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Ecological distribution of harmful epiphytic Oscillatoriales in Alexandria coast, Egypt, with special reference to DNA identification

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PEER REVIEW

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Comments

The paper is a traditional work concerning with epiphytic plants on macroalgal flora on the Alexandria coast, which is interesting and useful for further studies.
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ABSTRACT

Objective: To identify the potentially harmful epiphytic Oscillatoriales species and follow up their distribution along Alexandria coast.

Methods: Samples were collected bimonthly from April 2009 to February 2010 at three sites along Alexandria coast. Both morphological and molecular analyses were used for identifying the dominant species.

Results: Five species belonging to two families were identified; *Oscillatoria acutissima*, *Oscillatoria nigroviridis*, *Oscillatoria* sp., *Lyngbya majuscule* and *Phormidium formosum*. Their cell density ranged from 10^3 to 126×10^3 filament g^{-1} fresh weight macroalgae. The morphological study of the dominant species, *Oscillatoria* sp. (*Oscillatoria* sp. W1) showed much similarity with *Planktothrix agardhii* with no heterocysts and akinetes, while molecular ananlysis (16S rDNA) clustered the species in the same group with *Anabaena* sp.

Conclusions: The 16S rDNA genes are not suitable for identifying *Oscillatoria*les during the present study and another molecular method should be used instead.

KEYWORDS

Harmful algae, DNA analysis, epiphytes, *Oscillatoria*les, Alexandria, Egypt

1. Introduction

In the Egyptian Mediterranean waters, harmful algal blooms was initiated by Halim since 1956[1], who described the new genus and new species of dinoflagellates *Alexandrium minutum*. Since then a lot of investigations were occurred along the Egyptian coast. Although mass mortality of bottom feeding fish *Siganus rivulatus* in the Eastern Harbour of Alexandria was reported in March 2005[2], due to *Oscillatoria acutissima* (*O. acutissima*) proliferation, less attention has been given so far to the potentially harmful epiphytic cyanobacteria. The studies of cyanobacteria in the Egyptian waters were restricted to planktonic freshwater and brackish water species[3-5]. On the other hand, 14 Oscillatoriales species were recorded from the Western Alexandria coast with the perennial existence of *Oscillatoria brevis*[6].

The benthic potentially harmful species epiphytic on macroalgae in Alexandria coastal waters have been surveyed[7-9], four Oscillatoriales species were reported on macroalgae and its associated harmful microalgae in different habitat along Alexandria coast, namely, *O. acutissima*, *Oscillatoria agardhii*, *Oscillatoria formosa* and *Oscillatoria nigroviridis*[7-9]. While six Oscillatoriales species were associated with fish mortality in Alexandria water[2]; *O. acutissima*, *Oscillatoria limosa*, *Oscillatoria*

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nigroviridis, *Oscillatoria* sp., *Lyngbya* sp. and *Planktothrix* c.f. *agardhii* (*P. agardhii*).

Although identification by 16S rRNA gene is rapid and sensitive for the detection and genetic characterization of cyanobacteria, all of the previous studies were used only the morphological characters for identification.

Currently used classification system divides cyanobacteria into five sections (Section I: order Chroococcales, Section II: order Pleurocapsales, Section III: order Oscillatoriales, Section IV: order Nostocales, Section V: order Stigonematales[10,11]. Phylogenetic analysis of cyanobacteria based on 16S rDNA[12,13], indicated that Chroococcales (I) and Oscillatoriales (III) are polyphyletic.

The aim of this study is to survey the ecological distribution of potentially harmful epiphytic Oscillatoriales during a complete year in Alexandria coastal waters. The taxonomic positions of some isolated *Oscillatoria* species were determined by using both morphological characteristics and molecular analysis.

2. Materials and methods

2.1. Area of investigations

The area of investigation extends of about 40 km from the east to the west of Alexandria. Three sites were chosen representing different ecological entities along Alexandria coast; Abu-Qir, Mex beach and Eastern Harbour (Figure 1).

Figure 1. Sampling sites along Alexandria coast.

2.1.1. Abu Qir

This site is characterized by chains of natural rocks which are subjected to successive wave action and surrounded by pools[9,14]. These rocks provide excellent substrata for a rich algal flora.

