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Molecular and Morphological Characterization of Oil Polluted Microalgae

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ABSTRACT: Oil pollutions are widespread environmental pollutants. Most of the studies have been focused on the biodegradation of these pollutions. Unfortunately, the information on the microflore of the polluted regions especially for microalgae is limited. In this research, we have focused on Masjed Soleiman; one of the most oil polluted cities in Iran. Soil and water samples were collected from different stations and seasons and their microalgae identified morphologically and molecularly. TPH, PAH and heavy metals of these stations were analyzed. The relationship between TPH contents and micro algal populations has also been considered. Results showed microalgae, present in these regions belonged to Cyanobacteria, Chlorophyta and Diatoms. Among them Phormidium sp. was the most dominant species that was found in all polluted regions. Microalgae biomass, declined in high level polluted medium, whereas low levels of TPH showed no effect on microbial biomass. According to the results the isolated specimens have high resistance to environmental pollutions. So the type and frequency of the species, can lead us to estimate the amount of pollution in different sampling regions.

Key words: Cholorophyta,Contamination,Cyanobacteria, Morphology,16rRNA,18rRNA

INTRODUCTION

The extensive use of petroleum products leads to the contamination of almost all compartments of the environment by hydrocarbons (HC). Particularly in the zones of petroleum production, the environment is submitted to contamination by the crude oil produced at the site. The main process acting in the clean-up of HC contaminated ecosystems is microbial biodegradation, which has been extensively studied and reviewed (Atlas, 1984; Leahy and Colwell, 1990). In tropical crude oil production sites, cyanobacterial mats often develop on petroleum-polluted zones including surface soils and water environments. Cyanobacterial mats are microbial combination composed of photosynthetic and non photosynthetic bacteria and fungi embedded in cyanobacterial mucilage (Sorkhoh *et al.*, 1995; Abed *et al.*, 2002).

Cyanobacteria are a major and phylogenetically coherent group of prokaryotes possessing the unifying property of performing oxygenic plant-like photosynthesis with autotrophy as their dominant mode of nutrition (Castenholz and Waterbury, 1989). However, in spite of their typically aerobic photosynthetic nature, some of the cyanobacterial species can grow in the dark on organic substrate (Smith, 1983; Stal and Moezelaar, 1997) and others under anaerobic conditions with sulfide as electron donor for photosynthesis (Cohen *et al.*, 1986). Certain strains have the ability to fix atmospheric dinitrogen into organic nitrogen-containing compounds, so displaying the simplest nutritional requirements of all microorganisms. They can use as biosurfactants (Mazaheri Assadi and Tabatabaee, 2010) and also as heavy metals absorber (Murugesan *et al.*, 2008; Gaur and Dhankhar, 2009). These organisms have great ability to survive in most of natural illuminated environments, both aquatic and terrestrial, including several types of extreme environments (Whitton, 1992). This widespread distribution reflects a large variety of species, covering a broad spectrum of physiological

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properties and tolerance to environmental stress (Tandeau *et al.*, 1993).

The purpose of this work was collecting and identifying cyanobacterial strains from one of the most oil polluted regions of Iran "Masjed- soleiman". Masjed-soleiman (Fig.1) is a city in the khoozestan province in southwestern Iran. In 1908 the first oil well of the Middle East were discovered and drilled in the region "Naftoon" in the center of this city, so the 100th year anniversary of oil drilling in the Middle East was celebrated in 2008 in this city. In this city, oil is flooded in the surface of floor and rivers additionally, you can easily breathing the H_2S (Solfide dihydrogen) from its air. So it has been considered as one of the most oil contaminated parts of Iran. In this way studying the cyanobacterial strains of this region as tolerate species to oil pollution has been done.

MATERIALS & METHODS

Samples were collected from three polluted stations as follow: 1- Gas well 315; X=0343765, Y=3533899, Height=495m; 2- Masil number 2; X=0339659, Y=3536456, Height=243m; 3- Siberenj; X=0339545, Y=3535959, Height=250m. Also we collected samples from one unpolluted station; Sar masjed (Atashkade); X=0337746, Y=3539955, Height=312m; as control one. Sampling was taken in various seasons during November 2008 till September 2009 from soil and water of mentioned stations. Samples were collected and sealed in glass bottles and stored at 4ºC for various analyses in lab.

Soil samples were cultured by Sardeshpande and Goyal (1981). We have used various culture mediums to isolate different genera. These mediums were **BG11**: (mgl^{-1}) $NaNo_3$ 1.5, K_2HPO_4 40, $MgSO_4.7H_2O$ 75, $CaCl_2.2H2O$ 36, Citric acid 6, Ferric ammonium citrate 6, EDTA 1, Na_2CO_3 20 and trace metal solution containing ($mg\ l^{-1}$) H_3BO_3 2.86, $MnCl_2.4H_2O$ 1.81, $ZnSO_4.7H_2O$ 0.222, $Na_2MoO_4.2H_2O$ 0.39, $CuSO_4.5H_2O$ 0.079, $Co(NO_3).6H_2O$ 0.0494, **N_8**: ($mg\ l^{-1}$) $Na_2HPO_4.2H_2O$ 260, KH_2PO_4 740, $CaCl_2$ 10, FeEDTA 10, $MgSO_4.7H_2O$ 50, KNO_3 1000, Trace elements 1 ml as follow(gl^{-1}): $Al_2(SO_4)_3.18H_2O$ 3.58, $MnCl_2.4H_2O$ 12.98, $CuSO_4.5H_2O$ 1.83, $ZnSO_4.7H_2O$ 3.2, **Chu10:** Stock Solution($g.l^{-1}dH_2O$) $Ca(NO_3)_2$ 20, K_2HPO_4 2.5, $MgSO_4.7H_2O$ 12.5, Na_2CO_3 10, Na_2SiO_3 12.5, $FeCl_3$ 0.4, Trace metal

