

Nitrogen-Fixing Bacteria and change of the macrophyte vegetations in Bardawil Lagoon, Egypt

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ABSTRACT

Bardawil is a coastal lake/lagoon that stretches across most of the Mediterranean coastline of Egypt's Sinai Peninsula. The surrounding area is known as the eastern gate to Egypt, and has a long history of being a bridge between Africa and Asia. The lake's length is 76.37 km (extending from 31°03'N to 31°14'N, and 32°40'E to 33°30'E) and has a maximum width of 16.65 km, occupying a total surface of 518.99 km². Before 2011 the basic dominants flora in the lagoon were *Ruppia cirrhosa* and *Cymodocea nodosa*. In 2011, *Halophila stipulacea* became the most prominent species in the lake.

The aim of this study was to determine changes in the bacterial population of aquatic macrophytes, sediment and water samples of Bardawila lake and changes in the macro-vegetation and their possible reasons. For bacteriological analyses Samples of water, sediments and two seagrass roots were collected from two sectors of Lake Bardawil, Sector I at the eastern side with plant cover of *Halophila stipulacea* and Sector II at the water-circulated area in the northern middle and with plant cover of *Cymodocea nodosa*. Total bacteria and total diazotrophs were present in high densities in all samples. ANOVA analysis indicated significant differences attributed to the environmental niches. *Halophila stipulacea* roots support higher populations of diazotrophs (up to 10¹⁰ cfu g⁻¹).

This study demonstrates that diazotrophs are not randomly distributed in the lake and are either distributed by the host plant or adapted to different environmental niches. The reduction in the distribution of *Cymodocea nodosa* is a result of competitive displacement by *Halophila stipulacea*

INTRODUCTION

Bardawil is a coastal lake/lagoon that stretches across most of the Mediterranean coastline of the Egypt's Sinai Peninsula. The surrounding area is known as the eastern gate to Egypt, and has a long history of being a bridge between Africa and Asia. Bardawil lagoon is an important source for economical fish and salt production and a high diversity of habitat for wildlife. The lagoon is closely pure and is the slightest polluted in the entire Mediterranean area (Arvanitids *et al.*, 2009; Abd Ellah and Hussein 2009)

The Lagoon is located in an arid, semi-desert area, where rainfall is very scarce. The average annual rainfall in the middle of the lagoon is 82 mm. On the other hand, the

evaporation fluctuated between 72 mm in December and 246 mm in July (**El-Shabrawy and El Sayed, 2005**). The bottom sediments are texturally classified as three classes; clayey silt, silty clay and silty sand (**Samy and El-Bady, 2014**).

Symbiotic bacteria are important for growth and survival of plants (**Vandenkoornhuysen, et al., 2015**). Most environments where bacterial nitrogen fixation has been found are benthic, such as seagrass (**Smith and Hayasaka, 1982; Capone and Budin, 1982; Shieh et al., 1989; Pereg et al., 1994; Agawin et al., 2016**), salt marsh, mangrove, (**Gamble et al., 2010**), and a variety of estuarine and sediments (**Burns et al., 2002; Short et al., 2004**).

Dissolved organic carbon that is exuded from the roots and leaves of the seagrasses are the main carbon source for bacteria associated with seagrasses (**Wetzel and Penhale, 1979; Moriarty et al., 1986**) which includes bacteria that can provide nitrogen to the host plant (**Welsh 2000; Cole and McGlathery, 2012**). Biochemical processes in the nitrogen cycle, e.g., nitrification, denitrification and ammonification, occur at higher rates in the rhizosphere of seagrasses than in bare sediments, due to microbial activity (**Smith et al., 1984; Caffrey and Kemp, 1990**). Nitrogen-fixing prokaryotes found both in the phyllosphere (**Agawin et al., 2016**) and the rhizosphere can provide between 30% and 100% of the nitrogen requirement of seagrasses (**Welsh, 2000; Sun et al., 2015**).

