Journal of Phycological Research

Vol. 6, No. 2, Sep. 2022

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Effect of Azide on Growth Parameters of *Haematococcus pluvialis* in Green and Red Stages

Hakimeh Mansouri[1]*, Hamzeh Rezazadeh[1]

Received: 2022-04-20 Revised and accepted: 2022-08-15

Abstract

Haematococcus pluvialis can accumulate large amounts of astaxanthin under stress conditions. Azide as an effective respiratory inhibitor can induce oxidative stress. In this study, the effect of pre-treatment (7 days) and treatment (14 days) of different concentrations of azide (0, 25, 50, and 100 µM) were investigated on growth and biochemical parameters in green and red cells. Azide treatment and pre-treatment caused a decrease in all measured parameters except for the carotenoid in the green stage. Carotenoid content did not show any changes in azide treatment but pre-treatment with low concentration induced carotenoid accumulation in the green stage. The dry weight, protein, and carbohydrate amount did not change in red cells treated with azide, but carotenoid content decreased in these cells. Pretreated with azide hurt the amount of protein and carbohydrates but increased the carotenoid content. Azide pretreatment had better performance in increasing the carotenoid amount in red cells. These results showed that *H. pluvialis* has good potential for phytoremediation of azide and carotenoid accumulation.

Keywords: Sodium azide, Carbohydrate, Carotenoid, Protein, Pretreatment

Introduction

The green microalga *Haematococcus pluvialis* (Volvocales) is widely distributed worldwide, from brackish water to rock surfaces (Czygan 1970; Lorenz 1999). This microalga is the richest astaxanthin source, which exhibits a strong antioxidant effect and a powerful scavenging ability against singlet oxygen (O'Connor and O'Brien 1998). This microalga has a complex life cycle with two distinct cell forms. Under suitable conditions, the cells are green, pear-shaped, and motile with two flagella.. *H. pluvialis* cells are transformed to red, spherical, and immobile cells under stress conditions. The red color of the cells is due to the accumulation of a significant amount of astaxanthin This stage is usually accompanied by the transformation of green vegetative cells into red cysts. At first, astaxanthin is accumulated in lipid droplets in the cytoplasm surrounding the nucleus, then a massive accumulation of carotenoids occurs, and astaxanthin-containing lipid

1- Department of Biology, Faculty of Science, Shahid Bahonar University of Kerman, Iran
*Corresponding email address: h_mansori@uk.ac.ir

droplets eventually fill the whole cytoplasm. Several factors play a role in obtaining the maximum algal biomass and astaxanthin content, including quality and quantity of light, culture medium, growth regulators, and temperature. These factors usually have a contrary effect on biomass production and astaxanthin content. It means stress conditions that increase astaxanthin content, on the other hand, decrease growth and biomass production. Sometimes exposure to extreme stress can cause cell death in the short term (Su et al., 2014).

There are several ways to manipulate biological systems to increase biomass or specific bioactive compounds. However, biotechnology method permanent changes may cause detrimental effects on the ecosystem and humans (Hunt et al., 2010). Therefore, increasing the production of bioactive compounds and biomass using alternative methods such as chemicals can be a safe method in this regard. Studies have indicated that biochemical stimulators significantly increase microalgae productivity (Cheng et al., 2012). It has been shown that reactive oxygen species (ROS) can involve in the carotenogenesis and induction of astaxanthin accumulation in *H. pluvialis* (Kobayashi et al., 1993).

Azide is an effective respiratory inhibitor and irreversibly binds to the fourth complex of the electron transport chain. Inhibition in the electron transport chain causes ROS produced and induction of oxidative stress (Apel and Hirt, 2004). Azide can also increase ROS and induce oxidative stress by inhibiting the activity of catalase and superoxide dismutase enzymes. It was reported that azide induces massive accumulation of triglycerides and production of lipids in different microalgae species (Chen et al., 2019; Yahya et al., 2018; Zalogin and Pick, 2014).In the present study, the effect of azide on increasing the amount of biomass and carotenoids in *H. pluvialis* under proper growth and stress conditions (without nitrate to induce carotenogenesis) over two periods (7 and 14 days) was investigated.

Material and methods

Haematococcus pluvialis (UTEX) used in this study was obtained from Arian Gostar. *Haematococcus pluvialis* has grown autotrophically in Bold's Basal medium (Tripathi et al. 1999). Pure cultures were incubated under 50 µmol photons $m^{-2}s^{-1}$ fluorescent light intensity with a 16/8 h light/dark cycle at 25 ± 1°C. An equal number of cells (3.5 x 104 cells ml^{-1}) were used to inoculate 150 ml of fresh culture medium in 250 ml Erlenmeyer flasks for all treatments. Two methods were used for the experiment; the cultures were exposed to different concentrations of sodium azide (0, 25, 50, and 100 µM for 14 days), and algal cells were affected by the same azide concentrations for seven days and then transferred to a new culture medium without azide. To induce astaxanthin accumulation, the green cells were transferred from the growth conditions to the stress conditions (fresh medium of BG11 without nitrogen) after centrifugation (Siegień and Bogatek,

2006).

Growth was measured by counting cell numbers using a haemacytometer. The dry weight of the algal biomass was estimated after drying at 60 °C in a hot-air oven until a constant weight was obtained. For pigment analysis, a specific amount of biomass was extracted with 96% methanol, and chlorophyll and carotenoids were quantified as per the procedure given by Şükran et al. (Şükran et al., 1998).

Soluble protein concentration was obtained according to Bradford and Fales method (1976) was used to assay soluble carbohydrate content (Fales, 1951).

All experiments were performed in three replicates. Data are presented as mean ± standard deviation (SD) and analyzed by one-way analysis of variance. Besides, Duncan's multiple comparisons test was used to estimate the significance of the differences ($P< 0.05$).

Results and Discussion

Algae treated and pretreated with azide showed fresh weight loss of green cells (Fig. 1a).

However, these treatments did not have a significant effect on the fresh weight of red cells except for cells treated with 100 µM azide which showed less fresh weight compared to control cells (Fig. 1b).

Azide treatment significantly decreased dry weight of algae cells (Fig. 2). This effect was dose-dependent which it means that with increasing azide concentration, a greater

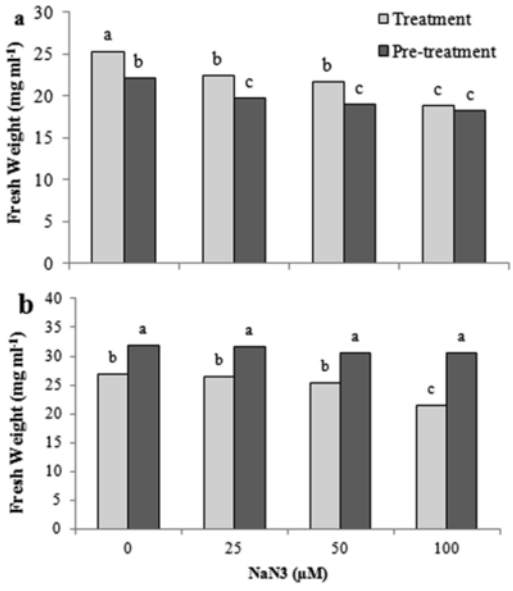

Fig. 1. Effect of azide treatment and pre-treatment on fresh weight in the green (a) and red (b) stage of *Haematococcus pluvialis*. Values are means of three-replication ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

decrease in dry weight was observed. The lowest dry weight was observed in culture treated with 100 µM azide with a 6-time decline. Pretreatment by azide also declined the cell dry weight of *H. pluvialis* in the green phase. However, it was not dose-dependent, and no difference was observed between different azide concentrations. Dry weight in the treated algae was about 60% control.

An incredible result was that azide treatment and pretreatment did not reduce the dry weight of microalgae in the red stage, just 100 µM azide treatment caused a 28% decrease in dry weight compared to control cells. Based on the decline in fresh weight and dry weight of the azide-treated algae in the green phase and no decline in the red phase, it can be concluded that the azide-treated cells grew better than the control cells in the stress phase (red stage). It was expected that this reduction would be observed in the red phase. Azide is a known metabolic inhibitor in plants and algae with many potential targets. Azide binds irreversibly to the heme cofactor in cytochrome C oxidase and inhibits catalase and superoxide dismutase, which scavenge ROS. Therefore, it enhances oxidative stress (Zalogin and Pick, 2014). The seedlings developed from treated seeds of Eruca sativa with sodium azide showed wide variation in plant growth (Al-Qurainy, 2009). In this study, azide was used to induce mutation in plants grown from treated seeds. Indeed, growth and biochemical parameters reduction can be due to oxidative stress

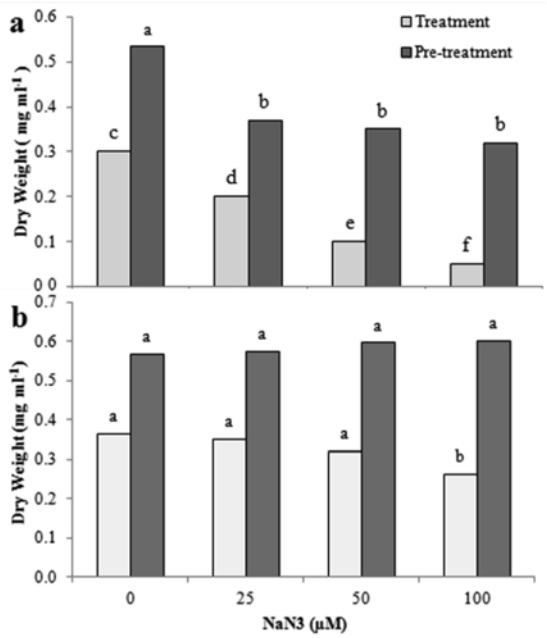

Fig. 2. Effect of azide treatment and pre-treatment on dry weight in the green (a) and (b) red stage of *Haematococcus pluvialis*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p< 0.05$ in one-way ANOVA and Duncan's tests

created by azide. It was reported that hydrogen peroxide production in *Anabaena nidulans* was increased many times by treatment with azide (Morales et al., 1992). The addition of azide had no significant effect on the cell biomass of *Anabaena* and *D. tertiolecta* (Chen et al., 2019; Nultsch et al., 1983). Contrary to our results, an experiment performed on different algae, reported that azide-treated cells had a higher rate of photosynthesis, more chlorophyll, and faster growth (Zalogin and Pick, 2014). Both treated and pre-treated algae with azide had lower amounts of chlorophyll a and b compared to control cells (Fig. 3). The lowest amount of chlorophyll a was observed in the treatment and pretreatment with 100 µM azide with a 50% decrease compared to the control.

The amount of chlorophyll b showed a 53 and 33% decrease in comparison to control in treatment and pretreatment by 100 µM azide, respectively. Similar to our results, a decrease in chlorophyll content decreased in *D. tertiolecta* treated with azide (Chen et al., 2019). Also, Al-Qurainy (2009) reported a significant decrease in chlorophyll content in Eruca sativa grown from seeds treated with azide.

Azide treatment had no significant effect on the carotenoid content of green cells (Fig. 4) . Pretreatment with an azide concentration of 25 µM resulted in a significant increase in carotenoid content, 28% increase compared to the pretreatment control and more than twofold compared to the treatment control.

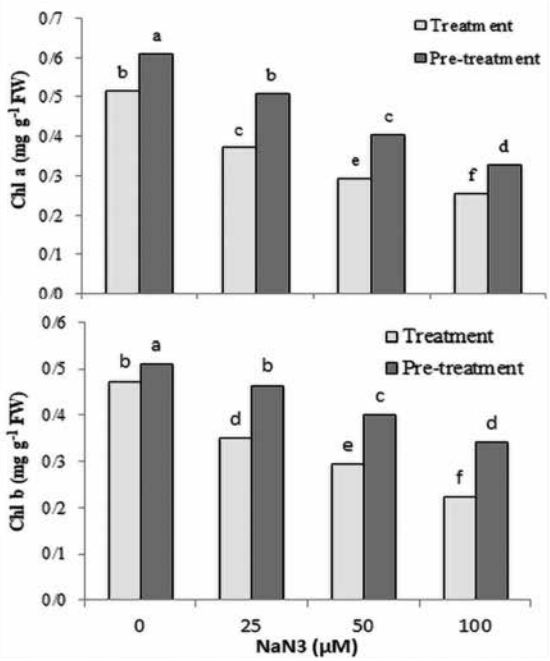

Fig. 3. Effect of azide treatment and pre-treatment on chlorophyll content in the green stage of *Haematococcus pluvialis*. Values are means of three-replications±standard deviation. Different letters show the significant difference with $p<0.05$ in one-way ANOVA and Duncan's tests

There was no significant difference between 50 and 100 µM concentrations of azide pretreatment, but these two concentrations significantly decreased the carotenoid level compared to the control. A decrease in the carotenoid content of Dunaliella tertiolecta and Eruca sativa was reported in cultures treated with azide (Al-Qurainy, 2009; Chen et al., 2019).

The results of the effect of treatment and pretreatment of azide on the carbohydrate content of green cells are shown in Figure 5. Although the number of carbohydrates significantly decreased at 100 µM azide, no significant difference was observed in carbohydrate content between the control, 25, and 50 µM azide treatments. In the pretreatment of sodium azide, the amount of carbohydrate in control and concentration of 25, 50, and 100 µM azide were 3.13, 2.35, 1.75, and 1.42 mg.g^{-1} FW, respectively. Carbohydrate content was reduced to half of the carbohydrate content in control by increasing azide concentration (by 100 µM azide).

Adding azide to the culture medium in the green phase for 14 days did not affect the carbohydrate content of cells in the red phase (Fig. 5). Azide pretreatment of cells in the green phase significantly reduced carbohydrate content in red cells. The effects of azide on the reduction of carbohydrate content were dose-dependent. The lowest amount of carbohydrate was observed in 100 µM azide with almost a 50% decrease compared to the control. Carbohydrate

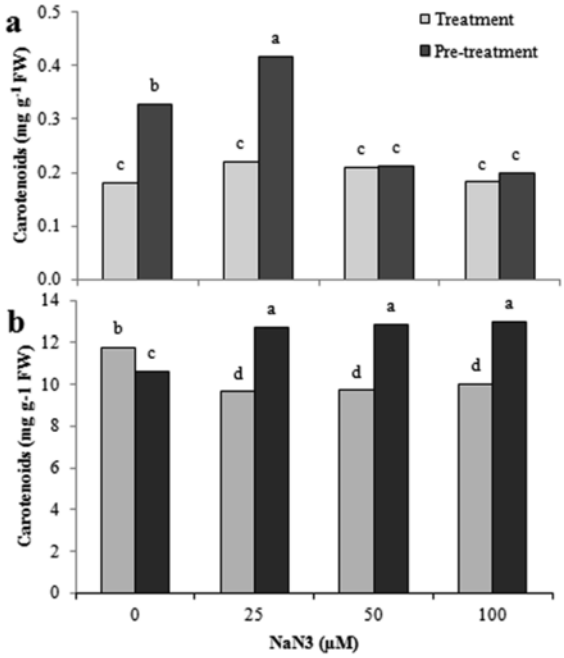

Fig. 4. Effect of azide treatment and pre-treatment on carotenoid content in the green (a) and (b) red stage of *Haematococcus pluvialis*. Values are means of three-replications±standard deviation. Different letters show the significant difference with $p<0.05$ in one-way ANOVA and Duncan's tests

reduction can be the result of the depletion of chlorophyll and photosynthesis rate. As a result, the inhibitory effect of azide on photosynthesis was proved in plants (Forti and Gerola, 1977). Besides, ATP synthesis decrease in the mitochondria due to the inhibitory action of azide is one reason for the decrease in the number of carbohydrates. The results showed that the amount of protein of green cells decreased in azide treatment and pretreatment (Fig. 6). The lowest protein content was observed in algae treated and pre-treated by 100 µM azide with 54 and 50% control respectively. The results of measuring the protein content of red cells *H. pluvialis* under sodium azide treatment showed that sodium azide treatment did not have a significant effect on the protein content of red cells. Sodium azide pre-treatment reduced the protein content of red cells significantly. The highest amount of protein was observed in pre-treatment red cells in the control group and the lowest amount of protein was observed in pre-treatment red cells at 100 µM concentration (50% reduction compared to control). As mentioned previously inhibitory effects of azide on nitrate reductase were confirmed (Zalogin and Pick 2014). This enzyme has a key role in nitrogen assimilation; therefore, reducing the amount of protein in the presence of azide is a predictable result.

Despite the negative effects of azide on the dry weight of cells in the green stage, the dry weight of the algae treated in the red stage was equivalent to the control sample. This shows that the treated cells show better performance than the control sample under red phase stress conditions. Azide with the concentrations used in this experiment showed negative effects on the biochemical parameters of the algae. Only the carotenoid content of green cells increased by 27% with 25 µM azide pretreatment and by 18% with

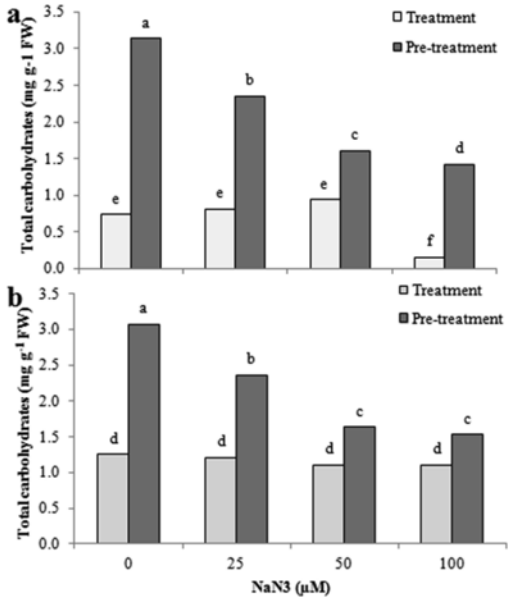

Fig. 5. Effect of azide treatment and pre-treatment on carbohydrate content in the green (a) and (b) red stage of *Haematococcus. pluvialis*. Values are means of

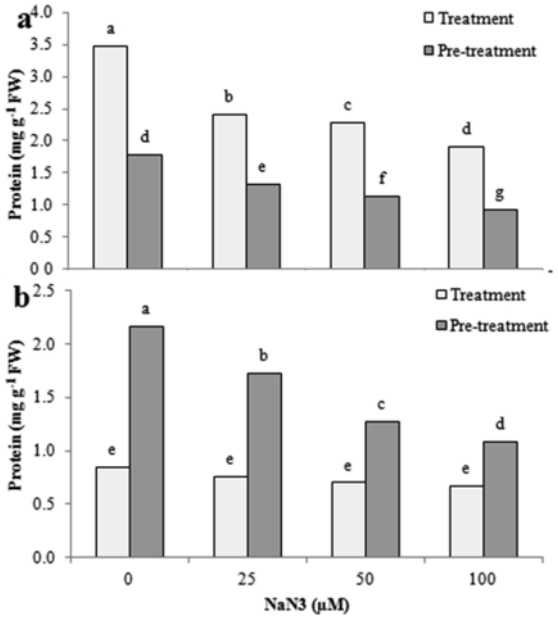

Fig. 6. Effect of azide treatment and pre-treatment on protein content in the green (a) and (b) red stage of *Haematococcus pluvialis*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p<0.05$ in one-way ANOVA and Duncan's tests

red pretreatment of all azide concentrations. Our results also suggested it may be that adding azide to induce carotenogenesis can be a good alternative for nitrogen deficiency conditions in this algae.

Acknowledgments

This work was carried out at Shahid Bahonar University of Kerman, Iran. There was no funding for this research.

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The Thallus Characteristics of Some Populations of *Chaetomorpha* and *Rhizoclonium* (Cladophoraceae) from the Persian Gulf

Nasrin Farasat[1]*, Hossein Riahi[1], Masoud Sheidai[1], Fahimeh Koohdar[1], Massoumeh Farasat[2]

Received: 2022-05-25 Revised and accepted: 2022-06-18

Abstract

Chaetomorpha and *Rhizoclonium,* two genera of the Cladophoraceae family, are rich in antioxidant, phenolic, and flavonoid compounds that have potential applications in the pharmaceutical, food, and cosmetic industries. This work examined thallus features in several populations of four *Chaetomorpha* species and one *Rhizoclonium* species. This study aimed to look into the morphological and anatomical characteristics of several populations of these taxa along the Persian Gulf and Oman Sea coasts. Good species separation in the studied species was indicated by the UPGMA dendrogram. According to a PCA-biplot of *Chaetomorpha* and *Rhizoclonium* based on equivalent morphological and anatomical parameters, the examined species can be distinguished by variables including cell wall thickness, diameter, thallus form, cell shape, and cell length. High agreement between the PCA biplot results and the heat map created using standardized morphological and anatomical features were observed. While CCA analysis of *Chaetomorpha* showed that the characteristic of cell wall thickness was affected by latitude, tallus form, and cell length were related to the longitude.

Keywords: *Chaetomorpha, Rhizoclonium,* UPGMA Dendrogram, PCA, CCA, Heatmap.

Introduction

Seaweeds are significant food, nutrient, and medicinal ingredient sources around the world. Moreover, they include significant amounts of nutritional fiber, fatty acids, vital amino acids, and vitamins A, B, C, and E. (Rajapakse and Kim, 2011). Seaweeds as functional food products that contain proteins, peptides, amino acids, polysaccharides, phenols, lipids, vitamins, and minerals (Mendis and Kim, 2011, Farasat et al., 2022). They have also been shown to have positive impacts on a variety of lifestyle disorders, including diabetes, hypertension, dyslipidemia, and obesity (Roohinejad et al., 2017). The Persian

1- Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.
2- Marine Pharmaceutical Science Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
* Corresponding author email: nas.farasat9@gmail.com, 09163025669
anatomical

Gulf is one of the strategically important aquatic ecosystems in the world, covering an area of 240,000 km2 (Tolouei, 1998). A significant quantity of green macroalgae has been found in the Persian Gulf, according to numerous studies on different marine algae (Gharanjik, 2000; Sohrabipour et al., 2004; Sohrabipour and Rabiei, 2007; Kokabi and Yousefzadi, 2015). Several of these have a high nutritional value and are employed in a variety of applications, including those in the food, feed, pharmaceutical, and healthcare sectors. Cladophoraceae is an important family of green algae (Osuna-Ruiz et al., 2016; Rani et al., 2018; Gahramzai and Taheri, 2021; Thanigaivel et al., 2021). *Chaetomorpha* Kützing and *Rhizoclonium* Kützing, two filamentous genera, exhibit antioxidant and antibacterial properties and are rich in phenolic and flavonoid compounds that are used in the pharmaceutical, food, and cosmetic industries. Due to the simplicity of the structure of these two filamentous algae and the small number of features that separate the species from each other, it is usually challenging to identify them accurately. For this reason, the thallus features of *Chaetomorpha linum* (O.F.Müller) F.T. Kützing, *C. aerea* (Dillwyn) F.T. Kützing, C. *brachygona* WH. Harvey, C. *crassa* (C. Agardh) FT. Kützing and *Rhizoclonium riparium* (Roth) WH. Harvey from selected areas of the Persian Gulf and Oman Sea were examined in this work. Since no comprehensive study has been done on these species in Iran, this work aimed to investigate the morphological traits and anatomical structure of the different populations of these genera in the coastal areas of the Persian Gulf and Oman Sea.

Materials and methods

Sampling

To *investigate* the *morphology* of the genera Chaetomorpha and Rhizoclonium, 29 populations were collected during low tide from the natural habitats of the coastal areas of the Persian Gulf. The geographical coordinates of each place were determined by GPS device (Table 1, Fig. 1). The collected algae were immediately washed with seawater to remove sand and possible epiphytic organisms. After washing and drying, the samples were identified and their morphological characteristics and anatomical structure were studied.

Anatomical and morphological analysis

Morphological and anatomical variables were examined for identification and data analysis. Characteristics like thallus form, branching type, thallus color, cell wall thickness, shape, diameter, length, and length/cell diameter (L/D) ratio were among the most crucial traits for identifying the species (Tables 2, 3). Microscopic images were acquired using an Olympus microscope and a Hund Wetzlar stereomicroscope, and Axiovision LE, Rel 4.5 software was used to measure the diameter, length, and width of the cells.

