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Effect of Different Levels of *Spirulina platensis* on Growth Performance, Intestinal Morphology, Gut Microflora, Carcass Characteristics and Some Blood Parameters in Broiler Chickens

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Abstract

The effect of different levels of *Spirulina platensis* on growth performance, intestinal morphology, gut microflora and some blood parameters in broiler chickens was investigated in this study. A total of 300 Ross 308 broiler chicks with initial weight of 36.28 ± 0.38 g were used in a completely randomized design with 5 treatments, 4 replicates and 15 broiler chicks in each replicate. Experimental diets included control diet (with no additive), 3 levels of algae (1, 1.5, 2 g/kg diet), and the birds were fed by one level of prebiotic (1g/kg diet) as a positive control from 1 to 42 day of age. Results showed that Feeding broilers with 2g/kg algae feed caused the highest weight gain compared to other experimental groups (P<0.05). The highest feed intake related to treatments include 1.5 and 2 g/kg algae diet and the lowest related to treatment include 2g algae/ kg diet (P<0.05). The concentration of white blood cells, IgY, IgM, Ca and Phosphate in blood serum of broilers fed with 2 g/kg alga diet was higher than other groups, however, the concentration of malondialdehyde (MDA) was lower (P<0.05). Supplementation of diet with alga increased the number of lactobacillus in gut (P<0.05). Also, villus height and the ratio of villus height/crypt depth in duodenum, jejunum and ileum of broilers fed with 2g/kg algae was higher than other groups (P<0.05). In conclusion, *S. platensis* improved growth performance, villus height, white blood cell (WBC) count and decreased MDA in serum of broiler chickens, so it can be considered as a useful additive in broiler chickens diet.

Keywords: Broiler chicken, Gut microflora, Intestinal morphology, *Spirulina platensis*

Introduction

Using of antibiotic growth promoters as feed additives has been banned by the European Union in 2006 due to cross-resistance against pathogens and residues in tissues, so scientists searched for alternatives to antibiotics (Hajati et al., 2011). In this view, medicinal plants and essential oils extracted from plants are becoming more important due to their antimicrobial characteristics and the stimulating effect on animal digestive systems (Ciftci et al., 2005). Beneficial ef-

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fects of herbal additives in farm animal may be due to the positive effects on feed intake and digestive secretions, immune stimulation, antibacterial, coccidiostatical, antihelmintical, antiviral or anti-inflammatory activity (Fotea et al., 2010).

Spirulina (Blue green algae) is a microscopic single cell algae which grows in fresh water and has a simple structure but a complex composition. It is a concentrated source of food containing various nutrients, antioxidants, and probiotics properties. Moreover, it is an important source of the blue photosynthetic pigmented protein C-PC, which has strong antioxidant and anti-inflammatory properties. Interestingly, *Spirulina* is known for its wide ranging biological activities, like prevention of anemia because of high iron and vitamin contents (Huang et al., 2005), inhibition of herpes simplex infection (Ferrira-Hermosillo et al., 2011). It was demonstrated that the ethanolic extract of *S. platensis* include alkaloids, flavonoids, glycosides, tannins and phenolic compounds, steroids and saponins (Anbarasan et al., 2011). Kaoud (2012) conducted a trial to investigate the effects of dietary supplementations of prebiotic (Lactose and Myco) and *S. platensis* on broiler performance, carcass yield, and organs weights. The researcher reported that the body weight, average daily weight gain, feed conversion ratio (FCR), and carcass yield of birds were significantly increased by the dietary inclusion of the prebiotic and *S. platensis* ($P<0.05$) as compared to the control group. Bonos et al. (2016) investigated the effects of dietary *Spirulina* on *growth performance*, meat oxidative stability and fatty acid profile of broiler chickens. They found that body weight gain (at 21th day and 42th day), feed conversion ratio, mortality, breast and thigh meat lipid oxidation did not differ among the groups. Also, the fatty acid profile of the thigh meat was enriched in polyunsaturated fatty acids, especially eicosapentaenoic acid and docosahexaenoic acid after *Spirulina* supplementation. Lokapirnasari et al. (2016) examined the effect of *Spirulina* as feed additive to myocardial necrosis and leukocytes which were infected by Avian Influenza H5N1 virus. They reported that there was no significant difference ($P>0.05$) in myocardial necrosis and significant difference ($P<0.05$) in leukocyte in the treatment of *Spirulina*. The researchers conclude that *Spirulina* can be used as feed additive to increase immunity of broiler chicken (Lokapirnasari et al., 2016).

Considering the beneficial effects of dietary *S. platensis* in broilers, this study was conducted to evaluate the effect of different levels of *S. platensis* on *growth performance*, intestinal morphology, gut microflora and some blood parameters in broiler chickens.

Materials and Methods

Diets and management

This experiment was carried out using a total of 300 Ross 308 (mixed-sex) broiler chicks. One day old chicks (initial weight, 36.28 ± 0.38 g) were obtained from a local hatchery and divided into 20 groups of 15 birds each. On arrival, chicks were weighed and randomly housed in wood shavings cov-

ered floor pens (each 1×1.2 m). There were 5 experimental diets including 0, 1, 1.5, 2 g/kg algae diet, and 1 g prebiotic/kg diet as a positive control. The feeding program consisted of a starter (1 to 10 day old), grower (11 to 22 day old), and finisher diet (23 to 42 day old). The basal diet was in mash form and prepared with the same batch of ingredients for starter, grower, and finisher periods and was formulated to meet the nutrient requirements according to Ross 308 guideline. All birds had free access to feed and water during the whole rearing period. The ingredients and chemical composition of the basal diets are

Table 1. Ingredients and nutrient composition of basal experimental diets.

Ingredient (g/kg)	1-10 d	11-28 d	29-42 d
Corn, ground	522.4	562.8	665
Soybean meal	410.1	361.5	259.9
Soybean oil	23.5	37.7	35.7
Dicalcium phosphate	18.2	15.8	17.2
Oyster shell	12.3	11.2	11.6
Common salt	3.6	2.8	2.9
Minerals mix[1]	2.5	2.5	2.5
Vitamins mix[2]	2.5	2.5	2.5
DL-Methionine	2.8	1.8	1.7
L-Lysine hydrochloride	2.1	1.5	1.4
$NaHCO_3$	0.0	1.0	1.0
Calculated composition			
ME (kcal/kg)	2880	3000	3100
CP (%)	22.5	21	18.89
Ca (%)	0.96	0.88	0.78
AP (%)	0.48	0.44	0.38
Lysine (%)	1.39	1.14	0.97
Methionine (%)	0.66	0.55	0.48
Methionine+Cystine	1.05	0.90	0.78

[1]Mineral mix supplied the following per kg of diet: Cu, 20 mg; Fe, 100 mg; Mn, 100 mg; Se, 0.4; Zn, 169.4 mg.

[2]Vitamins mix supplied the following per kg of diet: vitamin A, 18,000 IU; vitamin D3, 4,000 IU; vitamin E, 36mg; vitamin K_3, 4 mg; vitamin B_{12}, 0.03 mg; thiamine, 1.8 mg; riboflavin, 13.2 mg; pyridoxine, 6 mg; niacin, 60 mg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 0.2 mg; choline chloride, 500 mg.

shown in Table 1. Feed was prepared weekly and stored in airtight containers. Temperature was initially set at 34°C on day 1 and decreased linearly by 0.5°C per day. During the study, the birds received a lighting regime of 23 hours light and 1 hour dark (23L: 1D) from 1 to 42 day.

Spirulina platensis analysis

S. platensis algae samples were cultivated on July of 2017 in ACECR, Sari. Briefy, *S. platensis* was grown in modified Zarrouk's medium. Algae were incubated in a pond (12 m2) with paddle-wheels at mean temperature and irradiance of 29°C, 4 klux, respectively. Harvesting was performed after 12-14 days. The composition of *S. platensis* was measured by AOAC (Association of Official Analytical Chemists) procedures (AOAC, 1990).

Growth performance

The experimental period lasted 42 day. On day 1, 28, and 42, birds were pen weighed and feed consumption was recorded. Feed conversion ratio was calculated for each period.

Carcass characteristics

At 42 day old, two birds per pen close to the mean weight for the pen were selected and killed by cervical dis-location, to determine the carcass traits. The edible carcass (without viscera or feet), breast, drumstick + thigh, liver, empty gizzard and abdominal fat were weighed and expressed as percentages of live body weight.

Gut morphology

Intestinal tissues were obtained immediately after slaughter at day 42. Segments were removed from the duodenum, jejunum and ileum as follows. Intestine from the gizzard to pancreatic and bile ducts was referred to as the duodenum, the jejunum was defined as the portion of intestine extending from the bile duct entrance to Meckel's diverticulum and the ileum was defined as the region from Meckel's diverticulum to a point 40 mm proximal to the ileo-cecal junction. The relative length of duodenum, jejunum and ileum to 100 g live body weight was calculated. Jejunum samples (3 cm) were taken at the midpoint of each section and immersed in a phosphate-buffered formalin solution. Two portions per sample were cut perpendicular to the longitudinal axis of the intestine and embedded in paraffin wax. Transverse sections were cut (3 μm), stained with hematoxyline-eosin and analyzed under a light microscope (Model U-TV0.5 XC-2, Olympus corporatration, BX51, Japan) to determine morphometric indices using image-analysis software (DP2-BSW). Measurements for the villi height were taken from the tip of the villus to the villus-crypt junction.

Ileal microflora

The ileums were excised and contents were collected by gently pressing the fingers to move the content into tubes at 42 day of age. Digesta of birds within a replicate were pooled and put on ice until they were transported to the laboratory for enumeration of microbial populations. One gram of ileal content was homogenized in 9 ml sterile water. Each sample was serially diluted. Using these diluted sub-samples, *Lactobacillus* was enumerated on De Man-Rogosa-Sharpe (MRS) agar after incubation at 37°C in an anaerobic

chamber for 48 hours (Guban et al., 2006) and coli-forms and *E. coli* was counted on CHROM agar ECC (EF322-Paris France) after incubation at 37°C in an aerobic chamber for 48 hours (Sallam, 2007).

Hematological Parameters

At day 42, two birds from each replicate were selected and their blood samples were collected using sterile syringes (2 ml) to draw blood from the wing vein. Samples were collected in EDTA-containing tubes. Blood smears were prepared on slides and painted by Giemsa method. The white blood cell counts were determined by an improved Neubauer hemocytometer method (Jain, 1993). To prevent coagulation, blood samples were mixed with EDTA and centrifuged at 3000g for 10 minutes. Plasma glucose concentration was measured using commercial laboratory kits (zistshimi and parsazmoon) with god-pap method at 546 nm wavelengths. triglyceride, cholesterol, LDL-cholesterol and HDL-cholesterol were measured using commercial laboratory kits (Friedewald et al., 1972).

Statistical analysis

Data were analyzed by GLM procedures (SAS, 2001). Means were compared using Duncan's new multiple range test. The level of significance was reported at $P< 0.05$.

Results

Spirulina platensis analysis

The chemical composition (crude fat, crude protein, total carbohydrate, fiber, calcium, total phosphorus and ash) of the algae is shown in Table 2.

Growth performance

The effects of dietary supplementation of *S. platensis* on feed intake, body weight gain, feed conversion ratio, mortality and European production efficiency factor (EPEF) are shown in Table 3. In starter period, feed intake and body weight gain of broiler chicks fed with diet supplemented with 2g/kg *Spirulina* diet or prebiotic was higher than control group ($P<0.05$), however, feed conversion ratio of the chicks consumed 2g/kg *Spirulina* diet or prebiotic was lower than control group ($P<0.05$). In grower period, the body weight gain of broiler chicken fed with 1.5 or 2 g/kg *Spirulina* diet was higher than other groups ($P<0.05$). In finisher period and also whole period of rearing, broiler chicken fed with 2g/kg *Spirulina* or prebiotic had higher feed intake and body weight gain, however, their feed conversion ratio was lower than control group ($P<0.05$). Broiler chickens fed with 1.5 or 2g/kg *Spirulina* diet or prebiotic had lower mortality and higher European production efficiency factor in whole period of rearing.

Carcass characteristics

The effects of dietary supplementation of *S. platensis* on carcass characteristics are shown in Table 4. Supplement in diets with different

Table 2. Composition of the *Spirulina platensis* analyzed by AOAC methods.

Crude Protein (%)	Crude Fat (%)	Calcium (%)	Phosphorus (%)	Ash (%)
74	1.73	1.02	1.41	12.51

Table 3. Effects of *Spirulina platensis* on growth performance of broilers at different periods.

		Spirulina platensis (g/kg diet)					
Performance	Control	1	1.5	2	Prebiotic	SEM	Pr>F
1 to 10 day old							
FI (g)	218.35^{b}	224.11^{ab}	240.21^{a}	242.87^{a}	220.57^{ab}	7.75	0.03
BWG (g)	200.03^{d}	203.20^{c}	209.63^{b}	212.38^{a}	202.91^{c}	0.987	0.041
FCR (g/g)	1.31^{a}	1.22^{ab}	1.17^{b}	1.13^{c}	1.29^{a}	0.01	0.0003
11 to 28 day old							
FI (g)	1237.28^{b}	1240.06^{b}	1278.75^{ab}	1317.83^{a}	1234.38^{b}	12.46	0.02
BWG (g)	1151.5^{d}	1171.5^{c}	1238.25^{b}	1281.75^{a}	1167.75^{cd}	11.64	<0.0001
FCR (g/g)	1.53^{b}	1.44^{ab}	1.40^{bc}	1.45^{c}	1.58^{a}	0.013	0.014
29 to 42 day old							
FI (g)	3790.78^{b}	3844.17^{b}	3918.96^{a}	3960.7^{a}	3834.95^{b}	17.00	0.001
BWG (g)	1375.90^{c}	1303.40^{c}	1441.05^{b}	1488.71^{a}	1375.76^{c}	10.69	<0.05
FCR (g/g)	2.09^{a}	2.06^{a}	1.99^{b}	1.92^{c}	2.08^{a}	0.017	<0.05
1 to 42 day old							
FI (g)	3790.78^{b}	3844.17^{b}	3918.96^{a}	3960.7^{a}	3834.95^{b}	17.00	0.001
BWG (g)	2237.19^{c}	2266.25^{c}	2375.19^{b}	2468.06^{a}	2243.03^{c}	21.4	0.0011
FCR (g/g)	1.83^{a}	1.83^{a}	1.78^{b}	1.73^{c}	1.84^{a}	0.01	<0.0001
Mortality (%)	4.00^{a}	3.25^{b}	3.25^{b}	2.25^{c}	3.75^{ab}	0.4	0.05
EPEF*	279.4^{c}	285.3^{ab}	307.4^{b}	332.0^{a}	279.3^{c}	4.11	0.002

Means within the same row with uncommon superscript differ significantly (P < 0.05).

* European production efficiency factor

levels of *Spirulina* or prebiotic caused higher carcass yield at day 42 (P<0.05), however, breast (%), drumstick + Thigh (%) and abdominal fat pad (%) did not show significant differences among the groups (P>0.05).

Gut Gut morphology

The effects of dietary supplementation of *S. platensis* on gut morphology of broiler chicken are shown in Table 5. The villus height of duodenum in broiler chicken fed with 2g/kg *Spirulina* diet or prebiotic was higher than control group (P<0.05). The crypt depth and villus: crypt of duodenum in broilers fed with 1.5 or 2g/kg *Spirulina* diet or prebiotic was lower and higher than control group, respectively (P<0.05). The villus height of duodenum in broiler chicken fed with 1.5, 2g *Spirulina*/ kg diet or prebiotic was higher than control group (P<0.05). The crypt depth of broilers consumed different levels of *Spirulina* or prebiotic was lower than control group (P<0.05). The villus: crypt of duodenum in broilers fed with 1.5 or 2g/kg *Spirulina* diet or prebiotic was higher than control group, respectively (P<0.05). The villus height of ileum in broiler chicken fed with different levels of *Spirulina* was higher than control group (P<0.05).

Ileal microflora

As shown in Table 6, dietary supplementation of different levels of *S. platensis* or prebiotic decreased coliforms count of ileum content (P<0.05), however, the count of ileal *Lactobacillus subtilis* increased in broilers fed with different levels of *S. platensis* or prebiotic

Table 4. Effects of *Spirulina platensis* on carcass characteristics of broilers.

		Spirulina platensis (g/kg diet)					
	Control	1	1.5	2	prebiotic	SEM	Pr>F
42 day							
Carcass (%)	62.02^{c}	65.93^{a}	65.81^{a}	65.88^{a}	64.11^{b}	0.34	0.04
Breast (%)	24.97	24.91	24.9	24.91	24.94	0.016	0.67
Drumstick + Thigh (%)	15.84	15.71	15.78	15.78	15.80	0.023	0.63
Abdominal fat (%)	1.32	1.30	1.29	1.28	1.28	0.006	0.24

Means within the same row with uncommon superscript differ significantly (P < 0.05).

Table 5. Effects of *Spirulina platensis* on gut characteristics of broilers at 42 day.

		Spirulina platensis (g/kg diet)					
	Control	1	1.5	2	prebiotic	SEM	Pr>F
42 day old							
Duodenum							
Morphology (µm)							
Villus height	1857.61^{b}	1867.5^{b}	1995.75^{a}	2007.5^{a}	1861.5^{b}	17.93	0.0006
Crypt depth	199.37^{a}	185.52^{bc}	178.5^{c}	178.25^{c}	191.6^{ab}	2.23	0.0025
Villus: crypt	9.34^{c}	10.07^{b}	11.186^{a}	11.286^{a}	9.73^{bc}	0.18	<0.0001
Jejunum Morphology							
(µm)							
Villus height	1748.35^{c}	1833.25^{b}	1838.5^{b}	1927.5^{a}	1767.03^{c}	15.54	0.0001
Crypt depth	194.45^{a}	181.5^{c}	173.00^{d}	170.75^{d}	187.25^{b}	1.95	<0.0001
Villus: crypt	9.09^{d}	10.10^{c}	10.63^{b}	11.294^{a}	9.34^{d}	0.17	<0.0001
Ileum Morphology							
(µm)							
Villus height	851.93^{c}	1087.97^{a}	1081.05^{ab}	931.49^{bc}	1043.41^{ab}	2.078	0.0086
Crypt depth	121.67	147.94	157.26	134.60	142.04	0.875	0.0769
Villus: crypt	7.124	7.362	6.894	7.087	7.432	0.199	0.9117

Means within the same row with uncommon superscript differ significantly (P < 0.05).

(P<0.05).

Hematological parameters

The effects of dietary supplementation of *S. platensis* on hematological parameters of broiler chicken are shown in Table 7. Adding different levels of *Spirulina* or prebiotic to broilers diet increased hematocrit and phosphorus levels in blood (P<0.05), but it decreased the levels of MDA, cholesterol and triglyceride in blood of broilers (P<0.05). The concentration of calcium, white blood cell number and IgY titer increased in broilers fed with 1.5 and 2g/kg *Spirulina* diet or prebiotic (P<0.05). Broilers consumed 2g/kg *Spirulina* diet or prebiotic had higher levels of blood total protein and IgM titer (P<0.05).

Discussion

Growth performance

Today there is increasing interest for using natural feed additives such as non-digestible ingredients which are known as prebiotic or

Table 6. Effects of *Spirulina platensis* on ileal microbial population (log CFU/g of digesta) of broilers at 42 day

	Spirulina platensis (g/kg diet)						
	Control	1	1.5	2	prebiotic	SEM	Pr>F
Coliforms	7.94^{a}	7.21^{b}	7.11^{b}	6.05^{c}	7.27^{b}	0.12	<0.0001
Lactobacillus subtilis	5.5^{c}	6.67^{b}	6.58^{b}	7.58^{a}	6.87^{b}	0.13	<0.0001

Means within the same row with uncommon superscript differ significantly (P < 0.05).

Table 7. Effects of *Spirulina platensis* on hematological parameters in broiler chickens.

	Spirulina platensis (g/kg diet)						
	Control	1	1.5	2	prebiotic	SEM	Pr>F
Hematology, 42 day old							
WBC (10^3/ml)	153.11^{d}	154.22^{c}	156.18^{b}	159.18^{a}	153.1^{d}	0.52	<0.0001
RBC (10^6/ml)	2.17	2.30	2.35	2.34	2.32	0.02	0.23
Hematocrit (%)	29.2^{e}	30.37^{c}	33.41^{b}	35.42^{a}	30.22^{d}	0.53	<0.0001
MDA (μm/L)	1.72^{a}	0.915^{c}	0.895^{c}	0.892^{c}	1.14^{b}	0.07	<0.0001
Total protein	36.69^{c}	37.75^{bc}	41.56^{a}	39.47^{b}	37.5^{bc}	0.48	0.0011
Cholesterol	184^{a}	127.25^{b}	119.5^{b}	120.37^{b}	126.5^{b}	5.81	<0.0001
TG	46.65^{a}	38.00^{b}	26.78^{c}	26.04^{c}	37.75^{b}	1.8	<0.0001
Glucose	166.72	168.78	165.29	166.00	167.02	0.78	0.74
Calcium	2.64^{c}	2.95^{b}	3.05^{a}	3.107^{a}	2.60^{c}	0.05	<0.0001
Phosphorus	2.6^{d}	2.82^{b}	3.11^{a}	3.02^{a}	2.71^{c}	0.04	<0.0001
IgY	0.195^{c}	0.230^{b}	0.245^{ab}	0.225^{a}	0.207^{c}	0.006	0.0001
IgM	0.545^{b}	0.552^{b}	0.585^{a}	0.6^{a}	0.517^{c}	0.007	<0.0001

Means within the same row with uncommon superscript differ significantly (P < 0.05).

phytobiotics as growth promoters. Indeed, using antibiotics as growth promoters had been banned in 1999 by the European Union (European Commission) because of their detrimental effects such as microbial resistance to antibiotics, residues in chicken meat which might be harmful to human health and expansion of pathogenic microorganisms (Kaoud, 2015). In the present study, supplemental algae improved feed intake, body weight gain and feed conversion ratio of broilers. This is in contrast with the findings of Gognet et al. (2001) and Toyomizu et al. (2001). They did not find any significant effect of adding dietary *Spirulina* in the performance of broilers. However, Shanmugapriya et al. (2015) reported improvement of body weight gain and feed conversion ratio in broilers consumed *Spirulina* algae. Also, Mariey et al. (2014) reported that a low dietary level of *Spirulina* biomass (0.02 or 0.03%) improved performance of broiler chickens.