Por (1971) mentioned that the basic element of the marine flora in Bardawil lagoon is *Ruppia cirrhosa*. The seagrass *Ruppia spiralis* forms meadows distributed all over the lagoon in 1997–1998 (**Dewedar et al., 2009**). **El-Bana et al., (2002)** Found that *Ruppia cirrhosa* dominates the eastern coast of the lake, while *Cymodocea nodosa* is dominants on the western coast of the lake. *Halodule uninervis* was recorded as an associated species near the lake-sea connection. While **Abd El-Hady et al., (2007)** found *C. nodosa* and *R. cirrhosa*. The tropical seagrass, *Halophila stipulacea* entered the Mediterranean Sea from the Red Sea after the opening of the Suez Canal in 1869 (**Ruggiero and Procaccini, 2004**). The most remarkable is the replacement of the dominating seagrass by *Halophila stipulacea* in the 2011.

The aim of this study was to determine changes in the bacterial population of invasive and native seagrass, sediment and water samples of Bardawila lake and changes in the macro-vegetation and their possible reasons.

MATERIALS AND METHODS

Experimental sites

The lake's length is 76.37 km (extending from 31°03'N to 31°14'N, and 32°40'E to 33°30'E) and has a maximum width of 16.65 km, occupying a total surface of 518.99 km². Bardawil Lake is separated from the Mediterranean Sea by a sandbar that ranges from 0.3 to 1 km in width, and 80 km long. The sandbar has a natural opening: the eastern inlet in the Zaranik protected area, which has never been dredged and is closed occasionally due to silting. There are two artificial openings, which were created in 1927, Boughaz I and Boughaz II (Fig. 1).

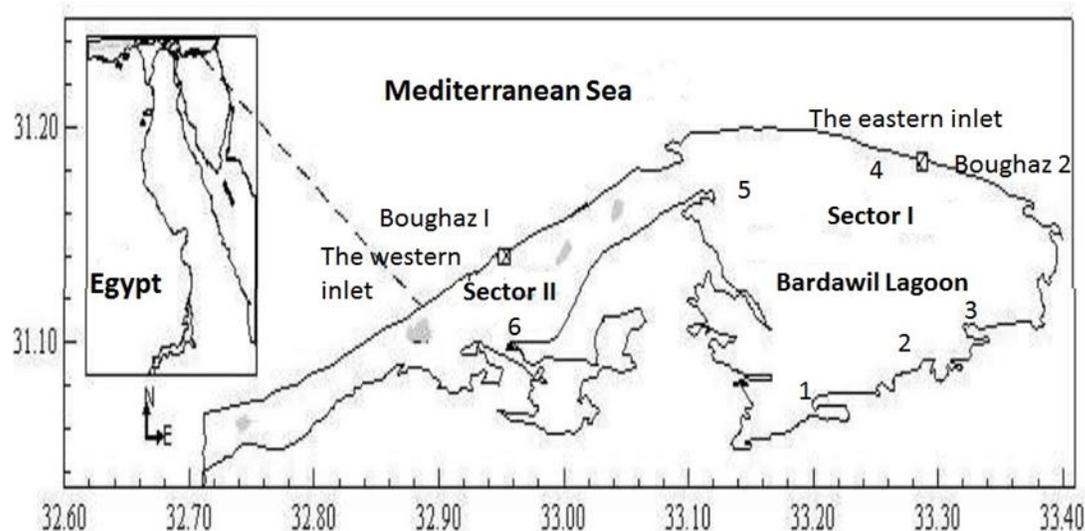


Fig. 1: A map of Lake Bardawil showing the two sampling sectors of operational sites.

Sampling

Samples of water, sediments and two seagrass roots were collected from two sectors of Lake Bardawil, Sector I at the eastern side with plant cover with *Halophila stipulacea* and Sector II at water-circulated area in the northern middle and with plant cover with *Cymodocea nodosa* (Figure 1).

Halophila stipulacea was collected from Sector I in all months as flowing, sites 1, 2 and 3 in October 2011, Sites 1 and 5 in December 2011, Sites 2 and 5 in March and April 2012, sites 1, 4 and 5 in July 2012 and 1 and 4 in September 2012. *Cymodocea nodosa* was collected from sites 6 (Sector II) in all months. Plants were collected intact with root zone sediments and transported to laboratory. Water samples were aseptically collected in sterile brown bottles (200 ml capacity), transported to laboratory, and stored at 4 °C until bacteriological analysis completed within 48 h of sampling.