2.1.2. Eastern Harbour

The Eastern Harbour of Alexandria is a shallow, semi-enclosed embayment covering an area of about 2.8 km^2, located along the central part of Alexandria. The southern part of the harbour has been reinforced by concrete blocks; the northern side is protected by an artificial breakwater with eastern and western inlets. It is bordered to the east by a land projection, El-Silsila, and to the northwest by a long causeway[15].

2.1.3. El Mex

El-Mex Bay is an exposed rocky area and lies to the east of El-Umom-Drain outlet[9], through which several types of wastes are discharged to the sea.

2.2. Environmental parameters

Water temperature, oxygen concentration, hydrogen ion concentration (pH) and salinity were measured *in situ* with multi parameter probe next to each macroalgal stand during sample collection. Water temperature and pH were measured by a digital thermometer (HANNA HI98127), salinity by a salintest (HANNA HI98203) and oxygen concentration oxymeter (HANNA HI9142W).

2.3. Macroalgae and benthic Oscillatoriales

Samples of macroalgae (about 100 g fresh weight) were collected via shore from depths between 0.5 m to 1.5 m. Three stations were collected from Abu Qir exposed area and two stations from both the Eastern Harbour and El Mex. Whenever possible, three samples of three different macroalgal species were collected. Macroalgal samples were carefully picked, placed into plastic bags, filled with local seawater and were vigorously shaken (to dislodge any epiphytic microalgae present). The macroalgae were removed from the plastic bags and weighted to determine wet weight. The water remaining in the plastic bags was transferred to graduated cylinder and the volume was recorded adjusted to 300 mL and fixed with Lugol solution[16]. The water samples were examined after sedimentation using Optika binocular microscope and Olympus research microscope[17]. *Oscillatoria* sp. was photographed using digital Olympus camera. The abundance of Oscillatoriales was determined by multiplying the number of species counted by a total volume of sample, and dividing by algal wet weight. The data were computed as cells/g fresh weight microalgae (fwm).

2.4. Culture of Oscillatorialles

Strains of *Oscillatoria* sp. were isolated from the Eastern Harbour using micropipette and cultured in enriched sea water with f/2 medium[18]. The culture was maintained for 1 week at 12D:12L cycle at 25 °C and subjected to DNA analysis.

2.5. DNA extraction, purification and amplification

DNA was extracted and purified using Gene JET Genomic DNA extraction and Purification (Fermentas Life Sciences). Polymerase chain reaction (PCR) was performed using a thermocycler (Mastercycler 5333) Eppendorf AG- Germany. PCR was carried out using CYA106F (5′-CGG ACG GGT GAG TAA

CGC GTG A-3′) and CYA781R (5′-GAC TAC TGG GGT ATC TAA TCC CAT T-3′) primers[13]. Primers were manufactured by Eurofins MWG Operon, Ebersberg, Germany. The PCR reaction was performed in a total volume of 50 μL by using 20 μL of 2.5× PCR MasterMix (5 prime GmbH, Hamburg, Germany), 3 μL from forward and reverse primer (10 pmol/ μL from each), 23 μL nuclease free water and 1 μL DNA (equivalent to 10 ng). The following temperature protocol was used: initial denaturizing step of 5 min at 95 °C, 40 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 48 °C, and extension for 1 min at 72 °C. Final extension was carried out at 72 °C for 7 min.

2.6. Gel electrophoresis

A total of 10 μL of the PCR product mixed with loading buffer, were loaded on a 1% (w/v) agarose gel and electrophoresed with 1X TAE (Tris-acetate EDTA) buffer. DNA was visualized by UV transillumination after staining with ethidium bromide (0.5 μg/mL). The molecular sizes of the amplified DNA fragments were estimated using DNA ladder of 100 bp.

2.7. DNA sequencing

PCR products were purified to remove excess primers using QIA quick PCR purification reagents (Qiagen, USA) and then sequenced with the BigDye Terminater cycle sequencing kit (Applied Biosystems, Foster City, CA) in ABI Prism 3700 sequencer (Perkin Elmer, Applied Biosystems, USA). Sequences were deposited in the GenBank (EMBL database) under the accession number JN899816.

