DETERMINATION OF NITROGEN FIXING RATE AT THE RHIZOSPHERIC SEDIMENT OF MANGROVE FOREST IN SHWE THAUNG YAN MANGROVE FOREST, MYANMAR

Myat Thwe Myint Aung[1], Htay Aung[2], Motoo UTSUMI[3]

Abstract

Mangrove forests dominate the world's tropical and subtropical coastlines, and provide a unique ecosystem. Mangrove sediments play a critical role in biogeochemical processes by behaving as both source and sink for nutrients and other materials. The interactions of mangrove plant's growth rate and the sediment nutrients are complex and dynamic. Nitrogen fixation is a process that converted the atmospheric nitrogen gas to ammonium and it is an important source of new nutrient in the mangrove ecosystem. However, the study of nitrogen fixation rate in rhizospheric sediment of mangrove forest is rare, and the factors that regulate N fixation within the biome remain largely unknown. In this study, the rate of nitrogen fixation in mangrove sediment was investigated using the acetylene reduction technique. Sediment samples were collected from Shwe Thaung Yan mangrove forest and were calculated the nitrogen fixation rate. The differences between sediment depths and nitrogen fixation rate, and nitrogen fixation ability at the mangrove forest were described.

Keywords: Mangrove, Nitrogen, Nitrogen (N) fixation, ARA assay, Shwe Thaung Yan

Introduction

Mangrove represents unique and ecologically important coastal habitats throughout the tropical and subtropical which is occupying around 180,000 km^2 around the world. Chapman (1940), Davis (1940) and MacNae (1969) defined mangrove as a general term applied to plants which live in muddy, loose, wet soils in tropical tidal waters. They are circum-tropical on sheltered shores and often grow along the banks of rivers as far inland as the tide penetrates. Mangroves are recognized as highly productive ecosystems that provide organic matter and shelters to adjacent coastal ecosystems. Despite their high production rates, mangrove habitats are often nutrient limited, particularly for combined nitrogen (Ryther and Dunstan 1971).

Many previous researches showed the mangrove habitats were often nutrient limited, particularly for combined nitrogen, while nitrogen fixation may be an important nitrogen input term to these ecosystems because of its potential to provide nitrogen in usable form to plants (Zuberer and Silver 1978, 1979). High rates of nitrogen fixation are associated with mangrove bark, decaying mangrove leaves, pneumatophores roots, and mangrove rhizosphere soil (Zuberer and Silver 1978, 1979). In Raymond *et al.* (2004) research, for organisms to fix nitrogen, either in association with plants or not, the presence and activity of the nitrogenase enzyme complex is required. In this study, we describe details of physicochemical properties in mangrove sediments and estimation the rate of nitrogen fixation that exist within different sites of the mangrove forest. These results may be helpful for guidance to determine the nitrogen fixation rate of interest within these distinct sites.

[1] Dr, Assistant Lecturer, Department of Marine Science, Pathein University
[2] Dr, Professor, Department of Marine Science, Pathein University
[3] Dr, Faculty of Life and Environmental Sciences, University of Tsukuba, Japan

Materials and Methods

Study sites

Mangroves sediment samples were collected from Magyi channel of Shwe Thaung Yan coastal, Ayeyarwady region. Site 1 (17º 04.222' N 94º 28.913' E) is located by the mouth of Magyi tidal creek and dominance mangrove specie by *Bugueria gymnorrhiza* spp. Site 2 (17º 04.324'N 94º 27.917'E) is located by the middle of tidal creek and dominance mangrove specie by *Ceriops tagal* spp. Site 3 (17º 04.701'N 94º 28.141'E) is located in the marine park (Mangrove conservation area of Shwe Thaung Yan coastal) and dominance mangrove specie by *Rhizophora mucronata* spp. All study sites are along the Magyi tidal creek (Figure 1).

