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# Effects of Filamentous Cyanobacterial Allelopathy on Phytoplankton Community in Miankaleh Wetland, North of Iran

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**Abstract**

This study investigated the effects of monocultures of three dominant cyanobacterial species *Nostoc spongiaeforme* C. Agardh ex Bornet & Flahault, *Anabaena vaginicola* F.E. Fritsch & Rich and *oscillatoria limosa* C. Agardh ex Gomont on a brackish water phytoplankton community in Miankaleh international wetland and peninsula, north of Iran. We compared the chlorophyll a concentration and cell numbers after treatment. As a result, among three cyanobacterial species, *N. spongiaeforme* had most allelopathic effects on phytoplankton community whereas *O. limosa* and *A. vaginicola* showed no noticeable effects. The effects of *N. spongiaeforme* on phytoplankton community could be divided into two types: Strong negative effects and positive effects.

**Keywords:** Allelopathy, Filamentous Cyanobacteria, Miankaleh Peninsula and Wetland.

## Introduction

Allelopathy is the release of organic and chemical compounds by plants, algae and bacteria that affect other organisms (Rice, 1984). Allelopathic interactions are widespread between all groups of algae (Gross, 2003). In aquatic ecosystems, allelopathy plays an important process that influence the shaping and structure of communities, plankton succession, competition and bloom formation (Leflaive and Ten-Hage, 2007). By two reasons allelopathic activity of phytoplankton and cyanobacteria is attractive. First, allelopathy is one of the factors promoting algal and cyanobacterial blooms in marine and freshwater ecosystem. Second, allelopathy is as biological agent that might be able to control harmful cyanobacterial blooms (HCBs) (Rudriguez, et al., 2007). From 800 different secondary metabolites originated from cyanobacteria only some compounds can be classified as allelochemicals. These chemicals include cyclic and non-cyclic peptides, polyketides, alkaloids, phenols and chlorinated aromatic compounds (Leao et al., 2009). Cyanobacterin, produced by *Scytonema hofmanni* inhibited the growth of cyanobacteria, algae and plants (Mason et al., 1982). Microcystins (MCs) belong to a family of cyclic heptapeptides produced by some strains of

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planktonic cyanobacteria such as *Microcystis, Anabaena, Oscillatoria* and *Nostoc* (Blanc et al., 2005). MCs have negative effects on phytoplankton, zooplankton and macrophytes (Blanc et al., 2005). *Nostoc spongiaeforme* produces a violet pigment named nostocine A, which inhibits a broad range of organisms from bacteria to animal cells (Hirata et al., 2003). A specific metabolite isolated from *Nostoc spongiaeforme* strain shows toxic and antibacterial properties (Leao et al., 2012). Bloor and England (1989) reported that a broad range of antimicrobial antibiotic is produced by *Nostoc muscorum* C.Agardh ex Bornet & Flahault, (Lancashire Polytechnic Culture Collection 23) during the post-exponential phase of growth. The secondary metabolite fischerellin, named after its source of isolation the cyanobacterium *Fischerella muscicola* Gomont, strongly inhibited the electron flow in photosystem II in cyanobacteria (Gantar et al., 2008). Recently, LEGE 05292 allelopathic compound has been isolated from *Oscillatoria* sp. This compound belongs to a new family of compounds named the portoamides A-D. LEGE 05292 inhibited the growth of *Chlorella vulgaris* Beijerinck (Leao et al., 2012).

The primary aim in this study was the assessment of the interactions between individual cultured strains of cyanobacteria and phytoplankton that were isolated from the Miankaleh wetland and peninsula. For this purpose, we examined the potential allelopathic of dominant Cyanophytes from the Miankaleh peninsula in microplanktonic community. Based on previous study, cyanobacteria like *Nostoc* spp., *Oscillatoria* spp. and *Anabaena* spp. have relatively higher growth rates than other species on the peninsula in last summer and early fall (Masoudi et al., 2012).

## Materials and Methods

### *Site Study*

The Miankaleh international wetland (Gorgan bay) is located at 36° 48′ to 36° 55′ N and 53° 25′ to 54° 02′ E, in the southeast of Caspian Sea in the north of Iran. It is almost entirely cut off from the open sea by the 60km long Miankaleh peninsula, a low lying sandy peninsula. The entire area of Miankaleh peninsula and wetland was designated as a protected region in May 1970 and was designated as a UNESCO Biosphere Reserve in June 1976. Its maximum water depth is 4.5 meters. Its average rainfall and temperature are 580mm and 21.8°C, respectively (Masoudi et al., 2012). Miankaleh wetland has a muddy bottom and is an oligotrophic ecosystem (Ramezannejad Ghadi, 2008).

### *Collection and Analysis of Phytoplankton Samples*

A natural phytoplankton community was collected from Miankaleh wetland and peninsula, in August 2010. The phytoplanktons were collected from 0.5 to 1.5m depth using a bottle. Water temperature and salinity during sampling were 17°C and 13ppm, respectively. Some of the samples were fixed with 4% formaldehyde for microscopic identification. All algae, except Bacillario-

phyta, were examined on temporary slides. Diatoms were cleaned using the Patrick and Reimer method (1975). Oxidation by hydrogen peroxide and potassium dichromate was carried out. Identification of algae was done using an Olympus (BH-2) microscope at different ×400 and ×1000 magnifications. Taxonomic identification was made according to Patrick and Reimer, 1975; Desikachary, 1987- 1988; Dillard, 1999; Hartley, 1996; Wehr and Sheat, 2002; John et al., 2002; Baker and Fabbro, 2002; Cronberg and Annadotter, 2006; Prescott, 1970.

*Cyanobacterial Culture*

Three cyanobacterial species were selected for examination of their effects on the natural phytoplankton community. The species of *Nostoc spongiaeforme, Oscillatoria limosa* and *Anabaena vaginicola,* were purified by method of alternate algal culture in Biology Laboratory of University of Shahid Beheshti, Tehran, Iran. All cyanobacterial species were grown in BG-11 medium at condition of 23°C, 2000 lux under a light/ dark cycle of 12:12 h, and continuously supplied with air. The culture medium was prepared from filtered water of Miankaleh wetland and peninsula (Whatman GF/C). The cultures used in this experiment were maintained in exponential growth.

*Measurements*

Effects of three cyanobacterial species have been tested by exposing the natural phytoplankton community to cell-free filtrates of the cyanobacterial cultures. Cell–free filtrates were obtained by gently filtering of cyanobacterial cultures through Whatman GF/C filters. 150ml freshly prepared filtrates were added to triplicate l Erlenmeyer flasks containing 350ml of the natural phytoplankton community. Triplicate controls were prepared by adding 150ml of BG-11 medium instead of filtrate from the phytoplankton community. Nitrate and phosphate concentration in the control medium were similar and close to nutrient concentration in all cyanobacterial filtrates (134µmol $NO^{3-}$ and 120µmol $PO_4^{-3}$). Afterwards, the bottles were incubated at 23°C, 2000 lux and 12:12 hour light/ dark cycle. The effects of the cyanobacterial filtrates on the natural phytoplankton community were assessed by daily measurements of chlorophyll-*a* concentration as well as phytoplankton cell counts at the beginning and end of the experiment (120h) (Suikkanen et al., 2005).

*Phytoplankton Enumeration*

Number of filamentous species was enumerated as total filament length per ml as the sum of the extension of each filament within a counting grid placed in the ocular of the microscope (Chorus and Bartram, 1999). The number of unicellular species was estimated by Neubauer Haemocytometer (Lobban et al., 1988).

*Measurement of Chlorophyll-a Concentration*

Chlorophyll-*a* concentration was measured using the spectrophotometric determination method (Marker, 1972). 1ml of algae suspension was ground in a mortar with 96% methanol and the extract was stored in the dark at 4°C for 24 h. The extract was centrifuged using the Hermle Z323K mod-

el centrifuge at 14000 rpm for 5 minutes. The absorbance of supernatant were read at 665nm on UV-2100 spectrophotometer. Following formula was used for calculation of chlorophyll *a*.

$C_{chl} = 13.1 \times OD665$

*Contamination test*

To check the bacterial and fungal contaminations in cultures, we used PDA and nutrient agar mediums to optimize growth condition of fungi and bacteria, respectively. After adding algal suspension, cultures were transferred to a 37°C incubator for 96 hours.

*Statistical analysis*

A one-way ANOVA was applied to find out whether there was a difference between the cell number of each phytoplankton taxon and chlorophyll a concentration treated with the three cyanobacterial filtrates and the control. ANOVA was performed using the software SSPS, version 11.5 for windows. The post hoc test used for this study was Tuky (Spss, 2002).

**Results**

The species of *Nostoc spongiaeforme, Oscillatoria limosa* and *Anabaena vaginicola* were purified by method of alternate algal culture. The cultures had no contaminant in the culture after 120 h. The phytoplankton community was composed of 16 species of cyanobacteria, Diatoms and Chlorophytes. All major groups present at the beginning of the experiment attended at the final experiment in all treatments (Table 1).

After 120 hours exposure of natural phytoplankton community with cell-free filtrates, the chlorophyll a concentration increased in the cyanobacterial filtrates and control treatment (Fig. 1). The growth rate of the phytoplankton community in the *N. spongiaeforme* filtrate was less than the control and other treatments (t-test, $p < 0.05$). The chlorophyll a concentration of the phytoplankton community in the *N. spongiaeforme* filtrate was significantly less than the controls (t-test, $p < 0.05$). Also the growth rate of the phytoplankton community in *A. vaginicola* and *O. limosa* filtrates were higher than the control, but it is not noticeable.

The effects of cyanobacterial filtrate additions on the different phytoplankton taxa, compared with the control, are listed in Table 1. In several cases, treatments with cyanobacterial filtrates stimulated the growth rate of other phytoplankton taxa. The numbers of *Cylindrospermum indicum* in the *N. spongiaeforme* and *O. limosa* treatments was significantly higher than in the control (Tuky's HSD, $p < 0.05$) (Fig. 1E). *N. spongiaeforme* filtrate significantly increased the number of *Monoraphidium minitum* (Tukey's HSD, $p < 0.05$) (Fig. 1F). The addition of *Nostoc spongiaeforme* filtrate significantly was decreased the numbers of *Merismopedia elegans* from colonial cyanobacter; *Scenedesmus opoliensis*, *Chlorella volgaris*, and *Monactinus simplex* from Chlorophyta and *Navicula causpidata* from Diatoms (Tuky's HSD, $p < 0.05$) (Table 1). Also, Figure 2 shows significant difference between the number of some species in control and *N. spongiaeforme* filtrate after 120h. It is observed that the abundance of *Monactinus simplex*, *S. opoliensis*,

*N. causpidata, Chlorella vulgaris*, in *Nostoc spongiaeforme* filtrate decreased rather than control, while the number of *Cylindrospermum indicum* and *Monoraphidium minitum* and *Nostoc spongiaeforme* increased in *Nostoc spongiaeforme* filtrate rather than control. In addition, Figure 4 shows different stages of life of *Monactinus simplex* and *S. opoliensis* after exposure to *N. spongiaeforme* filtrate and control. We observed that *N. spongiaeforme* devasted the cells of *Monactinus simplex* and *S. opoliensis*, when they were exposed to *N. spongiaeforme*, while the number of *Monactinus simplex* and *S. opoliensis* in control gradually raised (Fig. 2a, b). These results indicate that the addition of *N. spongiaeforme* filtrate affected algal species interactions.

## Discussion

**Table 1.** Statistically significant ($p < 0.05$) effects of Cyanobacterial filtrates on phytoplankton Species, compared with the control, as determined by one-way ANOVA (+: stimulatory; –: inhibitory; 0: no significant effects).

| Samples | *A. vaginicola* | *O. limosa* | *N. spongiaeforme* |
|---|---|---|---|
| **Colonial Cyanobacteria** | | | |
| *Merismopedia elegans* A. Braun ex Kützing | 0 | 0 | - |
| *Chroococcus minor* (Kützing) Nageli | 0 | 0 | 0 |
| **Filamenthus Cyanobacteria** | | | |
| *Cylindrospermum indicum* C. B. Rao | 0 | + | + |
| *Calothrix ghose*i Bharadwaja | 0 | 0 | 0 |
| *Anabaena vaginicol*a F. E. Fritsch & Rich | + | 0 | 0 |
| *Nostoc spongiaeform*e C. Agardh ex Bornet & Flahault | 0 | 0 | + |
| *Oscillatoria limos*a C.Agardh ex Gomont | 0 | + | 0 |
| **Chlorophyta** | | | |
| *Tetraedron minimum* (A. Braun) Hansgirg | 0 | 0 | 0 |
| *Scenedesmus opoliensi*s P. G. Richter | 0 | 0 | - |
| *Monoraphidium minitum* (Nageli) Komarkova- Legnerova | 0 | 0 | + |
| *Ankistrodesmus falcatu*s (Corda) Ralfs | 0 | 0 | 0 |
| *Chlorella vulgari*s Beijerinck | 0 | 0 | 0 |
| *Monactinus simplex* (Meyen) Corda | 0 | 0 | - |
| *Characium acuminatum* Braun | 0 | 0 | 0 |
| **Bacillariophyta** | | | |
| *Fragilaria crotonensi*s Kitton | 0 | 0 | 0 |
| *Navicula causpidata* Kützing | 0 | 0 | - |

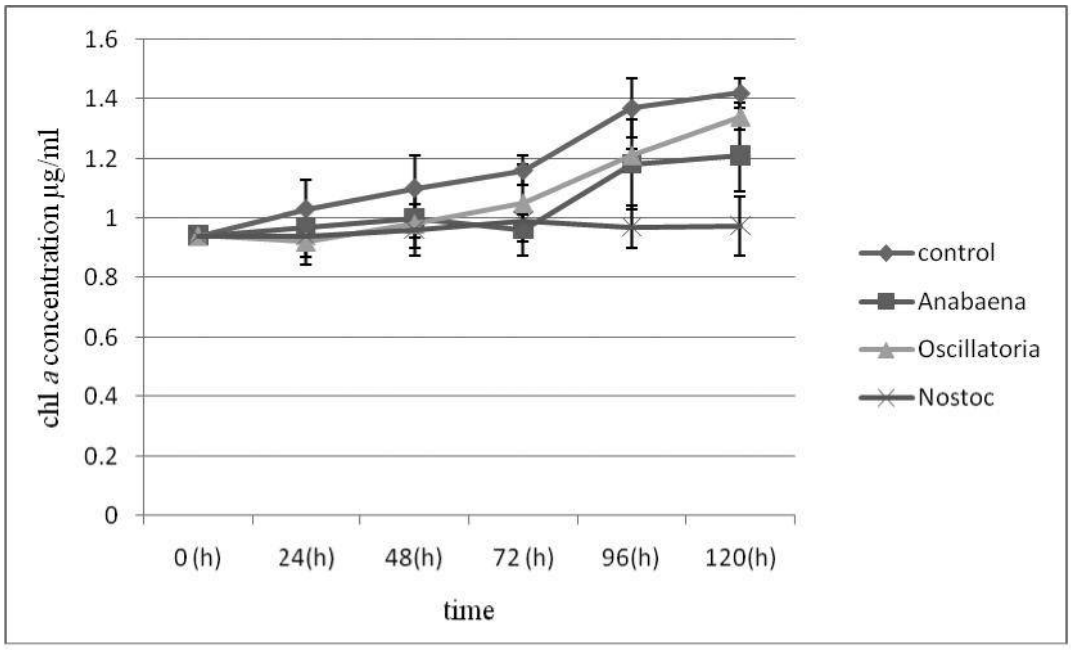


**Fig. 1.** Chlorophyll *a* concentration (µg/chl *a* l $^{1}$) of the phytoplankton community, treated with cell-free filtrates of *N. spongiaeforme*, *A. vaginicola* and *O. limosa*, as well as the control medium.

The concept of allelopathy is generally accepted among scientists as an ecophysiological process but, since the mechanism is difficult to demonstrate in the field, its importance on the ecosystem scale is still debated (Rodriguez et al., 2007). But some studies on cyanophytes explain this process in vitro (Fistarol et al., 2003; Hirata et al., 2003; Fistarol et al., 2004; Suikkanen et al., 2004; Suikkanen et al., 2005; Suikkanen et al., 2006; Zak et al., 2011). It has been proposed that changes in phytoplankton community structure are caused by the differential effect of allelochemicals on different targets. Target species may be completely eliminated, resistant to the allelochemicals or stimulated by them (Suikkanen et al., 2005).

