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Evaluation of in *vitro* Antioxidant Activities and Antibacterial Potentials of Two Brown Algae Extracts; *Lyengaria stellata and Padina boergesenii* Inhabiting the Persian Gulf, Iran

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Abstract

In-vitro antioxidant activities of semi-purified methanolic extract *of Lyengaria stellata* and *Padina boergesenii* were evaluated using DPPH radical-scavenging activity and reducing power. Total phenolic contents (TPC) and total flavonoid contents (TFC) were also investigated. The acetone fraction of *L. stellata* contained the highest value (126.04±8.65 mg GAE/g) of TPC, and the highest concentration of TFC (94±0.13 and 82±0.09 mg GAE/g) was obtained in ethanol fractions of *Lyengaria stellata* and *Padina boergesenii,* respectively. The highest DPPH scavenging activity (88.5%) was observed in the acetone fraction of *L. stellata* (1 mg/ml). The most significant reducing power was observed in the acetone fractions of both seaweeds at 100 mg/ml concentration (1.130±0.040 and 0.839±0.010, respectively). Ethanol and acetone fractions of *L. stellata* showed the greatest antibacterial activity (29.6±0.5 and 28.0±0.7 mm, respectively) against *Staphylococcus aureus*. The minimum value of MIC was observed in acetone fractions of both seaweeds against *S. aureus* (50.7 and 50.8 µg/ml), while *Salmonella enterica* showed the highest resistance to seaweeds extracts according to the– highest MIC values (>200 mg/ml) and the smallest IZ diameters. The extract of both algae showed considerable antioxidant activity according to DPPH radical scavenging activity and reducing power assays.

Keywords: Antibacterial activity, Antioxidant Potential, Persian Gulf, Seaweeds, *Lyengaria stellate, Padina boergesenii*

Introduction

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin (EQ), and propyl gallate (PG) have been used to prevent oxidative damage of aquaculture products in food industries. However, these chemical substances cause undesirable side effects such as liver damage and carcinogenesis in human consumers (Valko et al., 2007, Munir et al., 2013). Due to increased public interest in consuming healthy and natural foods, natural antioxidants are highly preferable

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in the food and pharmaceutical industry as a safe replacement for synthetic compounds (Balboa et al., 2013).

Macroalgae have been considered as an appropriate choice of bioactive compounds for natural antioxidants due to their high diversity, wide distribution, and easy accessibility (Munir et al., 2013). In the past decade, many researchers have investigated and improved the antioxidant activity of several types of macroalgae (Balboa et al., 2013). It is documented in the literature that macroalgae extracts, either in the form of crude extracts or chemically processed products, have potent inhibitory effects on the destructive processes of oxidative stress in biological systems (Zengin et al., 2011, Saeed et al., 2012). Among the three phyla of macroalgae, brown algae present higher antioxidant potential than red and green algae. A detailed summary of brown algal extracts' antioxidant activity has been made, confirming the potential of brown algae as a source of novel secondary metabolites as antioxidants (Balboa et al., 2013). *Alaria esulentaobia* (Zubia et al., 2009), *Fucus vesiculosus* (Wang et al., 2012, Wang et al., 2010), *Laminaria japonica* (Wang et al., 2010); *Ascophyllum nodosum* (Audibert et al., 2010, O'sullivan et al., 2011), *Callerpa lentillifera* (Matanjun et al., 2008), *Colpemnia sinuosa* (Kelman et al., 2012), *Eklonia radiate* (Kindleysides et al., 2012), *Sargassum horneri* and *Sargassum hyxtrix* (Luo et al., 2010); *Sargassum muticum* (González-López et al., 2012) and *Undaria pinnatifida* (Plaza et al., 2008) are some of the brown algae with antioxidant properties that have been recently investigated.

Although a variety of brown macroalgae species are widely distributed in the coastal area of the Persian Gulf in the south of Iran, limited attention has been given to explore their antioxidant potential. Macroalgae in the Persian Gulf live in complex habitats and even under extreme environmental conditions such as severe sunlight, high temperature, and salinity. It is expected that macroalgal exposure to such stresses could lead to the formation of free radicals and other oxidizing agents. Surprisingly, no oxidative damages have been reported for these algae until now. This fact suggests that the algae may synthesize antioxidant metabolites to protect their DNA materials and cellular membranes, which can be used as an alternative to synthetic antioxidants in the food and pharmaceutical industries (Huang and Wang, 2004).

In this study, we aimed to determine the antioxidant activity of two endemic brown algae, *Padina boergesenii* and *Lyengaria stellata* from the coast of the Qeshm Island, Persian Gulf, Iran. *P. boergesenii* and *L. stellata* are marine species, widespread in tropical seas, extending to subtropical and warm-temperate regions, 1 m depth (Ansari et al., 2014; Edgar et al., 2010; Gharamjik and Rouhani Ghadikolaee, 2010). The results of this work may serve as information enrichment for the antioxidant properties of macroalgae in the Persian Gulf, and a way to find new sources of natural antioxidants, dietary supplements and antibiotics. These natural antioxidants from seaweeds would

be an alternative safe source for antioxidant food and medicine ingredients.

Materials and methods

Sample collection and preparation

Endemic brown macroalgae, *Padina boergesenii* (Allender and Kraft, 1983), and *Lyengaria stellata* (Børgesen, 1939) (Fig. 1), were collected from coastal and shallow water areas of the north coast of Persian Gulf, Iran (Fig. 2); between October 2018

a b

Fig. 1. Seaweeds were collected from the Persian gulf coastal area, (a) *Padina boergesenii*, and (b) *Lyengaria stellate*

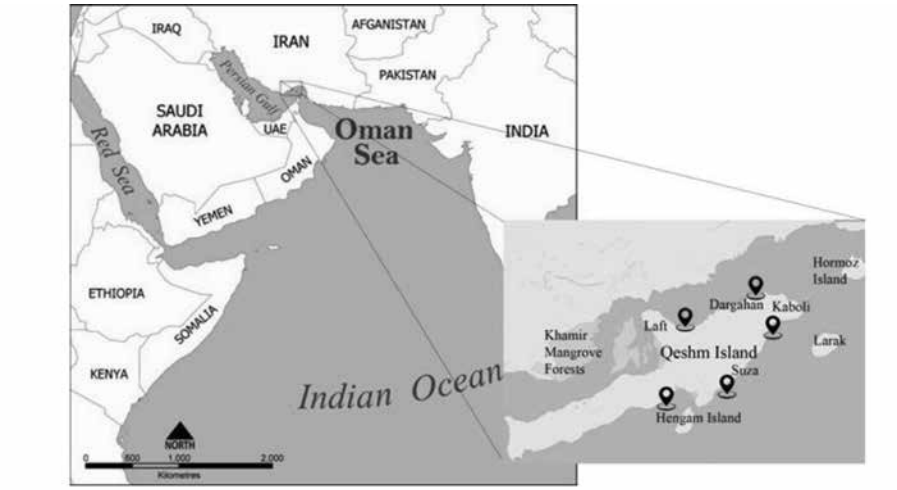

Fig. 2. Map of Persian Gulf indicating the collecting coastal area and the sampling stations in Qeshm island (Iran)

and February 2019.

Macro algae were collected in plastic jam containing seawater and transferred to the laboratory immediately, where the samples were washed thoroughly with deionized water to remove salts, sand, and epiphytes. Some samples were kept for further identification and confirmation of the species, which was carried out using available valid keys (Jones, 1986; Gharamjik and Rouhani Ghadikolaee, 2010; Richmond, 2011). The algae were dried in the shade until they reached a constant weight and then, dry samples were ground into powder and stored at -20° C.

Preparation of the crude extract

An accurately weighed 10 g sample was extracted with 50 ml of different solvents (sample: solvent=1:5). Methanol, Ethanol, and Acetone (80%) were used to compare their efficiency of extraction using three different methods of percolation, maceration (48 h), and ultrasound extraction (400W, 25 KHz, Scients-SB5200D, China) at 40° C in the water bath for 30 min (Zhou et al., 2018). Extracts were filtered and concentrated by rotary evaporation (DUAB-RE100pro, China). Each dry extract was reweighed, and the yield (extraction efficiency) was calculated and stored at -16° C (Zubia et al., 2007; Abdul Qadir et al., 2017).

Purification of the crude extract

The methanolic extract was semi purified to determine TPC and TFC as well as antioxidant potential. Semi-purified of crude extracts of *L. stellata* and *P. boergesenii* was performed using C18 cartridges by gradient elution with different organic solvents (Methanol, Ethanol, and Acetone). As a result, three semi-pure fractions were obtained, and the organic solvent of the fractions was removed using a rotary rotating evaporator at 40° C (Bergé et al., 2002; Wu et al., 2003). The fractions were stored at -20° C until analysis. To reach the favorite final concentration (1 mg/ml), 10% DMSO was used for all three fractions dilution.

Assessment of antioxidant activity

DPPH radical scavenging activity

DPPH (1, 1 Diphenyl 2- Picryl Hydrazyl) radical scavenging activity was determined using the earlier reported method with slight modification (Wu et al., 2003). Briefly, different concentrations (1, 0.5, 0.25, 0.1, 0.05 mg/ml) of sample solutions (1.5 ml) were mixed with an equal volume of 0.1 mM DPPH (in 95% methanol). The mixture was stirred vigorously for 30 min at room temperature. The absorbance was measured by spectrophotometer at 517 nm. DPPH is a stable free radical in powder form with red color, which turns yellow when scavenged. The lower the absorbance, the higher the DPPH scavenging activity. The scavenging effect is expressed as below:

[(blank absorbance–sample absorbance)/ blank absorbance] ×100%

The DPPH test was performed in triplicate, and ascorbic acid (10 mg/ml) was used as a reference.

Reducing power assay

The reducing power activity of algal extracts was determined by Fe^{3+} reduction, using protocols described by Lee and Kim (Lee and Kim, 2015), with slight modifications. 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$ were mixed with algae extracts. The resulting mixture was incubated for 20 min at 50° C. After adding 2.5 ml of 10% trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. Then, 2.5 ml of supernatant was diluted with an equal volume of deionized water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm by spectrophotometer (Philes-T6, China).

Determination of total phenolic contents (TPC)

To determine the total phenolic contents of algal crude extracts, the Folin-Ciocalteu

reagent was used as reported earlier (Zubia et al., 2007). Briefly, 0.5 mL of Folin-Ciocalteu reagent and 2.9 ml distilled water were added to 0.1 ml algae extract in test tubes. After 10 min at room temperature, 1.5 ml of 20% Na_2CO_3 was added into the test tubes and kept in the dark place at room temperature for one hour. Absorbance was measured at 725 nm. The total content of phenolic compounds was expressed in GAE mg/g dry weight based on a standard curve of gallic acid. Calibration curve was y=0.0584x+0.0077 (R 2 = 0.9926). Every sample was used in triplicate.

Total flavonoid content (TFC)

The total flavonoid content of the crude extract was determined by the aluminum chloride colorimetric method (Chang et al., 2002). Briefly, 500 µl of samples (1 mg/ml) was mixed with 1.5 ml methanol, and 3 ml distilled water, made up of 0.3 ml 10% AlCl3 solution, 0.3 ml potassium acetate (1 M). After 20 min incubation at room temperature, the mixture was allowed to stand for 15 min, and then, the absorbance of samples was measured at 415 nm using a spectrophotometer (PHILES-T6, China). The total flavonoid content was expressed as milligrams of quercetin equivalents (mg QE/g).

