]	0.05344	0.0111	4.81	<.0001*

Effect Summary

Source	LogWorth	P value
Mangrove	6.448	0.0000
Temperature	5.114	0.00001
Bioturbation*Mangrove	4.887	0.00001
Bioturbation	4.839	0.00001 ^
Bioturbation*Mangrove*Temperature	4.760	0.00002
Bioturbation*Temperature	4.712	0.00002 ^
Temperature*Mangrove	4.518	0.00003 ^

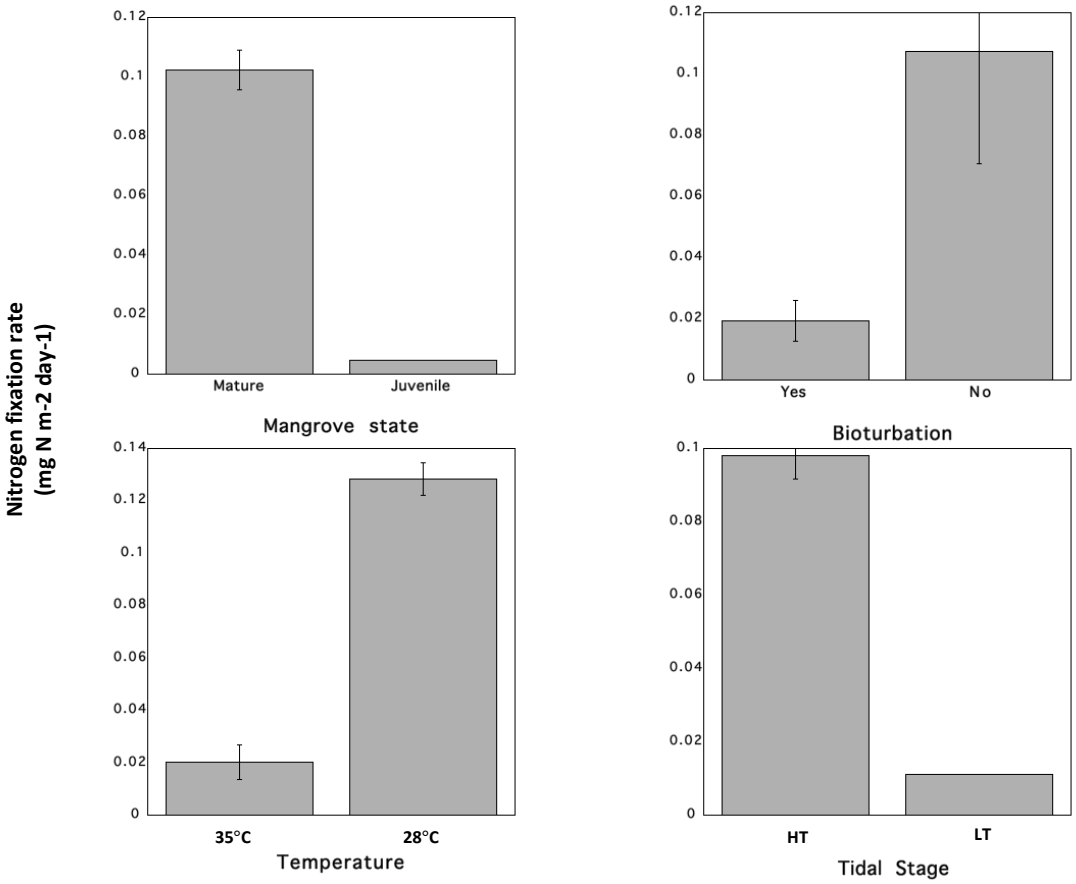
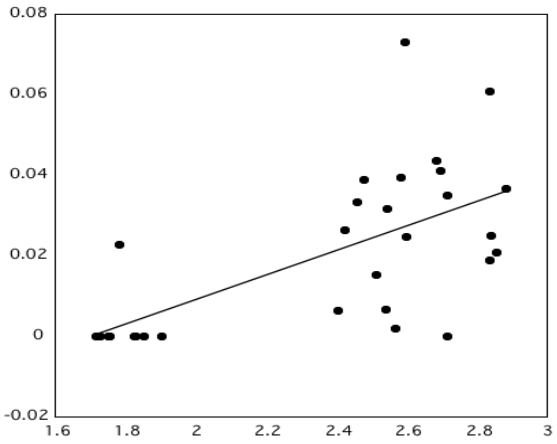
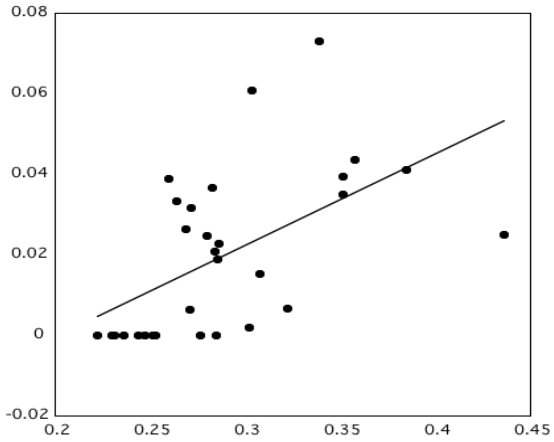