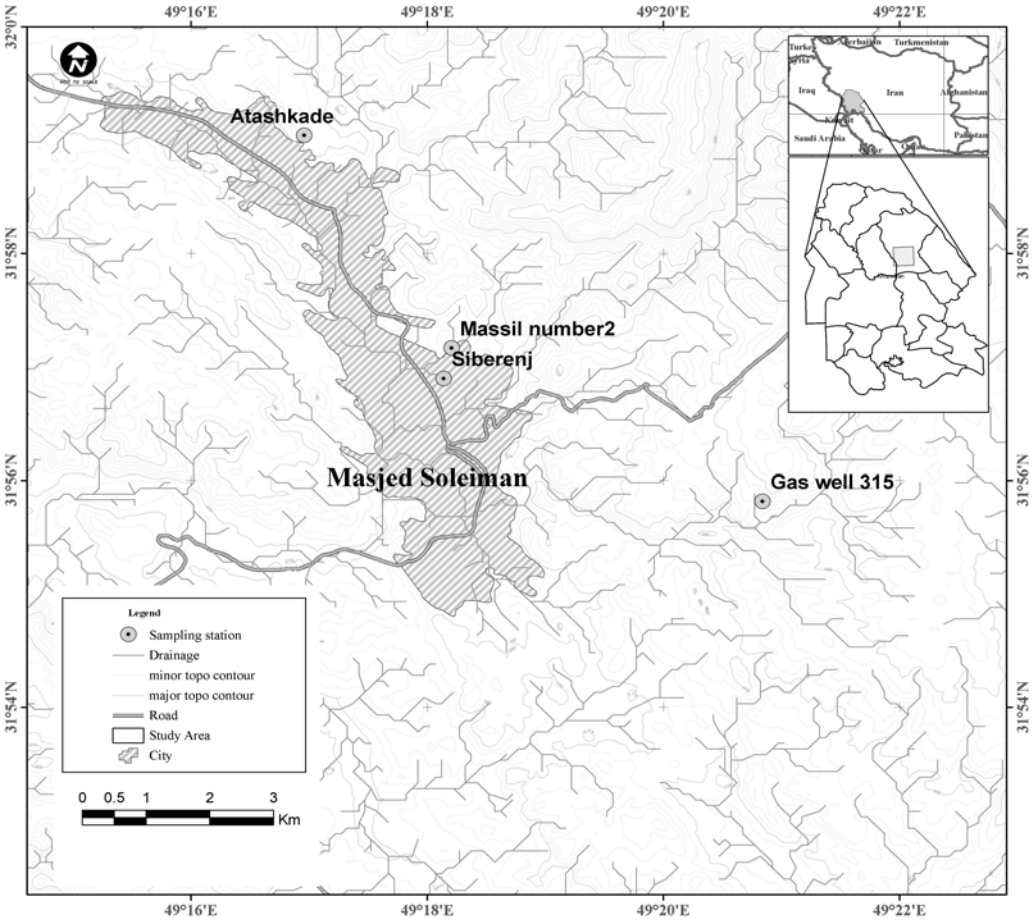

Fig. 1. Location of Masjed-soleiman and sampling stations

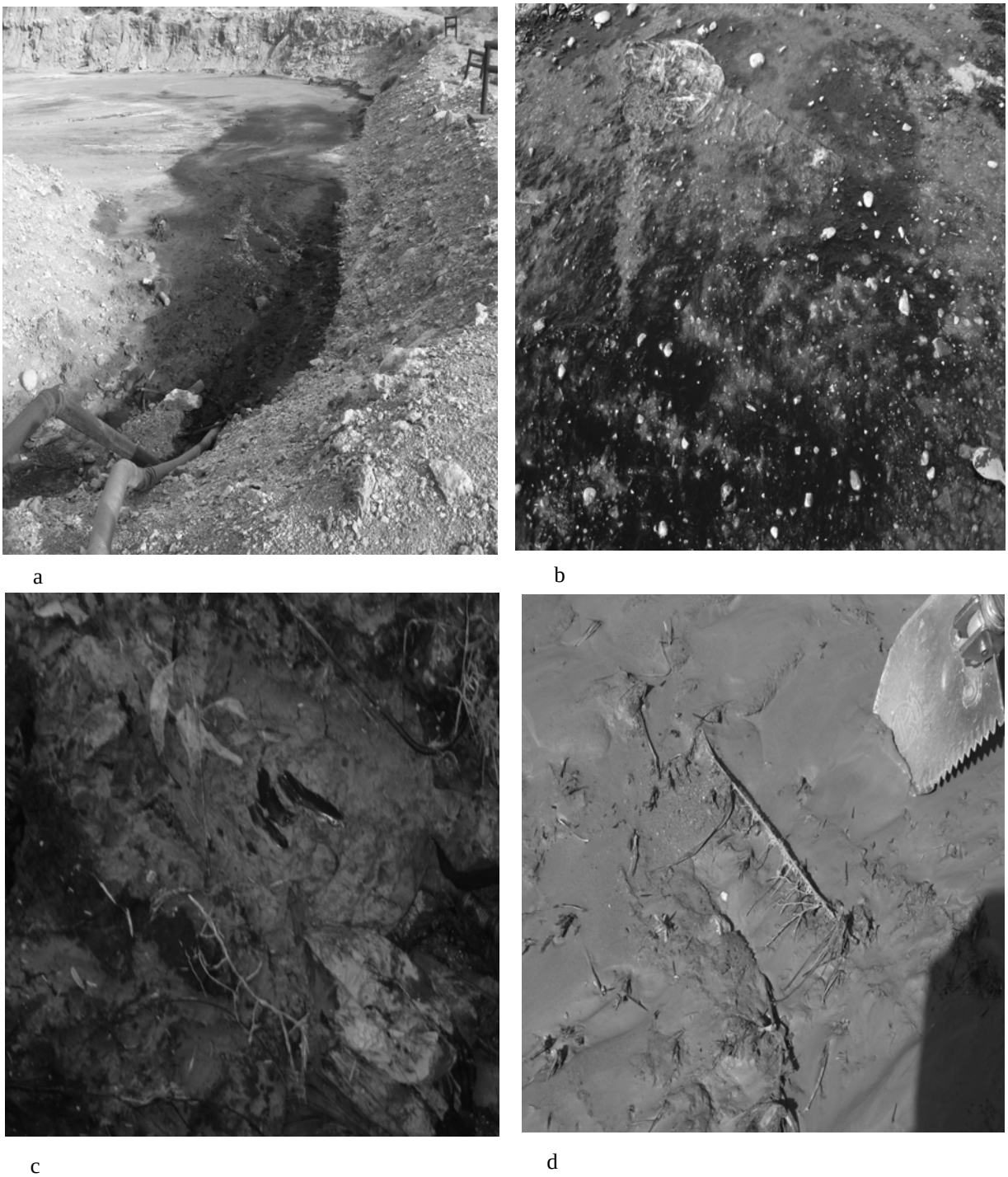

Fig. 2. Views of different station, a- Gas well 315; b-Siberenj; c-Masil number 2; d- Sarmasjed (Atashkade)