Our results indicate that among three cyanobacterial species, *N. spongiaeforme* had the most allelopathic effects on phytoplankton community, whereas *O. limosa* and *A. vaginicola* had no important effect. *N. spongiaeforme* could both stimulate and inhibit natural phytoplankton growth, depending on the effected species. Generally, the effects of *N. spongiaeforme* on the natural phytoplankton community could be eithre positive or negative. Positive effect: whereby the target species have a growth rate higher than control (for example cyanobacterium *C. indicum* and *M. minitum* from Chlorophyte). A few researchers have reported examples of stimulatory effects (Keating, 1977; Mohamed, 2002; Suikkanen et al., 2005). Suikkanen et al. (2005) indicated tested cyanobacterial filtrates tended to stimulate instead of inhibit the growth of natural phytoplankton species. The stimulatory effects may be due to an indirect effect related to a decrease in competition or compounds present in the cyano-

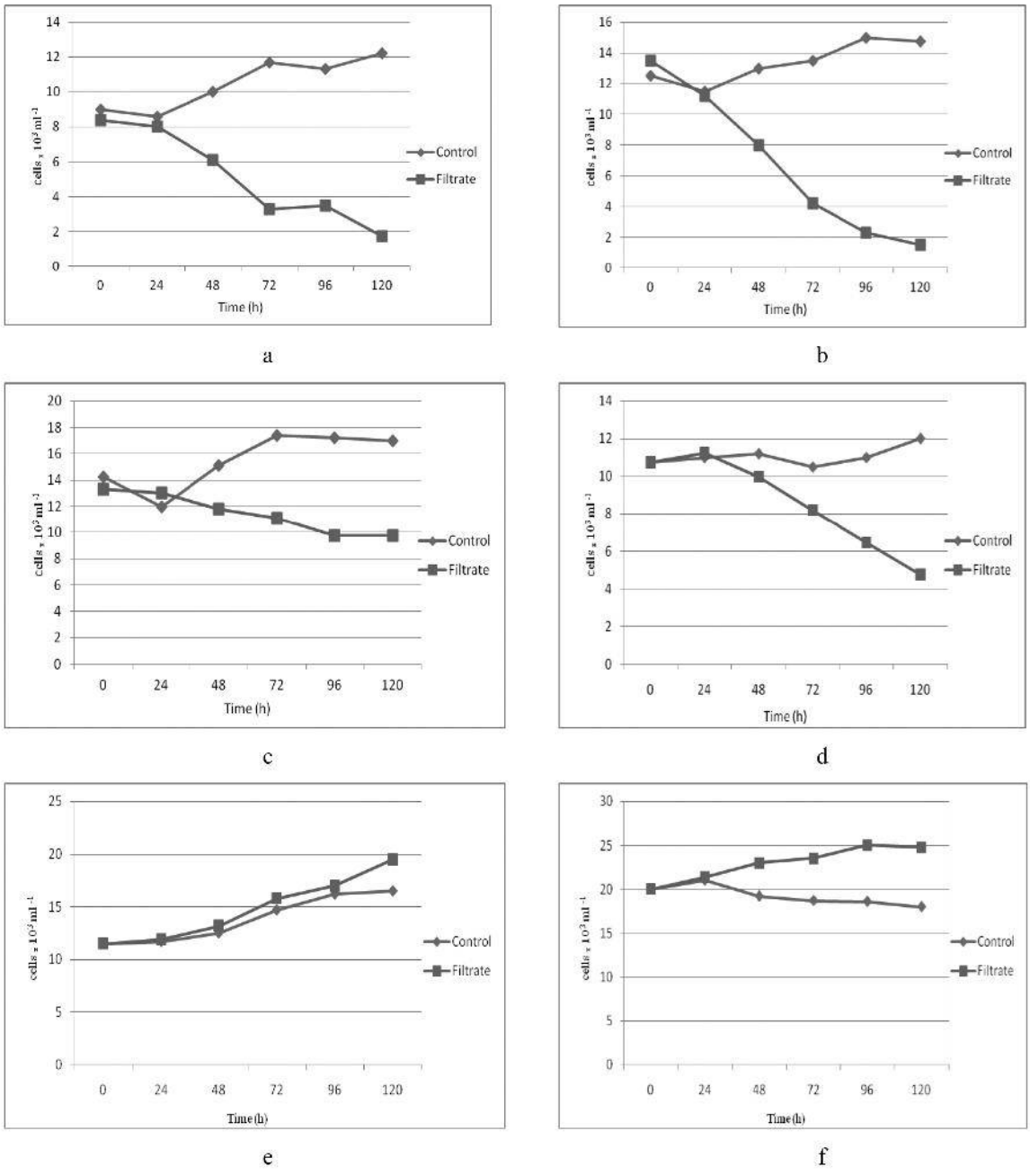


**Fig. 2.** Daily change of species in control and in exposing cell-free filtrate of *Nostoc spongiaeforme*. a) *Monactinus simplex*, b) *Scenedesmus opoliensis*, c) *Navicula causpidata* d) *Chlorella volgaris*, e) *Cylindrospermum indicum*, f) *Monoraphidium minitum*.

bacterial filtrate that may have proven useful to the other cyanobacteria because they may share the same primary metabolism (Suikkanen et al., 2005). Sharif et al. (2008) suggested that some cyanobacteria released quorum- sensing compounds to environment and compounds accelerated the growth of some species.

Negative effects: this led to a decrease in number and biomass of the affected phytoplankton species (for examples from chlorophytes: *S. opoliensis, C. vulgaris, Monactinus simplex*; cyanobacterium: *M. elegans* and a diatom: *N. causpidata*). When allelopathic algae caused strong negative effects, most of the phytoplankton species died and the phy-

toplankton community declined. Legrand et al. (2003) reported some allelochemicals were capable of inhibiting many essential functions of organisms. Hirata et al. (2003) indicated that *N. spongiaeforme* TISTR 8169, synthesizes and releases a violet pigment, nostocine A, into medium. The bioactivities of nostocine A on various organisms has been examined and were determined that the growth rate of some green algae (*Chlorella pyrenoidosa, Chlorella fusca, Dunaliella salina* and *Dunaliella tertiolecta*) and cyanobacteria *(Anabaena cylindrica and Nostoc commune*) were inhibited by Nostocine A. One of the *Nostoc* species produces an anti cyanobacterial named nostocyclamide which inhibits the growth of some of the cyanobacteria and Chlorophytes such as *Scenedesmus, Ankistodesmus* and *Nannochloris* (Smith and Doan, 1999). According to Hirata et al. (2003), growth inhibition by *Nostoc spongiaeforme* tends to be stronger towards green algae than towards cyanobacteria. But the reason why Chlorophytes inhibited more phytoplankton than other groups in the community remains unclear. One explanation for this might be the weaker adaptation of Chlorophytes to cyanobacterial compounds in comparison to others. Based on previous studies (Masoudi et al., 2012; Ramezannejad Ghadi, 2008), abundance of Chlorophytes in late summer and fall was lower, while at the same time abundance of cyanophytes, especially *Nostoc spongiaeforme,* increased. This is due to: some reasons (1) thermal condition, (2) superior light-capturing abilities and (3) nutrition limitation (Lee, 2008).

There are some studies on allelopathy in aquatic systems dealing with nutrient limitation (Johansson and Graneli, 1999; Graneli and Johansson, 2003; Fistarol et al., 2005). Under stress conditions (nutrition conditions) the allelopathic effect may be higher due to both the increase in the production of allelochemicals and in the sensitivity of the target species, increasing the competitive advantages of allelopathic algae (Graneli et al., 2008). It has been shown that cell-free filtrates of P-deficient cultures of *Prymnesium parvum* have a strong negative effect on other phytoplankton than non-P-deficient cultures (Graneli et al., 2008). In another study, the cyanobacterium *Cylindrospermopsis raciborskii* was suggested as an allelopathic species, but it was shown that when cultures of *C. raciborskii* grown under nutrient- replete condition did not have inhibitory effect on the target algae (Graneli et al., 2008). Therefore, nutrient- limiting conditions may increase allelopathic effects by making the target more susceptive to allelopathic compounds. However, the investigated studies do not explain how the allelopathic effects have been influenced by external nutrient conditions. But since the water of the Miankaleh wetland and peninsula is mainly phosphate-poor in fall rather than summer and spring (Laloie et al., 1993), it is possible that phosphate deficiency is an effective factor on allelopathic activity of *N. spongiaeforme*. Under the influence of phosphate limitation, *N. spongiaeforme* increases its own competitive strength by producing allelochemicals.

The important question that remains is

whether or not there is lytic activity among the extracellular allelochemicals of *Nostoc spongiaeforme,* and whether they act as a significant ecological factor for blooming in this area. Although knowledge is not completed because of the lack of information on the chemical nature and ecosystems complexity, allelopathy may be a successful strategy for phytoplankton species that occur in dense blooms (Rudriguez et al., 2007). A species that produces allelopathic compounds would have an advantage over its competitors (Wolfe, 2000). Thus, allelopathy, resulting in increased competition, could be an important factor in HCBs (Kearns and Hunter, 2001; Vardi et al., 2002). Oberhaus et al. (2008) suggested that allelopathy could have little effect on competition dynamics at the onset of a cyanobacterial bloom but with increasing biomass; it could have a greater influence on bloom maintenance due to production allelochemicals. Juan et al. (2010) reported that with low cell density of *A. tamarense* the allelopathic behavior of *A. tamarense* was weak. When the cell density of *A. tamarense* was high, the allelopathic effect got strong. Generally, since aquatic ecosystems are complex, definitive evidence for the role of allelopathy in the HCBs is an unclear topic. But according to our laboratory experiments and field observations, allelopathy suggests that *N. spongiaeforme* might provide competitive advantages on phytoplankton community by presence of allelochemical. The allelopathic compounds of *N. spongiaeforme* may not only reduce nutrient competition by eliminating competitors from community, but also increase the nutrient availability in the plankton community due to the lysis of microorganisms. As a result, *N. spongiaeforme* keeps the biomass of the other groups at a low level (organisms had a lower growth rate), while its own growth rate increases. Increasing of biomass *N. spongiaeforme,* due to suitable nutritional and environmental (temperature, pH) conditions, leads to more producing allelochmicals and finally forms spots by 3-7 meters in diameter in some parts of wetland in early fall (Fig. 3).

The mode of action on the target cells has seldom been described. For example, in several cyanobacterial species, allelochemicals can cause lysis, blistering and damaging to photosynthesize and plasma membrane (Ehlert and Juttner, 1997). But usually the lethal effects of allelochemicals involves the lysis of the target species (Graneli and Johansson, 2003). Figure 4 shows different phases of life of alga after exposing to *N. spongiaeforme* filtrate. These figures show how the cells change: in first they start to lose pigments, then cytoplasm and organelles aggregate, and finally lyses happens. Hirata et al. (2003) suggested that *N. Spongiaeforme* causes oxidative stress by acceleration of ROS generation such $O_2^-$.

Allelopathy in aquatic and marine environments is a new and exciting discipline that has emerged over recent years. Mechanistic studies are needed in order to clarify the complex relationships among between organisms in marine environments that help maintain, biodiversity and to increase our basic knowledge about allelopathy and nat-

**Fig. 3.** Algal bloom came from *Nostoc spongiaeforme* on the Miankale peninsula in early fall.

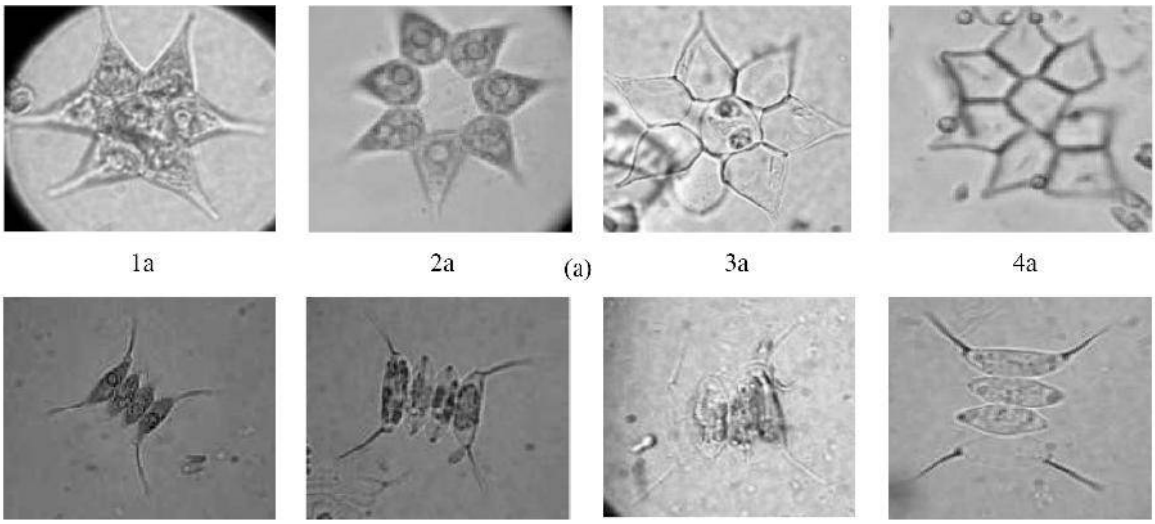


**Fig. 4.** The figures show how the cells change and the cell lysis. Different stages of cell's life of *Monactinus simplex* (a) and *Scenedesmus opoliensis* (b) exposed to *N. spongiaeforme* filtrate. First picture shows a cell that has not been exposed to filtrate. Pictures 2, 3 and 4 were after 2, 4 and 5 days, respectively. The pictures are from different cells.

ural adaptation to changing physical, chemical and biological factors in marine environment.

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# Effects of *Sargassum ilicifolium* (Sargassaceae, Phaeophyceae) Meal on Physico- Chemical Formulated Shrimp (*Litopenaeus vannamei*) Feed

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**Abstract**

Almost 309 species and infraspecific taxa of macroalgae including 78 Chlorophyta (within 15 families), 70 Ochrophyta (Phaeophyceae; within 7 families) are and 161 Rhodophyta (within 30 families) listed in coastal line of the Persian Gulf and iranian water of Oman Sea. Among them, *Sargassum ilicifolium,* a dominant brown seaweed, which was used as a part of aquatic animal feed in order to investigate its effect on physico chemical formulated feed. Two isocaloric diets (336 kcal metabolizable energy /100g diet) containing 33% crude protein, with and without inclusion of brown seaweed *S. ilicifilium* (0 and 15, replaced on protein resources of shrimp diet) were used. Seaweed supplement in formulated feed not only improved the humidity absorbance (110.20%±5.00%) and stability of pellet feed in seawater (98.11±3.23%) compared to the control but also acted as the best binder and increased the periods of leaching pellet (5.20±0.50) when they were dropped in seawater. It can be replaced instead of vitamin and mineral premixes when the feed is enriched by vitamin pyridoxine (6.4 mg.$100^{-1}$ seaweed DW) and minerals cobalt (0.06 mg.$100^{-1}$ seaweed DW) and zinc (1.1 mg.$100^{-1}$ seaweed DW).

**Keywords**: Physico- Chemical Characteristic Feed, Western White Leg Shrimp, *Sargassum ilicifolium*.

## Introduction

In recent decades, aquaculture activities affected by using algae as a part of aquatic animal diet not only change the physico-chemical properties of formulated feed, but also improve the quality and quantity of fish or shrimp fed with. Effect of dietary algae and adequate levels will probably vary with the species of both algae and fish or shrimp. During 1990 to 2010, more than 309 macroalgae species were described in Oman Sea and the Persian Gulf, IRAN (Gharanjic et al., 2010). Micro and macro algae, seaweed and aquatic plants with relatively high nutritional value and high production rate,act as a new dietary resources for cultured aquatic animals, specifically fish and shrimp (Nakagawa and Montgomery, 2007). Substitution of protein resources in fish and shrimp diets by algae has additional benefits for feed

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such as taste and binder improvement, flesh consistency, additional Omega 3 fatty acid content, growth rate promoting due to better digestibility caused on high mineral and vitamin consist (Hafezieh et al., 2017). Aquaculture seaweeds productivity in 2005 was more than 16.09 million tons (wet weight), (FAO, 2009) and because of their bioactive compounds, they produced different secondary metabolites which had biological activities even against aquatic animal pathogens and even human pathogens (Mahasneh et al., 1995; De Val et al., 2001; Liao et al., 2003). Nakagawa (1985) reported that the addition of small amounts of algae to fish and shrimp diet not only evaluated growth and survival rates, but also produced physiological improvement, vitality, disease resistance, desired body composition and carcass quality of fish and shrimp (Hamauzu and Yamanaka, 1997). Different algae composition reports revealed that algae and seaweed contain all essential amino acids (Hafezieh et al., 2017; Behairy and EL-Sayed, 1983; Qasim, 1991; FAO/WHO, 1991), nevertheless, Wong and Cheung (2000) reported that *Ulva lactuca* L. has not tryptophan amino acid. Fujiwara-Arasaki et al. (1984) analyzed 10–30% of amino acid in dry weight and showed they are rich in vitamins A, B1, B2, B6, B12, C and niacin. Fleurence (1999) measured 10-26% crude protein content in dry weight *Ulva* sp. and 47% in red seaweed, in order to be used as functional food and accelerator of nutrient absorption. They can also be replaced by animal protein and plant resources in fish and shrimp diet easily (Yone et al., 1986; Wong and Cheung, 2000). Moreover, Mustafa et al. (1994; 1995a, b) showed significant growth and feed utilization increase in fish *Pagrus majory* when they where fed only by small amount of algal powder in diets. Nakagawa et al. (1993) revealed optimum feed and protein in *Aconthopagrus schlegelii* (Bleeker) when they where supplemented by *Ulva* sp. Also, Mabeau and Fleurence (1993) reported higher content of the important minerals, calcium and iron in some seaweeds compared with vegetables and fruits. On the other hand, Basemir et al. (2004) and Nakagawa and Montgomery (2007) showed wide variety of EFA in lipids of different algae and seaweeds such as HUFA, PUFA specifically DHA and EPA which are the most important nutrients for neural function and health. Therefore, the objective of this work was to study the effect of seaweed (*S. ilicifolium*) supplemented to shrimp diet on physico- chemical properties of the pellet.

