# Nitrogen fixation in Red Sea seagrass meadows

Thesis by
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## EXAMINATION COMMITTEE PAGE

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# ABSTRACT

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Seagrasses are key coastal ecosystems, providing many ecosystem services. Seagrasses increase biodiversity as they provide habitat for a large set of organisms. In addition, their structure provides hiding places to avoid predation. Seagrasses can grow in shallow marine coastal areas, but several factors regulate their growth and distribution. Seagrasses can uptake different kinds of organic and inorganic nutrients through their leaves and roots. Nitrogen and phosphorous are the most important nutrients for seagrass growth. Biological nitrogen fixation is the conversion of atmospheric nitrogen into ammonia by diazotrophic bacteria. This process provides a significant source of nitrogen for seagrass growth. The nitrogen fixation is controlled by the *nif* genes which are found in diazotrophs. The main goal of the project is to measure nitrogen fixation rates on seagrass sediments, in order to compare among various seagrass species from the Red Sea. Moreover, we will compare the fixing rates of the Vegetated areas with the bare sediments. This project will help to ascertain the role of nitrogen fixing bacteria in the development of seagrass meadows.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ARA | Acetylene reduction assay |
| FID-MS | Flame Ionization Detector- Mass Spectrometer |
| KAEC | King Abdullah Economic City |
| LOI | Loss on ignition |

## LIST OF ILLUSTRATIONS

## Chapter 1. Introduction

Seagrasses are unique marine flowering plants adapted to grow in the sea, thriving across coastal and estuarine waters around the world. There are about 60 species of seagrasses, 12 of them reported in the Red Sea [1]. Whereas seagrass meadows abound in the Red Sea, there is a paucity of reports on their ecology and their ecosystem functions, with just a few papers on their distribution and ecology, with the highest number of species reported in the central Red Sea due to the habitat diversity.(Figure.1)[2].

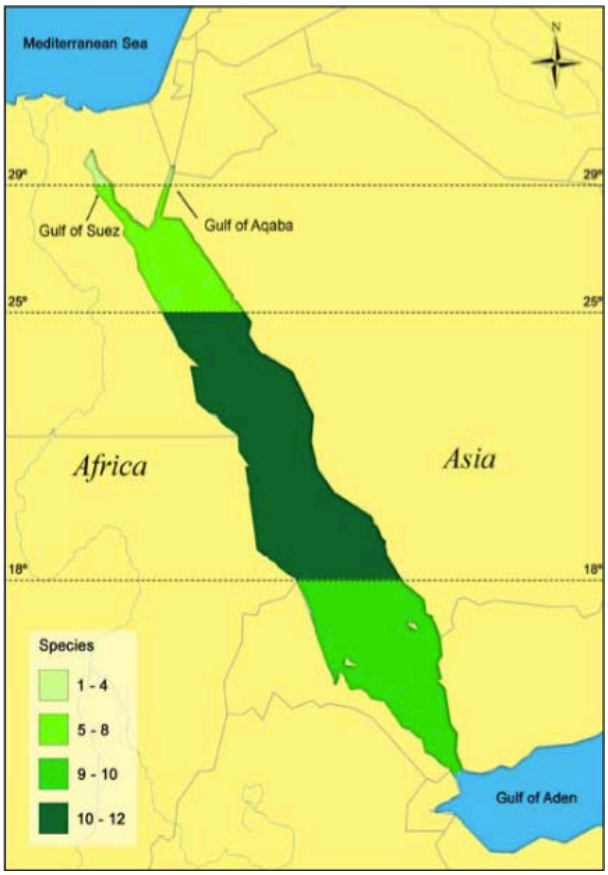



Figure.1 Schematic map represents the seagrass species diversity in the Red Sea. The figure was adapted from [2].

## 1.1 Ecosystem Services and ecosystem functions.

Seagrass has been used traditionally for a number of purpose, including packaging of delicate items and transportation of fresh fish, but understanding the major ecological function they perform is rapidly increasing. Seagrass meadows form highly productive and complex habitats that provide habitat for a large range of organisms, as demonstrated by elevated abundance of biota in seagrass meadows compared to adjacent bare sediments [3]. Seagrasses are primary food for sea turtles, dugongs, fishes and range of invertebrates, and their productivity fuels coastal food webs [2].

Seagrasses form extensive meadows submerged in seawater where they live and complete their life cycle [1], extending from shallow water and can exceed 60 m in depth in transparent waters [4]. Mixed seagrass meadows can be made of up to 14 species, but most of the meadows comprise only one seagrass species [5]. Seagrass meadows rank among the most productive ecosystems in the biosphere [6], despite often growing in nutrient-poor ecosystems. Seagrass are anchor to muddy and sandy sediments by root and rhizome systems, which play a critical role in their propagation and preservation. Their extensive root systems allow them to take up nutrients from the sediments [1], including nitrogen and phosphorous. Nitrogen is an essential element in seagrass development and often acts as limiting nutrient in their capacity to synthesize proteins, other molecules and structural material supporting their growth [7].
Whereas reduced nitrogen forms, which can be readily taken up by seagrass, are often present at low concentration in the environment. Nitrogen is abundant in the atmosphere, where it comprises about 79% of all gases. However, atmospheric nitrogen is inert and cannot be used

directly for a majority of organisms, as the triple bond linking the two nitrogen atoms in $N_2$ gas is difficult to break. However, a group microbes can break down atmospheric nitrogen form and convert nitrogen into reduced forms that can be used by other organisms. The conversion process of atmospheric nitrogen gas $N_2$ to ammonia called biological nitrogen fixation, which act as a critical link allowing atmospheric nitrogen to enter nitrogen cycle in the ecosystems [8]. Whereas seagrass cannot access atmospheric dinitrogen gas directly, microbes associated with seagrass roots and sediment can perform nitrogen fixation, providing ammonia that can then be used to support seagrass growth [7].