solution(Stock solution $g.l^{-1}dH_2O$): H_3BO_3 2.48, $MnSO_4.H_2O$ 1.47, $ZnSO_4.7H_2O$ 0.23, $CuSO_4.5H_2O$ 0.1, $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.07, $Co(NO_3)_2.6H_2O$ 0.14, Vitamins solution (Stock solution $g.l^{-1}dH_2O$): Thiamine.HCL 50 mg, Biotin 2.5, Cyanocobalamin 2.5, **BBM**: (Stock solution $g.l^{-1}dH_2O$): $NaNO_3$ 25, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 7.5, K_2HPO_4 7.5, KH_2PO_4 17.5, NaCl 2.5, Alkaline EDTA Solution: EDTA 50, KOH 31, Acidified Iron Solution: $FeSO_4.7H_2O$ 4.98, H_2SO_4 1ml, Boron Solution:H_3BO_3 11.42, Trace metal solution(Stock solution $g.l^{-1}dH_2O$): $ZnSO_4.7H_2O$ 8.82, $MnCl_2.4H_2O$ 1.44, MoO_3 0.71, $CuSO_4.5H_2O$ 1.57, $Co(NO_3)_2.6H_2O$ 0.49, **Zarrok**: (gl^{-1}) NaCl 1, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.04, $FeSO_4.7H_2O$ 0.01, EDTA 0.08, K_2HPO_4 0.5, $NaNO_3$ 2.5, K_2SO_4 1, $NaHCO_3$ 16.8, and 1ml of A_5 and B_6 as follow: $\mathbf{A_5}(gl^{-1})$: H_3BO_3 2.86, $MnCl_2.4H_2O$ 1.81, $ZnSO_4.7H_2O$

0.222, $CuSO_4.5H_2O$ 0.074, MoO_3 0.015, $\mathbf{B_6}$(gl^{-1}): NH_4NO_3 0.0229, $K_2Cr_2(SO_4)_4.24H_2O$ 0.096, $NiSO_4.7H_2O$ 0.0478, $Na_2SO_4.2H_2O$ 0.0179, $Ti(SO_4)_3$ 0.04, $Co(NO_3)_2.6H_2O$ 0.439. The cultures were kept in culture room that was illuminated continuously (50 $\mu Em^{-2}s^{-1}$) supplied by six fluorescent lamps and following incubation at 30 ± 1ºC. After one month, samples were grown on the surface of plates and identified both morphologically and molecularly.

Microalgal populations in the freshly collected soils were estimated by most probable number (MPN) method. The morphology of cells and filaments was studied using an OPTIKA N-400 Fl, fluorescence microscope with a digital camera. By using the semi-permanent microscopic slides of samples the following parameters were analyzed: length and width of vegetative cells; morphology of terminal cells; the presence or absence of heterocysts and akinetes; the distance between heterocysts and the distance between a heterocyst and the nearest akinete, and finally the shape of filaments and their potential aggregation into colonies. Microalgal taxonomy and nomenclature is primarily based on Desikachary (1959), Prescott (1970), John *et al.* (2003). Genomic DNA of microalgal isolates was extracted according to Sambrook *et al*. methods (2001). Since universal primers for direct sequencing of 16*and* 18rRNA genes are usually designed to be used with axenic cultures, specific primers were selected in order to obtain clean sequences for genes. Primers used in the amplification and sequencing of these genes are listed in Table 1.

Amplification was performed on a Programmable Thermal Controller (CR CorBett Research, USA) as it has been described by Nübel *et al.*, (1997). PCR amplified products were subjected to 1.5% (w/v) agarose gel using TBE buffer stained with 6 µg/ml DNA safe stain.

For photo documentation, an Uvi-DOC BTX-20-M, EEC system with MITSUBISHI P91E software was used. PCR products were purified with the Real Clean Spin Kit. Automated sequencing was determined using the TAG-Copenhagen Company with primers. The sequence data was analyzed using a similarity search by using the BLAST through the website of the NCBI. The BLAST tool of the National Center for Biotechnology Information (http://www.ncbi.nlm.nib.gov) was used to find homologous and other close sequences (97-100% identity and *E*-values d" 10^{-20}) to be included in the phylogenies as reference sequences. To achieve the final identification of the isolates, the following criteria were used. When the phylogenetic positioning of an isolates retrieved identification at the species level, this prevailed; if the phylogenetic positioning allowed only genus determination or no molecular identification was possible, then the identification at the species level obtained by morphological analysis was retained.The analyses of TPH in samples were carried out by IMPRC laboratories Ltd., Karaj, according to MOOPAM method. TPH in samples were extracted by GC and the analyses were fractionated according to their molecular weight. Heavy metal analyses were also carried out by IMPRC and DAR laboratories Ltd. according to ICP method.

RESULTS & DISCUSSION

Evaluation of changes of Total Petroleum Hydrocarbons (TPH) in polluted stations (Gas well 315, Masil number 2 and Siberenj) and unpolluted station (Atashkade) (Fig.3), shows that maximum range of TPH of polluted regions is seen in the autumn and winter. Gas well 315 had the highest TPH in autumn, meanwhile masil number 2 and Siberenj had the maximum TPH in winter.

Table 1. Primers used for 16 and 18 rRNA sequencing

Primer	Sequence(5'-3')	Ref. or source	Observation
CYA106F	CGG ACG GGT GAG TAA CGC GTG A	Nübel *et al.* (1997)	Universal for cyanobacteria
CYA781R(b)	GAC TAC AGG GGT ATC TAA TCC CTT T	Nübel *et al.* (1997)	Universal for cyanobacteria
CYA781R(a)	GAC TAC TGG GGT ATC TAA TCC CATT	Nübel *et al.* (1997)	Universal for cyanobacteria
F18	GTC AGA GGT GAA ATT CTT GGA TTT A	Nübel *et al.* (1997)	Universal for Chlorophytae
R18	AGG GCA GGG ACG TAA TCA ACG	Nübel *et al.* (1997)	Universal for Chlorophytae

Forward primer CYA106F was used in alternative reactions.

Reverse primer CYA781R was an equimolar mixture of CYA781R (a) and CYA781R(b)

R (reverse) and F (forward) designations refer to primer orientation in relation to the rRNA. A 40-nucleotide GC-rich sequence (59-CGC CCG CCG CGC CCC GCG CCG GTC CCG CCG CCC CCG CCC G-39) is attached to the 59 end of the forward primers.

