A taxonomic study of blue-green algae based on morphological, physiological and molecular characterization in Yazd province terrestrial ecosystems (Iran)

Received: 22.06.2015 / Accepted: 30.11.2015

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Abstract

Based on the limited resources available to algal flora in Yazd province in the center of Iran, cultivation, purification and identification of soil algae from seven sites was performed in summer 2013. The soil samples were processed for analysis of Cd, Pb contents. The present paper attempts to analyze survey of soil blue-green algal identification in different types of stations, using morphological, physiological and molecular characterization. Pb bioremediation assay, determination of chlorophyll, carotenoid and phycobiliproteins content measurements was investigated as physiological characterization. *Kamptonema animale*, *Leptolyngbya breviarticulata* and *Kamptonema* sp. are new to Iran and *Oscillatoria tenuis* with high heavy metal absorption could play an important role as biomonitoring agent.

Keywords: Bioremediation, Cd, heavy metals, morpho-species, Pb

مطالعه تاکسونومی جلبک‌های سبز-آبی براساس نشان ویژه‌سازی مورفولوژی، فیزیولوژی و مولکولی در اکوسیستم‌های خشکی استان یزد*

دریافت: ۱۳۹۴/۴/۱ / پذیرش: ۱۳۹۴/۹/۹

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خلاصه

استان یزد یکی از استان‌های خشک و کم آب واقع در بخش مرکزی ایران است که عمدتا از خاکی شور و قلیایی برخوردار بوده و مطالعات اندکی بر میکروفلور اکوسیستم‌های خشکی آن انجام شده است. با توجه به وجود گزارش‌هایی مبنی بر آلودگی خاک این استان به عناصر سنگین و نیز با در نظرگیری قابلیت جلبک‌ها در جذب عناصر آلاینده محیطی، در این مطالعه استفاده از جلبک‌های سبز-آبی به عنوان یکی از شاخص‌های مناسب جهت سنجش آلودگی محیطی مد نظر قرار گرفت. به همین منظور در کنار ارزیابی میزان آلودگی خاک منطقه به سرب و کادمیوم، کشت، جداسازی، خالص‌سازی و شناسایی نمونه‌های جلبکی موجود در خاک هفت ایستگاه مختلف مطالعاتی انجام شد. همچنین، بخشی از نمونه‌های جلبکی خالص‌سازی شده جهت نشان ویژه‌سازی مورفولوژیک و فیزیولوژیک انتخاب شدند. در این بخش، فاکتورهایی شامل قابلیت جذب سرب و سنجش رنگریزه‌ای (رنگریزه‌های کلروفیلی، کاروتنوییدی و فیکوبیلی پروتئین‌ها) جهت نشان ویژه‌سازی فیزیولوژیک مد نظر قرار گرفت. در نتیجه مطالعه حاضر، گونه‌های جدیدی شامل: *Kamptonema* sp., *Kamptonema animale*, *Leptolyngbya breviarticulata* برای فلور خاک‌های ایران گزارش می‌شوند و گونه *Oscillatoria tenuis* به واسطه برخورداری از بیشترین توان جذب سرب به عنوان نشانگر زیستی معرفی می‌گردد.

واژه‌های کلیدی: ریخت گونه، زیست پالایی، سرب، عناصر سنگین، کادمیوم

* بخشی از رساله دکترای نگارنده نخست به راهنمایی دکتر حسین ریاحی ارایه شده به دانشگاه شهید بهشتی

Introduction

Algae occupy a variety of terrestrial habitats, including soils, rocks and even caves (Lamprinou *et al.* 2013). Soil is the most important non-aqueous habitat for blue-green algae (Zenova *et al*. 1995). Due to their high capacity of these algae for morphological and physiological adaptations to different environments, blue-green algae often act as pioneer microorganisms in terrestrial ecosystems (Metting 1981, Hoffmann 1989). Blue-green algae are probably the oldest organisms with oxyphototrophic metabolism. In spite of their long existence, from Precambrian onwards, they not only haven't lost their vitality, but also they still are able to colonize all possible biotopes, including extreme environments (Komarek & Anagnostidis 1998).