### 1.2 Nitrogen fixation

The ability to perform nitrogen fixation is widely dispersed among prokaryotes. Diazotrophs are prokaryotes responsible for the conversion of atmospheric nitrogen into simpler and usable forms [8][9]. Sulphate- reducing bacteria are the most important heterotrophic diazotrophs in costal marine sediments. All diazotrophs contain at least one of the three structurally, mechanically subtypes related nitrogenase enzyme, which is a complex of two enzymes, dinitrogenase and dinitrogenase reductase, both containing iron while dinitrogenase reductase also contains molybdenum. The molybdenum core is solely responsible for the sustenance of a stable conversion process that keeps the process ongoing until it is complete [10]. Microbial community and seagrasses form a symbiotic relationships involving, among other processes, the supply of nutrients through nitrogen fixation, which represent a major source of nitrogen in seagrass meadows [6].

The reduction rate of nitrogen gas to ammonia by nitrogen fixation can be measured using different methods. The most widely applied methods to measure rates of marine $N_2$ fixation are the acetylene reduction assay (ARA), and the $^{15}N_2$ assimilation technique [11]. The ability of the nitrogenase enzyme to reduce a variety of substrates has made the ARA possible. ARA is an inexpensive, rapid, sensitive, and an accurate method [6]. The assay follows the reduction of acetylene ($C_2H_2$) to ethylene ($C_2H_4$) by the nitrogenase enzyme [12][13], where the ethylene is detected by flame ionizing detector after the gas chromatography separation. However, it is an indirect method, and since the formation of ethylene requires two electrons while the reduction of $N_2$ to ammonia need six electrons, a conversion factor of 3 $C_2H_2$: 1 $NH_4$ is typically applied to calculate nitrogen fixation rates [13].

Nitrogen fixation rates have been measured across a range of seagrass meadows, including temperate and tropical ecosystems [6], but have never been reported for the Red Sea, which ranks amongst the most oligotrophic ecosystems supporting seagrass growth. Hence, the role of nitrogen fixation in supporting the growth and production of Red Sea seagrass meadows is as yet unresolved, as well as the variability in rates among communities dominated by contrasting species in this ecosystem [2].

The main goal of this study is to quantify, for the first time, nitrogen fixation rates on Red Sea seagrass communities dominated by different species, and to assess the role of seagrass ecosystems as a source of nitrogen to the Red Sea ecosystems by comparing between vegetated and adjacent bare sediments.

# Chapter 2. Material and methods

## 2.1 study site and samples collection

Seagrass communities were sampled in different locations in the Saudi coast of the Central Red Sea, including King Abdullah Economic City Lagoon (latitude 22.386950$^{o}$ N, longitude 39.1305337$^{o}$ E), and a coastal area just north of Rabigh (latitude 22.910389$^{o}$ N, longitude 38.869722$^{o}$ E). Seagrass sediment samples were collected using Plexiglas cores, pushed 9-10 cm into the sediment with the help of a rubber hammer, and sealed with rubber stoppers while transported to the laboratory at KAUST. Three sites were sampled within King Abdullah Economic City lagoon, including vegetated and bare sediments. Vegetated sediments included *Cymodocea serrulata* and *Halodule uninervis* in one site sampled on 20/11/2016, *Cymodocea rotundata* in a second site sampled on 16/1/2017, and a mixed meadow of *Cymodocea rotundata* and *Halodule uninervis* sampled on 1/2/2017 in the third site (Figure.2). In order to maximize the number of seagrass species, in Rabigh seagrass sediments were collected from shallow nearshore waters, where monospecific patches of *Thalassia hemprichii, Halophila stipulace* and *Halodule uninervis* were sampled separately on 30/4/2017 (Figure.3).


Figure.2 Study sites at the Red Sea in King Abdullah Economic City Lagoon, Saudi Arabia.

1. *Cymodocea serrulata* and *Halodule uninervis,* 2. *Cymodocea rotundata,* 3. *Cymodocea rotundata* and *Halodule uninervis*

Figure.3 Study site at the Red Sea in Rabigh, Saudi Arabia.

**2.2 Samples preparation**

Seawater was siphoned out of the cores using a silicon tube to avoid disturbing the sediments. Then, the sediments in each core were extruded and placed in a plastic holder, where they were sliced into four sediment horizons (0-1, 1-2, 2-3 and 9-10 cm) to measure nitrogen fixation in different levels of sediment depth. Then from each horizon 80 ml of the sediment was placed in a 500 ml Pyrex glass incubation jar, and amended with 200 ml of seawater collected from the sampling location and stirred to produce a slurry. 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. Seagrass materials present in the samples were removed.

Bare sediment samples were processed as above, with a slight modification. Briefly, the top 10 cm of each sediment core was mixed and 80 ml of the mixture added to a Pyrex glass jar and amended with 200 ml of sea water to form the slurry, and incubated as described below.