**Materials and Methods**

This experiment was carried out at Off-Shore Research Center- Chabahar, IRAN. The prepared seaweed (*Sargassum ilicifolium* (Turner) (C.Agardh) was collected from nature along the coastal line of Tis, a village near Chabahar, Sistan and Baluchistan province, IRAN (25°21′36”, 60°36′26”). Rinsed, dried and powdered seaweed was measured for nutritional values in laboratory, based on standard method for statistical survey and was replaced by protein resources (fish, bone and meat meals) of shrimp

**Table 1.** Composition (gram per 100g DW feed) and proximate analysis of diets without (control) and with (treatment) seaweed *S. ilicifolium* used in this experiment.

| | Control | Treatment |
|---|---|---|
| *Sargassum* sp. meal | 0 | 15 |
| Fish meal | 40 | 21 |
| Fish Oil | 10 | 10 |
| Bone and meat meal | 7 | 7 |
| Soybean meal | 7 | 20 |
| Seaweed powder (*S. ilicifolium*) | 0 | 15 |
| Yellow corn | 8 | 7 |
| Wheat flour | 20 | 12 |
| Starch powder | 7 | 7 |
| Vitamins and minerals mixture | 0.3 | 0 |
| Iodized Salt | 0.5 | 0 |
| Moisture | 7.08 | 7.09 |
| Crude protein | 33.22 | 32.02 |
| M.E.*(kcal. kg) | 3555 | 3564 |
| NFE | 20.57 | 21.21 |
| Ash | 8.11 | 8.82 |

Fish meal (CP=54.06%, DM=92.89, EE=15.3, Fiber= 1.51, Ash= 22.92, M. E= 3335 kcal/kg); Bone and meat meals (CP=40.6%, DM=91.00, EE=16, Fiber= 1.51, Ash= 36.6, M. E= 2920 kcal/kg); Soybean meal (CP=44.84%, DM=88.22, EE=1.74, Fiber= 5.57, Ash= 5.73, M. E= 3005 kcal/kg); Seaweed (CP=9.18%, DM=82.89, EE=1.2, Fiber= 33.11, Ash= 11.11, M. E= 2301 kcal/kg); Yellow corn (CP=8.67%, DM=87.45, EE=3.84, Fiber= 2.17, Ash= 1.18, M. E= 3110 kcal/kg); Wheat flour (CP=16.67%, DM=87.74, EE=3.13, Fiber= 8.12, Ash= 4.57, M. E= 2930kcal/kg); Powdered Starch (CP=5.84%, DM=85.84, EE=0.55, Fiber= 13.83, Ash= 1.55, M. E= 2771 kcal/kg); Vitamin A, 4.8 MIU; Vitamin D, 0.8 MIU; Vitamin E, 4.0 g; Vitamin K, 0.8 g; Vitamin B1 , 0.4 g; Vitamin B2 , 1.6 g; Vitamin B6 , 0.6 g; Vitamin B7 , 20.0mg; Vitamin B12, 4.0g; Folic acid, 0.4 g; Nicotinic acid, 8.0g; Pantothenic acid, 4.0 g; Colin chloride, 200 g; Zinc, 22 g; Cooper, 4.0 g; Iodine, 0.4 g; Iron, 12.0 g; Manganese, 22.0 g; Selenium, 0.04 g.

*M.E= Metabolizable Energy

(*Litopenaeus vannamei*). Control diets were formulated from commercial ingredients of fish meal (wheat flour, wheat bran, soybean meal, yellow corn, bone meal, chemical binder, vitamins and minerals premixes) to achieve 33% dietary crude protein level with 336 kcal/100g. Metabolizable energy level of the diet (on dry basis) was based on feed-stuff values reported by NRC (1993) (Table 1), and the treatment diet included 15% of dried seaweed meal without using chemical binder and vitamins and mineral premixes. Dry ingredients were passed through a sieve (2mm diameter) before being mixed into the diets. Mixtures were homogenized in a food mixer. Boiling water (80°C) was then blend-

ed into the mixture at the ratio of 40% for pelleting. The diets were pelleted using meat grinder with a 2mm diameter.

*Analytical Measurements*

Analytical measurement of dried pellet diet was done using methods of AOAC (1990) to compare vitamin and mineral deficiencies of treatment diet and control. After preparing the treatment feed, samples of pellet feed were chosen for humidity absorbance, stability test and leaching time in seawater compared to control feed which was used in chemical binder (Sodium bentonite). The Student T-Test was done to compare samples.

**Results**

The results of the present study have shown that there are some deficiencies between vitamins (Pyridoxine) and minerals (Cobalt and Zinc) in the diet containing seaweed and control, which was prepared based on the amount required for shrimp (*L. vannamei*) diet. These deficiencies need to be enriched by adding to them the pure form (Table 2). Also, the physical characteristics of control and treatment pellet is shown in Table 3.

**Discussion**

several recent studies used seaweed as part of diet to improve growth performance, body composition, feed utilization and other quantitative traits of fish, such as Tilapia and western white leg shrimp which have become objects of interest for culturists and researchers throughout the world (Watanabe et al., 1990; El-Zaeem et al., 2009; El. Tawil and Amer, 2010).

Results of the work indicate that maximum percentage of *Sargassum ilicifolium* that can be replaced by protein ingredient in shrimp feed is 15%, which can act as binder agent,vitamin and mineral premixes when the final diet is enriched by pyridoxine vitamin and minerals, cobalt and zinc, based on vitamins and mineral requirements of shrimp juvenile (Ahamad Ali, 2001; Catacutan and De la Cruz, 1989; Mustafa and Nakagawa, 1995; Mustafa et al., 1994; Mustafa et al., 1995 a,b; Valente et al., 2006; Xu et al., 1993).

On the other hand, using seaweed in shrimp (*L. vannamei*) pellet diet can improve humidity absorbance (%), stability (%) and leaching time of pellet in seawater.

Previous scientists had confirmed this fact as they used different seaweed powders for shrimp and fish feed (Diler et al., 2007; Elmorshedy, 2010; Ergun et al., 2008; Fleurence, 1999; Guroy et al., 2007; Mabeau and Fleurence, 1993; Nakagawa, 1984, 2004).

It could be concluded that seaweeds (*S. ilicifolium*) can be used to enrich western white leg shrimp (*L. vannamei*) diet at optimum level of 15% to improve physico-chemical characteristics of formulated pellet. It also can be enriched by some minerals and vitamins.

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**Table 2.** Required level of vitamins and minerals in shrimp diet and enrichment amount of some deficiencies in treatment diet. (3 replication mean±sd).

| | Vitamins : mg.100 $^{-1}$ g feed DW (shrimp required) | Amount in treatment pellet | Required enrichment | | Minerals: mg.100 $^{-1}$ g feed (shrimp required) | Amount in treatment pellet | Required enrichment |
|---|---|---|---|---|---|---|---|
| Vitamin A | 150-200 | 170.90±11.11 | negative | Fe | >40 | 58.90±9.11 | negative |
| Vitamin E | 30-40 | 32.66±2.86 | negative | K | >80 | 82.66±2.81 | negative |
| Vitamin C | 100-800 | 890.20±98.04 | negative | Mn | 1-2 | 1.22±0.04 | negative |
| Thiamin | 40-50 | 45.37±6.03 | negative | Mg | >60 | 81.37±6.03 | negative |
| Riboflavin | 1-2 | 1.38±0.01 | negative | Zn | 3-4 | 2.38±0.1 | positive |
| Niacin | 10-15 | 12.04±0.09 | negative | Co | >0.1 | 0.04±0.01 | positive |
| Pyrodoxin | >10 | 0.61±0.03 | positive | Cu | >500 (µg in 100 g DW) | 700.04±11.01 | negative |
| | | | | I | >100 (µg in 100 g DW) | 140.14±12.01 | negative |

positive= needs enrichment

**Table 3.** Means ± standard error (SE) of humidity absorbance (%) and seawater stability (%) of pelleted feed

| Treatments | Humidity absorbance (%) | Seawaters stability (%) | Leaching time in seawater (h) |
|---|---|---|---|
| control | 95.20±4.03$^{a}$ | 95.20±2.73$^{a}$ | 3.12±0.33$^{a}$ |
| 15% | 110%.20±5.00$^{b}$ | 98.11±3.23$^{b}$ | 5.20±0.50$^{b}$ |

Means in each column followed by different letters are significantly different ($p< 0.05$)

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# *Spirulina* Production in Different Sources of Nitrogen

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**Abstract**

The Cyanobacteria *Spirulina* is an attractive target for its pigments, proteins, vitamins and other high-value cell components. Also, it can be easily and cheaply harvested by filtration from the cultivation medium. In this study a simple protocol was developed for *Spirulina* production by using different types of nitrogen in ammonium (urea and $(NH_4)_2SO_4$) and nitrate ($KNO_3$, $NaNO_3$) forms in combination with NPK fertilizer. Results demonstrated high amount of nitrogen in both forms inhibited *Spirulina* growth. Ammonium showed a stronger inhibitory role than nitrate in biomass production while increased phycocyanin content. Best phycocyanin content occurred in high ammonium or low nitrate concentration. In media based on 1% Urmia lake salt and 1 g/L NPK, a combination of low concentration (0.1 -0.5 g/L) of urea and $(NH4)_2SO_4$ obtained best results in biomass production. 1.2 g/L biomass during 14 days without any carbon source can be compared with Zarrouk/2 medium. This composition can be used economically for *Spirulina* production since little amount of cheap material make the possibility of *Spirulina* production.

**Keywords:** Ammonium, Fertilizer, Nitrogen, NPK, *Spirulina*

## Introduction

The current environmental conditions deteriorations, mental and physical stress, changes in the diet have been serious risk factors for the humans, increased the death rate and fatal diseases. These are the obvious reasons why new progressive trends are being extensively developed in modern medicine, pharmacology and biotechnology and more effective harmless medicaments are being sought for to treat and prevent various diseases. One of the trends in biotechnology is associated with blue green microalgae *Spirulina platensis* which have been widely employed as food and feed additives in agriculture, food industry, pharmaceuticals and perfume industry, (Saranraj and Sivasakthi, 2014).

Microalgae are a diverse group of microorganisms that have different morphological, physiological and genetic traits that have the potential to offer a variety of different biologically active metabolites like proteins, lipids, carbohydrates, carotenoids or vitamins for health, nutrient rich food and feed additives, cosmetics and for energy production (Priyadarshani and Rath, 2012).

In general, microalgae is able to provide a

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great variety of secondary metabolites, which do not happen in other organisms. The fundamental advantage of using microalgae for industrial production of valuable food ingredients depends on the fact that, for the majority of the species, cultivation is easy and growth is fast (El Baky et al., 2015).

The cyanobacteria *Spirulina* is widely commercialized as nutritional supplement for humans and as animal feed additives. *Spirulina* was approved by the Food Drug Administration (FDA) by the issuance of a generally recognized as safe (GRAS) certificate. *Spirulina* can be legally marketed as a food or food supplement without risk to human health (Costa and de Morais, 2013). It could be considered a luxury health food and a panacea for malnutrition since it is an excellent content of proteins (Colla et al., 2007), polyunsaturated fatty acids (PUFA) (Sajilata et al., 2008), pigments (Madhyastha and Vatsala, 2007), vitamins and phenolic (Ogbonda et al., 2007). Moreover, phycobiliproteins as a special group of pigments that are water-soluble occur only in cyanobacteria and Rhodophyta act as photosynthetic accessory pigments. C-phycocyanin (C-PC) is a blue pigment of phycobiliproteins with strong antioxidative and anti-inflammatory activities. Now a days, *Spirulina is* considered as the major source of phycocyanin with 20% of its dry weight (Benedetti et al., 2006). *Spirulina* is easy to culture and harvest in large- scale. Hence, these desired microalgae can be employed for commercial interest. Therefore, in this project, various types of nitrogen sources (urea, $KNO_3$, $NaNO_3$, $(NH_4)_2SO_4$) in composition with fertilizer (NPK) were studied to improve the biomass production and reduce the costs.

## Materials and methods

*Spirulina* was obtained from algae collection of research institute of applied science of ACECR, Tehran, Iran. *Spirulina* was cultivated at the Zarrouk medium and used as inoculum in the experiments. Various culture media were prepared according to various nitro-

**Table 1.** Different combination of nitrogen sources in media

| No. | Urea* | NPK* | $KNO_3$* | $NaNO_3$* |
|---|---|---|---|---|
| 1 | 1.5 | 1.0 | - | - |
| 2 | 1.5 | 2.5 | - | - |
| 3 | 1.5 | 5.0 | - | - |
| 4 | 1.5 | 1.0 | 1.0 | - |
| 5 | 1.5 | 1.0 | 2.5 | - |
| 6 | 1.5 | 1.0 | 5.0 | - |
| 7 | 1.5 | 1.0 | 1.0 | 1.0 |
| 8 | 1.5 | 1.0 | - | 1.0 |
| 9 | 1.5 | 1.0 | - | 2.5 |
| 10 | 1.5 | 1.0 | - | 5.0 |
| 11 | 1.0 | 1.0 | - | - |
| 12 | 0.5 | 1.0 | - | - |
| 13 | 0.1 | 1.0 | - | - |
| 14 | Zarrouk/2 | | | |

1-14 Different combinations on nitrogen
* g/l

gen sources $(NH_4)_2SO_4$, urea, $KNO_3$, $NaNO_3$ and NPK fertilizer (with 12:12:36 ratio) and followed the growth process for 2 weeks. In addition, C-phycocyanin content (C-PC) was evaluated on the 10th day growth (Wyman and Fay, 1986). The phycocyanin content (C-PC) was calculated according to the following equation:

C-PC= (A620 – 0.474 * A652)/ 5.34

where A is the absorbance of the substance at 620 and 652 nm.

The experiments were carried out in three steps as follow: initially, in order to select industrial culture media, we used distilled water, 1% Urmia lake salt solution and breeding fish effluent in combination with 1 g/L NPK, 0.32 g/L $KNO_3$ and 2.5 – 5.0 g/L urea. In the next step, various nitrogen sources with different concentrations were combined in 1% Urmia lake salt as follows to determine the type of nitrogen and their amounts for *Spirulina* growth. Different combination compared to find the best medium near to Zarrouk/2 medium production (Table 1).

Finally, new source of nitrogen ($(NH_4)_2SO_4$) helped urea to provide ammonium in media. Different amounts of NPK (2.5 and 0.5 g/L) in combination with urea (0.1 and 0.5 g/L) and $(NH_4)_2SO_4$ (0.5 and 2 g/L) in 10 treatments (numbers 15 to 24) were evaluated (Table 2). The concentration of 0.5 g/L of $KNO_3$ and 1% Urmia lake salt was considered in all experiments.

Experiments were carried out in 3 liters containers included 1 liter media, 29 ± 1 temperature, 8/16 photoperiod and permanent central aeration system. Zarrouk/2 medium (half of the total Zarrouk composition) was also used for all tests as an indicator to compare biomass production. For each treatment 30 ml of inoculum added to 1 L of media and the final pH adjusted to 9.2 ±0.3. The cultivation volume was 1 liter, which was maintained through the daily addition of distilled water to replace water loss by evaporation.

**Results**

*Spirulina* didn't grow in media based on distilled water and breeding fish effluent while 1% Urmia lake salt solution demonstrated *Spirulina* growth. Therefore, suitable substrate for growth of desired microalgae was considered as 1% sea salt. Among the various types of nitrogen sources in Table 1, which were mostly prepared on the basis of 1.5 g/L urea and 1 g/L NPK, the result showed a negative effect of $NaNO_3$ and $KNO_3$ on growth in different concentrations, somehow after one week, all the samples were wasted, but treatments containing different levels of NPK and urea were able to grow for up to 2 weeks (Fig. 1). The amount of urea in the media is a very important factor because a significant reduction for urea from 1.5 to 0.1 g/L has led to enhance in growth. Lower amount of NPK (with higher amount of nitrate than ammonium) increased growth; inhibition effect of nitrate observed after 10 days where 1 g/L NPK produce more biomass (1.2 g/L) than 2.5 g/L NPK (1.03 g/L). Reducing the amount of nitrogen in the environment (0.1 g/L urea, 1.0 g/L NPK) has resulted in the highest biomass production to 1.2 g/L. Although low total nitrogen levels have been

**Table 2.** Ammonium combination in media

| No. | NPK* | Urea* | $(NH_4)_2SO_4$* |
|---|---|---|---|
| 15 | 2.5 | 0.1 | 2.0 |
| 16 | 2.5 | 0.1 | 0.5 |
| 17 | 2.5 | 0.5 | 2.0 |
| 18 | 2.5 | 0.5 | 0.5 |
| 19 | 5.0 | 0.1 | 2.0 |
| 20 | 5.0 | 0.1 | 0.5 |
| 21 | 5.0 | 0.5 | 2.0 |
| 22 | 5.0 | 0.5 | 0.5 |
| 23 | 1.0 | 0.1 | 0.5 |
| 24 | | Zarrouk/2 | |

15-24 Different combination of ammonia
* g/l

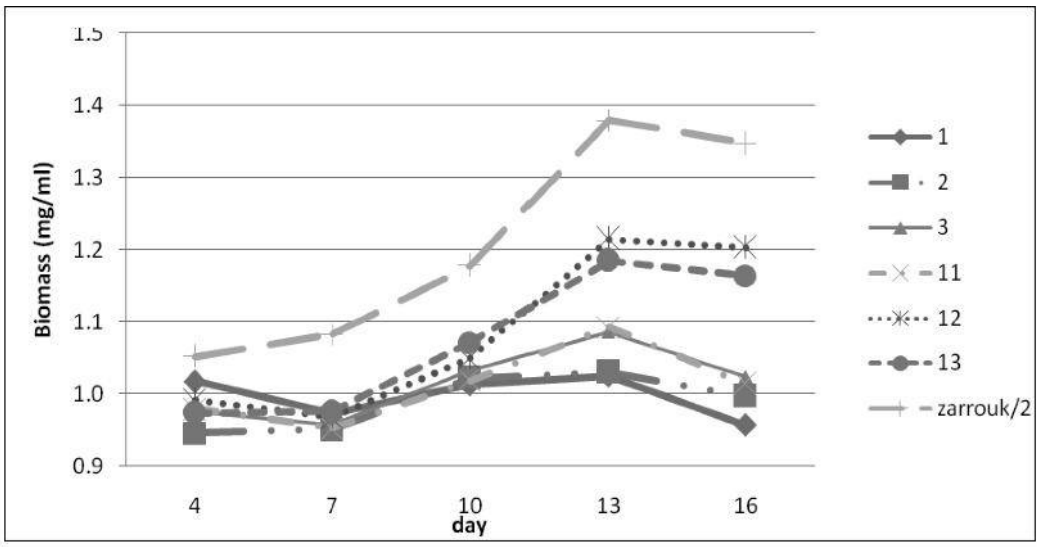


**Fig. 1.** *Spirulina* biomass production in different nitrogen sources.

able to stimulate growth as well as increase in phycocyanin levels there were some cases which along with an increase in the amount of nitrogen in the media (1.5-2.5 g/L urea or 1.0 g NPK), the production of phycocyanin also increased.