In vitro evaluation of antimicrobial activity

Microbial strains and growth conditions

Antimicrobial activity of semi-purified algae extracts was tested in both Gram-positive (*Staphylococcus aureus* (ATCC 29213), *Bacillus circulance* (ATCC 4516)) and Gram-negative bacteria (Escherichia coli (ATCC 25922), *Salmonella enterica* (ATCC 9150) obtained from the Microbial culture collection, Pasteur Institute, Tehran, Iran. Bacterial strains were cultured overnight at 37 °C in Mueller–Hinton agar (MHA).

Disk diffusion method

For the determination of the antimicrobial activity, the disk diffusion method was used as described by Kozekidou et al. (2008) with some modifications. Briefly, a suspension of the tested bacteria (10^8 CFU/ml) was spread on Sterile BHI Agar media plates. Sterile 6 mm filter paper discs were impregnated with 15 µl of extracts and placed on the inoculated plates and incubated at 37° C for 24 h. Negative controls were prepared using 15 µl of 30% DMSO.

Ampicillin (10 µg/disc) was used as positive growth control. Antimicrobial activity was determined by measuring the inhibition zone diameter around the discs using a metric scale and evaluation of the sensitivity of bacteria to extracts was interpreted by the inhibition zone diameter (IZD), according to the criteria mentioned by Sreepian et al. (2019); when IZD of the extract≤ 6 mm (No activity), 6 mm≤ diameter≤ 12 mm (weak activity), 12 mm≤ diameter≤ 20 mm (moderate activity) and≥20 mm (strong activity); and all the tests were conducted in triplicate.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was estimated by using the both micro dilution method (Akremi et al., 2017, Salah et al., 2019).

All tests were carried out in Mueller Hinton broth (MHB). Bacterial strains cultures were incubated at 37° C for 24 h in MHA, and the tested strains were suspended in MHB to reach a final density of 5.01 CFU/ml. All the extract's fractions were dissolved in 10% DMSO and geometric dilutions ranging from 12.5 to 500 µg/ml of each sample, were prepared in a 96-well micro-titer plate, including one growth control (MHB+10% DMSO). Plates were incubated at 37° C for 24 h. The MIC was determined as the lowest concentration of the extract at which the tested bacterial strains do not demonstrate any visible growth after incubation.

Statistical analysis

All experiments were performed in triplicates and expressed as mean± Standard deviation. A one-way ANOVA and LSD post hoc test were used to analyze the difference between groups. Data were analyzed statistically by using Statistix 10.0 and Origin 9.0 (Origin lab Corporation, Hampton, USA) software, and values $P \leq 0.05$ were considered significant.

Results

Crude extraction yield

Among the different methods and solvents, methanol and ethanol through the percolation method exhibited higher yields of 16.32% and 14.09%, respectively, followed by ethanol and methanol extractions of the maceration method (13.41% and 11.97%, respectively). The least efficiency belonged to the ultra-sonication method with all three solvents (Fig. 3).

Total phenolic (TPC) and flavonoids (TFC) contents

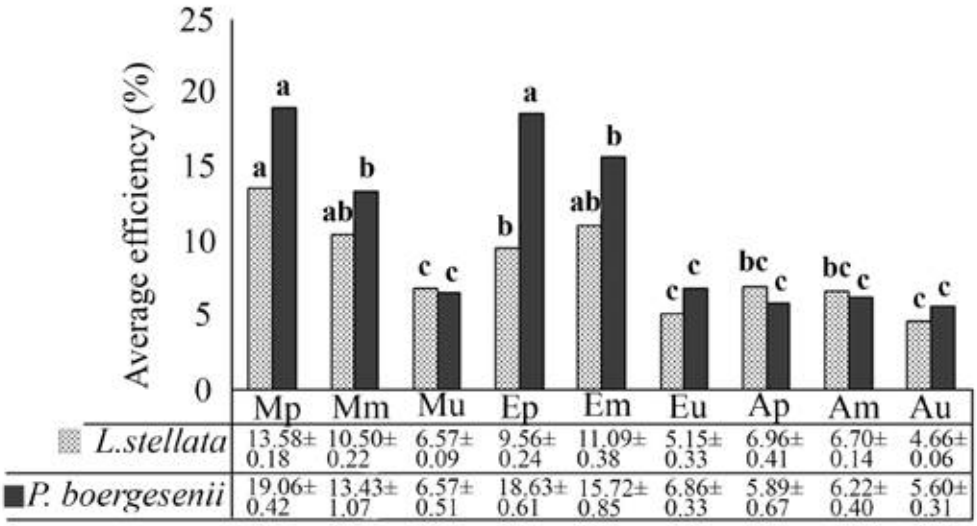

Fig. 3. Average efficiency (%) of extraction from two brown algae of Persian Gulf, Iran Expressed as percentage of extract to dry seaweed matter of triplicate. Solvent M: methanol; E: ethanol; A: acetone; method m: maceration; p: percolation; u: ultrasonication. Different letters (a, b, and c) represent significant differences

The measured values of total phenolic content (TPC) of *L. stellata* and *P. boergesenii* crude and semi-purified fractions are presented in Table 1.

Table 1 shows that a higher amount of TPC was obtained with acetone fraction for both *L. stellata* and *P. boergesenii* (126.04±8.65 and 110.22±6.14 mg GAE/g, respectively), which were significantly greater than ethanol and methanolic fractions as well as crude extract.

It was also found that the TFC was the highest (94±0.13) in the ethanolic fraction of *L. stellata* followed by the ethanol fraction of *P. boergesenii* (82±0.09). The remarkable point is that although acetone fractions showed the highest TPC content in both seaweeds, on the contrary, the lowest amount of flavonoids was measured in this fraction. Moreover, *L. stellata* crude extract and all fractions were observed to have a significantly higher amount of TPC and TFC as compared to *P. boergesenii.* The highest concentration of flavonoids in both *L. stellata* and *P. boergesenii* (94±0.13 and 82.00±0.09 mgQE/g) were measured in ethanolic fractions and the lowest one in acetone fractions (Table 1).

DPPH radical scavenging capacity

DPPH is a stable free radical with red color which turns yellow when scavenged by polyphenols and anthocyanins through the donation of hydrogen, forming the reduced DPPH-H (Luo et al., 2010). In this study, algae crude and semi-purified extracts with antioxidant activity to various degrees are shown in Figure 4.

As depicted in Figure 4, *L. stellata* extracts (1 mg/ml) had higher DPPH radical scavenging activity than the equivalent concentration of

Table1. Total phenol (TPC) and flavonoid (TFC) contents of *L. stellata* and *P. borgoensii* crude extract and its semi-purified fractions

Extract	seaweed	TPC[BC] (mg GAE/g)	TFC[DC] (mg QE/g)
Crude extract	*L. stellata*	66.36±5.86^{b}	73.01±0.08^{bc}
	P. boergesenii	50.60±1.69^{a}	65.20±0.07^{b}
Ethanol fraction	*L. stellata*	88.21±4.22^{c}	94.14±0.13^{d}
	P. boergesenii	61.73±2.56^{ab}	82.08±0.09^{c}
Methanol fraction	*L. stellata*	56.24±3.04^{a}	78.22±0.06^{bc}
	P. boergesenii	40.45±1.51^{a}	60.00±0.05^{b}
Acetone fraction	*L. stellata*	126.04±8.65^{d}	38.35±0.04^{a}
	P. boergesenii	110.22±6.14^{d}	26.18±0.06^{a}

[C] Mean of 3 determination ± SD. Mean within each seaweed for each parameter with different letter (a to d) differ significantly ($P \leq 0.05$)

[B] TPC = Total Phenolics Content, expressed as milligram GAE per g dry seaweed matter

[D] TFC = Total Flavonoid Content, expressed as milligram QE per g dry seaweed matter

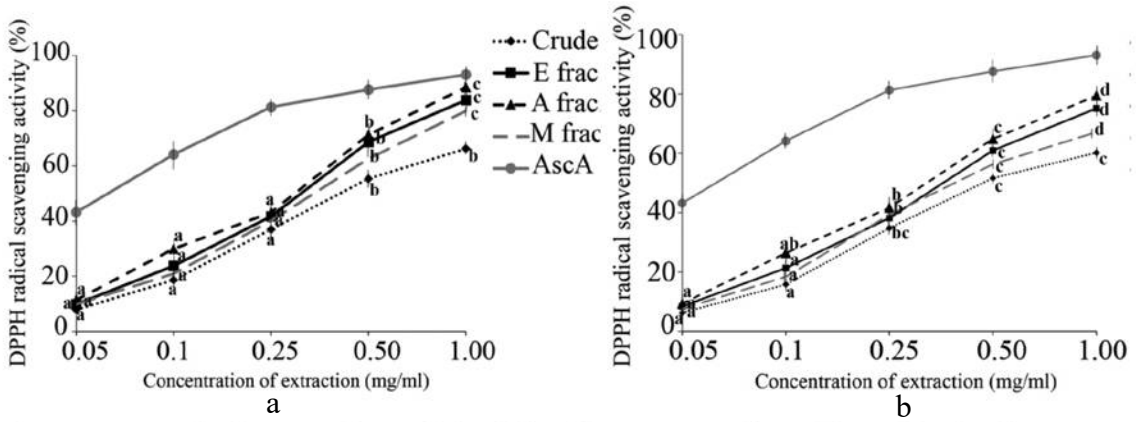

Fig 4. DPPH radical scavenging activity (%) of *Lyengaria stellata* (A) and *Padina boergesenii* (B) crude extract and its semi-purified fractions, from the Persian gulf, Iran (n = 3). AscA: Ascorbic acid. Values are the mean ± standard deviation of triplicate. Different letters (a to d) show a difference significantly ($P \leq 0.05$)

P. boergesenii extract. The highest DPPH scavenging activity (88.5%) was observed in the acetone fraction of *L. stellata* (1 mg/ml), followed by an ethanol fraction of this algae (83.83%) at the same concentration (1 mg/ml). Acetone and ethanol fractions of *P. boergesenii* at the concentration of 1 mg/ml also revealed the highest DPPH scavenging activity (79.46% and 75.18%) among other samples. The scavenging activity of crude extracts of both algae was significantly lower than acetone and ethanol fractions at 0.5 and 1 mg/ml concentrations. However, no significant difference was observed between crude extracts and semi-purified fractions at concentrations lower than 0.5 mg/ml/ ($P \leq 0.05$).

Reducing power

The reducing power of the extracts of two macroalgae, expressed in OD_{700} values, was analyzed by determining the color changes of the extract solution from yellow to various degrees of green and blue. The highest reducing power was observed in the acetone fractions of both *L. stellata* and *P. boergesenii* at 100 mg/ml concentration (1.130±0.040 and 0.839±0.010, respectively), followed by ethanol fractions of both seaweeds (0.828±0.020 and 0.794±0.070). It must be noted that the reducing power of the acetone fraction of *L. stellata* was greater than that of ascorbic acid, which was more evident in concentrations of 50 and 100 mg/ml. The lowest reducing power was observed in crude fractions of both seaweeds, which was more noticeable at a concentration of 50 mg/ml. In general, according to the results, the reducing power of *L. stellata* extracts was higher than that obtained for *P. borgoensii* extracts in all concentrations. Moreover, the reducing power of crude extracts and all fractions of both tested seaweeds showed a dose-dependent activity. In a concentration above 50 mg/ml, a sharp rising of curves is obvious (Fig. 5).