Carcass characteristics

Present experiment showed that *Spirulina* supplementation improved the carcass yield of broiler chickens. Raach-Moujahed et al. (2011) reported that *Spirulina* improved the carcass yield of Arbor Acres broiler at a rate of 2.5% of incorporation. Bellof and Alarcon (2013) reported that in organic farming, adding dietary *Spirulina* improved carcass performance parameters of broilers significantly.

Gut morphology

The critical role of villi height in the absorption of intestinal nutrients has been reported by Mekbungwan et al. (2002). Furbeyre et al. (2017) reported that *Spirulina* increased villus height of jejunum in weaned piglet. Also, Shanmugapriya et al. (2015) reported increased body weight gain and villus length in addition to fatty acid modification in broiler meat (Shanmugapriya et al., 2015). Present study revealed that dietary supplementation of *Spirulina* increased villus height in all segments of small intestine (duodenum, jejunum, ileum), which can increase nutrient uptake and cause higher digestibility of nutrients. So higher body weight gain in broilers consumed *Spirulina* may due to its positive effect on gut morphology.

Ileal microflora

Present study showed that *Spirulina* algae had positive effect on ileal micro flora. Previous researchers found that feeding dietary *Spirulina* may increase the Lactobacillus population and enhance the absorbance of dietary vitamins (Mariey et al., 2014).

Spirulina is one of the most important micro algae showing antimicrobial activity against many pathogenic bacteria and fungi (Kumar et al., 2013) and it contains active ingredients such as tocopherols, C-phycocyanin, and extracellular polysaccharide (Pradhan et al., 2012; Ciftci et al., 2005) which have antimicrobial activities against *Escherichia coli, Pseudomona* sp.*, Enterobacter* sp.*, Salmonella typhi, Klebsiella pneumoniae and Proteus vulgaris (Pradhan et al., 2012). Staphylococcus aureus, Staphylococcus epidermis* and *Aeromonas liquefaciens* (Shanmugapriya et al., 2015).

Hematological Parameters

This study showed that supplementing *Spirulina* in broiler diet increased RBC and hematocrit of birds. Zhang et al. (2001) found that

the polysaccharides of *Spirulina* increased the level of red blood cells, white blood cells, and hemoglobin in the blood, and also increased nucleated cells in bone marrow of dogs. They reported that Polysaccharide extract of S. platensis has chemo-protective and radio-protective capability, and may be a potential adjunct to cancer therapy (Zhang et al., 2001). It is reported that using phycocyanin after oxalate treatment significantly increased catalase and glucose-6-phosphate dehydrogenase activity ($p<0.001$) in RBC lysate suggesting phycocyanin as a free radical quencher (Farooq et al., 2006). So, the reduction in MDA concentration in this trial may due to free radical quenchering effect of *Spirulina*. Huang et al. (2005) reported that *Spirulina* polysaccharides decreased blood glucose and could protect the vascular of alloxan induced diabetic rats. This is in contrast with the findings of the present study as we don't see any different in blood glucose concentration. A reduction in serum cholesterol and increasing in IgG observed in the current study is in agreement with Zeweil et al. (2016). The antioxidant materials such as phycocyanin and phenolic compounds, and poly-unsaturated fatty acids in the microalgae *Spirulina* may be the cause of the properties of *Spirulina* on the decrease of serum lipids levels (Colla et al., 2008). Nagaoka et al. (2005) reported that S. platensis concentrates or phycocyanin, a pigment extracted of *Spirulina*, caused hypocholesterolemic activity in rats.

In conclusion, *Spirulina* improved *growth performance*, intestinal villus height, ileum Lactobacillus count, carcass yield and humoral immunity of broiler chickens. We recommend the addition of 2g ***Spirulina***/Kg diet to improve performance and EPEF of broiler chickens. It seems that further study is needed to clarify the exact effect of *Spirulina* on physiological mechanisms in broilers body.

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The Effects of Physico-Chemical Parameters and Situation of Dam Reservoirs on Their Phytoplankton Population in West Azarbaijan Province (North-West Iran)

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Abstract

This study was performed to determine relationship between dam reservoir physico-chemical parameters and the phytoplankton structure in 7 reservoirs of west Azarbaijan province. Samplings were carried out during July 2016. phytoplanktons were collected, identified and enumerated then chemical parameters analysed for each sampling site. Principal component analysis (PCA), Detrended Correspondent Analysis (DCA) and two-way Unweighted Pair Group Method with Arithmetic mean (UPGMA) were performed to determine the environmental variables affecting phytoplankton community dynamics. Seventy-three species belonging to five divisions were determined during this study. The result of PCA and DCA was confirmed by UPGMA analysis, in which three main groups were clustered on the basis of their correlation with phytoplankton community changes and environmental parameters. Totally, highly disturbed reservoirs contained different phytoplankton community than undisturbed ones.

Key words: PCA, DCA, Reservoirs, Phytoplankton, West Azarbaijan

Introduction

Reservoirs are artificial lentic water bodies, generally, associated with multiple objectives for human benefits such as water supply, irrigation, fisheries, hydroelectric power and recreation. Land use patterns are changing rapidly in many parts of the world (Sala et al., 2000). The phytoplankton composition can reflect the ecological status of reservoirs and respond both qualitatively and quantitatively changes (El-Otify, 2002). The dynamics and species diversity of phytoplankton are greatly influenced by physico-chemical variables (Harris, 1986; Reynolds, 1986; Sommer, 1989). Watershed land use affects the amount of nutrients exported into lakes and reservoirs via stream inflows (Knoll et al., 2003). Phytoplankton is affected by different environmental factors such as pH, light, and temperature (Buzzi, 2002; Çelekli et al., 2007).Watersheds dominated by agricultural or urban lands typically export nutrients at higher rates than undisturbed watersheds (Beaulac and Reckhow, 1982; Puckett, 1995). However, considerable variation exists in the relationship between land use and watershed nutrient export (Mueller et al., 1995; Puckett, 1995), as well as in the relationship between

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nutrient loading rate and nutrient concentrations, algal biomass, and algal density of different groups (Carpenter et al., 1998; Correll, 1998; Smith, 1998). Export of sediments from watersheds is also correlated with land use. Agricultural watersheds, particularly export considerable quantities of sediment as well as nutrients. Loading of sediments can reduce algal productivity by decreasing light intensity (Hoyer and Jones, 1983; Knowlton and Jones, 1995). Reservoirs can be especially influenced by inputs of nutrients and sediments because they have relatively large watersheds compared to natural lakes (Wetzel, 1990). Higher loadings of nutrients through watershed streams enhance algal blooms mainly cyanobacteria and impairment of water quality in freshwater ecosystems such as reservoirs and lakes. This may lead to change phytoplankton main groups ratios and reducing species richness and biodiversity.

At present, more than 50000 reservoirs with dams higher than 15 m exist all over the world. It was suggested that reservoirs serve as stepstones for phytoplankton, thus facilitating their dispersal (Dumont, 1999). The proliferation of cyanobacteria and the invasion of *Ceratium* hirundinella are mainly a consequence of building cascades of reservoirs on large rivers (Gil et al., 2012; Cavalcante et al., 2013).

To our knowledge, no multiple-lake studies have explicitly quantified the relationship between land use and phytoplankton primary productivity. In addition, we know of no studies explicitly relating land use to any water physical and chemical factors and eutrophication indicators in reservoirs. In this paper, we explore how agricultural land use in watersheds is related to phytoplankton primary productivity, and associatedwith water physical and chemical parameters in reservoirs.

Materials and Methods

Samplings were carried out during July 2016. One sample were taken from each reservoir, except for Aras reservoir which due to its great dimensions, three samples were picked out. Phytoplankton samplings was carried out by Ruttner model sampler from surface 0.5m water layer. Phytoplankton samples were immediately preserved with 4% formaldehyde solution for identifacetion and analysis. Phytoplankton samples were preserved in cold, dark conditions for 3-4 days before laboratory analysis for precipitation of microalgae. Additional discrete samples were collected from the same depths for chemical analysis) Greenberg et al., 1992). Water temperature, dissolved oxygen (DO), electron conductivity (EC) and pH were measured in situ at every sampling site in the superficial water layer (50 cm depth) with a WTW 320 Oxymeter, a WTW LF 320 EC meter and a Testo 320 pH meter respectively. Phytoplankton counts and identifications were made in three repeats with 5-mL settling chambers with a Nikon TS100 inverted microscope at 400× magnification by Utermöhl's (1958) method. At least 50 fields or 100 individuals of the most abundant species were counted in each sample.

The taxonomic composition, classes, orders, family and species and density of the phytoplankton community at each site were determined. The phytoplankton taxa were identified

based on Prescott (1962), Tiffany and Britton (1971) and Bellinger (1992); Cyanobacteria were identified according to the method of Komarek and Anagnostidis (1989, 2005). Dissolved total phosphorus were analyzed according to the methods described by Greenberg et al. (1992). Total phosphorus (TP(concentrations determined with a spectrophotometer model T80+ UV/VIS (PG Instruments Ltd., Leicestershire, UK). Water transparency was measured with a 30 cm diameter Secchi disc. Principal component analysis (PCA) was performed to observe sample waters on the basis of their environmental parameters and to reduce the phytoplankton data down to a few statistically significant taxa whose density distribution patterns were driving the total variance in the dataset. Two-way clustering of samples was carried out using the unweighted pair group method with arithmetic mean (UPGMA), according to the environmental parameters. The data were standardized (mean = 0, variance = 1) before running the analysis. The Euclidean distance was determined among the studied samples from standardized data. The distance matrix obtained was then used to construct the UPGMA tree. PCA and two-way clustering were performed by PAleontological STatistics (PAST) version 3.04 (Hammer et al., 2001) program.

Results

Totally, 33 phytoplankton species were recognized in studied reservoirs, belonged to 6 main phytoplankton groups including Chlorophyta (10 species), Cyanobacteria (4 species), Bacillariophyta (12 species), Pyrrhophyta (3 species), Desmidaceae (3 species) and Euglenophyta (1 species) (Table 1, Figs 1-7).

Some physico-chemical parameters of the reservoirs are indicated in Table 2. In the PCA model with all the selected environmental variables pc1, pc2 and pc3 explained 74.34, 17.27 and 6.9% of the variance in phytoplankton reservoirs communities, respectively.

The separation between the two types of reservoirs results mainly from the environmental variables correlated with the first PCA axis (Fig. 8). Reservoirs were positively correlated with component 1, mostly related to chlorophyta, EC and TDS. In general, these reservoirs presented smaller watersheds dominated by agriculture, with significant urban areas (Fig. 8). All reservoir types were clearly dominated by Bacillariophyta and Chlorophyta. *Cyclotella meneghiniana, Diatoma vulgaris, Pediastrum duplex, Navicula* sp., and *Microcystis botrys*, were positively correlated with Type 1 reservoirs and with the first PCA component (Fig. 8).

Sites on the right side of the first DCA axis lay in a fenced area. In general, these undisturbed sites were clearly dominated by non-tolerant taxa Bacillariophyta and Chlorophyta, mainly associated mesotrophic states of water bodies (Van Dam et al., 1994), Desmides and negatively correlated with axis 1 and associated with reference sites were mainly tolerant taxa, mostly *Scenedesmus* sp.*, Synedra ulna* and cyanobacteria.

Analysis of loading weights of reservoirs on two first axis of DCA indicated that Ar2 and Ara reservoirs had the lowest weights on axis2 of DCA respectively. On the other hand, Zola

Table 1. phytoplankton species list determined in the reservoirs.

Phytoplankton	Reservoir						
	Aras	Aras2	Ghigaj	Ghanbari	Barun	Zola	Derik
Chlorophyta							
Oocystis crassa Wittrock	+			+			+
Coelastrum microporum Nägeli	+						
Scenedesmus quadricauda (Turpin)	+	+	+	+			+
Brébisson			+				
Scenedesmus bijuga (Turpin) Lagerheim	+						
Gleocystis vesiculosa Nägeli	+	+		+			
Tetraédran minimum (A.Braun) Hansgirg	+			+			
Pediastrum duplex Meyen	+	+					
Dictyocepharium pulchelum H.C.Wood	+	+					
Coelastrum microporum Nägeli	+						+
Chlamydomonas sp.							
Cyanobacteria							
Microcystis botrys Teiling	+		+	+	+	–	
Oscillatoria sp.	+	+	+	+			
Anabaena spiroeides Klebahn	+						
Chroococcus turgida (Kützing) Nägeli				+			+
Bacillariophyta							
Cyclotella sp.	+	+		+	+	–	+
Nitzschia sp.	+	+	+	+	+		
Nitzschia closterium (Ehrenberg) W.Smith	+		+				
Diatoma vulgaris Bory	+		+				
Navicula lanceolata (Agardh.) Kütz.	+						
Navicula sp.		+		+	+		+
Cocconeis pediculus Ehrenb	+						
Synedra ulna (Nitz.) Her.	+		+		+		
Symbella sp1.		+					
Symbella sp2.		+	+				
Amphora oralis Kütz							
Gomphonema parvulum (Kützing) Kützing				+	+		
Pyrrhophyta							
Glenodinium quadridens (Stein) Schiller		+					+
Gymnodinium caudatum Prescott		+					
Dinobryon sp.					+		+
Desmidaceae							
Euastrum sp.		+					
Staurastrum gracille Ralfs ex ralfs		+		+			
Cosmarium subcostatum Nordst				+			
Euglenophyta							
Euglena proxima Dang.			+				+

and Ghigaj reservoirs had the highest weights on axis1 respectively (Fig. 9).

The result of PCA was confirmed by UPGMA analysis, in which three main groups were clustered on the basis of their correlation with phytoplankton community changes and environmental parameters (Fig. 10). The PAST software was used to determine the similarity and distance indices of reservoirs (Table 3).

Disturbed reservoirs were also dominated by tolerant taxa of Bacillariophyta namely *Navicula* sp. and Chlorophyta, mostly *Scenedesmus* sp. and species of cyanobacteria.

Discussion

The quality and availability of freshwater is one of the most essential determinants for the health of ecosystems and human societies worldwide. Human activities have exploited this resource heavily, and consequently se-

Table 2. physicochemical parameters of reservoirs in summer 2016.

Parameter / Resevoir	DO (mg/L)	Water Tem (°C)	pH	EC	Turbidity (m)	Salinity	TDS	%Oxygen	TP (mg/L)
Aras	7.23	23.57	8.88	218.33	0.50	0.0	139.7	91.9	24.73
Aras-2	6.53	23.2	9.12	1576	0.75	0.6	1014	83.0	31.9
Gheigaj	6.94	23.6	8.92	1283	0.35	0.5	825	89.3	14.6
ShahidGhanbari	9.56	26.2	9.56	1200	0.50	0.4	768	135.4	35.8
Barun	11.47	26.9	8.88	507	0.48	0.0	364	172.8	35.8
Zola	10.11	24.3	9.25	315	0.95	0.0	202	145.2	6.06
Derik	10.14	23.5	8.76	465	1.00	0.0	298	144.5	29.5

Table 3. Similarity and distance indices of reservoirs.

	Ara	Ar2	Ghi	Gha	Bar	Zol	Der
Ara	0	5911.8749	1911.0264	1976.6406	1226.1343	2480.2971	1901.3162
Ar2	5911.8749	0	6357.5169	6339.7227	6170.1606	6584.2441	5885.0478
Ghi	1911.0264	6357.5169	0	425.75194	2128.6647	2664.281	3116.5337
Gha	1976.6406	6336.7227	425.75194	0	2073.5283	2383.4947	3073.5021
Bar	1226.1343	6170.1606	2128.6647	2073.5283	0	1659.516	1121.7104
Zol	2480.2971	6584.2441	2664.281	2383.497	1659.516	0	2182.8676
Der	1901.3162	5885.0478	3116.5337	3073.5021	1121.7104	2182.8676	0

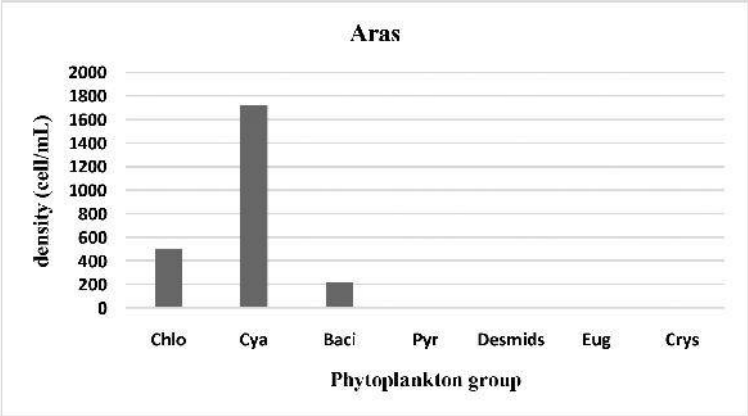

Fig. 1. Phytoplankton groups density in Aras reservoir summer 2016 (Chlo Chlorophyta; Cya Cyanobacteria; Baci Bacillariophyta; Pyr=Pyrophyta; Desmids=Desmidaceae; Eug= Euglenophyta; Crys Crysophyta).

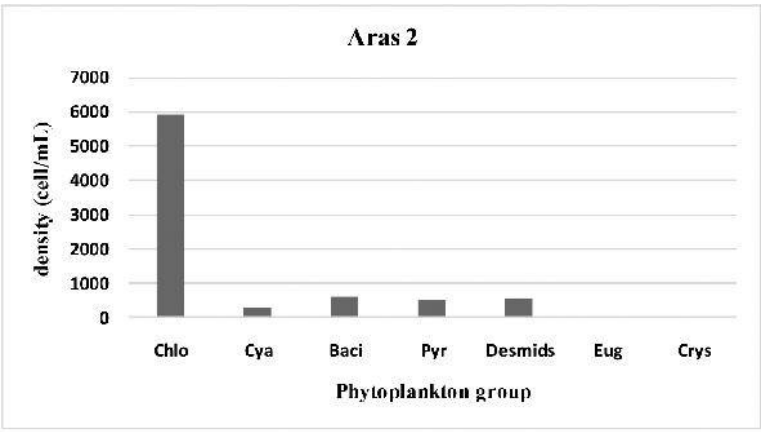

Fig. 2. Phytoplankton groups density in Aras2 reservoir in summer 2016 (Abbreviations as Fig. 1).

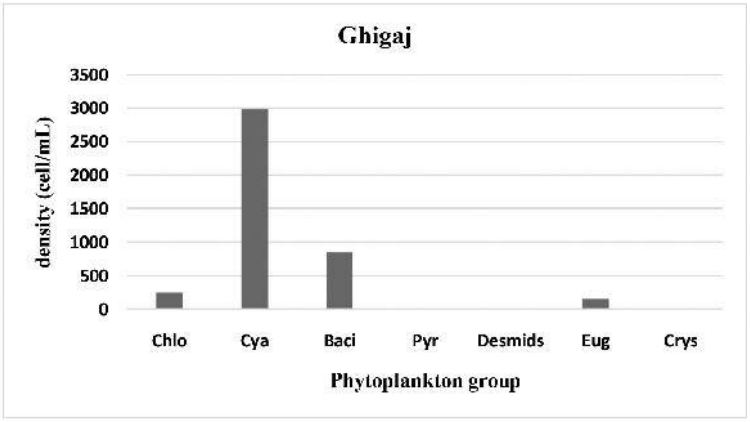

Fig. 3. Phytoplankton groups density in Ghigaj reservoir in summer 2016 (Abbreviations as Fig. 1).

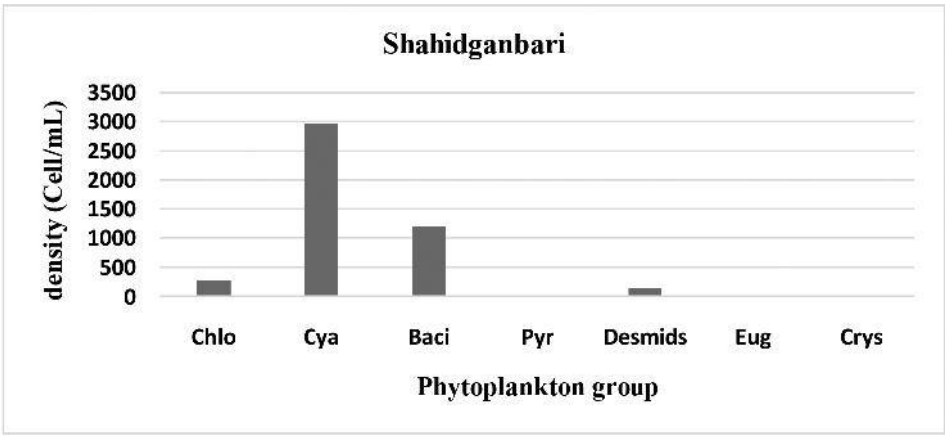

Fig. 4. Phytoplankton groups density in Shahid Ghanbari reservoir in summer 2016 (Abbreviations as Fig. 1).

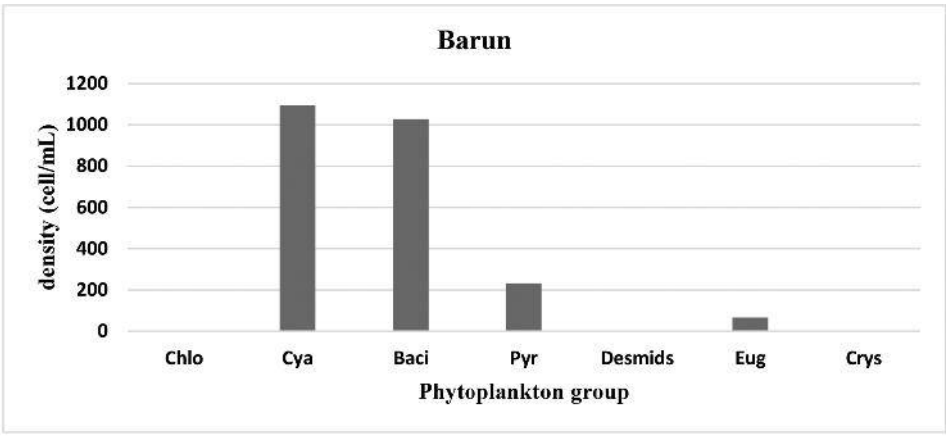

Fig. 5. Phytoplankton groups density in Barun reservoir in summer 2016 (Abbreviations as Fig. 1).