Structural analysis

Each specimen's morphological and anatomical information was collected, and quantitative features were coded according

Table 1. Specimens and related geographical and ecological data

Species	Type of substrate	Geographical location	Collection location	Code
C. aerea	Sandstone	51°53'48"E, 27°50'4"N	Bushehr: Southern Ouli	C.aSO
R. riparium var. *implexum*				R.iSO
C. aerea	Sandstone	51°53'15"E,27°50'18"N	Bushehr: Northern Ouli	C.aNO
R.riparium var. *implexum*				R.iNO
C. aerea	Sandy-mud	51°56'2"E, 27°49'56"N	Bushehr: Bandar-e Dayyer 1	C.aD1
C. aerea	Rocky-rock	51°56'9"E, 27°49'58"N	Bushehr: Bandar-e Dayyer 2	C.aD2
C. linum				C.lD2
R. riparium var. *implexum*				R.iD2
C. brachygona	Sandstone	50°53'23"E, 29° 3'40"N	Bushehr: Jazireh-ye Shif1	C.bSh1
R. riparium				R.rSh1
R. riparium	Sandstone	50°53'19"E, 29° 3'36"N	Bushehr: Jazireh-ye Shif2	R.rSh2
C. crassa	Rocky - sandy	50°50'33"E, 28°52'11"N	Hormozgan: Bandar-e lengeh, Park-e Dowlat 1	C.cSG
R. riparium var. *implexum*				R.iSg
C. linum	Sandstone	50°49'1"E, 28°58'5.6"N	Bushehr: Bandar-e Jofreh	C.lJO
R. riparium var. *implexum*				R.iJo
C. aerea	Sandy - Sandy	50°48'54"E, 28°54'36"N	Bushehr: Lian Park	C.aLP
C. brachygona	Sandstone with	50°52'44"E, 28°49'50"N	Bushehr: Halileh 1	C.bH1
R. riparium var. *implexum*	water ponds			R.iH1
C. linum	Sandy	51° 3'45"E, 28°42'41"N	Bushehr: Marjan Park	C.lMP
C. brachygona	Sandstone with	50°52'0.2"E, 28°50'30"N	Bushehr: Halileh 2	C.bH2
R. riparium var. *implexum*	water ponds			R.iH2
C. aerea	Sandy	50°48'32"E, 28°47'16"N	Bushehr: Morvarid Park	C.aMP
R. riparium var. *implexum*				R.iMP
C. brachygona	Sandy - Sandy	50°37'30"E, 29°29'6"N	Bushehr: Bandar-e Rig: Halieh1	C.bHa1
C. linum	Sandy - Sandy	50°37'32"E, 29°29'5"N	Bushehr: Bandar-e Rig: Halieh2	C.lHa2
C. brachygona				C.bHa2
R. riparium var. *implexum*				R.iH2
C. crassa	Sandy - Sandy	54°51'35.3"E, 26°32'2"N	Hormozgan: Bandar-e Lengeh: Park-e Dowlat1	C.cDP
R. riparium var. *implexum*	Clay mud	56°21'10"E, 27°11'18"N	Hormozgan: Bandar-e Abbas: Velayat Park 2	R.iV2

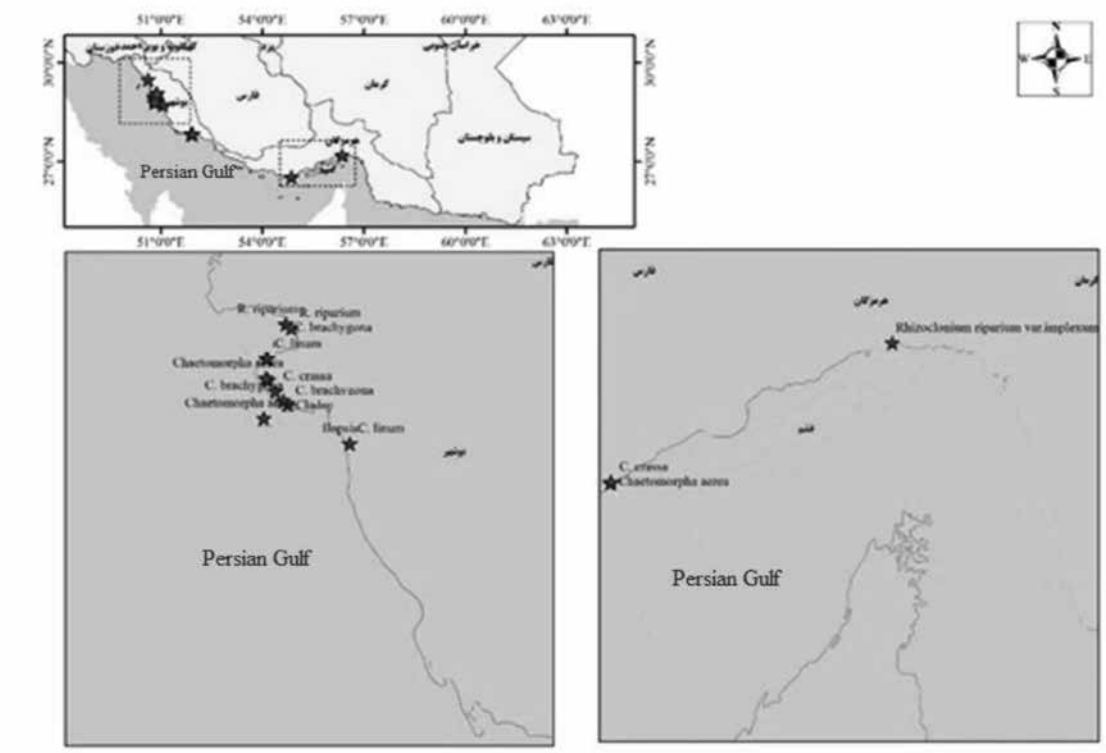

Fig. 1. Study area, sampling locations and species collected are marked on the map

to Table 2. The studied species were grouped by UPGMA (Unweighted paired group using the average method) after 1000 times bootstrap. Principal Component Analysis (PCA) was applied to investigate the species relationships and identify taxonomically discriminating features. CCA (Canonical Correspondence Analysis) was used to reveal the relationship between morphological characteristics and geographical coordinates by PAST version 2.0 software (Hammer et al. 2012). A heatmap was produced based on standardized morphological and anatomical characters by Heatmap software.

Results

Anatomical and morphological analysis

In this work, four species of *Chaetomorpha* and one species and one variety of *Rhizoclonium* were identified by the analysis of morphological and anatomical traits. While Table 4 lists the morphological and anatomical traits of each species, Figures 2–6 display the thallus

Table 2. Qualitative and quantitative morphological and anatomical traits of *Chaetomorpha* species were used in statistical analyzes

No	Traits	Type of traits	Code	No	Traits	Type of traits	Code
1	thallus form	straight, slightly curled skewer-like and straight, more or less twisted curly, cushion-like, rough to the touch twisted and tangled, cushiony, to the touch soft	1 2 3 4	5	cell shape	cylindrical swollen and barrel-like square to elongated rectangle	1 2 3
2	branching type	unbranched grouped	1 2	6	cell diameter	-	-
3	thallus color	light green dark green yellowish green	1 2 3	7	cell length	-	-
4	cell wall thickness	the length equal to the width the length 1.5-2 times the width the length less than the width	1 2 3	8	length/cell diameter (L/D) ratio	-	-

Table 3. Qualitative and quantitative morphological and anatomical traits of *Rthizoclonium* species were used in statistical analyzes

No	Traits	Type of traits	Code	No	Traits	Type of traits	Code
1	thallus form	filamentous in the form of felt or mass	1 2	5	cell diameter	-	-
2	thallus color	light green dark green yellowish green	1 2 3	6	length/cell diameter (L/D) ratio	-	-
3	cell wall thickness	-	-	7	Rhizoid	With rhizoid Without rhizoid	1 2
4	cell diameter	-	-			few rhizoid branches	3

Table 4. Morphological and anatomical traits of the examined species

Species	Thallus form	Branching type	Thallus color	Cell wall thickness (µm)	Cell length (µm)	Cell diameter (µm)	Cell shape	Length/cell diameter (L/D) ratio	Other charactristics
C aerea	straight, slightly curled	unbranched grouped	light green to slightly dark green, sometimes yellowish	(-5) 7-24 (-38)	(-42) 63-215 (-255)	(-52.5) 64-171 (-338)	cylindrical, in some cases oval	0.5-1.5	the height of the thallus is up to 9 cm, with needle crystals in some cells, compressed in the place of the transverse wall
C. linum	skewer-like and straight, more or less twisted	unbranched	light dark green	(-7.2) 9-24 (-33)	(-67) 84-240 (-440)	66-138 (-297)	Rectangular	0.5-0.75	cells slightly compressed in the transverse wall
C. brachygona	curly, cushion-like, rough to the touch	unbranched	light green to slightly dark green, sometimes yellowish	(-5)8-17 (-20)	(-40) 75-143 (-203)	84-166	square to rectangular	often the length is equal to the width, sometimes more than the width and rarely less than it	thallus is twisted and tangled
C. crassa	twisted and tangled, cushiony, soft to the touch	unbranched	light green	(-15)18-31(-38)	111-249	196-274	slightly swollen and barrel-like and cylindrical	often the length is less than the width, sometimes the length is more	cell wall often thick and bladed
R. riparium	filaments intertwined or involved with other algae	unbranched	light green to yellowish	1.5-4.4	19.3-75.8	12-37	rectangular	the length is usually 1.5-2 times the width, sometimes up to 3 times	with short and scattered irregular rhizoid branches or without them

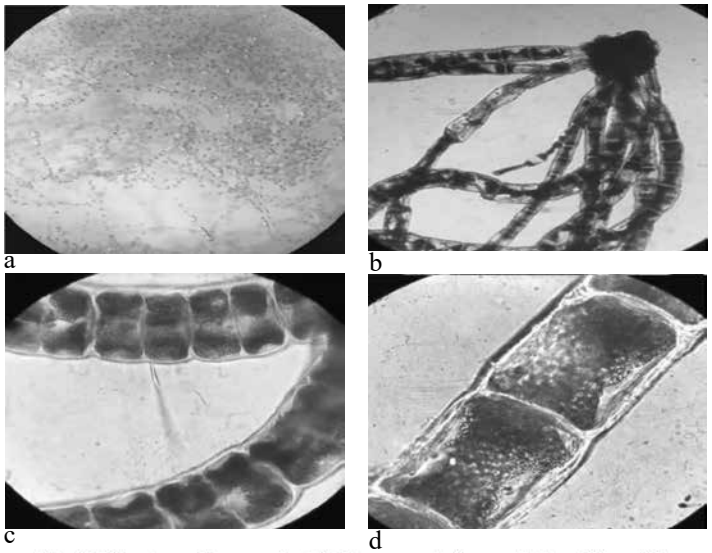

Fig. 2. *Chaetomorpha aerea* (a, b) Alga general view, scale bar=10 mm(a), 20 µm (20X) (b), (c, d) Cell structure, scale bar=10 µm (40X) (c), 10 µm (100X) (d)

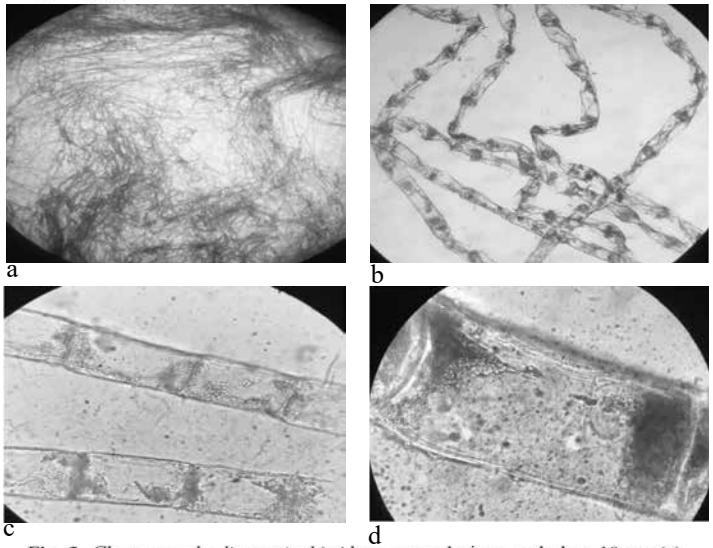

Fig. 3. *Chaetomorpha linum* :(a, b) Algae general view, scale bar=10 mm(a), 20 µm (20X) (b), (c,d) Cell structure, scale bar=20 µm (40X) (c), 10 µm

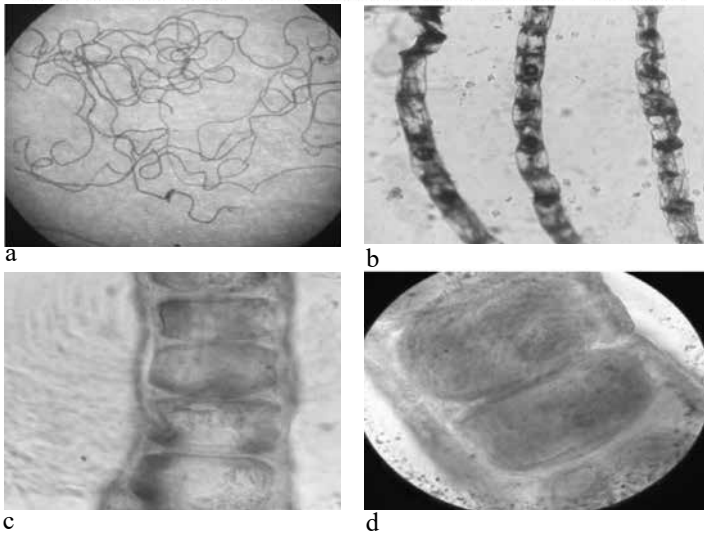

Fig. 4. *Chaetomorpha crassa* : (a, b) Algae general view, scale bar=15 mm (a), 20 µm (20X) (b), (c, d) Cell structure, scale bar=20 µm (40X) (c), 10 µm (100X) (d)

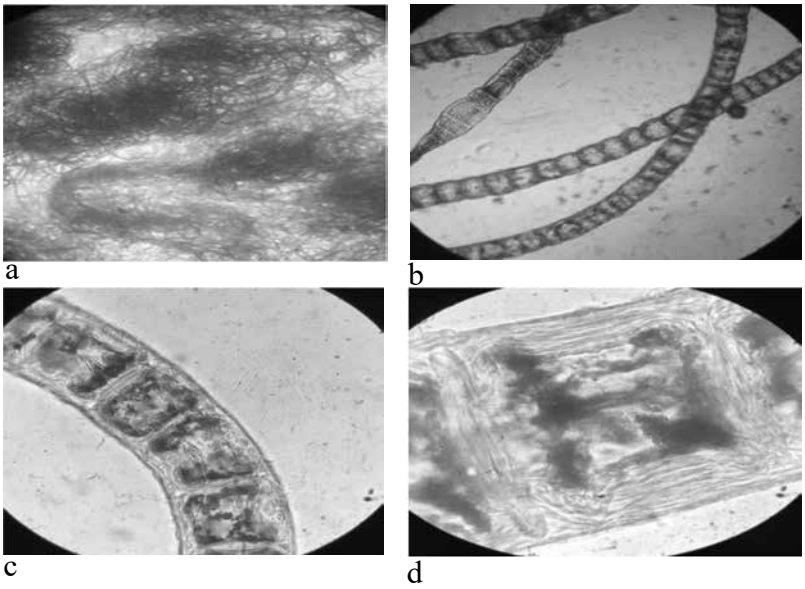

Fig. 5. *Chaetomorpha brachygona*:(a, b) Alga general view, scale bar=10 mm(a), 20 µm (20X) (b), (c,d) Cell structure, scale bar=20 µm (40X) (c), (10 µm (100X) (d)

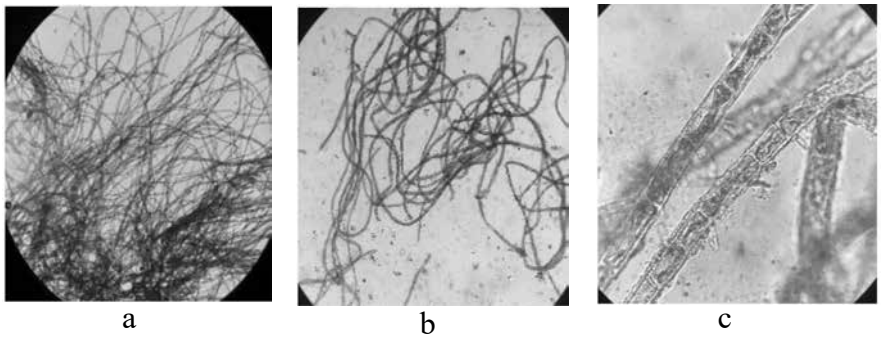

Fig. 6. *Rhizoclonium riparium*: (a,b) Alga general view, scale bar=20 mm (10X)(a), 20µm (20X) (b), (c) cell structure, scale bar= 10 µm (100X)

structures of each species.

Figures 7 and 8, display the UPGMA dendrograms based on combined morphological and anatomical data in the populations of the studied species. The UPGMA dendrogram demonstrated adequate species-level differentiation. In the UPGMA dendrogram of *Chaetomorpha* species, two completely distinct cluster was created. In the first group, there were *C. aerea* and *C. linum*, and second group including *C. brachygona* and *C. crassa.* However, due to the overlapping of some characteristics, some degree of interference of traits was also observed.

Based on morphological characteristics, a PCA-biplot of the *Chaetomorpha* species showed traits like X1; thallus form, X7; cell length, X3; thallus color, and X4; cell wall thickness, respectively, are diagnostic traits that can be used to distinguish the species from one another. According to PCA analysis, the first axes account for around 70% of the total variation. Characteristics of X1 and X7 have the highest positive correlations to this axis, with r values of 0.98 (Fig. 9).

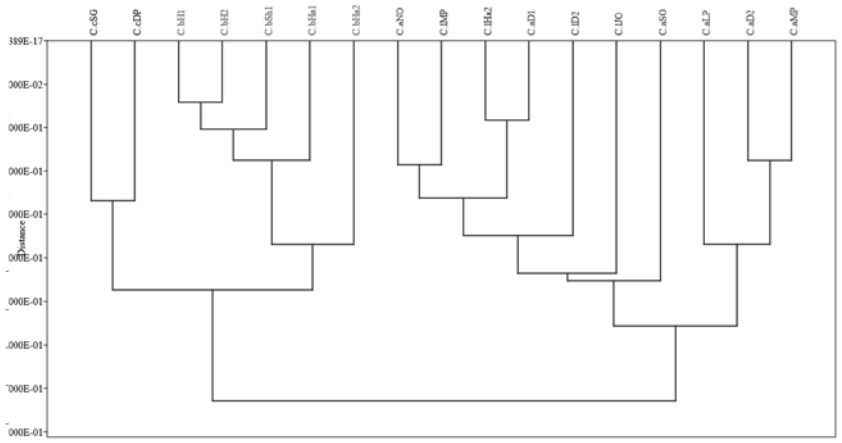

Fig. 7. UPGMA dendrogram of *Chaetomorpha* species based on morphological and anatomical features

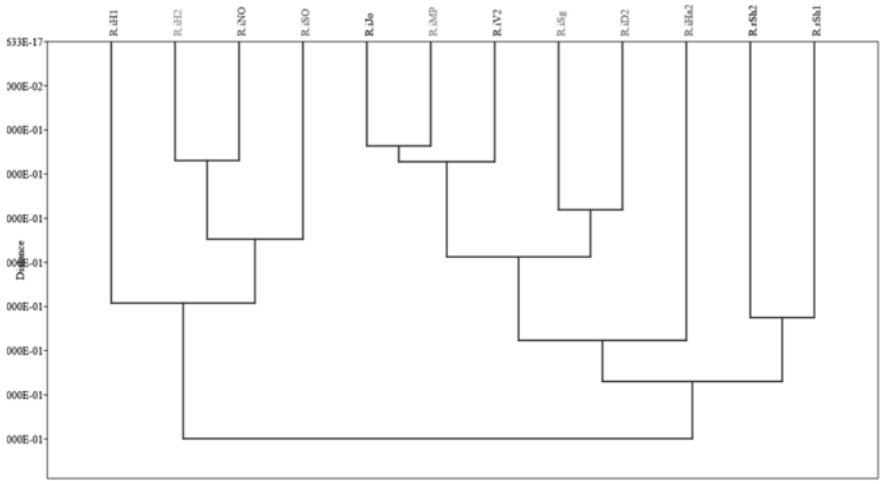

Fig. 8. UPGMA dendrogram of *Rhizoclonium* populations based on morphological and anatomical features

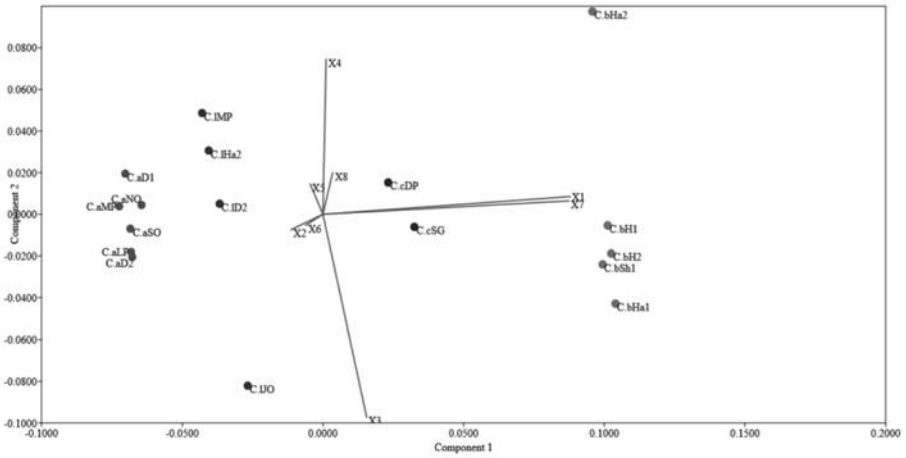

Fig. 9. PCA-biplot of *Chaetomorpha* species based on morphological and anatomical data. X1-thallus form,X2- branching type, X3-thallus color, X4- cell wall thickness, X5- cell shape, X6- cell diameter,X7- cell length, X8- length/cell diameter (L/D) ratio

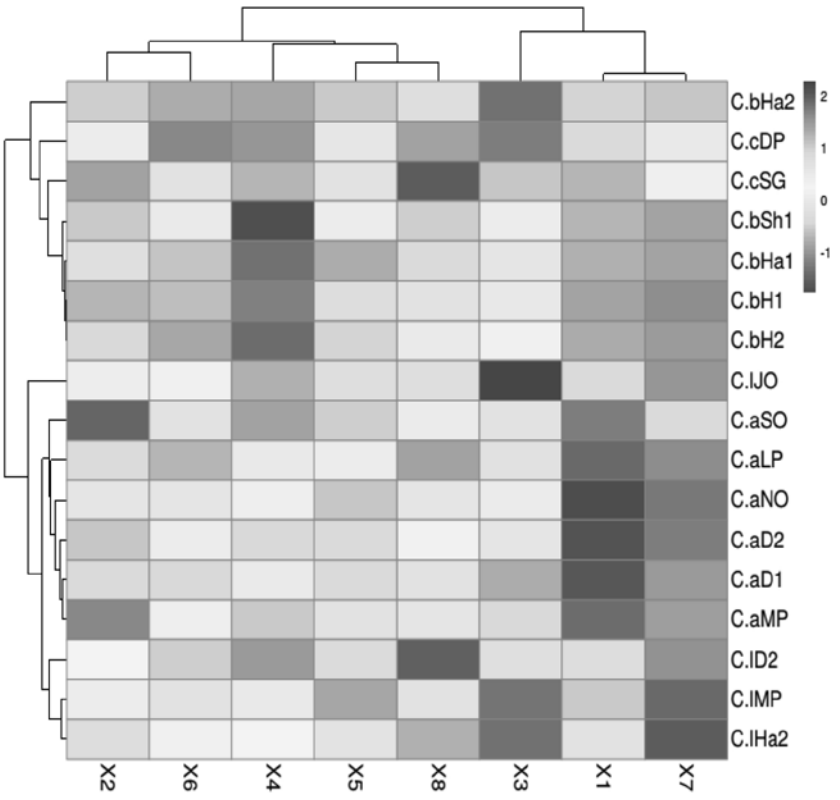

Fig. 10. Heat map of *Chaetomorpha* species based on standardized morphological characters. X1-thallus form,X2- branching type, X3-thallus color, X4- cell wall thickness, X5- cell shape, X6- cell diameter,X7- cell length, X8- length/cell diameter (L/D) ratio

The PCA biplot of the *Rhizoclonium* populations showed that features such as length/cell diameter, L/D ratio, cell length, and thallus form, more than other characteristics, can be differentiated the populations.

According to the heat map created using standardized morphological and anatomical characteristics (Fig. 10), *Chaetomorpha* species can be distinguished based on morphological characteristics such as thallus form, cell length, thallus color, and cell wall thickness, and *Rhizoclonium* populations can be separated based on length/cell diameter, L/D ratio, cell length, and thallus form. These findings were in high accordance with the PCA biplot findings.

According to the CCA analysis, latitude had an impact on the parameter of cell wall thickness, whereas longitude factor affected thallus form and cell length in *Chaetomorpha* species (Fig. 11). The CCA analysis in *Rhizoclonium* populations showed that thallus form, thallus color, rhizoid, and the cell length, were affected by latitude (Figures are not shown for *Rhizoclonium riparium*).

According to a PCA-biplot of *Chaetomorpha* and *Rhizoclonium* based on equivalent morphological and anatomical parameters, the species under study can be distinguished by variables including cell wall thickness, cell diameter, thallus form, cell shape, and cell length (Figure is not shown).