Preparation of samples for analysis

Root was washed with water to remove loosely associated sediment. Dilutions of root samples were prepared by transferring sufficient portions of root systems into sampling bottles containing sterile lake water. Bottles were shaken for 60 min. and further serial dilutions were prepared. Sediment samples were prepared by transferring 10 g sediment into sampling bottles containing 90 ml sterile lake water. Bottles were shaken for 60 min. and further serial dilutions were prepared. Dry weights of soil (105°C) were determined. Dilutions of water samples were prepared too.

Bacteriological analyses

(a) The pour plate technique (Parkinson *et al.*, 1971) was used for the enumeration of total culturable bacteria using polypepton-yeast agar medium (Shieh *et al.*, 1989) with 90% lake water; (b) Total diazotrophs were counted using the surface inoculated plate method and N-deficient combined carbon sources agar medium, CCM (Hegazi *et al.*, 1998) supplemented with 3% NaCl.

Isolation, purification and identification of diazotrophs

Three agar plates were inoculated from each suitable dilution and incubated at 30 °C for 72 h. Representative colonies were transferred to semi-solid CCM, and measured

for acetylene reduction (**Hegazi et al., 1980**). Isolates producing $> 5 \text{ nmol C}_2\text{H}_4 \text{ culture}^{-1} \text{ h}^{-1}$ were further purified by single colony isolation and successive streaking on CCM agar plates. Pure isolates were re-examined for acetylene reducing activity. Selected isolates were identified according to Bergey's Manual of Systematic Bacteriology (**Krieg and Holt, 1984**). The API microtube systems, API 20E (Enterobacteriaceae), API 20 NE (Non-Enterobacteriaceae) and API 50CH Bacillaceae were used as a standardized micromethod (**Logan and Berkeley, 1984**) in addition to conventional tests such as Gram test, sporulation and motility. The ability of isolates to produce indole acetic acid was quantitatively measured according to **Tang and Bonner (1947)**.

Media

The polypepton-yeast agar medium (**Shieh et al., 1989**)

Contains (g l^{-1}): 2.0 g polypepton, 0.5 g yeast extract, and 15 g agar in 1 liter of 90% lake water, adjusted to pH 7.6.

N-deficient combined carbon sources medium, with 3% NaCl CCM (**Hegazi et al., 1998**).

The media contained the following (g l^{-1}): glucose, 2.0; malic acid, 2.0; mannitol, 2.0; sucrose, 1.0; K_2HPO_4 , 0.4; KH_2PO_4 , 0.6; MgSO_4 , 0.2; NaCl, 30; MnSO_4 , 0.01; yeast extract, 0.2; fermentol (a local product of corn-steep liquor), 0.2; KOH, 2.0; CaCl_2 , 0.02; FeCl_3 , 0.015; Na_2MoO_4 , 0.002, ZnSO_4 , 0.00025; CuSO_4 , 0.00008; sodium lactate (60%, v/v) 0.6 ml^{-1} ; pH, 7.0. Filter-sterilized solutions of biotin (0.5 l g^{-1}) and para-amino benzoic acid (10 l g^{-1}) were added after sterilization.

Statistical analysis

Statistical analysis was carried out using STATISTICA v10 (**StatSoft Inc., 2011**).

RESULTS

Samples of the seagrass *Halophila stipulacea* and *Cymodocea nedosa* dominate in Bardawil Lake were collected. *Halophila stipulacea* occurred occasionally at all sites in Sector I, while *Cymodocea nedosa* was only found at site 6 (Sector II) in all months.

Total bacteria and total diazotrophs were present in high densities in all samples. ANOVA analysis indicated significant differences attributed to the environmental niches (Fig. 2).

Total bacterial counts ranged from 6.67×10^3 to $8.00 \times 10^6 \text{ cfu ml}^{-1}$, 7.85×10^4 to $1.47 \times 10^7 \text{ cfu g}^{-1}$ and 1.47×10^5 to $3.96 \times 10^{10} \text{ g}^{-1}$ for water, sediment and root respectively. On the other hand, total diazotrophs ranged from 1.00×10^3 to $9.46 \times 10^6 \text{ cfu ml}^{-1}$, 1.58×10^5 to $2.16 \times 10^7 \text{ cfu g}^{-1}$ and 3.53×10^5 to $6.07 \times 10^9 \text{ g}^{-1}$ for water, sediment and root respectively.