Results of the last experiment showed combination of urea and NPK could be increase the production of *Spirulina* and phycocyanin simultaneously. In the final experiment, the combination of two recent salts with $(NH_4)_2SO_4$ exhibited that the amount of nitrogen in the media played a significant role in the biomass production of *Spirulina*. The highest growth rate of microalgae occurs in the combination of 1.0 to 2.5 g/L NPK, 0.1 to 5.0 g/L urea, and 0.5 g/L $(NH4)_2SO_4$, which produced even higher biomass than Zarrouk/2 medium (Figure 2). The amount of phycocyanin in low nitrogen treatments was high and the maximum amount of phycocyanin was observed in treatment of 0.1 g/L NPK along with 0.1 g/L urea and 0.5 g/L $(NH_4)_2SO_4$.

**Discussion**

The results of the first part of the experiment showed that 1.0 g/L NPK, 0.32 g/L $KNO_3$ along with 2.5 g/L urea could be effective for *Spirulina* cultivation with 1% of Urmia lake salt solution while combination of above mixture with breeding fish effluent treatment and distilled water showed no growth. Stimulation of *Spirulina* growth in the composition of 1% salt of Urmia lake, in comparison with distilled water, due to the presence of mineral elements, even in low amounts of trace element in media but excess amount of ammonium in breeding fish effluent (Hargreaves and Tucker, 2004) severely inhibited the *Spirulina* growth.

Several investigations report inhibitory effect of ammonium in different ranges (2 mM till 15 mM) from various source of nitrogen like urea, $(NH_4)_2SO_4$, $(NH_4)Cl$, $(NH_4)NO_3$ (Costa et al., 2001; Danesi et al., 2002; Sassano et al., 2007; Rodrigues et al., 2010). The second experiment showed that in intervals less than 2.5 g/L urea (0.1 to 1.5 g/L), better growth could be seen but not more than Zarrouk/2. Zarrouk/2 contains nitrogen (1.25 g/L sodium nitrate) and 8g/L $NaHCO_3$ while we didn't include any carbon source except air aeration in our treatments. So the biomass production in our media (1 g/L NPK, 0.1 g/L urea and 0.5 g/L $(NH_4)_2SO_4$) can be considerable.

In the third experiment, increasing the nitrate concentration (NPK) up to 5.0 g/L inhibited the *Spirulina* growth. Therefore, *Spirulina* concentration less than 2.5 g/L could keep on growing for up to 2 weeks. Decreasing the nitrate concentration in NPK up to 1.0 g/L could increase the level of *Spirulina* growth even more than Zarrouk/2, but less amount of $(NH_4)_2SO_4$(0.5 g/L) and urea (0.1 g/L) should be included in media composition. As mentioned before, due to the lack of carbon source in desired mixture, the *Spirulina* growth was significant.

The usage of commercially available fertilizer and chemicals in the market for inorganic nutrition of plants is also economical for large-scale production of algae and could be considered as an appropriate alternative

for algal composition media in lab. Chemical fertilizers with various formulations and with the abbreviation NPK, which indicate the ratio of presence of nitrogen (N), phosphate (P), potassium (K) and sometimes magnesium (Mg) are easily available and can be used not only to resolve the requirement of three basic elements of alga cultivation, but also is a suitable basis for cultivating algae as a cultivation medium in combination with sea salt (in offshore areas) or seawater (in coastal areas). Supplementary usage of other nitrogen sources can be used to optimize growth based on the availability of ammonium and nitrate sources.

Nitrogen in various forms (ammonium or nitrate) along with other chemicals such as phosphate, sodium bicarbonate (as a carbon source), irradiance, etc. can play an important role in the growth of algae or the production of algal metabolites. Rodriguez et al. (2011) reported that the application of two sources of nitrogen (nitrate and ammonium) in the development of *Spirulina* showed more positive effects compared to separate use of nitrogen sources. The obtained results also showed urea and $(NH_4)_2SO_4$ in NPK-containing medium exhibited significant effect on growth, compared to the presence of $KNO_3$ or $NaNO_3$ salts. Some reports also indicate 25% nitrogen reduction in the Zarrouk medium and show no change in the final biomass (Colla et al., 2007; El-Baky et al., 2008). Castro et al. (2015) were able to have the highest amount of biomass (3.27 g/L) in concentration of 1.25-2.5 g/L sodium nitrate (Castro et al., 2015). Danesi et al. (2002) also suggested using urea instead of potassium nitrate. Urea, which contains two ammonium groups, was able to stimulate *Spirulina's* growth by less energy consumption in compare with other nitrogen sources. Also, it has a cheaper price (Castro et al., 2015).

High concentrations of nitrogen (either in the form of nitrates or ammonium) aren't always the reason of growth increasing (Castro et al., 2015; Gupta et al., 2017) and its inhibitory effects against *Spirulina* growth were observed. The inhibitory effects of ammonium have been reported with regard to the decomposition of urea in alkaline conditions in compare with nitrate consumption in *Spirulina* culture medium (Danesi et al., 2011; Rodriguez et al., 2011; Cruz-Martínez et al., 2015).

Our results demonstrated the concentration of 1.0 g/L nitrate in NPK and 0.5 g/L $KNO_3$ was stimulated and higher concentration was inhibited the growth. Costa et al. (2001) showed that $NaNO_3$ stimulated algae growth up to 50 mM and has reported the toxicity effect of $(NH_4)NO_3$ and urea above 10mM concentration.

Gupta et al. (2017) reported that higher amount of nitrogen (up to 100 mM $NaNO_3$) decreases biomass production while maximum C-phycocyanin produces in 40mM followed by 60, 80 and 100 mM $NaNO_3$. In second experiment, C-phycocyanin amount increased in higher amount of urea; while in the third experiment we preferred to use less amount of nitrogen to reduce inhibition effect. However, in new composition, higher value of C-phycocyanin obtained in media

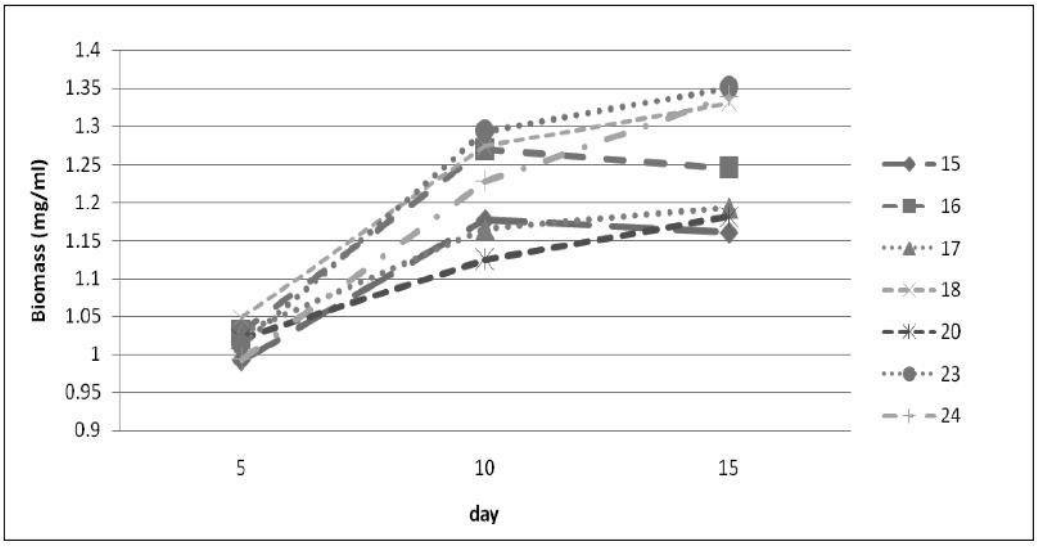


**Fig. 2.** Ammonium sulfate combination's effect on *Spirulina* production.

with lower nitrogen content.

According to the critical important role of nitrogen in the microalgae growth and quick consumption of ammonium by microalgae, it is suggested that ammonium could be added continuously in several steps to prevent its accumulative effect at the early phase of growth (Rodriguez et al., 2011). Urea is rapidly converted into ammonium and is evaporated; therefore, it could be suggested to feed algae at intervals and dividing nitrogen sources in the growth period to prevent ammonium shortage (Danesi et al., 2002).

Using NPK fertilizers, in combination with urea and $(NH_4)_2SO_4$ in 1% Urmia salt allowed to produce *Spirulina* microalgae with acceptable results similar to Zarrouk/2 medium. The results of this study can be used in wastewater treatment with different sources of nitrogen along with generating microalgae biomass.

**Acknowledgement**

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# Effect of Cyanobacterial Extract on Medicinal Plant *Mentha piperita* L.

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**Abstract**

As the simplest photosynthetic organisms, cyanobacteria are the interface between bacteria and plants in terms of evolutionary process. The positive effect of these photosynthetic microorganisms on plant growth is one of the important roles in biotechnology. Their production of various secondary metabolites is another known application of these microalgae in agricultural biotechnology. In this study, the effect of two species of heterocystous cyanobacteria, isolated from paddy fields of Golestan Province in Iran, on the growth of peppermint (*Mentha piperita* L.) was evaluated. A significant difference was observed between treated plants and control in terms of growth parameters. In addition, as one of the factors influencing the vegetative parameters of plants, the qualitative identification of phytohormones in cyanobacterial biomass was investigated by biochemical methods and High Performance Liquid Chromatography technique. The results indicated the presence of plant growth hormones such as indole acetic acid and indole butyric acid in extracts of studied cyanobacteria.

**Keywords:** Cyanobacteria, High Performance Liquid Chromatography, Phytohormones, Peppermint, Vegetative Parameters.

## Introduction

Cyanobacteria are the simplest photosynthetic organisms (Schopf, 2000). Most cyanobacteria are aerobic photoautotroph organisms that require water, carbon dioxide, minerals, and light during their life cycles. Over recent decade, researchers have focused mostly on the biotechnology aspects of this group of photosynthetic organisms (Bhadury et al., 2004; Dahms et al., 2006). The applications of cyanobacteria in various biotechnology fields have been reported, including the production of food, fuel, bio-fertilizer, color, and various metabolites such as toxins, vitamins, enzymes and pharmaceuticals (Abed et al., 2009). There are several reports on the production of secondary metabolites by most cyanobacteria (Gademann, and Portmann 2008; Rastogi and Sinha, 2009). Many researchers classify the phytohormones synthesized by cyanobacteria as secondary metabolites. In general, the production of phytohormones is one of the unique characteristics of plants, which is also found in some cyanobacteria.

Recent studies have shown that cyanobac-

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teria can be useful for plants through the production of the plant growth regulators (PGRs) such as auxin, cytokinin, gibberellin, ethylene, jasmonic acid and abscisic acid hormones (Rodgers et al., 1979). In vivo experiments on some plants treated with these microorganisms also clearly show the production of PGRs by cyanobacteria (Metting, 1988).

Medicinal plants are useful alternatives for chemical drugs. These plants can be used to reduce many adverse effects of synthetic chemical drugs. As a medicinal plant, peppermint with a scientific name of *Mentha piperita* L. is a hybrid of *Mentha spicata* L. and *Mentha aquatica* L.. This medicinal plant is a herbaceous perennial plant which belongs to a taxonomically Lamiaceae (Lamiales, Rosidae). The essential oil of this plant has antioxidant, anti-fungal and insecticidal properties which are used in medicinal applications (Gradiner, 2000).

Due to the importance of medicinal herbs in protecting human health and considering the high value of peppermint as a medicinal herb, the effect of cyanobacteria on peppermint (*Mentha piperita* L.) was investigated. Some purposes such as the evaluation of the effect of cyanobacteria on medicinal plants growth were followed in this study.

## Materials and Methods

*Sampling and purification*

Soil samples were collected from the farms of Hossein Abad village, 20 km from Gorgan city, Golestan Province (36° 55′ 50″ N; 54° 51′ 41″ E). 10 g of the collected soil was grounded in a porcelain mortar and moved to sterile Petri dishes containing culture media, nitrate-free BG-11 medium (pH of 7.1). Plates were placed in a culture chamber to form the algae colony for 14 days. Then, the colonies were purified in a solid culture medium (Stanier et al., 1971).

*Algae culture*

Two purified cyanobacteria species including *Hapalosiphon welwitschii* West & G.S.West and *Anabaena vaginicola* F.E. Fritsch & Rich were used for morphological and molecular identification. In this study, soil extract was used as cyanobacterial culture medium to obtain an algal inoculum in laboratory scale. In order to prepare the soil extracts, a suspension of 500g soil in water was prepared and extracts of mineral content were isolated. The extract was autoclaved at 121°C and pressure of 15p before inoculation of algae for 20 minutes.

The 50 ml of a three-week-old cyanobacterial stock was added to 1.5 liters of soil extract culture. It was kept under a condition of 25 ± 2°C and a light intensity of 4000 Lux (with a light period of 14 hours of light and 10 hours of darkness). The aeration was carried out manually during the culture period.

The cyanobacterial inoculum was adjusted based on 21-day culture density and concentration measurement. So that after three weeks, when the production of secondary metabolites and the cell exponential phase was recognized, the optical density of cyanobacterial culture was measured with spectrophotometer at a wavelength of 750 nm. Under culture optical concentration of 0.3

(OD = 0.3), algal suspension was homogenized with a blender.

*Pot culture and inoculation of cyanobacterial extract*

Pot culture was done in a greenhouse under the natural light and 20 to 25°C temperatures. peppermint (*Mentha pipertia* L.) rhizomes were prepared from the Research Institute of Medicinal Plants of Shahid Beheshti University. Then, they planted in pottery pots (with 15×15 diameter). For each pot, 1.5 Kg of soil was used (Soil contains 25% sand, 60% peat and 15% ordinary soil). In each pot, four peppermint rhizomes were planted. The pots were divided into three groups, first group was control pots which irrigated only with water; the second group was inoculated with cyanobacterial suspension, *Anabaena vaginicola*, and the third group was the plants which treated by cyanobacterial suspension, *Hapalosiphon welwitschii*. Six replicates were considered for each treatment. After two weeks planting rhizomes, the treated plants were irrigated with 200ml of cyanobacterial suspensions. The second and third irrigation were carried out with the same volume of cyanobacterial suspension four and six weeks after planting. The experiment was completed after 60 days. Vegetative growth parameters including leaf number, fresh and dry weight of leaf, stem length, fresh and dry weight of the plant, root length, fresh and dry weight of root were measured and calculated.

*Biochemical analysis*

The quality of hormones affecting vegetative growth parameters was determined, with the emphasis on auxin family including indole acetic acid (IAA), indole propionic acid (IPA) and indole butyric acid (IBA) in the extract of studied cyanobacteria. For this purpose, the standard solutions of the studied auxins, namely, IAA, IPA and IBA were prepared by dissolving 1 mg of each standard in 10 ml of water-methanol solution with a ratio of 80:20 (Shariatmadari et al., 2014). Then, the required concentrations were prepared from these stock solutions by successive dilution of 5, 10, 25, 50, 100 and 250 $ng.ml^{-1}$ for calibration curves. The device applied was a high performance liquid chromatography 1100 Model equipped with ultraviolet and fluorescent detectors as well as a HPLC 1200 Model equipped with a photodiode array detector. A wavelength of 225 nm was used in the analysis by using chromatography with a PDA detector. In addition, an excitation wavelength of 280 nm and an emission wavelength of 360 nm were applied with a fluorescence detector. The length of the chromatography column used was 25 cm with an internal diameter of 4.6 mm. Mobile phase was methanol-water and solid phase was Euros Pere 100-5 c18 from KNAUER company.

In order to carry out this part of the study, 21-days cyanobacterial solid culture, *Hapalosiphon welwitschii* and *Anabaena vaginicola* were collected. Then, the specimens were dried completely by a freeze dryer device. In the next step, 10 mg of each cyanobacterium were poured in 1 ml of water-methanol solution with a ratio of 80:20. Then, they were exposed to ultrasonic waves by ultrasonic

bath apparatus at different intervals. These time intervals were 15, 30, 45 and 60 minutes. In the next step, the suspensions were centrifuged and again, 1ml of fresh solvent (water-methanol) was poured in to cyanobacteria. Then the energy was received as the same way at 15-minute intervals, and the extraction operation was repeated. In each analysis, 20µl of the extract was injected into the HPLC apparatus. Then the results of the various chromatograms were compared.

*Statistical analysis*

Data from different experiments were analyzed using Excel 2007. Statistical analysis was done by one-way ANOVA and Tukey's test using SPSS (ver.16).

## Results

*Effect of Algal Treatment on Plant vegetative Parameters*

In this study, two taxa were collected from the studied sites. The identified taxa were: *Anabaena vaginicola* Fritsch & Rich, (Nostocaceae) and *Hapalosiphon welwitschii* West & GS. West (Stigonemataceae).