Analysis of Poly Aromatic Hydrocarbons (PAH) in different seasons (Fig.4), shows that maximum rate of changes of PAH occurs in spring and is related to Acenaph hydrocarbon. Results of this part also revealed that Siberenj station has the highest quantity of PAH than other ones. In Autumn PAH decreased significantly but in this season the rate of Acenaph and Florence in Siberenj and Anthracene in Masil number 2 has increased. We have seen the minimum rate of PAH in winter but among the various hydrocarbons, Acenaph and Anthracene in Masil number 2 have increased to some extent.

Comparison of the changes of heavy metals of different stations (Fig.5) showed that among different seasons, autumn has the highest range of heavy metals in all of the stations especially in Siberenj. These changes are clearly seen for Cr, Cu, Ni, Pb and Zn. In spring, Siberenj also has the high rate of the heavy metals but their quantities are much less than in the autumn. In summer the heavy metals decreased significantly in all stations but in Atashkade, they have a little increase. In winter, heavy metals increased just in Atashkade.

Generally during various sampling, 63 strains of water and soil were collected from our stations. Among them, 53 strains were isolated and purified. These results suggest that most of the species present in the stations (Gas well 315, Masil number 2, Siberenj and Atashkade) are diverse and responsible for bloom formation, and belong to eight cyanobacterial families: Oscillatoriaceae, Nostocaceae, Chroococcaceae, Scenedesmaceae, Chlorococcaceae, Oocystaceae, Cladophoraceae and Bacillariaceae (Table 2). Results indicate that all of the identified species belong to the blue-green, green algae and diatoms (Table 3). These strains were from 14 genera which 7 strains of them (13.2%) belong to green algae, 2 strains (3.8%) are from diatom and the rest of the species from cyanobacteria. Among the different isolates, the species predominantly found were from Oscillatoriaceae (56.6%) and Nostocaceae (24.5%).

The species composition of algae and cyanobacteria in polluted and unpolluted stations are presented in Table 2. Totally there were 14 genera of microalgae consisting of nine cyanobacteria and four green algae in polluted stations and also one species of diatoms in unpolluted station. Almost all the species were eliminated in the polluted and unpolluted stations whereas only *Phormidium* sp. was present in all the stations.

Molecular identification was done according to the 16S rRNA sequence for blue-green species and 18S rRNA sequence for green ones. Generally among the 53 purified strains; we have identified 19 strains from various polluted stations molecularly. Fig.7 shows fingerprinting profiles of isolated microalgae which are as follow:
1-*Phormidium* sp. ISC31, NCBI: GU138682; 2-*Phormidium* sp. ISC63, NCBI: GU477756;
3-*Phormidium* sp. ISC60, NCBI: GU584197; 4-*Phormidium* sp. ISC68, NCBI: GU560741;
5-*Leptolyngbya* sp. ISC25, NCBI: GU138681; 6-*Leptolyngbya* sp. ISC64, NCBI: GU560738;
7-*Leptolyngbya* sp. ISC69, NCBI: GU812287; 8-*Leptolyngbya* sp. ISC83, NCBI: GU937790,
9-*Leptolyngbya* sp. ISC67, NCBI: GU477759; 10-*Plectonema* sp. ISC33, NCBI: GU198918; 11-*Nostoc* sp. ISC62, NCBI: GU560740; 12-*Nostoc* sp. ISC90, NCBI: GU812288; 13-*Anabaena* sp. ISC55, NCBI: GU584196; 14- *Anabaena* sp. ISC93; 15-*Scenedesmus* sp. ISC73, NCBI: GU591757; 16-*Scenedesmus* sp. ISC79; 17-*Chlorococcum* sp. ISC98; 18-*Chlorococcum* sp. ISC66;