2.8 Data analysis and phylogenetics

After obtaining the sequences, homology search was performed against DNA Data Bank of Japan, using BLAST program to find the sequences producing significant alignment with the obtained sequences. Multisequence alignment was performed using Biology WorkBench software version 3.2. Molecular phylogeny was done using ClustalW2 (a distance-based free online analysis program at EMBL site, http://www.ebi.ac.uk/Tools/phylogeny/) program[19]. The tree topology was evaluated using the neighbor-joining method[20].

3. Results

3.1. Environmental parameters

During the present study, surface temperature showed the same pattern at the three sites, ranging from 18.0 °C to 29.3 °C. The minimum was recorded during December, while the maximum was in August except at Abu Qir, where the maximum was in June (Figure 2).

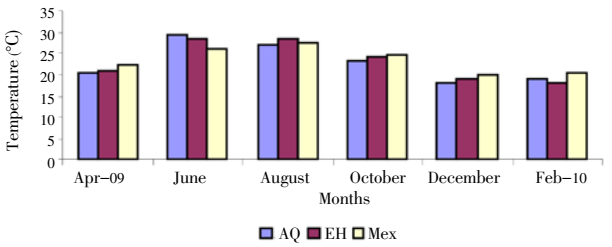

Figure 2. Bimonthly variations of water temperature.
AQ: Abu Qir; EH: Eastern Harbour; Mex: El Mex.

The pH values demonstrated negligible variations (8.1 to 8.3) at both Abu Qir and Eastern Harbour (Figure 3), as compared to that at El Mex (7.1 to 8.2). Salinity was the highest at Abu Qir with narrow variations (38.1 to 38.9 psu). At the Eastern Harbour, salinity was comparatively low (35.7 to 37.4 psu) as the harbour received domestic waste in its south-west margin while it sustained the lowest value with wide fluctuations at El Mex, (20 to 38.9 psu) due to the discharge of waste water from El-Umom-Drain (Figure 4). The three study sites showed high oxygen concentrations (2.6 to 13.3 mg/L) except during June and August (Figure 5).

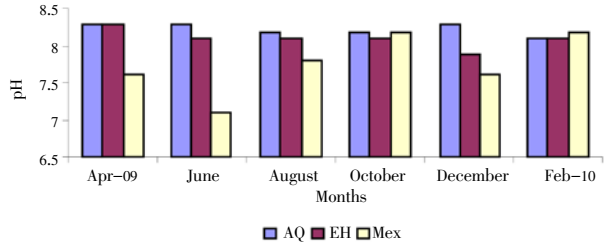

Figure 3. Bimonthly variations of pH.
AQ: Abu Qir; EH: Eastern Harbour; Mex: El Mex.

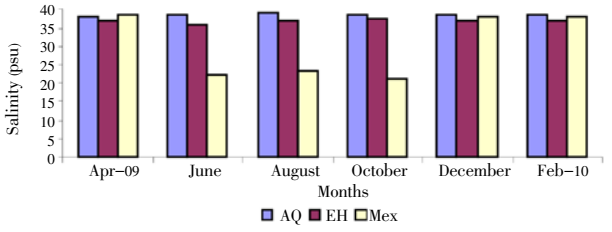

Figure 4. Bimonthly variations of salinity.
AQ: Abu Qir; EH: Eastern Harbour; Mex: El Mex.

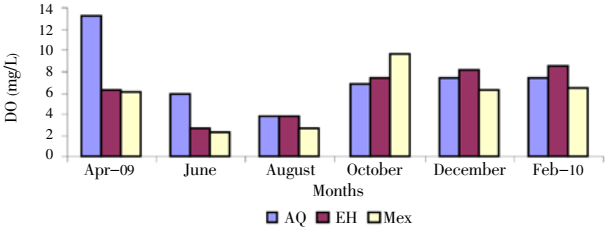

Figure 5. Bimonthly variations of oxygen concentration.
AQ: Abu Qir; EH: Eastern Harbour; Mex: El Mex.

3.2. Species composition and standing crop

The present study identified five species of epiphytic cyanobacteria belonging to two families; Oscillatoriaceae: *O. acutissima*, *Oscillatoria* sp. and *Lyngbya majuscule* (*L.*

majuscule); and Phormidiaceae: *Phormidium formosum*, *Phormidium nigroviridis* (*P. nigroviridis*).