Antibacterial activities

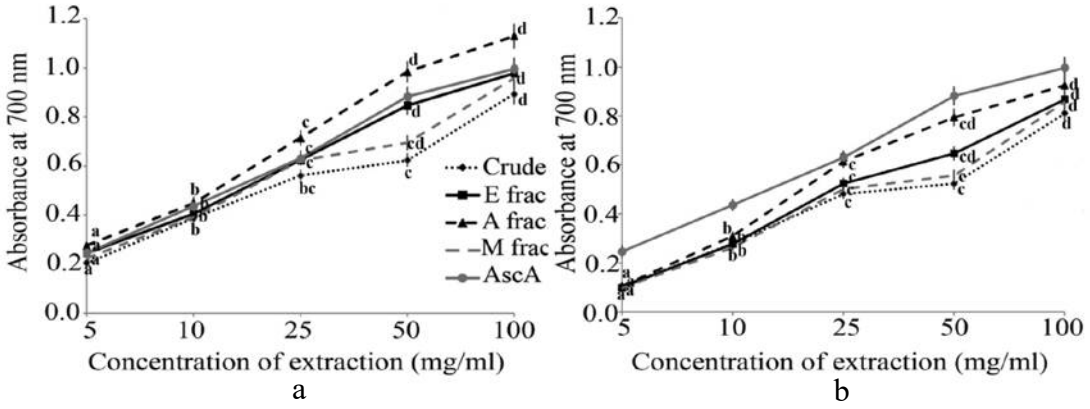

Fig. 5. Reducing power of extracts of *Lyengaria stellata* (A) and *Padina boergesenii* (B) crude extract and its semi-purified fractions from the Persian gulf, Iran (n = 3). AscA: Ascorbic acid. Values are the mean ± standard deviation of triplicate. Different letters (a to d) are showing a difference significantly ($P \leq 0.05$).

The Agar disk diffusion method was carried out to test the antibacterial activities of crude extracts and different fractions from two species of marine seaweeds, and the results are presented in Table 2. The greatest IZ (inhibition zone) diameters were observed in ethanolic and acetone fractions of *L. stellata* (29.6±0.5 and 28.0±0.7 mm, respectively) against *S. aureus*, and the lowest activity was recorded for crude extract (8.6±0.1) followed by ethanolic fraction extract (9.0±0.6) of *P. borgoensii* against *S. enterica* (Table 3).

According to the results from two tested seaweeds, ethanolic and acetone fractions showed maximum activity (according to IZ diameters) against all tested bacteria in comparison to crude extracts. There was no significant difference between the Inhibition activity of acetone and ethanolic fractions of both seaweeds against Gram-positive tested bacteria (*S. aureus* and *B. circulance*).

Discussion

The different methods of extraction of brown algae samples and the assays used for the analysis make it difficult to compare our results with others reported previously. The comparative extraction results showed a higher yield of total extracts for *Padina boergesenii* and *Lyengaria stellata* than other brown algae such as *Eisenia bicyclis* (9.91%, 1.80%), *Kjellmaniella crassifolia* (7.25%, 1.87%) as reported by Airanthi et al., 2011. It is also seen that the yields for methanol/chloroform-based extraction of *Sargassum fusiforme* and *S. hornery* were 5.83% and 5.42%, respectively (Luo et al., 2010), which might be due to different extraction methods or different species. Our results (in Fig. 3) are in agreement with the study performed by Airanthi et al. (2011) on *Alaria crassifolia* (14.61% extraction yield). Furthermore, significant differences were observed between the yield of methanol percolation and methanol maceration extracts,

Table 2. Overview of the phenolic content of some brown algae

Brown algae species	Solvent	Total phenolic contents (TPC) (mg GAE/gr)	Total flavonoid contents(TFC) (mg QE/g)	Reference
Sargassum siliquastrum	Methanol crude extract	51.00		
	Ethyl acetate	64.10		
Dictyota asiatica		13.71±1.20		
Padina arborescens		21.28±0.13	Not measured	(Lim et al., 2002)
Eklonia cava	Methanol (70%)	168.38±4.12		
Colpomenia sinusa		7.06±0.25		
Sargassum thunbergii		19.51±0.42		
Colpomenia crispus	Methanol	62.33±1.04	Not measured	(Cox et al., 2010)
	Ethanol	61.00±2.82		
Padina palmata	Methanol (60%)	42.83±3.26		
	Ethanol (60%)	30.00±0.00		
Padina sp.	Ethanol (70%) + ultrasonication	124.65±0.78	20.74±0.49	(Cox et al., 2010, Dang et al., 2018a)
Sargassum linearifolium		47.06±0.05	13.93±0.41	
Phyllosporea cemosa		67.78±1.01	9.89±0.41	
Dictyopteris membranacea	Methanol/Dichloromethan(1/1)	24.00±0.07	65.00±0.03	(Akremi et al., 2017)
Gracilaria gracilis	Ethylacetate	35.53±1.47	66.48±1.87	(Ebrahimzadeh et al., 2018)
	Mathanol	29.39±2.01	26.47±1.203	
Padina antillarum	Methanol(50%)	24.30	Not measured	(Generalić Mekinić et al., 2019)
Padina pavonica	Methanol	10.55		
Cystoseria crinita		261.53		
Fucus saratus	Methanol(80%)	80.70		

Table 3. Comparing antimicrobial activity measured by agar disk diffusion assay (Inhibition zone diameter or IZ), and Minimum inhibitory concentration (MIC) of the crude extract and its semi-purified fractions from *Lyengaria stellata* and *Padina borgoensii*

	Microbial strains	Ampicillin	seaweed	Inhibition zone (mm)±SD			
				Crude ext.	Ethanol frac.	Methanolic frac.	Acetone frac.
Gram +	*S. aureus*	30.0±0.4	*L. stellata*	20.5±0.4[a]	29.6±0.5[b]	19.5±0.4[a]	28.0±0.7[b]
			P.	18.0±0.0[a]	23.5±0.4[a]	16.6±0.4[a]	23.0±0.4[a]
	B. circulance	18.3±0.7	*L. stellata*	18.7±0.4[a]	18.2±0.6[a]	NA	19.6±0.6[a]
			P. boergesenii	14.6±0.8[a]	14.6±0.6[a]	NA	16.4±0.1[a]
Gram -	*E. coli*	20.4±0.6	*L. stellata*	14.5±0.8[a]	16.0±0.4[a]	NA	19.8±0.4[a]
			P. boergesenii	10.7±0.6[a]	12.5±0.4[a]	NA	16.6±0.3[a]
	S. enterica	17.0±0.6	*L. stellata*	10.6±0.4[a]	11.4±0.4[a]	NA	15.5±0.0[a]
			P. boergesenii	8.6±0.1[a]	9.0±0.6[a]	NA	11.4±0.1[a]
				MIC (µg/ml)			
Gram +	*S. aureus*		*L. stellata*	51.3±1.0[a]	65.8±2.4[a]	100.2±4.4[b]	50.8±1.7[a]
			P. boergesenii	62.2±2.4[a]	52.7±3.1[a]	125.4±1.0[b]	50.7±1.2[a]
	B. circulance		*L. stellata*	65.3±1.8[a]	63.2±2.0[a]	NA	65.3±2.0[a]
			P. boergesenii	75.4±0.9[a]	75.4±1.1[a]	NA	75.8±2.3[a]
Gram -	*E. coli*		*L. stellata*	100.0±0.0[b]	75.3±0.8[a]	NA	75.6±0.6[a]
			P. boergesenii	123.1±4.2[a]	100.4±2.0[a]	NA	100.5±3.5[a]
	S. enterica		*L. stellata*	151.3±2.0[a]	125.2±4.1[a]	NA	125.4±0.6[a]
			P. boergesenii	256.1±3.8[b]	255.4±3.7[b]	NA	206.3±3.1[a]

Values are the mean ± standard deviation of triplicate. Different letters (a and b) are showing the different significantly ($P \leq 0.05$)

while no significant difference was observed between methanolic and ethanol extracts. This suggests that the methods employed might significantly influence the extraction yield and the solvent type. Therefore, the solvents and procedures should be carefully selected for maximum yield.

Many reports exist about the antioxidant activity of polyphenols such as phlorotannins and fucoxanthin of algae extracts (Chandini et al., 2008, Wang et al., 2012, Sathya et al., 2017, Fernando et al., 2016). Considering the difference in structures and molecular weights of phenolic compounds of brown algae, they are highly similar in chemical properties (Lee and Kim, 2015), and it is presumed that phenolic compounds can act as a chemical shield against harmful UV irradiation and grazers (Swanson and Druehl, 2002). It needs to be noted that in many previous seaweed studies, the amount of TPC in the methanolic extract was higher than those assayed in ethanolic or aqueous extracts (Rastian et al., 2007, Airanthi et al.,

2011, Dang et al., 2018a, Neto et al., 2018). We presume that high temperature and long-term extraction may lead to different results in our case. There is a strong relationship between TPC and extraction methods (Tierney et al., 2013, Machu et al., 2015, Dang et al., 2018b). On the other hand, Lee et al. (2015) observed a comparatively higher phenolic content of 168.34 mg GAE/g in the methanolic extract of *Eklonia cava.*

Comparing our results with other reports mentioned in Table 2 also can prove that, total phenolic contents of *L. stellata* and *P. boergesenii* were higher than many other brown algae such as *Padina arborescens*, *Sargassum thunbergii*, *Dictyota asiatica*, and *Colpomenia sinusa*.

It's also known that flavonoids are responsible for a vast variety of biological activities (Cox et al., 2010, Dang et al., 2018a). According to the previous studies (Ganesan et al., 2008, Lee and Kim, 2015, Fellous et al., 2018, El-Sheekh et al., 2020), there was a wide range of TFC from different brown algae species and the highest evaluated amount was 66.48±1.87 mg QE/g in *Gracilaria gracilis;* while the highest value of TFC (94.00±0.13 mg QE/g) from ethanol fraction of *L. stellata* was measured in the present study was 40% more than that. It's known that each extract's TPC and TFC contents strongly depend on the extraction solvent and its polarity (Akremi et al., 2017), as well as the algae species. In our case, in addition to these factors, semi-purification of the extract also played the main role in the enhancement of TFC from the extract compared to other reports about crude extracts. Our results were supported by previous studies by Akremi et al., 2017 and Tierney et al., 2013, who mentioned that purification would increase the polyphenols and flavonoid contents of extracts.

Comparison of total phenolic and flavonoids contents of the extracts for these two brown algae, with the reports of other researchers on some other brown algae extracts, confirms that *L. stellata* and *P. boergesenii* had relatively higher contents of TPC and TFC (Table 2) and could be used as a natural source for bioactive compounds.

DPPH radical-scavenging capacity has been widely used to screen antioxidants from plants and algae (Luo et al., 2010, Ganesan et al., 2008, Lee and Kim, 2015). It is an easy, rapid, and convenient method (Nickavar et al., 2007). It was used in this research to evaluate the antioxidants and radical scavenging capacity of *L. stellata* and *P. boergesenii.* According to our results, *L. stellata* extracts showed higher DPPH radical scavenging activity as than *P. boergesenii* extract (Fig. 4). The scavenging activity of the crude extract of *P. boergesenii* was significantly lower than other extracts ($P \leq 0.05$).

Many studies have been done to determine the antioxidant capacity of seaweeds. Lee and Kim (2015) assayed the antioxidant activity of the methanolic (70%) extracts of more than 50 brown algae species of Korea based on the DPPH free radical-scavenging activity. They reported an activity range between 101.93±1.49 (*Callophyllis crispata*)

and 5.12±0.32 (*Sargassum macrocarpum*). According to the results of this research (Fig. 4), *L. stellata* acetone fraction showed relatively high radical scavenging activity among brown seaweeds. Dang et al. (2017) investigated six brown algae for their antioxidant activity and reported that *Hormosira banksii* and *Sargassum vestitum* showed DPPH levels almost near to ascorbic acid (positive control) at all concentrations, which is in agreement with our results of *L. stellata* (1mg/ml) (Fig. 4). However, Zhang et al. (2007), reported that the antioxidant activity of brown seaweeds was much lower than positive controls of ascorbic acid. The results of the present study also suggest that DPPH scavenging activities of the algae extracts were dose-dependent, which is in agreement with previous studies in the literature (Luo et al., 2010, Farasat et al., 2013, Liu et al., 2017).