Although, some soil algae have been studied in certain localities in various regions with the modern approach using the combined molecular, morphological and ecological criteria (Zancan *et al.* 2006, El-Gamal *et al*. 2008, Budel *et al.* 2009, Lin *et al*. 2013, Nimbhore & Jadhav 2014), information about the identification of soil algae in Iran is very limited.

For the first time, soil samples of Iran were collected by Kotschy from the territories between Persepolis, Shiraz and Bushehr which cover modern provinces, namely, Fars, Esfahan, Chaharmahal-o-Bakhtiari and Bushehr. Later, Ehrenberg (1854) studied them and revealed 45 species of algae, out of which 29 species became valid. In autumn of 1972, the Belgian multi-purpose expedition investigated deserts of the central, eastern and middle Iran, mainly Dašt-e Kavir, Dašt-e Lut and Jazmuriyān hollow. Botanist of the above-mentioned expedition, J. Leonard collected samples of algae, which became a subject of study in P. Compere's work (Compere 1981). The samples of algae were collected from 21 different places and about 300 species and varieties were presented.

In general, there are a few floristic data available for blue-green algae of Iran (Abarkar & Riahi 1995, Saadatnia & Riahi 2009, Shariatmadari *et al*. 2013, Siahbalaei *et al.* 2008, 2010, 2011). In addition, Jafari *et al*. (2014) have reported a new blue-green algae from the oil polluted soil in the south of Iran. With respect to the center of Iran, Moghtaderi *et al.* (2009) have reported *Microcoleous vaginatus* in soil crust of Chadormalu Desert area (Bafgh region in Yazd).

On the other hand, some industrial and anthropogenic activities result in the release of heavy metals as environmental contaminants (e.g. Cadmium and Lead). Soil algae play an important role in returning the environment to its original state altered by various contaminants (Rai *et al*. 1998, Whitton & Potts 2000) via bioremediation. Microalgae pose the ability to degrade or accumulate toxic heavy metals and organic pollutants from the environment, resulting in higher concentrations within themselves as compared to surrounding area (Shamsuddoha 2006). Recently, there has been increasing worldwide interest in using blue-green algae as an economic and low-maintenance remediation technology for contaminated and polluted sites (Bhatnagar & Kumari 2013). Considering limited information about soil microflora of Yazd province and also partial contamination of its soil (Rahmani 2009) with heavy metals, we decided to have a polyphasic study of four genera in this area using morphological, molecular and physiological assays.

Materials and Methods

- Field study, sampling and analyses

Soil samples were collected according to Rangaswamy methodology (1966) from seven different regions (cultivated and barren lands) from Yazd province in Aug. 2013 (Figs 1 & 2).

The soil samples were processed for the analysis of different physicochemical characteristics of the soils, viz., pH, EC, and Cd and Pb contents by Arian Fan-Azma Company, Tehran, Iran (Table 1).

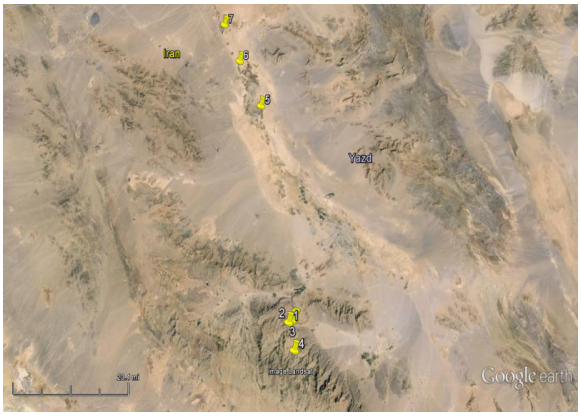

Fig. 1. Location of Yazd province and sampling stations.