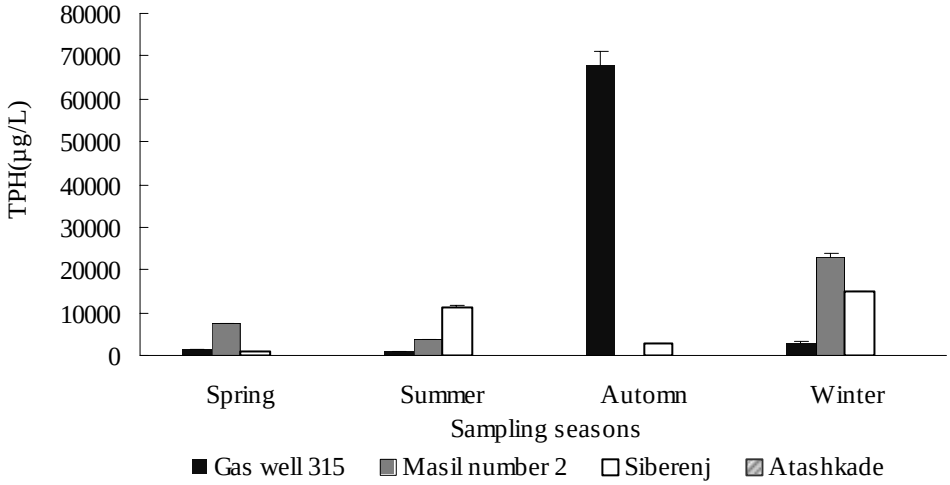

Fig. 3. TPH analysis of stations in different seasons

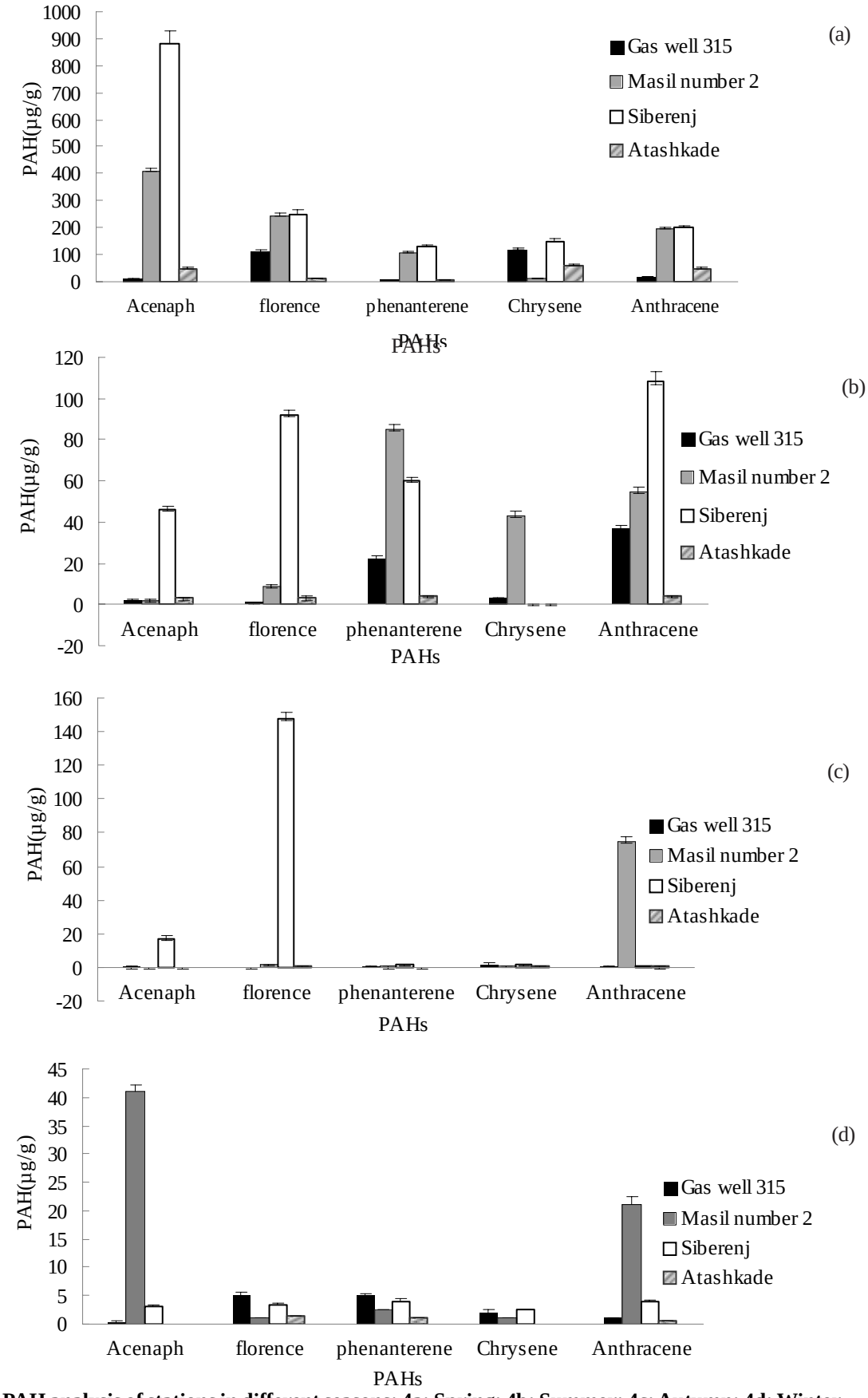

Fig. 4. PAH analysis of stations in different seasons; 4a: Spring; 4b: Summer; 4c: Autumn; 4d: Winter

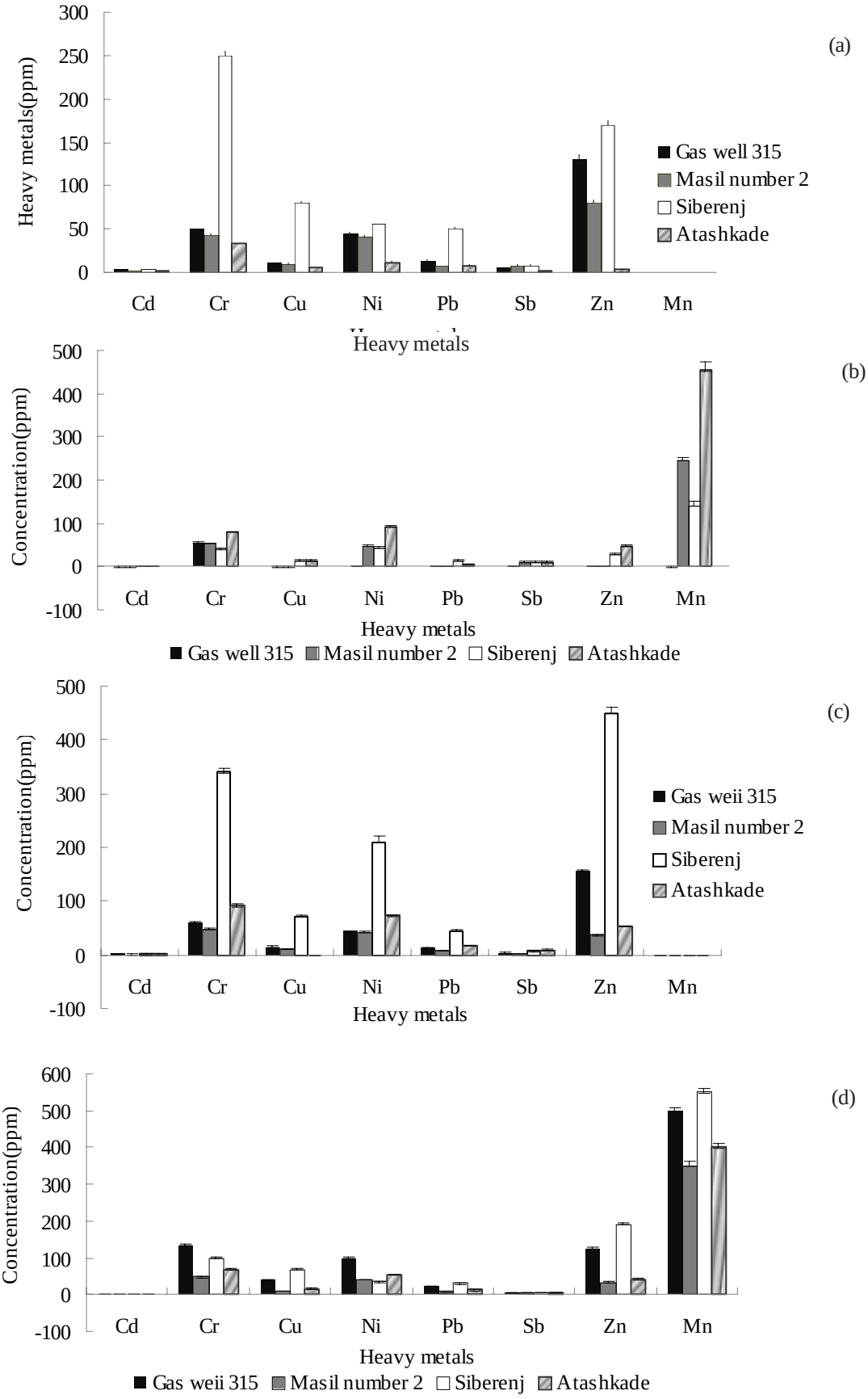

Fig. 5. Heavy metal analysis of stations in different seasons; 5a: Spring, 5b: Summer, 5c: Autumn, 5d: Winter