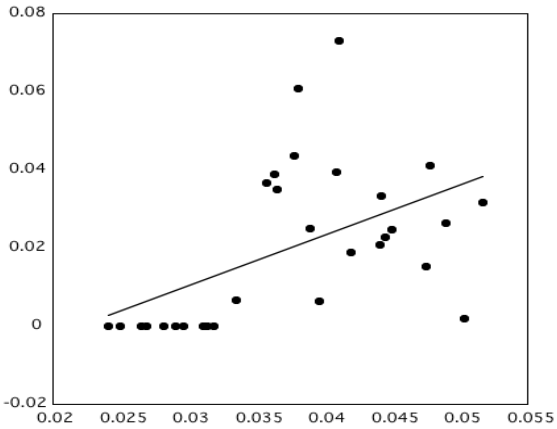
Figure 7: Relationship between the different treatments and nitrogen fixation rates.



a) Organic Matter (% dry weight)



b) Organic Carbon (% dry weight)



c) Nitrogen (% dry weight)

ogen fixation rate
g N m-2 day-1)

Figure 8: Relationship between sediment characteristics and nitrogen fixation rates. a) Organic matter (% OM), b) Organic carbon (% Org C) and c) Nitrogen (% N).

Table 2: Nitrogen fixation rates determined in central Red Sea mangrove stands at different temperature and under different mangrove maturity and bioturbation extent, along with the corresponding sediment organic content (OM, % dry weight) and organic carbon and nitrogen concentrations (% dry weight).

Sample	**Sampling date and temperature**	**N-Fix rate**	**%OM content**	**% Org C**	**% N**
Mature HT B1	Dec 6, 2016 at 35°C	0.007	2.541	0.322	0.034
Mature HT B2	Dec 6, 2016 at 35°C	0.035	2.717	0.352	0.037
Mature HT B3	Dec 6, 2016 at 35°C	0.025	2.840	0.436	0.039
Mature HT B4	Dec 6, 2016 at 35°C	0.073	2.594	0.339	0.041
Mature HT B5	Dec 6, 2016 at 35°C	0.041	2.698	0.385	0.048
Mature HT NB1	Dec 6, 2016 at 35°C	0.037	2.883	0.283	0.036
Mature HT NB2	Dec 6, 2016 at 35°C	0.061	2.837	0.304	0.038
Mature HT NB3	Dec 6, 2016 at 35°C	0.043	2.685	0.358	0.038
Mature HT NB4	Dec 6, 2016 at 35°C	0.019	2.835	0.285	0.042
Mature HT NB5	Dec 6, 2016 at 35°C	0.039	2.584	0.351	0.041
Juvenile LT B1	Dec 14, 2016 at 35°C	0	1.729	0.231	0.024
Juvenile LT B2	Dec 14, 2016 at 35°C	0	1.854	0.236	0.025
Juvenile LT	Dec 14, 2016	0	1.831	0.253	0.030