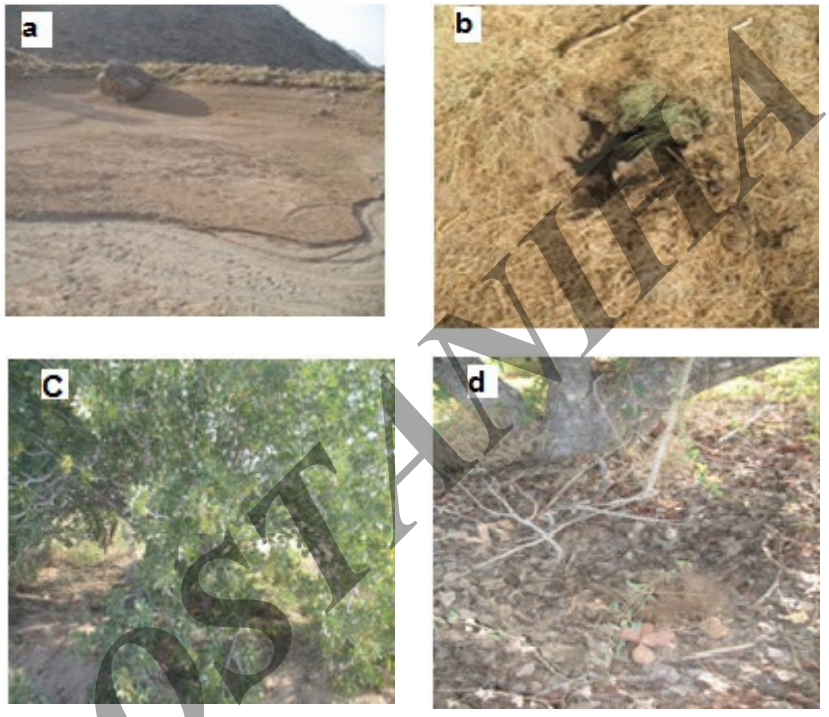

Fig. 2. Views of different stations: a. Barren land: Tezerjan, Barfkhane, b. Ardakan, *Tamarix* population, c. Cultivated land: Ardakan, d. *Pistachia* population.

- Culture and isolation

The collected soil samples were maintained by culturing in BG11 medium (Rippka *et al.* 1979) and incubated at 28 ± 2° C with continuous illumination at 60 µmol photon $m^{-2}s^{-1}$ prepared by three fluorescent lamps. After isolation, samples were purified by several subculturings on solid BG11 medium (Kaushik 1987).

- Morphological identification

Identification of samples was carried out based on accepted criteria (Prescott 1962, Desikachary 1959, Komarek & Anagnostidis 2005). Taxonomic determination was carried out by light microscopy (400–1,000×) and photographs were taken with a Canon camera 1200D. Axenic condition was checked daily by microscopic observation.

The most important vegetative and reproductive characteristics used in the taxonomic determination were: presence or absence of a sheath, the appearance of the filament and the color of cells, cell size and constriction at the cross walls, cellular granulation and cell shape.

- DNA extraction, amplification of the 16S rRNA gene and sequencing

The bulk of each blue-green alga was extracted by genomic DNA extraction kit Thermo. For DNA amplification, the 16S rRNA gene regions, approximately 600 bp in length, were amplified by PCR

using the 106F (5'CGG ACG GGT GAG TAA CGC GTG A 3') and 781Rb (5'GAC TAC AGG GGT ATC TAA TCC CTT T 3') primers (Nübel *et al.* 1997).

The PCR mixture contained 5 µl *Taq* commercial buffer, 5 µl purified DNA, 5 pM of each dNTP, 10 µM of each primer and 2.5 U *Taq* polymerase. An initial cycle consisting of 5 min. at 94° C, 34 cycles of amplification were started (1 min at 94° C, 1 min. at 60° C and 1 min. at 72° C). The termination cycle was 10 min. at 72° C.

PCR amplified products were subjected to 1.5% (w/v) agarose gel using TBE buffer stained with 6 µg/ml DNA safe stains. The sequence was determined by the CinaGene Company. The BLAST tool of the National Center for Biotechnology Information was used to find homologous and other close sequences (97–100% identity and *E*-values $\leq 10^{-20}$) to be included in the phylogenies as reference sequences.