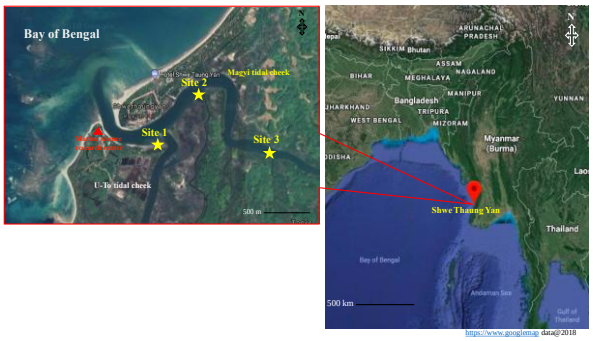


Figure 1 Study sites in Shwe Thaung Yan mangrove forest along the Magyi tidal cheek, the Republic of the Union of Myanmar.

Sampling

For physicochemical and microbial analysis, approximately 200 g of soil samples were collected from each of the three locations at low tide. Samples were collected at the rhizosphere region using by spade and triplicate from four different depth i.e, 1) 0 - 5 cm; 2) 5 - 10 cm; 3) 10 - 15 cm; 4) 15 - 20 cm of each sites. Soil samples were always taken from near parts of the roots (Figure 2). All collected samples were put into a plastic zipper bag and immediately transferred into an icebox transported to Japan for analyses.

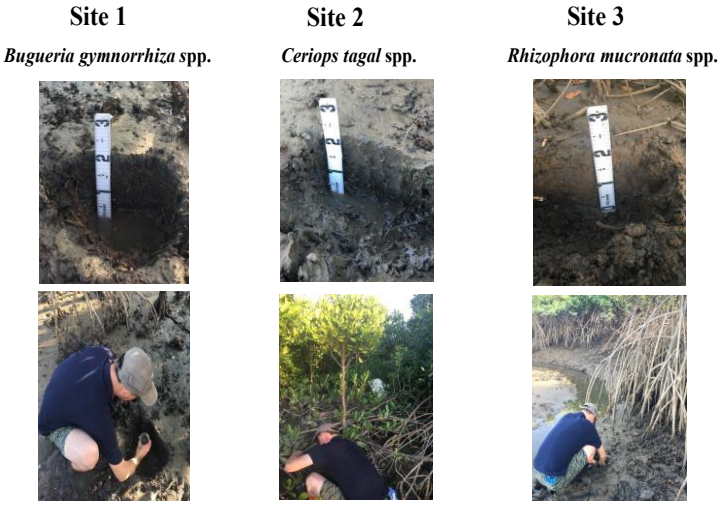


Figure 2 Study three sites information in Shwe Thaung Yan mangrove forest along the Magyi tidal cheek, Myanmar.

In situ measurement of physicochemical parameters in mangrove forest

Temperature, redox potential, and pH of the sediment samples were measured *in situ* using the thermometer, electric digital pH and conductivity meter (Horiba). And also, the soil samples were analyzed for their relative sediment structure because of microbe and vegetation types of mangrove are dependent on the soil types (e.g. muddy or sandy loam or silt-mud type).

Procedure for measuring soil nitrogenase activity (Acetylene Reduction Assay)

Nitrogen fixation rates were measured by the acetylene reduction technique (Capone and Montoya 2001) based on nitrogenase enzyme activity. Acetylene reduction assay (ARA) is a commonly used assay and is widely accepted as an effective and reproducible, instantaneous nitrogen fixation rate measurement. Before the incubation, 100 mL glass vials purge with Argon gas put into the flushing headspace vials (to create an anaerobic environment).

About 10 mL wet sediment sample was extruded from each layer sediment sample bag using 5 mL sterile syringes (with the top end of the syringe removed) put into the vials and sealed with an open cap over a Teflon - silicone septa. 10 % of the atmosphere of each vial containing 10 mL soil was substituted with gaseous acetylene (10 %) from a commercial cylinder inject to each vial (0.1 atm, 10 % C_2H_2 by volume) through the Teflon-silicone septa. The vials were shaken by hand in order to avoid heterogeneities within the gas phase. Samples were incubated in the dark at ambient water temperature (24^0C) for 24 h. From each incubation assay, 1 mL gas sample was taken from the vials at every 6 hours interval (specifically 0, 6, 12,18 and 24) and measured by gas chromatograph. The ethylene concentration was determined by a GC-14B gas chromatograph with FID detector (Shimadzu, Japan). Representative retention times in minutes are methane, 0.8; acetylene, 5.6; and ethylene, 7.8. 1 mL of gas sample from each vial was injected into GC-14B and Sincarbon ST (50/80 mesh) packed column with 3 m and 3 mm a stainless-steel column (column temperature 60^0C). Temperature at both the injector and detector was 210^0C while oven temperature was kept at 160ºC. The carrier gases flow rates were 60, 50 and 50 mL/min of helium (He), hydrogen (H_2) and oxygen (O_2) respectively (Table 1). The chromatograms were used to integrate the areas of the curves of acetylene (C_2H_2) and ethylene (C_2H_4) to estimate C_2H_4 production (Holguin *et al.* 1992, 2001). The acetylene reduction rates were calculated based on dry weight of sediments (samples were oven-dried at 60^0C for 48 h). Ethylene peak heights were measured and related to calibrations made with standard C_2H_2 (0.976%) and C_2H_4 (0.965%) concentration. The acetylene reduction rates were converted to rates of nitrogen fixation using the theoretical factor of three acetylene molecules equivalent to one nitrogen molecule (Turner and Gibson, 1980).

