

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) (Rodriguez, 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 $\times 400$ and $\times 1000$ 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 1 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 ($134\mu\text{mol NO}_3^-$ and $120\mu\text{mol PO}_4^{-3}$). 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*.

$$C_{\text{chl}} = 13.1 \times \text{OD}_{665}$$

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 Tukey (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 (Tukey's HSD, $p < 0.05$) (Fig. 1E). *N. spongiaeforme* filtrate significantly increased the number of *Monoraphidium minutum* (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 vulgaris*, and *Monactinus simplex* from Chlorophyta and *Navicula causpidata* from Diatoms (Tukey'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. causpadata, *Chlorella vulgaris*, in *Nostoc spongiaeforme* filtrate decreased rather than control, while the number of *Cylindrospermum indicum* and *Monoraphidium minutum* 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 devastated 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	<i>A. vaginicola</i>	<i>O. limosa</i>	<i>N. spongiaeforme</i>
Colonial Cyanobacteria			
<i>Merismopedia elegans</i> A. Braun ex Kützing	0	0	-
<i>Chroococcus minor</i> (Kützing) Nageli	0	0	0
Filamentous Cyanobacteria			
<i>Cylindrospermum indicum</i> C. B. Rao	0	+	+
<i>Calothrix ghosei</i> Bharadwaja	0	0	0
<i>Anabaena vaginicola</i> F. E. Fritsch & Rich	+	0	0
<i>Nostoc spongiaeforme</i> C. Agardh ex Bornet & Flahault	0	0	+
<i>Oscillatoria limosa</i> C. Agardh ex Gomont	0	+	0
Chlorophyta			
<i>Tetraedron minimum</i> (A. Braun) Hansgirg	0	0	0
<i>Scenedesmus opoliensis</i> P. G. Richter	0	0	-
<i>Monoraphidium minutum</i> (Nageli) Komarkova- Legnerova	0	0	+
<i>Ankistrodesmus falcatus</i> (Corda) Ralfs	0	0	0
<i>Chlorella vulgaris</i> Beijerinck	0	0	0
<i>Monactinus simplex</i> (Meyen) Corda	0	0	-
<i>Characium acuminatum</i> Braun	0	0	0
Bacillariophyta			
<i>Fragilaria crotonensis</i> Kitton	0	0	0
<i>Navicula causpadata</i> Kützing	0	0	-