To allocate the *Kamptonema* cluster to *Oscillatoriales*, the Maximum Likelihood method was employed based on the Tamura 3-parameter model in MEGA 5 (Tamura *et al*. 2007).

- Physiological assays

Pb removal was investigated using investigated blue-green algae. Algae biomass (0.02 g) was exposed to 15 ml of 15 ppm Pb stock for 60 min. (Nagase *et al*. 1997). Samples were then centrifuged (6000 rpm, 10 min.) and supernatants were separated for atomic absorption spectroscopy.

In order to chlorophyll determination, following Marker's methodology (1972), cells were extracted with pure methanol for 24 h at 4° C and the chlorophyll content was determined spectrophotometrically at 665 nm.

Carotenoid content was also measured spectrophotometrically (461 & 665 nm) by using the equations of Chamovitz *et al*. (1993). Phycobiliproteins were extracted after osmotic shock and measured spectrophotometrically at 562, 615, 652 and 750 nm (Wyman & Fay 1986).

- Statistical analysis

Data are the means and standard deviation of at least four replicates. Statistical differences were examined using the ANOVA test via SPSS (ver. 18) and Excel software.

Results

- Physicochemical properties of soil

The results of the physical analysis of the soil samples showed that, pH values in soil extract were mostly alkaline. The highest EC belonged to station 6 (5200 µmhos/cm) (Table 1).

Pb and Cd concentrations in soil samples were in acceptable range according to standards (Allaway 1990, Pendias & Pendias 1992).

Table 1. Geographical data and some ecological details of the sampling locations

Location	Latitude/Longitude	pH	EC (µmhos/cm)	Cd (mg/kg)	Pb (mg/kg)
Dehbala, Panjdukun	31.64 N/54.16 E	8.0	1990	0.1	1.42
Dehbala, Almas-e kavir	31.65 N /54.17 E	8.0	3200	0.1	12.00
Tezerjan, Barfkhane	31.57 N /54.16 E	7.36	1000	0.1	11.20
Meybod, Shahva	32.23 N/ 54.04 E	8.3	2030	0.1	1.45
Ardakan	32.37 N/ 53.95 E	8.4	5200	0.1	5.70

- Description of the taxa

In this study, three species belonging to *Kamptonema* and *Leptolyngbya* were identified as new records for Yazd province (Iran). Identified species characteristic is given in Table 2 and their photographs and Camera Lucida drawings can also be seen in Figures 3–4.

Kamptonema animale (C. Agardh ex Gomont) Strunecký, Komárek & J. Smarda (2014) (Fig. 3b)

Thallus expanded, thick, leathery, outside blackish, velvet-like on the surface, in lower layers black-brown, dark blue-green to brownish-green or olive-green, sometimes yellowish or violet. Filaments mostly straight, rarely (especially at the ends) curved, entangled. Sheaths thin, firm, distinct or diffluent, amorphous, mucilaginous, and sometimes lacking but often present. Trichomes bright blue-green or dirty green, (3.5)4–5 µm wide, motile with counterclockwise rotation and strong oscillation, not or sometimes slightly constricted at the frequently granulated cross-walls, mostly abruptly and strongly attenuated (narrowed trichomes to the end) at the ends, straight or weakly hooked (uncinate). Cells more or less isodiametric or up to 1/2 as long as wide, rarely longer than wide, 2-4(5) µm long; cell content sometimes keritomized. Apical cells frequently somewhat elongated, capitate, with rounded or truncated calyptras at the well develop trichomes.

Phormidium articulatum (Gardner) Anagnostidis et Komarek (1988) (Fig. 3e)

Thallus thin, blue-green or solitary trichomes among other algae. Sheaths thin, usually indistinct or lacking. Trichomes blue-green, 5–6(7) µm wide, long and straight or short and circinate, unconstricted or slightly constricted at the thick (preserved material!) cross-walls, not attenuated at the ends. Cells iso-diametric, 1–2 µm long, rarely longer. Apical cells rounded, without thickened outer cell wall.