In general, the two species, *Chaetomorpha linum* and *Chaetomorpha aerea* showed

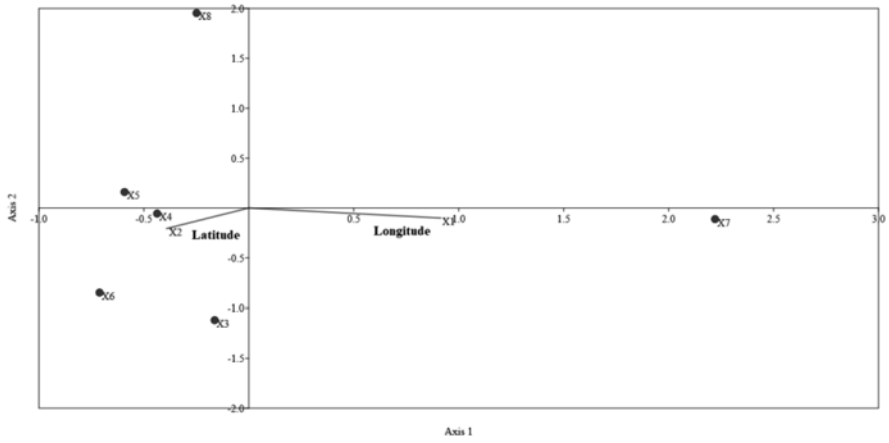

Fig. 11. CCA plot of the geographical distribution of *Chaetomorpha* species based on their morphological and anatomical data. X1-thallus form, X2- branching type, X3-thallus color, X4- cell wall thickness, X5- cell shapeX6- cell diameter, X7- cell length, X8- length/cell diameter (L/D) ratio

similarities in different characters, though the overlap of some traits was observed. However, *C. aerea* is light green to slightly dark green, sometimes yellowish, and *C. linum* is often light dark green. However, some variation was found in different populations. In this way, some thalli of *C. linum* in the Jofreh area (C.lJO) were yellow-green and slightly brown. For instance, in the Marjan Park population (C.lMP), the thallus was slightly twisted and light green whereas in the Dayyer population (C.lD), the filaments were observed as intertwined, bright green, and sometimes yellowish green.

In some *C. aerea* populations, such as Morvarid Park (C.aMP) and Lian Park (C.aLP) populations, some thalli were observed in a cluster, consisting of 2 to several thalli, attached to a large and distinct holdfast. The thallus color of *C. aerea* in Morvarid Park and Southern Ouli (C.iSO) populations were light green, sometimes dark green, and in the Northern Ouli population (C.iNO), was yellowish green and some filaments were springy. In the Lian population, the thallus was light or dark green, and some filaments were springy and twisted. In this population, mineral crystals on some thalli were observed. In some thalli, cells with a diameter greater than or equal to the length were also observed. In the Dayyer population, dark green filaments with yellow tendencies were seen. Most species were slightly compressed at the junction of the walls, and in some populations, the cells were slightly swollen.

For *C. aerea*, the highest length and diameter of the cell belonged to the northern Ouli population (255 and 337.7 µm, respectively), and the lowest length and diameter of the cell belonged to the southern Ouli population (41.6 and 52.5 µm, respectively). Also, the highest cell wall thickness was related to the Park Dowlat2 population (38 µm), and the Lian population

(5 µm) was the smallest in size. For *C. linum*, the highest cell length, and diameter related to the Marjan Park population (239.4 and 138.2 µm, respectively) and the lowest cell length was seen in Halieh 2 population (67 µm). The lowest cell diameter was observed in Dayyer 2 population (64.3 µm). However, a few cells with a length of up to 441.3 µm and a thickness of up to 33 µm were also seen in the Marjan Park population. The highest cell wall thickness was related to Halieh 2 population (24.7 µm), and the lowest cell wall thickness was recorded for Dayyer 2 population (7.2 µm).

Both *C. crassa* and *C. brachygona* are cushion-like. While *C. crassa*, is curly and rough to the touch and is usually free floating or involved with other algae, C. brachygona is soft and smaller in terms of cell dimensions and cell wall thickness. In addition, *C. crassa* cells are swollen and barrel-shaped but the cells of C. brachygona, are often square or rectangular, and the cell length is equal to or less than the cell diameter, and sometimes cell length reaches half the diameter. In Halileh 1 population, the thalli were yellowish green to light or dark green, some filaments were seen as skewer-like and smooth, and most of the cells had a cell diameter highest than the cell length.

For *C. brachygona*, the highest cell length and diameter belonged to Shif 2 population (C.bSh) around 203.2 and 166 µm, respectively. However, the lowest length and diameter of the cells were found in Halileh 1 population (C. bH1) about 53.2 and 60 µm, respectively. The highest and lowest thickness of the cell wall was found in the filaments of the Halileh 2 population (22.4 and 7 µm, respectively). In *C. carssa*, the highest cell wall thickness was seen in the population of Shegab Park (C.cSP) (8.26 µm), and the lowest one was observed in the Park-eDowlat1population (C.c DP) (3.8 µm).

The results showed that in *C. aerea*, cell wall thickness has an inverse relationship with latitude, whereas, in *C. linum*, *C. brachygona*, and *C. crassa*, this relationship is direct, that is, with increasing latitude, cell wall thickness increases in *C. aerea*. In *C. crassa*, the highest cell length was related to Shegab Park population (202.6 µm), and the lowest was seen in the Park-e Dowlat 1 population (81µm). The highest and lowest cell diameter belonged to the Park-e Dowlat 1 population (236.1 and 88 µm, respectively).

Discussion

Chaetomorpha and *Rhizoclonium* are unbranched filamentous algae, closely related to each other and belonging to Cladophoraceae, Cladophorales, and Chlorophyta (Zhao et al., 2018). *Chaetomorpha* is a common and widespread filamentous green macroalgae known as Spaghetti algae (Novaczek, 2001). Some species including *C. crassa*, *C. linum*, and *C. brachygona* are edible and consumed as salad or dessert in East Asian countries (Apaydin-Yagci and Turna, 2002). Also, this genus is currently the most popular macroalgal group

in saltwater aquariums (Odom and Walters, 2013). There are 141 species names in the genus *Chaetomorpha* including subspecies, variations, and forms, 78 of which are acknowledged taxonomically (Guiry, 2022). This genus includes filamentous, unbranched, connected by a holdfast, cylindrical or barrel-shaped, sometimes with lamellar walls, with reticulate chloroplast, multinucleate with abundant pyrenoids (Lawson and John, 1982; Teo and Wee, 1983). The genus *Rhizoclonium* was named by Kützing (1845), and *R. riparium* is considered the type species of this genus (Zhao et al., 2018). Thirty of the 97 species names for the genus *Rhizoclonium* including subspecies, variations, and forms are accepted taxonomically. This alga has a single-cell layer, a strong wall, and unbranched filamentous cells with many pyrenoids, nuclei, and chloroplasts with reticulation. The length/cell diameter (L/D) ratio of a cell is 4/1 (less than 6) (Lawson and John, 1982; Blair, 1983).

In some references, *R. riparium* var. *implexum* is known as R. implexum (Kützing, 1845). The length is two to five times the width, cell wall thickness from 1.5 to 2 µm, and few or no rhizoid branches are the characteristics that can be applied to identify this variety/species (Lawson and John, 1982; Farasat et al., 2013). Several populations of the examined species differed in cell wall thickness and the length/cell diameter (L/D) ratio.

The majority of the diagnostic characteristics used to identify the species demonstrated a high degree of agreement with those which were listed in the earlier reliable references (Borgesen, 1939; Lawson and John, 1982; Teo and Wee, 1983; Tseng, 1984; Ruangchuay et al., 2007; Alves et al., 2009; Milchakova, 2011). However, cell dimensions in some populations differed from earlier studies. For instance, Rath and Adhikary (2006) stated that the cell width in *C. linum* species obtained from India was smaller (15 to 30 µm) than our findings. Additionally, compared to the samples from the present study, Hinson and Kapraun (1991) found that the cell width of *C. brachygona* and *C. aere*a from the western Atlantic was lower (55 and 80-90 µm, respectively). They showed a correlation between cell dimensions and genome size so that species with a diameter of less than 100 µm, such as *C. brachygona* and *C. aerea* have almost half of the genomes of *C. antennina* and *C. melagonium* species with a diameter of over 400 µm. Contrary to these studies, some reports have stated larger cell dimensions for *Chaetomorpha* species. For example, Satpati and Pal (2016) reported greater cell width (400-620 µm) in samples of *C. aerea* from India.

Due to the morphological flexibility of the genera and the small number of species, *Chaetomorpha* and *Rhizoclonium* species generally create numerous systematic issues. The most variable characters that are measured to distinguish between various species include basal cell size, thallus form, cell diameter, and length/width ratio. Additionally, variations of the morphological

characters in these two genera are evident in the investigated populations due to changes in environmental conditions at different latitudes and longitudes.

The findings of this study are applicable for researchers looking for active compounds in these species because, with changes in plant morphology, it is not unlikely that biologically active compounds in populations from various areas will also change.

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Evaluation of the Effect of *Sargassum angustifolium* Extract on Growth and Yield Indices of *Lactuca sativa* Under Drought Stress Conditions

Afsaneh Mohkami

Received: 2022-05-15 Revised and accepted: 2022-07-20

Abstract

Lettuce (*Lactuca sativa*) represents a major horticultural crop in Iran and worldwide. Lettuce requires a high amount of water to grow well and is adversely affected by drought. Regarding the reports of the positive effect of seaweed extract on increasing the resistance of plants to abiotic stresses, the present study aimed to evaluate the effect of the seaweed *Sargassum angustifolium* extract on growth and yield indices of lettuce under drought stress. This study was conducted as a factorial experiment with three treatments of algae extracts concentrations and two treatments of drought stress, and non-stress treatment with three replications. To evaluate the effects of algae extract on lettuce under drought stress conditions, a combination of morphological and physiological characteristics including plant height and dry weight, photosynthetic pigment content, and antioxidant activities were measured. Results showed that treatment of seaweed extract significantly increased plant resistance to drought stress and improved morphological and physiological indices of lettuce ($p< 0.05$). The best results were obtained in the 1.5 g/l treatment of seaweed extract.

Keywords: Lettuce, Biostimulant, Seaweed, Physiological Characteristics, Proline

Introduction

The existence of environmental stresses, especially drought stress, has always been considered one of the factors limiting the growth and achieving optimal performance of agricultural and garden plants in arid and semi-arid areas. Stress is caused by the action of one or more living or non-living factors in the environment, which causes the physiological activities of the plant to be disrupted, which is reflected in the reduction of plant growth and performance. In other words, stress indicates the exposure of the plant to one or more environmental factors that cause the loss of growth and production efficiency and also decrease the value of the plant (Fang et al., 2015). The amount of water available to the plant is one of the most important climatic factors that affect the growth of plants all over the globe, and its deficiency causes morphological, physiological, and biochemical changes in agricultural and garden plants. Drought stress occurs when the plant's access to water decreases (Blum, 2017). Drought stress is one of the most crucial environmental

Research and Technology Institute of Plant Production, Shahid Bahonar University of Kerman, Kerman, Iran
*Corresponding author email: amohkami@uk.ac.ir

stresses that hurts plant growth and thus significantly reduces the level of agricultural production. Lack of rainfall and irregular distribution of rain during the growing season are the major causes of drought stress in garden plants. Drought stress is one of the most common and destructive non-living stresses in the agricultural sector, which causes a decrease in the yield of garden plants in different regions of the world, including Iran, most of which is under a semi-arid climate (Golbashy et al., 2010). When plant growth is reduced due to drought stress, a high yield of garden plants becomes challenging to achieve. According to reports, about 40% of the world's agricultural lands are located in semi-arid areas, which shows the importance of drought stress in reducing plant production and requires the measures adopted to protect plants from this stress (Mohammadi et al., 2017).

Lettuce (Lactuca sativa) is a garden plant with high nutritional and economic value, which has always seen high demand in domestic and international markets. On the one hand, this plant is a rich source of vitamins, minerals, and nutrients necessary for human growth and health, and on the other hand, it is highly marketable for various types of food production. Like other plants, lettuce is also sensitive to drought stress, and a vital part of the growth and performance potential is lost when faced with drought stress (Murtic et al., 2018). Plant resistance to stress is a polygenic trait when droughts cause stress, reactions such as the expression of stress-specific genes, the accumulation of metabolites, the expansion of the root network, and the reduction of leaf water level occur (Munné-Bosch et al., 2023). Because the metabolic pathway of stress resistance is very complex and different genes are involved in it; therefore, the use of methods based on genetic engineering to increase the resistance of plants to stress has not been very successful (Shukla et al., 2018). On the other hand, the use of biostimulant compounds is an effective, and at the same time economical approach to increasing the resistance of plants against drought stress. For this reason, in the last two decades, much attention has been paid to biostimulant compounds as a solution to overcome living and other stresses (Lephatsi et al., 2023; Hamedeh et al., 2022).

Seaweed extracts are one of the most important biological stimulants which are widely used in the production of agricultural and horticultural crops (Baltrusch et al., 2023). The use of seaweed extract in the soil or by spraying increases the chlorophyll content improves the efficiency of photosynthesis, and absorption of nutrients by plants, improves the ability to absorb and retain water and generally increases the resistance of plants to living and non-living stresses (Mousavi et al., 2023). The positive effects of the extract obtained from different species of *Sargassum* brown algae have been reported in various studies so far (Shahriari et al., 2021). Although the positive effects of seaweed extract on improving yield and improving the resistance level of the garden and agricultural plants to living and non-

living stresses have been reported by many researchers (Alharbi et al., 2022); However, many researchers regarding the molecular mechanisms and how the compounds present in seaweed extract affect the physiological and morphological characteristics of plants have not been done, and still many unanswered questions in this regard have remained. At the same time, knowing how algae extract affects the mechanisms involved in stress resistance will make it possible to adopt better strategies for using natural compounds to increase the resistance of garden plants to various stresses, including drought stress. Considering the above information and considering the potential ability of *Sargassum* seaweed extract in improving the resistance of plants to drought stress, or considering the importance of lettuce as a garden product with high market value, this research aims to evaluate the effect of *Sargassum angustifolium* seaweed extract on the growth and yield indices of lettuce under drought stress conditions. The aim of this research is to determine the mechanism of action of the mentioned algae extract in increasing the resistance level of lettuce plants to drought stress. In fact, this research is an attempt to determine the morphological, biochemical and molecular mechanisms that are involved in creating resistance to drought stress under the treatment of seaweed extract.

Materials and methods

Preparation of algae extract (AE)

The brown algae S. *angustifolium* was collected from the coast of Chabahar, and transferred to the laboratory, and its waste materials were removed through washing. Algae samples were dried under shade conditions and then powdered using a mill. 15 grams of algae powder was mixed with 300 ml of ethanol (70%) and stirred on a shaker for 24 hours at room temperature. The resulting extract was passed through filter paper and placed in a rotary evaporator to remove ethanol. The supernatant containing the algal extract was dried at 40 degrees Celsius and the algal pellet was re-suspended in distilled water and the dilution process was carried out to reach a concentration of 2 g/l.

Plant cultivation and applying drought stress

Lettuce seedlings var. Ferdos were grown in pots containing soil with pH=5.5-6.5 and electrical conductivity (EC) equal to 1.2-1.8mS/cm. To apply the treatments, algae extract solution (including three concentrations of zero (control), 1 g/l, and 1.5 g/l) was sprayed in the amount of 100 ml per seedling. Drought stress was applied at three levels: Field Capacity= 100%, Field Capacity=70%, and Field Capacity=50%. The weight method is used to determine FC. For this purpose, the pot was first filled with one kilogram of dry soil and then watered until the soil became saturated. After 24 hours, the pot was weighed, and the weight difference between before and after irrigation was considered as the maximum agricultural capacity (FC=100%). Other FC values were also determined according

to the weight of the pots. The experimental design is factorial.

Measurement of growth parameters

Plant height and dry weight

The height of two-week-old plants was measured in centimeters. At the end of the experiment, the lettuce seedlings were separated from the soil and transferred to the laboratory. First, the fresh weight of the seedlings was measured with a precise digital scale. Then the aerial parts of the seedlings were separated and placed in an oven at 80° C. Finally, the dry weight of the seedlings was measured.

The photosynthetic pigments content including chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content were measured using the method of Lichthen Thaler (1987). For this purpose, 0.2 grams of leaves of seedlings were treated with liquid nitrogen and mixed with 15 ml of 80% acetone. The amount of absorption (A) was measured using a spectrometer at three wavelengths of 646, 663, and 740 nm, and the number of photosynthetic pigments was calculated in micrograms per gram of fresh weight using the following formulas:

Chl a = 12.25 A663 -2.79 A646

Chl b = 21.21 A646 -5.1 A663

Chl T = Chl a + Chl b

Car = (1000 A470 -1.8 Chl a – 85.02 Chl b) /198

Where, Chl a, Chl b, Chl T, and Car represent chlorophyll a, chlorophyll b, total chlorophyll content, and carotenoid content, respectively.

Antioxidant activity

The power to eliminate free radicals was measured according to the method proposed by Shukla et al. (2018). For this purpose, 200 mg of leaf samples were mixed with 15 ml of 80% ethanol and centrifuged at 5000 rpm for 3 minutes. 250 µl of this extract was added to 250 µl of methanol and 500 µl of 1-1-diphenyl-2-picrylhydrazyl (DPPH). This mixture was kept in the dark for 30 minutes, and the absorbance was measured at 515 nm. Trolox reagent was used as a positive control, and the results were reported in Trolox equivalents per gram of dry weight.

Proline measurement

To measure the proline, the method of Batts et al. (1970) was used, in which proline concentration was determined in mg/g of fresh leaf tissue using a standard curve. The unit is expressed as milligrams per gram of body weight.

Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured using the method of Mansouri et al. (2014). The reaction mixture was added to 200 µL of an enzyme mixture. Then the reaction mixture was irradiated at 20° C, and the absorbance was measured at 560 nm. Inhibition percentage (%I) was measured using the following formula:

%I = Ab−As/As

In this formula, Ab represents the absorbance of the standard (blank), and As represents the absorbance of the sample. SOD activity is expressed in units per mg of protein (U/ mg protein), where each unit represents a

50% change in inhibition percentage.

Statistical analysis

This research was done as a factorial in a completely randomized design with three replications. The experimental factors included the first factor, three different levels of drought stress, and the second factor three different concentrations of algae extract in the form of foliar spraying. The statistical analysis of the data was done using SPSS 20 and comparing the average data with Duncan's multi-range test at the 5% level.

Morphological features

The results of the application of seaweed extract on the morphological characteristics of lettuce plants, including plant height and dry weight, are shown in Table 1. As can be seen, algae extract treatment significantly increased the height of lettuce plants under drought-stress conditions ($p< 0.05$). A similar trend was observed regarding the effect of seaweed extract on the dry weight of lettuce plants ($p< 0.05$).

Indeed, the effect of 1.5 g/l treatment had a greater effect on plant height and dry weight compared to the 1 g/l treatment ($p< 0.05$).

Photosynthetic pigments

Figures 1 to 3 show the effect of seaweed extract on the photosynthetic pigment content of lettuce. According to the results, the highest amount of chlorophyll a was observed in treated plants with a concentration of 1.5 g/l. The amount of chlorophyll in plants treated with both concentrations of algae extract was significantly higher than in the control group ($p< 0.05$). This means that AE treatment significantly increased chlorophyll in lettuce seedlings. The contents of chlorophyll b, total chlorophyll, and carotenoid were significantly higher in seedlings treated with AE compared to the control group ($p< 0.05$). In general, AE treatment significantly increased the content of pigments in all stages of drought stress.

The proline concentration

Figure 4 shows the changes made in the amount of proline of lettuce plants under stress in two cases of control and application of seaweed extract. Based on the obtained results, it was found that the use of seaweed extract at both levels of 1 g/l (treatment I) and 1.5 g/l (treatment II) significantly increased the amount of proline in lettuce ($p< 0.05$).

Antioxidant activity

The ability to eliminate free radicals in the treated plants as well as the plants of the control group was measured using the DPPH test. Based on the results shown in this graph, it is clear that seaweed extract treatment significantly increased the ability to remove reactive oxygen species (ROS) in treated plants compared to the control ($p< 0.05$). The highest amount of free radical elimination was observed in treatment II.

Table 1. Morphological traits of lettuce seedlings after 7 days drought

Indices	Height			Dry weight		
	T 2	T1	Control	T 2	T 1	Control
	43.01 ± 1.32^{c}	39.56 ± 2.22^{b}	34.33 ± 1.07^{a}	12.98 ± 1.76^{b}	12.45 ± 0.34^{ab}	11.21 ± 1.16^{a}

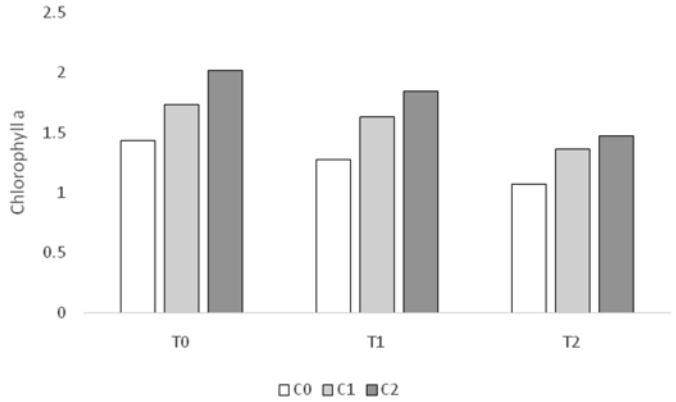

Fig. 1. Effect of stress and algae extract treatment on chlorophyll a content in lettuce seedlings. The values presented in this graph are the average of three replicates, C0, C1 and C2 stand for three levels of drought stress

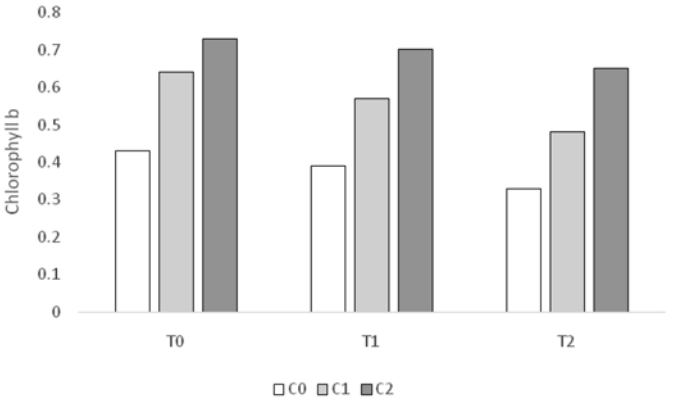

Fig. 2. Effect of stress and algae extract treatment on chlorophyll b content in lettuce seedlings. The values presented in this graph are the average of three replicates, C0, C1 and C2 stand for three levels of drought stress

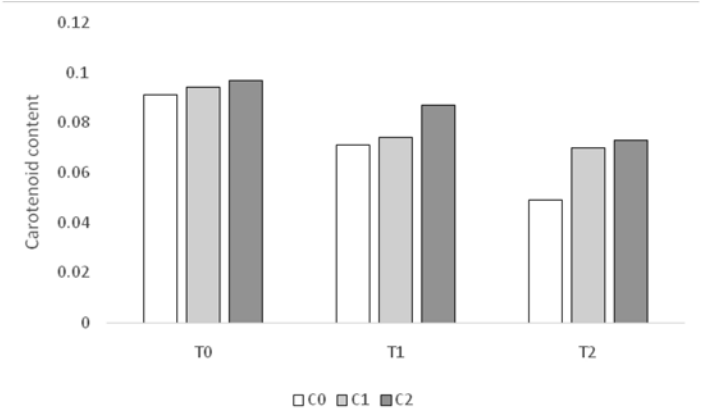

Fig. 3. Effect of stress and algae extract treatment on carotenoid content in lettuce seedlings. The values presented in this graph are the average of three replicates, C0, C1 and C2 stand for three levels of drought stress

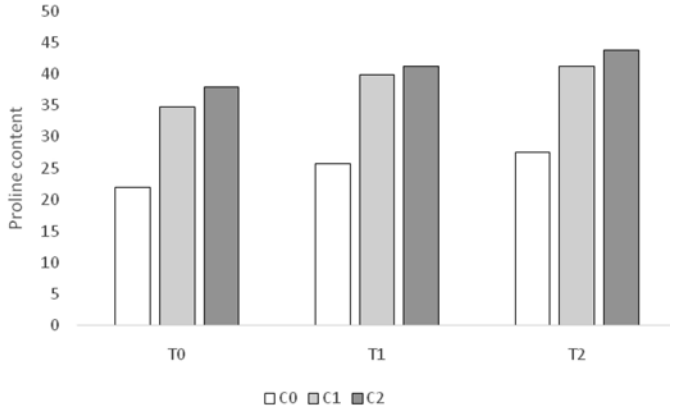

Fig. 4. Comparison of the amount of proline in lettuce plants under stress with and without the application of seaweed extract. The values presented in this graph are the average of three replicates, C0, C1 and C2 stand for three levels of drought stress

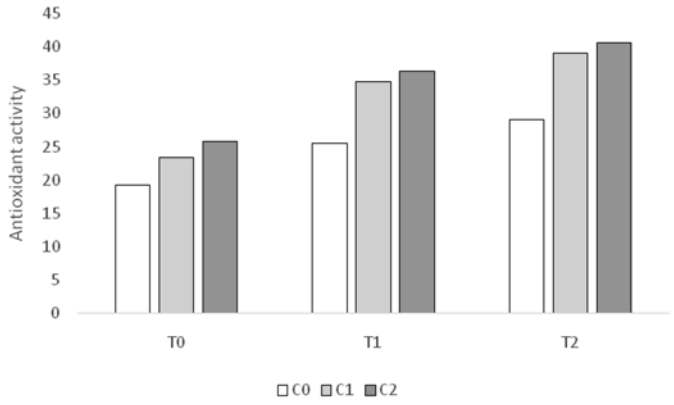

Fig. 5. Increase in the power of killing free radicals due to the application of seaweed extract in lettuce seedlings. The values presented in this graph are the average of three replicates, C0, C1 and C2 stand for three levels of drought stress

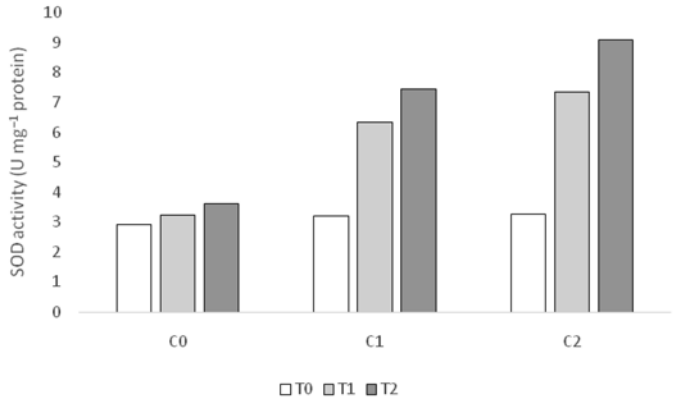

Fig. 6. Changes in superoxide dismutase activity. The values presented in this graph are the average of three replicates, C0, C1 and C2 stand for three levels of drought stress

Superoxide dismutase activity

The activity of the SOD enzyme as an antioxidant mechanism which is activated during plant stress was measured and its results are shown in Figure 8. According to antioxidant activity in SOD, the highest activity was observed in treatment II (concentration of 1.5 g/L of seaweed extract). The difference between treated and control plants in terms of superoxide dismutase (SOD) activity was significant ($p< 0.05$).