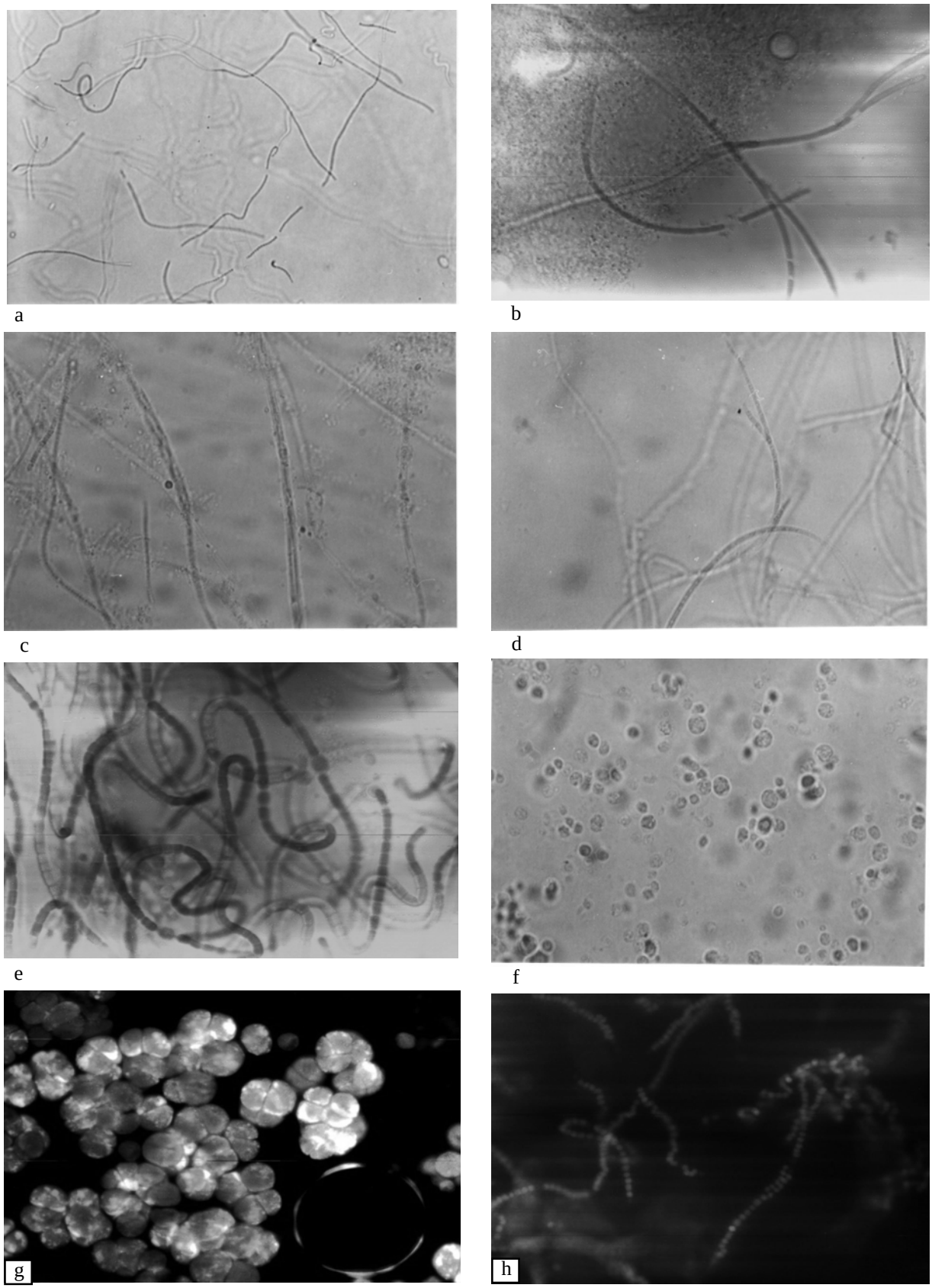

Fig. 6. Microphotograph of some straines isolated from different stations(40 X); a- *Phormidium* sp. ISC 33, b- *Leptolyngbya* sp. ISC 67, c- *Microcoleus* sp., d- *Plectonema* sp., e-Nostoc sp., f- *Chroococcus sp.*, *g- Chloroccocum* sp. ISC 84, h- *Anabaena* sp. ISC 55

Table 2. Algal distributions in different stations

Algal strains	Stations			
	1	2	3	4
Cyanobacteria				
1.Oscillatoria sp.	+	-	+	+
2.Phormidium sp.	+	+	+	+
3.Leptolyngbya sp.	+	+	+	-
4.Lyngbya sp.	+	-	+	+
5.Microcoleus sp.	-	-	+	-
6.Plectonema sp.	-	-	+	-
7.Nostoc sp.	-	+	-	+
8.Anabaena sp.	+	-	+	-
9.Chroococcus sp.	-	-	-	+
Chlorophyceae				
1.Scenedesmus sp.	+	-	-	+
2.Chlorococcum sp.	+	+	+	-
3.Chlorella sp.	-	-	+	-
4.Cladophora sp.	-	+	-	+
Bacillariophyceae				
Nitzchia sp.	-	-	-	+

1=Gas well 315, 2=Masil number 2, 3=Siberenj, 4= Sarmasjed(Atashkade)

Table3. Systematic position of isolated strains

Oscillatoria	*O. amphibia* *O.decolorata* *O.foreavi* *O.jasorvensis* *O.proteus* *O.terebriformis* *O.sp.*
Phormidium	*Ph. fragile* *Ph.* ISC27 *Ph.* sp. ISC31 *Ph.* sp. ISC33 *Ph.* sp. ISC60 *Ph.* sp. ISC63 *Ph.* sp. ISC68
Leptolyngbya	*L.* sp. ISC 25 *L.*sp. ISC 64 *L.*sp. ISC 67 *L.*sp. ISC 69 *L.*sp. ISC 83 *L.*sp. ISC 40
Lyngbya	*L.* sp. *L. subtilis* *L. allorgei* *L. aeruginea-coerulea* *L. semiplena* *L.hieronymusii* *L. kuetzingii* *L.cinerascens*
Microcoleus	*M.* sp. *M.vaginatus*
Plectonema	*P.*sp. ISC.33
Nostoc	*N.entophytum* ISC 32 *N. paludosum* *N.* sp. *N.* sp. ISC 26 *N.* sp. ISC 62 *N.* sp. ISC 90
Anabaena	*A.*sp. *A.*sp. ISC 55 *A.*sp. ISC 93
Chroococcus	*Ch.* sp.
Scenedesmus	*S.* sp. ISC 73 *S.* sp. ISC 79
Chlorococcum	*Ch.*sp. ISC 61 *Ch.*sp. ISC 66 *Ch.*sp. ISC 98
Chlorella	*C.*sp.
Cladophora	*C.*sp.
Nitzschia	*N. basilliformis* ISC 3 *N.* sp.