Table 1 Measurement condition of gas chromatography.

GC-14B	
Detector	FID detector (hydrogen flame ionization detector)
Column	Sincarbon ST (50/80 mesh), packed, length 3 m × inner diameter 3 mm stainless-steel
Sample injection volume	1 mL
Column temperature.	60°C
Injector temperature	210°C
Detector temperature	210°C
Oven	160°C
Carrier gas	He (60 mL/min), H_2 (50 mL/min), O_2 (50 mL/min)

Results

In situ measurement of physicochemical parameters in mangrove forest

The physicochemical properties of three sites of sediment samples were shown in Table 2. The pH of all sites was less than 7.0, indicating that the soil samples were slightly acidic. Electrical conductivity (EC) contents were 1750 µS/cm (Site 1), 1725 µS/cm (Site 2) and 1675 µS/cm (Site 3), respectively. Seawater and sediment porewater salinity at Site 1 (*B. gymnorrhiza* spp.) was much higher than Site 2 (*R. mucronata* spp.) and Site 3 (*C. tagal* spp.). Seawater and soil salinity ranged from 31 ‰ to 35 ‰. The sediment temperature measured at three depths (5 cm, 10 cm, and 15 cm) ranged from 27.9 ˚C to 29.2 ˚C. Three distinct sites, comprising three bulk sediment types, namely clay - loam (Site 1), mud - clay (Site 2), and mud - clay - loam (Site 3) rhizosphere soils. This indicated that the rhizosphere soil samples were substantially different from each other.

Table 2 Physico-chemical environmental parameters of sampling three sites at Shwe Thaung Yan mangrove forest, the Republic of the Union of Myanmar

Site	Dominant mangrove Species	Depth (cm)	Temp (℃)	pH	EC (µS/cm)	Sediment Texture	Salinity	
							Sediment	Seawater
1	*Bugueria gymnorrhiza*	5	28.5	5.9	1.750	Clay loam	35.0	32.0
		10	28.4					
		15	28.1					
2	*Ceriops tagal*	5	28.5	6.0	1.725	Mud-clay	34.5	31.0
		10	28.5					
		15	28.3					
3	*Rhizophora mucronata*	5	29.2	6.3	1.675	Mud- Clay- loam	33.5	32.0
		10	28.5					
		15	27.9					

Nitrogenase activity in mangrove sediments

Reduction of C_2H_2 to C_2H_4 by nitrogenase of mangrove sediment samples were examined with respect to a wide variety of characteristics, and the striking similarities between nitrogen fixation and C_2H_2 reduction were reported in these experiments. It follows that, for calibration of the relationship between acetylene reduction and nitrogen fixation, the acetylene concentration should be adjusted to give equimolar concentrations of acetylene and nitrogen in solution. To confirm the occurrence of nitrogen fixation in the samples of mangrove rhizosphere sediments with high nitrogen intake, additionally examined the acetylene-reducing activities in four-layer samples from each site. In this assay, each layer sample was divided into two: one was analyzed aerobic condition and the other was an anaerobic condition. By comparing these two types of sample, also evaluated the effect of both conditions on the nitrogen-fixing activity. In general, the anaerobic condition of C_2H_2 reduction activities was low and aerobic condition sediments generally exhibited high. All the samples of aerobic condition were showed acetylene-reducing activities (ranging from 0.14 to 2.36 nmol of C_2H_4/g (dry sediment)/h), but under the anaerobic condition, it was 0.05 to 0.88 (nmol of C_2H_4/g (dry sediment)/h). Aerobic activities showed 2.8 to 2.7 times higher than the values of the corresponding anaerobic condition (Tables 3, 4, 5). Rates of ethylene production showed a similar pattern for two sites (1 and 2) with relatively from 0 to