Discussion

Drought stress is one of the most important factors that reduce the performance of garden plants, which negatively affects garden production (Kuromori et al., 2022). In particular, this stress hurts the growth and functional characteristics of the lettuce plant and the climatic conditions of Iran. It is considered one of the main factors reducing the yield of the lettuce plant (Shahriari et al., 2021). On the other hand, considering the major benefits of seaweed as a source of micronutrients and reducing stress, the use of seaweed as biological fertilizers and growth stimulants has experienced a growing trend in recent decades. The present study was conducted to investigate the use of brown seaweed S. *angustifolium* as a biological agent to reduce the negative effect of water stress on the morphology and physiology of lettuce plants. The results obtained from this experiment showed that AE leaf spray caused a significant increase in shoot dry weight and seedling height compared to the control group. This positive effect is consistent with results reported for seaweed usage in other agricultural and horticultural crops. For example, in the research conducted by Haghparast et al. (2012), it was also observed that spraying with natural substances, especially seaweed extract, could increase the growth of chickpea plants under drought stress. Hernández-Herrera et al. (2018) also reported that the use of seaweed extract has a positive effect on the growth of plants under drought stress. The positive effect of seaweed on growth parameters may be attributed to the presence of auxin (Martynenko et al., 2016), cytokinin (Zhang et al., 2010), and other growth-promoting factors as well as micronutrients and micronutrients (Kumari et al., 2011).

he results obtained in this research show the positive effect of AE on photosynthetic pigments. The improvement of the performance of the photosynthesis apparatus may justify the increase in the dry weight of the branch and plant growth due to the application of seaweed extract. It is believed that the enhancement of the effect of seaweed extract on photosynthetic pigments is primarily due to the reduction of chlorophyll degradation during water deficit (Wahab et al., 2022). Betaine is a significant biological agent in seaweed extract that increases the chlorophyll content of plants, so the improvement in chlorophyll content of lettuce plants treated with AE may be due to the content of betaines in seaweed extract (Tinte et al., 2022). Considering that the loss of photosynthetic pigments is one of the main factors of growth reduction and

crop yield reduction during drought stress, it is suggested that the protective effect of AE on chlorophyll and other photosynthetic pigments affects the application of AE extrapolation in plant promotion.

Drought stress is one of the main factors that cause oxidative stress in plants, which disrupts the balance between ROS production and antioxidant and defense activities of the plant; this is why oxidative stress always occurs during drought stress (Bitarafan et al., 2019). Water stress leads to the accumulation of antioxidant compounds in the vacuoles of epidermal tissues of plants; however, sometimes the increase in the number of natural antioxidants is not high enough to prevent destructive oxidative reactions (Shukla et al., 2016). Therefore, it is necessary to increase the production of antioxidant compounds in plants under stress by using different treatments, to mitigate the destructive oxidative effects caused by drought stress. Seaweed extracts have attracted great attention in recent years due to their high potential to remove free radicals (Mansori et al., 2015). The results obtained in this research showed that the use of *Sargassum* seaweed extract has a positive effect on increasing the absorption power of free radicals and significantly increases the activity of the superoxide dismutase enzyme as a natural antioxidant agent. This finding is consistent with the results reported by other researchers regarding the effect of seaweed extract on improving the power of removing free radicals (Tinte et al., 2022; Zhang et al., 2010; Martynenko et al., 2016; Murtic et al., 2018).

Another result obtained in this research was an increase in the amount of proline with treatment by seaweed extract. Proline is an amino acid that plays a role in creating resistance against almost all non-living stresses. Based on the results obtained in this study, it was found that both levels of *Sargassum* algae extract have a significant effect on increasing proline concentration in lettuce. This finding is consistent with the results reported by other researchers (Elferjani et al., 2018; Erulan et al., 2009). In total, the results obtained in Sayer's research, by other published articles in this field, once again prove that seaweed extract can be a natural stimulant that significantly improves the resistance of plants against drought stress. This finding indicates that due to the prevalence of drought stress in many regions of the country, *Sargassum* algae extract can be used as a cheap and effective source to increase the resistance of garden plants to drought stress.

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Investigation of Microplastic Pollution in *Sargassum* sp. Macroalgae on Rocky Shores of Bushehr Province

Hasti Khosravi[1], Faedeh Amini [1]*, Nasrin Sakhaei[1], Bita Archangi[1], Sara Gholamipour[2]

Received: 2022-06-13 Revised and accepted: 2022-08-18

Abstract

Nowadays, the increase in microplastic pollution has become a global problem. The food chain can be polluted by the presence of microplastics in macroalgae. To measure the presence and amount of microplastics in macroalgae and water, sampling was performed from the rocky shores of Bushehr province during the winter season of 2021 and summer season of 2022 from four stations of Jofreh, Rishehr, Bojikdan, and Halileh. The result indicates the presence of microplastics in the macroalgae and the water, which were separated using a stereomicroscope. The microplastics were analyzed by FTIR-ATR device to determine the type of microplastic. The dominant species of the sampling stations were macroalgae *Sargassum* sp.

Although, according to the results, the microplastic pollution in macroalgae samples was higher in the summer than in winter, the microplastic pollution in the water was more in the winter season. The average abundance of microplastics was calculated as 173 ± 96.96 and 116.75 ± 63.36 microplastics/kg in the summer and winter seasons, respectively. Jofreh and Halileh stations, with a mean frequency of 225 ± 50.48 and 23 ± 5.69 microplastics/kg, were the most polluted and clean stations, respectively. The sphere form of the microplastic fragments was more frequent after the fiber type. The results of the FTIR-ATR analysis showed that the microplastic polymers in *Sargassum* sp. and water were polyamide (nylon)> polystyrene> polyvinyl chloride, respectively. Since the main activities that pollute the beaches of Bushehr province are fishing, shipping, tourism, etc., and they are more in the summer. So it can be seen as evidence for the results of this study.

Keywords: Microplastic, Marine Pollution, Macroalgae, Bushehr Province, *Sargassum* sp.

Introduction

Due to the expansion of plastic production techniques with high volume and low price following the invention of the first modern plastic in 1907, the production speed of plastic products has increased, and they are mass-produced (Cole et al., 2011). Today, it

1- Department of Marine Biology, Faculty of Marine Science and Oceanography, Khorramshahr University of Marine Science and Technology, Khorramshahr, Iran
2- Persian Gulf Oceanographic Station (Bushehr), Iranian National Institute for Oceanography and Atmospheric Sciences, Bushehr, Iran
*Corresponding Author email: f.amini@kmsu.ac.ir,

is impossible to imagine life without plastics and often used in various applications such as food and beverage packaging, drugs, cosmetics, detergents, chemicals, medicine, electronics, construction, automobiles, and aircraft parts (Kumar et al. al, 2020; Galgani et al., 2010). Plastic pieces smaller than 5 mm are called microplastics. Plastics that are produced in microscopic size are known as primary microplastics. These plastics are commonly used in facial cleansers and cosmetics (Zitko and Hanlon, 1991). Secondary microplastics describe small plastic fragments obtained from the breakdown of larger plastic residues, in the sea, and on land (Ryan et al., 2009). Coastal zones, around industrial units and harbors, have the highest abundance of microplastic fragments (Claessens et al., 2011; Desforges et al., 2014). Sources that directly introduce plastic into the marine environment include coastal tourism, marine fleets, recreational and commercial fishing, and marine industries e.g. oil and gas platforms, fish farming can pose a risk to both plants and animals (Thompson et al., 2009).

Plastics harm communities and their economies, as well as aquatic ecosystems. Also, there are concerns about human health and the impact of microplastics on organisms and their risks and side effects. Microplastic accumulation in marine macrophytes, such as macroalgae and seagrasses, is a potentially critical pathway that has been neglected. One of the ways how microplastics enter the marine food web is through macroalgae (Saeb Mehr et al., 2016). Previous research shows that microplastics are ingested by various marine organisms, from zooplankton to fish (Saeb Mehr et al., 2016). This phenomenon causes inhibition of growth, shorter life span, and reproductive capacity in these organisms. Fishes and crustaceans are the most studied groups, but studies on other organisms are much fewer. Microplastics also hurt the growth and psummerosynthesis of algae (Zhang et al., 2017). it has been stated that sometimes marine organisms mistake plastic waste for bait, and this causes plastics to enter the food chain (Cole et al., 2011).

Bushehr province, despite having more than 625 km of the coastal strip and proximity to sensitive coastal and marine areas and habitats such as coral reefs, mangrove forests, and estuaries, due to the establishment of economic and industrial centers of marine origin in its coasts and marine areas, it has always faced serious environmental risks. Oil and gas facilities located onshore and offshore, sources and facilities of thermal power plants located on land that cause severe thermal changes, agricultural and aquaculture activities, shipping, marine accidents, and urban sewage are one of the major causes of pollution and threats to the biodiversity of these beaches (Heidari et al., 2013).

Kurd and Naji (2018), intended to investigate the contamination of microplastics in 5 species of dominant fish in Chabahar Bay (Sistan and Baluchistan Province). They concluded that all fish sampled from Chabahar Bay contained microplastics. Naji and Esmaili

(2015) investigate the microplastic pollution in the coastal sediments of the tidal areas of Hormozgan province. They concluded that the highest frequency of microplastics was in the stations near the industrial zones, and the lowest microplastics were in the stations less influenced by human factors. Feng et al. (2020) have suggested that microplastics absorbed by edible macroalgae may be transferred to humans leading to a high potential risk to human health. Jiang et al. (2020) revealed the characteristics of microplastics in the surface waters of the South Yellow Sea under the influence of season. They concluded that the abundance of microplastics, especially in small sizes, was positively correlated with seawater salinity. These results show that microplastic pollution in the surface waters of the South Yellow Sea varies with different seasons due to differences in land resources and marine hydrological dynamics.

Considering the high biological capacities of Bushehr province as well as its many pollution sources, it is necessary to research their relationship. This study aims to investigate the relationship between microplastics in macroalgae and water as well as identify the polymers that make up microplastics trapped by *Sargassum* sp.

Materials and methods

Study Area

Bushehr province is located in the south of Iran and on the edge of the Persian Gulf. Algae have always existed on the rocky shores of this province as a stable ecosystem for many years (Saeb Mehr et al., 2016). After the investigations carried out along the rocky coasts of Bushehr province, four stations named Jofreh, Rishehr, Halileh village, and Bojikdan were selected as sampling stations, and their location was recorded by GPS (CX120) . The geographical location of these stations can be seen in Figure 1 and Table 1. The selection of stations was based on the number of human activities, incoming effluents, and the presence of algal species.

Table 1. Coordinates of research stations

Station No.	Station	Longitude	Latitude
1	Jofreh	50° 82' 52.4" E	28° 97' 45.57" N
2	Rishehr	50° 82' 60.48" E	28° 90' 00.41" N
3	Halileh	50° 87' 40.5" E	28° 83' 57.2" N
4	Bojikdan	51° 07' 54.19" E	28° 64' 51.17" N

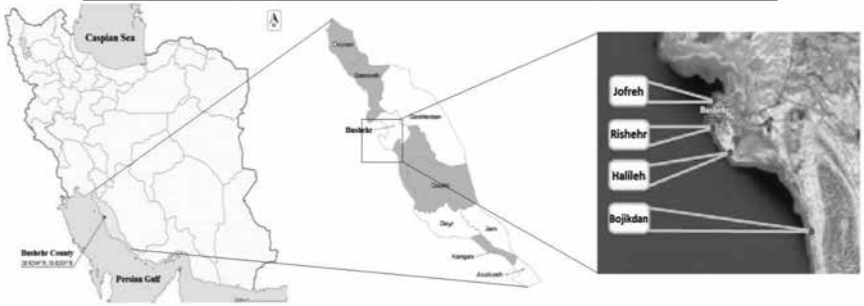

Fig. 1. Bushehr province map

Physicochemical factors

Environmental factors such as temperature, salinity, pH, and electrical conductivity of water were measured by a Hach multimeter. The measurement results in the two winter and summer seasons are shown in Tables 2 and 3. These factors are measured to understand the changes and relationship between environmental factors and microplastics in both seasons.

macroalgae and water sampling

Sampling was done during the winter and warm seasons from November 2021 to June 2022 from four stations in the studied area. Among the algae observed, the macroalgae *Sargassum* sp. of different sizes and healthy appear in tidal areas, and during the full tide, were manually sampled. Then samples were carefully placed in aluminum foil, and the station specifications and sample code were specified. Samples were transferred to the laboratory in a flask filled with ice (Feng et al., 2020). Water samples were taken at a distance of 10 meters from the coast of each station in the amount of two numbers of 5 L barrels from the coastal waters and transferred to the marine biology laboratory of Khorramshahr University of Marine Sciences and Technologies for filtration inside the glacier containing ice.

Identification of macroalgae and microplastics in the laboratory

In the laboratory, macroalgae samples were identified using a stereomicroscope (Olympus SZ40) and using an Olympus TL2 microscope, as well as using an algae identification key)Baldock et al., 2009; Bast et al., 2014 Shams et al., 2013). Then, the microplastics in the macroalgae were transferred to the Petri dishes of each sample using forceps. After checking the appearance of microplastics, they were psummerographed (figure 2-b,c,d). Also, 10 liters of water were collected from each station and transported to the laboratory. In the laboratory, using Gff (glass fiber filter) filter paper, the microplastics in the water sample were separated from the water (Prata et al., 2018). The microplastic samples in filter paper were observed and psummerographed by stereomicroscope and microscope. Both types of microplastics found in macroalgae and water were transferred to the laboratory of Amirkabir University (Tehran, Iran) to measure organic and polymeric compounds and determine functional groups in microplastics and were measured by FTIR infrared spectrometer.

Statistical methods

To investigate the presence or absence of significant differences between physicochemical factors and the abundance of microplastics in macroalgae samples, ANOVA statistical method and Pearson coefficient were used in SPSS ver. 22, and Excel 2010 was used to draw the graphs.

Results

Physicochemical factors

The results related to the physicochemical factors of the water of each of the studied stations in the two winter and summer seasons are given in tables 2 and 3, respectively. The water temperature ranges from 21.9 degrees

to 23.7 degrees Celsius in the winter season and 29.1 degrees to 30.5 degrees Celsius in the summer season. In winter and summer seasons, the range of water salinity changes between 38.6 to 39.7 and 39.1 to 41.2 ppt, the water acidity from 8.06 to 8.11 and 8.06 to 8.22 ppt, respectively. Moreover, the electrical conductivity of water is between 51.6 to 53.3 and 52.1 to 54.6 mS/cm.

Microplastics in macroalgae

The results showed that the dominant species of the study area is *Sargassum* sp. (Figure 2a). It also showed that microplastics were found in this species in all stations (Figure 2b, c, d).

The abundance of microplastics found in macroalgae *Sargassum* sp. is shown for 4 stations in the two seasons (Figure 3). Jofreh station has the highest amount of microplastic pollution with an average frequency of 180 ± 10.14 microplastics/kg in the winter season and 270 ± 9.84 microplastics/kg in the summer season (Figure 3). Also, Halileh station, which had the lowest amount of microplastic contamination, 19 ± 3.46 and 27 ± 4.58 microplastics/kg in winter and summer seasons, respectively, was considered as a control station. According to the graph of abundance in summer and winter, the average of microplastic trapped in the summer is 173 ± 96.96 microplastics/kg, which is more compared to the winter with an average of 116.75 ± 63 microplastics/kg of macroalgae (Figure 3).

The examined microplastics have different shapes including the film, piece, bullet, and fiber shape, which can be seen in Figure 4 in terms of abundance percentage. Fiber-shaped microplastics with 71% and film-shaped with 5% had the highest and lowest form of microplastics, respectively.

Also, the abundance percentage of each

Table 2. Physicochemical factors of water in the winter season

Station	Temp (°C)	pH	Salinity (ppt)	Electrical Conductivity (mS/cm)
Jofreh	21.9[a]	8.06[a]	38.8[a]	52.6[a]
Rishehr	22.1[a]	8.10[b]	38.6[a]	51.6[b]
Halileh	22.4[b]	8.11[b]	39.7[b]	53.3[c]
Bojikdan	23.7[c]	8.11[b]	39.5[b]	53.1[c]

Dissimilar letters in each column indicate a significant difference ($P < 0.05$)

Table 3. Physicochemical factors of water in the summer season

Station	Electrical Conductivity (mS/cm)	Salinity (ppt)	pH	Temp (°C)
Jofreh	29.5[a]	8.06[a]	40[a]	53.7[a]
Rishehr	30.1[b]	8.07[b]	39.1[b]	52.1[b]
Halileh	29.1[c]	8.22[c]	40.1[a]	54.1[c]
Bojikdan	30.5[d]	8.11[d]	41.2[c]	54.6[d]

Dissimilar letters in each column indicate a significant difference ($P < 0.05$)

Fig. 2. Sample of *Sargassum* sp. macroalgae (a). The forms of microplastics in the sample of *Sargassum* sp. macroalgae (b,c,d)

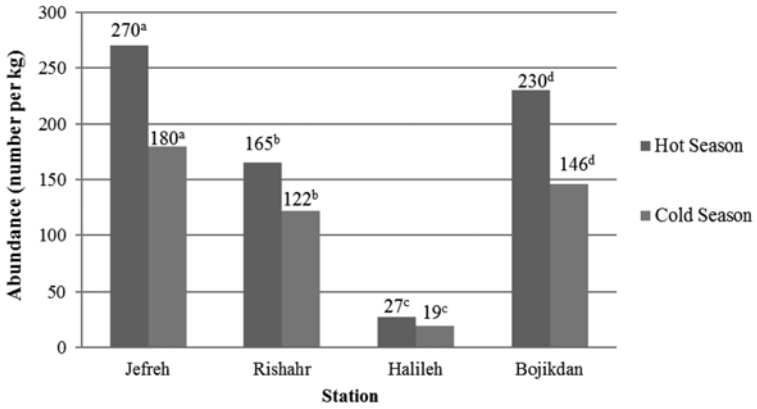

Fig. 3. Abundance of microplastic/Kg of *Sargassum* sp. in winter and summer seasons

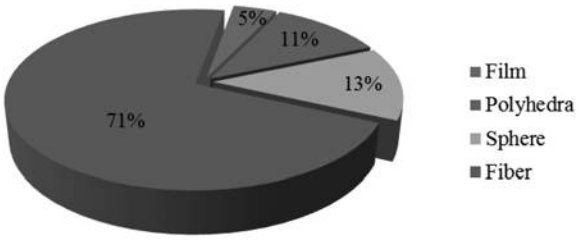

Fig. 4. The percentage of abundance of microplastic found in *Sargassum* sp. based on appearance

microplastic form in the macroalgae Sargassum sp. can be seen at different stations in Figure 5.

Forms and abundance of microplastics in water

Microplastics were also observed in filtered water (Figure 6). The abundance of microplastics in water is also listed in Table 4. The highest amount of microplastics in water was determined in both seasons at Jofreh station. Also, the frequency of microplastics in the winter was higher than in the summer (Table 4).

Identification of types of polymers

The results of FTIR-ATR analysis showed that the most abundant polymers found in microplastics are polyamide (PA) or nylon, polystyrene (PS), and polyvinyl chloride (PVC) (Tables 5 and 6). According to Table 5, polyamide polymer (nylon) was found in the macroalgae samples of all stations, followed by polystyrene polymer, which was the predominant percentage of macroalgae contamination. Also, according to Table 6, most polymers are polystyrene type, but the information in this Table cannot be expanded due to the low abundance of microplastics found in water samples.

The spectrum diagram can be seen in Figures 7, 8, and 9. The output spectrum of the FTIR

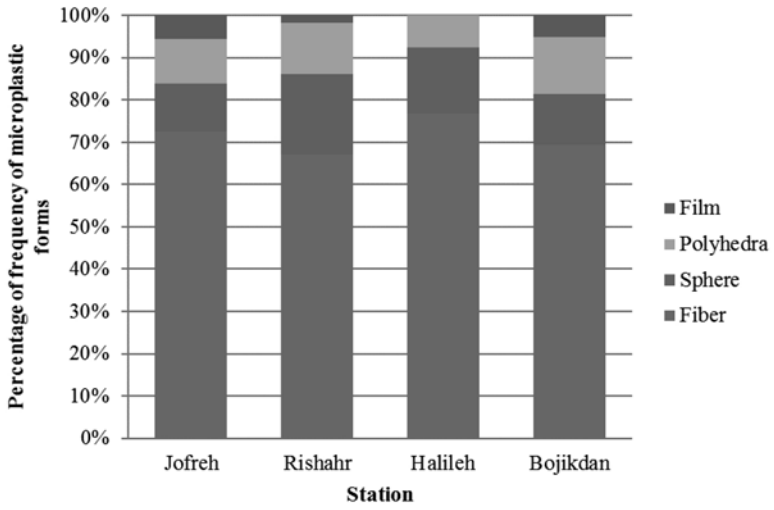

Fig. 5. The abundance percentage of different forms of microplastics found in samples of *Sargassum* sp.