Oscillatoria tenuis Agardh ex Gomont (1892) (Fig. 3a)

Thallus flat, in form of mats or clusters, blue-green or olive-green, usually thin mucilaginous. Trichomes ± straight or very slightly irregularly curved, not constricted (or very slightly constricted) at cross-walls, usually without granulation at cross-walls, blue-green or grayish-violet, 5–6 µm wide, cylindrical, not attenuated, but very rarely slightly curved at the ends. Cells always shorter than wide, 2–3.5(5–6) µm long, sometimes with scattered solitary granules; apical cells rounded, not capitate, with slightly thickened outer cell wall in well developed trichomes. Occurrence: Freshwater, benthic and metaphytic, sometimes recorded also from wetted rocky walls; not very common, but distributed worldwide, possibly cosmopolitan. Several records are known from tropical regions.

Kamptonema sp. Strunecký, Komárek & Smarda (2014) (Fig. 3d)

Filaments solitary without sheaths or with very fine, facultative and diffluent sheaths, sometimes with several to many trichomes associated more or less in parallel without common firm sheaths. The fascicles are not enveloped in slime. Continually narrowed trichomes towards ends, simple, cylindrical along their whole length (up to the end), uniseriate, blue-green, slightly constricted or unconstricted at cross-walls, motile, 2.5 µm wide. The cells are more or less isodiametric or a little longer or shorter than wide, the terminal cell is rounded and usually without calyptra and the very fine sheaths are developed only exceptionally.

Leptolyngbya breviarticulata (Claus) Anagnostidis (2001) (Fig. 3c)

Thallus expanded, lamellate, olive-green. Filaments with diffluent sheaths. Trichomes pale blue-green, 1–2 µm wide, not constricted at cross-walls, not attenuated at the ends, straight. Cells isodiametric or shorter than wide, 1.5–2 µm long; apical cells rounded, without calyptra.