24 h during incubation. The highest rates observed were 2.36 and 1.10 (nmol of C_2H_4/g (dry sediment)/h) for surface layer (0 - 5 cm) and the second layer (5 - 10 cm) in Site 3. There was no statistically significant difference in nitrogenase activity between all sediment samples during the incubation (R_2= 0.878 (Adjusted R_2= 0.857). The highest nitrogen fixation rate was occurred in Site 3 under the two conditions (aerobic and anaerobic) (Figure 5; A and B), followed by Site 1 (Figure 3; A and B), and Site 2 (Figure 4; A and B). In contrast, the activities in the deepest sediment layer (15 - 20 cm) of all sites were very low. The differences of nitrogen fixation rates in aerobic and anaerobic conditions were statistically significant (Multiple Regression: $p < 0.001$, $n = 4$). Aerobic and anaerobic rates were highly significantly different, and the acetylene-reduction rates were correlated positively with the time during the incubation periods. The nitrogen fixation rates were estimated the theoretical reduction ratio C_2H_2:N_2 = 3:1 was used.

Table 3 Rates of ethylene production and nitrogen fixation in Site 1 sediments incubated with 0.1 atm acetylene under (a) aerobic condition, (b) anaerobic condition

(a) Aerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	2.03±0.31	0.68	3:1	(n=4)
5-10	2.38±0.48	0.80	3:1	(n=4)
10-15	0.52±0.13	0.17	3:1	(n=4)
15-20	0.73±0.13	0.24	3:1	(n=4)

(b) Anaerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	0.66±0.09	0.22	3:1	(n=4)
5-10	0.72±0.11	0.24	3:1	(n=4)
10-15	0.35±0.05	0.12	3:1	(n=4)
15-20	0.33±0.05	0.11	3:1	(n=4)

Table 4 Rates of ethylene production and nitrogen fixation in Site 2 sediments incubated with 0.1 atm acetylene under (a) aerobic condition, (b) anaerobic condition.

(a) Aerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	3.53±0.77	1.18	3:1	(n=4)
5-10	0.80±0.14	0.27	3:1	(n=4)
10-15	0.85±0.14	0.28	3:1	(n=4)
15-20	0.43±0.08	0.14	3:1	(n=4)

(b) Anaerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	0.53±0.77	0.18	3:1	(n=4)
5-10	0.64±0.10	0.21	3:1	(n=4)
10-15	0.40±0.06	0.13	3:1	(n=4)
15-20	0.15±0.01	0.05	3:1	(n=4)

Table 5 Rates of ethylene production and nitrogen fixation in Site 3 sediments incubated with 0.1 atm acetylene under (a) aerobic condition, (b) anaerobic condition.

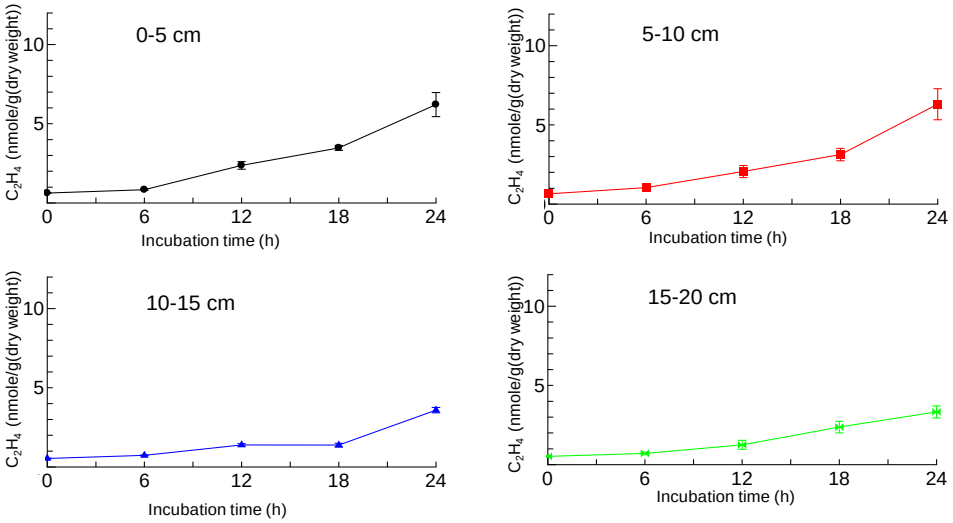
(a) Aerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	7.08±1.38	2.36	3:1	(n=4)
5-10	3.31±0.69	1.10	3:1	(n=4)
10-15	1.84±0.33	0.61	3:1	(n=4)
15-20	2.32±0.45	0.77	3:1	(n=4)