The abundance of Oscillatoriales showed wide temporal fluctuations at the three sites (Figure 6). Both the Eastern Harbour and Abu Qir were characterized by higher abundance than El Mex (not exceed 9×10^3 filament/g fwm). The maximum abundance over the whole area (126×10^3 filament/g fwm) was recorded at the Eastern Harbour in June while the lowest abundance (10^3 filament/g fwm) occurred at both Abu Qir and Eastern Harbour during April and at El Mex in February.

Oscillatoria sp. was almost the dominant component of cyanobacteria at the three sites, forming large mate particularly in the Eastern Harbour and in association with the bloom of the benthic diatoms *Licmophora* sp. This bloom was associated with drop in salinity and increased in temperature (Table 1). On the other hand, *O. acutissima* and *Phormidium formosum* shared the dominance with *Oscillatoria* sp. during August, December and February at Abu Qir.

Table 1

Maximum cell density of Oscillatoriales with environmental parameters.

Parameters	Abu Qir	Eastern Harbour	Mex
Maximum abundance ($\times10^3$)	20	126	9
Duration	August	June	December
Temperature °C	27.1	28.6	19.9
Salinity (psu)	38.9	35.7	38.3
pH	8.2	8.1	7.6
Oxygen concentration (mg/L)	3.80	2.60	6.42

The macroalgal species as a host played an important role in the distribution of their epiphytic Oscillatoriales. Fifteen macroalgal species were recorded; *Enteromorpha clathrata*, *Enteromorpha compressa*, *Enteromorpha flexuosa*, *Enteromorpha intestinalis*, *Ulva fasciata*, *Ulva lactuca*, *Cladophora rupestris*, *Padina pavonia*, *Sargassum salicifolium*, *Gelidium latifolium*, *Pterocladia capollacea*, *Corallina mediterranea*, *Corallina officinalis*, *Jania rubens* and *Hypnea musciformis*. Seven species appeared to be the preferred hosts for the Oscillatoriales. The relation between Oscillatoriales and macroalgal species was not clear at El Mex which was inhibited only by *Ulva fasciata* and *Ulva lactuca*. Meanwhile, several algal species were found at the other two sites, the green algae *Ulva* spp. and *Enteromorpha* spp. were covered by about 71% of the total Oscillatoriales, followed by the red algal species *Corallina* spp., *Jania rubens* and *Pterocladia capollacea* (23%) and the brown algae *Padina pavonia* and *Sargassum salicifolium* (6%) (Figure 7).

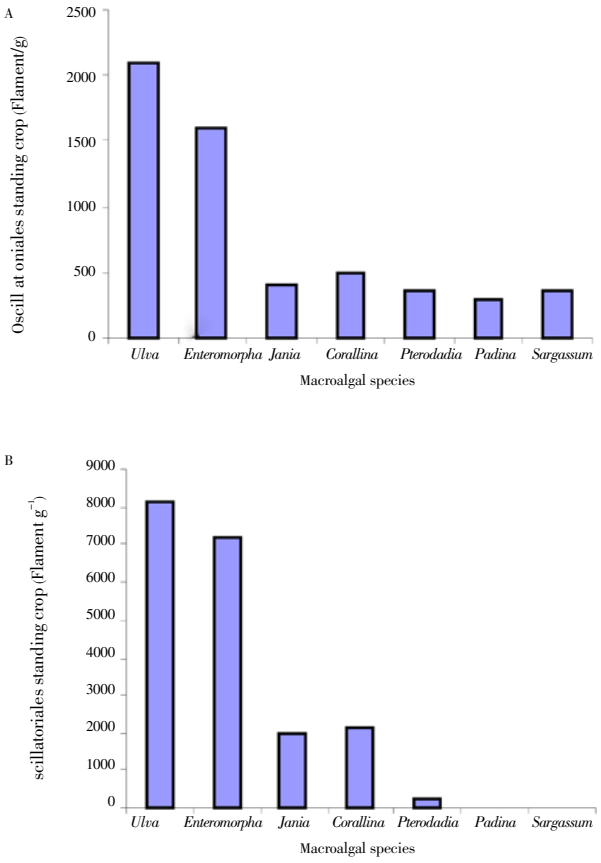

Figure 7. Abundance of Oscillatoriales on macroalgal species.
A: Abu Qir; B: Eastern Harbour.