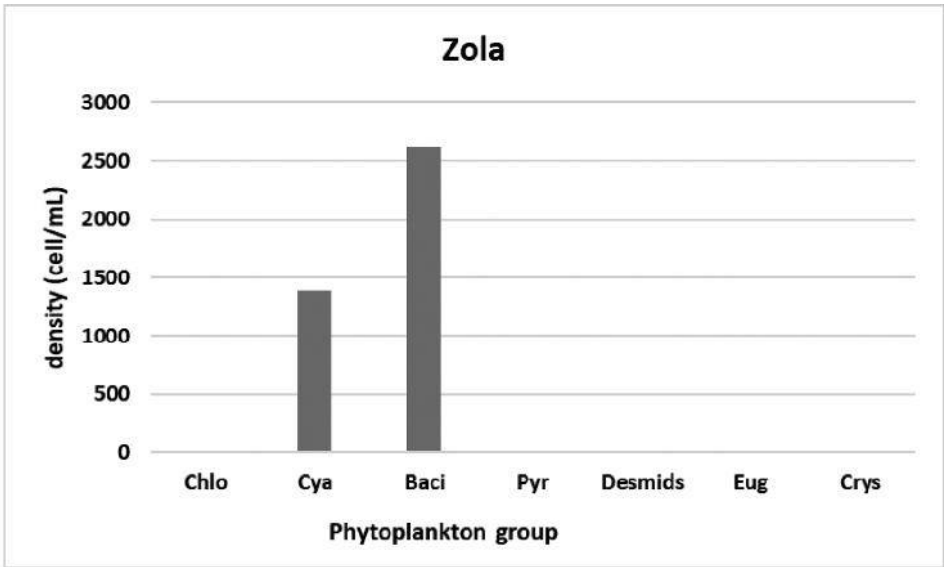

Fig. 6. Phytoplankton groups density in Zola reservoir in summer 2016 (Abbreviations as Fig. 1).

verely deteriorated freshwater ecosystems. Hydrological changes, physical disturbances, point and non-point sources of pollution, from both rural and urban activities, are all examples of processes responsible for the large-scale deterioration of freshwater systems and lentic waters, such as, for instance, reservoirs (Cabecinha et al., 2009).

Land use change is one of the major anthropogenic influences on aquatic systems, which affects the water quality and phytoplankton community of reservoirs and rivers.

The destruction of pastured areas, stimulated by economic exploitation and demographic expansion, should be closely observed. In the past 50 years, a considerable loss of biodiversity and ecosystem impairment has been observed primarily because of non-sustainable agricultural practices (Pinheiro et al., 2015). Natural vegetation suppression in drainage basins can change the chemical characteristics of reservoirs, rivers and streams. Phytoplankton productivity is also affected by land use change. Conductivity, hardness, phosphate concentrations were strongly related to agricultural activity. This was not too surprising given the wide range of agricultural land use in both the lowland and higher catchments, namely extensive and intensive agriculture.

Consistent with hierarchy theory; namely that species composition at a site is the product of environmental filters operating at successive spatial scales (Poff, 1997). Frisell et al. (1986), defined the "hierarchical concept of landscape", where spatial scales are related or even interdependent, at least unidirectionally from large to small spatial scales. This was reflected in our study by the strong shared effects of spatial scales in the reservoir types.

Electron Conductivity (EC) and Total Dissolved Oxygen (TDS) have the highest influence on pc2 of PCA (Table 3). This reflects that these two parameters separate the reservoirs in this study. Aras 2 reservoir has the highest values of EC and TDS than other reservoirs (Table 2). On the other hand, Zola contains the lowest Total Phosphorus (TP) which separated this reservoir from other reservoirs on PCA

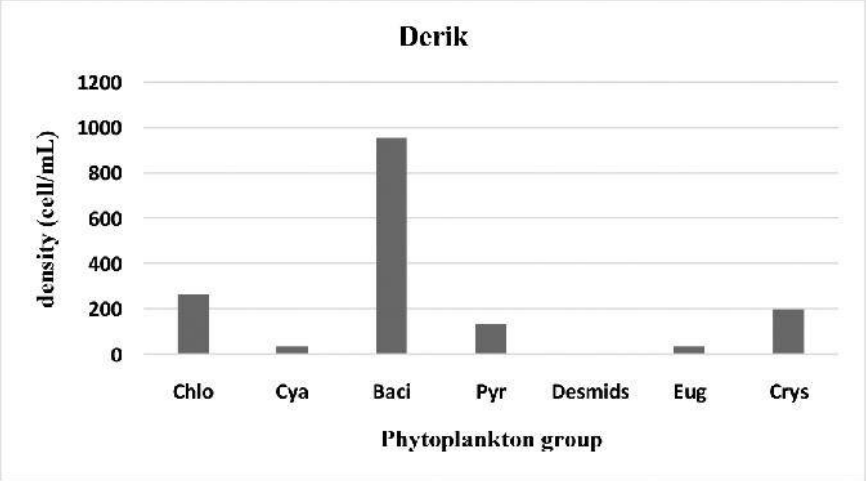

Fig. 7. Phytoplankton groups density in Derik reservoir in summer 2016 (Abbreviations as Fig. 1).

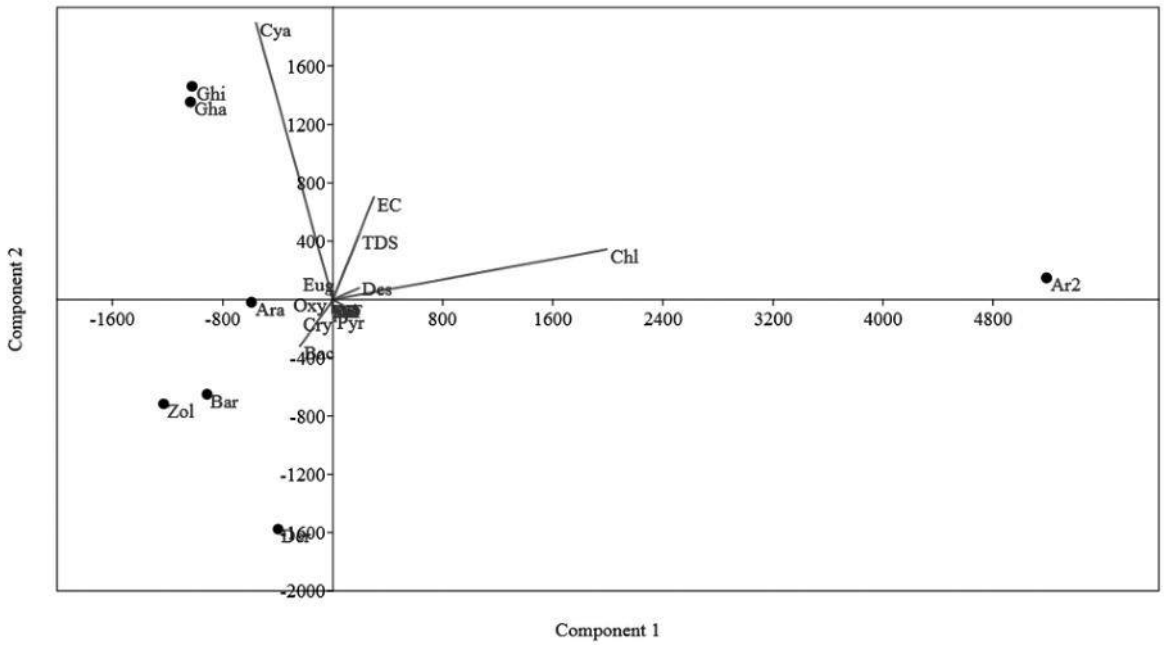

Fig. 8. Principal Component Analysis (PCA). The circles indicate reservoirs, the vectors show phytoplankton groups and physical and chemical parameters. (Ara= Aras, Ar2= Aras2, Bar= Barun, Der= Derik, Gha= Ghanbari, Ghi= Ghigaj, Zol= Zola; Bac= Bacillariophyta, Chl= Chlorophyta, Cya= Cyanobacteria, Cry= Cryptophyta, Des= Desmidae, Eug= Euglenophyta, Pyr= Pyrophyta; Do= Dissolved Oxygen, EC= Electron Conductivity, Oxy= Oxygen%, Sal= Salinity, TDS= Total Dissolved Solids, Tur= Turbidity, WT= Water Temperature).

(Fig. 8) and DCA (Fig. 9).

Cyanobacteria had the highest effect on the first component of PCA (Fig. 8) by separating Ghigaj and Ghanbari reservoirs. Indeed, this algal group had the highest density in these two reservoirs (Figs 3, 4). Furthermore, chlorophyta had the highest density in Aras 2 (Fig. 2), so that has taken this reservoir to a far distant point on PCA, DCA and UPGMA dendrogarm (Figs 8, 9 and 10).

In general, disturbed sites were clearly dominated by tolerant taxa of Bacillariophyta and Chlorophyta. Therefore, meso-eutrophic to hypertrophic taxa (Van Dam et al., 1994), namely *Cocconeis placentula, Cyclotella* sp., *Diatoma vulgaris, Navicula* sp., *Nitzschia palea, Synedra ulna* were positively associated with an anthropogenic pressure gradient.

The society must consider the disturbance that vegetation suppression will have on endemic species of aquatic biota. Pinheiro et al. (2015) suggested that the replacement of natural vegetation formations, with species used in agricultural activities over the years, interferes considerably with biota longevity and aquatic food chains.

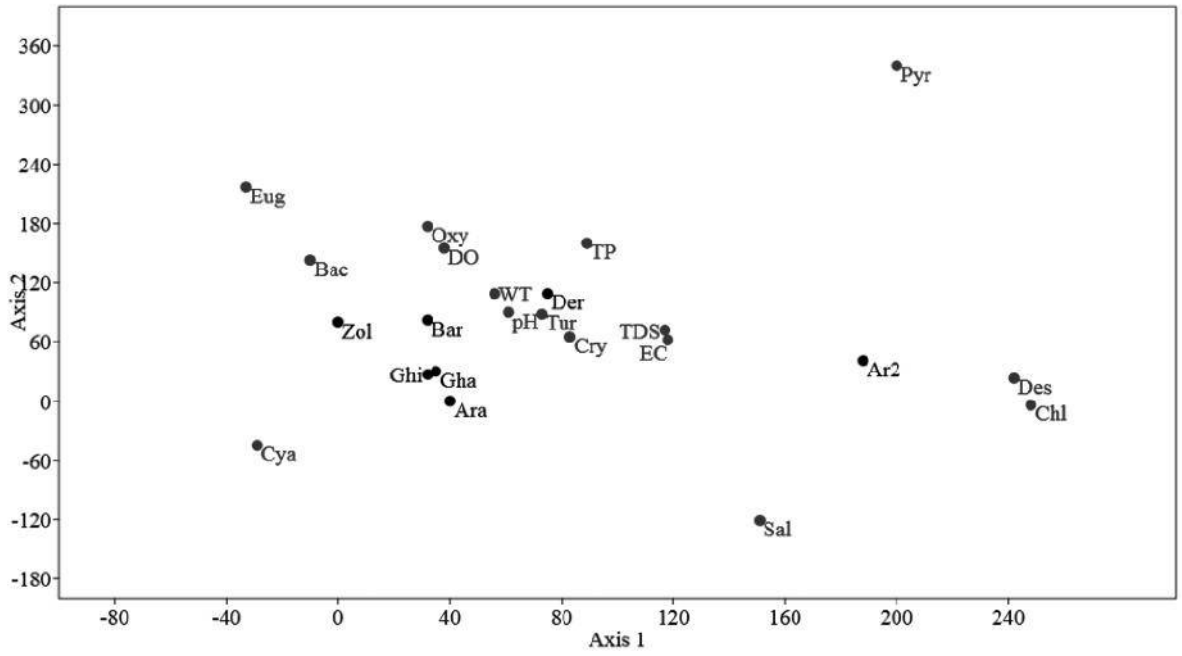

Fig. 9. Detrended Correspondence Analysis (DCA) of dam reservoirs against phytoplankton groups and some physical and chemical variables (abbreviations as Fig. 8).

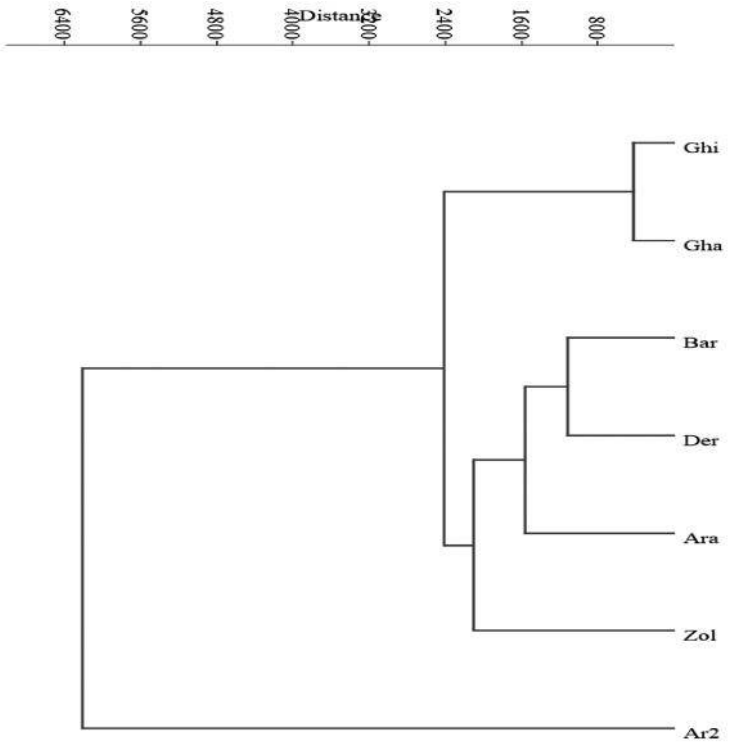

Fig. 10. Unweighted pair group method with arithmetic mean clustering of sampling stations (dam reservoirs) according to phytoplankton groups and physicochemical parameters.

With these studies, we may predict of how human alterations affect lentic water ecosystems and herewith the planning and implementation of conservation and management programs can be improved.

The small number of studies in the country makes it difficult to determine the main factors contributing to long-term effects of land use changes in small watersheds. However, removal of natural vegetation would tend to contribute to higher nutrient export to reservoirs promoting changes in N and P cycles, thus altering the phytoplankton structure.

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Evaluation of Different Extraction Methods for Phycocyanin Extraction from *Spirulina platensis*

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Abstract

C-Phycocyanin is a natural pigment that has several applications in food and cosmetic industry. Recent studies have also revealed the medicinal effects of this natural blue dye. In this study, Comparison effect of mechanical (freezing and thawing, sonication and bead milling) along with chemical (inorganic acid, buffers and sea salt) extraction methods were carried out on C-Phycocyanin concentrations to achieve the optimum conditions of phycocyanin extraction from *Spirulina platensis*. Our result authenticated that repeating temperature cycles obtained higher phycocyanin concentration which could observed clearly by unaided eye. The optimum condition for extraction is freezing and thawing method under -20°C condition in five repeated cycles in comparison with liquid nitrogen at the same condition. The best solvent was determined sea salt solution then distilled water, PBS and TE buffer, respectively. Under optimize condition phycocyanin was extracted with a concentration of 0.29 mg/ml and purity ratio (A_{620}/A_{280}) of 3.42.

Key words: *Spirulina,* C-phycocyanin, Solvent, Freeze-thaw extraction, Sonication

Introduction

Currently, the demand of natural colorants is growing rapidly due to their beneficial applications in food and pharmaceutical industry (Chethana et al., 2015; Patil et al., 2006). Among of different colorant, the natural blue dye is more important than the others in confectionary and drinks industry due to rarity in nature (Martelli et al., 2014).

Phycobiliproteins are an excellent colorant water-soluble proteins bearing covalently attached with linear tetrapyrrole prosthetic groups known as phycobilins. Based on their visible absorption properties, the phycobiliproteins have been classified to four spectroscopic classes: phycoerythrocyanin, phycocyanins, allophycocyanins and phycoerythrins (Antelo et al., 2008). phycobilins are found not only in cyanobacteria but also in red algae and crytomonads (Moraes et al., 2011). They are mainly different in their protein structure and pigment color: red (phycoerythrins), purple

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(phycoerythrocyanin, R-Phycocyanin), dark blue (C-Phycocyanin) and blue-green (allophycocyanins) (Silva et al., 2009)

Among of all microalgae, the *Spirulina* genus is applied as a rich, inexpensive and natural source of phycocyanin pigment for food and cosmetics (Bhaskar et al., 2005; Furuki et al., 2003) which approved legally by Europe, Japan, USA and Brazil (Colla et al., 2016). It was estimated up to 20% of protein fraction of *Spirulina platensis* is phycocyanin (Silveira et al., 2007). Phycocyanin has been demonstrated some therapeutic properties including anti-inflammatory and anti-cancer effects (Dupuis et al., 2017; Mitra et al., 2015). The purity of C-PC which is figured out by the absorbance ratio of A620/A280, is essential factor to determine final application, so as purity of 0.7, 3.9 and above 4 consider as food, reactive and analytical grade, respectively (Patil et al., 2006; Rito- Palomares et al., 2001).

a wide range of C-PC extraction methods through physical or enzymatic treatments, have been reported which an industrial process involve physically cell disruption (Furuki et al., 2003). However, it is essential to present an appropriate technique for phycocyanin extraction from *Spirulina platensis* in large scale which is able to produce C-PC with high purity and concentration without any further purification requirement.

Due to many applications of phycocyanin in various fields, the aim of this study was to examine an extraction method with maximum yield of pigment and improvement of efficiency and purity.

Materials and Methods:

Culture conditions of Spirulina platensis

The algae was donated from algae collection of research institute of applied science of ACECR, Tehran, Iran. Algal cell cultivated in Zarrouk (Vonshaket al., 1982) medium at pH 9 using white lamp, shaked 100 rpm and aerated with atmospheric air.

Extraction methods

C-PC was extracted from the wet biomass of *Spirulina platensis* by using following methods:

Inorganic acid extraction

The wet biomass was treated with hydrochloric acid (5 M) in the proportion 5:1 (g biomass: ml hydrochloric acid) and thenwas subjected three alternative different methods: Without any treatment was placed in room temperature for 24 hours (Moraes et al., 2011). Repeated freeze- thaw in -20°C (3 cycles) Ultrasonic treatment for 30 min, temperature control to be under 30°C by exchanging with freshwater.

Mechanical extraction

The *Spirulina* cells were subjected 2 methods to disrupt cell wall mechanically: either using glass beads with mortar for homogenizationor freeze-thaw at first in -20°C (3 cycles) and followed by liquid nitrogen (3 cycles). TE buffer was used as a solvent in both methods.

Freeze-thaw optimization

In the present of Tris-HCl buffer (pH=8), *Spirulina* biomass was frozen in liquid nitrogen (3 cycles) or -20°C (3 or 5 cycles) and thawing at room temperature in order to compared together.

Comparing different solvents

To find out the best solvent for phycocyanin

extraction, *Spirulina* biomass was suspended in distilled water, different buffer (50mM sodium phosphate buffer pH 7 and Tris-HCl buffer in pH 8) and 1% sea salt separately. It was followed by Freezing (-20°C) and thawing (4°C) three repeated cycles.

In all above experiments the extracts were centrifuged at 10000 rpm for 5 min to remove cell debris and phycocyanin concentration ($mgml^{-1}$) was evaluated in their supernatants using the following equation 1 (Moraes et al., 2011; Sarada et al., 2011; Siegelman et al., 1978):

(1) $$CPC : \frac{(OD620 - 0.474OD652)}{5.34}$$

The purity and yield were calculated by following equation 2 and 3, respectively:

(2) $$\text{Purity: } \frac{OD620}{OD280}$$

(3) $$\text{Yield: } \frac{(CPC)V}{DB}$$

Where in OD620 is the maximum absorbance of C-Phycocyanin and OD280 is the total protein absorbance. V is the total solvent volume (ml) and DB is the dry biomass (g).

Results

This study compared three different types of extractions using solvent, ultrasonic and freeze-drying extraction.

Using HCL (5M) as an inorganic acid solvent in different PC extraction method (without any treatment, Sonication and freeze-thaw) showed no phycocyanin absorption and all of supernatants were colorless. The result exhibited that proper extraction method was freezing and thawing method with applied of TE buffer. However, applying homogenization in mortar and pestle with glass bead in TE buffer showed low amount of PC which we could not consider as a valuable data.

In comparison of freezing and thawing in-different temperature, the highest amount of C-PC was obtained by applying freeze-thaw in -20°C (5 cycles). Furthermore, The purity in this method was the highest in compared to other methods were used. According to Table 1, liquid nitrogen method yield was significantly higher than others, almost two times of -20°C (3 cycles). Our research experiments showed 3–5 cycles freeze–thaw were quite enough to achieve the high extraction efficiency. Although liquid nitrogen frozen in the presence of extraction medium for 3 cycles obtained the highest yield but the purity is lower than one. To choose suitable buffer for maximum phy-

Table1. Comparison of different freeze-thaw methods for C-Phycocyanin extraction

Amounts	Method		
	-20 (3 Cycles)	-70 (3 Cycles)	-20 (5Cycles)
CPC* (mg/ml)	0.20	0.31	0.33
yield (mg/g)	2.89	4.46	3.39
purity	0.80	0.97	1.31

*CPC means phycocyanin

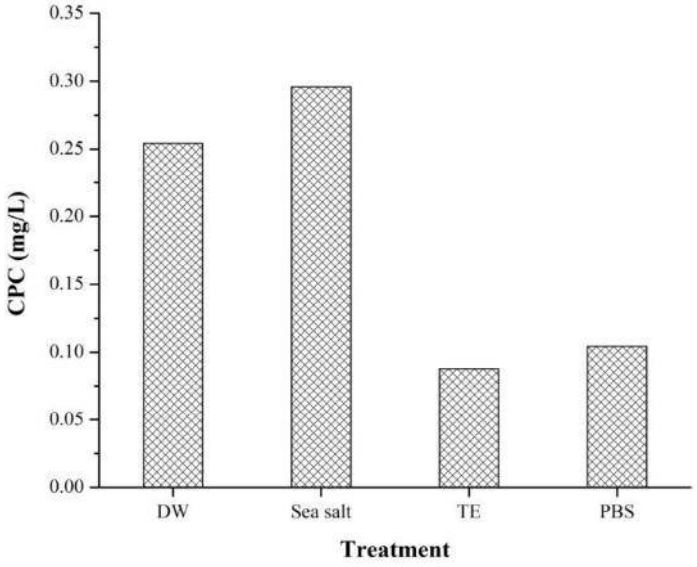

Fig. 1. C-Phycocyanin extraction by using different solvent

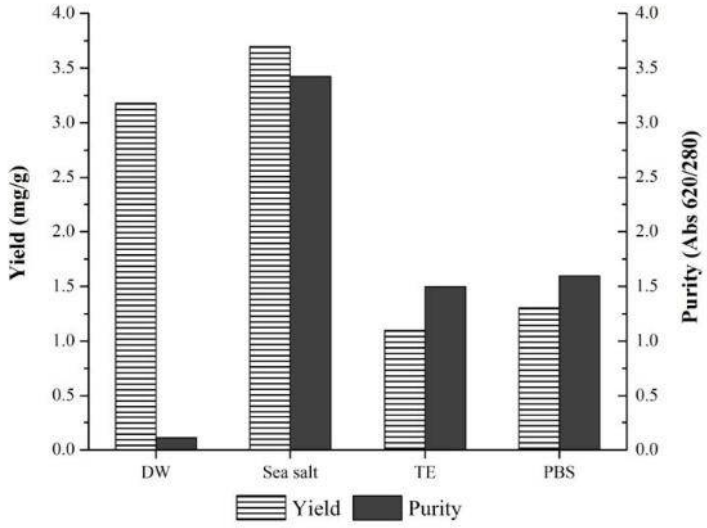

Fig. 2. Comparison of extraction purity and yield of C-phycocyanin by applying different solvent.

cobiliproteins extraction, Freezing (-20°C) and thawing at 4°C was done in present of PBS, TE, distilled water and sea salt. The highest amount of C-Phycocyanin was extracted when sea salt and distilled water were used as a solution, respectively (Fig. 1).