Studied plants were treated with these cyanobacteria and control was treated with water following peppermint potting. The vegetative parameters of root, stem and leaf were measured after 60 days. The results showed statistically significant difference between treated plants and control in some vegetative parameters (Table 1). The leaf number as well as fresh and dry weight of leaves in the plants treated with *Anabaena vaginicola* showed significant differences with compared control plants. *Hapalosiphon welwitschii* treatments also showed a significant difference with controls, especially in stem length and fresh weight of root (Figure 1).

*Qualitative and quantitative evaluation of metabolites affecting growth with emphasis on auxin family*

Using high performance liquid chromatography (HPLC) technique for qualitative identification of the auxin family metabo-

**Table 1.** Effect of Cyanobacterial suspension on vegetative parameters of peppermint (*Mentha pipertia* L.).

| | Leaf | | | Stem | | | Root | | |
|---|---|---|---|---|---|---|---|---|---|
| | Number | F.W | D.W | Length | F.W | D.W | Length | F.W | D.W |
| Control | 545.33±57.83 | 17.98±1.44 | 3.427±.33 | 34.7±1.5 | 32.03±22.23 | 5.83±.53 | 22.5±1.58 | 20.44±2.94 | 0.168±0.052 |
| *Hapalosiphon* | 639.33±48.39 | 22±1.63 | 4.029±.26 | 41.47±1.04* | 39.23±3.09 | 7.02±.5 | 25.34±1.58 | 34.4±2.94* | 0.13±.028 |
| *Anabaena* | 854.33±57.3* | 43.63±4.03* | 4.96±.54* | 36.83±.88 | 43.63±4.03 | 8.32±.92 | 26.47±1.72 | 26.88±1.72 | 0.16±.018 |

DW: Dry Weight, FW: Fresh Weight

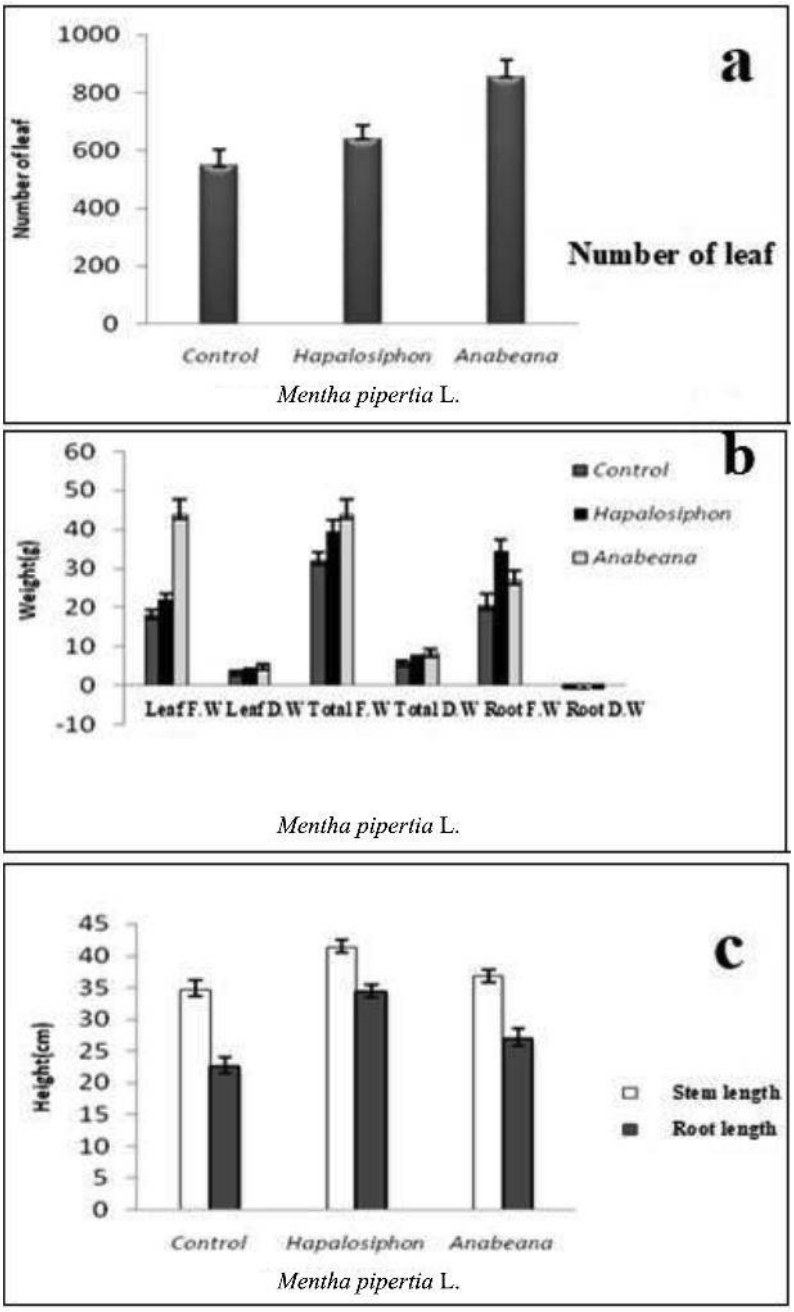


**Fig. 1.** a) The effect of cyanobacterial suspension on the number of peppermint leaves; b) The effect of cyanobacterial suspension on leaf fresh weight, leaf dry weight, total fresh weight, total dry weight, root fresh weight, and root dry weight; c) The effect of cyanobacterial suspension on the stem length and root length of peppermint.

lites in cyanobacterial biomass of *Hapalosiphon welwitschii* and *Anabaena vaginicola* was performed. The comparison of the obtained chromatograms have been shown that IBA was the most observed hormones in 30 min. (Figs 2 and 3). Therefore, the optimum duration for extraction is 30 minutes. In addition to the IBA, Indole Acetic Acid (IAA)

was also found in the extracts.

The comparison between the levels of IBA and IAA in cyanobacterial extracts showed a significant difference between these two hormones in different cyanobacterial extracts. In other words, the quantitative analyses performed on these two cyanobacterial extracts showed higher levels of IBA, as a

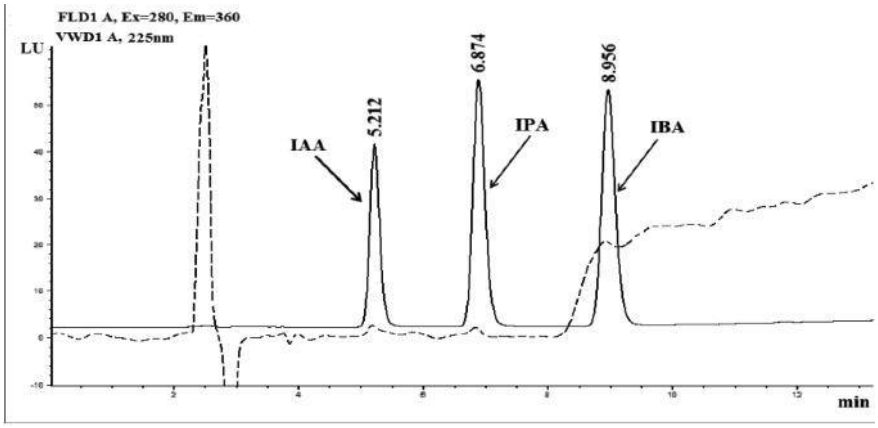


**Fig. 2.** The chromatogram of isolating four standard compounds of auxin and detection by fluorescence detector.

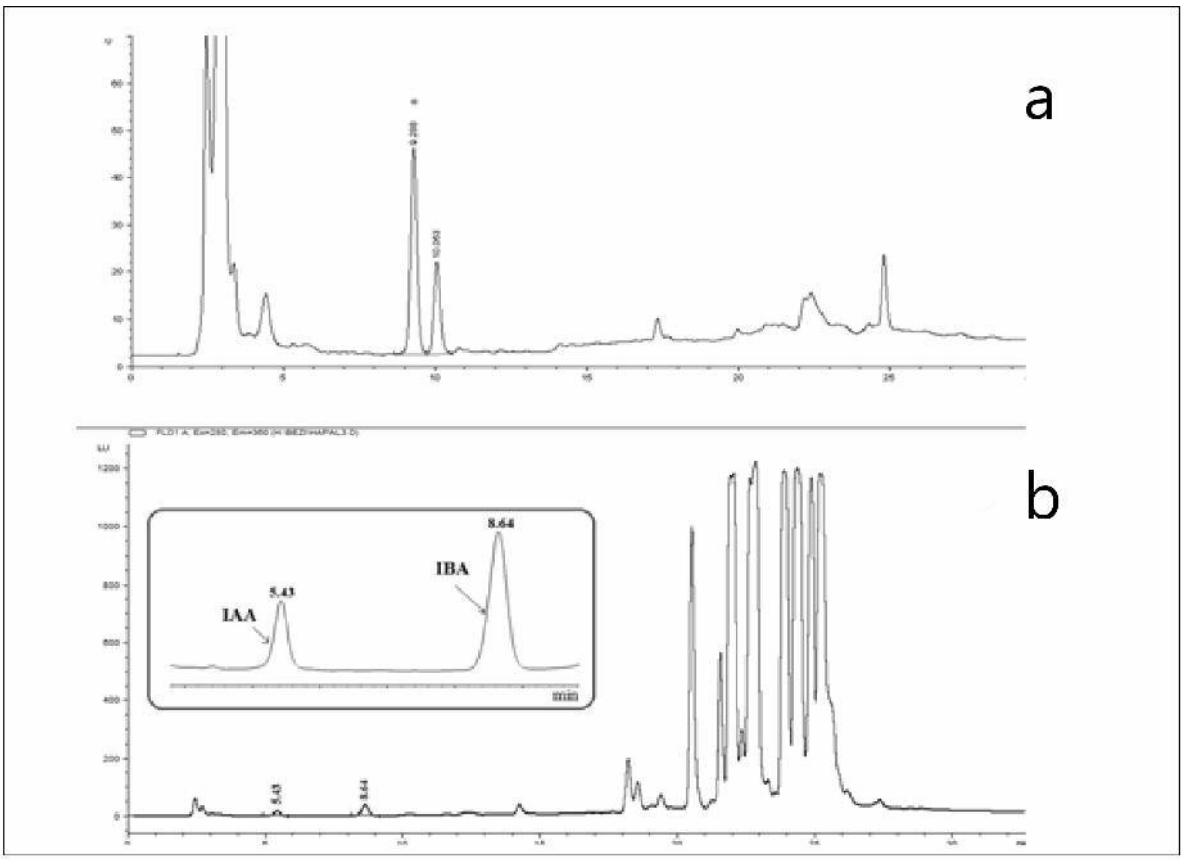


**Fig. 3.** a) The chromatogram of the ultrasound *Anabaena vaginicola* specimen in methanol-water (20:80) for 30 minutes and detection by the fluorescence detector; b) HPLC chromatogram of *Hapalosiphon welwitschii* specimen extracted by ultrasonic waves for 30 minutes (detected by fluorescence detector).

**Table 2.** Estimated concentrations of three auxin compounds in the studied Cyanobacteria.

| | Estimated Concentration (µg/g) in DW (FW) | | |
|---|---|---|---|
| | **IAA** | **IPA** | **IBA** |
| *Hapalosiphon welwitschii* | 0.292 | - | 0.616 |
| *Anabaena vaginicola* | 0.020 | - | 1.275 |

growth-promoting hormone, in *Anabaena vaginicola* in comparison with *Hapalosiphon welwitschii* (Table 2).

## Discussion

In this study, the effect of cyanobacterial extracts on vegetative growth parameters of medicinal herb, peppermint (*Mentha piperita* L.) was studied. Inoculation of cyanobacterial extract had a positive effect on leaf number, fresh and dry weight of leaf, stem length and fresh weight of root comparing with control. In plants treated by *Anabaena vaginicola*, the maximum growth was observed in leaf characters (leaf number and leaf fresh and dry weight). Plants treated by *Hapalosiphon welwitschii* also showed the maximum growth in parameters such as stem length as well as fresh weight of root.

Over the last few years, researchers observed acceleration of some plants growth by Plant Growth Promoting Rhizobacteria (PGPR) (Asghar et al., 2002; Morissey et al., 2004; Richardson et al., 2009). Soil cyanobacteria have a particular importance in nutrient and organic biogeochemical cycles as well as in maintaining the quality of soil (Kennedy et al., 2004; Khalid et al., 2004). Most studies have been performed on the growth-promoting role of cyanobacteria on *Oryza sativa* L. (Saadatnia and Riahi, 2009). Cyanobacteria are a distinct and primary group of photosynthetic prokaryotes that can supply 86% of nutrient needs of this plant (Venkataraman, 1972). Also, several species of cyanobacteria shown different effects on the vegetative characters of plants such as rice, chick pea, cucumber and squash (Shariatmadari et al., 2011; Prasanna et al., 2008; Yanni and Abd El-Rahman, 1993; Jagannath et al., 2002; Mostafa and Soha, 2009; Tiedemann et al., 1980; Pachpande, 1990; Nanda et al., 1991). Prasanna et al. (2012) reported the performance of metabolites such as IAA derived from cyanobacteria including *Anabaena* sp., *Hapalosiphon* sp., *Nostoc* sp. and *Calothrix* sp. on rice. Mostafa and Soha (2009) used two kinds of fertilizers, *Spirulina platensis* and potassium-humic acid on sesame. They observed that cyanobacterial fertilizer increases plant length, number of branches, number of capsules and weight of the seeds. Some researchers have argued that relationship between cyanobacteria and plants is a mutual relationship for exchanging the nutrients, particularly fixation of carbon or nitrogen by cyanobacteria (Rai and Bergman, 2002; Jaiswal et al., 2008; Karthikeyan et al., 2009). Khanjir (2011) studied the effects of heterocystous cyanobacteria on medicinal herbs, water mint (*Mentha aquatica* L.) and summer savory (*Satureia hortensis* L.). She

justified the incremental effects of cyanobacterial treatments on vegetative parameters and essential oil content with the production of phytohormones by these microorganisms. In addition to studies on growth-promoting effects of cyanobacteria, identification of auxins and their inactive analogues in other algae groups such as Phaeophyceae (*Macrocystis, Laminaria*), Rhodophyta (*Botryocladia*) and Chlorophyceae (*Enteromorpha, Chlorella*) were also performed in 1960s and 1970s (Schiewer, 1967). Similar studies suggest that hormones of the auxin family stimulate formation of rhizoid in green algae *Bryopsis plumose*, and accelerate growth in cyanobacteria and some microalgae (Provasoli and Carlucci, 1974; Arendrachuk, 1974). Auxins are involved in various biological cell cycles, such as cell division, elongation, differentiation and root extension. IAA is known as an important type of auxin which directly affects plant growth (Spaepen et al., 2007). IBA promotes the growth of root cells by stimulating division of the first priming cells. In fact, increasing the concentration of IBA leads to an increase in the number and length of the root. It is due to the effect of this regulator which stimulates adventitious roots and promotes development of latent and pre-formed root primers (Khoshkhooy, 2003).

In this study, the hormones of auxin family (IAA and IBA) which stimulate plant growth, were identified in the extract of *Hapalosiphon welwitschii* and *Anabaena vaginicola*. Due to the higher content of IBA in *Anabaena vaginicola* than *Hapalosiphon welwitshii*, this cyanobacteria were considered as a more appropriate stimulator for treated plants. Therefore, it can be concluded that production of growth stimulating phytohormones can improve growth of treated plants by these cyanobacteria along with the ability to nitrogen fixation. However, it should be considered that other factors such as increasing in organic content of soil, exchanges between cyanobacteria and plants, improving aggregation and soil structure can also be effective.

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# Impact of Salinity and pH on Several Species of *Anabaena* (Nostocaceae, Nostocales) Isolated from Rice Fields in Iran

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**Abstract**

The purpose of this study is to develop a biofertilizer based on filamentous nitrogen-fixing cyanobacteria selected from rice fields and to generate a technological package compatible with its use for the rice crop in Iran. Cyanobacteria was isolated and purified from rice fields in Kalate Naderi. In this research we studied the effect of salinity (NaCl, 0, 1, 2 and 4%) and pH (5, 7, 9 and 11) on growth and chlorophyll-a contents in six species of *Anabaena*. Results showed that *Anabaena sphaerica* Bornet & Flanault possessed the best adaptation to pH changes. It could be more active in 5-11 pH values. *A. vaginicola* F.E. Fritsh & Rich and *A. variabilis* Kutzing ex Bornet & Flanault were remarkable for salinity tolerance. They adapted to salinity stress up to 2% salt concentration in the medium. Our results indicated that the growth of all strains decrease by 4% salt concentration and pH 11. Indeed, *Anabaena* is a cyanobacterium with nitrogen fixation ability and high potency of adaptation to environmental stress. So, it can be a useful candidate for biofertilizer in agriculture, particularly in rice fields.