3.3. Identification of Oscillatoria sp.

Oscillatoria sp. could be identificated by the characteristics such as trichomes slightly curved, tapered towards apex, blue-green in colour, and cells short cylindrical. Cell length was slightly shorter than wide, 4-6 μm wide, 3-4 μm long (Figure 8). No heterocyst and akinetes were observed. The species is much similar to *Planktothrix agardhii*.

3.4 DNA analysis

The DNA of the isolate, *Oscillatoria* sp. (*Oscillatoria* W1), was subjected to extraction and purification in order to amplify around 700 bp (Figure 9) of its gene using CYA106F and CYA781R primers. Partial sequencing of the 16S rDNA genes was performed using the same primers. The sequences showed very far homology from *Oscillatoria* species available in the database (Figure 10).

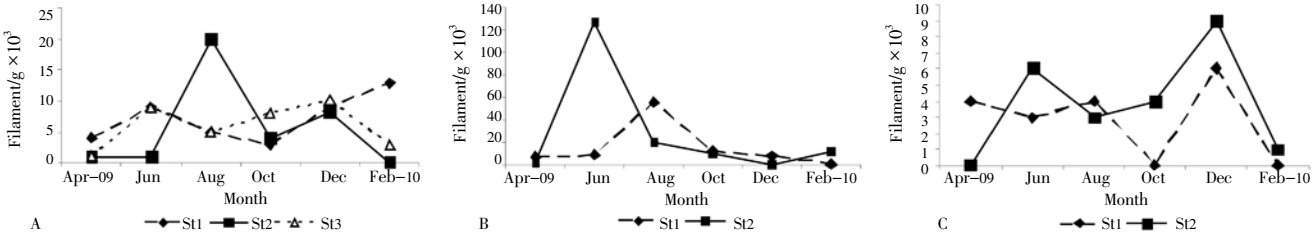

Figure 6. Abundance of Oscillatoriales three sites.
A: Abu Qir; B: Eastern Harbour; C: El Mex.

Figure 8. *Oscillatoria* sp. (*Oscillatoria* sp. W1).

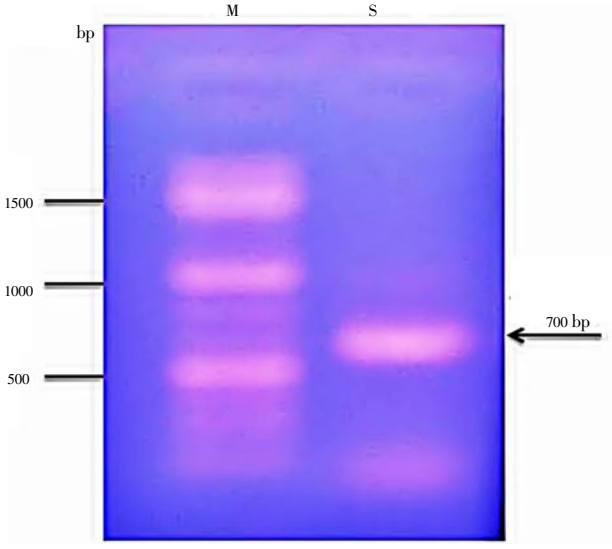

Figure 9. Gel electrophoresis of the 16S rDNA gene of *Oscillatoria* sp. W1. S: approx 700 bp; M: 100 bp genetic marker.

CGGATCAGCATTCGCGCATGGGCTGTCAGTTGCGGCCAGTGAAGCGCCTTCGCCTCGGTGTTCTTCC
TGATCCCTAGAATTTTACCGCTACCTTGGAATTCCCTCTGCTCTACCATACTCTAGCTCACCAGTTTCCA
CTGCCTATCAGAAGTTAAGCCCCCGGATTTAACAGCAAACTTGAGGCCGACCTGAGGACGCTTTACG
CCCAAATTTTCCGGATAACGCTTGCCTCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGAG
GCTGATTCCTCAGGTACCGTCTTTTTCTTCCCCCCTGAAAAAAGGGGTTTACAACCCAAAGGCTTTCCT
CCCCCAGCGAACTGGCTCATCAGAGTTTTGGCCCATTGCGGAAAATTCCCCACTGCTGCCTCCCGTAG
GAGTCAGGGCCGTGTCTCAGTCCCCTTGTGGCTGATCATCCTCTCAGACCGGCTACTGATCGTCGCCT
TGGTAGGCTTTTACCCCACCAACTACCTAATGGGACGCAAGCTCCTCCTCAAGCGAATATTCTTTTACT
TTTCTTCCCCACGCGAGATACTCATAATTTCCCTCTGTTGTCGCCGTCTCAAGGGTATATCCTCGTCCA
TTACTCACCTTCTTCACGCGTTACTCACCCGTCCGA

Figure 10. Partial sequences of the 16S rDNA gene of *Oscillatoria* sp. W1.