Both seaweeds extracts showed reducing power almost at the same degrees (Fig. 5), and it can be expressed in descending order as acetone> ethanol> methanolic fraction> crude extract. Interestingly, the acetone fraction extract of *L. stellata* presented higher reducing power than the positive control (AscA). Figure 4 reveals that the reducing power of all extracts showed a dose-dependent response at 700 nm. Similar results have been reported in the literature (Ganesan et al., 2008, He et al., 2016, Xu et al., 2018).

The reducing properties are generally associated with reluctant, which exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (He et al., 2016). Comparing TPC and reducing the power of these two seaweeds, demonstrates that higher TPC of extracts leads to higher reducing power. Our results are thus in agreement with Luo et al. (2010), He et al. (2016), and Akremi et al. (2017), who reported a relation between TPC and the reducing power of seaweed extracts.

Crude extract and all prepared fractions showed high inhibition activity against *S. aureus*, and altogether tested gram-negative bacteria, were less negatively affected by the extracts compared to gram-positives.

All the extracts revealed antimicrobial activity but only methanolic fractions, as shown in Table 2. Methanolic fractions of both seaweeds had inhibition activity only against *S. aureus* and did not show any effect against other bacteria (*B*. *circulance*, *E. coli*, and, *S. enterica*).

The analysis of variance revealed that the effect of MIC was significantly different for gram-positive and gram-negative tested bacteria. At the same time there was no significant difference between the two seaweed extracts ($P \leq 0.05$). The minimum value of MIC was observed in acetone fractions of both seaweeds against *S. aureus* (50.7 and 50.8 µg/ml), while, *S. enterica* showed the highest resistance to seaweeds extracts according to the highest MIC values (> 200 mg/ml) and the smallest IZ diameters (Table 3).

The results also indicated that gram-negative tested bacteria were more resistant to the seaweed extracts than gram-positive ones;

which may be due to their cell wall structure (Eliuz et al., 2019, El-Sheekh et al., 2020, Maadane et al., 2021). The outer membrane of gram-negative bacteria is covered by a slim layer, which in turn hides the antigens of the cell wall and does not allow the penetration of antibiotics into the cells (Vergalli et al., 2020). Generally, acetone fractions with the highest phenolic and flavonoid contents showed the most significant antimicrobial activity. On the contrary, the methanolic fraction showed a lack of antimicrobial effect but only against *S. aureus* despite the relatively high TPC and TFC content levels. This result can be explained by the synergy outlined by Freeman et al. (2010) and Parker et al., (2010). The synergic effect is caused by changes in concentrations of the compounds and their proportions in different fractions (García et al., 2015). So, it can be concluded that antimicrobial solid activity depends not only on the high content of antimicrobial components such as polyphenols and flavonoids but also on the synergy between all components of each extract (Akremi et al., 2017).

Concerning our findings on *Lyengaria stellata* and *Padina borgoensii,* the antimicrobial activity of crude extract can be improved by purification to semi-purified acetone, and ethanol fractions, and our results were supported by previous findings that purification of extracts leads to increasing the antimicrobial potency (García et al., 2015, Parker et al., 2010).

The results of the present study indicated that the percolation method with methanol and ethanol solvent produced the most outstanding extraction efficiency from brown seaweeds *L. stellate* and *P. borgoensii*. Acetone fractions of both analyzed seaweed were the richest fractions of phenolic, while the ethanol fractions showed the highest amounts of flavonoids. *L. stellate* and *P. borgoensii* were found to be potent sources of natural antioxidants and showed good radical scavenging activity and reducing power. Nowadays, the world's human community faces a significant challenge regarding the antimicrobial resistance of bacteria against existing antibiotics. These seaweed extracts also showed good antibacterial activities against pathogenic bacteria, which suggests them as potent source of alternative antibiotics. So identification and characterization of bioactive compounds from natural sources are highly regarded; as a source of producing a new class of therapeutics that might be used as new antibiotics.

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References

Abdul Qadir M, Shahzadi SK, Bashir A, Munir A, Shahzad S. (2017). Evaluation of phenolic compounds and antioxidant and antimicrobial activities of some common herbs. International Journal of Analytical Chemistry. 6 p. DOI: 10.1155/2017/3475738.

Airanthi, MWA, Hosokawa M, Miyashita K.

(2011). Comparative antioxidant activity of edible Japanese brown seaweed. Journal of Food Science. 76 (1): C104-C111. Doi: 10.1111/j.1750-3841.2010. 01915.x.

Akremi N, Cappoen D, Anthonissen R, Verschaeve L, Bouraoui A. (2017). Phytochemical and in vitro antimicrobial and genotoxic activity in the brown algae *Dictyopteris membranacea*. South African Journal of Botany. 108: 308-314. DOI: 10.1016/j.sajb.2016.08.009.

Ansari Z, Seyfabadi J, Owfi F, Rahimi M, Allee R. (2014). Ecological classification of southern intertidal zones of Qeshm island, based on CMECS model. Iranian Journal of Fisheries Science. 13 (1): 1-19.

Audibert L, Fauchon M, Blanc N, Hauchard D, Ar Gall E. (2010). Phenolic compounds in the brown seaweed *Ascophyllum nodosum*: distribution and radical-scavenging activities. Phytochemical Analysis. 21 (5): 399-405. DOI: 10.1002/pca.1210.

Balboa EM, Conde E, Moure A, Falqué E, Domínguez H. (2013). In vitro antioxidant properties of crude extracts and compounds from brown algae. Food Chemistry. 138 (2-3): 1764-1785. DOI: 10.1016/j. foodchem.2012.11.026.

Bergé JP, Debiton E, Dumay J, Durand P, Barthomeuf C. (2002). In vitro anti-inflammatory and anti-proliferative activity of sulfolipids from the red alga *Porphyridium cruentum*. Journal of Agricultural and Food Chemistry. 50 (21): 6227-6232. DOI:10.1021/jf020290y.

Chandini SK, Ganesan P, Bhaskar N. (2008). In vitro antioxidant activities of three selected brown seaweeds of India. Food Chemistry. 107 (2): 707-713. DOI:10.1016/j. foodchem.2007.08.081.

Cox S, Abu-Ghannam N, Gupta S. (2010). An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. International Food Research Journal. 17 (1): 205-220.

Dang TT, Bowyer MC, Van Altena IA, Scarlett CJ. (2018a). Comparison of chemical profile and antioxidant properties of the brown algae. International Journal of Food Science and Technology. 53 (1): 174-181. DOI: 10.1111/ijfs.13571.

Dang TT, Bowyer MC, Van Altena IA, Scarlett CJ. (2018b). Optimum conditions of microwave-assisted extraction for phenolic compounds and antioxidant capacity of the brown alga *Sargassum vestitum*. Separation Science and Technology. 53 (11): 1711-1723. DOI:10.1080/01496395.2017.1414845.

Ebrahimzadeh MA, Khalili M, Dehpour AA. (2018). Antioxidant activity of ethyl acetate and methanolic extracts of two marine algae, *Nannochloropsis oculata* and *Gracilaria gracilis*-an in vitro assay. Brazilian Journal of Pharmaceutical Sciences. 54 (01). Doi: 10.1590/s2175-97902018000117280.

Edgar GJ, Davey A, Kelly G, Mawbey RB, Parsons K. (2010). Biogeographical and ecological context for managing threats to coral and rocky reef communities in the Lord Howe Island Marine Park, south-western Pacific. Aquatic Conservation: Marine and Freshwater Ecosystems. 20 (4): 378-396. DOI: 10.1002/aqc.1075.

El-Sheekh MM, Mousa ASH, Farghl AA. (2020).

Antibacterial efficacy and phytochemical characterization of some marine brown algal extracts from the red sea, Egypt. Romanian Biotechnological Letters. 25 (1): 1160-1169. DOI:10.25083/rbl/25.1/1160.1169.

Eliuz E, Börekçi NS, Deniz A. (2019). The antimicrobial activity of Enteromorpha sp. methanolic extract and gelatin film solution against on some pathogens. Marine Science and Technology Bulletin. 8 (2): 58-63. DOI: 10.33714/masteb.640614.

Farasat M, Khavari-Nejad RA, Nabavi SMB, Namjooyan F. (2013). Antioxidant properties of some filamentous green algae (*Chaetomorpha Genus*). Brazilian Archives of Biology and Technology. 56 (6): 921-927. DOI: 10.1590/S1516-89132013000600005.

Fellous S, Abd el Halim Boussaa MY, Benzaoui RD, Amar Y. (2018). Optimization of extraction conditions of total flavonoid content from *cystoseira. amentacea* var. stricta using response surface methodology. Journal of Applied Environmental and Biological Sciences. 8 (2): 46-52.

Fernando IS, Kim M, Son KT, Jeong Y, Jeon YJ. (2016). Antioxidant activity of marine algal polyphenolic compounds: a mechanistic approach. Journal of Medicinal Food. 19 (7): 615-628. DOI: 10.1089/jmf.2016.3706.

Ganesan P, Kumar CS, Bhaskar N. (2008). Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. Bioresource Technology. 99 (8): 2717-2723. DOI: 10.1016/j.biortech.2007.07.005.

García BF, Torres A, Macías FA. (2015). Synergy and other interactions between polymethoxy flavones from citrus byproducts. Molecules. 20 (11): 20079-20106. DOI: 10.3390/molecules201119677.

Generalić Mekinić I, Skroza D, Šimat V, Hamed I, Čagalj M, Popović Perković Z. (2019). Phenolic content of brown algae (Pheophyceae) species: extraction, identification, and quantification. biomolecules. 9 (6): 244. DOI: 10.3390/biom9060244.

Jones DA. (1986). A field guide to the seashores of Kuwait and the Arabian Gulf. University of Kuwait, Kuronuma K, Abe Y. (1972). Fishes of Kuwait. Kuwait Institute for Scientific Research. 123: 1-9.

Gharanjik BM, Rouhani Ghadikolaee K. (2010). Atlas of the macrophytic algae of the Persian Gulf and Oman Sea. Iranian Fisheries Research Organization. Tehran. 170 p.

González-López N, Moure A, Domínguez H. (2012). Hydrothermal fractionation of *Sargassum muticum* biomass. Journal of Applied Phycology. 24 (6): 1569-1578. DOI: 10.1007/s10811-012-9817-1.

He J, Xu Y, Chen H, Sun P. (2016). Extraction, structural characterization, and potential antioxidant activity of the polysaccharides from four seaweeds. International Journal of Molecular Sciences. 17 (12): 1988-2005. DOI: 10.3390/ijms17121988.

Huang HL and Wang BG. (2004). Antioxidant capacity and lipophilic content of seaweeds collected from the Qingdao coastline. Journal of Agricultural and Food Chemistry. 52 (16): 4993-4997. DOI: 10.1021/jf049575w.

Kelman D, Posner EK, McDermid KJ, Tabandera NK, Wright PR, Wright AD.

(2012). Antioxidant activity of Hawaiian marine algae. Marine Drugs. 10 (2): 403-416. DOI: 10.3390/md10020403.

Kindleysides S, Quek SY, Miller MR. (2012). Inhibition of fish oil oxidation and the radical scavenging activity of New Zealand seaweed extracts. Food Chemistry. 133 (4): 1624-1631. DOI: 10.1016/j.foodchem.2012.02.068.