19- *Chlorococcum* sp. ISC61; 20- *Microcheate tenera* ISC13, NCBI: JF290484; 20- *Nostoc* sp. ISC17, NCBI: JF290485; 21- *Leptolyngbya* sp. ISC38, NCBI: JF290485.

Soil samples from different stations were cultured and their growth analyzed meanwhile the relation between TPH contents and growth of species were investigated (Fig. 8, a-d). In Gas well 315 (Fig.8,a), growth of species has increased during various seasons but this increase isn't correlated to the TPH contents. It shows that in this station, by decreasing the pollution of oil hydrocarbons, growth percent has

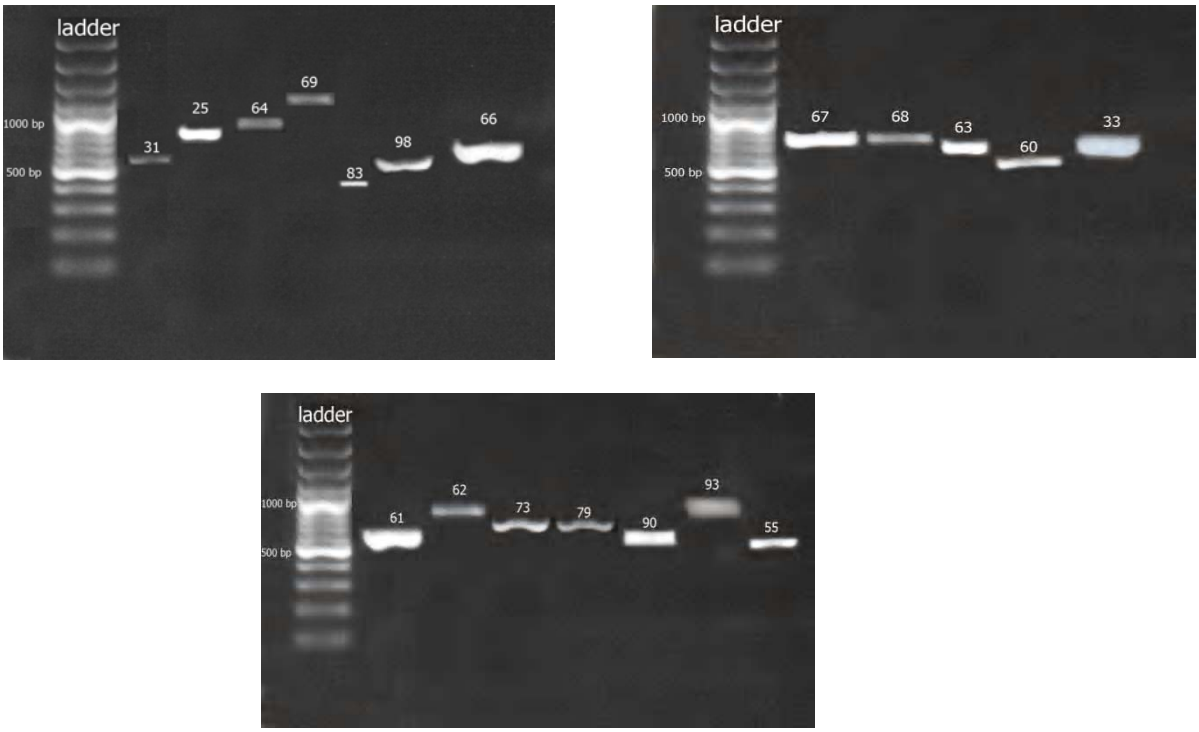

Fig.7. Fingerprinting profiles of isolated microalgae, (ISC 31) *Phormidium* sp.; (ISC25) *Leptolyngbya* sp.; (ISC 64) *Leptolyngbya* sp.; (ISC 69) *Leptolyngbya* sp.; (ISC 83) *Leptolyngbya* sp.; (ISC 98) *Chlorococcum* sp.; (ISC 66) *Chlorococcum* sp.; (ISC 67) *Leptolyngbya* sp.; (ISC 68) *Phormidium* sp.; (ISC 63) *Phormidium* sp.; (ISC 60) *Phormidium* sp.; (ISC 33) *Phormidium* sp.; (ISC 61) *Chlorococcum* sp.; (ISC 62) *Nostoc* sp.; (ISC 73) *Scenedesmus* sp.,; (ISC 79) *Scenedesmus* sp.; (ISC 90) *Nostoc* sp.; (ISC 93) *Anabaena* sp.; (ISC 55) *Anabaena* sp

a significant increase.Fig.8.b shows these changes in Masil number 2. In this station, minimum content of TPH is seen in autumn but growth percentage has increased gradually from spring till autumn. In winter, the average of TPH is maximum but growth percent is as the same as autumn. These results revealed that specimens which are presented in this station have a high resistance to pollution. Accordingly although the TPH has the highest rate in winter but growth percent of species remain constant as in autumn.

In Siberenj (Fig.8, c), like the other stations, lower oil pollution is corresponded with higher growth percent. In winter the TPH has the maximum rate (like Masil number 2) but species have a little increase rather than the autumn. By the way we can see the highest growth percent of specimens in winter.

In Atashkade (Fig.8, d), although the TPH rate is very low rather than the polluted stations, but we can see the highest growth percent in autumn. In this station we have the same relation between TPH and growth percent it means that by decreasing the TPH content, growth percent will increase.Considering the identified specimens in this research, concluded that all of the mentioned species belong to resistant ones in environmental pollutions. By reviewing other related research, It has been obvious that some genera like *Oscillatoria*, *Leptolyngbya* and *Phormidium* have special forms that helps them to be more flexible than other species in floristic studies of polluted regions (Cerniglia *et al.* 1979; Satheesh Kumar *et al*. 2009). But some genera like *Spirogyra*, *Zygnema*, *Ulothrix*, *Cosmarium* are so sensitive and can be found in or unpolluted waters and soils.