In 2011, *Halophila stipulacea* became the most prominent species in the lake, Figure (2) show significant differences between the numbers of N_2 -fixing bacteria present at any given sites associated with *Halophila stipulacea* root and those of *Cymodocea nedosa*. *Halophila stipulacea* roots support higher populations of diazotrophs in summer months (up to $10^{10} \text{ cfu g}^{-1}$).

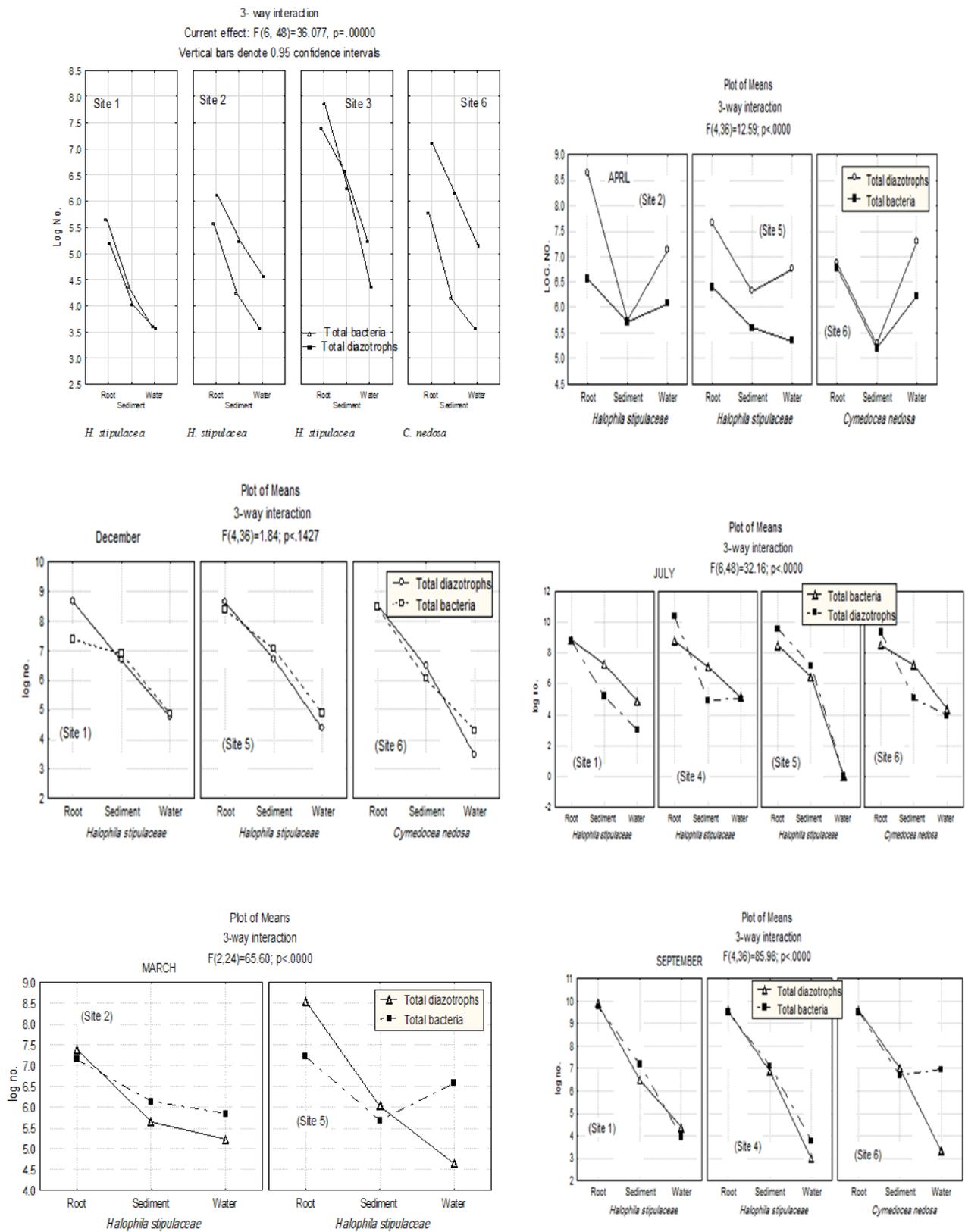


Fig. 2: Combined statistical analysis of population of culturable bacteria in different months of water sample along Bardawil lake.