(b) Anaerobic condition

Depth (cm)	C_2H_4 (nmole/g(dry weight)/h)	N_2 – fixation rate (nmole/g(dry weight)/h)	Molar ratio	
0-5	2.65±0.44	0.88	3:1	(n=4)
5-10	1.45±0.25	0.48	3:1	(n=4)
10-15	1.59±0.27	0.53	3:1	(n=4)
15-20	0.58±0.09	0.19	3:1	(n=4)

(A) Aerobic condition



(B) Anaerobic condition

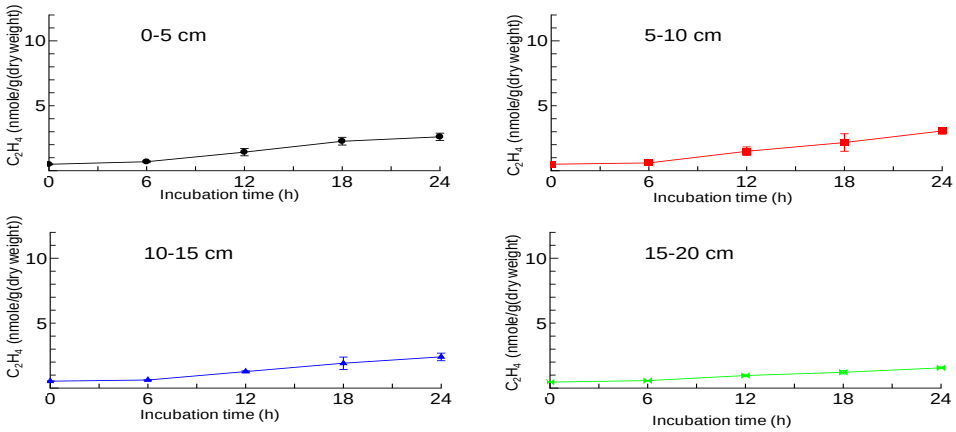
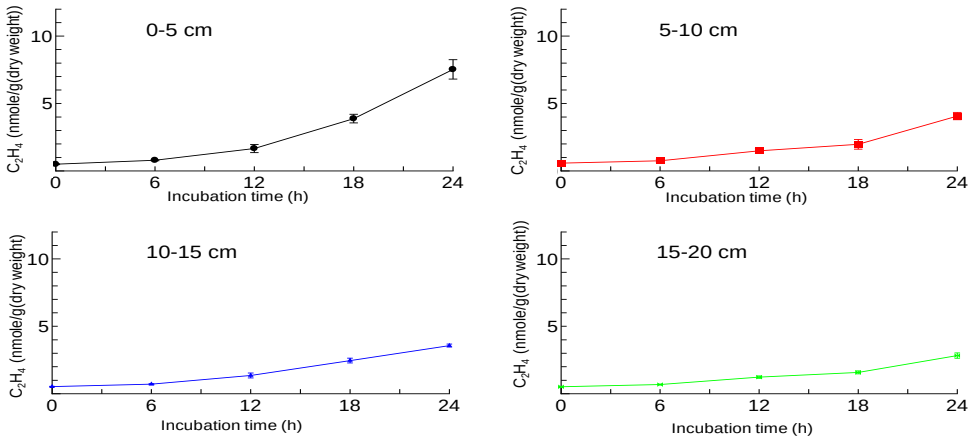