Table 4. The abundance of microplastics in one cubic meter of water

Station	Summer Season	Winter Season
Jofreh	100	100
Rishehr	0	100
Halileh	0	0
Bojikdan	0	100

Fig. 6. Microplastic found in water filter sample

Table 5. The number of microplastics found in samples of *Sargassum* sp. at each station according to the type of polymer

Station	The most abundant polymers found	Polyvinyl chloride (PVC)	Polystyrene (PS)	Nylon
Jofreh	Nylon – PS – PVC	15	24	103
Rishehr	Nylon – PS	7	12	39
Halileh	Nylon	1	2	10
Bojikdan	Nylon	8	10	41

Table 6. The number of microplastics found in water samples at each station according to the type of polymer

Station	Polymers found in water	Polystyrene (PS)	Nylon
Jofreh	Nylon – PS	1	1
Rishehr	PS	1	0
Halileh	-	-	-
Bojikdan	PS	1	0

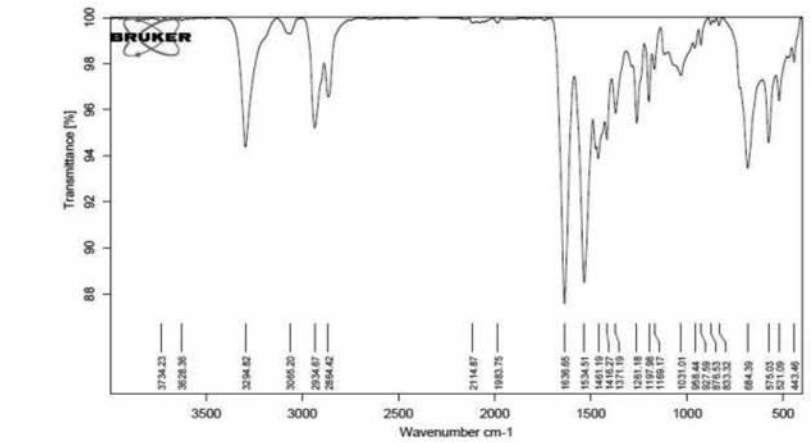

Fig. 7. The result of FTIR-ATR analysis of the microplastic sample in the *Sargassum* sp. which is similar to the standard spectrum of PA (Nylone) polymer

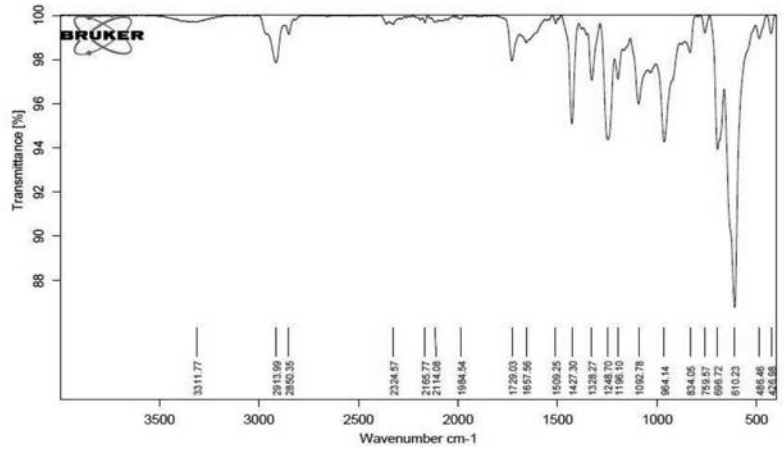

Fig. 8. The result of FTIR-ATR analysis of the microplastic sample in the *Sargassum* sp. which is similar to the standard spectrum of PVC

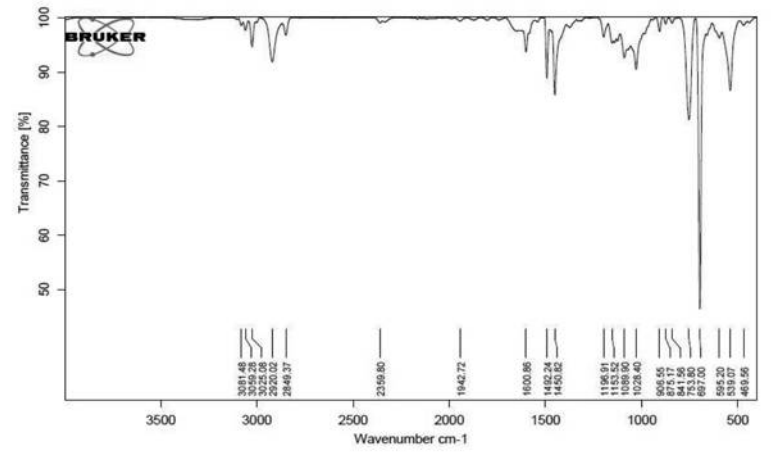

Fig. 9. FTIR-ATR analysis of the microplastic sample in the *Sargassum* sp. macroalgae which is similar to the standard spectrum of PS

device, which belongs to the microplastics in the Sargassum sp. sample, is shown in Figure 7. The peaks can be seen at the wavelengths of 3294.82, 2934.67, 1636.65, and 1534.51 cm-1, and the resulting spectrum is similar to the standard polyamide (nylon). Figure 8 also shows a similar spectrum of another type of microplastic found in macroalgae Sargassum sp. and the polyvinyl chloride polymer.

Correlation between physicochemical and microplastic factors

According to Pearson's statistical test results, the correlation between salinity and temperature factors with microplastics was not significan. The one-way ANOVA statistical test showed a significant difference in the abundance of microplastics in the stations in both seasons.

Discussion

The area investigated in this research is the shores of Bushehr province, which includes industrial and fishing wharves. By their nature, they can be a source of pollution in the environment. The presence

of microplastics, plastic particles smaller than 5 mm in marine environments, has caused a growing global concern due to their low weight, long shelf life, and risk to related marine communities. Considering the importance of microplastic pollution, monitoring the release of plastics by the tourism and fishing beaches of Chabahar Bay can be proposed as a topic for future studies. Pollution of the marine environment is a global phenomenon, and plastics are one type of pollutant introduced by humans into aquatic ecosystems, especially in the sea environment (Naji et al., 2017). Sea-based resources such as shipping, transportation activities, fishing, and land-based resources such as factories and industries adjacent to the sea and tourism, introduce plastics into the sea and ocean.

According to the results of measuring the abundance of microplastics in macroalgae Sargassum sp. in both seasons (Figure 3), Jofreh station, which is near urban residential areas, surface water inlets, urban sewage, fishing, and recreational piers, as well as coastal parks, has the highest abundance of microplastics in two seasons, on average 225 ± 50.48 microplastics/kg of macroalgae. After Jofreh, Bojikdan station has the highest level of microplastic pollution, 188 ± 48.01 microplastics/kg of macroalgae. Bojikdan station is in the vicinity of the coastal village, which seems today development in the tourism industry, the creation of eco-tours and residences, as well as the lack of proper waste management and proper culture, has caused the disposal of plastic waste and the transfer of untreated sewage to the sea. Rishehr station is near urban residential areas with a lower population concentration than Jofreh, the swimming area, and the surface water inlet, hand as an average of 143.5 ± 25.82 microplastics/ kg of macroalgae. Finally, Halileh station is located in the vicinity of rural residential areas, a local fishing pier, and a coastal park outside the city, and due to the small scale of the fishing industry in this place compared to other fishing piers, with an average of 23 ± 5.69 microplastics/kg of macroalgae. It is the cleanest station and witness station.

According to the results of the one-way ANOVA statistical test (Table 7), there is a significant difference in the abundance of microplastics in the stations in both seasons. The reason for this can be seen from what was said about the type and amount of pollution sources at each station. ($P< 0.05$).

By comparing the abundance of microplastics in macroalgae in both seasons, it can be seen that the entrapment of microplastics in macroalgae was more in the summer. The reason for this can be seen as the decrease in the viscosity of seawater due to heat and as a result, the reduction in the buoyancy of microplastics in water. Also, the onset of heat and fishing activity does not affect this difference. Other reasons include the high tourism capacity of Bushehr province in April and May and the increase in the amount of plastic waste entering the sea.

The results showed that about 71% of the found microplastics are fibers, 13% are spherical, 11% are multifaceted, and 5%

are in the form of films (Figure 4). The origin of fibrous and stringy microplastics can be from nets, ropes, and threads used in fishing (Jang et al., 2014) or clothing made from plastic fibers (Frias et al., 2016). Based on the studies, it has been shown that stringy microplastics are often produced in urban areas, and spherical and polyhedral microplastics are produced more often in industrial areas (Abbasi et al., 2019). Also, considering that one of the critical industries of the shores of the study area of Bushehr province is fishing, it seems that fishing nets containing plastic fibers are one of the sources of increasing microplastics in the region.

The amounts of microplastics found in the water of the study area in both seasons were insignificant. The results showed that more microplastics were found in surface water in the winter than in the summer, with 75 ± 50 microplastics/m^3 of water in the winter season and 25 ± 50 microplastics/m^3 of water in the summer season (Table 4). The main reason is that viscosity has an inverse relationship with microplastic sedimentation. Due to the low temperature in the winter season, the viscosity is high which increases microplastic sedimentation, and more microplastics can float on the water. But in the summer season, this situation is reversed, the increase in temperature reduces the intermolecular force of water particles, and the viscosity reduction may result in microplastic sedimentation increases, and this is the reason why fewer microplastic particles are seen in surface water in the summer season (Kooi et al., 2017).

The results of the polymer analysis of microplastics showed that the polyamide polymer (nylon) was found in macroalgae Sargassum sp. Polystyrene and polyvinyl chloride were the most abundant in macroalgae and water samples. Nylon is used as raw material in manufacturing fishing nets and ropes, clothing, plastic fibers, electrical insulation, consumer or industrial goods, electronic industries, etc. (Deopura et al., 2008). Polystyrene is mainly used in disposable containers, sports equipment, toys, winter insulation (Styrofoam), and the packaging of goods. (Marsh and Bugusu, 2007). Polyvinyl chloride is also used in making all kinds of pipes, construction works, etc. (Titow, 2012).

Based on the results (Tables 5 and 6), polyamide polymer (nylon) is the most abundant in macroalgae and water in the Jofreh station. Residents of the areas around Jofreh station have many fishing activities. Therefore, amounts of fishing nets and ropes can be placed on macroalgae and water surfaces in the form of microplastics.

Considering the fishing activity and the entry of rural and urban residential sewage sampling stations, the presence of nylon polymer is probable.

Also, the results of Table 5 showed that polyvinyl chloride is more in the macroalgae of the Jofreh station than in other stations. One of the justifications for the increase of this polymer can be mentioned in the increasing urban constructions and residential areas in the Jofreh region. Also, according to Table

6, this type of polymer was not found in water samples. According to Tables 5 and 6, polystyrene polymer was the most abundant in macroalgae and water samples in Jofreh station due to the increase in plastic wastes such as disposable containers, toys, tourism, and water activities.

Few studies have been done regarding the investigation of microplastics in macroalgae (Table 8). In the present study, the amount of microplastic pollution in the macroalgae Sargassum sp., on average, is 145 pieces per kilogram. Feng et al. conducted research on several species of macroalgae in 2020, in the Yellow Sea region, Haizhou Bay. Their results showed that *Ulva prolifera* had the highest microplastic contamination of 190 pieces/kilogram. *U. pertusa* also had the lowest amount of contamination with microplastic contamination of 60 pieces/kg. Also, Feng and his colleagues conducted another study in 2020 in the Yellow Sea, as a result of which microplastic contamination of 660 pieces/kilogram of *U. prolifera* macroalgae was recorded. According to the research conducted in this study, the amount of microplastic found shows a lower level of pollution in Bushehr province. The reason for this can be seen as less connection with open waters, fewer industries and sources of pollution (tourism, fishing, factories, etc.), less population, and therefore less plastic consumption.

Relatively extensive research has been done on the microplastic pollution in water bodies. According to the present study, this type of pollution in the Persian Gulf, the shores of Bushehr Province, is 50 microplastics/m3 of water. According to Table 8, it was found that there is a significant difference in the amount of this pollution all over the world. Microplastic pollution in the water is as high as 5300 microplastics/m3 in the southern Yellow Sea (Jiang et al., 2020). Besides, Aytan et al. (2016) recorded a pollution level of 1100 microplastics/m3. On the other hand, Doyle et al. (2011) concluded the contamination at the level of 0.004-0.19 microplastics/m3 in the North East Pacific Ocean. These results show that the pollution

Table 8. Abundance of microplastics in macroalgae in other studies in the world

Region	Species	Abundance (number per kg)	Reference
Haizhou bay	*Ulva. prolifera*	190	Feng et al., 2020
Haizhou bay	*Pyropia yezoensis*	170	Feng et al., 2020
Haizhou bay	*Sargassum horneri*	140	Feng et al., 2020
Haizhou bay	*Ulva Pertusa*	60	Feng et al., 2020
Yellow sea	*Ulva. prolifera*	660	Feng et al., 2020
Bushehr province	*Sargassum* sp.	145	Recent study

of Bushehr beaches is in the moderate range compared to other regions of the world (Table 9).

It is expected that in the waters of Iran, this amount of pollution will be lower than the global level due to the less population, fewer factories and plastic production industries, etc. However, due to the advancement of technology and proper management in other countries of the world to eliminate these pollutions, including advanced purification systems, the use of suitable alternatives instead of plastic, extensive training, etc., one should not rely only on the current data and take action to eliminate this. The problem did not become global.

As stated by the results of the present study, it can be seen that macroalgae play a significant role in trapping microplastics. Also, the beginning of the summer and the increase in fishing, and the tourism industry cause an increase in microplastic contamination in the current study areas. Jofreh station had the highest microplastic pollution, which can be attributed to the higher input volume of surface water, urban sewage, fishing, recreational activities, etc. Also, the most microplastics found in all the samples were polyamide (nylon) and string-shaped (fiber), which can make the main tools of fishing activity in these areas.

Acknowledgment

The authors are grateful to the Department of Marine Biology, Faculty of Marine Science and Oceanography, Khorramshahr University of Marine Science and Technology to facilitate this research. Also, special thanks to Mohammad Kolahi for his close cooperation.

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Table 9. Abundance of microplastics in water in other studies in the world

Region	Abundance (number per m^3)	Reference
Black sea	1100	Aytan et al. 2016
Mediterranean sea	0.15	De Lucia et al. 2014
Hong Kong beach	3.973	Cheung et al. 2018
North east Pacific Ocean	0.004-0.19	Doyle et al. 2011
Seto inland sea	0.39	Isobe et al. 2014
California waters	3.92	Lattin et al. 2004
Xiangshan bay	8.91	Chen et al. 2018
Southern Yellow sea	5300	Jiang et al. 2020
Haizhou bay	1450	Feng et al. 2020
Chabahar bay	0.49	Khamarzadeh et al. 2019
Bushehr province	50	Recent study

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The Effect of Cyanobacterial Bioelicitors on Total Phenolic Content of *Echinacea purpurea* L.

Zahra fallah hosseini[1], Hossein Riahi[1], Majid Ghorbani Nohooji[2], Zeinab Shariatmadari[1]*

Received: 2022-07-17 Revised and accepted: 2022-09-12

Abstract

Cyanobacteria have the ability to nitrogen fixation, and the production of plant growth-stimulating substances, which increases plant growth and productivity as biological elicitors. Considering the medicinal value of *Echinacea purpurea* (L.) Monch, the effect of two species of heterocystous cyanobacteria, *Nostoc punctiforme* Hariotand, and *Nostoc calcicola* Brébisson ex Bornet & Flahault, on growth factors and phenolic content of this medicinal plant were evaluated. For this purpose, four pots were considered for each treatment and four others for the control plants. *E. purpurea* cultivated in a randomized complete block design in an experimental greenhouse condition. Then the treated plants were irrigated with 120 ml of 0.2% cyanobacterial suspensions at 20-day intervals. After 60 days, plants were collected and dried under shade and at room temperature. The total phenolic content of the aerial part and root of treated and control plants were evaluated using the Folin-Ciocaltiu method. The results showed that the total phenolic content of cyanobacterial-treated plants, especially plants treated with *N. calcicola*, significantly increased. This metabolite improvement was observed both in the aerial part and the root system of the *N. calcicola* treated plants. The results also showed that plant growth parameters such as root and stem length, as well as number of leaves in both treatments increased significantly compared to the control. As a result of this study, cyanobacteria can be improving the phenolic content and growth indices of the medicinal plant *E. purpurea*.

Keywords: Cyanobacteria, *Echinacea purpurea,* Secondary metabolite, Bioelicitor, Nitrogen fixation

Introduction

Echinacea purpurea (L.) Moenchis is an herbaceous and perennial plant that belongs to the Asteraceae family and its origin is reported in North America. All plant parts contain valuable substances, including caffeic acids (cicuric acids, chlorogenic acids, cynarine), alkamides, polysaccharides, polyphenols, proteoglycans, lipophilic alkylamides, and other phenolic compounds (Attarzadeh et al., 2020). This medicinal herb has immunostimulatory and anti-inflammatory properties, especially to

1- Department of Plant Sciences and Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran.
2- Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.
*Corresponding author email address: z_shariat@sbu.ac.ir

alleviate cold symptoms. The plant chemical compounds have several pharmacological properties such as anti-cancer, antianxiety, antidepression, and antimutagenicity (Manayi et al., 2015). The antioxidant activity of this plant, as well as antimicrobial, antifungal, and antiviral properties also have been reported in several studies (Thygesen et al., 2007; Sharma et al., 2010; Manayi et al., 2015).

Phenolic compounds are one of the most important chemical constituents of this medicinal plant that stimulate and strengthen the body's immune system (Noori et al., 2017). Various biological functions have been proposed for phenolic compounds, the most important of which are: antioxidant, antimicrobial, anti-inflammatory, and anti-cancer effects (Fazeli-Nasab et al., 2018; Noori et al., 2017). Phenolic compounds with antioxidant and free radical scavenging properties can be crucial in preserving food products and maintaining human health (Noori et al., 2017). The antioxidant activity of Echinacea extract has been reported in previous studies (Sloley et al., 2001).

One of the methods for the elevation of the plant's secondary metabolite content is the use of elicitors. The direct and indirect effects of elicitors on plant metabolic pathways can amplify the production of some secondary metabolites and could serve as a defense mechanism for the plant (Kokate et al., 2002). Cyanobacteria or blue-green algae are the most primitive group of photosynthetic organisms that played an important role in the evolution of Early Earth and the biosphere (Demoulin et al., 2019). Today, this group of photosynthetic organisms plays a specific role in biotechnological research and can produce a wide range of valuable organic compounds (Soleimani et al., 2022). Cyanobacteria are found in various habitats such as terrestrial ecosystems (Gademann and Portmann, 2008). Soil is the habitat of some cyanobacteria species that improve soil fertility by fixing atmospheric nitrogen, helping to maintain moisture, preventing soil erosion, and producing hormonal and non-hormonal plant growth-promoting substances (Shariatmadari et al., 2013; Abinandan et al., 2019). Therefore, soil microflora plays a significant role in the carbon and nitrogen cycle of terrestrial ecosystems. For this reason, this group of photosynthetic microorganisms can act as a suitable stimulus in the pathway of plant growth (Johnson et al., 2013; Miralles et al., 2011; Parikh, 2006).

In addition, cyanobacteria can increase soil phosphate due to the production of organic acids. Phosphorus is the second essential element in agriculture, which is needed for the growth and development of plants (Zahra et al., 2020).

According to available reports, the proper use of biofertilizers can increase crop production in many plants by 20-30%, and significantly reduce the occurrence of plant pests (Ananya and Ahmad, 2014). In general, biofertilizers, such as fertilizers derived from cyanobacteria, are the more suitable alternative for agricultural purposes due to limited production costs and energy

consumption compared with synthetic fertilizers such as urea (Zahra et al., 2020). The results of the previous studies showed the improvement of growth indices, as well as quantitative and qualitative improvement of the essential oil and metabolites in cyanobacteria-treated plants (Chookalaii et al., 2020).

Considering the economic value of *E. purpurea*, it is necessary to find ways to improve the chemical composition of this plant. Therefore, the present study aimed to investigate the effect of native cyanobacteria as biological elicitors in increasing the plant biomass and total phenolic content of *E. purpurea* as widely used and an economically crucial medicinal plant in Iran.

Materials and methods

Isolation, purification, and identification of cyanobacteria

For the isolation of cyanobacterial species employed in this study, soil samples were collected from several central fields of Iran under the cultivation of medicinal plants. The purification of the cyanobacteria was done through repeated subculturing of the colonies on the nitrate free BG11 solid medium (Andersen, 2005). The cyanobacteria were cultured under controlled laboratory conditions and artificial light illumination (74 µmol photons/m^2 s), with a 16:8 hours light-dark cycle, and 25 ± 2 ºC temperature. Finally, purified taxa were identified by optical microscope (Olympus, Model BH-2) based on valid identification key books (Komárek, 2013).

Cyanobacterial suspension preparation

The suspension of two isolated and purified cyanobacterial species, *Nostoc punctiforme* Hariot and *Nostoc calcicola* Brébisson ex Bornet & Flahault, were prepared through homogenizing 2 g of cyanobacterial biomass, after four weeks of culturing, in one liter of sterilized distilled water (0.2% cyanobacterial suspensions).

Pot culture and growing condition

E. purpurea plant was received from Karaj Medicinal Plants Research Institute. Healthy and similar plants were grown in pots containing 40% soil, 40% sand, and 20% perlite for 60 days. The experiment was performed in a randomized complete block design in an experimental greenhouse. All pots of treated and control plants were irrigated similarly for 60 days and for cyanobacterial treatments, 125 ml of cyanobacterial suspensions (2 g biomass in one-liter distilled water) was added to each treated pot on the first day of planting and every 20 days thereafter. The control plants were irrigated only with distilled water.

Total phenolic assay

To evaluate the total phenolic content (TPC), the dried leaves and roots were extracted with methanol for 24 hours with three-time repetitions. The TPC of the total extracts was measured using the Folin-Ciocaltiu method. To analyze the content of total phenolic content, Folin reagent 10% in the amount of 200 µl, and $NaHCO_3$ in the amount of 800 µl were added to 100 µl of methanolic extract (three replicates of each sample) and placed at ambient temperature

for three hours then absorption was recorded at 765 nm. From the calibration curve, the total amount of phenolic compounds (mg. ml^{-1}) was calculated and determined with gallic acid equivalent (mg GA dry matter/g) (Karimi et al., 2018; Ashouri Sheikhi et al., 2016; Kamtekar et al., 2014; Singleton et al., 1999).

Statistical analysis

Statistical analyses were conducted using SAS version 9.1. Analyses of variance (ANOVA) were performed to compare the data. All determinations were done at least in triplicate, and all were averaged. The confidence limits used in this study were based on 95% ($p \leq 0.05$).

Results

Considering the medicinal importance of phenolic compounds in this medicinal plant, the total phenol content of the treated and control plants was measured and compared. The results showed that the TPC of seedlings treated with *N. calcicola* significantly increased compared to the control plants. Seedlings treated with this strain (*N. calcicola*) showed a significant increase of TPC in the root and aerial parts (Figure 1). It should be noted that this increase was not the same in both sections of plants, and the TPC in the root section was more than the aerial part of treated plants.

Also, the growth parameters such as root length and number of leaves have increased significantly in both treatments compared to the control (Figure 2, 3). However, the leaf area was significantly different only in the *N. calcicola* treated plants compared to the control plants (Figures 2 and 4).

The highest root length was recorded for plants treated with *N. punctiforme* with 84 % increase compared to the control. The *N. calcicola* treated plants also showed a 32 % increase in the root length compared to the control.

The highest increase in the length of aerial parts of plants was also recorded for *N. punctiforme* treated plants with a 21 % increase compared to the control plants, followed by those inoculated with *N. calcicola* (Figure 2).

The highest leaf area was recorded for

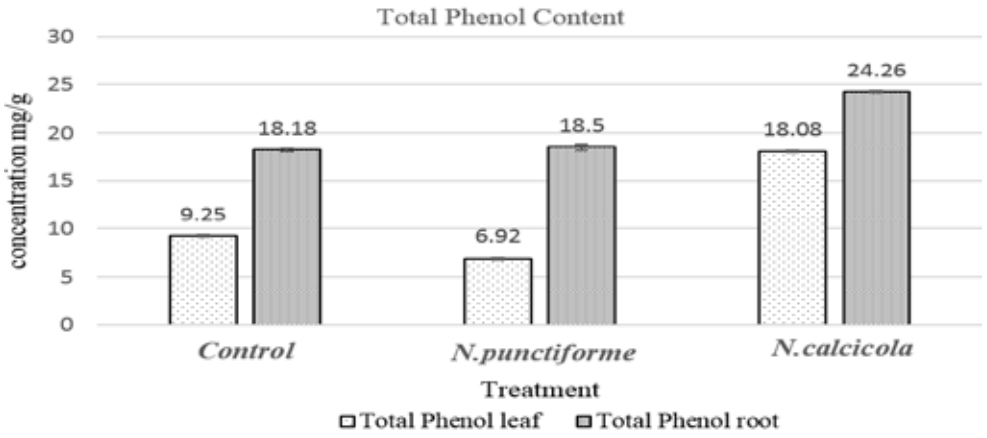

Fig. 1. The total phenol content of *Echinacea purpurea* in control and Nostoc-treated plants

plants treated with *N. calcicola* with a 155% increase compared to the control. Plants treated with *N. punctiforme* showed an 22% increase compared to the control.