Similarly, the highest yield and purity showed in sea salt extraction. The comparison result of different C-phycocyanin extraction methods demonstrated in Figures 1 and 2, as shown the highest C-Phycocyanin extraction concentration (Figure 1), yield and purity (Figure 2) were estimated approximately 0.29 $mgml^{-1}$, 3.69 mgg^{-1} and of 3.42, respectively. The highest phycocyanin purity obtained from sea salt solution. PBS buffer at pH 7.0 exhibited phycocyanin yield slightly.

Discussion

One of the most important requirements to obtain phycobiliproteins from algae is optimizing the extraction method. A perfect method should be included rapid and sufficient disruption for a quantitative extraction and recovery of the released pigment. Our study showed that among different physically cell disruption methods, the best method for PC extraction are freeze-thaw which is similar with Moraes (2011) and Sarada et al. (1999) research reports. They indicated that HCl concentration lower than 8M resulted in the least amount of PC and also proved that the best method for disrupting cell wall was freezing and thawing. In addition, another study showed that high concentration of HCl was not suggested for separation of pigment from phycobiliprotein (O'hEocha, 1963; Sarada et al., 1999) and also proved that phycocyanin had low stability in pH below 5 (Sarada et al., 1999). Siavasankari and Ravindran (2014) reported that HCl (12M) showed significantly poor yield of phycocyanin in comparison with freezing and thawing method. Moreover, some papers reported that the freeze-thaw was the best method for C-phycocyanin extraction from wet biomass (Hemlata and Fareha, 2011; Kissoudi et al., 2017; Soni et al., 2008). The freeze–thaw method exhibited all mention advantages in addition to cost-effective without significant deprivations of the protein biological capacity (Moraes et al., 2011). In comparison withliquid nitrogen or -20°C, Freeze-thaw in -20°C showed the highest purity. As PC concentration depends straightly on the cell envelope disruption (Avila Jerley and Prabu, 2015), whenever the cell is frozen, the ice crystals formed during freezing and resulting in damage to the cell wall, promoting a better extraction of the phycobiliprotein (Soniet al., 2008). The more cell disruption, the higher protein extraction which effect on phycocyanin purity, however maximum 5 cycles freeze–thaw were enough to achieve the highest purity. It was sameas Zhu et al. (2007) and Horváth et al. (2013) reports.

Silveria et al. (2007) obtained the highest amount of phycocyanin using water as a proper solvent among applying different solvents which is similar to our result. It was concluded that the highest yield and purity was obtained by applying sea salt solution whereas the main content of sea salt is sodium nitrate and sodium hydrogen carbonate. Herrera et al. (1989) findings have already confirmed that sodium

nitrate solution is the best solution for phycocyanin extraction with high level of purity. On the contrary, our result about PBS extraction, Kissoudi et al. (2017) reported, sodium phosphate buffer show the best function in extraction.

In the present investigation, a new method has been examined for the C-PC extraction, which is not only simple but also is rapid and more efficient in compare with existing methods.

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Taxonomy of the Brown Algal Genus *Padina* With the Description of the New Species *Padina* sp. PG nov. (Dictyotales, Pheaophyceae) from the Northern Coast of Persian Gulf

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Abstract

Padina is a marine brown macro algal genus, comprising of about 37 species. Representatives genus data along the Persian gulf coast is limited to a few floristic surveys. The present study introduces the *Padina* species along the Persian gulf coast, with emphasis on the molecular taxonomy. Sequences of the large subunit of RuBisCo (rbcL) have been used in the molecular analysis of species and for phylogenetic purposes. Based on the rbcL sequences, four species are recognized along the Persian gulf coast: *Padina* sp. FA, *Padina* sp. PG, *Padina* sp. INDEGRO32 and *Dictyota ciliolata*. A new species, *Padina* sp. PG nov. is described based on morphology and molecular analyses. Twelve new sequences were generated among the samples examined.

Keywords: Macroalgea, *Padina* sp. PG nov., Molecular Systematic

Introduction

Species of the marine brown algal genus *Padina* are widely distributed through out the tropics and recognize in the field with their "fan-like" blade. According to AlgaeBase (Guiry and Guiry, 2011) 37 species are currently recognized worldwide, in which 6 species were recorded in the northern of Persian gulf coast of Iran based on morphological studies (Børgesen, 1939; Nizamuddin and Gesner, 1970; Sohrabipour and Rabiei, 1996, 1999, 2005, 2008). Several studies about systematic of macroalgae in Persian gulf along Arabian coasts heve been done (Al-Hasan and Jones, 1989; Basson et al., 1992, 1989; Basson, 1979a, 1979b; Børgesen, 1939; DeClerk et al., 1997; Newton, 1955a, 1955b; Abdel-Kareem, 2009). Taxonomic studies of *Padina* species in the coast of Persian gulf, until recently were usually consisting of revisions or descriptions of the genus in this area, exclusively based on morphological characters (e.g. thallus shape, size and color) which might be variable (Trono, 1969; Ni-Ni-Win et al., 2011 a,b). Furthermore, several recent studies dealing with European and Mediterranean taxa have indicated that common morphological data without the support of DNA sequence data are an insufficient basis for estimates species diversity and knowl-

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edge of species boundaries (De Clerck et al., 2005, 2006). However a few papers, based on taxonomic sources, were published dealing with the marine algal flora of the Persian gulf, like the molecular studied on three *Sargassum* species that were done by Noormohammadi et al. (2011 a, b) in which they used RAPD and ISSR markers for analyses. In addition, *Padina* could be employed as a possible environmental bioindicator in Persian gulf (Amini et al., 2013). In this study, we execute molecular phylogenetic analyses using chloroplast rbcL gene sequences. The chloroplast encoded large subunit of the RuBisCo gene (rbcL) has been used in molecular phylogenetic studies of brown algae and has been demonstrated to be a useful molecular marker by authors (Cho et al., 2004; Hoshina et al., 2004; De Clerck et al., 2006; Cho et al., 2007; Bittner et al.. 2008; Ni-Ni-Win et al., 2008, 2010, 2011b; Phillips et al., 2008). The aim of the present study was to characterize the molecular diversity from the new sequences of *Padina* species for the first time in Persian gulf and compare them with the *Padina* species in the other regions. Additionally, to describe the genus based on newly generated sequences from already available sequence data and describe two new species based on morphological and molecular analyses.

Materials and Methods

Padina samples (Phaeophyceae, Dictyotales, Dictyotaceae) were collected from the intertidal regions along the southern coast of Iran (the northern coast of Persian gulf) that showed in Figure 1 namely: Kish island (26° 51' N, 53°59' E), Lengeh area (26° 28' N, 54°78' E), Qeshm island include Shib Deraz (26° 42' N, 56°04' E), Messen (26° 49' N, 53°23' E), Behind Farmandari (27° 04' N, 56°59' E) from Persian gulf during May, June, August, September 2011 and March 2012 (Figure 1). The samples were obtained by hand and diving. Standard collecting and preserving proceeds follow Tsuda (1972) and molecular procedures carried out according

Fig. 1. Location of sampling for seaweeds on the intertidal Coast of Persian Gulf of Iran. 1-Kish island, 2- Lengeh area, 3-ShibDeraz (Qeshm island), 4-Messen (Qeshm island), 5-Behined Farmandari (Qeshm island).

to Siemer et al. (1998) and Ni- Ni-Win et al. (2008, 2011a). Collected samples were deposited in HSBU (Herbarium of Shahid Beheshti University). All specimens used for this study are listed in Tables 1 and 2.

Total genomic DNA was extracted from tissue samples, dried in a silica gel. DNA extraction, amplification (PCR) of the rbcL region and sequencing carried out using an extraction protocol detailed by Ni-Ni-Win et al. (2008 and 2011b). PCR conditions for rbcL were as follows: an initial denaturation step at 94°C for 3 min, followed by 94°C for 0.5 min, annealing at 58°C for 0.5 min, extension at 72°C for 2 min for 28 cycles, and final extension at 72°C for 10 min. PCR products were checked for length and yielded by electrophoresis on 1.5% agarose gels dyed with ethidium bromide. In order to minimize possible errors during PCR, three independent PCR reactions were performed for each DNA sample. Primer sequences, annealing temperatures, and bibliographic sources are provided in Table 3.

For anatomical observations, specimens were sectioned by hand and micrographed using a Dino capture Ver. 3 digital camera attached to a microscope (Olympus, Tokyo, Japan). For each specimen, morphological and anatomical characters (vegetative and reproductive) analyzed. According to Ni-Ni-Win et al. (2011a) some of the morphological characters such as shape, size, color, and thickness of the thallus are highly variable within the species and are depend on environmental conditions and age of the speciemens. But other characters like the number of cell layers, presence or absence and degree of calcification, the position and arrangement of hair lines and sporangial sori, and presence or absence of an indusium, the presence or absence of groups of rhizoid-like hairs on the thallus surface, the structure and arrangement of hair lines and reproductive sori were considered less variable within the species. The main anatomical characters used for species identification have been summarized in Table 4.

DNA sequences are deposited in DNA Data Bank of Japan (DDBJ). The 12 newly generated sequences were complemented with 31 sequences downloaded from GenBank and aligned using Thompson et al. (1994) and the alignment was refined manually. Phylogenetic analyses were carried out by maximum parsimony (MP), maximum likelihood (ML) and neighbor joining (NJ) methods, using MEGA5.1 (Tamura et al., 2011). An appropriate model of sequence evolution for maximum likelihood (ML) analysis was selected the best-fit models based on AICc values criterion with MEGA's built-in model testing suite. A ML tree was inferred using the selected GTR model using nearest neighbor interchange tree rearrangements. A neighbour-joining (NJ) distance-based tree was constructed (Saitou and Nei, 1987) using a Kimura 2-parameter method. Maximum parsimony (MP) analysis was obtained using a standard heuristic search with tree-bisection-reconnection (TBR) branch swapping options. Bootstrap resampling was carried out with 100 replicates for ML and 1000 replicates for NJ and MP (Felsenstein, 1985*). Dictyota ciliolata* and *Dictyota dicoto-*

Table1. Species used in this study, from which all new sequencing is obtained. Herbarium of Shahid Beheshti University (HSBU).

Sequence entry	Species	Origin	Voucher No.	DDBJ code for rbcL	Reference
1	*Padina* sp. PG, haplotype: 1	Qeshm Island, IR Iran	HSBU-2011300	AB793713	This study
2	*Padina* sp. FA haplotype: 2	Kish Island, IR Iran	HSBU-2011301	AB793714	This study
3	*Padina* sp. INDGR032 haplotype: 3	Kish island, IR Iran	HSBU-2011302	AB793715	This study
4	*Padina* sp. FA haplotype: 4	Kish Island, IR Iran	HSBU-2011303	AB793716	This study
5	*Padina* sp. FA haplotype: 5	Kish Island, IR Iran	HSBU-2011304	AB793717	This study
6	*Padina* sp. FA haplotype: 6	Qeshm Island, IR Iran	HSBU-2011305	AB793718	This study
7	*Padina* sp. FA haplotype: 7	Qeshm Island, IR Iran	HSBU-2011306	AB793719	This study
8	*Padina* sp. FA haplotype: 8	Kish Island, IR Iran	HSBU-2011307	AB793720	This study
9	*Padina* sp. FA haplotype: 9	Legeh Port, IR Iran	HSBU-2011308	AB793721	This study
10	*Padina* sp. FA haplotype: 57f	Kish Island, IR Iran	HSBU-2011309	AB793724	This study
11	*Padina* sp. FA haplotype: 2	Kish Island /LengehPort/ Qeshm Island, IR Iran	HSBU-2011310	AB775783	This study
12	*Dictyota ciliolata*	Qeshm Island, IR Iran	HSBU-2011311	AB775782	This study

Table 2. List of species from other studies investigated in this study, including collection site and GenBank accession number.

Sequence entry	Species	Origin	Voucher No.	DDBJ code for rbcL	
1	*Padina antillarum*	India	INDGR032	AB096907	
2	*Padina antillarum*	Diani Beach, Kenya	ODC1508	JQ364044	Silberfeld et al., 2013
3	*P. australis* Hauck	Baie de Gadji, Île des Pins, New Caledonia	IRD233	JQ364054	Silberfeld et al., 2013
4	*P. australis* Hauck	Sawang, Siquijor, Philippines	ODC1459	JQ364056	Silberfeld et al., 2013
5	*P. australis* Hauck	Japan	OKNNG019	AB096901	Hoshina et al., 2004
6	*P. australis* Hauck	Awase, Okinawa I., Okinawa Pref., Japan	SAP105579	AB358907	Ni-Ni-Win et al., 2008
7	*P. australis* Hauck	New Caledonia	IRD241	JQ364055	Silberfeld et al., 2013
8	*P. australis* Hauck	Urazoko, Okinawa I., Okinawa Pref., Japan	SAP105580	AB358906	Ni-Ni-Win et al., 2008
9	*Padina australis*	Awase, Okinawa I., Okinawa Pref., Japan	SAP105579	AB358907	Ni-Ni-Win et al., 2008
10	*P. australis* Hauck	Ngapalibeach,Thandwel(Sandoway),Myanmar		AB489914	Ni-Ni-Win et al., 2010
11	*P. australis* Hauck	Newcastle, NSW, Australia		AB489913	Ni-Ni-Win et al., 2010
12	*Padina australis*	Karang Jong E,KepulauanSeribu, Indonesia	L0609534	AB489912	Ni-Ni-Win et al., 2010
13	*Padina australis*			AB512524	Ni-Ni-Win et al., 2011b
14	*Padina australis*	Ouano, New Caledonia	IRD167	JQ364052	Silberfeld et al., 2013
15	*Padina australis*	Australia		AB512525	Ni-Ni-Win et al., 2011b
16	*Padina australis*	Poindimié, New Caledonia	IRD158	EU579959	Silberfeld et al., 2013
17	*Padina australis*	Balabio, New Caledonia	IRD172	JQ364053	Silberfeld et al., 2013
18	*Padina boergesenii*	Dickwella, Sri Lanka	HEC15869	JQ364057	Silberfeld et al., 2013
19	*Padina boergesenii*	Dickwella, Sri Lanka	HEC15913	JQ364058	Silberfeld et al., 2013
20	*Padina boergesenii*	Cahuita, Costa Rica	LBC0930	JQ364059	Silberfeld et al., 2013
22	*Padina boergesenii*	Nungwi, Zanzibar, Tanzania	TZ0520	JQ364061	Silberfeld et al., 2013
23	*Padina boergesenii*	Paje, Zanzibar, Tanzania	TZ0848	JQ364063	Silberfeld et al., 2013
24	*Padina boergesenii*	Makunduchi, Zanzibar, Tanzania	TZ0863	JQ364064	Silberfeld et al., 2013
25	*Padina boergesenii*	Makunduchi, Zanzibar, Tanzania	TZ0872	JQ364065	Silberfeld et al., 2013
26	*Padina tetrastromatica*	Malaysia		AB512554	Ni-Ni-Win, 2011b
27	*Padina tetrastromatica*	Indonesia:KepulauanSeribu, Kelor		AB512553	Ni-Ni-Win, 2011b
28	*Padina tetrastromatica*	Thailand:Nakhon Si Thammarat, Huasai		AB512552	Ni-Ni-Win, 2011b
29	*Dictyota ciliolata*	Canary Islands	D191	GQ425109	Tronholm, 2010
30	*Dictyota dichotoma*	Korea		AY748311	Lee, 2004
31	*Dictyota dichotoma*	Japan: Kanagawa, Aburatsubo		AB358934	Ni-Ni-Win, 2008

ma (Dictyotales) were considered as outgroup to root the trees.

Table 3. The name and references of the primers used in this study.

Primer name	Gene	Direction	Sequence (5'a→3')	Annealing T°C	Reference
rbcL-P1	*rbc*L	Forward	GGGTAATTTGTAAGTGGATGCG	64	Ni-Ni-Win et al. (2008) Kawai et al. (2007)
rbcL-D2	*rbc*L	Reversed	CGACGAAGTCAGGAGTATCTG	61.4	Ni-Ni-Win et al. (2008) Kawai et al. (2007)
Fa(57-76)	*rbc*L	Forward	GTGGACTGTTGTTTGGACTG	60.6	Present study
Ra500-519	*rbc*L	Reverse	ACATTTACGAAGAAGCCC	59.7	Present study

Results

Morphological observations

***Padina* sp. PG, haplotype:** 1 nov. AB793713 (HSBU-2011300)

Habitat: Qeshm Island

The erect thalli with 2-4 cell layers, (4 cells layered at the base) is yellowish brown in color, the length between 5 to 10cm, and the width up to 4 cm, blades much divided, attached by branched rhizoidal stipe. Thalli moderately calcified on both surfaces. Sporangia rows are closely alternate with hair rows at different intervals without indusia, sometimes as isolated patches between two hair lines on the lower surface when both surfaces are viewed together. The species resembles *P. tetrastromatica Hauck* but the blades of *P. tetrastromatica Hauck* showed no calcification (Wynne et al., 1999) but this species has light calcification on two sides. In cross sections of the blades, both in mid region and in more basal portions, showed a 4-layered organization but *Padina* sp. PG has 2 cell layer and 4 layers at the base.

Ecology: This species is a new species, usually grows in the lower portions of the intertidal zone on rocky substrates or shallow subtidal zones.

Etymology: The species epithet refers to Persian gulf.

***Padina* sp. FA** INDGR032, haplotype: 3AB793715 (HSBU-2011302)

Habitat: Qeshm Island, Hormozgan province

The thallus is bright brown with 2-4 cell layers,

Table 4. Main morpho anatomical characters used in the taxonomic identification of *Padina* species of Persian gulf.

Species	Color	Length (Cm)	Width (Cm)	Cell layer (thallus)	Calcify on Lower/Upper surface	Sporangial surface	Indosia	Phaeophyceae hairs	Stip with Rust-colored Fibrous hairs	Origin	Accession number
Padina sp. PG haplotype: 1	Yellowish	5-10	4-10	2-4	light / light	Upper/Lower	Absent	Upper/Lower	Present	Qeshm Island	AB793713
Padina sp. FA haplotype: 2	Dark brown	4-10	4-10	2	Light/ heavy	Upper	Present	Upper	Present	Kish Island	AB793714
Padina sp. INDGR032 haplotype: 3	Bright brown	5-9	5-9	2-4	Light/ light	Upper/Lower	Absent	Upper/Lower	Present	Kish Island	AB793715
Padina sp. FA haplotype: 4	Dark brown	4-6	5-9	2-6	Light/Light	Upper	Present	Upper	Absent	Kish Island	AB793716
Padina sp. FA haplotype: 5	Bright brown	10-15	5-8	2	Light/Heavy	Upper/Lower	Present	Upper	Absent	Qeshm Island	AB793717
Padina sp. FA haplotype: 6	Yellowish	4-6	6-10	2-6	Heavy/Heavy	Upper	Present	Upper/Lower	Absent	Qeshm Island	AB793718
Padina sp. FA haplotype: 7	Bright brown	5-15	8-15	2	Heavy/Heavy	Upper/Lower	Present	Upper/Lower	Absent	Kish Island	AB793719
Padina sp. FA haplotype: 8	Bright Brown	4-6	6-9	2-4-6	Heavy/Heavy	Upper	Present	Upper	Absent	Lcgch Port	AB793720
Padina sp. FA haplotype: 9	Dark brown	5-9	4-8	2-4	Heavy/Heavy	Upper	Present	Upper/Lower	Absent	Kish Island	AB793721
Padina sp. FA haplotype: 57f	Darkbrown	10-17	10-13	2-4-6	Heavy/Heavy	Upper	Present	Upper	Absent	Kish Island /Lengeh Port/ Qeshm and Kish Island /Lengeh Port/ Qeshm Island	AB793724 AB775783
Padina sp. FA haplotype: 2	Brightbrown	5-7	4-6	2-4-6	Heavy/Heavy	Upper/Lower	Present	Upper	Absent		

the range of the wide is the same as the long, 5-9 cm. Thallus attached by a thick, discoid holdfast, stipe short with Rust-colored Fibrous hairs, lightly calcificated on both surfaces of the thallus, sporangial sori without indusium is alternating with hair lines on both surface.
Ecology: This species is a new species, usually grows in the lower portions of the intertidal zone on rocky substrates.

Padina sp. FA
Habitat: Qeshm Island, Hormozgan province
The sequences obtained for the rbcL of 11 *Padina* sp. FA samples collected along the coast of Persian gulf yielded 9 distinct haplotypes. There are high similarities among the haplotypes of *Padina* sp. FA and their morphological characters are very similar to those of *P. boergesenii* and *P. australis*. The following characters are similar to more samples of haplotypes. Sporangia with indusium, relatively high calcified on lower and upper surface. They all have more than five blades.
Etymology: The species epithet refers to the name of author.
Ecology: This haplotype is a new reported for the first time in Iran, usually grows in the lower portions of the intertidal zone on rocky substrates.