Lee JH, Kim GH. (2015). Evaluation of antioxidant activity of marine algae-extracts from Korea. Journal of Aquatic Food Product Technology. 24 (3): 227-240. DOI: 10.1080/10498850.2013.770809.

Lim S, Cheung P, Ooi V, Ang P. (2002). Evaluation of antioxidative activity of extracts from a brown seaweed, *Sargassum siliquastrum*. Journal of Agricultural and Food Chemistry. 50 (13): 3862-3866. DOI: 10.1021/jf020096b.

Liu X, Yuan W, Sharma-Shivappa R, Van Zanten J. (2017). Antioxidant activity of phlorotannins from brown algae. International Journal of Agricultural and Biological Engineering. 10 (6): 184-191. DOI: 10.25165/j.ijabe.20171006.2854.

Luo HY, Wang B, Yu CG, Su Cl. (2010). Evaluation of antioxidant activities of five selected brown seaweeds from China. Journal of Medicinal Plants Research. 4 (23): 2557-2565.

Maadane A, Merghoub N, Mernissi NE, Ainane T, Amzazi S, Bakri IW. (2021). Antimicrobial activity of marine microalgae isolated from Moroccan coastlines. Journal of Microbiology, Biotechnology and Food Sciences. 2021: 1257-1260. DOI: 10.15414/jmbfs.2017.6.6.1257-126.

Machu L, Misurcova L, Vavra Ambrozova J, Orsavova J, Mlcek J, Sochor J, Jurikova T. (2015). Phenolic content and antioxidant capacity in algal food products. Molecules. 20(1): 1118-1133. DOI: 10.3390/molecules2001111.

Matanjun P, Mohamed S, Mustapha NM, Muhammad K, Ming CH. (2008). Antioxidant activities and phenolics content of eight species of seaweeds from north Borneo. Journal of Applied Phycology. 20 (4): 367-373. DOI: 10.1007/s10811-007-9264-6.

Munir N, Sharif N, Naz S, Manzoor F. (2013). Algae: a potent antioxidant source. Sky Journal of Microbiological Research. 1: 22-31.

Neto R, Marçal C, Queirós A, Abreu H, Silva A, Cardoso S. (2018). Screening of *Ulva rigida*, *Gracilaria* sp., *Fucus vesiculosus* and *Saccharina latissima* as Functional Ingredients. International Journal of Molecular Sciences. 19 (10): 2987. DOI: 10.3390/ijms19102987.

Nickavar B, Kamalinejad M, Izadpanah H. (2007). In vitro free radical scavenging activity of five Salvia species. Pakistan Journal of Pharmaceutical Sciences. 20 (4): 291-294.

O'sullivan A, O'Callaghan Y, O'grady M, Queguineur B, Hanniffy D, Troy D, O'Brien N. (2011). In vitro and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland. Food Chemistry. 126 (3): 1064-1070. DOI: 10.1016/j.foodchem.2010.11.127.

Parker TL, Miller SA, Myers LE, Miguez FE, Engeseth NJ. (2010). Evaluation of synergistic antioxidant potential of complex mixtures using oxygen radical absorbance capacity (ORAC) and electron paramagnetic resonance (EPR). Journal of Agricultural and Food Chemistry. 58 (1): 209-217. DOI: 10.1021/jf903080f.

Plaza M, Cifuentes A, Ibáñez E. (2008). In the search of new functional food ingredients from algae. Trends in Food Science and Technology. 19 (1): 31-39. DOI: 10.1016/j.tifs.2007.07.012.

Rastian Z, Mehranian M, Vahabzadeh F, Sartavi K. (2007). Antioxidant activity of extract from a brown alga, Sargassum boveanum. African Journal of Biotechnology. 6 (24): 2740-2745. DOI: 10.5897/AJB2007.000-2438.

Richmond M. (2011). A field guide to the seashores of Eastern Africa and the Western Indian Ocean Islands. Revised third edition. Sida/WIOMSA: Sweden. ISBN: 9987-8977-9-7. 464 pp.

Saeed N, Khan MR, Shabbir M. (2012). Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. BMC Complementary and Alternative Medicine. 12 (1): 221-233. DOI: 10.1186/1472-6882-12-221.

Salah HB, Bouaziz H, Allouche N. (2019). Chemical composition of essential oil from *Rhanterium suaveolens* Desf. and its antimicrobial activity against foodborne spoilage pathogens and mycotoxigenic fungi. Journal of Essential Oil Bearing Plants. 22 (3): 592-603. DOI: 10.1080/0972060X.2019.1624199.

Sathya R, Kanaga N, Sankar P, Jeeva S. (2017). Antioxidant properties of phlorotannins from brown seaweed *Cystoseira trinodis* (Forsskål) C. Agardh. Arabian Journal of Chemistry. 10: S2608-S2614. DOI: 10.1016/j.arabjc.2013.09.039.

Swanson AK, Druehl LD. (2002). Induction, exudation and the UV protective role of kelp phlorotannins. Aquatic Botany. 73 (3): 241-253. DOI: 10.1016/S0304-3770(02)00035-9.

Tierney MS, Smyth TJ, Hayes M, Soler-Vila A, Croft AK, Brunton N. (2013). Influence of pressurised liquid extraction and solid–liquid extraction methods on the phenolic content and antioxidant activities of Irish macroalgae. International Journal of Food Science and Technology. 48(4): 860-869. DOI: 10.1111/ijfs.12038.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry and Cell Biology. 39 (1): 44-84. DOI: 10.1016/j.biocel.2006.07.001

Vergalli J, Bodrenko IV, Masi M, Moynié L, Acosta-Gutiérrez S, Naismith JH, Winterhalter M. (2020). Porins and small-molecule translocation across the outer membrane of Gram-negative bacteria. Nature Reviews Microbiology. 18 (3): 164-176. DOI: 10.1038/s41579-019-0294-2.

Wang J, Zhang Q, Zhang Z, Song H, Li P. (2010). Potential antioxidant and anticoagulant capacity of low molecular weight fucoidan fractions extracted from

Laminaria japonica. International Journal of Biological Macromolecules. 46 (1): 6-12. DOI: 10.1016/j.ijbiomac.2009.10.015.

Wang T, Jónsdóttir RS, Liu H, Gu L, Kristinsson HG, Raghavan S, Ólafsdóttir GN. (2012). Antioxidant capacities of phlorotannins extracted from the brown algae *Fucus vesiculosus*. Journal of Agricultural and Food Chemistry. 60 (23): 5874-5883. DOI: 10.1021/jf3003653

Wu HC, Shiau CY, Chen HM, Chiou TK. (2003). Antioxidant activities of carnosine, anserine, some free amino acids and their combination. Journal of Food and Drug Analysis. 11(2): 148-153. DOI: 10.38212/2224-6614.2720.

Xu P, Tan H, Jin W, Li Y, Santhosh kumar C, Li P, Liu W. (2018). Antioxidative and antimicrobial activities of intertidal seaweeds and possible effects of abiotic factors on these bioactivities. Journal of Oceanology and Limnology. 36 (6): 2243-2256. DOI: 10.1007/s00343-019-7046-z.

Zengin G, Aktumsek A, Guler GO, Cakmak YS, Yildiztugay E. (2011). Antioxidant Properties of Methanolic Extract and Fatty Acid Composition of *Centaurea urvillei* DC. subsp. *hayekiana Wagenitz*. Records of Natural Products. 5 (2): 123-132.

Zhou P, Wang X, Liu P, Huang J, Wang C, Pan M, Kuang Z. (2018). Enhanced phenolic compounds extraction from *Morus alba* L. leaves by deep eutectic solvents combined with ultrasonic-assisted extraction. Industrial Crops and Products. 120: 147-154. DOI: 10.1016/j.indcrop.2018.04.071.

Zubia M, Fabre MS, Kerjean V, Le Lann K, Stiger-Pouvreau V, Fauchon M, Deslandes E. (2009). Antioxidant and antitumoural activities of some Phaeophyta from Brittany coasts. Food Chemistry. 116 (3): 693-701. DOI: 10.1016/j.foodchem.2009.03.025.

Zubia M, Robledo D, Freile-Pelegrin Y. (2007). Antioxidant activities in tropical marine macroalgae from the Yucatan Peninsula, Mexico. Journal of Applied Phycology. 19 (5): 449-458. DOI: 10.1007/s10811-006-9152-5.

Biosynthesis of Gold Nanoparticles by Medicinal Cyanobacterium *Spirulina platensis* Geitler

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Abstract

The biosynthesis of nanoparticles using microorganisms as emerging bionanotechnology has received considerable attention due to a growing need to develop environmentl-friendly technologies in materials synthesis. Nanoparticles produced by a biogenic enzymatic process are far superior in biomedical applications to those produced by chemical methods. This study explored the biosynthesis of gold nanoparticles by *Arthrospira platensis* Gomont. Two series of experiments hence the dose dependency (chloroauric acid solution with different concentrations) and the temperature dependency (room, 75º C, and 90º C temperature) of Au nanoparticles formation, were studied. Optimizing the synthesis of gold nanoparticles and gold nanoparticles concentration determination was done. The results showed that the gold nanoparticles' size is reduced by reducing the gold concentration and raising the reaction temperature. In addition, the size of spherical shape nanoparticles has decreased from 80 nm to 20 nm, and as the concentration increased, nanoparticles became more stable. Extracted nanoparticles solutions were examined by UV-visible Spectroscopy, scanning electron microscopy (SEM), dynamic Light Scattering (DLS), and EDAX or EDS (Energy-dispersive X-ray spectroscopy) analysis. Results indicated that algae extract is very suitable for biosynthesis and are more efficient than biomass. The maximum production efficiency with this method is 98%, which is excellent and economical.

Keywords: Cyanobacteria, Green Synthesis, Biological production, Optimization, Bionanotechnology

Introduction

The first report on the synthesis of gold nanoparticles by Alfalfa sprouts was done by Gardea-Torresdey (2002), who showed that at the beginning, Au (III) ions are reduced to Au (0) ions by the alfalfa plant, and then the metal atoms are absorbed by the plant, which leads to It will be revived later. Present observation lends support to a

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previous study where formation of triangular gold nanoparticles was reported using the *Spirulina* (Chandran et al., 2006). Shankar et al. (2003) demonstrated the neem leaf mediated synthesis of Ag, Au and bimetallic Au core–Ag shell nanoparticles. Further, silver and gold nanoparticles have been synthesized using bacteria, fungi, yeasts (Gericke and Pinches, 2006; Deendayal et al., 2006) and amino acids (Selvakannan et al., 2003). There are also reports on the microbes-mediated synthesis of alloy nanoparticles, both extra and intracellular (Nair and Pradeep, 2002; Ahmad et al., 2005). The synthesis of gold nanoparticles in algae has also been reported, including *Chlorella vulgaris* (Ting et al., 1995), *Sargassum wightii* (Singaravelu et al., 2007) and *Plectonema boryanum* (Lengke et al., 2006b). In addition, Chakraborty et al. (2006, 2009), Nayak et al. (2006) synthesized nanoparticles by cyanobacteria, green algae, and diatoms. Recently, cyanobacterium-mediated platinum nanoparticles' synthesis by the reaction of filamentous *Plectonema boryamum* with platinum (IV) chloride complex has also been reported. Biological production systems are of special interest due to their effectiveness and flexibility. One of the issues raised in connection with algae, especially blue-green algae, is the ability to use them for biological production. Today, microorganisms such as bacteria, fungi, yeasts, actinomycetes, and algae are used to produce gold nanoparticles. Green synthesis of nanoparticles using microorganisms as an emerging technology has received much attention due to the growing need to develop environmentally friendly technologies in material synthesis (Rai et al., 2011).Variant nanoparticles are produced by three physical, chemical and biological methods; the first two methods are problematic, expensive and cause environmental pollution, hence the use of biological production procedure due to not cause environmental pollution is of excessive significance (Faramarzi et al., 2010; Rastgar Farajzadeh et al., 2010). Biological production systems are of particular interest due to their effectiveness and flexibility. Nanoparticles produced by a biogenic enzymatic process (the product of the activity of livingorganisms) are far superior to those produced by chemical methods in biomedical applications (Li et al., 2011; Mandal et al., 2006).