Figure 3 Acetylene reduction assay under aerobic condition (A), anaerobic condition (B) using sediment samples of Site 1

(A) Aerobic condition



(B) Anaerobic condition

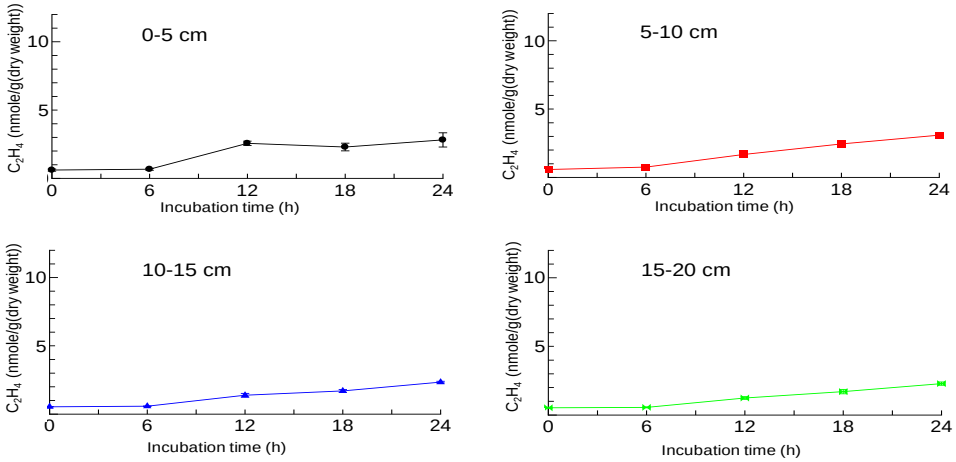
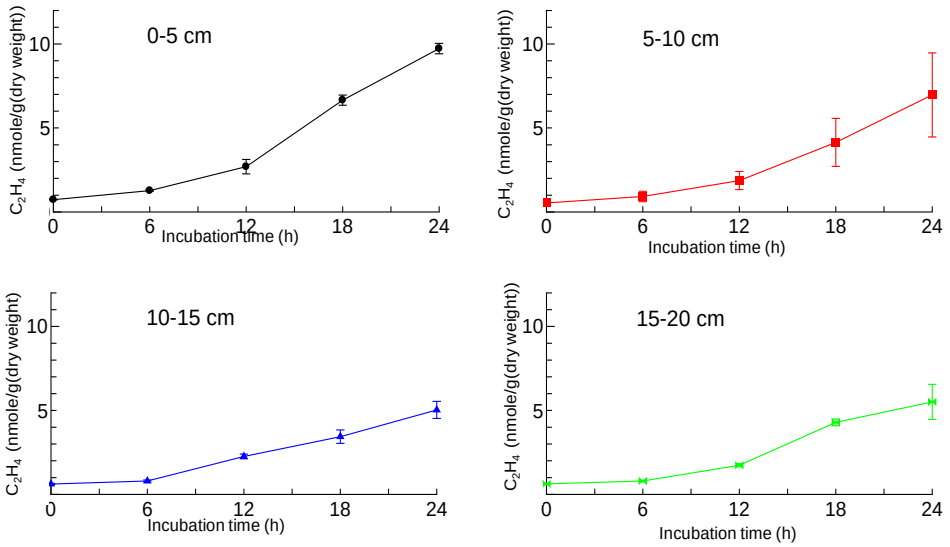


Figure 4 Acetylene reduction assay under aerobic condition (A), anaerobic condition (B) using sediment samples of Site 2