Discussion

In this study, the potential of two

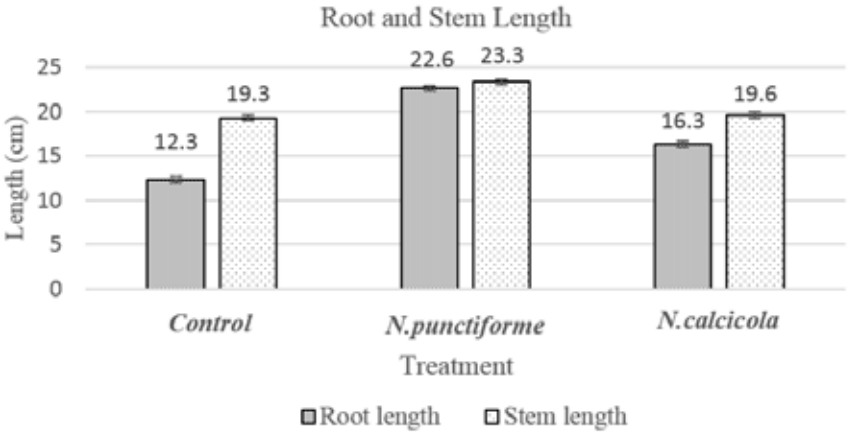

Fig. 2. The length of the root and stem of *Echinacea purpurea* in

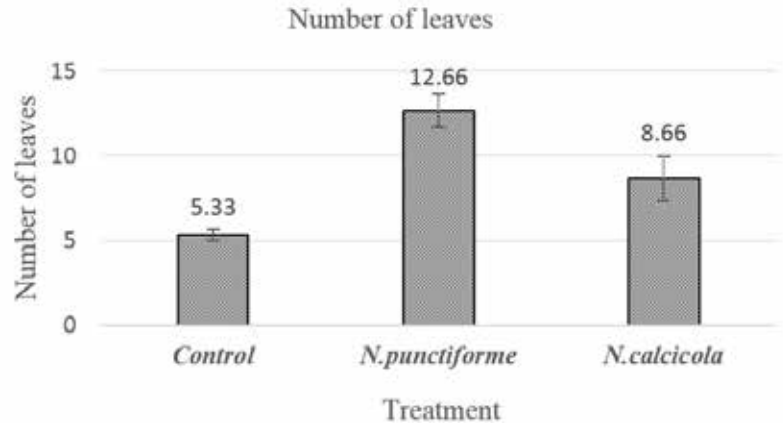

Fig. 3. The number of *Echinacea purpura* leaves in control and Nostoc-treated plants

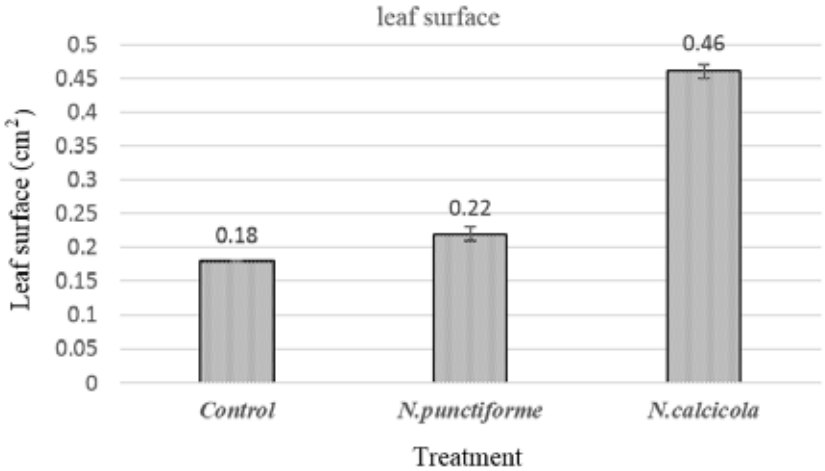

Fig. 4. Leaf surface of *Echinacea purpura* in control and Nostoc-treated plants

cyanobacterial strains as biological stimulants in *E. purpura* was investigated. As a result, treatment of this medicinal plant with heterocystous cyanobacterium *Nostoc calcicola*, has increased the amount of phenol content in the root and aerial parts of the plant. Phenolic compounds are one of the most important compounds that stimulate the immune system, and these compounds are found in the root and aerial part of this medicinal plant and are considered a metabolite that stimulates the immune system (Noori et al., 2017).

The results of the present study showed that the cyanobacterium *N. calcicola* significantly enhances the amount of total phenol in the roots and shoots of *E. purpurea*. The results also showed that several species of cyanobacteria do not have the same ability, and the cyanobacterial suspension of *N. punctiforme* has not shown a similar and positive performance about the level of TPC. In the previous studies, the improvement of plant growth and phenolic content of *Vicia faba* by using some biofertilizers was reported. Another study also showed that the treatment of bean plants with biofertilizers increases the phenolic compounds of treated plants (Ragaa at el., 2013). Previous studies have also shown that cyanobacterial biostimulants increased the flavonoid content of *Plantago major* L. and improved the medicinal properties of this plant (Chookalaii et al., 2020).

Some researchers believe that biological factors can increase the production of phenolic compounds in plants by stimulating the secretion of organic acids and the production of growth regulators (Attarzadeh et al., 2020). Several studies have shown that phenols and flavonoids increase as a result of plant interactions with the stimulating agents such as plant pathogens (Pusztahelyi et al., 2015). Researchers also reported that biofertilizers improve plant growth and increase phenolic compounds in plants by increasing the absorption of nutrients and biosynthesis of phytohormones (Atsami et al., 2018), which findings are consistent with the results of the present study.

Based on the studies conducted in this field, it can be concluded that cyanobacteria can be used as natural and effective agents to increase the quality and quantity of valuable metabolites of medicinal plants and can be used purposefully to increase the efficiency of medicinal plant production. As a result, the *N. calcicola* strain can increase phenol production in *E. purpurea* plant. In summary, the selected cyanobacteria, isolated domestically, can act as potential biofertilizer candidates to promote the production of *E. purpurea* as a medicinal plant.

Considering the ability of this taxa to fix nitrogen and produce growth-stimulating compounds, these cyanobacteria are suitable biological stimulating agents for the quantitative and qualitative improvement of the phenolic content of medicinal plants such as *E. purpurea*. It suggested that further studies be conducted to determine the effects of cyanobacteria on other metabolites of *E. purpurea*.

Acknowledgment

The authors wish to thank University of Shahid Beheshti for funding this project. Thanks are also due to Dr. Seyedeh Batool Hassani for her kind assistance during the research.

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Design and Production of an Algal Biofilter for Industrial Wastewater Treatment

Sasan Ghobadian[1]*, Neda Soltani[2], Maryam Ameri[3], Mehdi Bolfion[4]

Received: 2022-07-12 Revised and accepted: 2022-09-25

Abstract

The increasing need for water resources and other factors in reducing these resources, along with the health and environmental problems of wastewater, make it clear that our linear water economy must evolve into a resilient circular water economy, where water is continuously reused and "contaminants" become the feedstocks for other economically valuable processes.

Biofilters as an important emerging technique, which utilizes biological living things as catalysts to harvest valuable components.

Cyanobacteria and microalgae's ability to be mixotrophs provides a competitive advantage against bacteria and fungi to be used in biofilters. Due to the reduction of environmental nutrients, heavy metals, and pathogens, the oxygen productionfor aerobic organisms, and the consumption of carbon dioxide, microalgae play a prominent role in purification processes.

In this research, by examining different types of algal species and immobilization methods, the appropriate species were chosen for biofilters production. The performance of the optimally produced biofilter to reduce the pollution indicators of the industrial effluent was investigated.

The results show the appropriate performance of the biofilter produced with AFC008 and AFC110 species to perfect removal of nitrate and phosphate and 76% COD reduction and 79% reduction of BOD in less than a week (along with aeration pretreatment).

Keywords: Biofilter, Industrial Wastewater, Phycoremediation, Wastewater Treatment, Algae

Introduction

Despite of regional nature, global processes exacerbate water stress. These global-scale influences include climate change, population and economic growth, food production, and trade. Because of these global-scale influences and the importance of the related problem, it is profitable to find a way of evaluating the efficiency of any policies aimed at mitigating water stress at the global level (Wada et al., 2014). So

1Assistant Professor, Department of Civil Engineering, Faculty of Civil Engineering and Architecture, Malayer University, Malayer, Iran
2Department of Research and Development, Afaghzist Company
3Industrial Microorganisms Biotechnology Research Department, Industrial Biotechnology Institute, ACECR, Mashhad, Iran
4Department of Petroleum Microbiology, Research Institute of Applied Sciences, ACECR, Shahid Beheshti University, Tehran, Iran
*Corresponding Author: s.ghobadian@malayeru.ac.ir

our linear water economy must evolve into a resilient circular water economy, where water is continuously reused and "contaminants" become the feedstocks for other economically valuable processes (Mauter and Fiske, 2020).

The environmental and health problems of wastewater, such as the role of nitrates in blue baby disease and methemoglobinemia (Jain et al., 2010), the effect of phosphorus in Eutrophication (Le Moal et al., 2019), the effect of various pathogens in diseases such as typhoid and dysentery, are well known. In contrast, the recent changes in the public perception of wastewater have led to several paradigm shifts: i) Water reuse considers wastewater as a water resource rather than a hazardous waste, ii) Wastewater-based epidemiology considers wastewater as a source of information regarding the overall health of a population through the analysis of specific biomarkers, iii) Circular economy through the implementation of treatment processes aiming to harvest valuable components such as precious metals or produce valuable goods such as biofuel (Villarín and Merel 2020).

According to the Country's Water and Sewage Engineering Company Report (2020), only 2078 million cubic meters of wastewater were collected out of 6647 million cubic meters of produced water. However, assumption of an 80% water-to-wastewater conversion factor, the produced wastewater estimation will be 5318 million cubic meters. Meanwhile, industrial wastewaters with extreme quantitative and qualitative fluctuations, including BOD and COD chemical parameters, harmful and toxic chemicals, and heavy metals are critical. Biochemical oxygen demand (BOD), suspended solids, nutrients (NO_3, NO_2, NH_4, PO_4), coliform bacteria, and toxicity removal are the main goal of perfect wastewater treatment. Common wastewater treatment processes include physical, chemical, and biological treatments. Chemical treatment is often inefficient, and expensive, which leads to toxic waste production due to physical or chemical replacements. Amidst, the Bio-based techniques, biofilters are found to be suitable, sustainable technology, and easy to operate for various contaminants removal in the aquatic environment (Pachaiappan et al., 2022). Biofilters usage is an important emerging technology that utilizes biological living things as catalysts for algae, bacteria, plants, protozoa, viruses, yeast, and mixed microbes (Delhomenie and Heitz, 2005). In simple terms, the biofiltration process can be represented as follows (Bressani-Ribeiro et al., 2,018).

Pollutants + Photosysthetic creatures + Oxygen → Biomass + Water + Carbon dioxide

The biofilter consists of a microbe immobilized on the substrate material. Immobilization of the microbes could be done by natural attachment or artificial immobilization of microbes to the biofilter bed materials (Baltrėnas et al., 2020). The performance of biofilters depends upon the microorganism. They are responsible for the phase transformation and degradation of

contamination in input-polluted air or water. Several parameters such as Biological organisms, biofilter bed, Supply of nutrients, pH, operating temperature, moisture contents, and pressure drop determine the efficiency of the biofiltration process (Pachaiappan et al., 2022).

There are several areas of bioremediation as mycoremediation by fungi and phycoremediation by algae depending on the organism or microorganism used. Bacteria as bioremediation agents have more advantages due to their adaptability and rapid growth, higher surface-to-volume ratio, potential ability to horizontally transfer genes of catabolic enzymes, and ease of genetic manipulations (Subashchandrabose, Ramakrishnan et al. 2013). Cyanobacteria and microalgae can live as autotrophs, heterotrophs, or mixotrophs. The ability to be mixotrophs provides a competitive advantage against bacteria and fungi to decompose organic pollutants (Subashchandrabose et al., 2013).

If algae are used, the environmental trinity is established. It meant wastewater treatment, CO_2 stabilization, and biomass production. Treatment is accomplished under light conditions without oxygen demand, and biologically biomass is produced by carbon stabilization. Phycoremediation has many applications; removal of food from municipal wastewater and streams rich in organic matter. Removal of xenobiotics and food compounds with the help of algal absorbents, treatment of acidic and metal wastes, CO_2 consumption, transformation, and decomposition of xenobiotics. Detection of toxic compounds with the algal biosensors' assistance (Rawat et al., 2011).

Due to the reduction of environmental nutrients, heavy metals, and pathogens, the production of oxygen for the consumption of bacteria and aerobic organisms, and the consumption of carbon dioxide, microalgae played a prominent role in purification processes (Muñoz and Guieysse, 2006).

The heavy metals are removed by various mechanisms such as rapid surface absorption (biosorption) and intracellular absorption depending on metabolism (bioaccumulation).

Several environmental or nutritional factors are effective on the growth of algae and consequently on the efficiency of the treatment process. The pH value determines the solubility of CO_2 in the culture medium, and its high values are responsible for the depletion of NH_4-N (Bouldin et al., 1974) and the deposition of PO_4-P (da Silva Cerozi and Fitzsimmons 2016). Therefore, the mechanisms of carbon concentration and nitrogen and phosphorus removal by microalgae cells are most affected by the pH value. The light intensity required by algae is lower compared to higher plants. Photosynthetically active solar radiation (400 to 700 nm), used as an energy source for algae, is 43-45% of the total incoming radiation. Theoretically, the maximum conversion of this radiation to carbohydrates is 27% (Prajapati et al., 2013). An increase in temperature leads to higher metabolic activities and nutrient absorption,

as well as a decrease in the solubility of some nutrients such as CO_2 and NH_4-N (Gonçalves et al., 2017).

The main sources of carbon include atmospheric CO_2 or the output of industrial gases or soluble carbonates. Although carbon is often 60% of the algae's total nutrient requirement, levels higher than optimal level inhibit the algae growth (Goldman et al., 1972, Chen and Xu, 2021).

Microalgae plays a crucial role in the treatment of the third stage of domestic wastewater in large or small ponds which related process can remove phosphorus and nitrogen in a brief period, even about 1 hour. In addition to the basic demand for nitrogen and phosphorus, small amounts of micronutrients such as Na, Mg, Ca, Mn, Zn, Cu, Fe, and Mo are also needed for optimal algae growth.

The proper species was investigated by examining different types of algal species and immobilization methods for biofilters production in this research. In the next stage, the performance of the optimally produced biofilter was examined to reduce the pollution indicators of the industrial effluent.

Materials and methods

Wastewater collection

Exemplification of industrial wastewater was investigated between May and July 2017. The Effluent Analysis is indicated in Table 1. The effect of sterilization on pathogens removal in the growth rate of stabilized microalgae and the bead's decay in 70% and 100% dilutions wastewater were investigated. The growth of free microalgae in non-sterile wastewater was also analyzed as a control in the experiments. Changes in the chemical characteristics of the effluent such as pH and EC were recorded at the end. The samples were cultured in a volume of 100 ml, 150 rpm, and 3000 lux illumination with three replications.

Screening and strain selection

After four days of continuous aeration, the green effluent was cultured on agar plates to purify the samples. In addition to conventional culture media (BG_{11}, N8, BBM, WALN, F26, BG_{110}), 1% cefotaxime antibiotic was used to control the growth of pathogens. Finally, after proving the purity of the species with a light microscope DNA extraction, and final identification were done.

In the first phase four strains out of tens of ones screened, which were cultivated in actual wastewater, were selected and cultivated in four different dilutions of wastewater with similar illumination.

Based on the 8-day growth curves, microalgae with the highest growth in high effluent concentrations were chosen for stabilization experiments. 100 ml samples were cultured at 150 rpm, constant illumination of 3000 lux with three replications. The absorbance was recorded at a wavelength of 680 nm every other day. Cyanobacterial genomic DNA was extracted using a DNA extraction kit (Thermo ScientificK0512, Lithuania) according to the instructions, and the quality of extracted DNA was evaluated by

electrophoresis on 0.8% agarose gel. The genus of the extracted samples was the green alga morphologically. Gene duplication was done through 16S rDNA and 18S rDNA sequences to determine the genus accurately. The PCR reaction was performed on the sample's DNA with a thermocycler (Corbet, Australia) and a pair of specific primers in a volume of 50 microliters. To observe the band pattern of the PCR product, one hour of electrophoresis was performed on a 1% (w/v) agarose gel at 70 V. 1Kb molecular size marker (Fermentase GenRuller SM0373) was used to determine the size of PCR products. Photography was taken by GelDoc device (UVP, USA).

After the cultivation of the isolated species isolated from the wastewater, two species, AFC 008 and AFC 111, which had the highest growth rate and resistance in the wastewater, were selected for future experiments.

Immobilization

Alginate was used as a fixation substrate for algae stabilization due to its cheapness, high efficiency, and biodegradability. Variable efficient factors in the preparation of matrices are as follows.

Alginate concentration (2 and 3%), solidification solution 2% ($BaCl_2$, $CaCl_2$), adding some salts ($CaCO_3$ 1%), and using other polymers (PVP, Chitosan and 3-methyl hydroxy cellulose) in combination with alginate or to cover the Beads.

Secondary Polymer was used according to reports on the coating of calcium alginate microcapsules by polycations for various purposes, such as the immunoprotective of carriers in cell transport, enzyme stabilization, and drug release systems. It has been demonstrated that the attachment and stability of alginate-polycation capsules depend on the composition of the alginate gel and the molecular weight, flexibility, and charge density of the polycation (Thumvijit et al., 2013).

Chitosan in concentrations of 1% to 0.1%,

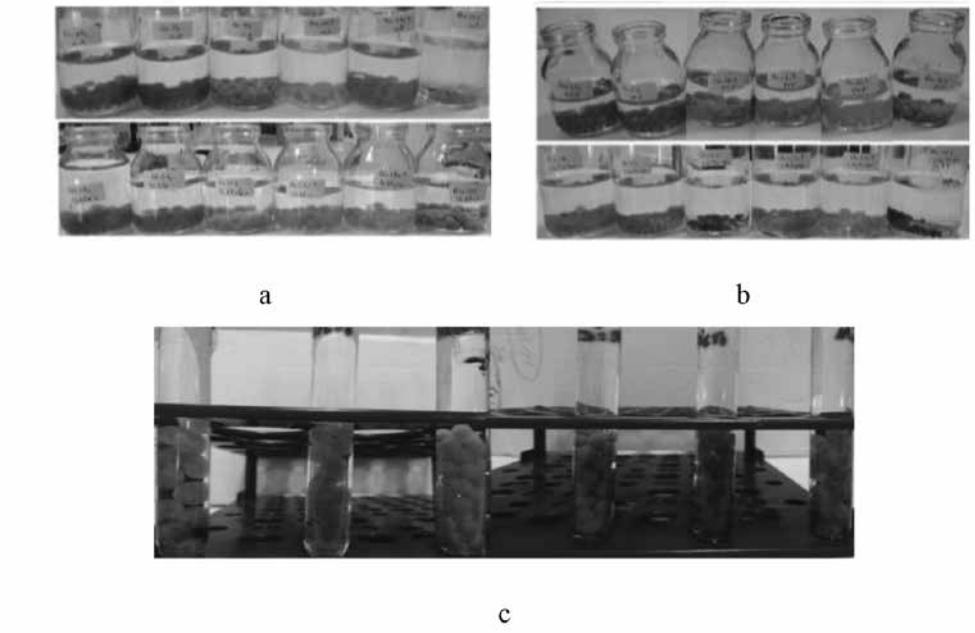


Fig. 1 a, b, c. The appearance and coating of beads

PVP 1%, and hydroxymethyl cellulose 1% in combination with 1% and 2% alginate were added after alginate was dissolved. Then algal biomass was added. The viability of the beads was checked with 48 hours of pretesting in sewage and radiation, and the appropriate substrate was selected. As in all experiments, beads without algae were used as controls. The appearance and coating of beads are showed in Figure1.

Alginate calcium beads are highly unstable when exposed to chelators such as phosphate, lactate, citrate, or non-gel cations such as sodium or manganese ions. Introducing calcium into phosphate and forming calcium phosphate causes disorder in the beads' structure. Therefore, it is necessary to check the stability of formed beads in different concentrations of phosphate buffer. In addition, in some studies, sodium chloride ions have been mentioned as a disintegrating agent. Different phosphate buffer concentrations were used for three days, and a mixture of 1% sodium chloride was used on the shaker for two days to check the disintegration of the beads along with phosphate ion treatments. Beads with a resistant structure were selected as stable patterns after several days of exposure to high phosphate concentrations.

The optical density on the 8th day of cultivation in the wastewater was recorded along with the effluent color and the color and health status of the stabilization structure so that the microalgae selected from this section could be used for forming experiments.

Shaping of stabilization structures

In the first part of the biofilter production, the microalgae stabilization formulation was obtained according to factors such as the algal survivorship and its active metabolism to absorb nutrients and gas exchange, the stability of this structure in the presence of anions and various chemical agents in actual wastewater. Then different immobilization structures, like bead, sheet, sponge, and net, were compared to establish the obtained composition.

Bead

Titration of alginate suspension in Barium Chloride solution produces uniform seeds with the same diameters called beads. The wastewater was treated with 2 to 4% of these beads.

Sheet

A plate or sheet refers to a fixed flat structure that covers a wide area and is used in the industry for purification in pools and bioreactors. Considering the stability of the structure, they should be designed as thinly as possible both in terms of the possibility of food and gas exchanges and economic issues (the amount of alginate).

To manufacture the sheet, two thicknesses of alginate suspension were poured on the bottom of the container. Then Barium Chloride solution was added and kept at room temperature for one day. Two concentrations of selected microalgae were also used in this experiment. In another attempt to prepare the sheet, the solution was poured into hollow molds, and the solidification solution was slowly added. The formed sheet had a

uniform and acceptable diameter.

Sponge

Due to the resistant infrastructure of sponges, slices were prepared and immersed in alginate suspension until they were completely saturated. After washing with Sodium Chloride solution and distilled water, they were placed in wastewater (70 and 100%) and distilled water. After one week, the degree of opening of the structure and color change of the effluent was checked.

Net

In this experiment, pieces of plastic mesh plates were used. Four treatments were considered for two selected algae and a consortium of the two algae together with purified Cyanobacteria at the same ratio. After adding alginate-containing algae, the filters were placed in a $BaCl_2$ solution for 4 hours. Then, it was washed with $NaCl_2$ solution and kept in distilled water for an average of 3 hours until the beginning of the treatment.

Wastewater treatment and analysis

Industrial wastewater was aerated for two days. After straightening with a cleaning cloth, it was divided into 10 L containers and were transferred to the laboratory. The filters were suspended in the effluent with the help of a string for three weeks at 25 ± 2° C and illuminated at 16: 8 h light/dark, and aerated constantly and slowly.

The levels of BOD, COD, PO_4^{3-}, and NO_3^- were analyzed in the laboratory (RehanAzma Iranian). The primary effluent samples were tested immediately after preparation, after two days of aeration (before treatment), and on days 6, 13, and 21 after treatment with screen filters. In addition, the final sample was sent to this laboratory for microbial analysis. ICP analysis of all the elements of the untreated wastewater was also performed in Aria Chemical Laboratory.

Results

Wastewater effluent

The results of the effluent analysis using ICP-MS and ICP-OEC devices are shown in Table 1. Among heavy metals, chromium showed the highest amount of 1.7 mg/L in the wastewater sample.

Among other metals, the amount of sulfur,

Table 1. Analysis of the main elements of the treatment plant effluent

Element	S[1]	Ca[23]	K[4]	Mg[5]	Si[6]	P[7]	Na[8]	B[9]	Sr[10]
Concentration (ppm)	902	295	82	62	26	73	5.1	1.9	1.8
Element	Cr[11]	Li[12]	Cu[13]	Fe[14]	Al[15]	Zn[16]	Ni[17]	Mn[18]	B[19]
Concentration (ppb)	1700	350	260	215	200	90	60	30	20

[1]Sulfur, [2]Calcium[3], Potassium, [4]Magnesium, [5]Silicon, [6]Phosphor, [7]Sodium, [8]Boron, [9]Strontium

[10]Chromium, [11]lithos, [12]Cuprum[13], Ferrum, [14]Aluminum, [15]Zinc, [16]Nickel, [17]Manganese, [18]Barium

Table 2. Analysis of the acidity and EC of the effluent and Cr and BOD/COD

Test	Unit	Value (1397)	Value (1398)
BOD	ppm	5200	3736
COD	ppm	10000	7950
pH	-	8.1	8.3
TSS	ppm	589	-
Cr	ppb	1700	2300
N	ppm	780	-
EC	mho/cm	27250	-
Total Caliform	ppb	-	< 3

calcium, potassium, and magnesium due to the functional role of acids and bases in leather-making processes is considerable.

The results of the output acidity and EC are shown in Table 2. According to the high BOD/COD, re-sampling was done to re-examine BOD/COD, Cr, and total microbial load.

Isolated microalgae

Several microalgae samples grew in the aerated effluent, mainly belonging to the blue-green algae and green algae. The absorbance of four selected species in four dilutions of 10, 30, 50, and 70% at 680 nm is shown in Figure 2. Finally, After eight days of cultivation of the isolated species in different wastewater dilutions, two species with the highest growth rate were chosen for this study. As it is illustrated in Figure 2, all species had better growth in 10 and 30% dilutions. The genomic DNA band in agarose gel electrophoresis, the specific band obtained from PCR for the 16S rRNA gene, and the 18S rRNA gene are presented in Figure 3 a-c, respectively.

Algal cultivation

Cultivation of the two selected species in 100% and 70% effluent dilutions and sterile and non-sterile conditions indicated the stability and survival of willows in species AFC008 compared to species AFC010. After six days, the chemical characteristics of the effluent, especially the pH were between 7.9 and 8.2 and did not change much.

The plate shape of alginate initially led to the formation of heterogeneous sheets. For this reason, other sections of the sheets were exposed to effluent. Due to the high agitation intensity, dissolution started after one day, and free algae were released into the effluent. An aeration system with a lower stirring speed should be used in future studies.