Padina **sp. FA**, haplotype: 2 AB793714 (HSBU-2011301)
Habitat: Kish Island, Intertidal
The thallus is two cells thick through out, up to 7 cm high, usually become 3 cells layered at the base. Generally blades dark brown in color, more deep develops from a stipe short withRust-colored Fibrous hairs. Sporangia rows

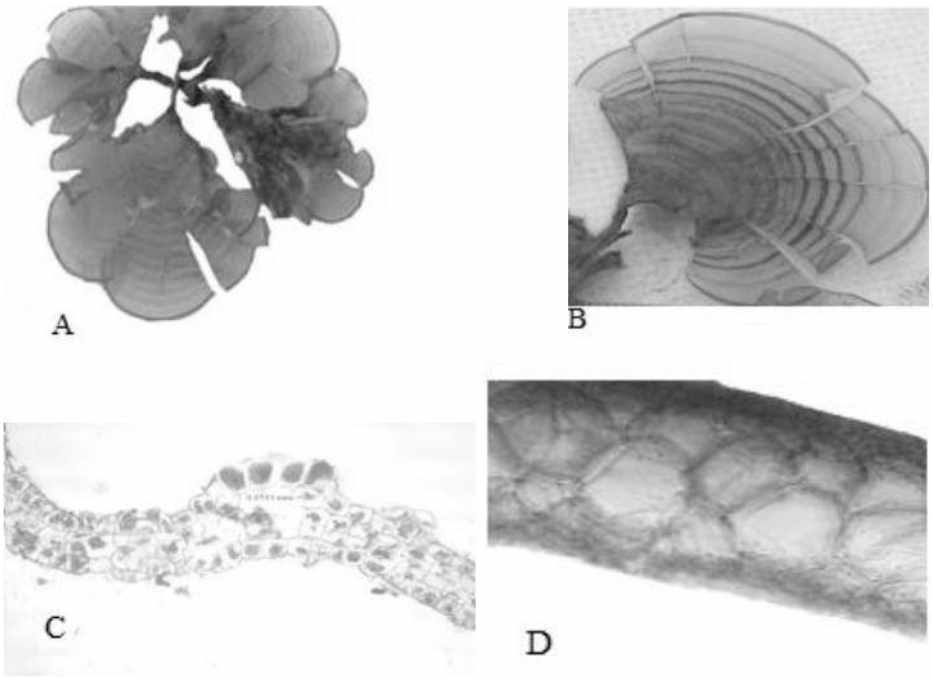

Fig. 2. A and B. Habitat, C. 2 cell layers transverse section of the middle portion of thallus, D. The view of 3 layers with hair lines (arrow) ×40.

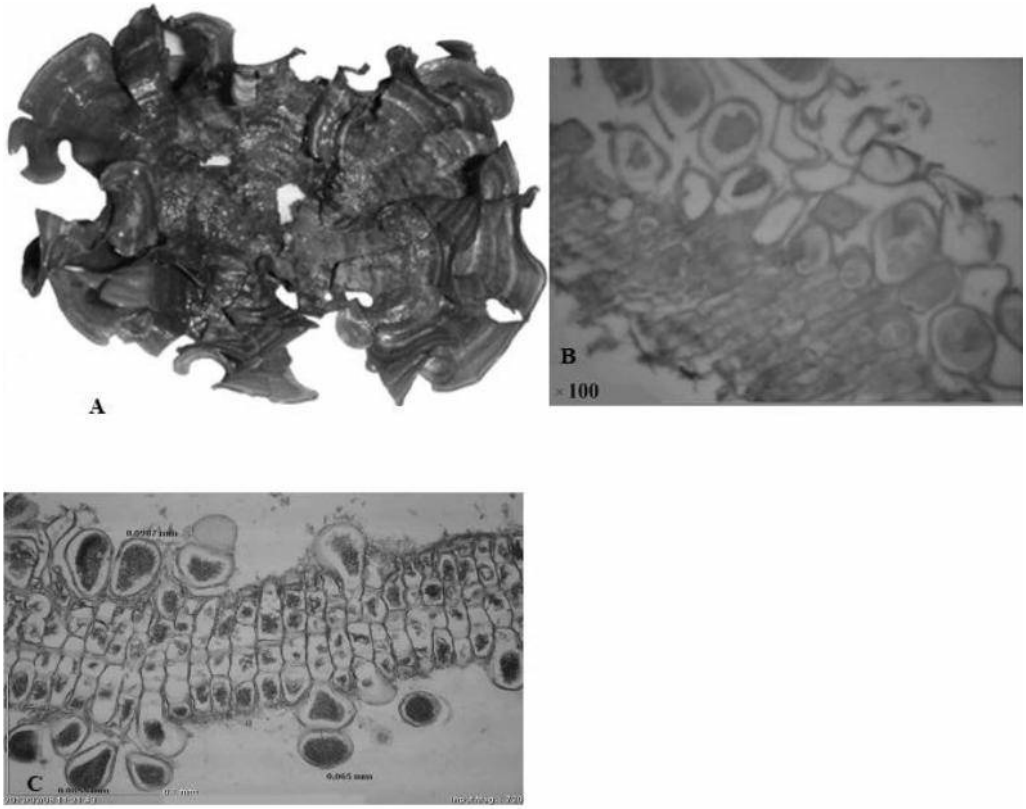

Fig. 3. A. Habitat, B. The view surface, C. Longitudinal section of the thallus×100.

were always situated on the upper surface with indusium, closely alternate with hair rows at equal intervals. Hairs in concentric lines only on the upper surface. The calcification is heavy on upper surface and light calcificated on the lower surface.

***Padina* sp. FA**, haplotype: 4AB793716 (HSBU-2011303)

Habitat: Kish Island, Intertidal

The erect dark brown thalli with 2-6 cell layers, are relatively small, wider than tall at 4-6 cm long and 5-9cm wide. Calcification islight on both surfaces. Sporangia with indusium principally on the outer surfacealternating with hair lines, usually in small clusters with a thin indusium. Concentric hair lines on the upper surface of the thallus.

***Padina* sp. FA**, haplotype: 5AB793717 (HSBU-2011304)

Habitat: Qeshm Island, Intertidal

The bright brown thallus (2 cell layers) is relatively large with 10-15 length and 5-8 width, lightly calcified on the lower surface and moderately to heavily on the upper surface, sporangia principally on the outer surface and sometimes also on the inner surface, usually in small clusters with indusium, hair lines on upper

***Padina* sp. FA**, haplotype: 6AB793718 (HSBU-2011305)

Habitat: Qeshm Island, Intertidal

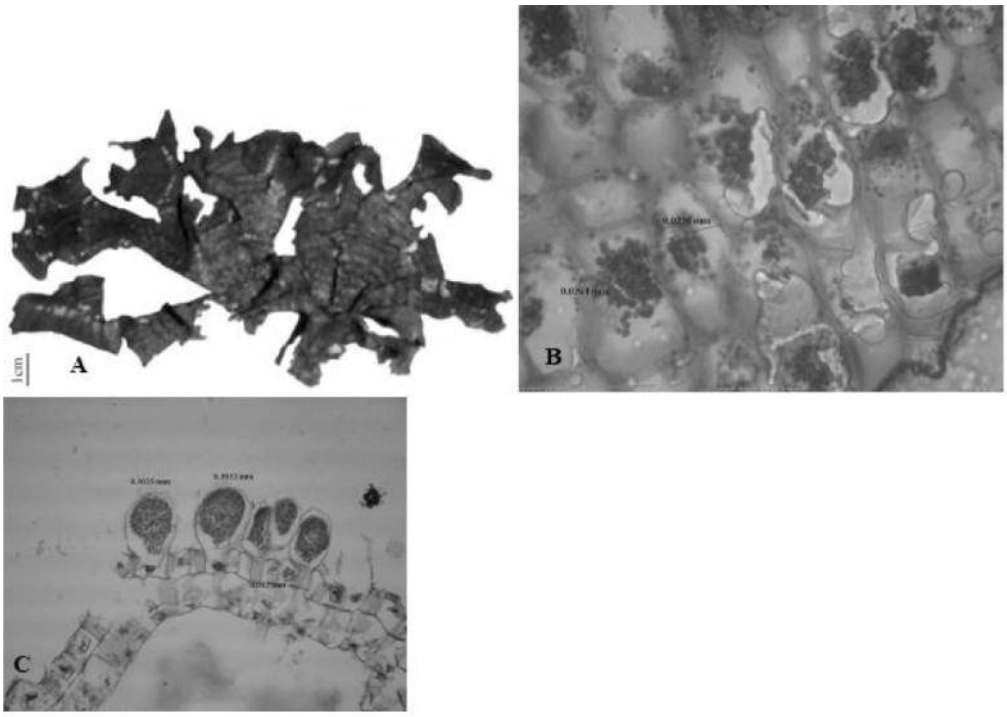

Fig. 4. A. Habitat, B. The surface view with the length of cell ×40, C. Cross section showed two cell layers ×40.

The yellowish thallus (2-6 cell layers) has 4-6 cm long and 6-10 cm wide. Upper and lower surfaces with heavy calcification. Sporangial sori is alternating with hair lines.

Padina **sp. FA**, haplotype: 7 AB793719 (HSBU-2011306)
Habitat: Kish Island, Intertidal
Thalli is bright brown with 2 cell layers and 5-15 cm long and 8-15 cm wide , stipe short without rust-colored fibrous hairs, heavily calcified on both surfaces except for hair lines, indusium present, hair lines on both surfaces sporangial sori alternating with hair lines, sporangial sori on both surface.

Padina **sp. FA,** haplotype: 8AB793720 (HSBU-2011307)
Habitat: Lengeh Port, Intertidal
Thalli (2-4-6 cell layers) with bright brown in color,4-6 cm long and 6-9 cm wide, hair lines on upper surface and alternating with sporangial sori without indusium, heavy calcification is on upper and lower surfaces.

Padina **sp. FA**, haplotype: 9AB793721 (HSBU-2011308)
Habitat: Kish Island, Intertidal
The length of bright brown thallus (2-4 cell layers) is 5-9 cm and the width is 4-8cm. Calcification is heavy on Upper and lower surfaces, sporangial sori on upper surface. Thallus dark brown with 2-4cell layers, 5-9 length, 4-8 width, high calcified on two sides spo-

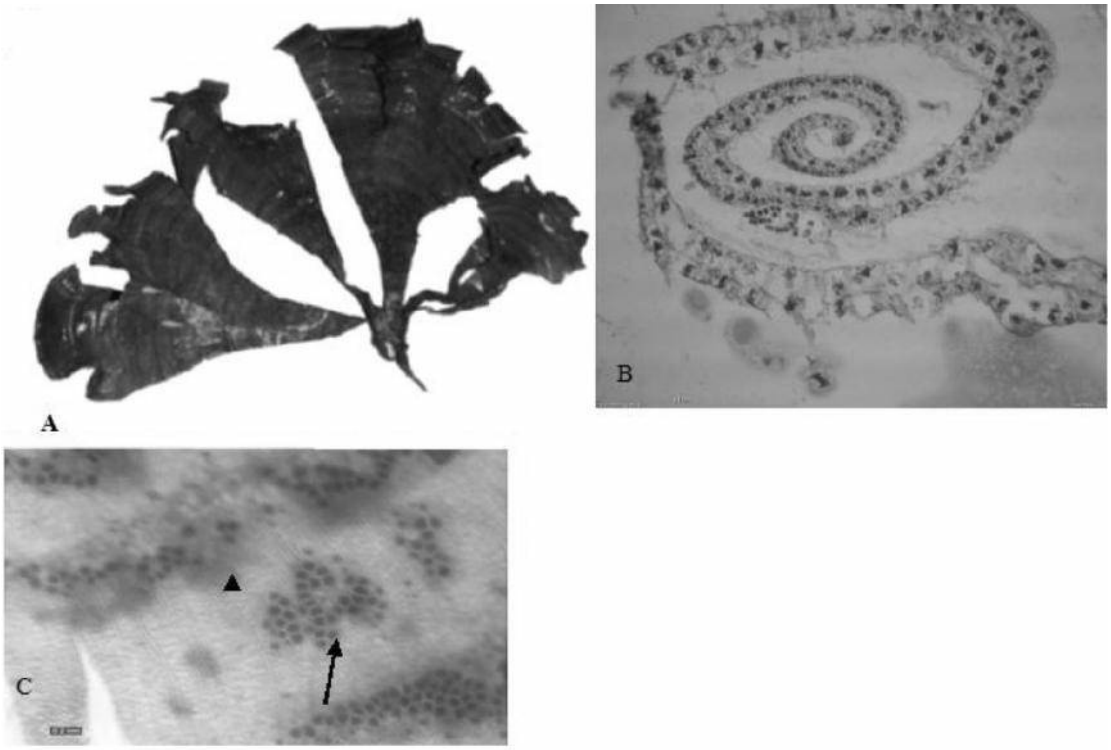

Fig. 5. A. Habitat, B. Longitudinal section×40, C. Hair lines (arrowhead) sporangium line (arrow) ×40.

rangial on upper surface, reproductive organs and Phaeophycean hairs present in concentric zones.

***Padina* sp. FA**, haplotype: 57FAB793724 (HSBU-2011309)

Habitat: Kish Island, Lengeh Port, Qeshm Island, Intertidal

Thallus color is dark brown with 2-6 cell layers, up to 10-17cm length and 10-13cm width, hair lines on upper surface, sporangial sori alternating with hair, calcification is on upper and lower surfaces heavily.

***Padina* sp. FA,** haplotype: 2AB775783 HSBU-2011310

Habitat: Qeshm island, Intertidal

Thalli (2-6 cell layers) has 5-7 long and 4-6 wide color is bright brown, hair lines on upper surface sporangial sori alternating with hair lines sporangial sori on upper and lower surfaces, upper and lower surfaces is heavily calcified. They are yellow greenish in color. Apices are acute to round and sometimes somewhat incurved is completely erect with more than 10 cm.

Molecular phylogenetic analysis

The chloroplast-encoded rbcL gene has been extensively used in molecular phylogenetic studies of brown algae and has been demonstrated to be a useful molecular marker by authors (Hoshina et al. 2004; De Clerck et al., 2006; Lane et al., 2006; Cho et al., 2007; Bittner et al., 2008; Ni-Ni-Win et al., 2008 and

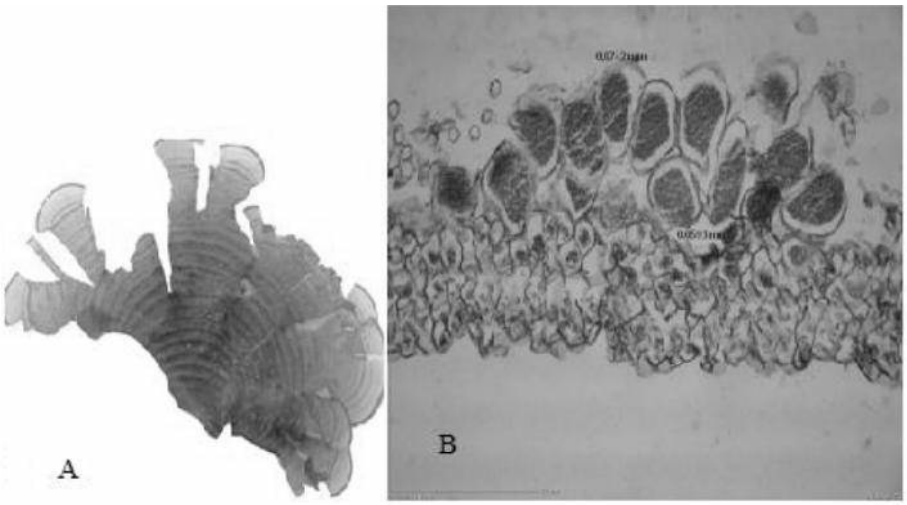

Fig. 6. *Padina* sp. FA haplotype 6: A. Habitat, B. Longitudinal section×40.

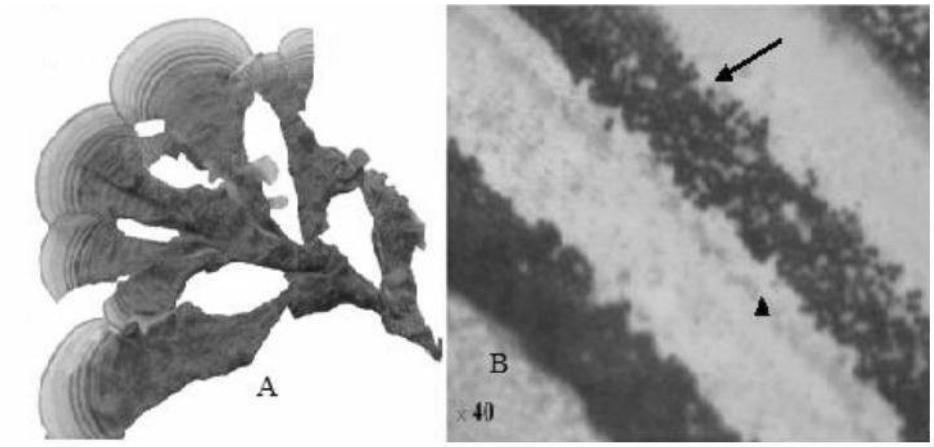

Fig. 7. *Padina* sp. FA haplotype 7: A. Habitat, B. Hair lines (arrow head) sporangium line (arrow) ×40.

2010). The rbcL alignment consisted of 12 sequences representing three Padina species and one out group taxa, including twelve new sequences and yielded 10 distinct haplotypes along the coast of Persian gulf (Table 1). Figures 13 and 14 showed the main monophyletic groups were constant in performed analyses. The first clade (Fig. 14) presented high bootstrap values (99-100% support) in all analyses (ML, NJ, MP) and included 9 haplotypes of *Padina* sp. FA from Persian gulf which grouped with *Padina boergessenii.*

Phylogenetic trees constructed from the ML and NJ analyses showed a similar topology. P. boergessenii specimens and *Padina* sp. FA haplotypes were closely allied, and formed a strongly supported monophyletic group with high bootstrap confidence of 90%. Padina boergesenii with JQ364063 and JQ364065 accession numbers are clearly separated from the

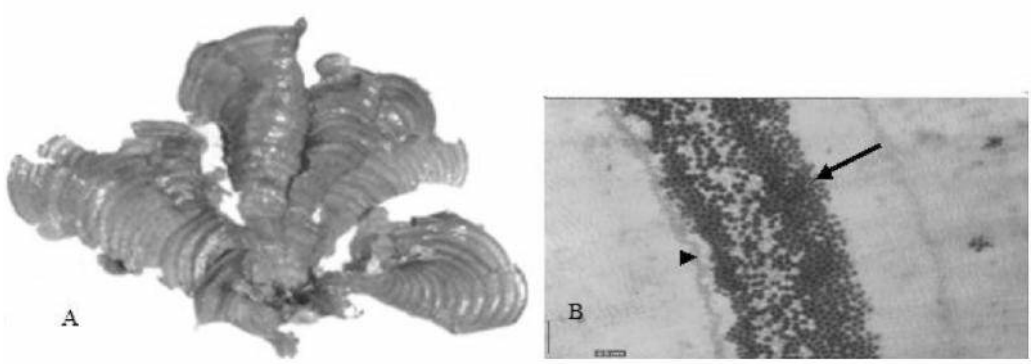

Fig. 8. *Padina* sp. FA haplotype 8: A. Habitat, B. view surface hair lines (arrow head) of tetrasporangium line (arrow) ×40.

strongly monophyletic group consisting of the other *P. boergessenii* specimens and Padina sp. FA haplotypes. *Padina* sp. FA haplotypes are morphologically similar to *Padina* australis which have 2-6 layers but they are molecularly similar to *P. boergesenii* from Zanzibar, Tanzania which has 0-7 nucleotide differences. However, these similarities cover the partial of the nucleotides. In other words, all nucleotides of this study are almost 490 bp, but this is a comparison among 275 nucleotides. *Padina* sp. FA haplotypes are morphologically similar to *Padina* australis which have 2-6 layers but they are different from *P. Australi*s with more than 10 nucleotides.. Other taxa *Padina* sp. PG and Padina sp. INDEGRO32 included in clade 2 in Figure 13 and in calde 3 of Figure 14. Morphological study showed that *Padina* sp. PG is closely to *P. antillarum* (*P. tetrastromatica*). As previously noted the differences between species are the calcification of blades and the number of cell layers. The phylogenetic trees confirmed *Padina* sp. PG was always nested in the diverse clade of the *P. antillarum*. This placement was highly supported with 92% bootstrap confidence (Fig. 14). *Dictyota ciliolata* and *Dictyota dicotoma* is used as outgroup.

Discussion

Molecular phylogenetic analyses using rbcL sequences, combined with morphological observations, showed the occurrence of two undescribed Padina species in Persian gulf coasts. *Padina* sp. FA most closely related *P. australis* and *P. boergesenii.* The members of the *P. boergesenii* and *Padina* sp. FA haplotypes are the most strongly grouped in the trees constructed in the present study (Fig. 14). This result suggests that these species might have evolved closely to each other, but separately from the other species of the *P.boergessenii*. Given the close similarity of *Padina* sp. FA haplotype sequences to the *P.boergessenii* sequences, it seems safe to assume that these samples are conspecific, in addition, there are some differences in nucleotides between 0 to 8 positions. In order to investigate wheth-

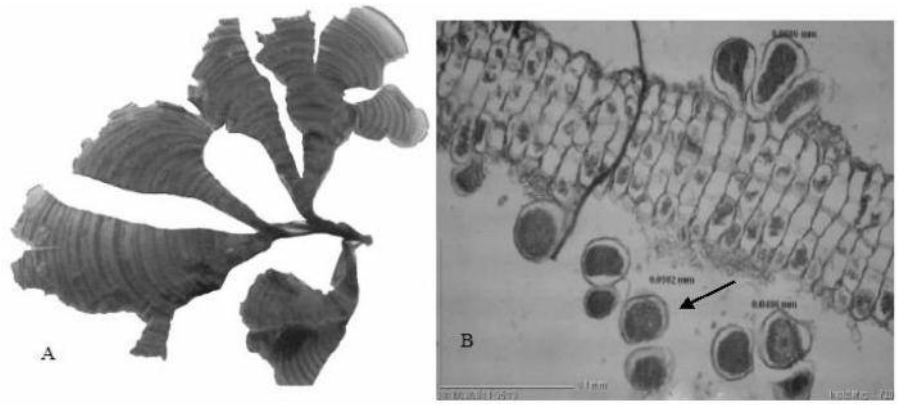

Fig. 9. *Padina* sp. FA haplotype 9A: Habitat, B. Longitudinal section Transverse section of tetrasporangial sori, showing obovate tetrasporangia (arrow) ×40.