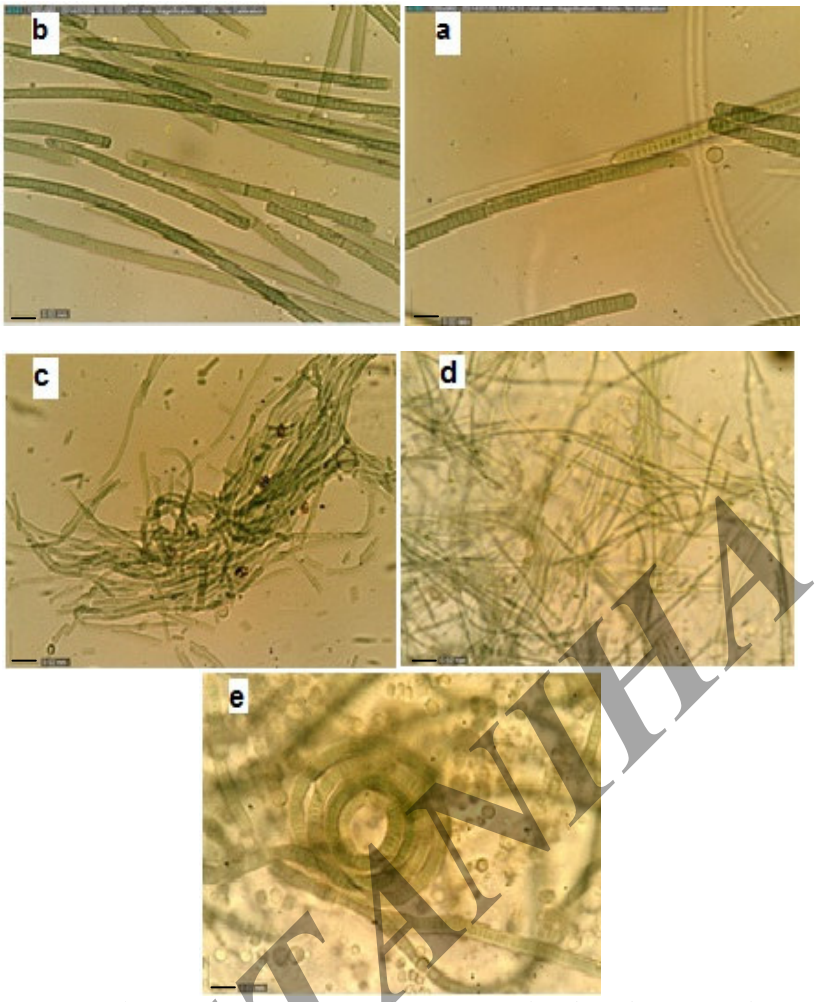

Fig. 3. a. *Oscillatoria tenuis*, b. *Kamptonema animale*, c. *Leptolyngbya breviarticulata*, d. *Kamptonema* sp., e. *Phormidium articulatum* (Bar = 0.02 mm).

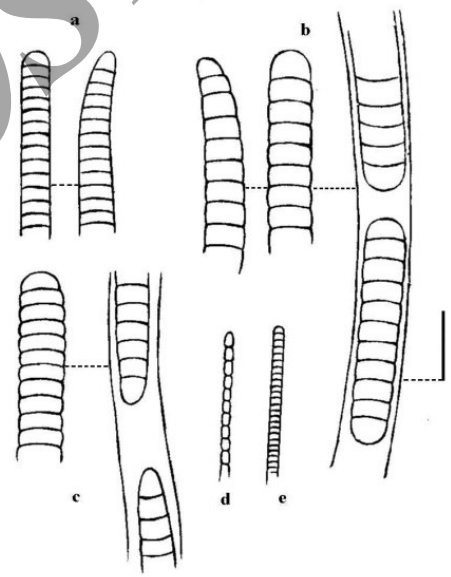

Fig. 4. a. *Kamptonema animale*, b. *Phormidium articulatum*, c. *Oscillatoria tenuis*, d. *Kamptonema* sp., e. *Leptolyngbya breviarticulata* (Bar = 10 µm).

The results of this study showed that, no species could grow in the station seven with highest EC (data not shown). All species identified in this study belonged to order *Oscillatoriales*. In the genera of this order, heterocysts was not present, apical cell had different shape and hormogonial sheath was seen in some genera such as

Oscillatoria and *Phormidium*. Cell sizes of thallus in these algae also differed from 1 to 7 µm (Table 2).

Table 2. Selected morphological characteristics of five species

Criteria	Sheath	Hormo-gonial sheath	Appearance of the filament	Filament width (µm)	Color	Cell size (µm)	Cell shape	Constr-iction at the cross walls	Cellular gran-ulation
Oscillatoria tenuis	-	+	Creeping filaments	5–6	Green	2–5 × 5–6	Rectangular, Quadrangular	Limited	-
Kamptonema animale	-	+	Creeping filaments	4–5	Blue-Green	2 × 4	Rectangular	-	+
Leptolyngbya breviarticulata	+	-	Adherent to Agar	1–2	Green	1.5–2 × 1–2	Quadrangular	-	-
Kamptonema sp.	+	-	Adherent to Agar	1.5–3	Blue-Green	1–3 × 1.5–2	Rectangular	+	-
Phormidium articulatum	-	+	Adherent to Agar	5–7	Green	1–2 × 5–7	Rectangular	Limited	-

* Numbers (min. and max.) are length × width

- Phylogenetic analysis of the blue-green algae

The sequence of the 16S rRNA gene was determined for *Oscillatoria*, *Phormidium*, *Leptolyngbya* and *Kamptonema*. The sequences were compared with those of representative non-heterocystous cyanobacteria available in GenBank (http://www.ncbi.nlm.nih.gov/BlAST). The 16S rRNA sequences were combined with other *Oscillatoriales* species available in the database (Casamatta *et al.* 2003, Ezhilarasi & Anand 2009). The nucleotide sequences described in this study have been submitted to the NCBI under the accession numbers NCBI: KP297408, KP297409, KP297410, KP297411, KP297412.

The phylogenetic tree, generated for the samples isolated in this study and the related sequences from the NCBI database is shown in figure 5. The *Escherichia coli* 16S rRNA sequence was used as the out-group to root the tree.