Gold nanoparticles play a significant role in nanotechnology due to their potential use in industry and medicine (Sadowski, 2010). When microorganisms take metal ions from the environment, nanoparticles are synthesized, and then they are converted into nanoscale particles by cell-produced intracellular or extracellular enzymes (Lengke et al., 2011). The particles produced by these processes have a higher catalytic reaction, more specific surface area, and improved contact between the enzyme and the desired metal salt due to the presence of the matrix (field) carrying the bacteria. They have been used in various applications such as targeted drug delivery, cancer treatment, gene therapy, and DNA analysis, antibacterial agents, biosensors,

increasing reaction speed, separation science (Khosravi-Darani et al., 2017). In many types of research, *Spirulina* has been used to produce nanoparticles. Cyanobacterial such as *Plectonema* can convert gold chloride solution into gold metal (Lengke et al., 2006 .(Lengke et al. (2006 a, b) studied the synthesis of gold nanostructures with different shapes (spherical, cubic, and octagonal) by filamentous cyanobacteria and analyzed their formation mechanism. Bakir et al.(2018) showed that the cyanobacterium *Lyngbya majuscula* in contact with a solution of 1500 mg/ml of gold chloride produces gold intracellular nanoparticles for a day and can produce gold extracellular nanoparticles after two months of incubation. Tikariha et al. (2012) showed that the cyanobacterium *Plectonema boryanum* and green algae *Chlorella* can produce gold nanoparticles, and *Sargassum* seaweed can produce gold nanoparticles with dimensions of 8 to 12 nm from gold chloride solution. Govindaraju et al. (2008) have studied the extracellular biosynthesis of silver, gold, and mixed metal nanoparticles using *Spirulina*. These researchers have studied the synthesis of silver and gold nanoparticles by *Spirulina* because of its nutritional and medicinal importance. *Spirulina* is a filamentous cyanobacterium or multicellular spiral filamentous algae. It is one of the most valuable natural nutritional sources known in the world. This microalga contains 60-70% vegetable protein, is perfectly balanced in terms of amino acids, rich in beta-carotene, iron, the richest natural source of vitamins, essential fatty acids, and other biologically active beneficial substances in the world (Doshi et al., 2007). *Spirulina* blue-green microalgae is widely used as a medicinal matrix and also as a food additive for humans and animals. The production of complexes that are easily absorbed by the human organism is one of the distinctive features of *Spirulina*. Considering that *Spirulina* is a valuable medicinal alga and can be used for the green synthesis of gold nanoparticles, this research is devoted to investigating the biosynthesis ability of gold nanoparticles by *Spirulina*. So far, no such research has been done in Iran, and it is a green approach in the field of synthesis of gold nanoparticles in the country.

In this research article, which is the result of the first author's postdoctoral research course, we report the use of *Spirulina platensis* biomass and extract for the biosynthesis of pure metallic gold nanoparticles by simultaneous reduction of aqueous $HAuCl_4$.

Materials and methods

Preparation of S. platensis stock and its cultivation

In order to conduct the experiments, the green stock of *Spirulina platensis* Geitler was purchased from Parsjolbak Company of Shiraz, then it was cultivated in BG11 medium. This blue-green algae culture medium containing (0.1 Na_2Mg EDTA, 0.6 Ferric ammonium citrate, 0.6 Citric acid. $1H_2O$, 3.6 $CaCl_2.2H_2O$, 1.5 $NaNO_3$, 7.49 $MgSO_4$. $7H_2O$, 0.02 Na_2CO_3, 4 $K_2HPO_4.3H_2O$, 2.86

H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.22 $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, 0.05 $CoCl_2.6H_2O$, 0.39 $NaMoO_4.2H_2O$ g/L) is used to produce abundant biomass (Stanier et al., 1971).

Gold nanoparticles production using S. platensis biomass

Before experimentation, the biomass was washed thrice in deionized water to remove the unwanted materials. For all the synthesis of gold nanoparticles, 613 mM chloroauric acid ($HAuCl_4$) was used as received. Gold nanoparticle formations were carried out by taking 0.1 gr of *S. platensis* biomass in a 50 mL Erlenmeyer flask with 0.613 mM aqueous $HAuCl_4$ and incubated at room temperature. The pH was checked during the course of the reaction, and it was found to be 3 (Kalabegishvili et al., 2012).

Spirulina extraction by methanol solvent

10 gr *S. platensis* powder in 20 ml methanol (100%) twice used to prepare methanolic extract. After 24 hours, the solution was filtered with filter paper and the extract was concentrated with a rotary device (Saad AM. et. al., 2020)

Biosynthesis of gold nanoparticles by extract of S. platensis with a high concentration of gold salt

After placing 0.1 g of fresh extract of *S. platensis* exposed to 100 micro liters of 613 mM gold solution for 24 hours, the color change of the solution and the biosynthesis of nanoparticles were investigated (Xie et al., 2007).

Measurement of an absorption spectrum of nanoparticles by UV-Visible Spectrophotometer

After the biosynthesis of gold nanoparticles, the absorption spectrum of the solution at the wavelength of 531 nm, which is the absorption wavelength of gold nanoparticles, was determined by the UV-Visible spectrophotometer.

Determining the shape and size of gold nanoparticles by electron microscopy

The size of nanoparticles was checked by SEM electron microscope. In order to determine the shape and size of the samples, the biosynthesized sample was centrifuged at 5000 rpm for ten minutes and after being concentrated and placed in a 60° C oven, it was delivered to the electron microscope room of the physics department of Yazd University.

EDAX analysis or EDS

EDAX analysis or EDS (X-ray Energy Diffraction Spectroscopy) is an add-on to SEM devices to detect elements in solid samples. This analysis can detect the type of element and its weight or atomic percentage by using the unique X-ray energy emitted from the sample. In this study, biosynthetic gold nanoparticles were transferred to Beam Goster Taban material analysis laboratory for EDAX analysis.

Biosynthesis by S. platensis extract with a low concentration of gold salt and estimate the hydrodynamic size (DLS)

One of the samples of *S. platensis* extract, which was exposed to less gold salt for the biosynthesis of nanoparticles, was subjected to DLS analysis to determine the size and size distribution. Sample size and distribution of biosynthesized particles were checked by

the DLS device. Gold nanoparticles were analyzed by Dynamic Light Scattering (DLS). Extracted gold nanoparticles were analyzed using Malvern Nano ZS to estimate the hydrodynamic size of the particles.

Gold nanoparticles concentration determination

The concentration of gold nanoparticles was determined by an atomic absorption spectrophotometer (AAS) (Analyst 400; Perkin Elmer, Waltham, MA, USA).

Sample stability test

The stability test of biosynthetic gold nanoparticles was also performed. In this way, the absorption of the sample was recorded during biosynthesis and after one month after biosynthesis, and if the absorption number has not changed, it indicates the stability of the nanoparticles and is one of the most critical indicators that determine the quality of the gold nanoparticles sample are the stability index.

Results

Solution color change and synthesis of gold nanoparticles

The biosynthesis of nanoparticles by biomass and *S. platensis* extract can be detected only by changing the color of the solution from yellow to red. Synthesis of gold nanoparticles at room temperature showed that after 24 hours, the extract solution and wet biomass changed from yellow to red, and red gold nanoparticles were made.

The result of the measurement of an absorption spectrum of gold nanoparticles

The addition of *S. platensis* biomass to 0.613 mM aqueous $HAuCl_4$ solutions led to the appearance of red color in biomass after 48 h of reaction, indicating the formation of gold nanoparticles. These colors arise due to the excitation of surface plasmon vibrations in the metal nanoparticles (Singaravelu et al., 2007). Figures 1 shows the UV–Vis spectra recorded from the aqueous auric chloride and *S. platensis* reaction medium as a function of the reaction time. The gold surface plasmon resonance (SPR) band occurred at 530 nm. Function of the time of reaction was recorded on a UV–Vis 1601 Schimadzu spectrophotometer which was operated at a resolution of 1 nm. The gold nanoparticles produced did not show an absorption peak at the wavelength of 531 nm, which is the absorption wavelength of gold nanoparticles in the biosynthesis section with biomass. This indicates that the intracellular gold nanoparticles are synthesized and are not in free form, and no extracellular nanoparticles have been synthesized. In the biosynthesis section with extract, the sharpie absorption peak at 530 nm is observed, which indicates the presence of extracellular biosynthetic nanoparticles in free form.

SEM electron microscopy

The size of nanoparticles was checked by SEM electron microscope. The shape of biosynthesized gold nanoparticles is spherical between 20- 80 nm (Figure 2).

Dynamic Light Scattering test result

DLS study supports the presence of gold nanoparticles of different sizes in the extracted solution. The average size distribution of

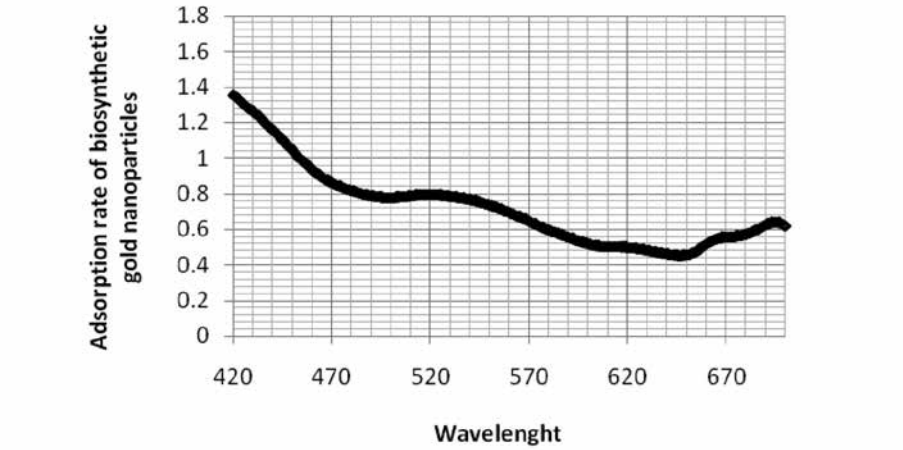
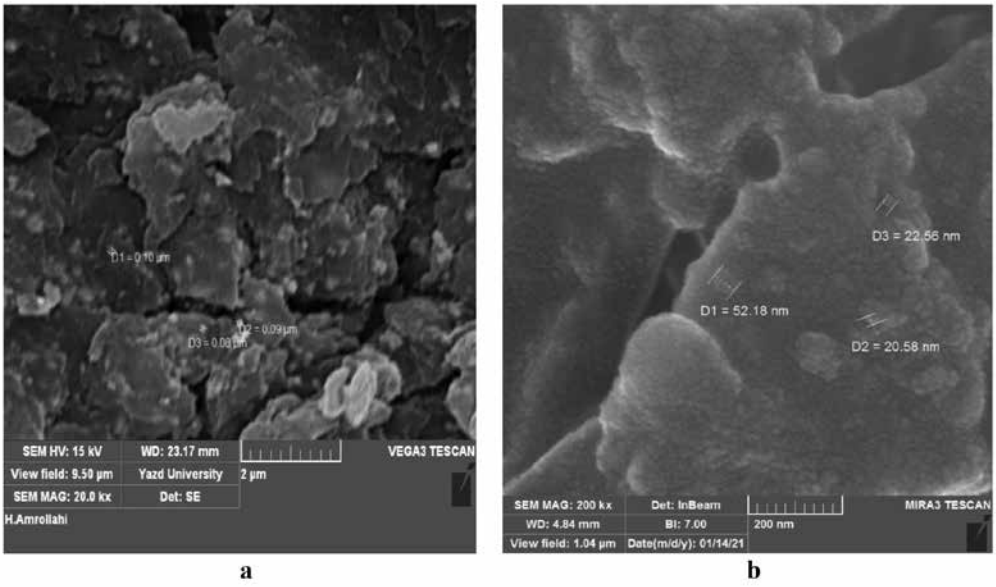


Fig. 1. UV–Vis spectra recorded as a function of time of reaction of the aqueous solution of chloroauric acid with *S.*



a b

Fig. 2. SEM images of gold nanoparticles formed by reduction of Au ions using *S. platensis* biomass and extract; (a) intracellular nanoparticles (with a high concentration of gold) and (b) extracellular nanoparticles (with a low concentration of gold)

biosynthesized gold nanoparticles is about 56 nm (Figure 3).