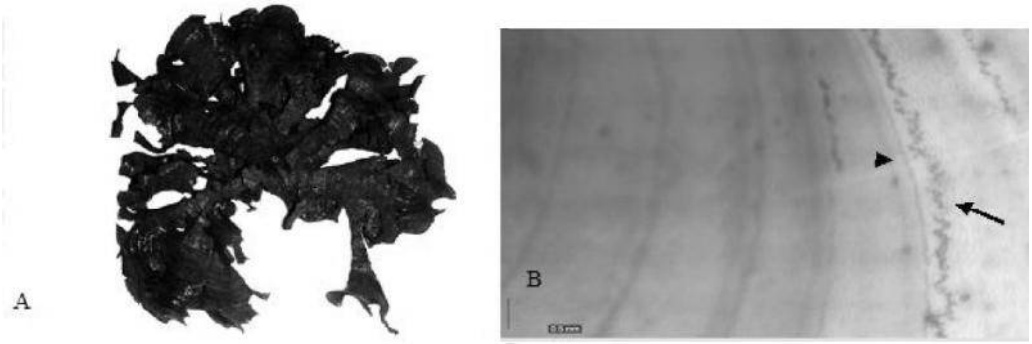

Fig. 10. *Padina* sp. FA haplotype 57FA: Habitat, B. view surface view surface hair lines (arrowhead) Tetrasporangium line (arrow) ×40.

er the *Padina* sp. FA is a new species or the conspecific with the *Padina boergesenii* it is necessary to make further studies. Also more samples and using different molecular markers is needed. Until further studies clarifing their taxonomic status, we prefer to set the *Padina* sp. FA samples as a new species, since they were indistinguishable in morphology and positioned in a monophyletic clade in all analyses of rbcL gene and other markers.

Phylogenetic relationships among the *Padina* sp. INDEGRO32 and *P. antillarum* species can be clear. The phylogenetic analyses of the rbcL data show the monophyly groupings. The monophyly of Padina was fully supported (De Clerck, 2006).

Padina sp. PG is very similar to *Padina antillarum* (Kützing) Piccone = *P. tetrastromatica* Hauck but the blades of *P. tetrastromatica* showed no calcification (Wynne et al., 1999). In cross sections of the blades, both in mid region and in more basal portions, *Padina* sp.

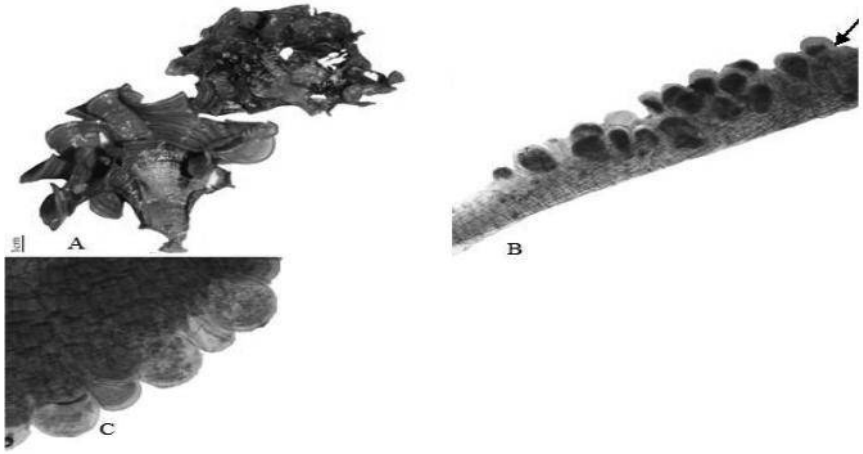

Fig. 11. *Padina* sp. FA haplotype 2: A. Habitat, B. Transverse section of tetrasporangial sori, showing obovate tetrasporangia and C. Tetrasporangium on surface *Dictyota ciliolata* AB775782.

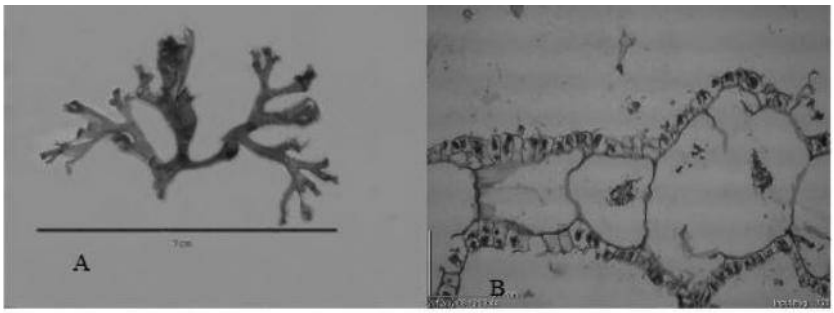

Fig. 12. Dictyota ciliolate AB775782: A. Habitat, B. Transverse section.

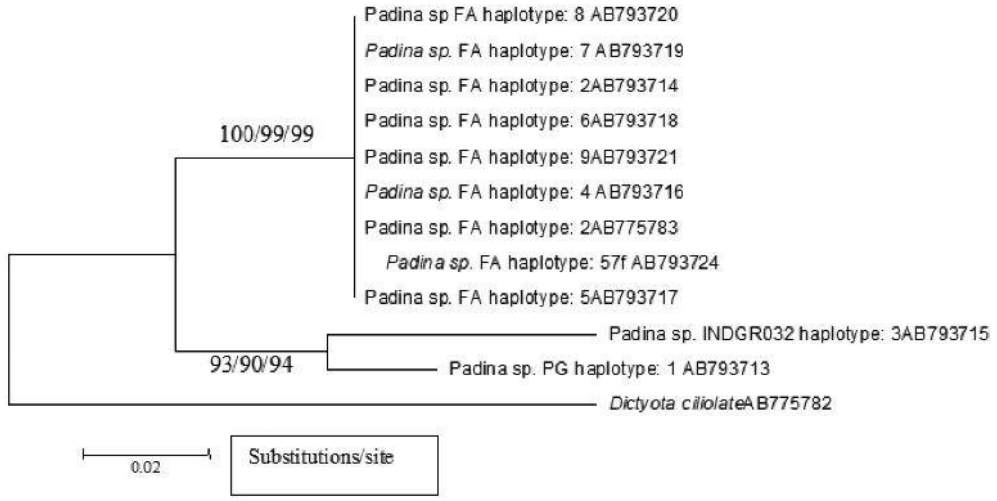

Fig. 13. Maximum likelihood tree (-1n L = 531.2360) based on rbcL gene sequences. Numbers at each node indicate bootstrap values (>50%) for maximum

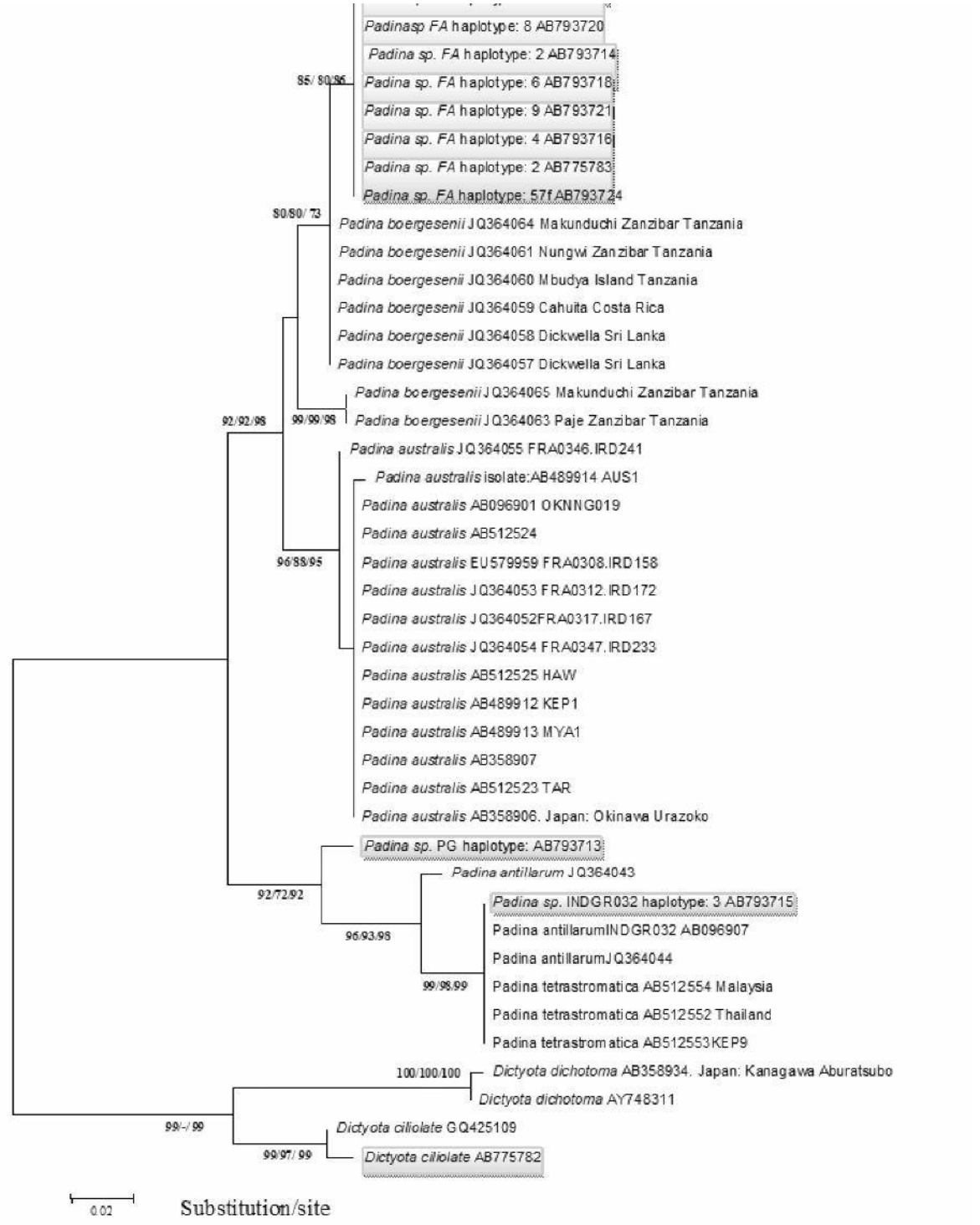

Fig. 14. Maximum likelihood tree (-1n L= 702.1252) based on rbcL gene sequences. Numbers at each node indicate bootstrap values with only values>70% being shown for maximum likelihood (ML) (left), maximum parsimony (MP) (middle) and neighbor joining (NJ) (right). The Persian gulf specimens whose sequenced were determined in the present work are in green boxes.

PG showed a 4 layered organization but this species has 2 cell layer and 4 layers at the base. Molecular studies confirmed this dividing. A sequence of *Padina* sp. FA is genetically identical with those of *P. australis* and *P. boergesenii*. Therefore, we consider *Padina* sp. FA to be conspecific with *P. boergessenii.* Many Padina species may remain to be discovered. This species is possibly endemic to Persian gulf. However, additional sampling in other regions might be able to confirm either its endemism northern or southern of Persian gulf.

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Taxonomic Study of Some Cyanobacteria Species in Natural Habitats of *Tanacetum parthenium* Emphasising on *Wollea* and *Cylindrospermum* Morphological Characters

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Abstract

Floristic study of cyanobacteria in the terrestrial habitats of Iran, revealed several taxa belonging to seven genera. Soil samples were collected from natural habitats of medicinal plant, *Tanacetum parthenium*, located in four provinces of Iran (Qom, Isfahan, Lorestan, Razavi Khorasan Provinces). All studied provinces are located in semi-arid regions of the country. The results of this study indicated the presence of 13 species belong to three families of cyanobacteria. Among these families, Nostocaceae with four genera and eight species showed the highest diversity, whereas Rivulariaceae with one genera and one species exhibited the lowest diversity. Among several heterocystous and non heterocystous taxa, *Nostoc* and *Oscillatoria* were found to be the most dominant genera in almost all the studied sites. *Wollea* and *Cylindrospermum* with limited distribution in terrestrial habitats of Iran were found. Therefore, part of the current systematic study has been done with emphasis on these two specific taxa. A distribution of local area and camera lucida pictures of identified taxa is subjected in this study.

Keywords: Blue-green algae, Diversity, Iran, Nostocaceae, Terrestrial habitat, *Tanacetum parthenium*

Introduction

Algae are an important part of the surface soil and play a special role in soil health and dynamics (Metting, 1981). Blue-green algae (cyanobacteria) are one of the most important and most efficient groups of algae, because they can affect properties of the soil and play a special role in the growth and productivity of plants through their ability to stabilize nitrogen and the production of growth-promoting metabolites. Cyanobacteria are also capable to release complex organic carbon compounds in the rhizosphere (Chamizo et al., 2018), which can improve soil density, texture, permeability, and water holding capacity through the binding to soil particles (Kaushik, 2007). So that, the positive effects of cyanobacteria as a biofertilizer for some crops and medicinal plants such as saffron (*Crocus sativus* L.), mint (*Mentha aquatica* L.), peppermint (*Mentha piperita* L.) and savoury (*Satureja hortensis* L.) were emphasized in previous studies (Riahi et al.,

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2013; Zand et al., 2015; Shariatmadari et al., 2015).

Due to the practical value of these photosynthetic microorganisms as bio-fertilizers and regarding the importance of medicinal plants in human societies, in recent decade, the study of the cyanobacteria present in bed soil of medicinal plants has been emphasized by several researchers (Riahi et al., 2013, Riahi et al., 2017). *Tanacetum parthenium* (L.) Sch. Bip. or fever few known as a perennial herbaceous medicinal plant with a right stem and pinnatifid leaves and belongs to *Asteraceae* family. This species is expanded throughout the northern, western, central and eastern parts of Iran and is used to treat diseases such as fevers, migraine headaches, rheumatoid arthritis, stomach aches, toothaches and insect bites (Pareek et al., 2011). In addition, this plant has antibiotic and anticancer agent (Pareek et al., 2011; Mathema et al., 2012). Like any other plant, the growth and productivity of feverfew can be improved by applying the biofertilizers, including algae based fertilizers, because of the ability of these organisms to produce growth stimulants such as auxins, amino acids, sugars and vitamins (Sergeeva, 2002; Singh et al., 2016). There are also reports implying the ability of these photosynthetic organisms to produce antifungal and antibacterial compounds (El-Mougy and Abdel-Kader, 2013).

The study of algal flora, especially cyanobacterial flora of terrestrial ecosystems of Iran has no long history and returns to last decade. Due to limited information present about the algal flora of terrestrial ecosystems, especially the microflora of several plants rhizosphere, this study can be considered as a step towards the completion of algal flora of Iran. In addition, according to the results of previous studies regarding the positive effect of cyanobacteria on the growth and productivity of some crops and medicinal plants (Saadatnia et al., 2009; Riahi et al., 2012), also considering the medicinal value of the *Tanacetum parthenium*, the present study provides an opportunity to introduce effective cyanobacterial taxa for improving growth of this medicinal plant.

Materials and Methods

Study sites

Soil samples were collected from natural habitats of *Tanacetum parthenium* located in four provinces of Iran (Table 1), according to Rangaswamy method (1966). Soil sampling was done in the summer of two consecutive years 2012 to 2013. All investigated provinces are located in semi-arid regions of the country, and altitude of 1400 to 2500 meters.

Cyanobacterial species Identification

The sieved soils from different sites were transferred to sterile petri dishes containing sterile liquid nitrate free BG-11 medium (Stanier et al., 1971). The petri dishes were incubated in a culture chamber at 25±2°C for two weeks of artificial light illumination (74 µmol photons $m^{-2}s^{-1}$) with a 12/12 hours light-dark cycle. After colonization, isolates were transferred to agar plates for purification. For taxonomic determinations, the semipermanent slides of colonies were

Table 1. Geographic details of the sampling locations.

Sites	Origin	Altitude (m)	Latitude/Longitude
1	Iran, Isfahan, Abyaneh	1820	33° 15′ N 51° 49′ E
2	Iran, Qom, Avel	2501	34° 16′ N 50° 59′ E
3	Iran, Razavi Khorasan, Neyshabur	1680	36° 64′ N 59°28′ E
4	Iran, Qom, Veshnavah	2472	34° 15′ N 50° 59′ E
5	Iran, Lorestan, DareDurak	1400	33° 11′ N 49° 56′ E

prepared and morphometric study was performed by light microscopy (Olympus, Model BH-2) and based on Desikachary (1959), Prescott (1970) and Komárek (2013). The microscopic images were provided by the digital camera (Canon, Model G10) and were drawn by means of camera lucida (Olympus, Japan).

Results

In this study, 13 specimens belonging to seven genera were identified (Figs 1, 2). The comparison of cyanobacterial diversity of *Tanacetum parthenium* bed soil in studied sites showed that higher cyanobacterial diversity occurred at the Veshnaveh village of Qom with five taxa compared to other stations. The stations, Avel village of Qom and Abyaneh in Isfahan provinces with four taxa was in the second level of diversity. All species and their distribution are located in Table 2.

1. ***Nostoc verrucosum*** Vaucher ex Bornet & Flahault 1886. (Fig. 1a)

Syn.: *Nostocella verrucosa* (Vaucher) Gaillon., *Nostoc rothii* C. Agardh 1824.

General Distribution: Europe (Whitton, 2011), Asia (Desikachary, 1959; Hirose et al., 1977; Shariatmadari et al., 2013).

Distribution in studied sites: Isfahan: Abyaneh (33°15′ N 51°49′ E).

2. ***Nostoc calcicola*** Brébisson ex Bornet & Flahault 1888. Brébisson in Meneghini, Monographia *Nostochinearum italicarum*, 121, 1843. (Fig. 1b).

General distribution: Europe (Alvarez-Cobelas and Gallardo, 1988), Asia (Desikachary 1959; Hu and Wei 2006; Shariatmadari et al., 2011), Australia and New Zealand (Day et al., 1995).

Distribution in studied sites: Isfahan: Abyaneh (33°15′ N 51°49′ E), Razavi Khorasan: Neyshabur (36°64′ N 59°28′ E).

3. ***Nostoc linckia*** Bornet ex Bornet & Flahault 1886: 193 (as 'Linckia').

Syn.: *Nostoc confusum* C. Agardh, *Monormia intricata* Berkeley, *Nostoc* intricatum (Berkeley) Meneghini, *Anabaena* intricata (Berkeley) Kützing, *Nostoc* piscinale Kützing ex Bornet & Flahault, *Nostoc* rivulare Kützing ex Bornet & Flahault

General distribution: Europe (Whitton, 2011), Asia (Desikachary, 1959; Hirose et

Table 2. List of soil cyanobacterial species recorded from natural habitats of *Tanacetum parthenium* and their distributions.

Species	Site 1	Site 2	Site 3	Site 4	Site 5
Nostocales					
I. Nostocaceae					
Cylindrospermum voukii Pevalek					•
Nostoc verrucosum Vaucher ex Bornet & Flahault	•				
Nostoc calcicola Brébisson ex Bornet & Flahault	•		•		
Nostoc linckia Bornet ex Bornet & Flahault		•			
Nostoc punctiforme Hariot			•	•	
Nostoc carneum C.Agardh ex Bornet & Flahault				•	
Trichormus fertilissimus (C.B.Rao) Komárek & Anagnostidis				•	
Wollea saccata Bornet & Flahault					•
II. Rivulariaceae					
Calothrix stagnalis Gomont		•			
III. Oscillatoriaceae					
Oscillatoria perornata Skuja	•			•	
Oscillatoria chilkensis Biswas	•	•		•	
Oscillatoria subbrevis Schmidle					•
Phormidium articulatum (N.L.Gardner) Anagnostidis & Komárek		•			

Site 1. Abyaneh; Site 2. Avel; Site 3. Neyshabur; Site 4. Veshnavah; Site 5. DareDurak

al., 1977).

Distribution in studied sites: Qom: Avel (34 ˚16′ N 50 ˚59′ E).

4. ***Nostoc punctiforme*** Hariot 1891. (Fig. 1c).

Syn.: *Nostoc hederulae* Meneghini ex Bornet & Flahault, *Polycoccus punctiformis* Kützing.

General distribution: Europe (Alvarez-Cobelas and Gallardo 1988; Whitton et al., 1998; Caraus, 2002; O′Brien et al., 2006), Asia (Desikachary, 1959; Shariatmadari et al., 2013), Pacific Islands (Sherwood, 2004), Australia and New Zealand (O′Brien et al., 2006).

Distribution in studied sites: Razavi Khorasan: Neyshabur (36 ˚ 64′ N 59 ˚28′ E), Qom: Veshnavah (34 ˚15′ N 50 ˚59′ E).

5. ***Nostoc carneum*** C. Agardh ex Bornet & Flahault 1886. (Fig. 1d)

Syn.: *Nostoc rufescens* C. Agardh, *Anabaena rufescens* (C. Agardh) Kirchner, *Nostoc spongiaeforme* C. Agardh ex Bornet & Flahault.