**Key words**: Biofertilizer, Heterocyst cyanobacteria, pH stress, Rice field, Salinity stress.

## Introduction

It is known that cyanobacteria supply more nitrogen in wetland rice fields in tropical regions (Singh, 1985, 1988) than in dryland fields (Yamaguchi, 1979), and this is attributable to the unique characteristics of wetland rice fields: along with water, there is a natural supply of plant nutrients, especially nitrogen, which encourages general plant growth when soil pH is neutral. Additionally, there is considerable literature indicating that in rice-flooded soils of temperate regions, cyanobacteria increase soil nitrogen through nitrogen fixation (Vaishampayan et al., 2001), such as, for example, in Japan and in most Southeast Asian countries (Watanabe, 1973). However, this behavior is not shown in all rice soils in temperate regions, since the environmental conditions such as moisture, soil pH, combined nitrogen levels and temperature are factors that can play an ecologically determinant role on the abundance of cyanobacteria and nitrogen fixations (Pereira et al., 2009). On the other hand, salinity of soil is an important ecological variable and a serious problem in agriculture. The widespread distribution of cyanobacteria indicates that they can tolerate a wide spectrum of global environmen-

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tal stress such as temperature, pH, desiccation. Salt stress is one the limiting factors on growth and productivity of microorganisms. They have developed a number of mechanisms by which cyanobacteria defend themselves against environmental stress (Rajendran et al., 2007). The physiological basis for the adaptation to high salinities in several cyanobacteria species includes three main sub processes: 1) active extrusion of inorganic ions, leading to relatively unchanged internal salt concentrations; 2) accumulation of large internal amounts of organic osmo-protective compounds; and 3) expression of a set of salt stress proteins (Soltani et al., 2007). Iranian rice fields are a source of enormous biological diversity-which is scarcely studied. Characterization of biotechnology value in cyanobacteria has been reported by Soltani et al. (2005, 2006). In this study, we investigated six species of *Anabaena* that were isolated from four rice fields as biofertilizer in the salinity or acidic/alkaline agriculture soils in Iran. Growth rate was measured under salinity and pH stress.

## Materials and Methods

*Sampling and cyanobacterial culture*

This study is focused on Kalat Naderi district of Khorasan Razavi Province. The Kalat Naderi town is located in the northeast of Iran between 59° 9′ to 60° 27′ E longitude and 36° 24′ to 37° 17′ N latitude. Soil samples were collected from four sites (Amirabad, Sarrood, Qaleno and Kalat). The depth of sampling was 5 cm. The collected soil samples were transferred to sterilized nitrate free BG-11 medium (Stanier et al., 1971). The Petri dishes were placed in a culture chamber (Model SB5520) at 25±5°C and 12/12 h light/dark cycle under fluorescent illumination of 40μmol photon $m^{-2}$ $s^{-1}$ for two weeks. After colonization, the cyanobacterial strains were studied by optical microscopy (Olympus, Model BM-2) and different taxa were identified based on morphological characteristics using standard key books and articles (Desikachary, 1959; Prescott, 1970; Komárek and Anagnostidis, 1989). Morphology of filaments, shape and dimensions of vegetative cells, heterocyst and akinetes were some of the characters used for identification of these taxa.

*Physiological analysis*

The axenic culture of *Anabaena* was prepared by repeated subculture on solid medium. Stock cultures were grown in nitrate free BG-11 (BG-110). Cells in logarithmic phase of growth were collected from stock cultures and inoculated. BG-110 medium in different salinity and pH was made for inoculation of *Anabaena* species.

The required salinity was obtained by adding sodium chloride (NaCl). The six strains of *Anabaena* were grown in a salinity gradient from 0, 1, 2 and 4%. The pH gradient was prepared by KOH and HCl. The pH values 5, 7, 9, 11 were adjusted. The flasks were maintained for 10 days at 25±5°C and 12/12 h light-dark cycle under fluorescent illumination of 40 μmol photon $m^{-2}s^{-1}$. Growth rate of each strains in different treatments was measured as the content of chlorophyll-*a* according to Soltani et al. (2007).

Each treatment consists of three replicates; the results presented are mean values. Moreover, the morphological character's variation and changes due to stress conditions, salinity and pH were investigated.

## Results

Six nitrogen-fixing species of *Anabaena* were isolated from paddy fields in Iran (Table 1). In order to select a suitable candidate as bio-fertilizer, we investigated their tolerance to salinity and pH stress. The growth curves for six species under salinity stress were obtained (Fig. 1). The results showed that *A. torulosa* grows in no salt and 1% salinity. *A. variabillis* exhibited good increasing growth with no salt, cells became colorless and the filament was degenerated in higher than 2% salt concentrations. *A. vaginicola* was relatively resistant, its growth rate continued up to 2% of salinity. Although it could tolerate 2% salt in the medium, finally the cells died. *A. sphaerica* was able to grow up to 1% of salinity. *A. ambigua,* at the beginning of the growth phase, showed delay. After a few days, adaptation was found and became resistant to 1% salt concentration. *A. oscillarioides* in the control treatment (0% salinity) showed the maximum growth rate and resistant to 1% salinity (Fig. 1). *A. oscillarioides* in the acidic pH 5 and alkaline pH 11 became deformed; the cells were destroyed and the filaments changed. The maximum growth rate of *A. torulosa* obtained in pH 9. *A. variabillis* was able to grow in pH 5 and 7 but the best condition was in pH 9. *A. sphaerica* adapted to pH 11. *A. vaginicola* showed the maximum grow rate in pH 9. *A. ambigua* became completely deformed in pH 5, optimum pH value for this strain was 9 (Fig. 2).

## Discussion

Industrial-scale microalgae cultivation, needs selection of algae strain with high production of target biochemical and tolerance to a wide range of environmental conditions, such as salinity, temperature, pH and nutrient or pollutant loads. Such algal 'super-species' should also show high biochemical productivity, which would be considerably simple production regarding

**Table 1.** List of *Anabaena* species identified from the soil of rice fields of Kalat-Naderi

| | Locations | | | |
|---|---|---|---|---|
| Species | Amirabad | Qaleno | Sarrood | Kalat |
| *A. vaginicola* | - | + | + | + |
| *A. torulosa* | - | + | + | + |
| *A. oscillarioides* | - | + | + | + |
| *A. ambigua* | - | - | + | - |
| *A. sphaerisca* | - | - | + | - |
| *A. variablis* | - | - | + | - |

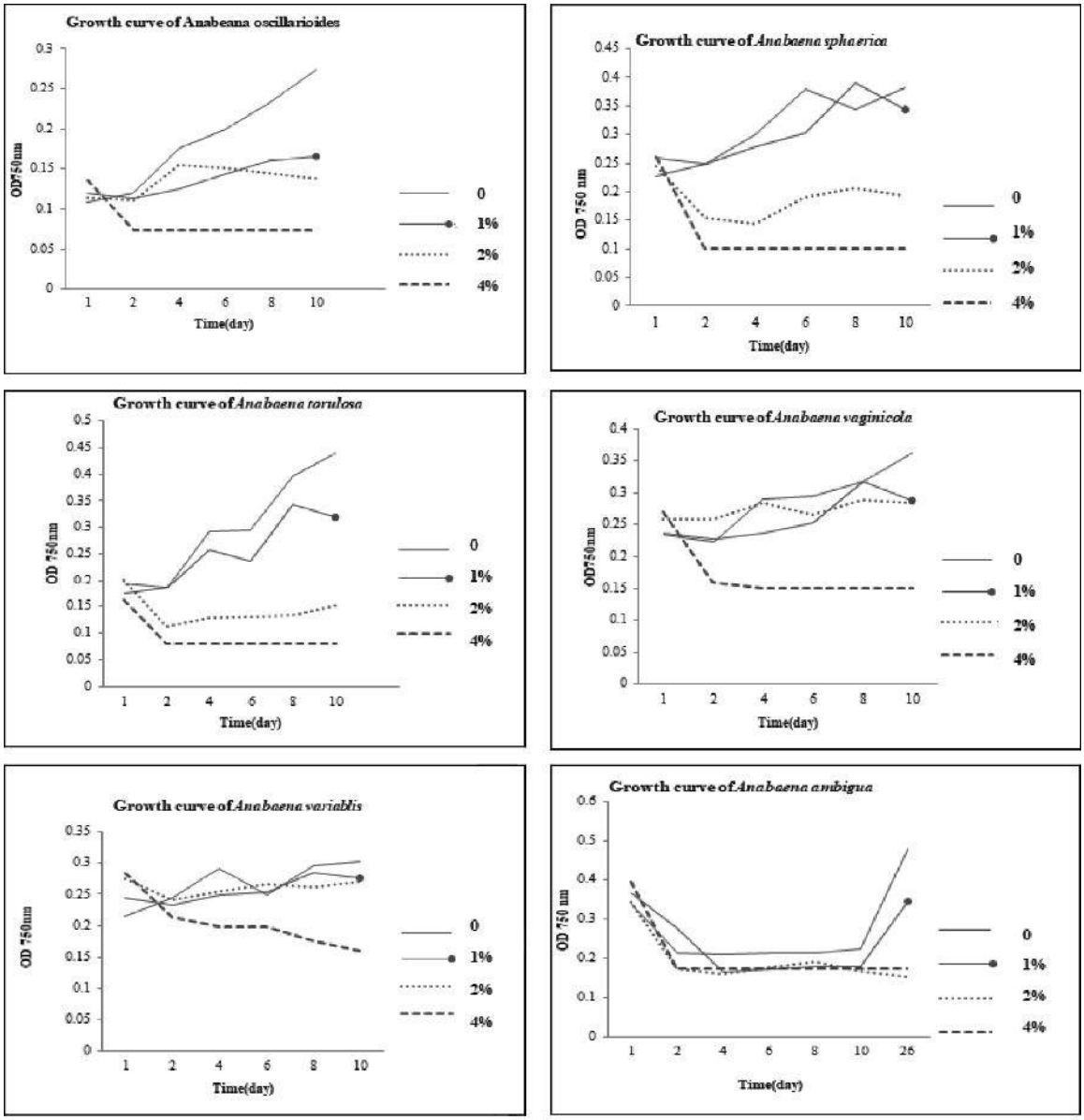


**Fig. 1.** Growth curve of six species of *Anabaena* studied under salinity stress.

standardization of product quality, across a range of production sites (Alvensleben et al., 2013). Two potentially important factors that can regulate growth and biomass of cyanobacteria are salinity and pH. This study revealed six species of *Anabaena* species, isolated from different salinity and pH in rice fields. Although metabolic requirement of sodium for physiological activities like nitrogen fixation, growth, photosynthesis and intracellular pH regulation, transport of carbon and energy, transductions and maintenance of RuBisCo are documented in cyanobacteria, hyper-salinity is found to induce a number of adaptive responses (Sekar and Subramanian, 1999). The halotolerant cyanobacteria are known to synthesize a variety of osmolites to balance hypersaline stress in response to the concentration of NaCl in the external media (Reed et al.,1986). Many agricultural ecosystems increasingly become salt affected, thus rendering them in-

hospitable to crops. Such salt affected soils cover an estimated 7 million hectares of potential crop land in India (Roychoudhury et al.,1985). So, indication of halotolerance cyanobacteria can be important for use as a biofertilizer and improvement of salty soils conditions.

Regarding physiological responses of *Anabaena* species to NaCl, as shown in figures 1, 3 and 4, growth rate decreased with salinity increase in but it continued up to 2%. These results also reported for *Anabaena aphanizomenoide* which grew in NaCl up to 15 $gL^{-1}$ and 20 $gL^{-1}$ had inhibiting effect (Moisander et al., 2002). These results confirm the variation of chlorophyll content in different salinity. Figure 2 shows a similar growth rate pattern. It confirms the role of chlorophyll in cyanobacteria growth which changes by varied environmental factors.

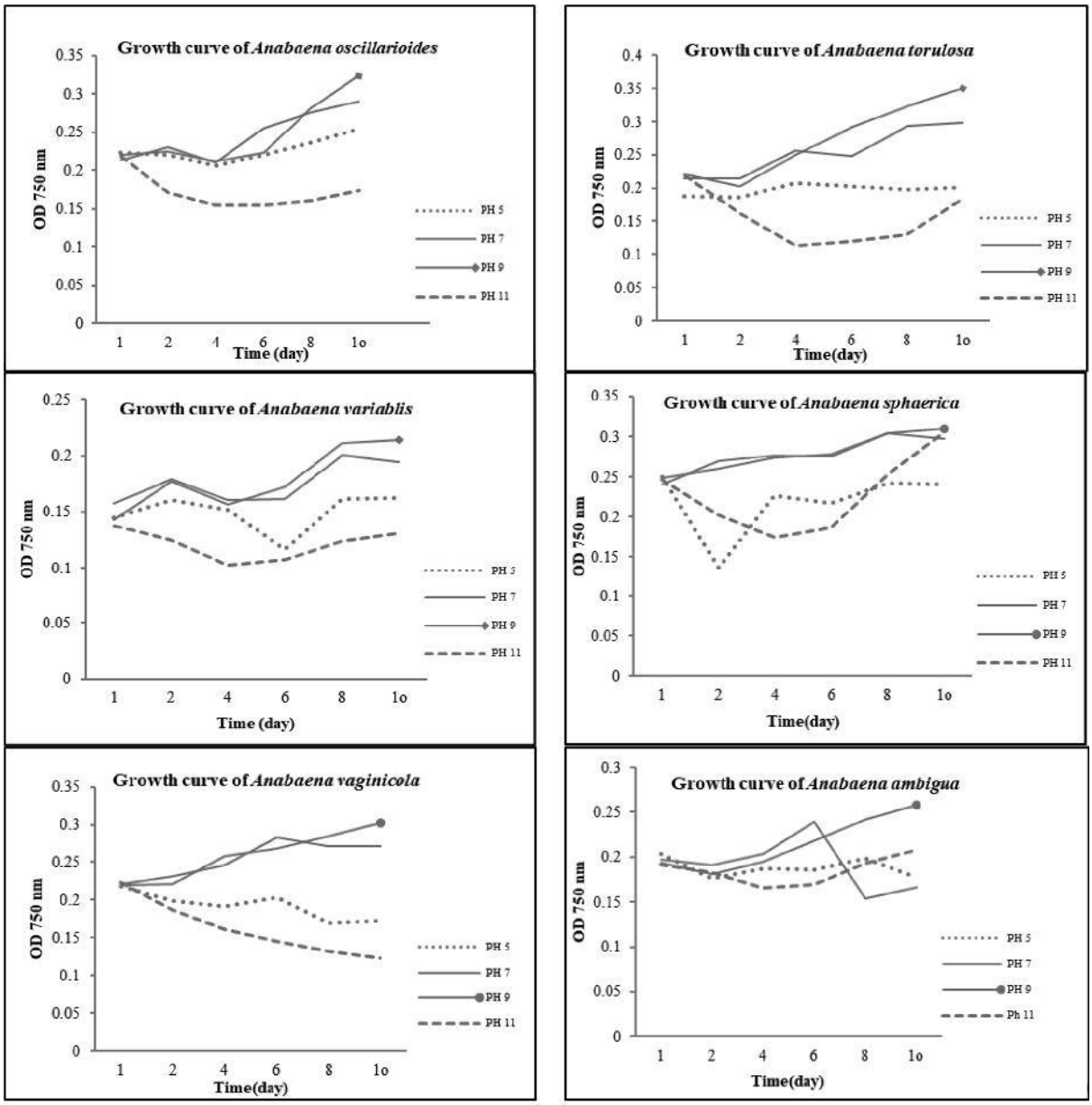


**Fig. 2.** Growth curve of six species studied of *Anabaena* under pH stress.

Maximum photosynthesis rate was seen in control. Our results confirm the salinity has a significant effect on growth and photosynthesis in some of the species. These cyanobacteria need more light for survive in saline environment. There have been relatively few studies showing how cyanobacteria tolerate acidic or alkaline stress, while cyanobacteria are found in nearly all ecosystems. Ecological observations have shown that cyanobacteria have a preference for alkaline conditions. Steinberg et al. (1998) showed that cyanobacteria were completely absent in picoplanktons when the pH fell below 4.5 in ten lakes. Acidic pH is remarkable considering the physiological changes caused by increasing in external proton concentration. Changes in the proton concentration of a cell's environment can affect the dissociation rate of $CO_2$, the electrical charge of the cell-wall surface, ion transport systems and membrane potentials (Jean et al., 2002). Acidic pH can interfere with the function of outer-surface cell components such as pili, chemoreceptors, cell walls, exopolysaccharide, periplasmic proteins and flagella. The pH changes can cause disruption of the plasma membrane, protein denaturation and loss of enzyme function, as well as damage to macromolecules or ionization of nutrient molecules, which affects the availability of these compounds to the cell (Park et al., 1996). The dissociation of protein functional groups is directly affected by pH stress and changes; also pH can interfere with enzyme activity. Chakraborty et al. (2011) showed the effect of pH on Chl a concentration in the freshwater system. Concentration of Chl a gradually increased from acidic to neutral and reached at its maximum level at the pH 8.15. They also reported that salinity resistance range in cyanobacteria was attributed to enhance synthesis of zeaxanthin as a protective xanthophyll against the osmotic stress. However, the effect of pH on green algae and cyanobacteria growth rate was not as dramatic as salinity and they showed a considerable acclimation towards fluctuating pH. Cyanobacteria are not only able to fix atmospheric nitrogen, but also have an important role in rice fields by their frequency. Soltani et al. (2007) reported that nitrogenase activity varies depending on the strain. Accordingly, *Anabaena* has higher nitrogen fixation than *Nostoc* in saline water. According to our results in this study, *A. vaginicola*, *A. variabilis* and *A. sphaerica* are more capable of tolerating environmental stress. We suggest that mentioned *Anabaena* species as suitable candidates for biofertilizer in saline and alkaline stressed soils.