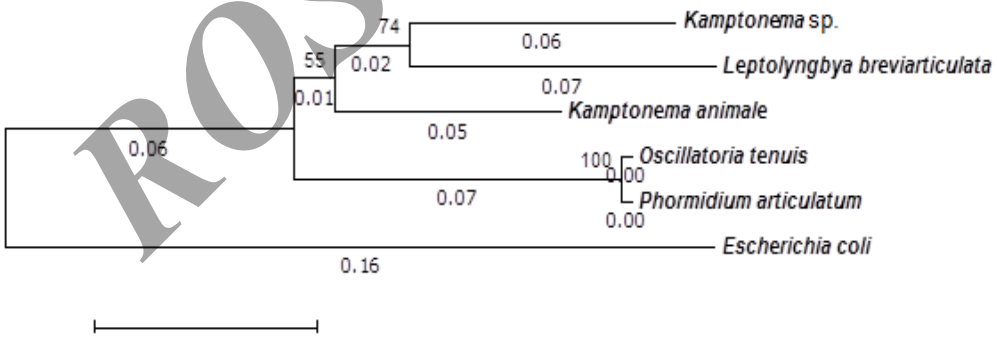

Fig. 5. Phylogenetic tree from 16S rRNA gene sequences of blue-green algae using maximum likelihood method (*Escherichia coli* is the out-group).

- Physiological results

Batch equilibrium sorption experiments were used for screening for cost-effective soil blue-green algal biomass. Biosorption of lead by five algae was investigated. Biosorption of lead was rapidly occurred onto algal biosorbents and most of the sorbed metal was bound in <60 min. of contact. *Oscillatoria tenuis* species, removed lead most efficiently from aqueous solution with 77.3% absorption. The applicability of the Langmuir models for the different biosorbents was also tested (Table 3).

Table 3. Pb content in soil and Pb absorption by algae after 1 hour under experiment condition

Sample	Absorption	ppm	Q (mg/g blue-green algae)	SD	Pb (mg/kg)
Control	0.3	15	-	0.001	-
Oscillatoria tenuis	0.03	3.40	8700	0.008	12.00
Kamptonema animale	0.09	10.26	3555	0.001	11.20
Leptolyngbya breviarticulata	0.086	9.26	4305	0.001	5.70
Kamptonema sp.	0.096	10.39	3457.5	0.001	1.45
Phormidium articulatum	0.101	11.03	2977.5	0.001	1.42

The results showed that, the highest chlorophyll content belonged to *Kamptonema animale* and there was no significant difference with *Kamptonam* sp. with respect to phycobili proteins (PBP) and carotenoids same results were achieved. The maximum content was belonged to *Kamptonema animale* too (18.34 & 4.08, respectively). The pigment contents were higher in *kamptonema animale* (Table 4).

Table 4. Selected physiological characteristics of five species

Sample	Chl ($\mu g\ mL^{-1}$)	PBP ($\mu g\ mL^{-1}$)	Carotenoid ($\mu g\ mL^{-1}$)
Oscillatoria tenuis	0.84	4.61	0.18
Kamptonema animale	2.53	18.34	4.08
Leptolyngbya breviarticulata	1.27	2.44	0.33
Kamptonam sp.	2.03	4.40	0.80
Phormidium articulatum	0.96	1.56	0.28

Data are mean values of four experiments

Discussion

The algal flora of soil has attracted the attention of phycologists for past few decades and cyanophycean algal forms were found dominant. Among several genera of these algae, those such as *Oscillatoria*, *Phormidium*, *Leptolyngbya*, *Calothrix*, *Limnothrix*, *Pseudanabaena*, *Schizothrix*, *Sytonema*, *Anabaena*, *Cylandrospermum* and *Microcoleus* were found dominant in soil microflora (Zancan *et al*. 2006, El-Gamal *et al*. 2008, Budel *et al*. 2009, Nimbhore & Jadhav 2014). Lin *et al.* (2013) observed different kind of *Osillatoria* such as *O. tenuis* and *O. chlorine* in different farmlands in mid-Taiwan.