**2.3 Acetylene Reduction Assay**

Nitrogen fixation activity in the collected sediment samples was indirectly estimated using the acetylene reduction assay (ARA). ARA measures the nitrogenase enzyme activity, which reduces acetylene gas ($C_2H_2$) into ethylene gas ($C_2H_4$) [14][15][16]. Acetylene reduction rates are converted to nitrogen fixing rates following the theoretical ratio 3:1 as mentioned before. Acetylene was added to the incubation bottles in the form of acetylene-enriched seawater.

Acetylene enriched-seawater was prepared by bubbling acetylene gas, from a cylinder, through a gas regulator into 1000 ml Pyrex glass jar fitted with two gas inlets in the lid containing 300 ml

of filtered seawater. A stream of acetylene gas was introduced through one of the gas inlets, with second inlet acting as a vent, for 5 mins. Acetylene enriched-seawater was prepared independently for each seagrass species tested.

The slurries were then injected with 20 ml of Acetylene-enriched seawater and gently mixed to allow the diffusion of acetylene through the sample. Immediately, 3 ml of the headspace gas was taken from each seagrass sediment sample using a plastic syringe and the gas samples were injected into gas tight vacuum tubes ($T_0$). Samples were then incubated under dark conditions at 29 °C (*in situ* temperature). Gas samples were collected again after 12h and at different time points during the incubation period (24h).

**2.4 Quantification of ethylene using gas chromatography.**

Measurement of $C_2H_4$ were conducted with a gas chromatograph equipped with FID-MS (Flame Ionization Detector- Mass Spectrometer). The gas samples were separated with Gs- carbon plot column (60m in length, 0.320 in diameter and 1.50 microfilm). The total analytic time per sample was 14.08 min. Three replicates of known ethylene gas concentration (93 ppm, 9 ppm and 1.5 ppm) were used as standards and to stablish a calibration curve. The ethylene peak appeared in the standards following a retention time of 7.5 mins. Rates of $C_2H_4$ production of the seagrass sediment samples were determined from the increase of $C_2H_4$ concentration over time and converted to nitrogen fixation rates following [11]. 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.

**2.5 Calculation of nitrogen fixate on rates**

Nitrogen fixation rates were estimated based on the ethylene production rate measured by the GC, following the described formulas by [11].

1. The $C_2H_4$ concentration (ppm) in the equilibrated headspace was calculated from the $C_2H_4$ peak area in the GC, using the calibration curve, Y = mX + b, established using the standards.

$$\text{where the } concentration\ (x)\ =\ peak\ area\ (y)\ -\ (b/a).$$

2. Total dissolved $C_2H_4$ concentration ($\{C_2H_4\}_w$ ml of $C_2H_4$ /ml of $H_2O$) in the water after equilibration by:

$$\{C_2H_4\}_w = 10^{-6} \text{ x Bunsen solubility coefficient x } C_2H_4 \text{ in headspace (ppm) x } p$$

where Bunsen solubility coefficient of $C_2H_4$ is 0.075 at 29$^{o}$C and 40% [17], and *p* is atmospheric pressure (atm) of dry air = 1

3. $C_2H_4$ concentration in the initial seawater ($\{C_2H_4\}_{aq}$ in ml of $C_2H_4$ /ml of $H_2O$) was calculated, as follows:

$$\{C_2H_4\}_{aq} = (\{C_2H_4\}_w \text{ x } V_w + 10^{-6} \text{ x } C_2H_4 \text{ in headspace x } V_a) / V_w$$

where $V_w$ is the water sample volume= 300 ml, and $V_a$ is the volume of the headspace = 200 ml

4. The conversion of $\{C_2H_4\}_{aq}$ in ml of $C_2H_4$ /ml of $H_2O$ to nmol/L as follows:

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

where R is the gas constant = 0.08206 atm liter mol$^{-1}$K$^{-1}$, and T is temperature (K) = 273+29.

The ethylene production rate was calculated from the increase in concentration over the incubation time. Finally, ethylene production rates (nmol $L^{-1}h^{-1}$) were converted to nitrogen fixing rates (nmol N $L^{-1}$ $h^{-1}$) by dividing the values by 3.