(A) Aerobic condition



(B) Anaerobic condition

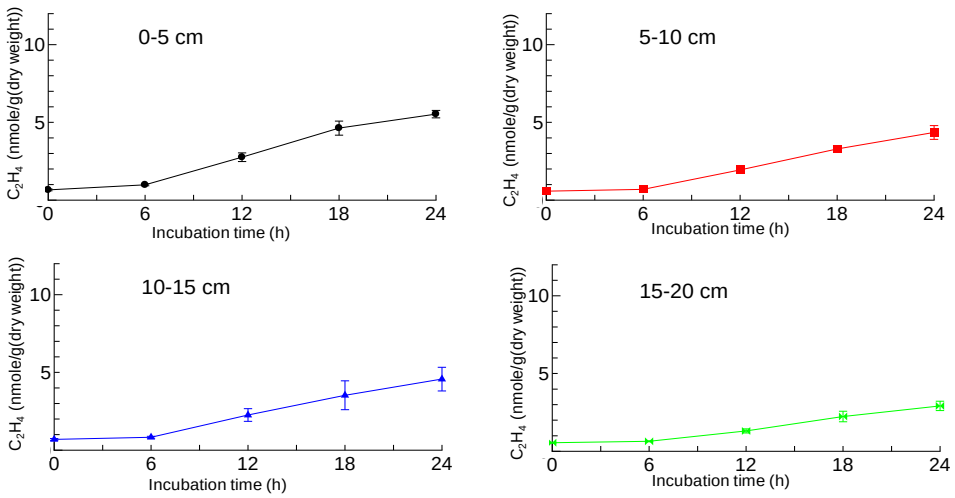


Figure 5 Acetylene reduction assay under aerobic condition (A), anaerobic condition (B) using sediment samples of Site 3

Discussion and Conclusion

The physicochemical indicators in seawater and sediment (pH, EC, and salinity) were similar to those of each site, with the exception of the extreme salinity at Site 1 (mouth of the channel). And then, the relatively high salinity was found at Site 3. Possibly, circulation of water is poor, and high air temperature (29.2˚C) leading to higher salinity at Site 3. However, acetylene reduction assay (ARA) showed that nitrogen fixation was taking place at all sites. Site 1 had low N_2-fixing activity, probably a consequence of extremely high salinity in the sediment, and the sediment structure was clay loam type. So far, Site 1 and Site 2 showed the similar pattern of N_2-fixing activity and these sites sediment also appeared similar physicochemical characteristics. The highest nitrogen-fixing activity was detected at Site 3, perhaps because of the relatively high concentration of mud loam soil and sediment salinity was lower than other two sites. Although,

the best predictor for the activity of nitrogen fixation in all sites sediment pH was around 6.1. On the other hand, one of the strong effects is pollution. Site 1 and 2 were located on the tidal cheek and it is very near to the local village. And Site 3 was located in the park and it is far from local village. Some reports (Dias *et al.* 2010; Holguin *et al*. 2001) were suggested a correlation between nitrogen pollution and low nitrogen fixation activities in mangrove forests. Also, Mohammadi *et al.* (2012) reported that the environment factors such as temperature, pH, nutrient availability and soil condition had a significant difference in nitrogen fixation activities. Zuberer and Silver (1978) recommenced, nitrogenase activity was associated with many different components of the mangrove ecosystem. These included sediments, mangrove root systems, mangrove leaf litter, and litter from macro-algae and seagrasses, as well as low activity were found in fresh and healthy mangrove leave. Evidence for nitrogen fixation has been confined chiefly to the sediments, with highest rates found within the surface layer (0-5 cm) and mostly in aerobic condition. Expressed in terms of ammonia nitrogen fixed, rates in this layer ranged from 0.22 to 1.18 nitrogen fixation rate (nM/g (dry weight)/h). The mangrove sediment habitat can occur nitrogen fixation because the main reason is the oxygen gradient, thus including the anaerobic conditions, the presence of carbonaceous and other nutrients (Andreote *et al.* 2012; Li *et al.* 2011).

This research implied its effectiveness for measurement of nitrogen fixation rate in Shwe Thaung Yan mangrove forest. The higher nitrogen fixation rate was occurred in mangrove park (Site 3) than other two sites. In this study, Site 1 was more frequently suffers waste pollution from the village, that would be likely to increase exogenous nitrogen inputs (Zedler *et al.* 2008) and inhibit nitrogen fixation rates (Shi *et al.* 2006). However, these phenomenon is probably not important as a nitrogen source in the mangrove sediments because of low fixation rates found at deep layers. The results of these studies indicated that N_2 fixation in mangrove communities is quite widespread, and this study indicates that the level of activity is of significance to the well-being of the community, especially in areas subject to nitrogen limitation. All of these results suggested that nitrogen fixation in sediments would be used as an indicator of the health of a mangrove forests ecosystem in the tropical environment.

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