B3	at 35°C				
Juvenile LT B4	Dec 14, 2016 at 35°C	0	1.757	0.222	0.027
Juvenile LT B5	Dec 14, 2016 at 35°C	0	1.715	0.248	0.028
Juvenile LT NB1	Dec 14, 2016 at 35°C	0	2.718	0.252	0.027
Juvenile LT NB2	Dec 14, 2016 at 35°C	0	1.752	0.276	0.032
Juvenile LT NB3	Dec 14, 2016 at 35°C	0	1.828	0.230	0.031
Juvenile LT NB4	Dec 14, 2016 at 35°C	0	1.902	0.243	0.029
Juvenile LT NB5	Dec 14, 2016 at 35°C	0	1.723	0.285	0.031
Mature LT B1	Dec 26, 2016 at 35°C	0.015	2.514	0.307	0.048
Mature LT B2	Dec 26, 2016 at 35°C	0.025	2.599	0.280	0.045
Mature LT B3	Dec 26, 2016 at 35°C	0.032	2.544	0.271	0.052
Mature LT B4	Dec 26, 2016 at 35°C	0.026	2.426	0.269	0.049
Mature LT B5	Dec 26, 2016 at 35°C	0.002	2.568	0.302	0.050
Mature LT NB1	Dec 26, 2016 at 35°C	0.023	1.783	0.286	0.045
Mature LT NB2	Dec 26, 2016 at 35°C	0.039	2.479	0.260	0.036
Mature LT NB3	Dec 26, 2016 at 35°C	0.033	2.460	0.264	0.044
Mature LT NB4	Dec 26, 2016 at 35°C	0.021	2.856	0.284	0.044
Mature LT NB5	Dec 26, 2016 at 35°C	0.006	2.407	0.271	0.040
Mature HT B1	Jan 31, 2017 at 28°C	0.006	3.207	0.432	0.056
Mature HT B2	Jan 31, 2017 at 28°C	0.051	3.324	0.472	0.057
Mature HT B3	Jan 31, 2017 at 28°C	0.038	3.141	0.447	0.057
Mature HT B4	Jan 31, 2017 at 28°C	0.034	3.334	0.431	0.055
Mature HT B5	Jan 31, 2017 at 28°C	0.024	3.290	0.501	0.057
Mature HT	Jan 31, 2017	0.506	2.911	0.349	0.047

NB1	at 28°C				
Mature HT NB2	Jan 31, 2017 at 28°C	0.159	2.821	0.286	0.039
Mature HT NB3	Jan 31, 2017 at 28°C	0.315	2.799	0.272	0.040
Mature HT NB4	Jan 31, 2017 at 28°C	0.552	2.867	0.293	0.043
Mature HT NB5	Jan 31, 2017 at 28°C	0.787	2.662	0.309	0.051
Juvenile HT B1	Feb 28, 2017 at 28°C	0.013	2.175	0.250	0.030
Juvenile HT B2	Feb 28, 2017 at 28°C	0.005	2.132	0.224	0.039
Juvenile HT B3	Feb 28, 2017 at 28°C	0.009	1.941	0.266	0.050
Juvenile HT B4	Feb 28, 2017 at 28°C	0.011	1.929	0.271	0.054
Juvenile HT B5	Feb 28, 2017 at 28°C	0.013	2.102	0.219	0.039
Juvenile HT NB1	Feb 28, 2017 at 28°C	0.010	2.022	0.226	0.034
Juvenile HT NB2	Feb 28, 2017 at 28°C	0.016	1.955	0.223	0.047
Juvenile HT NB3	Feb 28, 2017 at 28°C	0.012	2.178	0.277	0.044
Juvenile HT NB4	Feb 28, 2017 at 28°C	0.006	2.084	0.245	0.041
Juvenile HT NB5	Feb 28, 2017 at 28°C	0.000	1.987	0.242	0.041