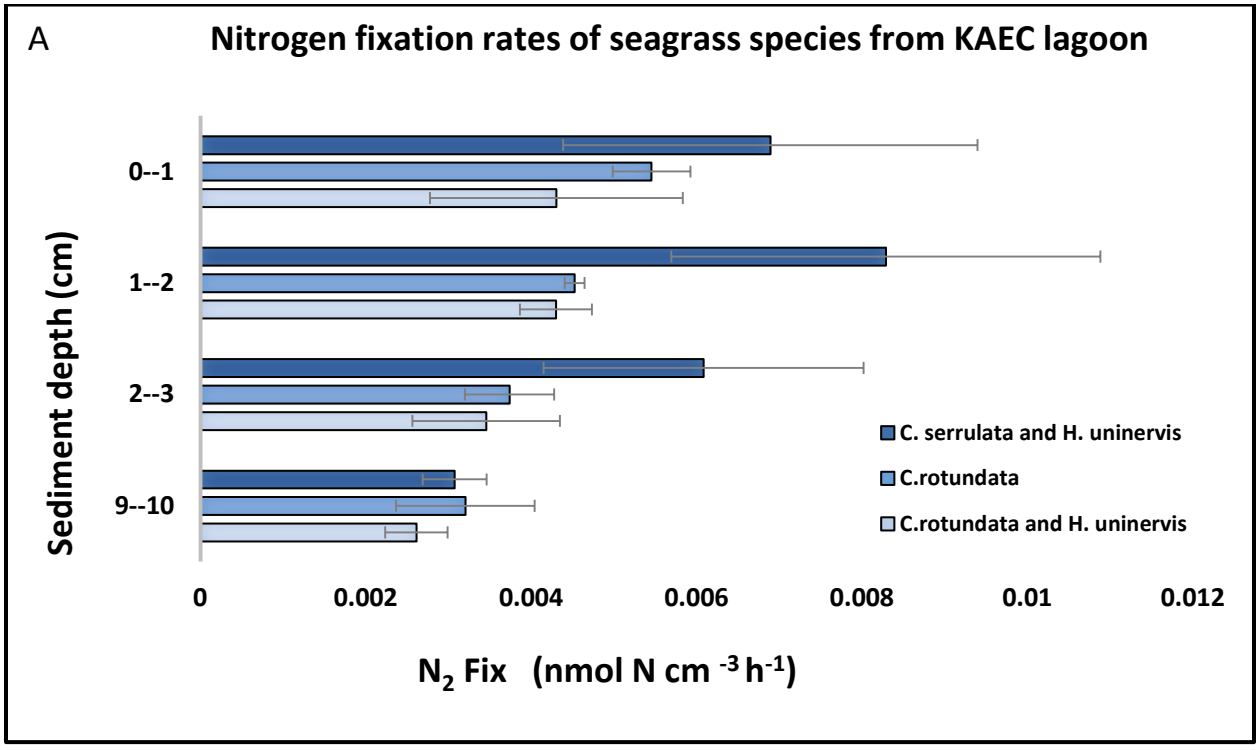
**2.6 Sediment Analysis**

The organic matter (%OM) in seagrass sediment samples was calculated from loss on ignition (LOI). Sediment subsamples from the layers used to assess nitrogen fixation rates were dried at 60°C x in a drying oven and then grinded using a Mill – Grinder to a size of approximately 2mm particles. Then porcelain crucibles were heated in a furnace oven for 1 hour. at 400°C, crucibles weights were measured one by one using a high precision balance. A subsample of 3 g of dried, grinded sediments was then placed on a porcelain crucible and combusted for 5 hour at 450$^{o}$C in the furnace oven to determine the percent of organic matter from the loss on ignition. Measurements of dry and burned sediment were used in the below formula to calculate the organic matter content in the samples:

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

## 3. Results

Nitrogen fixation rates were detected in all the tested seagrass meadows, and tended to be higher in surface sediment horizons (0-3 cm) than in deeper sediments. Nitrogen fixation rates in surface horizons ranged between 0.0034 and 0.0082 nmol N $cm^{-3}$ $h^{-1}$ in the seagrass stands sampled at KAEC lagoon and between 0.0008 and 0.0024 nmol N $cm^{-3}$ $h^{-1}$ in the seagrass stands sampled at Rabigh. Nitrogen fixation rates in deep sediments (9-10 cm), where few roots penetrated, were associated with low rates, ranging between 0.0026 and 0.0032 nmol N $cm^{-3}$ $h^{-1}$ in the seagrass stands sampled at KAEC and 0.0009 to 0.0019 to nmol N $cm^{-3}$ $h^{-1}$ in the seagrass stands sampled at Rabigh (Figure.4). Nitrogen fixation rates of *C. Serrulata and H. uninervis, C. rotundata and C rotundata and H. uninervis* were greater in surface than in deeper sediments. Nitrogen fixation rates in Rabigh, tended to be similar although the horizons are different.