Contaminants reduction

After two days of aeration, COD and BOD values decreased from 2600 and 1300 to 1700 and 760 mg/L, respectively. After that, by adding algae treatments in the form of two separate species and a combined consortium, COD and BOD decreased by about 55%

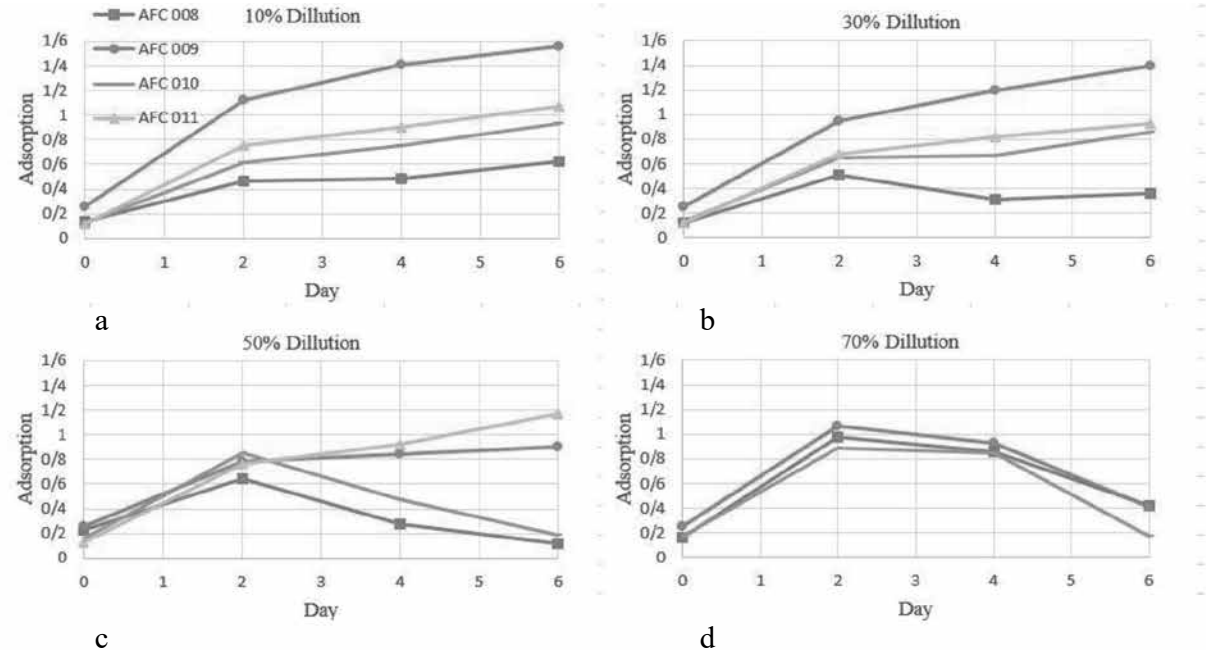


Fig. 2 a, b, c, d. Several samples of microalgae grew in the aerated effluent; the absorbance at 680 nm wavelength for four selected species in different dilutions of 10, 30, 50 and 70%

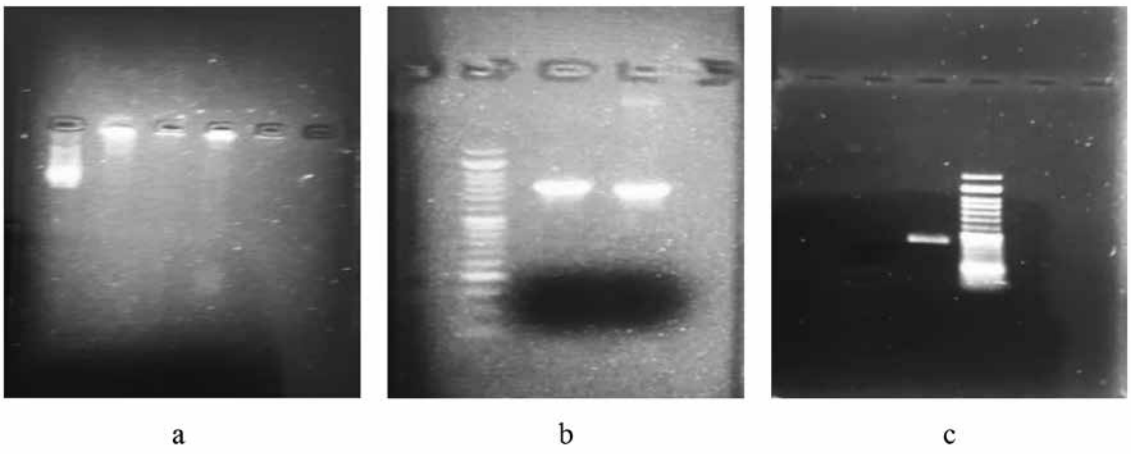


Fig. 3. (a) The bands obtained from genomic DNA electrophoresis (Chlorella sp.), (b) specific band obtained for 16S rRNA gene (*Chlorella sorokiniana*), (c) specific band obtained 18S rRNA gene (*Chlorella* sp.)

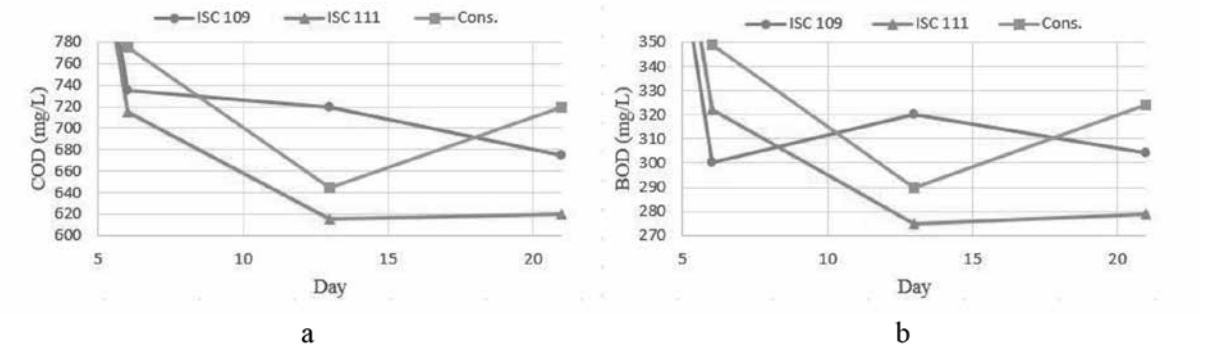
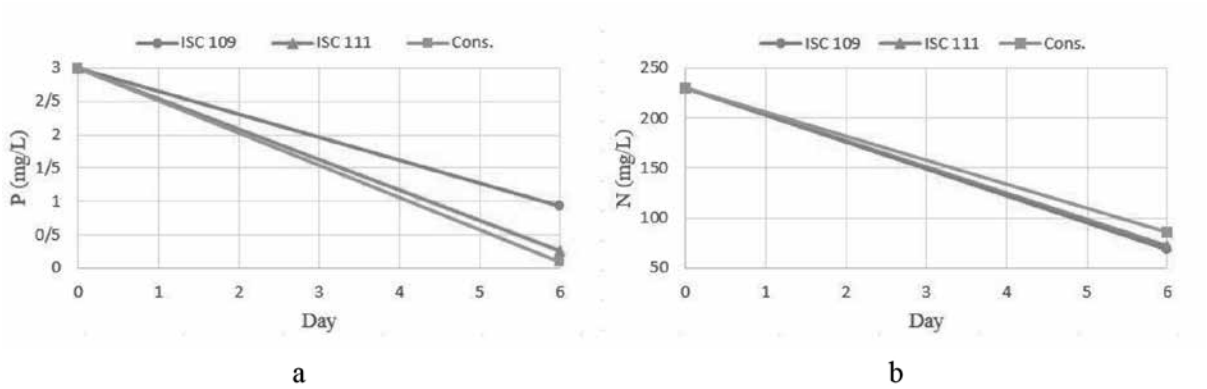


Fig. 4. BOD (a) and COD (b) reduction by algal treatment (initial value: BOD= 760, COD=1700 mg/L)



a b

Fig. 5 a, b. Nitrate and Phosphate reduction by algal treatment. (initial value: NO_3= 230, PO_4=3.00 mg/L)

during the first six days. The other values up to day 21 have changed according to Figure 4. The highest reduction of COD and BOD (about 68%) was achieved by the strain SC 111 on the 13th day.

Due to the relationship between BOD and COD with nitrate and phosphate reduction, and their slow decline after the sixth day, phosphate and nitrate values were measured on the sixth day. However, phosphate and nitrate values increased two days after initial aeration from 2.55 and 190 to 3 and 230 mg/L, respectively. The separation of some sediments, dissolution of phosphates in particles, and release of nitrate reserves in wastewater sediments may be the reasons for this increase. The highest reduction of phosphate and nitrate was achieved by consortium and ISC109, respectively. The reduction values are shown in Figure 5.

According to the report of Rehan Azma laboratory, the amount of total and Fecal coliform in the effluent was lower than the standard release limit. The pH and EC levels in the effluent sample after two days of aeration were 8.1 and 5050 mg, respectively, which did not change significantly during the treatment with algae.

Algae AFC 010 was separated from the filter in the first days and grew abundantly in the effluent. The consortium sample, which contained AFC 010 algae, was released in the wastewater a few days after sample 2. After two weeks, other algae were released into the environment and grew both on the biofilter and in the water environment. Therefore, according to the process of reducing nutrients, the time of one week is optimal.

Discussion

Among more than 1000 algal taxa (240 genera, 725 species) that have treatment potential, the most widely used include *Euglena*, *Oscillatoria*, *Chlamydomonas*, *Scenedesmus*, *Chlorella*, *Nitzschia*, *Navicula*, *Stigeoclonium* (Abdel-Raouf et al., 2012).

The use of native microalgae is suggested in conditions where the climate changes in the region and the possibility of competition is high.

Cultivation of several algal species with special metabolic abilities such as mixotrophy, the ability to bind to specific metals, the tendency to use a specific form of nitrogen compounds and eutrophy improves the total treatment capacity when exposed to fluctuating effluent in flows. For example, the superiority of freshwater (*Botryococcus braunii*, *Chlorella saccharophila*) and saltwater (*Dunaliella tertiolecta*, *Pleurochrysis carterae*) microalgae, without symbiotic bacteria, for carpet factory wastewater treatment has been reported (Sriram and Seenivasan, 2012). Also, the problems of monoculture in the presence of various organic and inorganic compounds, as well as toxic substances and microbial communities are mentioned (Fouilland, 2012). In this study, algal consortium cultivation in immobilized conditions is as effective as single cultivation in reducing wastewater pollutants within one week.

In this research, the appropriate ability to grow and absorb nutrients and some heavy elements by different species of *Chlorella* microalgae are in line with the successful results of other studies in removing or absorbing a wide range of pollutants. Effective biosorbents for the absorption of heavy metals using algal biomass have been produced on an industrial scale, of which ALGASORB and AER are examples. Bioabsorption in brown and green algae has been considered due to the presence of alginate in their cell walls, which have functional groups such as carboxylic acids (Mahmoud and Mohamed, 2017) (guluronic and mannuronic acid) as metal binding sites through electrostatic forces and complexation (Blanes et al., 2011). The results of removing copper, nickel, and cadmium metals from drinking water with the immobilized structure of chlorella in alginate (Petrovič and Simonič, 2016) and the higher tendency of alginate beads for chromium compared to lead and copper (Sriram and Seenivasan, 2012) are examples of these studies.

The mechanism of metal absorption in microalgae includes fast surface and Intracellular absorption and prolonged absorption inside the cytoplasm dependent

Table 2. Main binding groups in brown algae

Binding chemical group	Ligand atom	Biopolymer
Carboxyl	Oxygen	Alginic acid
Thiol	Sulphur	Amino acids
Sulfonate	Sulphur	Sulfate polysaccharides, fucoidan
Amine	Nitrogen	Amino acids, peptidoglycan
Amide	Nitrogen	Amino acids

on intracellular metabolism (Kim, 2011). The carboxyl groups of cell wall polysaccharides play a key role in the absorption of heavy metals by algae or cyanobacteria. Other functional groups like sulfonate and amino have a lesser role in this field (Olguín, 2003). Functional groups in the algal absorption of brown algae are listed in Table 3.

Immobilized culture systems have been introduced as an alternative to suspended cell culture systems to overcome the problems of expensive and time-consuming harvesting methods (Aslan and Kapdan, 2006; He and Xue, 2010; Pires et al., 2013). Natural immobilization is based on the inherent ability of cells to attach to a specific surface. Artificial or active immobilization technique includes adsorption, entrapment in liquid-liquid emulsion, sequester in a semi-permeable membrane, covalent attachment, and entrapment in polymers (He and Xue, 2010). The current methods to stabilize microalgae are cell entrapment in a polymer matrix, usually alginate and carrageenan, and cell adhesion and biofilm formation on a solid surface (Eroglu et al., 2015).

Al-Rub et al. (2004) reported that the immobilization of *Chlorella* algae increased the absorption of Nickel from the environment. Lau et al. (1997) studied the growth of *C. vulgaris* in the carrageenan alginate matrix and showed that it does not decrease. They also demonstrated that the immobilization of the cells causes more chlorophyll production than the free cells. However, some reports have mentioned the higher potential of metal absorption in free algae compared to immobilized algae. Changing the wall structure during immobilization and lack of access to a part of the wall surface caused by gelling materials may be barriers to absorption (Sarada et al., 2006). Some studies have compared the increase in biomass efficiency and pigment and lipid content with the high cost of the immobilization matrix (Christenson and Sims, 2011).

De-Bashan et al. (2004) reported using the *C. vulgaris* fixation method in beads alginate matrix. *C. vulgaris* removed 93% of NH_3-N within eight days in synthetic wastewater treatment (De-Bashan and Bashan, 2010). According to this research, algae immobilization can effectively remove nutrients from wastewater. The nutrients are first absorbed on the surface of the matrix, then penetrate the matrix, and are continuously absorbed by the cells (Tam and Wong, 2000).

In this research, an alginate bed was used to immobilize algae cells that facilitate algal harvesting, and better control the effluent current changes. Based on this, immobilized microalgae in undiluted wastewater increased compared to free forms that could not survive in diluted wastewater by 70%. We also tried to increase economic efficiency by using the minimum amount of alginate. However, in this study, the cyanobacterial forms of *S. aeruginosus* and *L. fragilis* could not show stable immobilization structures, and the alginate structures were lost after a while.

Due to the many reports of the high

potential of alginate and the properties of this algal metabolite, this material was used in this research. The results showed that continuous shaking of alginate, especially in high volumes, could create a uniform suspension. Also, at least one hour of heating in the temperature range of 70-80° C has been confirmed as the main factor of polymerization resistance in other studies (Sarada et al., 2006). Because of using different polymers, the beads created in this research had a diameter of 1 to 4 mm with the external and internal application of chitosan, respectively. Their desirable size is listed between 0.7 and 1.5 mm depending on the size of commercial resins to remove metal ions (Sarada et al., 2006).

The bead's surface is one of the most significant absorption factors. For example, the exit of carbon dioxide gases and the development of more pores in the bead's structure lead to an increased cadmium absorption capacity (McGinn et al., 2012). In this study, it has been tried to create thin and resistant plates to increase access levels. Double ions such as Calcium and Barium play an influential role in the consistency and strength of alginate-based structures. According to some studies, the morphology of Calcium and Barium alginate beads has shown the same appearance and behavior in the environment (Ibáñez and Umetsu, 2002). In this study, the Barium influence on the alginate strength and algae structures was confirmed.

Ibáñez and Umetsu (2002) reported that the stability of alginate-chitosan capsules depends on the amount of chitosan binding to them (Ibáñez and Umetsu, 2002). In the one-stage production of the beads, the alginate solution is headed directly into the chitosan solution. All the chitosan was deployed as a thin layer on the surface. These beads are much weaker than the wills made in two steps in chitosan and calcium chloride solutions. In 2-stage production, the connection is 100 more than 1 stage production (Thumvijit et al., 2013). Chitosan's absorption capacity is reported to be 5 to 6 times compared to chitin. That is mainly due to amino T-distillation groups, hydrophilic properties of hydroxyl factor groups, and the flexible structure of the polymer chain. This study also showed the presence of chitosan in the alginate composition when used as an internal application. Although it was effective in increasing the stability of the immobilization systems, algae biomass was destroyed.

Gradual changes in the environment acidity affect the solubility of ammonium or phosphorus in the medium, and its high levels lead to ammonium deficiency or phosphate deposition. Acidity stimulates between 9 and 11 phosphorus deposits in the form of calcium phosphate (Tam and Wong, 2000). The structure of the beads in the pH range of 5 to 9 is stable. This structure does not show very high resistance at pH above nine and less than three. Alginate beads begin to break at pH 11, and treatment with intense acid solutions resulted in the loss of stabilized biomass (Thumvijit et al., 2013).

The initial concentration of pollutants can

be one of the main factors in the output of work and standards, especially when there is a metal composition in the environment. In one study in the stabilized yeast in alginate, as a result of chromium concentration (VI) from 200 to 1000 mg/l, chromium absorption percentage showed a significant decrease (Mahmoud and Mohamed, 2017). The difference between algae growth at different wastewater concentrations confirms that in this study.

The presence of multiple metals leads to competing in active positions and attracts different absorption of a single element. The ability to interfere with other elements is also different. For example, 1.5 mM of iron reduces the cadmium's uptake by 24%, but the same cadmium concentration causes a 45% reduction in iron absorption by *Sargassum sp*. (Sarada et al., 2006). Alginate-containing alginates almost completely absorb Zinc and nickel solutions, but when the zinc was added to the nickel solution, both absorption was reduced by free and immobilized algae. Competition on the same algae connection stations has led to a reduction in absorption (Mihranyan, 2011). Similar results are observed on zinc and cadmium (Bootsma et al., 2004). The same inhibitory effect was observed in the process of growth and absorption of algae in this study.

Algae have been used either in a single form or in different algal and bacterial consortia to remove nutrients and reduce BOD/ COD. There are several advantages to using microalgae for removing nutrients including nitrogen and phosphorus absorbed by microalgae can be recycled as biofertilizers from microalgae biomass, Production of bioenergy, food, animal feed, and medicines from algal biomass (Aslan and Kapdan, 2006; Renuka et al., 2013).

According to the results of this research, microalgae *Chlorella* sp. among algal species is generally used in basic research for wastewater treatment for its potential for high growth rate, ability to survive in wastewater environment, short reproduction time, and effective removal of nutrients (Pittman et al., 2011; He et al., 2013). It has been reported that *Chlorella vulgaris* can remove 55-88% of nitrogen and 12-100% of phosphorus from municipal wastewater (Ruiz-Marin et al., 2010). The nutrient removal percentages obtained in the research are comparable with similar cases: González et al. (1997) reported 55% phosphorus uptake from industrial, agricultural wastewater of total phosphorus concentration by *C. vulgaris* and *Scenedesmus dimorphus* (González et al., 1997). When *C. vulgaris* is fixed together with *Azospirillum brasilense* for two days, it removes 91% of ammonium (from 3.2 mg/L) (De-Bashan et al., 2002). Liang et al. (2013) reported 78% nitrogen removal (from 20 mg/L) after six days using co-culture of microalgae *C. vulgaris* and bacteria *Bacillus licheniformis* (Liang et al., 2013). The mechanisms related to the removal of carbon, nitrogen, and phosphate using microalgae are discussed by Gonçalves et al. (2017).

Alone, microalgae are not able to remove

nutrients efficiently from wastewater. Growth of Microalgae growth promoting bacteria, starvation, and effluent dilution are different pathways that increase the rate of nutrient removal (Sriram and Seenivasan, 2012). Co-cultivation of activated sludge with algae and higher removal of nutrients (Su et al., 2012), use of plant growth-promoting bacteria alongside algae (De-Bashan and Bashan, 2010), and simultaneous stabilization of *Chlorella vulgaris* species with *Azospirillum brasilense* (McGinn et al., 2012) are all examples of multiple cultures.

The cellular mechanisms of nitrogen removal are as follows. Firstly, the enzymes involved in the nitrogen metabolism of microalgae like as glutamate dehydrogenase and glutamine synthetase increase. Secondly, PGPB can produce growth hormones to enhance microalgae growth.

Starvation also plays a significant role with a synergistic effect on phosphorus absorption from wastewater (De-Bashan and Bashan, 2010). The same integrated cultivation systems have also had efficient removing BOD and COD. Increasing the efficiency of BOD removal in the co-culture system of algae and activated sludge (Vasseur et al., 2012) and removing 80% of COD using the algae-bacteria system (Hoffmann, 1998) are examples of these studies.

The fine performance of AFC 008 and AFC 010 species in reducing BOD/COD like nitrate and phosphate absorbing in less than a week is confirmed with several similar studies. *Chlorella* can treat wood-based pulp industrial wastewater and remove 58% COD, 84% color, and 80% absorbable organic xenobiotics (AOX) (Tarlan et al., 2002). Mixed culture of *Chlorella* vulgaris and removal of 88% of COD from the initial concentration (250 mg/L) in 190 hours (Travieso et al., 1996), removal with the efficiency of 61%, 76.6% and 28% for COD, nitrogen, and phosphorus respectively, by *C. vulgaris* in the treatment of diluted ethanol and citric acid produced in industrial wastewater (Valderrama et al., 2002), reduction of 84% BOD and 89% COD using *C. vulgaris* and *Scenedesmus* sp. in a batch system (Hammouda et al., 1995); reduction of 89% BOD and 88% COD using *C. vulgaris* in 48 hours (Azeez, 2010).

Based on the results of this research, it is possible to reduce 80% of BOD and COD using a microalgae system within a week. Nevertheless, giving more time without effective change in pollutant reduction will cause algae destruction and release.

Acknowledgments

This research was done with the support of Research Institute of Applied Sciences, ACECR, in the molecular identification segment.

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The Algae of Urmia Lake (Northwest Iran): a Brief Review

Fereidun Mohebbi[1]*, Masoud Seidgar[1]

Received: 2022-08-29 Revised and accepted: 2022-09-29

Abstract

This study tries to review the algal assemblage of Urmia Lake in different environmental circumstances. There was an inconsistency about the phytoplankton of Urmia Lake in references. For example, *Enteromorpha intestinalis* a macroscopic green alga was already reported from the lake and permanently vanished in the 1990s. On the other hand, various researchers have reported different algae from different sampling sites. These variations are related to limited and irregular samplings or increased salinity during recent years, which has eliminated some intolerant species. *Dunaliella* salt-tolerant green alga is responsible for more than 90% of primary production in hypersaline environments. This two-flagellate unicellular alga, in Urmia Lake, composed 92.1% and 99.6% of algal population density in high-stand and low-stand periods, respectively. In drought conditions, eight species of algae were observed in Urmia Lake. Chlorophyll as an indicator of primary production was lower in Urmia Lake than in the sister Great Salt Lake. So, it can be categorized as an oligotroph lake from this point of view. This study showed the significance of an algal herbarium on the national or regional scale to record and preserve algae species that may be vanished someday from the ecosystems.

Keywords: *Dunaliella*, Urmia Lake, Phytoplankton, Chlorophyll a, Hypersaline

Background

Urmia Lake hosts diverse bacterial communities, hyperhalophilous phytoplanktons, the macrozooplankton crustacean, and the brine shrimp *Artemia urmiana.* Thus, about its ecological significance, unique biodiversity, and indigenous communities, Urmia Lake has been recognized as a Protected area since 1967 and was designated as a National Park in 1976 as one of 59 biosphere reserves by UNESCO (Eimanifar and Mohebbi, 2007). In 1975, it was also registered in the Ramsar Convention on Wetlands as a wetland of international importance (Djoined, 1970).

The world has been witnessing the violent withdrawal of Urmia Lake, the second-largest hypersaline lake in the world during the last two decades. Despite this rapid shrinking, recording the Urmia Lake organisms, as an extreme environment, either extant or extinct, particularly algae

1- National Artemia Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Urmia, Iran
*Corresponding author email: mohebbi44@gmail.com

as crucial primary producers is significant. There is not an integrated study on the algae of Urmia Lake as an important extreme habitat in the eastern hemisphere. Therefore, this study tries to review the algal communities of the lake in various environmental circumstances.

Introduction

Early investigations on hypersaline lakes' phytoplankton structure were mostly related to that of the Great Salt Lake. Even an algal herbarium was established in the nineteen century (Harvard University, 2018). Perhaps the first systematic study of algae in the Great Salt Lake was conducted in the 1890s by an algae expert and a famous woman, Josephine Tilden, the first female professor at the University of Minnesota (Horsfield, 2016). She started a scientific study in the western United States that involved sampling and isolating algae from extreme environments such as Yellowstone Park and the Great Salt Lake (Tilden, 1898). In addition, she stored samples in the herbariums that are still available.

In Urmia Lake, there was a limited systematic study of algae. However, with algae as the main primary producers and food source for the brine shrimp, *Artemia* has participated in most of the investigations performed on ecology, monitoring, or stock assessment programs on the lake.

Algal flora of Urmia Lake

The food web of hypersaline environments is very simple. In a seemingly simple food web, birds who visit the lake eat brine shrimp and brine flies, and the fly larva and shrimp eat the algae and other microorganisms in the brine. As a hypersaline environment, Urmia Lake complies from this rule. So, algal species is expected to contain lower richness than that of freshwater.

It seems that there is a seasonal fluctuation in phytoplankton population structure in hypersaline lakes of temperate regions of the world. For example, the results of studies in Urmia Lake indicated that the highest algal species richness was observed in spring and early summer and the lowest in the late summer, autumn and winter (Mohebbi et al., 2020; Esmaeili Dahesht et al., 2010). Besides, the same results was reported from Mighan Lake and the Great Salt Lake (Hafezieh et al., 2020; Barret and Belovsky, 2020).