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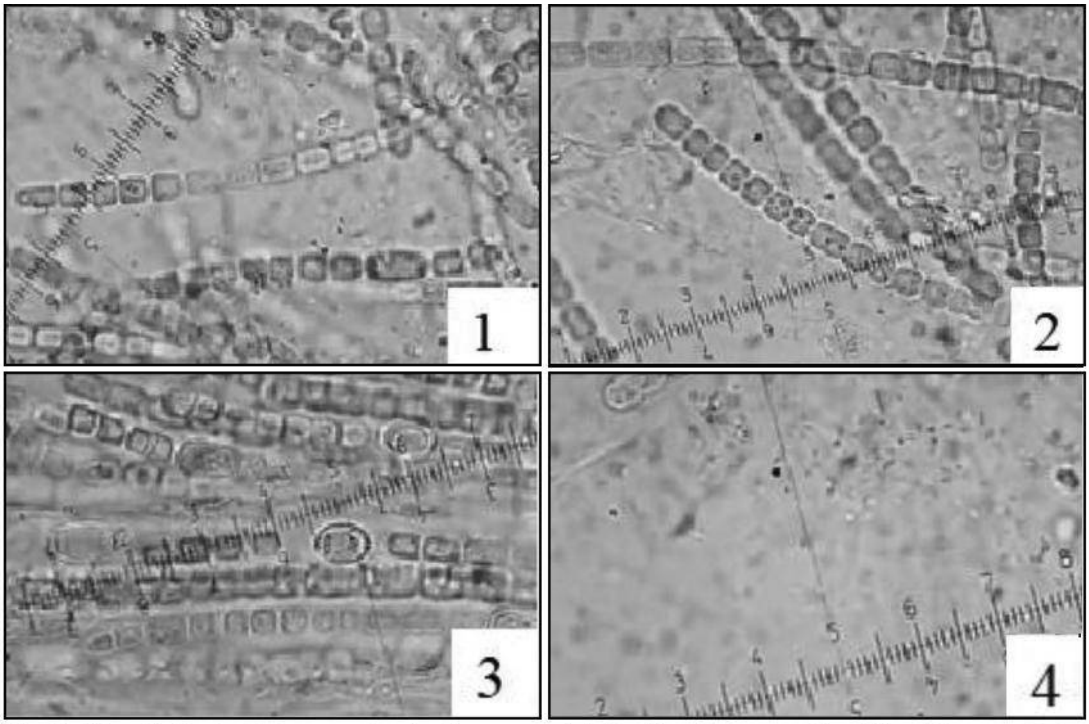


**Fig. 3.** Morphological changes in *Anabaena variablis* after 2 days under :(1) without salinity, (2) 1% salinity, (3) 2% salinity, (4) 4% salinity.

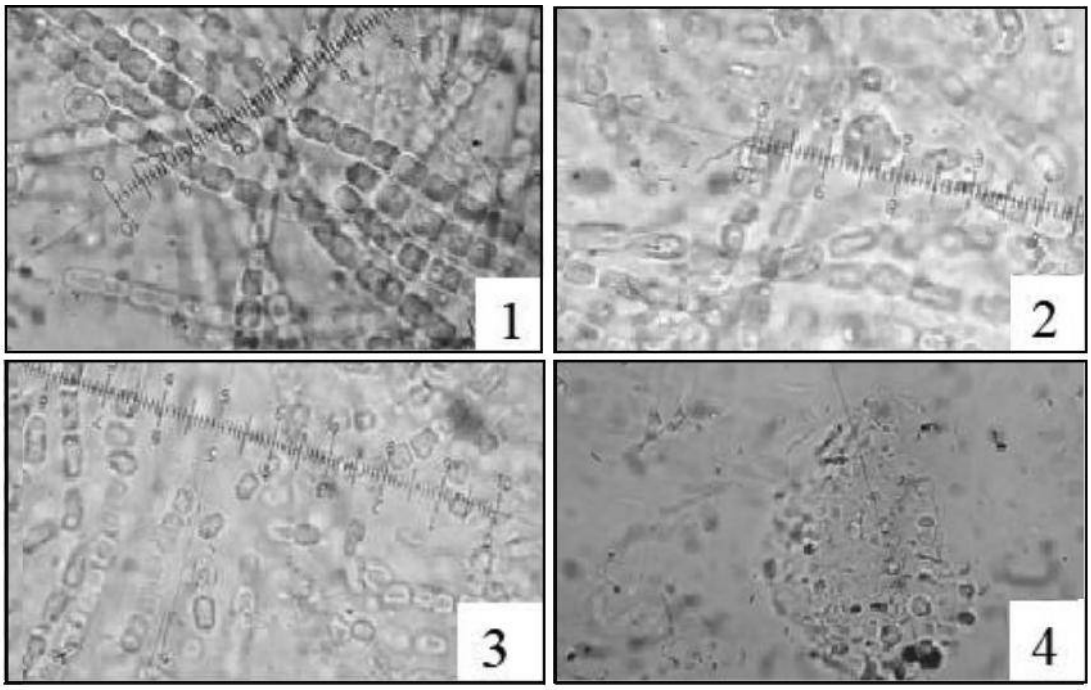


**Fig. 4.** Morphological changes of *Anabaena variablis* after 10 days under: (1) without salinity, (2) 1% salinity, (3) 2% salinity, (4) 4% salinity.

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# Bayesian Analysis of Population Structure and Gene Flow in *Chara* (Chryophyceae) Species

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**Abstract**

*Chara* is a morphologically variable genus.Molecular analysis (ISSR) of genetic diversity and population structure was performed an *Chara* species. Population groups identified based on geographical provinces showed a significant genetic difference, but Mantel test did not show isolation by distance in the studied species. Bayesian analysis of population structure grouped *Chara* species in 2 distinct genetic groups differing in allelic composition and frequency. STRUCTURE analysis revealed genetic admixture among species which was supported by reticulation analysis. Coalescence analysis showed the occurrence of gene duplication and extinction as possible evolutionary changes along with polyploidy as the main forces of speciation in the genus *Chara*.

**Key words**: *Chara*, Gene exchange, Isolation by Distance.

## Introduction

Characeae is a large and unique family of algae with about 300 species, characterized by the complexity of their morphological features, including the structure of their gametangia and their axis differentiated into nodes and internodes (Picelli-Vicentim et al., 2004). They grow in freshwater, in brackish and semi-terrestrial environments. They range in size from a few millimeters to over a meter in length. The filament internodes are unicellular (sometimes may be covered by subsequent growth of corticated filaments), while filament nodes have a complex, parenchyma-like organization.

The Charales have an excellent fossil record extending back far over 420 million years, and two extant lineages. The Characeae and Nitellaceae can be traced back to roughly 200 million years ago (Mattox and Stewart, 1984). These algae are considered the closest living relatives of land plants (Karol et al., 2001) and recent molecular study of mitochondrial DNA and chloroplast DNA showed that despite important differences in size and intron composition, *Chara* mtDNA strikingly resembles *Marchantia* mtDNA and genome comparisons and phylogenetic analyses based on mtDNA and cpDNA provided unequivocal support for a sister-group relationship between the *Charales* and the land plants (Turmel et al., 2003, 2006). These green algae also play an important ecological role in aquatic ecosystems as their assemblage increases water transpar-

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ency and they act as efficient nutrient sinks and influence zooplankton and phytoplankton biomasses (Meurer and Bueno, 2012). In some places, Characeae species constitute much of the submerged aquatic plant life, and thus contribute through photosynthesis to the oxygen balance in natural waters.

Charophytes are a taxonomically difficult group, both with regards to species identification and in relation to other algal groups (Griffin, 1963). High variability within species separated by small morphological differences can prevent the determination of species and subspecies, varieties and forms. Further, the morphology of members of Characeae are often affected by their ecological environment (Urbaniak, 2010). Extensive reshuffling occurs in the taxonomic status of Charophytes; large number of species were reduced to the status of subspecies, variety or forma; while some distant species were merged together (Abrol and Bhatnagar, 2006).

Few molecular studies have been performed to study genetic diversity among and within Characeae species; for instance Mannschreck et al. (2002) used amplified fragment length polymorphism (AFLP) to study biosystematics of *C. hispida, C. intermedia* and *C. tomentosa* from the genus *Chara*, while Abrol and Bhatnagar (2006) used Random amplified polymorphic DNA (RAPD) markers to investigate biodiversity of 12 Indian charophyte taxa.

Schaible et al. (2011) used microsatellite markers (SSRs) and Bayesian assignment method to determine population genetic structure in sympatric sexually and Parthenogenetically reproducing population of *C. canescens* (Charophyta) in Europe; while Lewis and Lewis (2005) performed molecular phylodiversity of *Chara* species growing in desert area by using 18S rDNA and Bayesian analysis showing substantial molecular diversity in these taxa and that desert lineages are distantly related to their nearest aquatic relatives. Schneider et al. (2015) used barcodes of the ITS2, matK and rbcL regions to test if the distribution of barcode haplotypes among individuals of 91 speciemens from 10 European countries, Canada and Argentina. They found out that herbarium specimens also for aquatic plants like *Chara* are useful as a source of material for genetic analyses and rbcL and matK had highest sequence recoverability, but matK and ITS2 had higher discriminatory power than rbcL.

Limited systematic studies are available on charophytes of Iran only recently reports concerned with morphological and micromorphological diversity of *Chara* species have been published (Ahmadi et al., 2012a,b,c). The present study considers cytology, genome size and molecular diversity of some *Chara* species of Iran for the first time and tries to use these data to investigate species relationship.

## Materials and Methods

*Plant material*

Studies were performed on 18 populations of *Chara* species: *Chara gymnophylla* (A. Br.) A. Br. var. *gymnophylla*, *C. gymno-*

*phylla* var. *rohlenae* (Vilh.) Fil., *C. vulgaris* var. *longibracteata* (Kutz.) J. Gr. & B.-W., *C. vulgaris* L. var. *vulgaris*, *C. contraria* A. Br. ex Kutz., *C. kirghisorum* Less., *C. crassicaulis* Schl. ex A. Br., *C. socotrensioides* R.D.W., *C. tomentosa* L., *C. connivens Salz.* ex A. Br., *C. pedunculata* Kutz, *C. fibrosa* C. Ag. ex Br., *C. kohrangiana* Ahmadi et al. and *Nitella hyalina* (DC.) C. Ag., as outgroup.

*ISSR assay*

Ten ISSR primers: (AGC)5GT, (CA)7GT, (AGC)5GG, UBC 810, (CA)7AT, (GA)9C, UBC 807, UBC 811, (GA)9A and (GT)7CA commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25 µl volume containing 10 mM Tris- HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of a single primer; 20 ng genomic DNA and 3U of Taq DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 1 min at 50°C and 1 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany). The experiment was replicated 3 times and constant ISSR bands were used for further analyses.

*Data analyses*

ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). Based on geographical distribution of species and populations studied, they were divided into 6 population groups for genetic diversity analysis.

Genetic diversity parameters determined were percentage of allelic polymorphism, allele diversity (Weising et al., 2005), Nei's gene diversity (H), Shannon information index (I) (Weising et al., 2005; Freeland et al., 2011), number of effective alleles and percentage of polymorphism. Furthermore, AMOVA (Analysis of molecular variance) test (with 1000 permutations) as performed in GenAlEx 6.4 (Peakall and Smouse, 2006), was used to show molecular difference among the population groups. Mantel test (Podani, 2000) was performed to study association between molecular distance and geographical distance of the populations.

Dice as well as Nei's genetic distance (Weising et al., 2005; Freeland et al., 2011) was determined among trees and used for the grouping of the genotypes by unweighted paired group method with arithmetic average (UPGMA) and Neighbor Joining (NJ) clustering methods after 100 times bootstrapping (Freeland et al., 2011). Similarly, ordination plot was utilizedbased on principal co-ordinate analysis (PCoA), as well as Multidimentional scaling (MDS) (Podani, 2000), using PAST ver. 2.17 (2012, Hammer et al., 2001) and DARwin ver. 5 (2012).

Bayesian clustering method was performed to elucidate the populations, genetic structure by using STRUCTURE v. 2.3 (Pritchard et al., 2000). The program structure imple-

ments a model-based clustering method for inferring population structure using genotype data consisting of unlinked markers. For this reason, we first performed linkage disequilibrium test for SSR loci as implemented in POPGENE ver. 1.32 (2000).

The model applied in STRUCTURE analysis assumes the existence of *K* clusters (Pritchard et al., 2000). The Markov chain Monte Carlo simulation was run 20 times for each value of *K* (5) for $10^6$ iterations after a burn-in period of $10^5$. All other parameters were set at their default values. Data were entered as suggested by Falush et al. (2007) and data sample provided in STRUCTURE home page. STRUCTURE Harvester web site (Earl and von Holdt, 2012) was used to visualize the STRUCTURE results and also to perform Evanno method to identify proper number of *K* (Evanno et al., 2005). The choice of the most likely number of clusters (*K*) was carried out comparing log probabilities of data [Pr(X|K)] for each value of *K* (Pritchard et al., 2000), as well as by calculating an *ad hoc* statistic $\Delta K$ based on the rate of change in the log probability of data between successive *K* values, as described by Evanno et al. (2005). Genetic differentiation of population subgroups was determined by Fst determined by STRUCTURE. Reticulation was performed by T-REX (Tree and Reticulogram Reconstruction) ver. 3 (2000), and DARwin ver. 5 (2012) which infer reticulogram from distance matrix. For reticulation, we first built a supporting phylogenetic tree using Neighbor Joining (NJ), followed by a reticulation branch that minimizes the least-squares at each step of the algorithm (Legendre and Makarenkov, 2002). Furthermore, coalescence analysis was performed for molecular data after 1000 reiterations (Liu et al., 2009), as suggested for SSR polymorphic data by Wilson and Balding (1998) and performed in Mesquite (Maddison and Madison, 2011). Gene tree heterogeneity and discordance with the species/population tree was checked by parameters provided in Mesquite (Maddison and Madison, 2011), including deep coalescence, gene duplication and extinction.

## Results

*Genetic diversity analysis*

All ISSR primers used produced 67 bands in 18 *Chara* species and populations studied. ISSR band No. 17 was present in all *Chara* species studied but was absent in outgroup species *Nitella,* while band 20 occurred only in *Nitella hyalina* and was absent in all *Chara* species. ISSR loci 35, 50 and 69 were private band for *C. fibrosa*., while band 49 occurred only in *C. crassicaulis.*

Dentrented correspondence analysis plot obtained including Convex hulls as well as 95% Ellipses methods (Fig. 1), showed scattered distribution of all ISSR loci in the plot, indicating they are well scattered in the genome and possibly are not correlated to each other. Gst analysis revealed that ISSR loci have Gst values of 0.05 to 0.80 with mean Gst value of 0.42, which is considered a low to moderate value. This indicates the presence of shared common alleles and possible gene exchange among *Chara* species

(which will be discussed more in following paragraphs).

Genetic diversity analysis performed among 5 population groups based on geographical provinces (Table 1) revealed a higher degree of genetic polymorphism in population group 2 (Markazi province) (83.58%), followed by population group 4 (Chaharmahal and Bakhtiari province) (71.64%), compared to other population groups. Similarly, Markazi province had a higher value of effective alleles (Ne) (1.41) and expected heterozygosity (He, 0.26) compared to other provinces. Nei's genetic identity determined among 5 provinces varied from 0.70 between populations 3 (Kohgiluyeh and Boyer-Ahmad ) and 5 (Kurdestan) to 0.87 between populations 1 (Esfahan) and 3 (Kohgiluyeh and Boyer-Ahmad). Similarly, these populations had the highest and lowest Nei's genetic distance values (0.35 and 0.14 respectively). When UPGMA tree based

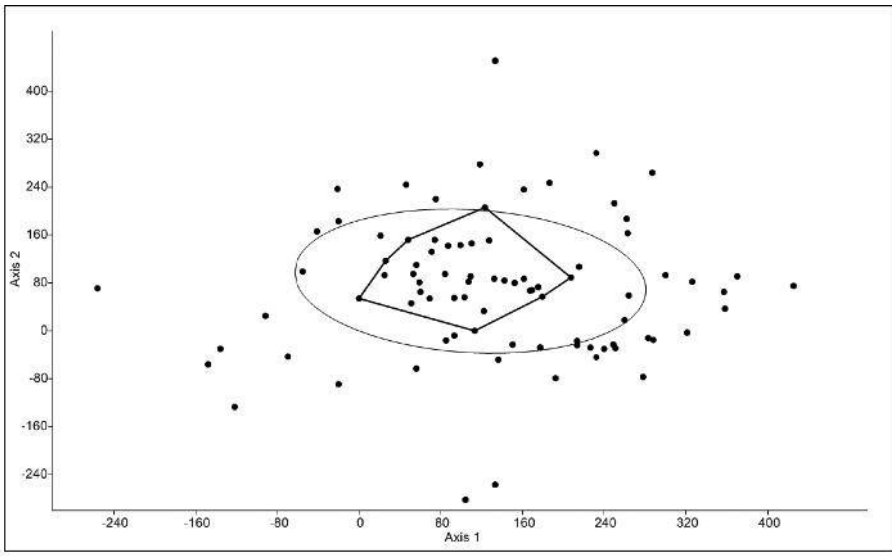


**Fig. 1.** Dentrented correspondence analysis of ISSR loci.

**Table 1.** Genetic diversity parameters in studied provinces.

| Pop | Province | N | Na | Ne | I | He | %P |
|---|---|---|---|---|---|---|---|
| Pop1 | Esfahan | 4.000 | 1.373 | 1.314 | 0.298 | 0.194 | 59.70% |
| Pop2 | Markazi | 6.000 | 1.672 | 1.429 | 0.401 | 0.261 | 83.58% |
| Pop3 | Kohgiluyeh and Boyer-Ahmad | 2.000 | 0.970 | 1.253 | 0.217 | 0.148 | 35.82% |
| Pop4 | Chaharmahal and Bakhtiari | 5.000 | 1.478 | 1.347 | 0.337 | 0.216 | 71.64% |
| Pop5 | Sananndaj-Kurdestan | 1.000 | 0.299 | 1.000 | 0.000 | 0.000 | 0.00% |
| Total | | 3.600 | 1.158 | 1.269 | 0.250 | 0.164 | 50.15% |

N = No. of populations/species in the group, Na = No. of Different Alleles,

Ne = No. of Effective Alleles = 1 / (p^2 + q^2), I = Shannon's Information Index

= -1* (p * Ln (p) + q * Ln(q)), He = Expected Heterozygosity = 2 * p * q,

%p = Polymorphism percentage.

on Nei's genetic distance of provinces was drawn (Fig. 2), Markazi and Charmahal and Bakhteiari (pops 2 and 4) were placed close to each other, followed by Esfahan and Kuhgiluyeh and Boyer-Ahmad provinces (pops 1 and 3), while Kurdestan (pop 5) differed from other provinces and joined the others with a greater distance.