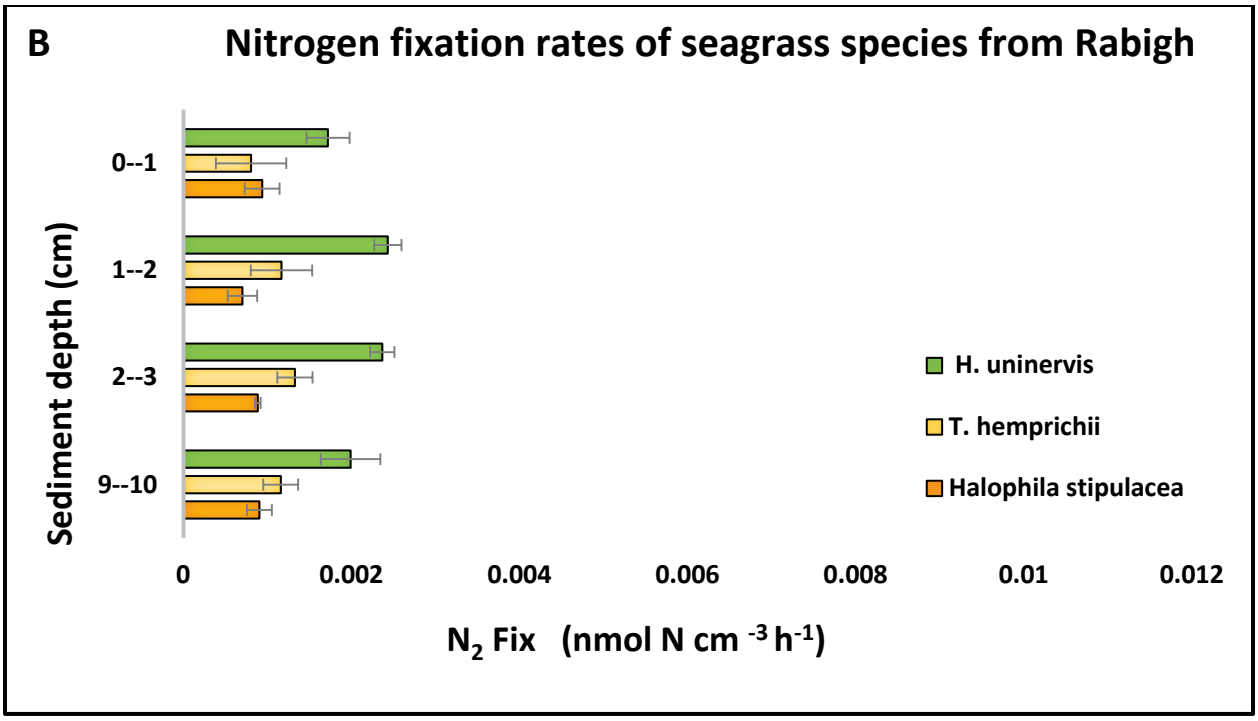
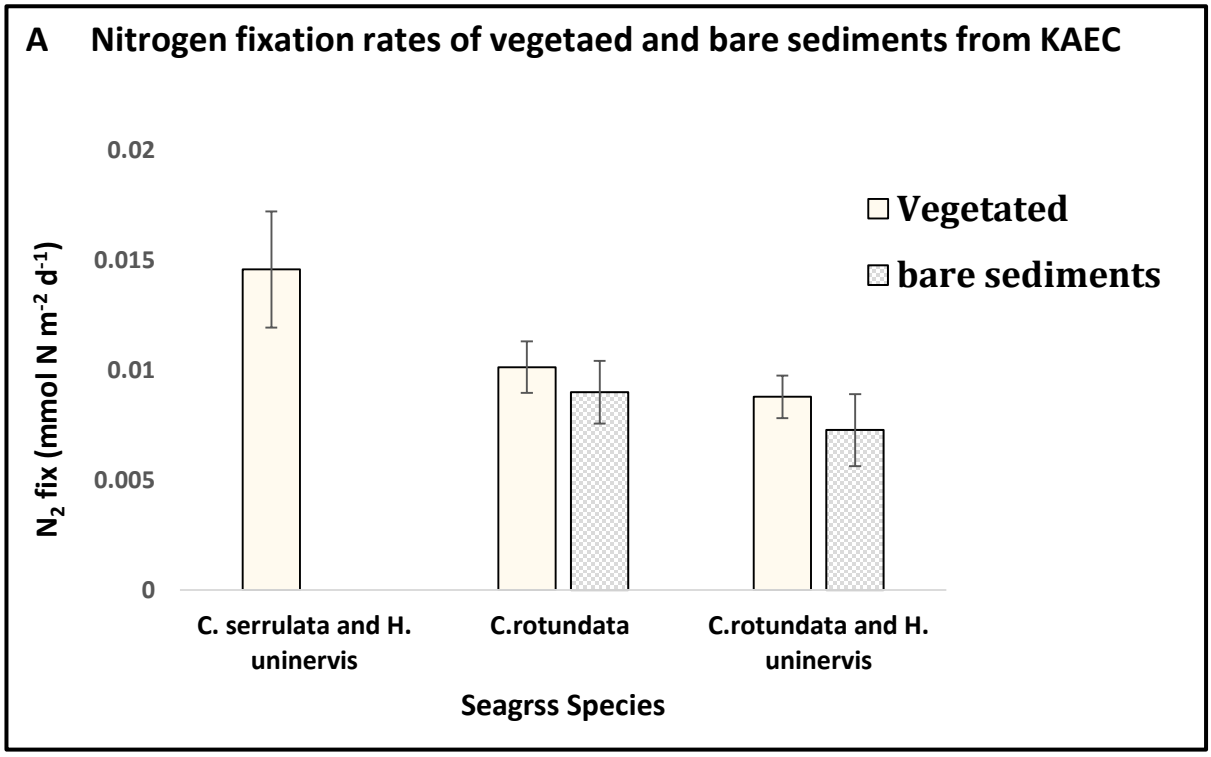



Figure 4. Nitrogen fixation rates (mean ± SD) of seagrass sediment horizons. (A) samples from King Abdullah Economic city lagoon. (B) Samples from Rabigh. Standard deviations calculated from three cores taken from each meadow.