Chapter 4

Discussion

Rates of nitrogen fixation in mangrove communities reported in literature ranged from 0.03 to 2.6 g N m^{-2} $year^{-1}$ [26][36]. The range of nitrogen fixation rates detected in the Central Red Sea *Avicennia marina* mangrove stand examined here were within the low range, averaging 0.007 to 0.168 g N m^{-2} $year^{-1}$. Indeed, Central Red Sea stands are stunted and relatively unproductive when compared to mangrove stands elsewhere [11], due to critical nutrient limitation [10]. Indeed, experimental assessment of nutrient limitation in Central Red Sea mangroves concluded that they are iron-limited [10], which is consistent with the low rates of nitrogen fixation detected here, as the nitrogenase enzyme requires an iron cofactor. However, the results presented here revealed a number of drivers affecting nitrogen fixation rates in mangrove sediments. First, nitrogen fixation process appeared to be strongly temperature dependent, as it had much higher rates, 10 fold greater on average, during the colder period studied (28° C) than at maximum temperatures (30° C). This is consistent with the optimal temperature

for nitrogen fixation rates, which range between 15 and 30°C, with reduced nitrogen fixation rate starts at 35°C [37][38]. Hence, higher temperatures affect nitrogenase enzyme activity produced by nitrogen fixers (diazotrophs) through the repression of the enzyme synthesis [39], consistent with the low rates observed at the highest temperatures reached in the Central Red Sea. In addition, temperature can control the microbial community structure [40], which include microorganisms responsible for nitrogen fixation process. Some studies reported thermophilic microorganisms that can tolerate high temperatures and act as nitrogen-fixers [41][42]. According to the low nitrogen fixation rates we have obtained at higher temperature (35°C), we speculate that the microbial composition in our samples might not have thermophilic nitrogen fixers. To prove this point, further studies on the microbial community in the mangrove sediment should be done.

Second, nitrogen fixation rates were significantly affected by crab bioturbation, with non-bioturbated sediments showing higher nitrogen fixation rates than bioturbated sediments do. Crab bioturbation can affect nitrogen fixation activity in several ways. First, crab burrowing activity transports oxygen to deeper, anoxic sediment layers, which should results in unfavorable conditions for N_2 fixation, which requires anaerobic conditions, and inhibits nitrogenase enzyme activity [2][30]. Burrowing activity will also oxidize and release iron from the sediment, which is a limiting factor for mangrove growth [43][10]. In addition, crabs can limit nitrogen fixation through their feeding behavior, which alters the microbial community in the sediments. Cyanobacteria is one

of the important microorganisms that are responsible for nitrogen fixation in mangrove ecosystems [44]. Hence, feeding of crabs on cyanobacteria might reduce nitrogen fixation activity in the system. Bioturbation also changes the sediment composition such as organic matter content, which tend to be lower in bioturbated sediments and this affects the benthic nitrogen cycle [29][43], as demonstrated by the positive relationship between nitrogen fixation rates and sediment organic carbon, and nitrogen, concentration.

Lastly, colonization of sediments by juvenile mangroves is associated with much lower nitrogen fixation rates than those in sediment supporting mature mangrove stands. This suggest that mature mangrove stands condition, possibly through increasing the organic carbon stocks of the sediments, the biogeochemical conditions and the microbial communities able to perform nitrogen fixation rates [45].

In conclusion, in this study we estimated, for the first time, nitrogen fixing rates in central Red Sea mangrove ecosystems, and confirmed that they are toward the low range of rates reported for mangrove ecosystems. The low rates were also associated with the high temperatures in the Red Sea, which, for the highest annual temperatures, exceed the optimal temperature for nitrogen fixation, leading to a sharp decline in rates. Furthermore, we demonstrate here that nitrogen fixation was negatively impacted by the activity of burrowing crabs. These different drivers act in isolation, but also interact,

to determine a complex regulation of nitrogen fixation rates in central Red Sea mangroves.

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