Optimization of the synthesis of gold nanoparticles

Three factors in temperature, the amount of gold sample in the environment, and the amount of algae biomass are significant in optimizing the synthesis of gold nanoparticles in different dimensions. The results show that the temperature is very effective in the duration of the synthesis of nanoparticles and significantly reduces the time required for the synthesis; also, the temperature affects the size of the nanoparticles, and as the temperature increases, the size of the nanoparticles decreases. In addition, with the reduction of gold concentration, the size of gold nanoparticles becomes smaller. In the

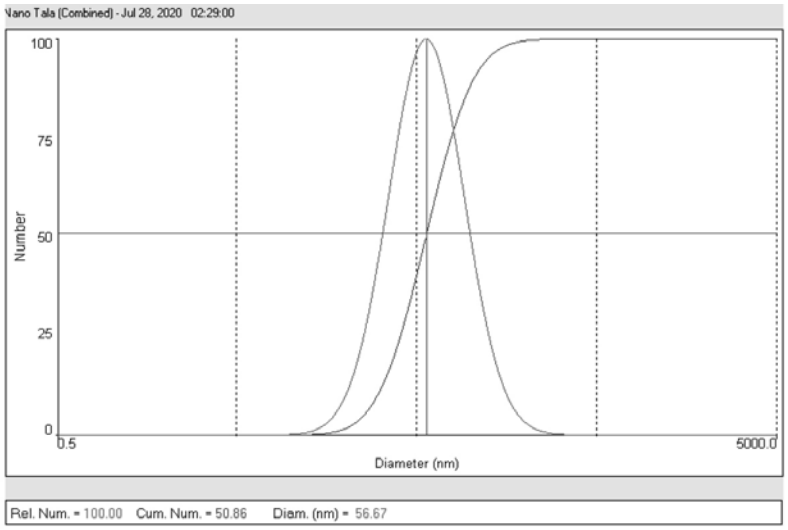


Fig. 3. Dynamic light scattering test result

present experiment, the size of nanoparticles has decreased from 80 nm to 20 nm by halving the gold concentration compared to previous experiments.

Gold nanoparticles concentration determination

An atomic absorption spectrophotometer analysis determined the concentration of gold nanoparticles. The results show that more biosynthetic nanoparticles are synthesized with *S. platensis* algae extract at high temperatures and low volumes of gold solution. The maximum production efficiency with this method is 98%, which is excellent and economical.

Table 1. Determining the concentration of biosynthetic gold nanoparticles by the gold standard

Number	Test temperature	Initial concentration of gold solution (ppm)	sample	pity	Concentration of gold nanoparticles (ppm)	Production efficiency (%)
1	25° c	71.2	*Spirulina platensis* 1	1	A: 28.60 O: 28.60	47.35
2	75° c	71.2	*Spirulina platensis* 2	25	A: 2.368 O: 59.19	98
3	90° c	85.6	*Spirulina platensis* 3	25	A: 2.755 O: 68.88	95.14

(A: The concentration value of the diluted solution, O: The concentration value of the original solution)

(A: The concentration value of the diluted solution, O: The concentration value of the original solution)

EDAX analysis or EDS (Energy-dispersive X-ray spectroscopy)

an EDAX (energy dispersive X-ray analysis) pattern of gold nanoparticles synthesized by treating *S. platensis* with chloroauric acid aqueous solution is proved the presence of gold nanoparticles in Figure 4. The energy of X-rays is characteristic of the elements from which these X-rays are emitted. A spectrum of the energy versus relative counts of the detected X-rays is showed in Figure 4. Two peaks of Au were observed for biomass of *S. platensis*, Edax analysis confirmed the particles only with gold.

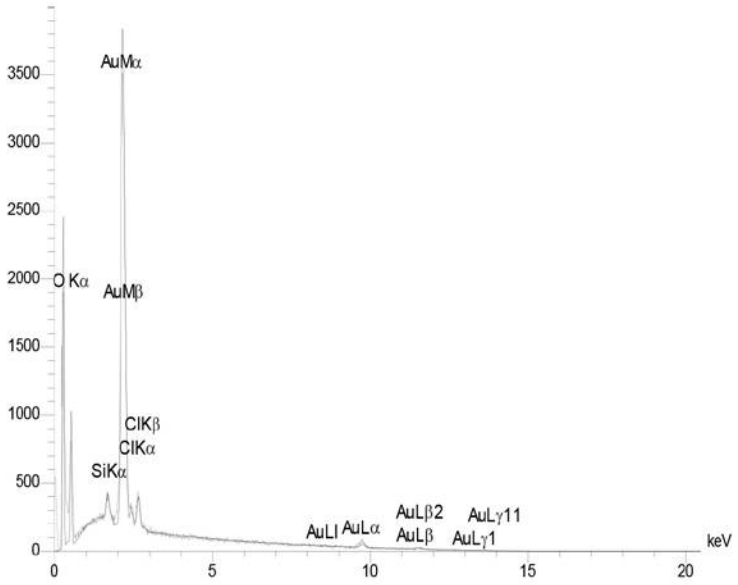


Fig. 4. Edax analysis of biosynthetic gold nanoparticles confirmed the particles only with gold

Discussion

Green synthesis of AuNPs has become of great interest using bacteria, fungi, and algae because of its numerous benefits, such as low-cost medium for microbial growth, efficiently handling process, and its ability to absorb gold ions (Lee et al., 2020; Akintelu and Folorunso et al., 2021). Several material scientists have synthesized various types of gold nanoparticles through physical and chemical methods (Hu et al., 2007; Panigrahi et al., 2005; Wang and Shi, 2007). Ahmad et al. (2005) envisaged that chemical synthesis may still lead to the presence of some toxic chemical species adsorbed on the surface that may have adverse effects in medical applications. On this basis, the present study has its essentials. Although the therapeutic potential of *S. platensis* is promising, its bio-reduction property of inorganic materials is yet to be exploited. Kalabegishvili et al. (2012) studied the synthesis of gold nanoparticles by *S. platensis* at different doses and at different time intervals. This study showed that after 1.5-2 days of exposure to gold salt, spherical

intracellular nanoparticles were formed, and the peak size of the particles was between 20-30 nm.

The results showed that the concentration of gold accumulated by the *S. platensis* biomass was proliferating at the beginning, followed by some increase over the next few days. In the UV-visible absorption spectrum of *S. platensis* suspension after the addition of gold chlorate solution at different concentrations (dose-dependent), it has been shown that a broad gold Surface Plasmon Resonance (SPR) peak appears at 530 nm for its concentrations (10^{-3}-10^{-4} M). At higher concentrations of $HAuCl_4$ 10^{-2} M such a peak was not observed. The results show that at the concentration of $HAuCl_4$ 10^{-3} M, the size of gold nanoparticles is ≈ 14 nm, at 10^{-3} M ≈ 20 nm, and at 10^{-2} M ≈ 100 nm. In the present research, the size of gold nanoparticles becomes smaller with the reduction of gold concentration. In the current experiment, the size of nanoparticles decreased from 80 nm to 20 nm by halving the gold concentration compared to previous experiments, which is consistent with the results of Kalabegishvili et al. (2012). In the current research, by increasing the concentration of *S. platensis* extract and decreasing the attention of the gold solution used for biosynthesis, as well as increasing the temperature, nanoparticles with a smaller size and higher concentration are synthesized, as seen in Table 3, which indicates The results are similar to the research of Kalabegishvili et al. (2012) on the synthesis of gold nanoparticles by the cyanobacterium *S. platensis*.

The results of the studies by Sharma et al. (2009) show that the color of gold nanoparticles changes from red to blue and this color change depends on the shape and size of the nanoparticles. The nanoization of gold particles by microorganisms is caused by the reduction of Au(III) to Au(0) and its production in intracellular and extracellular form, which is entirely consistent with the present research; *S. platensis* makes intracellular and extracellular nanoparticles brown to red depending on the size by absorbing gold solution in different temperature and concentration conditions (Duff et al., 1987; Chow and Zukoski, 1994; Lujan et al., 1994; Chakraborty et al., 2009; Parial et al., 2012).

Gerick and Pinches (2006) reported that the shape of particles is spherical if the amount of regeneration (reduction) is low. If the amount of regeneration is high, the form of particles becomes nanorods and nanoplates. In addition, their investigation showed that the high amount of regeneration at low pH might lead to the production of nanorods. Therefore, we find that according to the results of this research, which shows that biosynthetic nanoparticles are spherical, so the amount of reduction is low.

According to the Miefis Theory, only one SPR band is expected in the absorption spectrum of spherical nanoparticles. At the same time, anisotropic particles can produce two or more SPR bands depending on the particle shape (Sosa et al., 2003). In the present case, a single band was observed, which shows evidence of the presence of

spherical gold nanoparticles, which was confirmed by TEM and SEM images. In the present research, the synthesis of spherical nanoparticles is proved by having a sharp band. As all the reports state that biosynthesis with algae extracts is better than biomass, this research also shows that algae extract very suitable for biosynthesis and has more efficiency (Shankar et al., 2016). The maximum production efficiency with this method is 98%, which is excellent and economical.

The "green route" of biosynthesis of extracellular gold nanoparticles in *S. platensis* is a very simple, economically viable, and environmentally friendly process, which has a significant advantage over the intracellular synthesis process in terms of applications in medicine, pharmaceuticals, and other technological fields and has a chemical synthesis process.

Acknowledgement

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References

Ahmad A, Senapati S, Khan MI, Kumar R, Sastry M. (2005). Extra- and intra-cellular biosynthesis of gold nanoparticles by an alkalotolerant fungus, *Trichothecium* sp. Journal of Biomedical Nanotechnology. 1 (1): 47-53. DOI: 10.1002/smll.200400053.

Akintelu SA, Yao B, Folorunso AS. (2021). Bioremediation and pharmacological applications of gold nanoparticles synthesized from plant materials. Heliyon. 7 (3): e06591. DOI: 10.1016/j.heliyon.2021.e06591.

Bakir EM, Younis NS, Mohamed ME, El Semary NA. (2018). Cyanobacteria as nanogold factories: chemical and anti-myocardial infarction properties of gold nanoparticles synthesized by *Lyngbya majuscula*. Marine Drugs. 16 (6): 217. DOI: 10.3390/md16060217.

Chakraborty N, Banerjee A, Lahiri S, Panda A, Ghosh AN, Pal R. (2009). Biorecovery of gold using cyanobacteria and an eukaryotic alga with special reference to nanogold formation–a novel phenomenon. Journal of Applied Phycology. 21 (1): 145-152. DOI: 10.1007/s10811-008-9343-3.