Identified species mostly have protective systems. A kind of these systems is the presence of sheath around cells or colonies. Genera such as *Chroococcus*, *Nostoc* and *Nodularia* in our study are concluded in these forms. Microscopical examination of algal cells indicated that the crude oil lead to an increase in algal biomass, although it caused heterocysts separation from the filament of *Anabaena*. In the case of *Oscillatoria* the algal cells aggregated in clusters like a ball covered with oil (Gamila *et al*. 2002).

CONCLUSION

Traditionally, the classification of microalgae (cyanobacteria) has been based on morphological characters such as trichome width, cell size, division planes, shape and arrangement, pigmentation and the presence of characters such as gas vacuoles and a sheath (Valerio *et al.*, 2009). Beyond the considerable

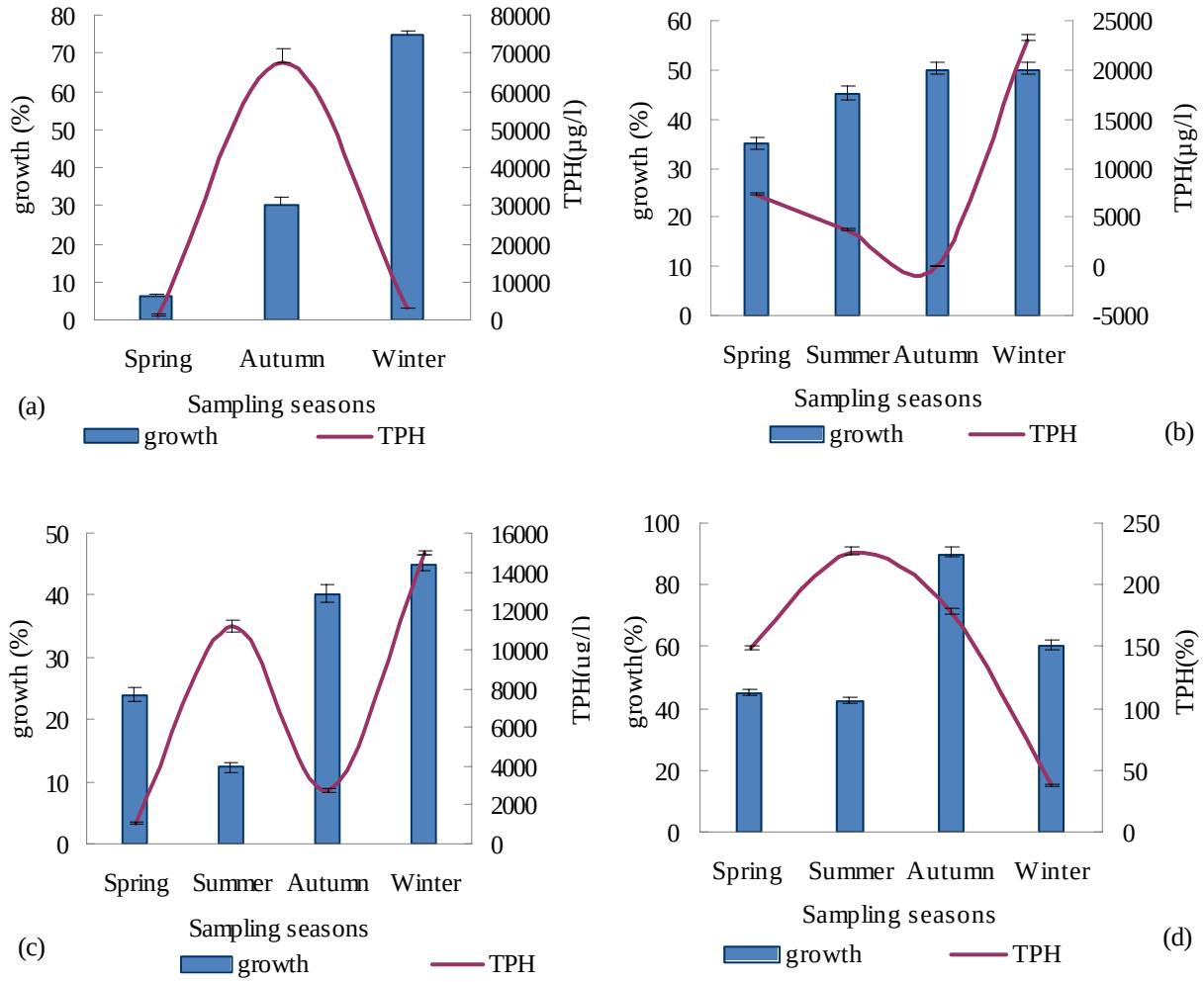

Fig. 8. Comparison between growth of specimens and TPH in different stations; a: Gas well 315, b: Masil number 2,c: Siberenj, d:Atashkade

expertise required to identify species by such characters, subjective judgment by operators can lead to errors, resulting in incorrect assignment of isolates. Moreover, some diagnostic features, such as gas vacuoles or akinetes, can show variations with different environmental or growth conditions and even be lost during cultivation. Such limitations of phenotypic characters have highlighted the requirement for more reliable methods and promoted molecular approaches in microalgae and cyanobacterial taxonomy, including DNA base composition (Kaneko *et al.*, 2001), DNA hybridization (Kondo *et al.*, 2000), gene sequencing (Nübel *et al.*, 1997) and PCR fingerprinting (Rasmussen and Svenning, 1998; Versalovic *et al.*, 1991). In this way, molecular phylogeny has become a powerful tool in elucidating evolutionary pattern. Our analysis based on 16S rRNA sequences indicate that filamentous cyanobacterial without heterocyst (Oscillatoriales), filamentous cyanobacteria that have heterocystes (Nostocales) and true-branching filaments (Stigonematales) are phylogenetically coherent. These findings are based on other researches on diversity of cyanobacteria (Tomitani *et al.*, 2006).

By reviewing other related research it is obvious that microbial biomass, and microalgae declined in high level polluted medium, whereas low level of TPH pollution showed no effect on microbial biomass. These findings are in agreement with Megharaj *et al.* (2000). Among the identification studies of species, *Phormidium* sp. is the most frequent species that is present in various polluted and even unpolluted stations. Totally changes in soil algal community can be used to identify potential environmental hazards at polluted sites and may be useful to establish guideline for soil quality. In view of the limited knowledge on exposure and toxicity of pollutants to microorganisms in terrestrial environments, the soil algal tests assume special importance.

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