A total of 68 different isolates of N₂-fixing bacteria was secured from roots and sediments. Most isolates obtained from sites 1, 2, 3 and 5 in December, March and October (Table 1).

Table (1): Taxonomic position of Halotolerant isolates associated to plants of Bardawil lagoon

Code	Host plant	Environmental niche	ARA*	IAA**	Best matched identity
1r	<i>Halophila stipulacea</i>	Root	59	7.5	<i>Bacillus coagulans</i>
1s	<i>Halophila stipulacea</i>	Sediment	44.7	8.3	<i>Bacillus circulans</i>
1s	<i>Halophila stipulacea</i>	Root	47.4	22.5	<i>Enterobacter cloacae</i>
2s	<i>Halophila stipulacea</i>	water	19	11.4	<i>Pantoea</i> spp 3
2w	<i>Halophila stipulacea</i>	Root	19.7	25.0	<i>Pseudomonas luteola</i>
3r	<i>Halophila stipulacea</i>	Root	43.3	6.5	<i>Serratia liquefaciens</i>
4s	<i>Halophila stipulacea</i>	Sediment	38.1	10.0	<i>Bacillus circulans</i>
4s	<i>Halophila stipulacea</i>	Sediment	28	8.5	<i>Bacillus pumilus</i>
4r	<i>Halophila stipulacea</i>	Root	22.6	27.3	<i>Enterobacter sakazakii</i>
5r	<i>Halophila stipulacea</i>	Root	53.2	28.1	<i>Enterobacter cloacae</i>
5rb	<i>Halophila stipulacea</i>	Root	33	12.2	<i>Bacillus pumilus</i>
5w	<i>Halophila stipulacea</i>	Water	15.5	5.5	<i>Klebsiella oxytoca</i>
5s	<i>Halophila stipulacea</i>	Sediment	66.6	16.0	<i>Bacillus pumilus</i>
6r	<i>Cymodocea nodosa</i>	Root	39.8	14.8	<i>Bacillus circulans</i>
6s	<i>Cymodocea nodosa</i>	Sediment	26.5	9.5	<i>Bacillus coagulans</i>

* nmoles C₂H₄h⁻¹culture⁻¹; **mg/mL culture

Representative isolates of diazotrophs were single-colony purified and tested for their acetylene reducing activities. Potential isolates, having > 5 nmol C₂H₄ culture⁻¹ h⁻¹, were identified by API profiles, being Gram negative *Enterobacter sakazakii*, *Pantoea* spp (3), *Klebsiella pneumoniae* ssp *pneumonia*, *Pseudomonas luteola*, *Serratia liquefaciens*, *Serratia marcescens*, and Gram positive *Bacillus pumilus*, *Bacillus circulans*, *Bacillus coagulans*. All isolated were halotolerant, i.e., able to grow and fix N₂ in media containing 3% NaCl.

DISCUSSION

The results revealed that occurrence of diazotrophs depend on the presence of plants for a supply of organic matter. This supply may come directly from the plant as excretions of photosynthesis (Moriarty and Pollard, 1982; Moriarty *et al.* 1986). Energy sources for the heterotrophs may also come from the decomposition of organic matter in the sediment, as suggested by Kenworthy *et al.*, (1987); O'Neil and Capone (1989). Communities on the surface of aquatic plant roots might be influenced by the host plant and environmental factors.

Seagrass rhizosphere have higher number of diazotrophic bacteria than in sediments (O'Donohue *et al.*, 1991, Hamisi *et al.* 2009) and bacteria in vegetated sediments usually show significantly higher abundance of N-cycling genes than those in bare sediments (Vila-Costa *et al.*, 2016, Trias *et al.*, 2012). *Halophila stipulacea* roots support high populations of diazotrophs (up to 10^{10} cfu g⁻¹). Plant metabolites such as dissolved inorganic carbon, gaseous exchange, and chemical composition are known to be important factors controlling attached communities in aquatic ecosystems (Moriarty *et al.*, 1986; Wetzel, 1993; Pereg *et al.*, 1994).