General distribution: Europe (Whitton, 2011; Loza et al., 2013), Asia (Desikachary,

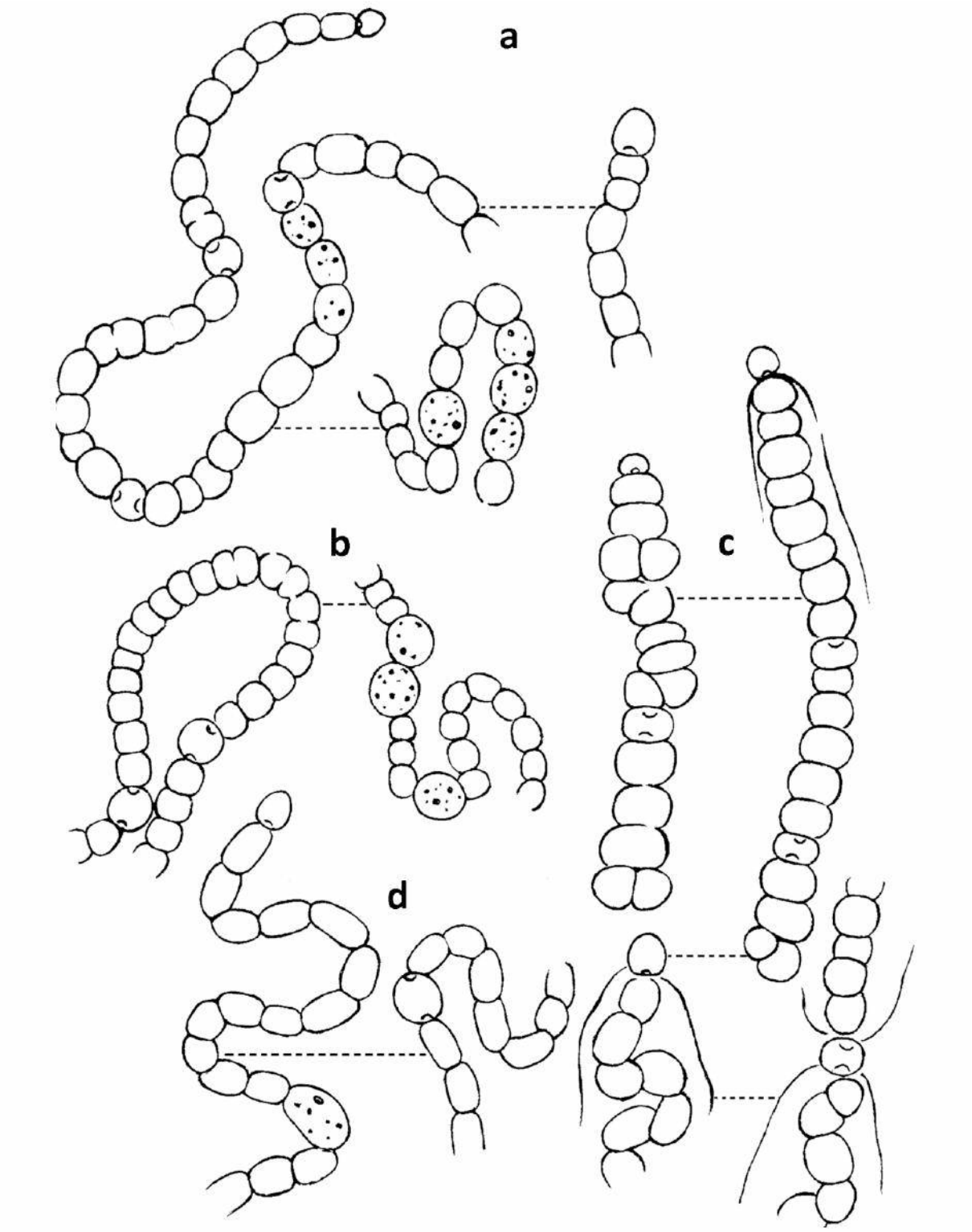

Fig. 1. Camera lucida images of taxa: a. *Nostoc verrucosum*, b. *Nostoc calcicola*, c. *Nostoc punctiforme*, d. *Nostoc carneum* (Scale: 10 μm).

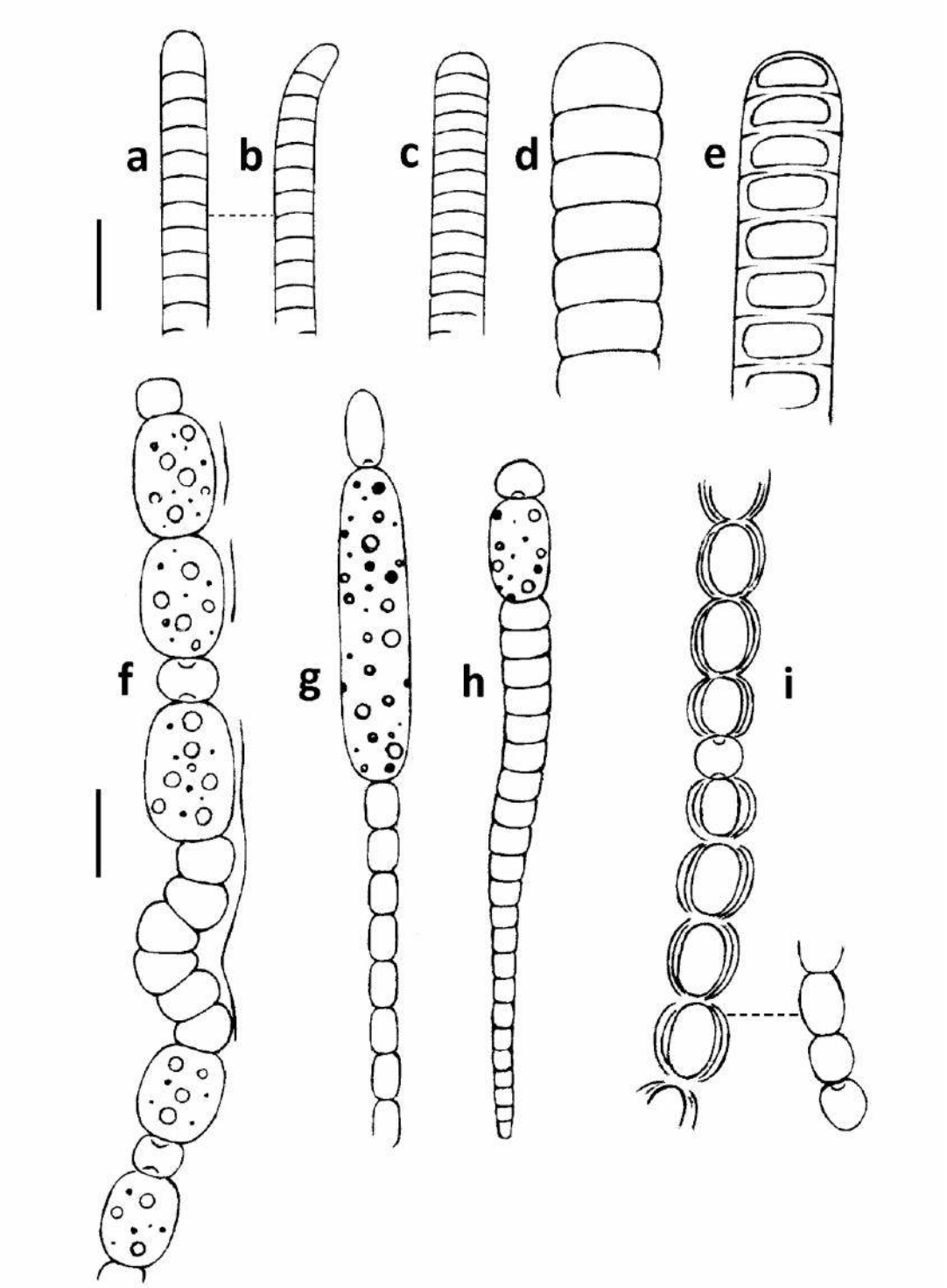

Fig. 2. Camera lucida images of taxa: a, b. *Oscillatoria chilkensis*, c. *Oscillatoria subbrevis*, d. *Oscillatoria peronata*, e. *Phormidium articulatum*, f. *Wollea saccata*, g. *Cylindrospermum voukii*, h. *Calothrix stagnalis*, i. *Trichormus fertilissimus* (Scale: 10μm).

1959; Hirose et al., 1977).
Distribution in studied sites: Qom: Veshnavah (34˚15′ N 50˚59′ E).

6. ***Wollea saccata*** Bornet & Flahault 1886. (Fig. 2f)
Syn.: *Sphaerozyga saccata* Wolle
General distribution: Northern of eastern Siberia, North America (Kozhevnikov and Kozhevnikova, 2011), Asia (Shariatmadari et al., 2018)
Distribution in studied sites: DareDurak (33 ˚11′ N 49˚56′ E).

7. ***Trichormus fertilissimus*** (CB Rao) Komárek & Anagnostidis, 1989. (Fig. 2i)
Syn.: *Anabaena fertilissima* CB Rao.
General distribution: America (Dawes, 1974), Asia (Desikachary, 1959; Shariatmadari et al., 2011).
Distribution in studied sites: Qom: Veshnavah (34˚15′ N 50˚59′ E).

8. ***Cylindrospermum voukii*** Pevalek 1916. (Fig. 2g).
Syn.: No synonyms are currently included in AlgaeBase.
Distribution in studied sites: Lorestan: DareDurak (33 ˚ 11′ N 49 ˚ 56′ E).

9. ***Calothrix stagnalis*** Gomont 1895. (Fig. 2h)
Syn.: No synonyms are currently included in AlgaeBase.
General distribution: Europe (Whitton 2011), Asia (Leghari et al., 2005; Shariatmadari et al., 2013).
Distribution in studied sites: Qom: Avel (34˚ 16′ N 50˚59′ E).

10. ***Oscillatoria perornata*** Skuja 1949. (Fig. 2d).
Syn.: *Planktothrix perornata* (Skuja) Anagnostidis & Komárek
General distribution: North-Eastern Australia (McGregor, 2007), Asia (Desikachary, 1959).
Distribution in studied sites: Isfahan: Abyaneh (33˚15′ N 51˚49′ E), Qom: Veshnavah (34˚15′ N 50˚59′ E).

11. ***Oscillatoria chilkensis*** Biswas 1932. (Figs 2a, b).
Syn.: No synonyms are currently included in AlgaeBase.
General distribution: South-west Asia (Siddiqui et al., 2009, Silva et al., 1996, Gupta, 2012, Kesarwani et al., 2015, Rao and Gupta, 2015)
Distribution in studied sites: Isfahan: Abyaneh (33˚15′ N 51˚49′ E). Qom: Avel (34˚ 16′ N 50˚59′ E), Qom: Veshnavah (34˚15′ N 50˚59′ E).

12. ***Oscillatoria subbrevis*** Schmidle 1901. (Fig. 2c).
Syn.: No synonyms are currently included in AlgaeBase.
General distribution: Asia (Vynogradova, 2014; Park, 2012; Gul et al., 2007; Hirose et al., 1977), North-Eastern Australia (McGregor 2007), Europe (Whitton 2011).
Distribution in studied sites: Lorestan: DareDurak (33 ˚11′ N 49 ˚56′ E).

13. ***Phormidium articulatum*** (NL.Gardner) Anagnostidis & Komárek 1988. (Fig. 2e).
Syn.: *Oscillatoria articulata* NL.Gardner, *Oscillatoria grunowiana* var. *articulata* (Gardner) Drouet.
General distribution: Asia (Park, 2012; Desikachary, 1959).
Distribution in studied sites: Qom: Avel (34 ̊16′ N 50 ̊59′ E).

Discussion

Algal flora in terrestrial habitats of Iran indicate the importance of floristic study of these special ecosystems. Considering to complete the data bank of algal flora, cyanobacteria presented in bed soil of medicinal plant, *Tanacetum parthenium*, was studied. A total of 13 taxa of cyanobacteria belonging to the Nostocales and Oscillatoriales were identified, from which the family Nostocaceae with 61.5% of total taxa include the largest number and the families Oscillatoriaceae and Rivulariaceae with 30.5 % and 8% of total taxa, respectively, were in the next places. *Nostoc* and *Oscillatoria*, with 38.5% and 23% of total specimens, counted as highest biodiversity among identified taxa. *Wollea, Trichormus, Cylindrospermum, Calothrix* and *Phormidium,* each with one species, constituted only 7.8% of the total taxa, thus accounted for the least species diversity in the studied microflora. The results of this study are consistent with another study on cyanobacteria in agricultural lands, such as rice fields of Iran, which reported that the Nostocacea family was more diverse than other identified families (Shariatmadari et al., 2013). Similar results also were reported by Prasanna and Nayak (2007) in agricultural soils of India.

In another part of present study, the selection of efficient characteristics for identification of studied taxa were considered. Separating characteristics such as thallus structure, existence or absence of heterocysts, akinete position, size and shape of akinetes and presence or absence of gelatinous sheath around the trichomes have shown the importance of morphometric studies. It should be noted that in relation to some taxa, such as Wollea Bornet et Flahault, the morphological characteristics is even more effective than genetic traits. Wollea is a poorly known genus which is most morphologically similar to genera *Anabaena* and *Nostoc* (Komárek 2010; Kozhevnikov and Kozhevnikova, 2011). Several species of this genus was reported from aquatic and terrestrial habitats of Iran (Table 3). From these taxa can be pointed out to species, *Wollea ambigua* (Rao) Singh and *Wollea vaginicola* (Fritsch & Rich) Singh, which are reported from rice fields of Iran (Shariatmadari et al., 2014). *Wollea saccata* is another species of this genus which is reported from *Tanacetum parthenium* habitats in present study. The difference in the shape and size of akinetes as well as akinete color in maturity stage are the most important differences of this taxon with the other species (Shariatmadari et al., 2018).

Cylindrospermum voukii is another species that have limited distribution in terrestrial habitats of Iran and reported from *Tanace-*

tum parthenium habitats (Shariatmadari et al., 2018). One of the distinguishing characteristic of this taxon is its long and narrow akinete. *Cylindrospermum zonatum Komárek* and *Cylindrospermum minutissimum* Collins are other taxa with cylindrical akinetes (Komárek, 1989). But length/width ratio of akinetes (3.7-4.0 respectively) is far more than these taxa and is consistent with the *Cylindrospermum voukii* (Tables 4, 5, 6). Among the identified taxa, genera such as *Nostoc, Wollea, Trichormus, Cylindrospermum* and *Calothrix* are classified as heterocystous cyanobacteria. The heterocystous cyanobacteria can fix molecular nitrogen due to the presence of specific cells called heterocyst. It should be noted that some of the heterocystous taxa, such as *Nostoc linckia* Bornet ex Bornet & Flahault, are known as poisonous specimens (Hallegraeff et al., 2003). Therefore, it can be said that any specimens is not suitable for the production of bio-fertilizers, despite its nitrogen fixation capabilities. On the other hand, among the identified taxa, another species *Nostoc calcicola* Brébisson ex Bornet & Flahault, can be considered as a biofertilizer (Shariatmadari et al., 2013). The ability to add total nitrogen, nitrite, nitrate, ammonium and cations such as Na^+, K^+ and Ca^{2+} into the rhizosphere of plants, as well as the relatively high compatibility of this taxon against environmental stresses such as salinity and pH stresses make this cyanobacteria an appropriate choice for fertilizer production (Obana et al., 2007). In addition, the ability to biosynthesize secondary metabolites effective in plant growth, such as hormonal compounds, indole butyric acid and indole acetic acid (Seyed Hashtroudi et al., 2012) and other undeniable capabilities such as improving soil texture, facilitating soil moisture retention, increasing the amount of organic matter in the soil have made this species with high practical value in the production of bio fertilizers. Therefore, using this taxon as bio fertilizer for feverfew can be a starting point for further research in this regard.

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Table 3. Comparision of main morphological characteristics of the *Wollea* species has been reported from Iran.

Characters	***Wollea saccata* Bornet & Flahault**	***Wollea ambigua* (Rao) RY.Singh**	***Wollea vaginicola* (Fritsch & Rich) Singh**
Vegetative cells			
Form	barrel shaped or discoid	discoid	sub-quadrate
Length (µm)	3.0-5.0	5.0-7.0	4.5-5.0
Width (µm)	4.0-6.0	8.5-9.0	4.5-5.0
Terminal cells			
Form	conical-rounded	widely rounded	conical with rounded apex or spherical
Heterocyst			
Form	barrel shaped or slightly longer than wide	spherical or sub-spherical	spherical or elongated spherical
Length (µm)	4.0-6.0	9.0-10.0	5.5-7.0
Width (µm)	4.0-5.0	9.0-10.0	5.0-6.0
Akinete			
Form	ellipsoidal or ovate	ellipsoidal or widely oval	oblong
Color	yellowish brown in maturity stage	yellowish green in maturity stage	yellowish green in maturity stage
Position	solitary or several in both sides of heterocysts	solitary aside of both sides of heterocystes	2-4 in rows aside of both sides of heterocystes
Length (µm)	12.0-17.0	14.0-27.0	8.0-11.0
Width (µm)	6.0-8.0	14.0-16.0	6.0-8.0
Ecology	bed soil of *Tanacetum parthenium*	submerged soils and aquatic ecosystems (Shariatmadari et al., 2011)	submerged soils and aquatic ecosystems (Shariatmadari et al., 2011)

Table 4. Comparision of main morphological characteristics of the some *Cylindrospermum* species has been reported from Iran

Characters	***Cylindrospermum voukii* Pevalek**	***Cylindrospermum catenatum* Ralfs ex Bornet & Flahault**	***Cylindrospermum minutissimum* Collins**
Vegetative cells			
Form	barrel-shaped or slightly longer	sub-quadrate or sub-cylindrical	sub-quadrate or cylindrical
Length (µm)	4.0-5.0	5.0-7.0	5.0-6.0
Width (µm)	3.0-4.0	4.0-4.5	3.0-3.5
Heterocyst			
Form	obovoid	obvoid or oblong-ovate	oblong-ovate
Length (µm)	7.0-8.0	8.0-11.0	6.0-11.0
Width (µm)	4.0-4.5	4.5-5.0	5.0-8.0
Akinete			
Form	oblong	ovate	cylindrical
Color	epispore colorless	epispore yellowish brown	epispore colorless
Position	solitary adjacent to the heterocystes	several adjacent to the heterocystes	solitary adjacent to the heterocystes
Length (µm)	20.0-27.0	13.0-25.0	11.0-22.0
Width (µm)	5.0-8.0	8.0-9.0	6.0-8.0
Ecology	bed soil of *Tanacetum parthenium*	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)

Table 5. Comparision of main morphological characteristics of the *Cylindrospermum* species has been reported from Iran.

Characters	***Cylindrospermum marchicum* (Lemmermann) Lemmermann**	***Cylindrospermum michailovskoense* Elenkin**	***Cylindrospermum muscicola* Kützing ex Bornet & Flahault**
Vegetative cells			
Form	barrel-shaped or sub-cylindrical	quadrate or slightly longer than broad	sub-quadrate or slightly longer than broad
Length (µm)	6.0-6.5	4.0-5.0	5.0-6.0
Width (µm)	4.0-4.5	4.0-4.5	4.0-6.0
Heterocyst			
Form	ovoid with rounded apex	sub-sherical or ovoid	oblong or ovoid
Length (µm)	10.0-10.5	8.0-9.0	6.0-9.0
Width (µm)	5.0-5.5	7.0-7.5	4.0-6.0
Akinete			
Form	ovate to barrel-shaped	ovate	oval or broadly oval
Color	epispore colorless	epispore colorless	epispore brownish or yellowish brown
Position	catenate series adjoining the heterocyst	solitary adjacent to the heterocyste	solitary adjacent to the heterocyste
Length (µm)	6.0-9.0	21.0-27.0	17.0-23.0
Width (µm)	4.0-6.0	10.0-15.0	7.0-10.0
Ecology	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)

Table 6. Comparision of main morphological characteristics of the *Cylindrospermum* species has been reported from Iran.

Characters	***Cylindrospermum majus* Kützing ex Bornet & Flahault**	***Cylindrospermum sphaericum* B.N.Prasad**	***Cylindrospermum stagnale* Bornet & Flahault**
Vegetative cells			
Form	sub-quadrate	sub-quadrate or cylindrical	sub- cylindrical
Length (µm)	5.0-6.0	5.0-8.0	6.0-6.5
Width (µm)	5.0-5.5	4.0-4.5	3.5-4.5
Heterocyst			
Form	sub-spherical or ovoid	ovoid, sub-spherical or ellipsoidal	elongate or ovoid
Length (µm)	5.0-6.5	6.0-7.0	8.0-10.0
Width (µm)	6.0-6.5	4.0-5.0	5.0-6.0
Akinete			
Form	ovate	spherical	oblong or sub-cylindrical
Color	epispore brownish with distinct papillae	epispore brownish	epispore brownish
Position	solitary adjacent to the heterocyste	singly or in pair adjacent to the heterocyste, rarely with intermediate akinet	solitary adjacent to the heterocyste
Length (µm)	20.0-33.0	7.0-17.0	20.0-29.0
Width (µm)	10.0-14.0	7.0-17.0	13.0-18.0
Ecology	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)	submerged soils and aquatic ecosystems (Shariatmadari and Riahi, 2012)

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Effects of Light Intensity, Photoperiod and Nitrate Levels on Biomass Production in Green Algae *Scenedesmus dimorphus*

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Abstract

The effects of three different photoperiods 12:12, 16:8 and 8:16 hours light: dark (LD), light intensity of 3000, 5000 and 7000 lux and nitrate levels of 1.47, 2.94 and 4.41 mmol/l on growth rate (cell number) and biomass of *Scenedesmus dimorphus* was studied. The algal cells were cultured at 28±2°C in BBM culture medium during one month and cells were counted every three days. By the end of experiments the lowest cell concentration (9.3×10^6 cells/ml) was observed at 12:12 LD, 1.47 mmol/L nitrate and 3000 lux light intensity and the highest cell concentration at 8:16 LD, 2.97 mmol/L nitrate and 3000 lux light intensity. The lowest algal biomass (1.38 g/l) was observed at 12:12 LD, 3000 lux light intensity and 1.47 mmol/l nitrate levels while the highest biomass (5.2 g/l) at 8:16 LD, 7000 lux light intensity of and 2.94 mmol/l nitrate level.

Keywords: *Scenedesmus dimorphus*, Light intensity, Photoperiod, Biomass production, Light quality, Growth rate

Introduction

Algae are photosynthetic organism divided into major groups of micro and macro algae (Rosenberg et al., 2008). Microalgae are considered as the most basic energy sources of an aquatic ecosystem (Walker et al., 2005). Environmental factors such as pH and light availability strongly influence the growth rate and biomass production in microalgae. Growth rate is the most important indicator of ecological success or adaptation of a species to environmental changes (Rivkin, 1989; Isik et al., 2006). Currently several groups of algae are used in industries, and green algae are more important in this aspect (Mata, 2010). Nowadays, the science of biotechnology have focused on developing effective stimuli to improve growth rate, properties of biochemical components and pigments in algae. As a photosynthetic organism green algae require an aqueous medium in the form of water, light, CO_2 and range of certain minerals (Balat, 2010). Algae contain valuable chemicals like vitamins, carotenoids, proteins, poly-

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saccharides, fatty acids with anti-inflammatory, anti-cancer, antifungal, antioxidative and antibacterial activities (Pereira, 2018; Shalaby, 2011). They also sustain immune system stimulatory features (Hanna et al., 2008). *Scenedesmus* has significant potentials in biotechnology (Soltani et al., 2002) with astonishing tolerance to acid condition (Soltani et al., 1992). Due to high protein contents the algae is mass cultured in aquaculture as a protein source (Kianmehr, 2005). This species is used as standard organism in water technology and management (Zochleder et al., 1986). Several authors have studied the effects of light quality and quantity on growth and biochemical properties of algae. Velichkova et al. (2013) cultured *Scenedesmus dimorphus* in two different culture media (BBM and 3N-BBN) and studied its potentials for biofuel production. Liang et al. (2009) used glucose as carbon source in *Chlorella vulgaris* biomass production under light and dark condition and achieved maximum productivity under light condition. Dittamart et al. (2014) applied different organic carbon supplements in mixotrophic culture of *Scenedesmus* sp. to enhance biomass and lipid production in AARL G022 and reported a photoperiod of 16:8 LD condition as optimum. The aim of the present study was finding out optimum light intensity and photoperiod to induce higher growth rate in *Scenedesmus dimorphus* to be used in larger and industrial scales.