Using the partial sequences of the 16S rDNA genes for both *Oscillatoria* W1 and the most homologous species from the database, a phylogenetic tree was drawn (Figure 11). The tree topology illustrated that there were three main clusters (trichotomous tree) that included four different clades. The first cluster included different *Anabaena* species: 1-*Anabaena oscillarioides* (strains BECID32 and BECID22), 2-*Anabaena cylindrica* (XP6B), and 3-*Anabaena flos-aquae* (itu35s12). *Oscillatoria* W1 was clustered in the same group with *Anabaena* species. The second cluster included two different clades, *Aphanizomenon* (*Aphanizomenon issatschenkoi* 1469 and *Aphanizomenon issatschenkoi* 1470) and *Lyngbya* (*Lyngbya semiplena*, *Lyngbya aestuarii* PCC 7419, *Lyngbya* sp. strain PCC 749, *Lyngbya* sp. SCyano73, and *Lyngbya aestuarii* kopara-LY). Finally, the third cluster included one clade that belonged to the genus *Trichormus* (*Trichormus variabilis* GREIFSWALD and *Trichormus variabilis* HINDAK).

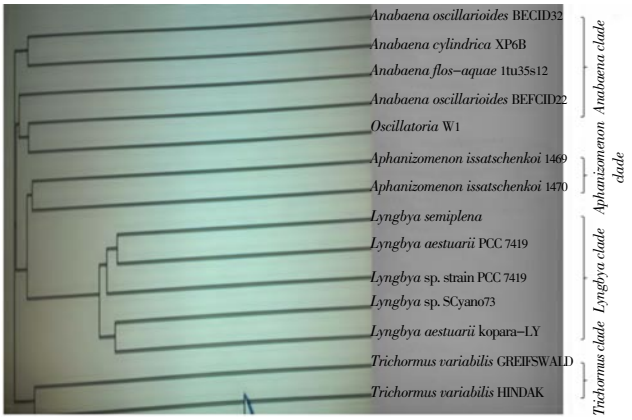

Figure 11. Phylogenetic tree showing the phyletic relationships among the partial sequences of the 16S rDNA genes of *Oscillatoria* sp.W1 and other homologous species in the database.

4. Discussion

The present study revealed clear difference in species composition and abundance of Oscillatoriales between Alexandria coast and its western coast[2,6,9]. *O. acutissima* and *P. nigroviridis* are common in Alexandria coast; in addition, *L. majuscule* was recorded for the first time during this study. The maximum abundance of Oscillatoriales in the present study was greatly higher than some earlier (8.6×10^3 filament/g wet weight macroalgae)[9], but considerably lower than others (4.8×10^6 filament/g wet weight macroalgae)[2].

Cyanobacterial blooms persist in water that contain adequate levels of essential inorganic nutrients such as nitrogen and phosphorus, water temperature generally between 15 and 30 °C, and pH between 6 and 9[21]. Nutrients, pH, CO_2, salinity and dissolved oxygen are the main chemical factors that contribute to the development of dominant cyanobacterial blooms[22]. Although nutrient salts were not analyzed during this study, the data available in earlier studies indicated high nutrient concentrations in the sampling sites[9,14,23]. Nitrate and phosphate ranged from 0.03 to 18.87 μg at NO_3-N/L and 0.01 to 1.5 μg at PO_4-P/L at Abu Qir, from 0.04 to 29.75 μg at NO_3-N/L and 0.02 to 2.52 μg at PO_4-P/L at the Eastern Harbour, and from 5.69 to 54.72 μg at NO_3-N/L, 0.17 to 5.34 μg at PO_4-P/L at El-Mex[9,14,23,]. Accordingly these sites became a suitable environment for algal blooms both benthic and planktonic.