There is an inconsistency about phytoplankton of Urmia Lake in references (Eimanifar and Mohebbi, 2007). For example, *Enteromorpha intestinalis* macroscopic green alga which was reported from the lake by Plattner (1960) and Saberi (1977) has completely disappeared from the lake in late 1990s. During the years when the salinity was lower, this alga was so abundant that the whole lake looked like a plant soup. On the other hands, various researchers have reported different algae from different sampling sites. Riahi et al. (1994) have reported six species of Cyanophyta; *Anacystis* sp., *Chroococcus* sp., *Lyngbya* sp., *Oscillatoria* sp., *Synechococcus* sp., *Anabaena* sp. Four species of green algae; *Dunaliella* sp., *Monostroma* sp.,

Ankistrodesmus sp., *Pandorina* sp., two species of Bacillariophyta; *Amphora* sp., *Navicula* sp. from the lake. Mohebbi et al., (2006) have reported three species of Cyanophyta; *Anabaena* sp., *Oscillatoria* sp., *Synechococcus* sp., two species of green alga; *Dunaliella* sp., *Ankistrodesmus* sp., eleven species of Bacillariophyta; *Navicula* sp., *Nitzschia* sp., *Cyclotella* sp., *Symbella* sp., *Synedra ulna*., *Pinnullaria* sp., *Diatoma* sp., *Amphiprora* sp., *Surirella* sp., *Cymatopleura* sp., *Gyrosigma* sp. during whole year (June 2005- May 2006). Samplings was done from the eight stations. These variations can be related to limited and irregular samplings or increased salinity during the recent years which has eliminated some intolerant species (Table 1).

The main difference between two studies was laid in the presence of *Monostroma* sp. a macroalga belonging to Chlorophyta in study performed by Riahi et al., (1994) and absence of the macroalga in the later study (Mohebbi et al. 2006). *Monostroma* sp. is green macroalga that has many similarities to *U. intestinalis,* both have nutritional values and belonging to Ulvophyceae class. Therefore, it may be the same species. This was confirmed when we considered the sampling time of the *Monostroma* sp. by Riahi et al. (1994), in which either the Urmia Lake salinity was about 165 g/l and met the time when this alga was present in the lake, *U. intestinalis* was disappeared from the lake in the late 1990s. The salinity of Urmia Lake in the second study was about 310 g/l and its level was decreased compared to the first study period. When comparing two above mentioned studies from Bacillariophyta species number point of view, we see that there is few species in the first than the second study (2 vs. 11 species). This high number difference has two reasons; the first study was based on one sampling performance probably in warm season and the second study had monthly samplings during one year round, and Bacillariophyta species mostly were observed in cold season, the first study samplings was probably performed in warm season.

Ulva intestinalis

It was the only macroscopic alga in Urmia Lake that completely vanished from the lake in the late 1990s. Although there are no studies on this macroalgae's history, morphology, taxonomy, and ecology and could not determine it initially, there are few resources on its pigments and applications in food industries and medicine. Gunther (1899) first described a macroscopic lichen from Urmia lake that was probably *U. intestinalis*. Plattner (1960) a lecturer at Tabriz University, described *E. intestinalis* of Urmia Lake as dark green strips several meters long. *E. intestinalis (U. intestinalis)* originally described by Linnaeus (1753), is a cosmopolitan species and considered a euryhaline species (Reed and Russell, 1979; Edwards et al., 1987; Kamer and Fong, 2000). Therefore, its occurrence in the hypersaline

Table 1. Phytoplankton species taxonomy of Urmia Lake

phylum	class	order	familly	genus	species	Reference
		Bacillariales	Bacillariaceae	*Nitzschia*	sp.	(5) ،(3) ،(1)
		Symbellales	Symbellaceae	*Symbella*	*Prostrata*	(5) ،(3) ،(2)
			Naviculaceae	*Amphiprora*	sp.	(3) ،(1)
				Navicula	sp.	(5) ،(3) ،(1)
Gyrista		Naviculales	Plurosigmaceae	*Gyrosigma*	sp.	(3)
			Pinnulariaceae	*Pinnularia*	sp.	(3)
	Bacillariophyceae	Surirellales	Surirellaceae	*Cymatopleura*	sp.	(3)
				Surirella	sp.	(3)
		Thalassiophysales	Catenulaceae	*Amphora*	sp.	(1)
		Thalassiosirales	Stephanodiscaceae	*Cyclotella*	sp.	(5) ،(2) ،(1)
		Achnanthales	Cocconeidaceae	*Cocconeis*	*pediculus*	(5)
		Frgilariales	Fragilariaceae	*Diatoma*	sp.	(3)
				Synedra	*ulna*	(5) ،(3)
		Sphaeropleales	Selenastrceae	*Ankistrodesmus*	sp.	(3) ،(1)
	Chlorophyceae	Volvocales	Dunaliallaceae	*Dunaliella*	sp.	(5) ،(3) ،(2) ،(1)
Chlorophyta			Volvocaceae	*Pandorina*	sp.	(1)
	Ulvophyceae	Ulotricales	Monostromaceae	*Monostroma*	sp.	(1)
		Ulvales	Ulvaceae	*Enteromorpha*	intestinalis	(4)
		Oscillatoriales	Oscillatoriaceae	*Lyngbya*	sp.	(1)
				Oscillatoria	sp.	(5) ،(3) ،(1)
Cyanobacteria	Cyanophyceae	Notocales	Nostocaceae	*Anabaena*	sp.	(3) ،(1)
		Chroococcales	Chroococcaceae	*Synechococcus*	sp.	(3) ،(1)
				Chroococcus	sp.	(1)
				Anacystis	sp.	(1)

(1) Riahi et al., 1994; (2) Shoa hasani et al., 1996; (3) Mohebbi et al., 2006; (4) Plattner (1960); (5) Mohebbi (2020)

Urmia Lake was not so surprising.

There is solid evidence that *Ulva* and *Enteromorpha* are not distinct evolutionary entities and should not be recognized as separate genera (Hayden et al. 2003). As *Ulva* is the oldest name, *Enteromorpha* is here reduced to synonym.

Salinity may be one of the parameters that impacted the disappearance of this alga from Urmia Lake in the 1990s. We do not know if natural or anthropogenic influences might have vanished this alga from Urmia Lake. *E. intestinalis* produces considerable amounts of β-carotene, a red-colored carotenoid with antioxidant properties (Djoined, 1970).

Even though *Dunaliella* is responsible for more than 90% of primary production in hypersaline lakes so, the whole ecosystem depended on its carbon fixation a few studies have focused on this alga's ecology. However, as *Dunaliella* capability in beta-caroten production, its physiological and biochemical characteristics have been studied extensively. A few studies have been

performed on the seasonal fluctuations of *Dunaliella* in the Great Salt Lake (Stephens and Gillespie, 1976; Post, 1977).

In 1838, *Dunaliella* was observed in salt evaporation ponds on the Mediterranean coast of Montpellier (southern France) by Michel Felix Donal for the first time (Dunal, 1838). In the 116 years since *Dunaliella* was formally identified, it has emerged as a traditional model microorganism for studying the algal adaptation to salinity. The observed organism was called *Hematococcus salinus* and *Protococcus* by Donal. The discovery of this alga was carried out following research regarding the cause of the red color of salt ponds by the French Academy of Sciences in Paris.

Dunaliella population structure in salt production pools has been poorly studied. So, a detailed investigation goes back to the 1920s in salt ponds on the Atlantic coasts of France (Labbé, 1921). In high salinities or during dilution asexual cells with a thick wall called an aplanospore may be formed. These cells may be contributed to facilitating severe conditions as they were observed in the cold season at the bottom of the lakes.

Labbé mistakenly proposed that large red and small green cells of *Dunaliella* were two stages of development of the same creature. However, we know that these cells may be present simultaneously red cells are produced in the warm and dry seasons.

When the Romanian botanist Emanoil C. Teodoresco (1949-1866) described the characteristics of the new halophile unicellular algal genus *Dunaliella*, the alga had been identified from salt lakes and ponds around the Mediterranean and Black Seas (1, 3). He named the alga *Dunaliella* in honor of Felix Donal, who had described these red unicellular algae from salt ponds in 1838 (Dunal, 1838; Teodoresco, 1905). The first definition of *Dunaliella* by Hamburger (1905) refers to aplanospores (dormant cysts) and palmeloid cell formation in the alga life cycle.

The green alga *Dunaliella* was first reported from the Great Salt Lake by Daines in 1910 (Daines, 1910). *Dunaliella salina* in high salinities (332 g/l) of the northern part of The Great Salt Lake was reported in the range of 1000-10000 cells/ml in the 1970s (Post, 1977).

High concentrations of Mg and Ca and the reduced water level have turned the dead lake into an unsuitable environment for *Dunaliella*, which was present in the past.

However, *Dunaliella* is an aquatic alga, and a few species were observed in terrestrial ecosystems. Buchheim et al. (2010) reported a *Dunaliella* population from saline soils of Oklahoma deserts based on the 18S rRNA findings. More surprisingly, a new strain of *Dunaliella* was observed in the spider's web of a cave in Chile. Molecular analysis based

on the 18S rRNA and chloroplastic genes, this alga was related to *D. atacamensis* (Azúa-Bustos et al., 2010).

Dunaliella is a key primary producer in hypersaline ecosystems which consumer components depend on it. Glycerol is an important compound that is produced inside the *Dunaliella* cells and accumulated to regulate osmotic pressure in high salinities.

Molecular analyses of an algal bloom in Urmia Lake were provided by the study of the 18srDNA gene and sequencing of the ITS region (Manaffar et al., 2015). The results of this study revealed the main reason for bloom-forming was the dominant species of the *Dunaliella tertiolecta* alga in the concentration of 1.2×106 cells/ml.

Hejazi et al., (2016) applied the intron-sizing method to compare the 18S rDNA fingerprint between *Dunaliella* isolates of Urmia Lake. According to the results, the genetic variation in the *Dunaliella* genus depends on different areas of Urmia Lake. PCR with species-specific primers in the population confirmed the existence of at least four species of *D. tertiolecta*, *D. parva*, *D. salina*, and *D. bardawil*, and some types of isolates which were similar to *Dunaliella* sp. ABRIINW-M1/2. In summary, this study indicated that this method was appropriate to differentiate between some species of *Dunaliella* and rapidly identify them.

Dunaliella spp. was observed in the whole body of Urmia Lake. In a study performed in 2005-2006, 14 phytoplankton species were reported from the lake among which ten species of Diatoms, two species of Chlorophyta (green algae), and two species of Cyanophyta (blue-green algae) (Esmaili Dahesht et al., 2010; Mohebbi et al., 2006). During 2005-2006 the *Dunaliella* density reached 92.1% of the total phytoplankton density of the lake. In this study, *Navicula* sp. and *Synedra ulna* contained the highest density with 23963 and 12283 cells/l, respectively.

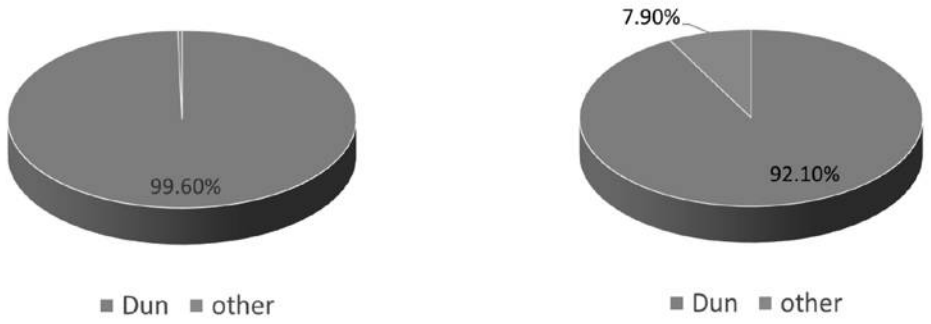
Phytoplankton communities of Urmia Lake in shrinkage

Urmia Lake is the largest natural habitat of a unique brine shrimp species, *A. urmiana* which is the main food source for migratory birds (Ahmadi et al., 2011; Asem et al., 2014, 2016; Aghakouchak et al., 2015). The lake water level has dropped dramatically which can be compared to the fate of the Aral Sea in Eurasia, where its size was decreased to 10% of its original size due to diverting the two main rivers for agricultural irrigation (Aghakuchak et al., 2015). The main morphology characteristics of Urmia Lake in the Low-stand and high-stand periods were indicated in Table 2.

According to the latest study (Mohebbi, 2020), eight species of algae were observed in Urmia Lake: one species of Chlorophyta ; *Dunaliella* sp., one species of Cyanophyta; *Oscillatoria*

Table 2. Morphometric characteristics of Urmia Lake before and after shrinkage

Characteristics	High-stand period	Low-stand period	References
Watershed area (km^2)	51876	51876	Marden et al., 2014; Delju et al., 2012
Surface area (km^2)	4800-6100	1730-2200	http://agrw.ir
Mean depth (m)	4.5	0.5	Sima and Tajrishi, 2013
Volume (m^3)	26×10^9	1.5×10^9	http://agrw.ir
Stream flow (m^3/yr)	972×10^6	250×10^6	Nourani et al., 2018
Mean evaporation (mm/yr)	1156	1629	Heidari et al., 2010
Salinity (ppt)	150-180	>300	Eimanifar and Mohebbi, 2007
pH	6-8	6-8	Alipour, 2006



a b

Fig. 1. Phytoplankton population structure in Urmia lake during 2018-2020 (a) and 2005-2006 (b)

sp., and six species of Bacillariophyta; *Cocconeis pediculus*, *Nitzschia* sp., *Synedra ulna*, *Symbella prostrate*, *Cyclotella* sp.; N*avicula* sp. (Table 3).

The phytoplankton population density of Urmia Lake during the shrinkage period is indicated in Table 3.

A crucial aspect of the shrinkage that should be pointed out is that *Dunaliella* sp. shared higher density of total algae (99.6% vs. 0.4%), while in periods the water level was higher, *Dunaliella* had about 92.1% of total algal density in Urmia Lake (Esmaeili et al., 2010) (Fig. 1). Actually, this is because *Dunaliella* is tolerant against harsh environmental conditions so can replace with low tolerant species.

Algal flora of Urmia Lake vs. Mighan Lake

When the relationship between water salinity and algal composition in Urmia Lake and Mighan Lake (Arak province, center of Iran) was compared, we got fascinating results (Mohebbi, 2020; Hafezieh et al., 2020), which are indicated in Table 4.

Table 3. phytoplankton population structure and density of Urmia Lake in water withdrawal condition (2018-2020)

Density (%)	Density (No/l)	species	Spr 2018	Sum 2018	Fall 2018	Win 2018	Spr 2019	Sum 2019	Fall 2019	Win 2019	Spr 2020
99.6	1923025	*D. salina* (Dunal) Teodoresco	+	+	+	+	+	+	+	+	+
	2172	*Oscillatoria* sp.	+	+	-	-	+	-	-	-	-
	2573	*Navicula* sp.	+	+	+	+	+	-	-	+	+
	271	*Cocconies pediculus* Her.	-	+	-	+	-	-	-	-	-
	1801	*Nitzschia* sp.	-	-	+	+	+	-	-	-	+
0.4	219	*Synedra ulna* (Nitzsch) Ehrenberg	-	+	+	-	-	-	-	-	-
	62	*Symbella prostrate* (Berkeley) Cleve	-	-	+	-	-	-	-	-	-
	287	*Cyclotella* sp.	-	-	+	-	-	-	-	-	+

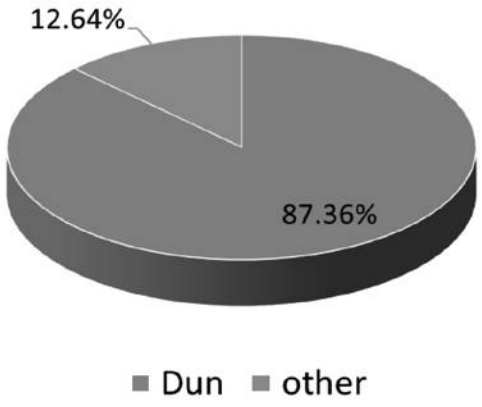


Fig. 2. Phytoplankton population of Mighan lake (Mar-Dec 2019)

Comparing the two lakes, salinity as a key factor plays a crucial role in phytoplankton population structure in both ecosystems comes true. However, it should be noted that above a definite salinity level, the species diversity (the number of species) declined sharply in hypersaline waters.

As shown in Table 4 as salinity rises *Dunaliella* density and dominance increase, and other algae density decreases accordingly and vice versa. However, salinity is not the sole factor influencing the algal composition in hypersaline lakes and researchers believe that integrated conditions and factors are involved in shaping the algal composition in these environments.

In Mighan Lake, the highest and the lowest *D. salina* density was observed in June and October 2018, respectively (Hafezieh et al., 2020). Dunaliella density, water level, and nutritional ions were simultaneously increased in the spring, which was agreed with observations made in other hypersaline lakes such as Urmia Lake (Mohebbi et al., 2020). However, the role of brine shrimp as a filter-feeding organism in phytoplankton population fluctuations should not be ignored. In this regard, Brine shrimp plays a

Table 4. Comparison of phytoplankton structure in Urmia lake (2018-2019) and Mighan lake (Mar-Dec 2019)

parameter	Urmia Lake	Mighan Lake (Arak)
Salinity (g/l)	220-350	30-120
Phytoplankton species number	8	10
Dunaliella abundance (%)	99.6	87.36
Other microalgae (%)	0.4	12.64

crucial role in the definite season of the year.

Primary production in Urmia Lake

In 1995-96 chlorophyll concentration in Urmia Lake was measured as 0.5-0.8 µg/l and seldom exceeded 1 µg/l (Van Stappen et al., 2001). This was lower than that of the Great Salt Lake (0.5-3.5 µg/l) reported by Gliwicz et al. (1995). Therefore, Urmia Lake may be categorized as an oligotroph from a phytoplankton production point of view. Quantitative analysis of chlorophyll and phytoplankton has indicated that primary production in Urmia Lake is lower than that of the Great Salt Lake (Van Stappen et al., 2001), and *Dunaliella* was the dominant species in both lakes.

All types of chlorophylls showed similar fluctuations, indicating that continuous blooms of different species did not occur. Therefore, there were uniform phytoplankton species in the whole lake. Chlorophyll concentrations in December-January rose to a maximum which then during late winter a gradual decline occurred during the spring and early summer. Minimum chlorophyll values were observed in June 1996 (Van Stappen et al., 2001).

Minimum chlorophyll values were observed in June 1996. This indicates that phytoplankton density reached an approximate bloom. Phytoplankton was eliminated by Artemia with a steady process that occupy the lake from late winter onward. Algal bloom did not occur like in other hypersaline lakes such as Mono Lake and the Great Salt Lake.

There was an evident variation in the primary production of various stations. Generally, chlorophyll values were higher near shore and river deltas than offshore sampling sites, which can be related to nutrients loading into the lake in these regions. However, recorded values for delta regions of Urmia Lake were well below the values (60 µg/l) reported for similar regions of the Great Salt Lake (Van Stappen et al., 2002).

Seasonal fluctuations of the lake phytoplankton were matched with chlorophyll variations: maximum Dunaliella density was observed in winter and minimum

in May - September. A similar pattern was observed for *Cyclotella* sp. and *Nitzschia* sp. but with a lower density. According to this study, we can declare that the Urmia Lake microalgae composition is similar to that of the GSL, which mainly included *Dunaliella* with a fraction of Diatoms.

In 1995-96 *Dunaliella* was the dominant microalga of the lake, with a density more than 10 times higher than the sum of other microalgae. In August and December 2005, *Dunaliella* density in Urmia Lake reached 480×103 and 80×103 cells/L, respectively.

Red coloration of Urmia Lake

During warm and dry seasons, as the water level drops below 1372 m (Fig. 1), the density of halophilic bacteria in the family of Halobacteriaceae rises to more than 108 cells/ ml in the lake. These are prokaryotic organisms and have pigments distributed evenly on the cell membranes. So, absorb light more efficiently than carotenoids of eukaryotic *Dunaliella* as a dominant alga (Mohebbi et al. 2011). Although β-carotene derived from *Dunaliella* is the most abundant carotenoid pigment in hypersaline water, its dense packaging within granules inside the cell's chloroplast greatly decreases its participation in the overall light absorbance in the water. As for Urmia Lake (e.g. Arash Rad, 2000; Asgarani et al., 2006; Bahari et al., 2009), the presence of Halobacteriaceae family has been reported by Post (1977) and Baxter et al. (2005) in the North Arm of Great Salt Lake, Utah, which shows similar color changes in high salinities. Urmia Lake's color changes seasonally, and as drought and agricultural water overuse persist in the region, a ruddy hue may become a more common sight.

The Urmia Lake vegetation

The Urmia Lake ecosystem includes two particular plant communities; the vegetation of salt marshes around the lake and the island's vegetation which may be observed in Kaboodan, Spir, Ashk, and Arezoo islands. The island's vegetation is composed mostly of steppe and sub-shrub, and shrubs and is estimated around 4810 ha (Baghaee, 2009). Salt marshes around the lake and islands included 177 and 174 plant species, respectively. The largest plant families were Asteraceae and Poaceae with 33 and 21 species, respectively. The largest plant genus was *Euphorbia* and *Trigonella foenum-graecum*. Among the Urmia lake national park plant families, 12 were endemic to Iran. However, about 50% of plant species belonged to the Irano-Touranian phytogeographic region (Alipour, 2009).

According to the latest study (Ghorbanalizadeh, 2022), 24 plant communities were distinguished regarding floristic and ecological characteristics in Kaboodan Island, the largest island of Urmia Lake. They were categorized into

three groups: First of all, Plant communities formed on the dried bed of Urmia Lake *Salicornietum iranicae*, *Halimocnemis rarifolia* comm. Frankenia hirsuta comm., *Halocnemetum strobilacei*. Secondly, Plant communities developed on the island's former shorelines: *Atraphaxis spinosa*, *Ephedra major* subsp. *procera* comm., *Alhagi maurorum* comm. Lastly, Plant communities found on hills adjacent to shorelines, steppe areas, and valleys of the island: *Caroxylon dendroides* comm., *Halothamnus glaucus* comm., *Artemisia spicigera, Ephedra major* subsp. *procera* comm., *Rhamnus pallasii*, *Artemisia spicigera* comm., *Pistacia atlantica* subsp. *mutica-Rhamnus pallasii* comm., *Peganum harmala* comm. For example, Kaboodan Island has an unspoiled ecosystem and no anthropogenic activities over decades which is home to various plant species and vegetation types.

It was found that Urmia Lake shrinkage had influenced the vegetation of salt lands around it. During 21 years, from 1995 to 2015 about 67.3% of plant communities dried up, other communities have replaced, and only 32.7% could retain their life power (Ahmadi et al., 2018). On the other hand, the crown cover has decreased by a minimum of 10.4% and a maximum of 73.3% for Halantietum rarifolii and Iridetum musulmanicae communities, respectively.

Discussion

Twenty-four algal species were reported from Urmia Lake by various authors. However, there is an inconsistency about the phytoplankton of Urmia Lake in references. These variations can be related to limited and irregular samplings or increased salinity during the last two decades which has eliminated some intolerant species. *Ulva intestinalis* macroscopic alga was reported from the lake in the 1960s, and 1970s even until the early 1990s completely disappeared from the lake in the late 1990s.

Urmia Lake phytoplankton is comprised mainly of *Dunaliella* spp. as a dominant genus. So, in high stand periods, it included more than 90% of phytoplankton abundance. In low stand periods of the Urmia Lake water level, *Dunaliella* spp. composed even higher components of phytoplankton assemblages (98-99%). *Dunaliella* species are euryhaline and can tolerate high salinities and are so the dominant species in hypersaline environments worldwide.

Primary production in Urmia Lake is lower than its sister The Great Salt Lake located in Utah in the United State of America (0.5-0.8 µg/l vs. 0.5-3.5 µg/l). Therefore, Urmia Lake was considered oligotrophic from Chlorophyll a values point of view. Urmia Lake water withdrawal has influenced the vegetation of saline marshes and areas around the lake. According to studies, During 21 years, from 1995 to 2015 about 67.3% of plant communities have dried up and other communities have replaced, and only 32.7% could retain their life power.

Despite the rapid shrinkage of Urmia Lake during the last two decades, its island vegetation has not been impacted by this disaster and could maintain its plant species diversity. This was improved by the fact that human accessibility was limited to the lake's islands due following reasons. The Environment Agency protected the Urmia Lake and its islands as a protected region and had difficulty accessing the islands human for cattle grazing.

Regarding the anthropogenic and climate changes that rapidly impact the natural ecosystems, particularly crucial ecosystems such as Urmia Lake, this study showed the significance of an algal herbarium on the national or regional scale to record and preserve specimens of algae that may be vanished someday from the ecosystems.

Acknowledgments

The authors would like to thank Iranian Fisheries Research Institute and National *Artemia* Research Center for their support to do this study.

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