Materials and Methods

Scenedesmus dimorphus was provided by the Clean Nature Explorers Company (CNE Company, Rasht, Iran). All equipment was sterilized prior to commencement of the experiments to eliminate potential contaminant risk.

Culture condition

Light intensity was adjusted to 3000, 5000 and 7000 lux by Lux Meter TES 1336A. Mean water temperature was 28±2°C. The BBM culture medium with slight modification was prepared for culture. The Design-Expert software suggested 17 experimental treatments and 3 factors. The first factor (light) was adjusted between 3000-70000 lux with binary level of 5000 lux. The second factor (nitrate) was used with normal concentration 2.94 mmol/l, half normal (1.47 mmol/l) and 1.5 normal (4.41 mmol/l). The third factor was different photoperiod (light/dark) with 3 levels of 12:12 (LD), 8:16 (LD) and 16:8 (LD) hours light: dark. The samples were cultivated in 4 liter containers using BBM medium. Culture container aerated regularly to maintain algal cells suspended. Culture period lasted for 30 days and algal cells were counted twice in a week in this period using a Thoma counting chamber. The cultured algae harvested by keeping the containers in dark room. Cells started to settle down at the bottom of the containers overnight. The settled algae cells were released into a falcon, centrifuged at 2500 rpm and dried by freeze drier and then weighted.

Results

The biomass of algae cells in light intensities of 3000, 5000 and 7000 lux as variable factor were plotted against nitrate levels (2.94, 1.47 and 4.41 mmol/l) and photoperiod (12:12, 8:16 and 16:8 hours LD). Considering the rotations in figures there is no direct relationship between nitrate concentration and darkness at fixed light intensity. The red areas reflect the highest yield obtained and cell number at 7000 lux light intensity. The results are presented in Figure 1. The BC variables (light intensity and photoperiod) were kept constant and the variable A or nitrate was changed. As shown in Fig-

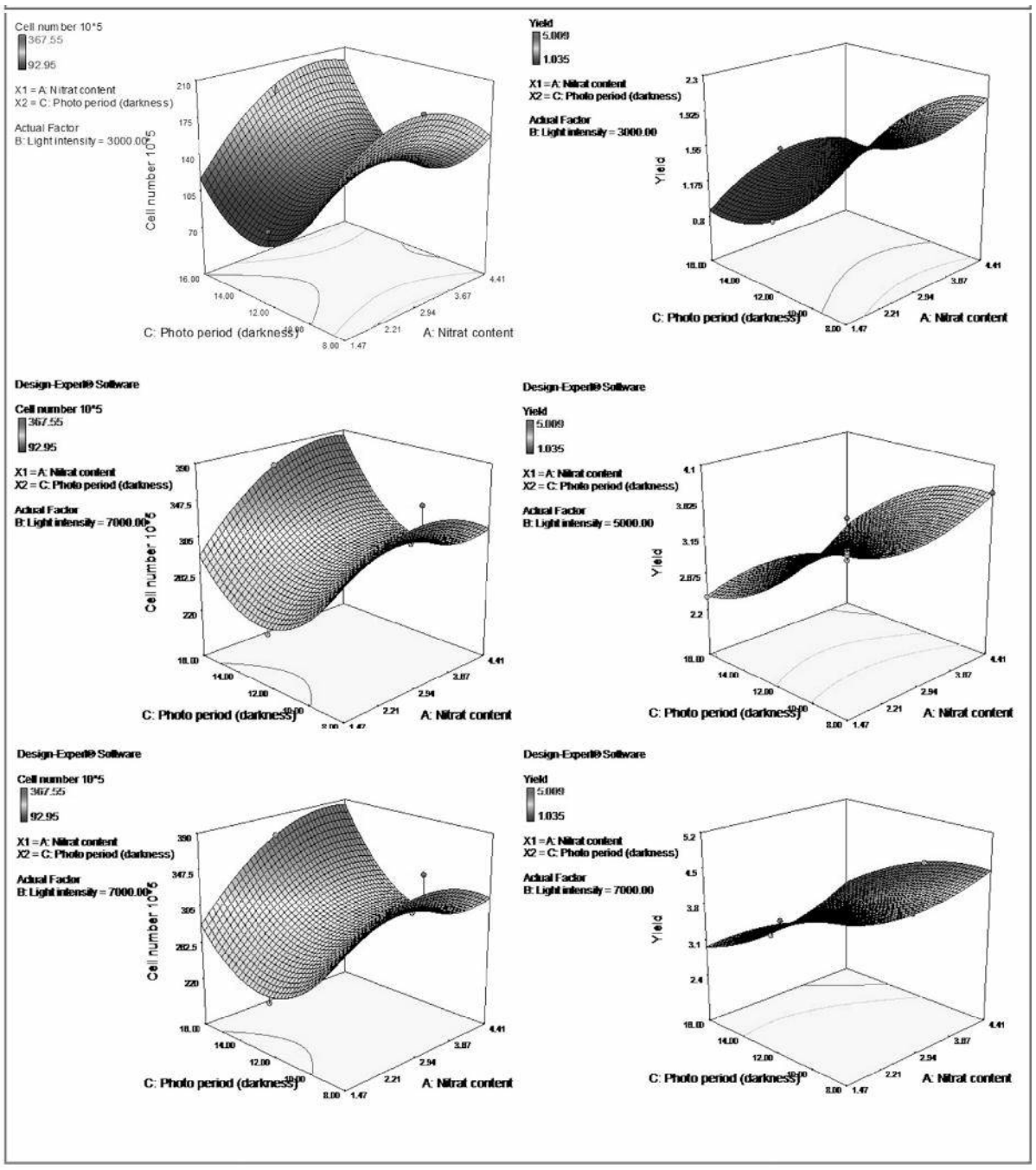

Fig. 1. Surface plot of the algal biomass (g/l) and algal concentration (cell/ml) light intensity as variable factor (3000, 5000, 7000 lux) and photoperiod and nitrate as fixed factors.

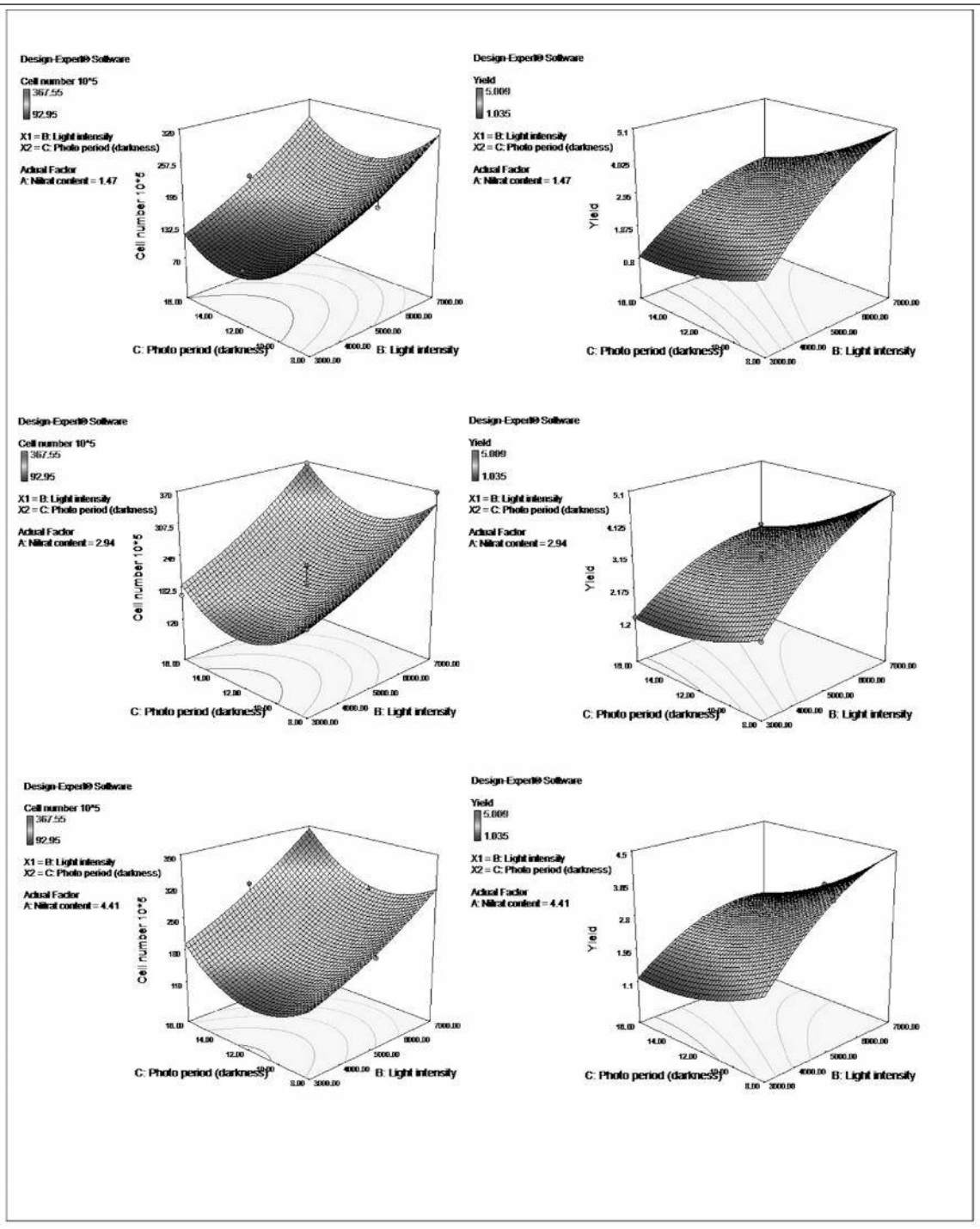

Fig. 2. Surface plot of the algal biomass (g/l) and algal concentration (cell/ml) with nitrate level as variable factor (1.47, 2.94, 4.41 mmol/l) and photoperiod and light intensity as fixed factors.

ure 2 increases in light intensity at different nitrate concentration resulted in higher biomass and yield. The highest cell number was observed at 4.41 mmol/l (red areas). The effects on cell number are plotted in Figure 2.

Biomass obtained at different photoperiod as variable factor were plotted against nitrate levels and light intensities. As shown in Figure 3 at 8 hours darkness increase in light intensity resulted in higher cell number and

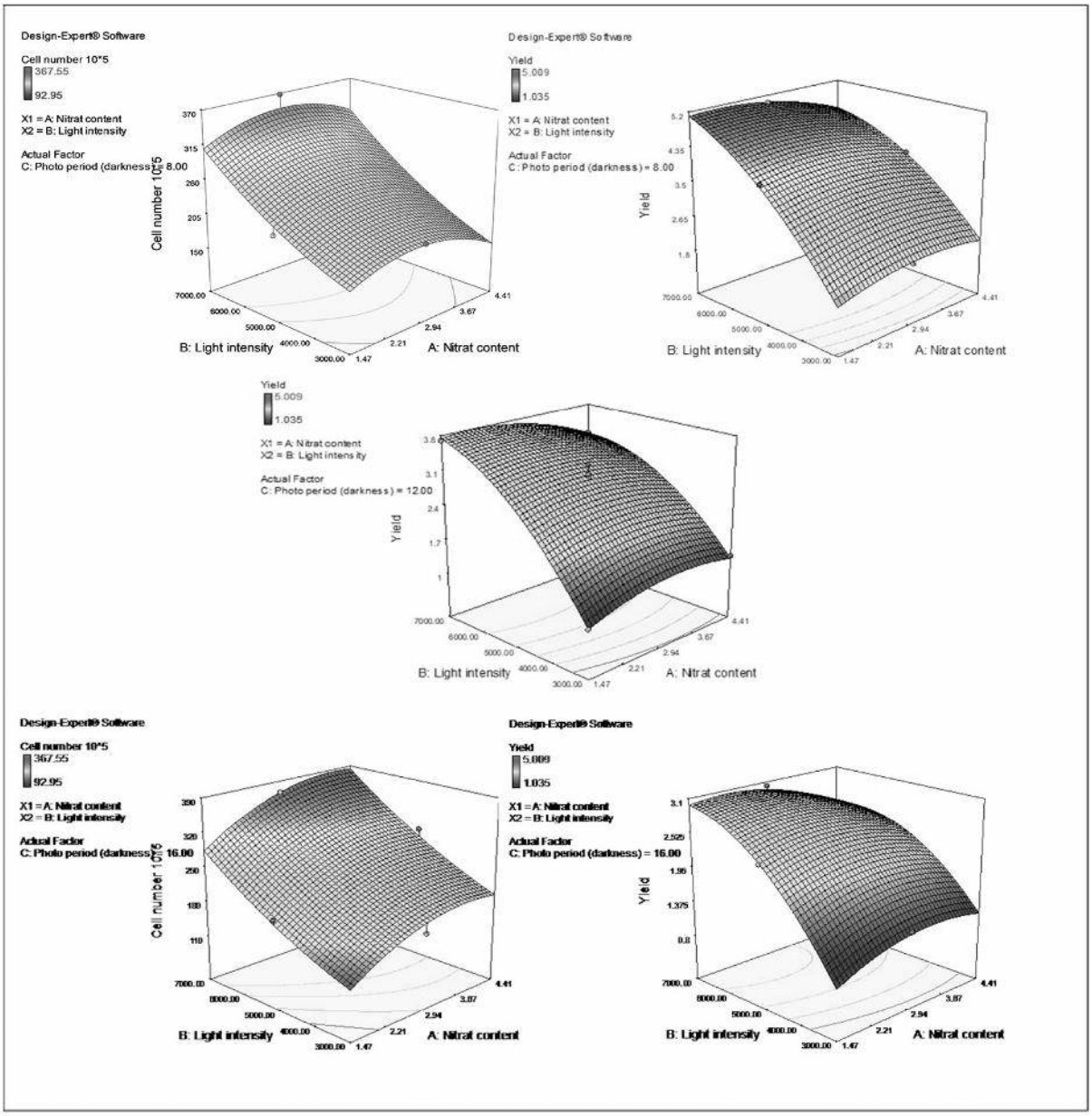

Fig. 3. Surface plot of the algal biomass (g/l) and algal concentration (cell/ml) at 8:16, 16:8, 12:12 photoperiod as variable factor and light intensities and nitrate levels as fixed factors.

the highest cell number was observed at 2.94 mmol/l nitrate concentration. Under this condition (upper right graph) biomass was also increased. Increaseing in light intensity resulted biomass increasing and yield. The results are presented in Figure 3.

Biomass of algae cells and growth rate related to photoperiod 12:00, 8:16 and 18:6 hours and nitrate levels, 1.47, 2.94 and 4.41 mmol/l were recorded for the period of 30 days and plotted which are presented. As light intensity increased the number of cells increased, correlations 0.787 and 0.782 yield. Also, a negative correlation coefficient occurs in longer dark period and high nitrate concentrations (Figure 4).

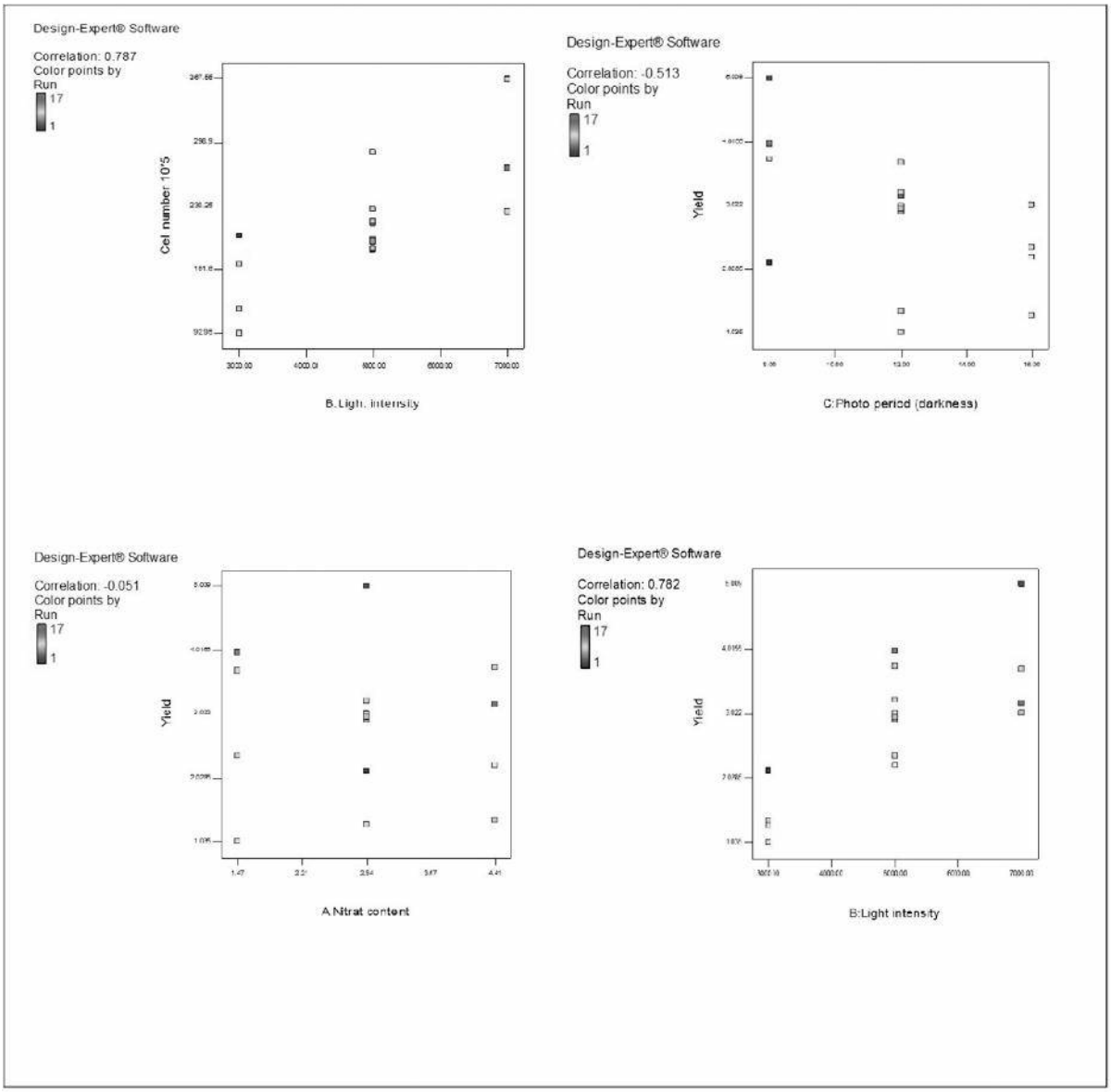

Fig. 4. Correlation matrix between cell number, light intensity, photoperiod and nitrate levels.

Discussion

Environmental factors such as light intensity and photoperiods influence the growth pattern of algae (Rai and Gupta, 2016), therefore vigilant regulation of these parameters is indispensable for optimal operation of algae culture system. The quality and quantity of light control grow rate, metabolism (Ruangsomboon, 2012) and biomass of algae (Sun et al., 2014). As a limiting factor, light should be adjusted properly (Xue et al., 2011). Nitrate levels are also critical in algae culture according to Pribyl et al. (2016). They changed nitrate level from 1.47 to 4.41 mmol/l and biomass production decreased. A similar result was achieved in present study where cell number increased but the total biomass decreased. Similar result has been reported by Guedes et al. (2010). Although increase in cell number was appar-

ent, it seems that algal cell did not grow optimally at lower photoperiod. Therefore reduction in final biomass could be justified which was the case in a study by Liang et al. (2013). The Response Surface Method (RSM) with Box- Behnken Design (BBD) was used to determine the optimum growth condition for *S. dimorphus*. In these types of graphs the interactions between parameters, responses and variables-responses are illustrated. Higher correlation coefficient shows effective interaction between the parameters. According to regression results, light intensity positively associated with cell number and growth rate of algae. Three dimensional plots illustrate the zones and extent of effects of each parameters on response. Considering the effects of photoperiod on cell concentration, a significant increase in cell concentration was observed at lower nitrate level (1.47mmol/l), highest light intensity (7000 lux) and 16: 8 LD. Singh and Singh (2015) and Pancha et al. (2015) reported similar results where increase in light intensity resulted in higher biomass production and yield. The lowest cell concentration was observed at 12:12 LD with 9.3×10^6 cells/ml and the highest at 16:8 LD and nitrate level of 2.94 mmol/l with 3.91×10^7 cells/ml. As shown in Figure 2 growth rate and biomass increased in various nitrate levels under 8:16 LD which resulted in significant increase in cell concentration. George et al. (2014) have reported similar trend in their study. Analysis of the effects of light intensity on growth rate showed the lowest growth rate at 3000 lux light intensity (2×10^6 cells/ml) and the highest growth at 7000 lux with 3.9×10^7 cells/ml a result which was observed in Scott et al. (2010). Nitrate also influenced the growth rate and cell concentration in combination with other environmental parameters. The lowest cell concentration was observed at the lowest nitrate level (1.47 mmol/l), 3.2×10^7 cells/ml and the highest at the 4.41mmol/l nitrate concentration about 3.9×10^7 cell/ml. Similar result has been reported by Sun et al. (2014) and confirmed that nitrate levels influence the production of algal biomass. In this study the lowest cell biomass was observed at 4.41 mmol/l nitrate (Mandotra et al., 2016) with 4.5 g/l and the highest at 2.94 mmol/l nitrate (5.2 g/l). As for light intensity and its effect on algal biomass the lowest biomass was recorded with 2.3 g/l at 3000 lux light intensity similar to Liang et al. (2013) and the highest with 5.2 g/l at 7000 lux which is in agreement with findings of Yeesang and Cheirsilp (2011). Photoperiod also influenced the biomass production. The lowest biomass (3.1 g/l) was observed at 16:8 light/dark condition and the highest (5.2 g/l) at 8:16 hours light/dark photoperiod.

Combination of environmental parameters such as light intensity, photoperiod and nitrate levels affect on growth rate and biomass production in different and rather unpredictable way. The lowest biomass for example was observed at higher nitrate level where the highest biomass was observed at lower nitrate level and lower photoperiod 8:16 LD. There are multiple interactions which directly or indirectly affect algal cell growth and

biomass production. Here we shed light into some of these parameters which impact algal growth individually and with other factors combination. Obviously there are several other factors or combinations of factors which impact growth rate and biomass production in algae. Therefore further study on this subject with different species of algae and different parameters suggested.

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