AMOVA test showed significant genetic difference ($p<0.05$) among provinces and revealed that 11% of total genetic variation is due to among group difference, while 89% is due to within group genetic variation. This indicates the presence of high degree of genetic difference among as well as within each province.

Mantel test (Fig. 3) showed no significant correlation ($p = 0.40$) between geographical distance and genetic distance. Therefore, isolation by distance does not occur in *Chara* species studied. This is also supported by Fst values of population groups determined by STRUCTURE. These values varied from 0.00-0.07, which are very low Fst values showing gene flow among geographical regions. Fst values obtained for population groups are in agreement with Gst results of ISSR alleles presented earlier, both indicating presence of shared common alleles and gene exchange among populations.

*Genetic affinity of Chara species and populations*

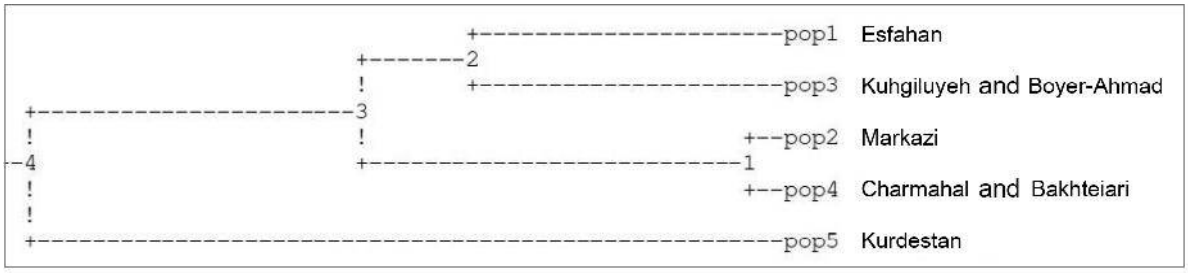


**Fig. 2.** UPGMA tree of provinces based on Nei's genetic distance.

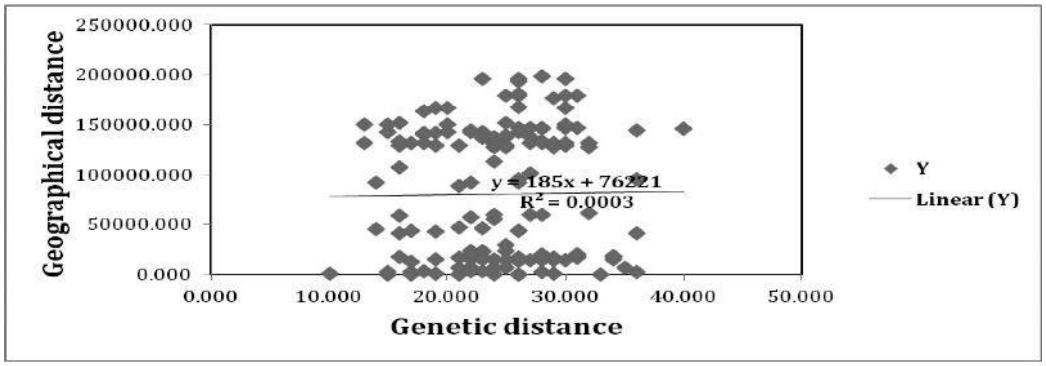


**Fig. 3.** Mantel plot between geographical and genetic distance.

NJ and UPGMA tree of ISSR data produced similar results, but since NJ tree had a higher cophenetic correlation (0.93), it is discussed here (Fig. 4). Outgroup species, *Nitella hyallina,* is placed separate from *Chara* species studied, while our ingroup taxa are placed in 2 major clusters. In the first major cluster, *C. vulgaris* var. *vulgaris* and *C. vulgaris* var. *longibracteata*, and populations of *C. tometosa* are placed close to each other and along with *C. crassicuais, C. connivens* and *C. peduculata* form this major cluster.

The second major cluster contains 3 sub-clusters, showing closer affinity between *C. gymnophylla* var. *rohlenae* and *C. gymnophylla* var. *gymnophylla*, *C. contraria* and *C. kohrangia* which along with 1 population of *C. vulgaris* var. *longibracteata* comprise the first sub-cluster. *C. fibrosa* and *C. kirghisorum* form the second sub-cluster, while *C. socotrensioides* and 1 population of *C. gemnophylla* comprise the third sub-cluster.

Close affinity between *C. vulgaris* and *C. crassicuais, C. contraria* and *C. kohrangiana*, as well as between *C. gymnophylla* and *C. contraria* is in agreement with our earlier morphometrical study (Ahmadi et al., 2012b). Moreover separation of *C. fibrosa, C. kirghisorum* and *C. socotrensioides* from *C. vulgaris* and *C. gymnophylla* and their position in a separate cluster is also supported by morphology.

MDS and PCoA plots separated different species and populations in 2 major groups (Figures not given), supporting NJ tree. *Nitella hyallina*, the outgroup species was separated from the other *Chara* species and populations of *C. vulgaris* and *C. gymnophylla* were placed far from each other due to genetic variation. Test for linkage disequilibrium performed for ISSR loci, as implemented in POPGENE, did not show any significant association between them supporting Dentrented correspondence anal-

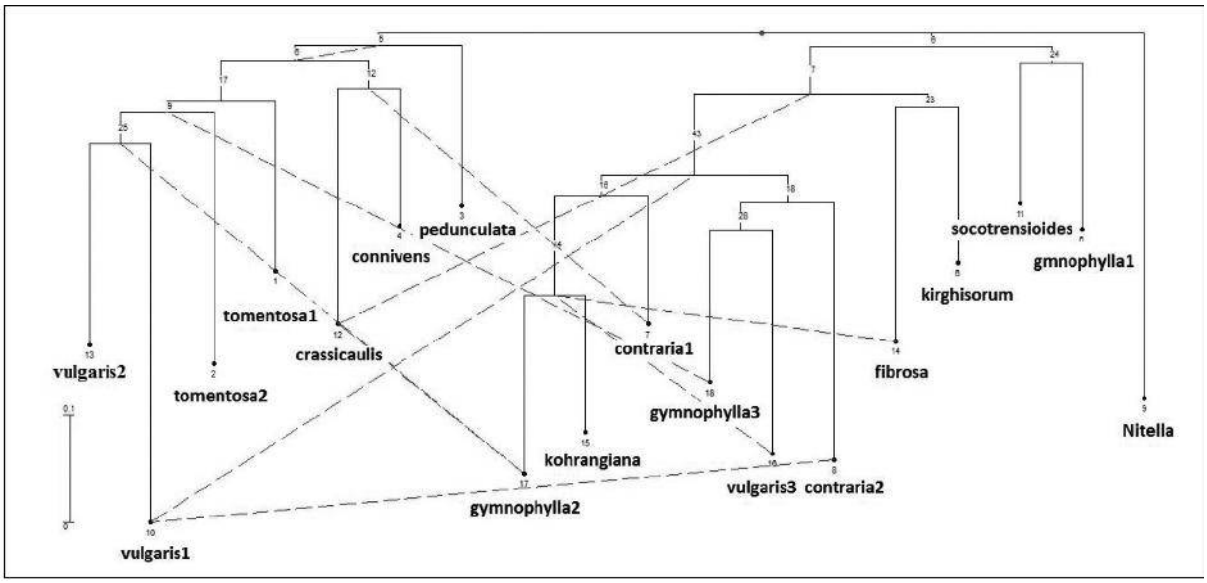


**Fig. 4.** NJ tree and reticulation of *Chara* species and populations. (numbers above branches are bootstrap values, dashed lines indicate possible gene exchange).

ysis. Therefore, these molecular markers were considered to meet the assumptions for applying the Bayesian method implemented in the program STRUCTURE to assign individuals to population groups.

Q-matrix plot of STRUCTURE analysis (Fig. 5) showed presence of 2 major subgroups. Evanno method and ad hoc statistic ΔK also showed k = 2 as the best number of population subgroups (figure not given). STRUCTURE plot showed that populations of *C. tomentosa* (No. 1 and 2) and *C. contraria* (No. 9 and 10) populations differed only in allele frequencies (different proportion of segments with similar colors), while *C. gymnophylla* differed in allelic composition (different colours). This holds true particularly for *C. gmnophylla* var. *rohlenae* collected from Markazi province (No. 5 in Fig. 5). Similarly, *C. vulgaris* populations differed in both allelic composition and frequency. Mannschreck et al. (2002) used AFLP markers to study 33 samples of 3 *Chara* species, *C. hispida*, *C. intermedia* and *C. tomentosa* and found great interspecific molecular diversity.

CCA plot (Fig. 6), showed that all 3 ecological factors studies contribute to distribution of *Chara* species, however, we may say that latitude has prominent effect on *C. tomentosa*, while combination of both latitude and altitude affect *C. gymnophylla* and in particular combination of longitude and altitude affect *C. contraria* and *C. vulgaris*.

Reticulogram (Fig. 4) showed gene exchange/shared common genetic loci among most of the *Chara* species and populations studied. Gene exchange occurred between

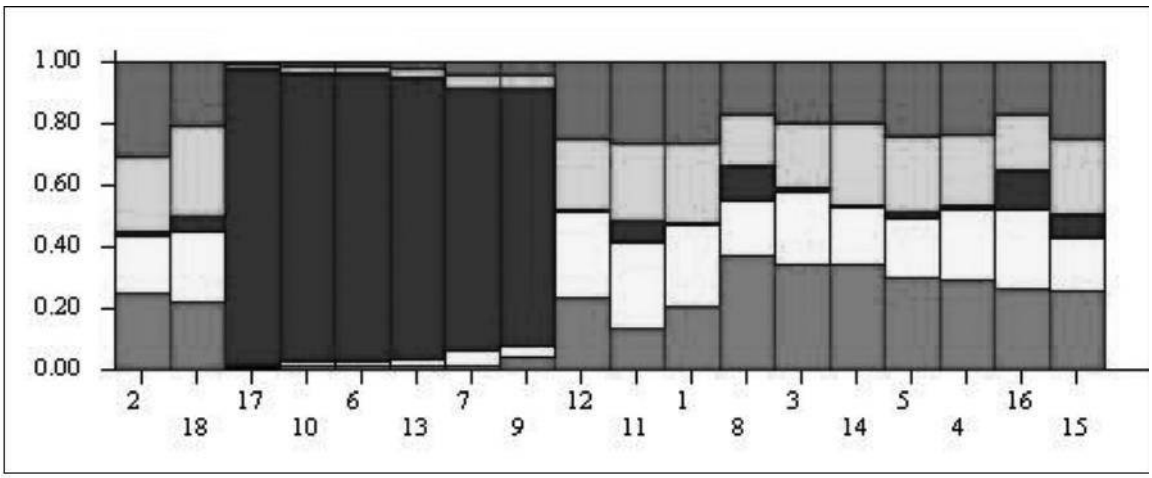


**Fig. 5.** Q-matrix plot of STRUCTURE.

Populations abbreviations: 1 & 2 = *C. tomentosa*, 3 = *C. pedunculata*, 4 = *C. connivens*, 5-7 = *C. gymnophylla*, 8 = *C. kirghisorum*, 9 & 10 = *C. contraria*, 11-13 = *C. vulgaris*, 14 = *C. socotrensioides*, 15 = *C. crassicaulis*, 16 = *C. fibrosa*, 17 = *C. kohrangiana*, and 18 = *Nitella hyalina*.

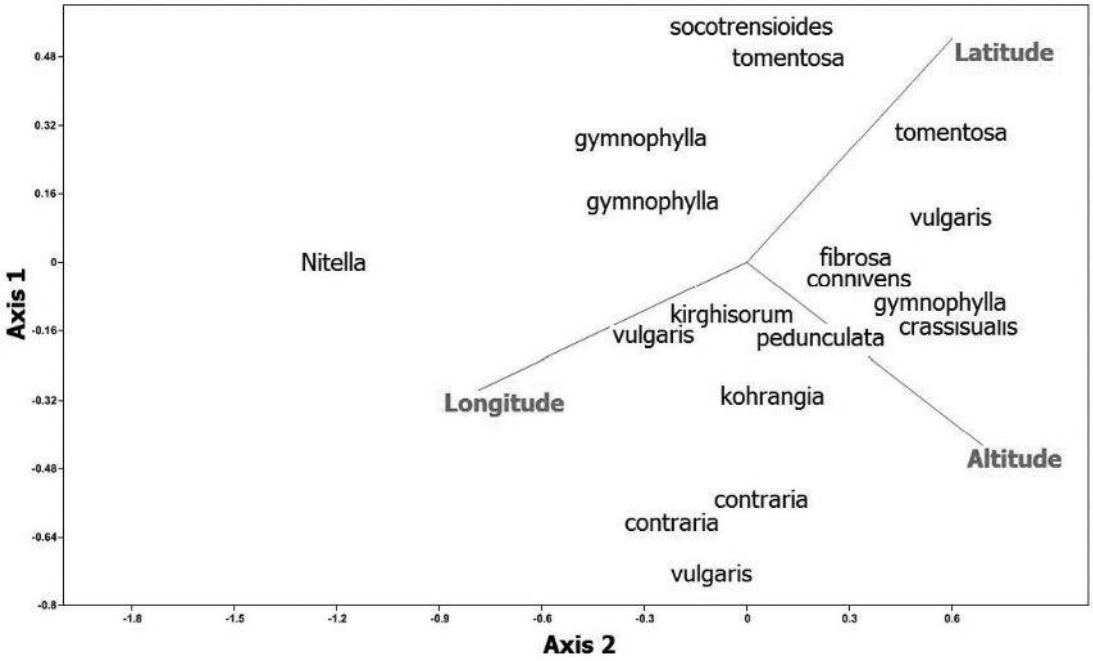


**Fig. 6.** CCA plot of ISSR data.

species from both clusters of NJ tree. These results support STRUCTURE analysis results and further illustrated the source of common allelic composition identified in STRUCTURE plot.

Coalescence analysis produced gene trees which grouped *Chara* species somewhat different from NJ tree and when gene trees were contained in population tree, the best result obtained showed deep coalescence cost of 23 with 8 duplication and 39 extinction (Fig. 7).

To summarize the findings, we may say that ISSR markers showed genetic diversity among and within *Chara* species. These molecular markers discriminate *Nitella hyalina* from *Chara* species and also can reveal *Chara* species relationship. Bayesian analysis of data showed genetic differences of the species studied and grouped them in 2 separate genetic groups and indicated great genetic admixture among species as also supported by reticulation analysis. Coalescence results showed that along with cytological change in *Chara* species which is a main evolutionary mechanism, gene duplication and extinction have played a role in species diversification.

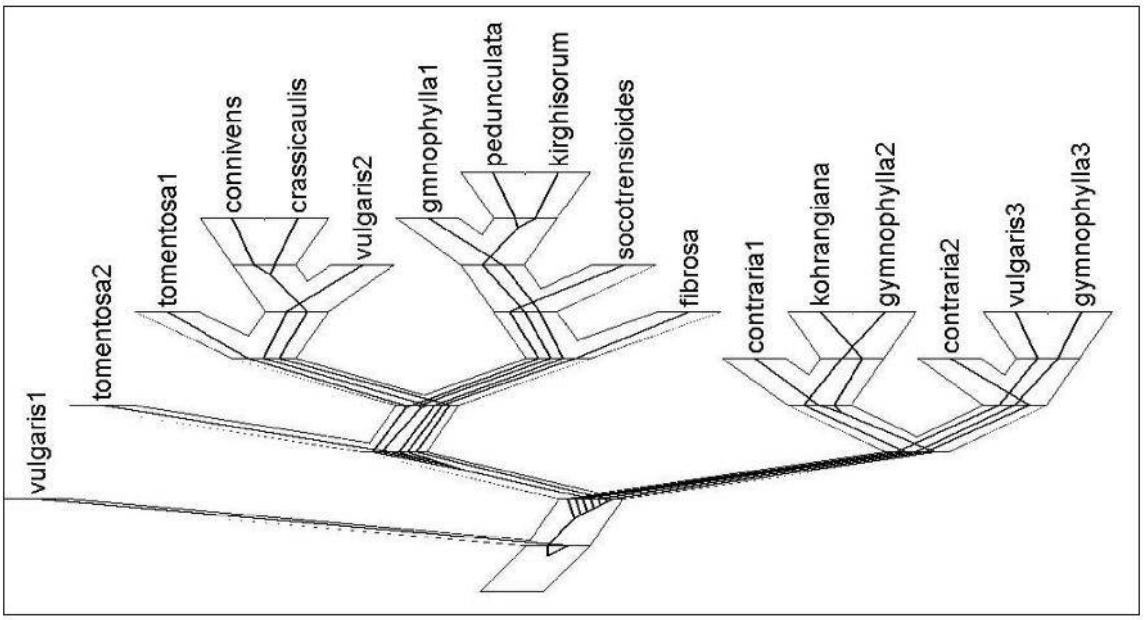


**Fig. 7.** Coalescence tree of *Chara* species. (gene tree is contained in species tree).

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