The results were then integrated to 10 cm in order to allow comparisons of the rates between vegetated and bare sediments. Nitrogen fixation rates observed in seagrass colonized sediments were generally greater than those reported for adjacent unvegetated sediments. Nitrogen fixation rates of seagrass meadows in KAEC were similar to the rates in adjacent bare sediment (Figure. 5A). Nitrogen fixation rates in *C. rotundata* meadow were 0.010 ± 0.001 and 0.008 ± 0.00 mmol N $m^{-2}$ $d^{-1}$ in the *C. rotundata and H. uninervis* meadow, while the rates of adjacent bare sediments were somewhat lower, but not significantly, so, averaging 0.009 ± 0.001 mmol N $m^{-2}$ $d^{-1}$ and 0.007 ± 0.001 mmol N $m^{-2}$ $d^{-1}$, respectively in contrast, Nitrogen fixation rates of seagrasses meadows from Rabigh were higher than the rates of bare sediments


A Nitrogen fixation rates of vegetaed and bare sediments from KAEC
0.02
0.015
0.01
0.005
0
N2 fix (mmol N m-2 d-1)
Vegetated
bare sediments
C. serrulata and H. uninervis
C.rotundata
C.rotundata and H. uninervis
Seagrss Species

(Figure.5B).

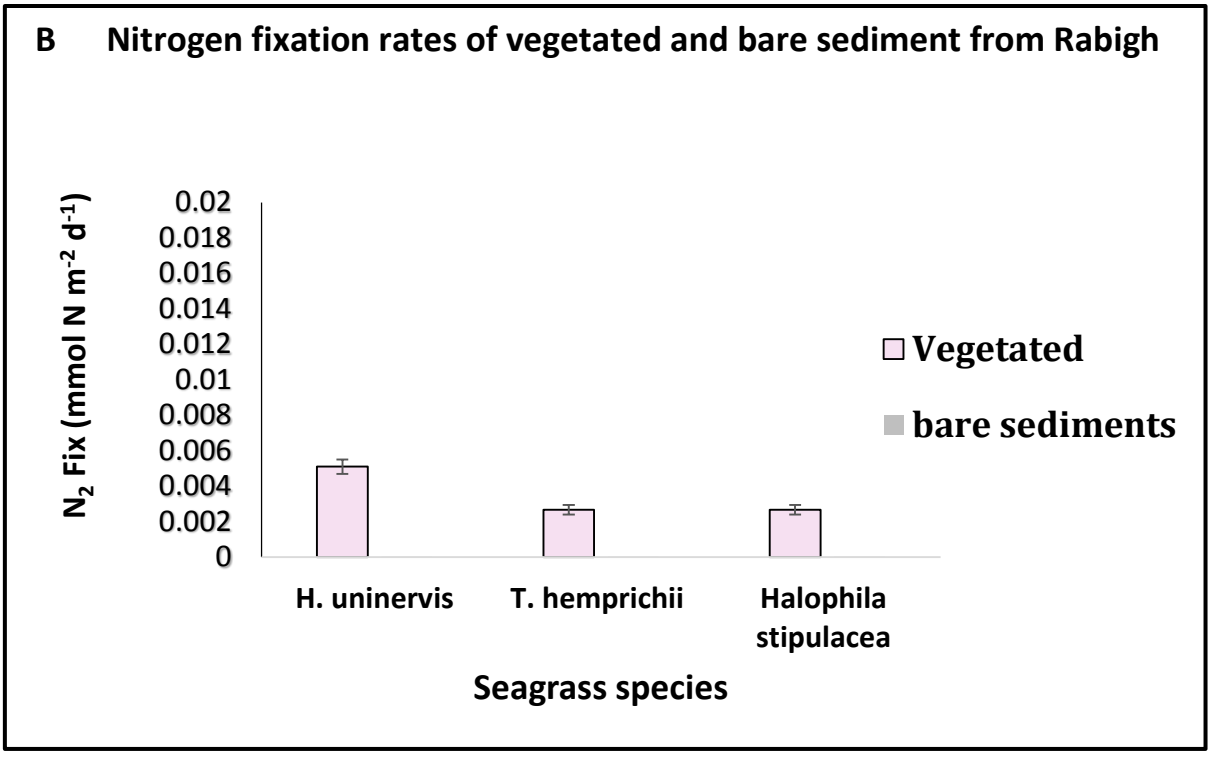

B Nitrogen fixation rates of vegetated and bare sediment from Rabigh
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
N2 Fix (mmol N m-2 d-1)
Vegetated
bare sediments
H. uninervis
T. hemprichii
Halophila stipulacea
Seagrass species

Figure 5. Nitrogen fixation rates of vegetated and bare sediment samples A) Nitrogen fixation rates of seagrass and bare sediment samples from KAEC. B) Differences in nitrogen fixation rates between seagrass and bare sediment samples in Rabigh. Stander deviations calculated from three cores taken for each species.

When comparing the two sites, nitrogen fixation rates of seagrass meadows in KAEC lagoon were more than threefold greater than those in the seagrass meadow sampled near Rabigh, with average ± SD rates of 0.011 ± 0.001 mmol N $m^{-2}$ $d^{-1}$ in KAEC and 0.003 ± 0.0008 mmol N $m^{-2}$ $d^{-1}$ in Rabigh, respectively (Figure 6). Nitrogen fixation rates resulted from sediment samples in Rabigh were generally low. Overall nitrogen fixation rates differed among seagrass species, with these differences accounting for 50% of the variance (ANOVA test, $p<0.0001$).