The cyanobacteria blooms may increase in distribution, duration and intensity, as global temperature rise[24]. Beyond the direct effects on cyanobacterial growth rates, rising temperature may cause change in the physical characteristics of aquatic environments in ways that may be favorable for cyanobacteria. For instance, higher

temperature will decrease surface water viscosity and increase diffusion towards the cell surface, an important process when competition for nutrients between species occurs[25]. It seemed that temperature played an important role in the dominance of Oscillatoriales during the present study, whereas their blooms at the Eastern Harbour and Abu Qir were recorded during summer (June and August). These results are compatible with the earlier studies, as the blooms of Oscillatoriales were recorded at temperature ranged from 20.1 to 30.2 °C[2,9].

Species of some benthic cyanobacteria, including *Lyngbya* and *Oscillatoria* are well adapted to freshwater or saline conditions[26]. Their proliferations commonly occur at all latitude but the composition of species generally differs among these habitats because of salinity[27]. Therefore changes in salinity may affect community composition as well as potential toxin concentration and distribution[28]. This is partially in agreement with the present observations particularly at El Mex, where the marine *P. nigroviridis* was completely absent. However, Oscillatoriales blooms during the present study were recorded at salinity compatible with previous studies (37.5 and 39 psu)[2,9].

Bacterial 16S rRNA genes contain nine "hypervariable regions" (V1-V9) demonstrated considerable sequence diversity among different bacteria. Species-specific sequences within a given hypervariable region constitute useful targets for diagnostic assays and other scientific investigations[29]. During the present study, the species was successfully identified as *Oscillatoria* W1 by sequencing of the 5′ end hyper variant region of the 16S rDNA gene, approx 700 bp. This reflects the significance of this vital region as a molecular chronometer for a precise identification and determination of the bacterial taxonomic position[30].

The phylogenetic analysis revealed four clusters of related sequences (*Anabaena*, *Aphanizomenon*, *Lyngbya*, and *Trichormus*). The sequences of these four species showed high homology degree with *Oscillatoria* sp. W1. However, its relatedness to *Anabaena* sp. was the greatest as it was clustered with it in the same group rather than with other cyanobacteria. This relatedness between *Oscillatoria* sp. W1 and *Anabaena* sp. was previously found[31]. They figured the homology between the two genera according to the 16S rDNA restriction and restriction fragment length polymorphism patterns. On the other hand, anatoxin-a synthetase genes required for anatoxin-a production in the genomic sequence of *Anabaena* sp. was identified[32]. This gene resembled that identified in *Oscillatoria* sp. strain PCC 6506[30] and its anatoxin-a synthetase gene content was the same, with high sequence identity. In addition, the phylogenetic relationship between *Anabaena* and *Aphanizomenon* in the present study was emphasized in previous studies[31,33] which stated that species of these two genera were found closely related according to their 16S rDNA sequences.

During the present study, the morphological results showed different trend from those of the molecular analysis. The 16S rDNA gene sequences were successful only at the group level and identified the species as *Anabaena*, while the morphological characteristics identified it as *Planktothrix agardhii* depending on the absence of heterocysts and akinetes. This indicated that the 16S rDNA genes are not suitable for identifying Oscillatoriales and other genes have to be used for more precise identification of the taxonomic position.

Conflict of interest statement

The authors have no conflict of interest.

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Comments

Background

Cyanobacteria are present in all aquatic environments both in planktic and benthic forms. Many genera of this group are known to produce a wide variety of toxins and can threat marine habitat. Therefore, monitoring of cyanobacteria in marine environment is very important and needed.

Research frontiers

The present research is concerned with the study of the distribution of potentially harmful epiphytic species of order Oscillatoriales along Alexandria coast. Molecular analysis was also used for identification of unidentified species of *Oscillatoria*.

Related reports

Epiphytic cyanobacteria are reported as potentially harmful species along Alexandria water. Cyanobacteria blooms have become more intensive during the last two decades, due to the increasing anthropogenic inputs of nutrients to water environment.

Innovations and breakthroughs

In the present study, authors used both traditional and modern techniques. They concluded that the 16S rDNA genes were not suitable for identifying of Oscillatoriales.

Applications

From the literature survey, it has been found that, cyanobacteria species are increased. The present study supports this statement. With one new recorded species *L. majuscule* during this study.

Peer review

The paper is a traditional work concerning with epiphytic

plants on macroalgal flora on the Alexandria coast, which is interesting and useful for further studies.

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