In this study, the species of four genera, viz., *Oscillatoria*, *Phormidium*, *Leptolyngbya* and *Kamptonema* were found and selected for Bioremediation assays.

In Yazd, 20,000 cubic meter of industrial sewage are generated daily and discharge into old abundant Qanat, absorption well, and used for irrigation. The practice of this tradition has caused soil and surface and underground water pollutions (Rahmani 2008). Rahmani *et al*. (2001) believed that, the concentration of any pollutants such as Cr, Cu, Zn, Cd, and Pb in the underground water in Yazd province does not exceed the acceptable level, while other studies exhibited contamination of soil as well as surface and ground water in this province (Rahmani 2012). Also, the concentrations of pollutants such as zinc and cadmium in soil samples have been reported in critical range (Rahmani 2009). The results of present study showed that, Cd and Pb content in the soil samples of Yazd province are lower than critical range.

In another part of this study, algae from five stations with different concentrations of Pb have been selected for bioremediation assay. For this purpose, blue-

green algae such as *Oscillatoria tenuis*, *Kamptonema animale*, *Leptolyngbya breviarticulata*, *Kamptonema* sp. and *Phormidium articulatum* used for heavy metal absorption. Previous studies of role of algae in the removal of heavy metals from the soil showed that, algal species such as *Fischerella* sp., *Lyngbya spiralis*, *Tolypothrix tenuis*, *Stigonema* sp., *Phormidium molle*, *Lyngbya heironymusii*, *Gloeocapsa* sp., *Oscillatoria jasorvensis*, *Anabaena inaequalis*, *Chlorella* sp., *Stigeoclonium tenue*, *Synechococcus* sp. and *Westiellopsis prolifica* were resistant to heavy metals and thus used for the removal of heavy metals (Kannan *et al.* 2012, Bajguz 2011, Rahman 2011).

Inthorn *et al.* (2002) studied adsorption of lead from aqueous solutions and showed in blue-green algae; highest Pb removal was by *Nostoc punciforme*, *Oscillatoria agardhii*, *Gloeocapsa* sp., *Nostoc piscinale*, *Nostoc commune* and *Nostoc paludosum* (98%, 96%, 96%, 94%, 94% and 92%, respectively).

The status of metal concentrations including Pb, Cd, Cu, Ni, Zn and Fe in six species of *Padina* were determined on the coast of the Persian Gulf by Amini *et al.* (2012). The positive correlations between metal contents in the sediment and almost in all species indicated that, these algal species could absorb metals from sediments and play an important role as biomonitoring agents. Also in this research *Oscillatoria tenuis* with high metal biosorbtion (77.33%), which existed in the station 3 with Pb high concentration content, could play a key role as biomonitoring agent.

The blue-green algae show the most evident response in different agro-ecosystems and consequently seem to be the most suitable group to adopt as a soil bioindicator of land use (Zancan *et al.* 2006). As suggested by Paoletti (1999), however, there is a general need to improve our knowledge of micro-organisms, including algae, to better appreciate the many benefits that humans derive from their existence. Further information of this kind, using standard quantification methods and precise identification procedures should, therefore, be obtained in order to draw general conclusion about the potential role of soil algae as bioindicators.

Taxonomic results showed that, *Phormidium articulatum* was reported by Shariatmadari *et al.* (2011) from the paddy soils of north of Iran and *Oscillatoria tenuis* have been reported from Gilan paddy soil by Soltani *et al.* (2005). Consequently, *Phormidium articulatum* and *Oscillatoria tenuis* are found new for Yazd province (Iran).

Results of the present study showed that, three blue-green algae, namely, *Kamptonema animale*, *Leptolyngbya breviarticulata* and *Kamptonema* sp. are new to Yazd and Iran and *Oscillatoria tenuis* with high heavy metal absorption played an important role as biomonitoring agent.

Acknowledgments

Authors thank the Department of Botany of Shahid Beheshti University (Tehran, Iran) for facilities provided for research performance. Authors also appreciate Department of Petroleum Microbiology, Research Institute of Applied Science (ACECR), Tehran, Iran. Thanks are also due to Prof. Komarek for his kind assistance during this research.

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