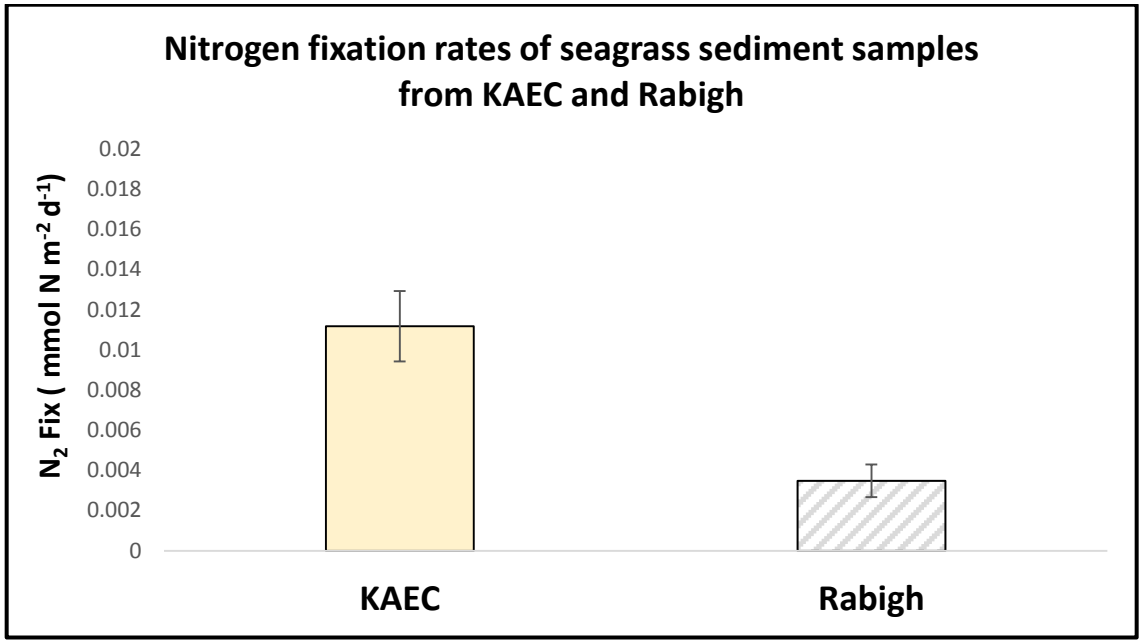
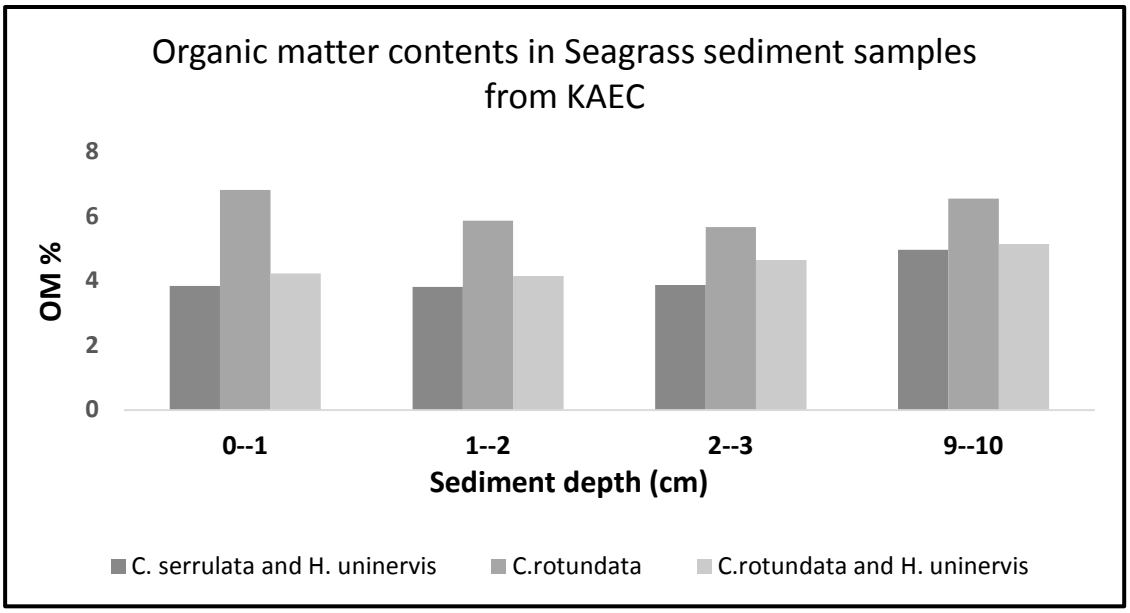


Figure 6. Mean ± SE nitrogen fixation rates in seagrass meadows sampled in king Abdullah Economic City Lagoon and Rabigh, central Red Sea.

The organic matter content in the seagrass sediments ranged between 2 and 6 % of dry weight, with the values at KAEC lagoon tending to be somewhat higher over the top 3 cm compared to those in Rabigh (Figure.7). We found no significant relationship between the organic matter content of the sediment and the




nitrogen fixation rates in the meadows ($P > 0.5$).