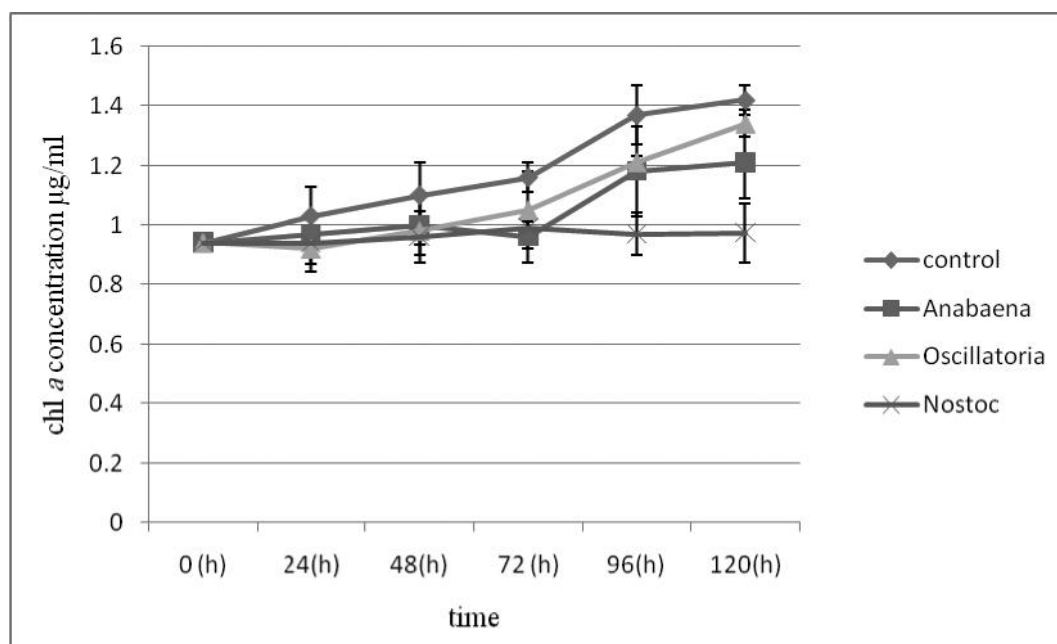


Fig. 1. Chlorophyll *a* concentration ($\mu\text{g}/\text{chl } a \text{ l}^{-1}$) of the phytoplankton community, treated with cell-free filtrates of *N. spongiaeforme*, *A. vaginicola* and *O. limosa*, as well as the control medium.

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 either positive or negative. Positive effect: whereby the target species have a growth rate higher than control (for example cyanobacterium *C. indicum* and *M. minutum* 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-