Nielsen *et al.*, (2001), who studied *Zostera noltii* and *Spartina maritima*, proposed that high sulfate and acetylene reduction rates observed on the rhizomes and roots indicate the importance of these habitats for sulfate-reducing and nitrogen-fixing bacteria.

A decline in seagrasses has been observed worldwide, partly due to climate change, human activities, diseases, and increased sulfide concentrations in the coastal porewaters (Ugarelli *et al.*, 2019) Possibly, subsequent decline in *Cymodocea nedosa* in lake may be due to the exhaustion of sediments or by competition. Nitrogen, especially ammonium in sediment is considered to be more limiting for macrophyte growth compared with phosphorus (Barko *et al.*, 1991). ANOVA analysis indicated no significant differences in total phosphorus among sites and months.

The high production of *H. stipulacea* is sustained by the high uptake rates of ammonium both in the leaves and the roots (Alexandre *et al.*, 2014). Also, nitrogen fixation, provided by a stable microbiome, might supply this seagrass with enough nitrogen to sustain growth (Viana *et al.*, 2019).

The invasive seagrass capable of rapid expansion, with the displacement of the native seagrass beginning in 10–12 weeks. Invasive species are able to accelerate the decline of native populations under environmental stress. (Gurevitch and Padilla, 2004; Willette and Ambrose, 2012).

Most isolates obtained from Sector I that covered with *Halophila stipulacea* in December, March and October. This might be due to the low of ammonium concentration in these sites (76.5 -96.9 $\mu\text{g l}^{-1}$) compared to other sites (Up to 740 $\mu\text{g l}^{-1}$). Table (2) represented a comparison of some water quality parameters of Bardawil lagoon in different time. Ammonium is proposed to inhibit nitrogenase activity by acting as a decouple of the membrane potential and thereby reducing the supply of electrons to

nitrogenase (Laane *et al.*, 1980). However, ammonium concentration may be inhibitory above a concentration of 100-200 μM (Yoch and Whiting, 1986). This is confirmed by significant negative correlation coefficients ($r = -0.90$, -0.99 $n=4$, $p<0.05$) computed between total diazotrophs and the ammonium concentrations in October and December. Using ANOVA, we found Ammonia levels were significantly higher ($p = 0.031^*$) in July than others months

Table (2): Range and mean of water parameters compared to those of other studies.

Parameters	Present Study (2011-2012)	2004	2004	2006-2007	2013- 2014
		(Ali <i>et al.</i> , 2006)	(Sabae, 2006)	(El-Halag <i>et al.</i>)	El-Kassas <i>et al.</i> , 2016
Water Temp. $^{\circ}\text{C}$	13.6-31.4*	-	17.4-29.7	15-31.8	15.9-27.4
Salinity ‰	36.67-58.30*	-	-	37.5-53.8	38.18-62.4
EC mS /cm	57.3-91.1*	-	-	-	-
pH	8.09-8.48*	7.95-8.8	7.94-8.8	7.6-8.8	8.12-8.9
$\text{NO}_2^- \text{-N } \mu\text{g}^{-1}$	0.0-26.37*	0-19	0.0-18.9	14-53	1.06–6.28
$\text{NO}_3^- \text{-N } \mu\text{g}^{-1}$	7.92-263.18*	13-89	12.92-89.2	44-198	0.00–40.00
$\text{NH}_4^- \text{-N } \mu\text{g}^{-1}$	83.30-741.20*	9-138	18-223.5	ND	0.00–140.00
TP μg^{-1}	90.00-135.60*	10-90	-	18-148	0.54–6.44
TBC (cfu ml^{-1})	6.67 x 10^3 - 8.00 x 10^6	-	8 x 10^5 - 71 x 10^7	-	-
TD (cfu ml^{-1})	1.00 x 10^3 - 9.46 x 10^6	-	0-460 (Azotobacter)	-	-

*Report project of “Survey and ecological studies of Bardawil Lagoon”. (Mohamed El-Sherif Goher, National Institute of Oceanography and Fisheries)

This study demonstrates that diazotrophs are not randomly distributed in the lake and are either distributed by host plant or adapted to different environmental niches. The reduction in the distribution of *Cymodocea nodosa* is a result of competitive displacement by *Halophila stipulacea*

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