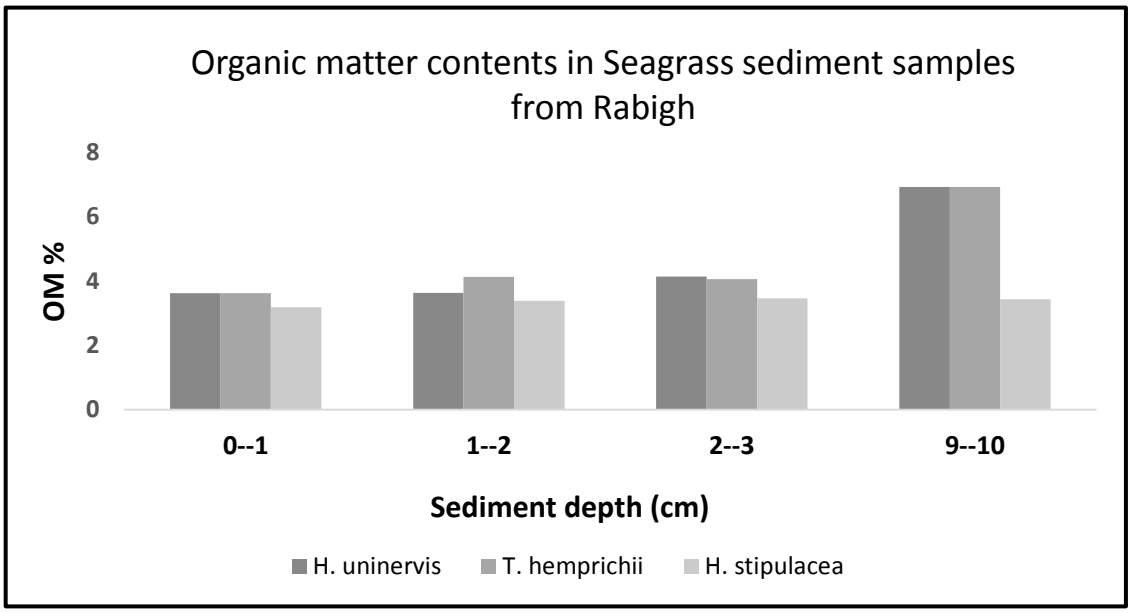



Figure.7 Organic matter percentage in KAEC and Rabigh.

## 4. Discussion

The results presented provide the first estimates of nitrogen fixation rates in Red Sea seagrass sediments, and did so for a number of species growing in two Central Red Sea locations. Nitrogen fixation rates were higher in surface sediment horizons than deeper ones (9-10 cm), indicating high activity of diazotrophs in the surface sediment layers. There is a tight relationship between seagrass and diazotrophic bacteria in the seagrass rhizosphere, based on nutrient and organic substrate release by the seagrass, available to large number of bacteria attached directly to the plant root and rhizome surface. Sulfate reducing bacteria, which are major components of the nitrogen fixing microflora, are also important components of the seagrass rhizosphere [6]. Overall the results obtained showed relatively high nitrogen fixation

rates in surface sediments, which correlates with the presence of diazotrophs associated with the plant's rhizomes [18], which are typically positioned close to the sediment surface.

Our findings indicate that nitrogen fixation rates in sediments vegetated with *T. hemprichii, H. stipulace* and *H. uninervis* are more than 1700 times fold higher, on average than those in bare sediment. This finding is in agreement with previous studies on several seagrasses from different locations, such as *Zostera capricorni* from Moreton Bay (Queensland, Australia), or *Thalassia testudinum* from Florida and Barbados [19]. Our findings, along with similar findings in the literature, confirm that diazotrophs establish a close interaction with seagrass, as indicated above, conducive to highly elevated rates compared to those in bare sediments.

Nitrogen fixation rates of seagrass meadows in KAEC lagoon were greater than those supported by seagrass meadows in Rabigh. The difference we observed can be related to different environmental factors such as sediment type, grain size, seagrasses biomass and seawater depth. In particular, seagrass plants in KAEC lagoon were larger than those in Rabigh, which may help explain the relatively higher rates of nitrogen fixation in KAEC lagoon as nitrogen fixation rates have been found to be positively related to seagrasses biomass in the past [20]. Microbial flora and the sediments type of each location play a role in nitrogen fixation rates. The abundance of sulfate reducer bacteria, many of which may also fix nitrogen, correlate with plant biomass and increased sulfate reduction rates, which explain the observed black color of seagrass sediments from KAEC lagoon. The muddy nature of sediments in KAEC lagoon may also affect nitrogen fixation rates compared to the sandy sediments in Rabigh.

Finally, nitrogen fixation in seagrasses sediments range between 0.03 and 140 mg N $m^{-2}$ $d^{-1}$ among meadows, dependent upon the seagrass species and the locality. Based on the data we obtained, nitrogen fixation rates in the Red Sea seagrass meadows ranged between 0.05 and 0.408 mg N $m^{-2}$ $d^{-1}$, suggesting seagrass sediments in the Red Sea support comparatively low rates of Nitrogen fixation. Critical nutrient limitation especially iron-limited which identified in the Red Sea play an important role in the ecosystem nature [21]. Iron detected to limit nitrogen fixation in previous studies [22], which is consistent with the low rates of nitrogen fixation detected here, as the nitrogenase enzyme requires an iron cofactor. Another factor that might decrease the nitrogen fixation rates in the red sea is temperature, NifA protein is a central transcription activator of the *nif* genes. It binds to activator sequences (UASs) that lie about 100 bp upstream of the *nif* gene promoters. The N-terminal domain of the NifA protein showed to be sensitive to the temperature, with nitrogen fixation rates restricted due to the domain sensitivity at temperatures higher than 37℃ [23]. Hence, the extreme temperatures of the Red Sea may provide, together with its oligotrophic nature, unsuitable conditions to support high nitrogen fixation rates.

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