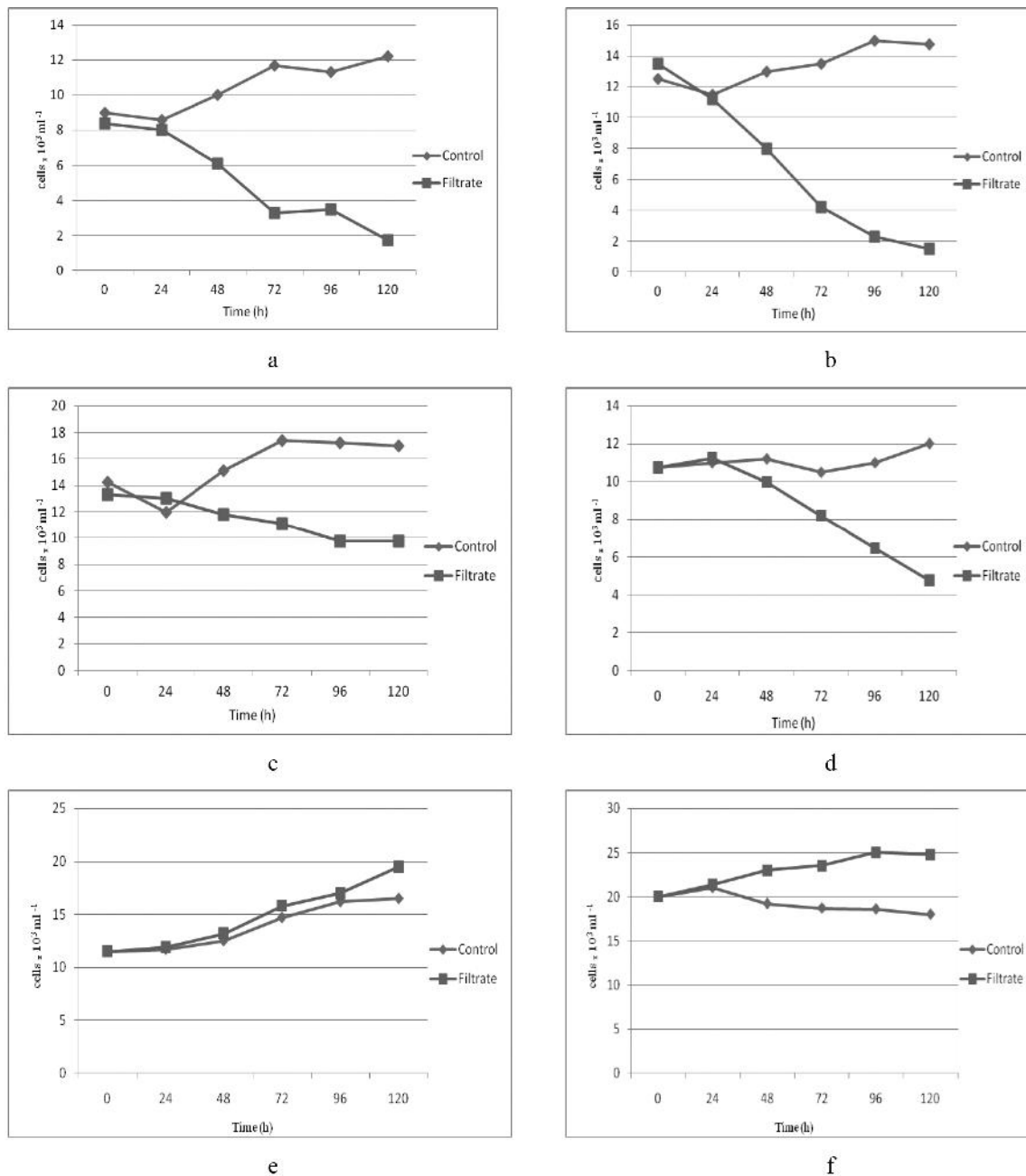


Fig. 2. Daily change of species in control and in exposing cell-free filtrate of *Nostoc spongiaeforme*. a) *Monactinus simplex*, b) *Scenedesmus opoliensis*, c) *Navicula causpidata* d) *Chlorella vulgaris*, e) *Cylindrospermum indicum*, f) *Monoraphidium minutum*.

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 susceptible 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 (Rodriguez 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. tamarensis* the allelopathic behavior of *A. tamarensis* was weak. When the cell density of *A. tamarensis* 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 allelochemicals 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.

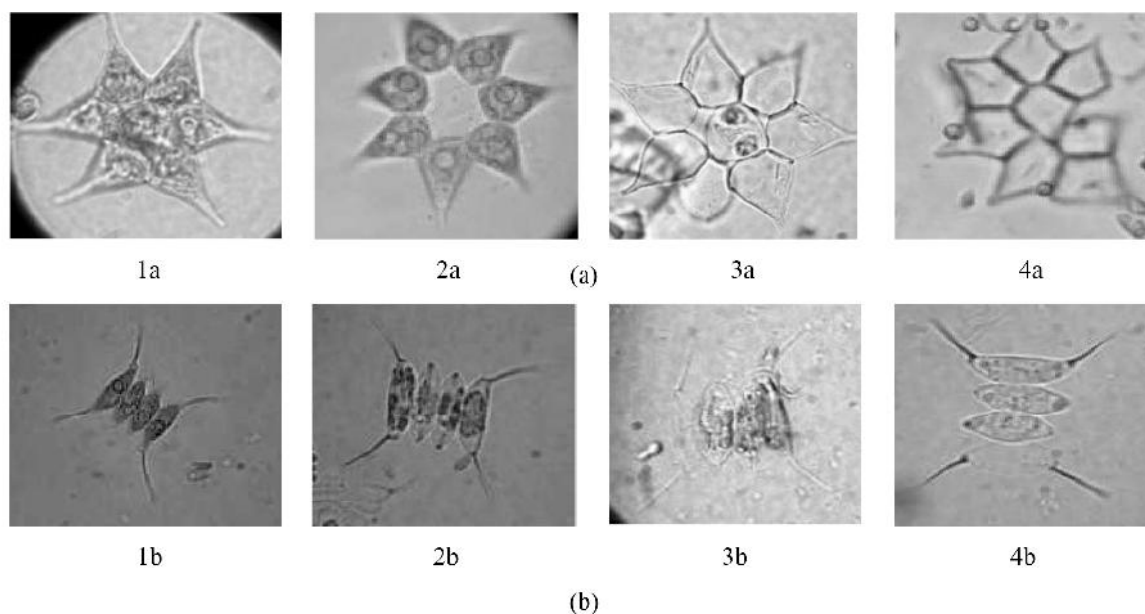


Fig. 4. The figures show how the cells change and the cell lysis. Different stages of cell's life of *Monactinus simplex* (a) and *Scenedesmus opoliensis* (b) exposed to *N. spongiaeforme* filtrate. First picture shows a cell that has not been exposed to filtrate. Pictures 2, 3 and 4 were after 2, 4 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) 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. ilicifolium* (0 and 15, replaced on protein resources of shrimp diet) were used. Seaweed supplement in formulated feed not only improved the humidity absorbance ($110.20\% \pm 5.00\%$) and stability of pellet feed in seawater ($98.11 \pm 3.23\%$) 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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