Chakraborty N, Pal R, Ramaswami A, Nayak D, Lahiri S. (2006). Diatom: a potential bio-accumulator of gold. Journal of Radioanalytical and Nuclear Chemistry. 270 (3): 645-649. DOI: 10.1007/s10967-006-0475-0.

Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M. (2006). Synthesis of gold nanotriangles and silver nanoparticles using *Aloe vera* plant

extract. Biotechnology Progress. 22 (2): 577-583. Doi: 10.1021/bp0501423.

Chow MK and Zukoski CF. (1994). Gold sol formation mechanisms: role of colloidal stability. Journal of Colloid and Interface Science. 165 (1): 97-109. DOI: 10.1006/ jcis.1994.1210.

Deendayal M, Bolander EM, Mukhopadhyay D, Sarkar G, Mukherjee P. (2006). The use of microorganisms for the formation of metal nanoparticles and their application. Applied Microbiology and Biotechnology. 69: 485-492. DOI: 10.1007/ s00253-005-0179-3.

Doshi H, Ray A, Kothari IL. (2007). Bioremediation potential of live and dead Spirulina: spectroscopic, kinetics and SEM studies. Biotechnology and Bioengineering. 96 (6): 1051-1063. DOI: 10.1002/bit.21190.

Duff DG, Curtis AC, Edwards PP, Jefferson DA, Johnson BF, Kirkland AI, Logan DE. (1987). The morphology and microstructure of colloidal silver and gold. Angewandte Chemie International Edition in English. 26 (7): 676-678. DOI: 10.1002/anie.198706761.

Faramarzi MA, Fortunfar H, Shakibaei M. (2010). Biotechnology of microalgae. Tehran University of Medical Sciences.

Gardea-Torresdey JL, Parsons JG, Gomez E, Peralta-Videa J, Troiani HE, Santiago P, Yacaman MJ. (2002). Formation and growth of Au nanoparticles inside live alfalfa plants. Nano Letters. 2 (4): 397-401. DOI: 10.1021/nl015673+.

Gericke M and Pinches A. (2006). Biological synthesis of metal nanoparticles. Hydrometallurgy. 83 (1-4): 132-140. DOI: 10.1016/j.hydromet.2006.03.019.

Govindaraju K, Basha SK, Kumar VG, Singaravelu G. (2008). Silver, gold and bimetallic nanoparticles production using single-cell protein (*Spirulina platensis* Geitler). Journal of Materials Science. 43 (15): 5115-5122. DOI: 10.1007/s10853-008-2745-4.

Hu Y, Li C, Gu F, Zhao Y. (2007). Facile flame synthesis and photoluminescent properties of core/shell TiO2/SiO2 nanoparticles. Journal of Alloys and Compounds. 432 (1-2): L5- L9. DOI: 10.1016/j.jallcom.2006.05.134.

Kalabegishvili T, Kirkesali E, Rcheulishvili A. (2012). Synthesis of gold nanoparticles by blue-green algae *Spirulina platensis*. JINR-E--14-2012-31. Frank Laburatory of Neutron Physics.

Khosravi-Darani K, Sohrabvandi S, Zoghi A. (2017). Intra-cellular Biosynthesis of Gold Nanoparticles by Fungus Penicillium chrysogenum. Medicinal Journal of Tabriz Univesity of Medicinal Sciences Health Services. 39 (3): 32-38.

Lee KX, Shameli K, Yew YP, Teow SY, Jahangirian H, Rafiee-Moghaddam R, Webster TJ. (2020). Recent developments in the facile biosynthesis of gold nanoparticles (AuNPs) and their biomedical applications. International Journal of Nanomedicine. 15: 275. DOI: 10.2147/IJN.S233789.

Lengke MF, Sanpawanitchakit C, Southam G. (2011). Biosynthesis of

gold nanoparticles: a review. Metal Nanoparticles in Microbiology. 37-74. DOI: 10.1007/978-3-642-18312-6-3.

Lengke MF, Fleet ME, Southam G. (2006a). Morphology of gold nanoparticles synthesized by filamentous cyanobacteria from gold (I)− thiosulfate and gold (III)− chloride complexes. Langmuir. 22 (6): 2780-2787. DOI: 10.1021/la052652c.

Lengke MF, Ravel B, Fleet ME, Wanger G, Gordon RA, Southam G. (2006b). Mechanisms of gold bioaccumulation by filamentous cyanobacteria from gold (III)− chloride complex. Environmental Science and Technology. 40 (20): 6304-6309. DOI: 10.1021/es061040r.

Li X, Xu H, Chen ZS, Chen G. (2011). Biosynthesis of nanoparticles by microorganisms and their applications. Journal of Nanomaterials. 2011 (8): 1-16. DOI: 10.1155/2011/270974.

Lujan JR, Darnall DW, Stark PC, Rayson GD, Gardea-Torresdey JL. (1994). Metal ion binding by algae and higher plant tissues: a phenomenological study of solution pH dependence. Solvent Extraction and Ion Exchange. 12 (4): 803-816. Doi: 10.1080/07366299408918239.

Mandal D, Bolander ME, Mukhopadhyay D, Sarkar G, Mukherjee P. (2006). The use of microorganisms for the formation of metal nanoparticles and their application. Applied Microbiology and Biotechnology. 69 (5): 485-492. DOI: 10.1007/s00253-005-0179-3.

Nair B and Pradeep T. (2002). Coalescence of nanoclusters and formation of submicron crystallites assisted by Lactobacillus strains. Crystal Growth and Design. 2 (4): 293-298. Doi: 10.1021/cg0255164.

Nayak D, Nag M, Banerjee S, Pal R, Laskar S, Lahiri S. (2006). Preconcentration of 198 Au in a green alga, Rhizoclonium. Journal of Radio analytical and Nuclear Chemistry. 268 (2): 337-340. DOI: 10.1007/s10967-006-0170-1.

Panigrahi S, Kundu S, Ghosh SK, Nath S, Pal T. (2005). Sugar assisted evolution of mono-and bimetallic nanoparticles. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 264 (1-3): 133-138. DOI: 10.1016/j.colsurfa.2005.04.017.

Parial D, Patra HK, Dasgupta AK, Pal R. (2012). Screening of different algae for green synthesis of gold nanoparticles. European Journal of Phycology. 47 (1): 22-29. DOI: 10.1080/09670262.2011.653406.

Rai M, Gade A, Yadav A. (2011). Biogenic nanoparticles: an introduction to what they are, how they are synthesized and their applications. In Metal nanoparticles in microbiology. Springer, Berlin, Heidelberg. 1-14. DOI: 10.1007/978-3-642-18312-6_1.

Rastgar Farajzadeh M, Bardania H, Rahmati Shadabad K. (2010). Preparation of microbial nanoparticles. Nano Technology Monthly. 7 (156).

Saad AM, Mohamed AS, Ramadan MF. (2021). Storage and heat processing affect flavors of cucumber juice enriched with plant extracts. International Journal

of Vegetable Science. 27 (3): 277-287. DOI: 10.1080/19315260.2020.1779895.

Sadowski Z. (2010). Biosynthesis and application of silver and gold nanoparticles. Silver Nanoparticles. 22: 257-277.

Selvakannan PR, Mandal S, Phadtare S, Pasricha R, Sastry M. (2003). Capping of gold nanoparticles by the amino acid lysine renders them water-dispersible. Langmuir. 19 (8): 3545-3549. DOI: 10.1021/la026906v.

Shankar PD, Shobana S, Karuppusamy I, Pugazhendhi A, Ramkumar VS, Arvindnarayan S, Kumar G. (2016). A review on the biosynthesis of metallic nanoparticles (gold and silver) using bio-components of microalgae: Formation mechanism and applications. Enzyme and Microbial Technology. 95: 28-44. DOI: 10.1016/j.enzmictec.2016.10.015.

Shankar SS, Ahmad A, Sastry M. Geranium leaf assisted biosynthesis of silver nanoparticles. (2003). Biotechnology Progress. 19 (6): 1627-1631. DOI: 10.1021/bp034070w.

Sharma V, Park K, Srinivasarao M. (2009). Colloidal dispersion of gold nanorods: Historical background, optical properties, seed-mediated synthesis, shape separation and self-assembly. Materials Science and Engineering: R: Reports. 65 (1-3): 1-38. DOI: 10.1016/j.mser.2009.02.002.

Singaravelu G, Arockiamary JS, Kumar VG, Govindaraju K. (2007). A novel extracellular synthesis of monodisperse gold nanoparticles using marine alga, *Sargassum wightii* Greville. Colloids and surfaces B: Biointerfaces. 57 (1): 97-101. DOI: 10.1016/j.colsurfb.2007.01.010.

Sosa IO, Noguez C, Barrera RG. (2003). Optical properties of metal nanoparticles with arbitrary shapes. The Journal of Physical Chemistry B. 107 (26): 6269-6275. DOI: 10.1021/jp0274076.

Stanier RY, Kunisawa R, Mandel MC, Cohen-Bazire G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological Reviews. 35 (2): 171-205. DOI: 10.1128/br.35.2.171-205.1971.

Tikariha S, Singh S, Banerjee S, Vidyarthi AS. (2012). Biosynthesis of gold nanoparticles, scope and application: a review. International Journal of Pharmaceutical Sciences and Research. 3 (6): 1603-1615. DOI: 10.13040/IJPSR.0975-8232.3(6).1603-15.

Ting YP, Teo WK, Soh CY. (1995). Gold uptake by Chlorella vulgaris. Journal of Applied Phycology. 7 (1): 97-100. DOI: 10.1007/BF00003557.

Xie J, Lee JY, Wang DI, Ting YP. (2007). Identification of active biomolecules in the high-yield synthesis of single crystalline gold nanoplates in algal solutions. Nano Micro Small. 3 (4): 672-682. DOI: 10.1002/smll.200600612.

Wang S and Shi G. (2007). Uniform silver/polypyrrole core-shell nanoparticles synthesized by hydrothermal reaction. Materials Chemistry and Physics. 102 (2-3): 255-259. DOI: 10.1016/j.matchemphys.2006.12.014.

Effect of Soil Physico-Chemical Characteris tics on Cyanobacterial Communities in Arid Lands

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Abstract

Cyanobacteria are a group of prokaryotes that can live under stressful environmental conditions due to their high metabolic flexibility. In this study, we examined the terrestrial cyanobacterial communities in the wheat fields adjacent to the indus trial areas of Yazd province. The physical and chemical properties of the soils were evaluated, including pH, EC, salinity, and the concentration of two heavy metals, lead, and cadmium. In addition, the diversity and abundance of cyanobacteria were investigated in the soil of the studied stations. The frequency of taxa was determined based on the colony count method. According to the results, the amount of cadmium was very low (lower than 0.1 ppm), but the lead concentration in the s tudied stations varied between 1.602 ppm and 4.044 ppm. The sodium concentration in the soil ranged from 16.18 to 89.54 $mEqL^{-1}$. The present results show that with a slight increase in the concentration of lea, the diversity of cyanobacteria does not decrease. Still, an increase in the concentration of information from a specific range causes a reduction in their biodiversity. Moreover, the cyanobacteria abundance does not show any significant decrease in this range of lead concentration. This result may indicate the relative resistance of the dominant taxon to lead metal and the development of these taxon communities in stressful conditions.

Keywords: Cyanobacteria, Diversity, Heavy metal, Salinity, Soil texture

Introduction

Terrestrial ecosystems occupy a large part of each country's natural habitats. These ecosystems are the exclusive habitat of many microorganisms, which have developed biological communities. The abundance and diversity of soil microbial communities depend on various factors including the soil Physico-chemical properties, moisture, organic matter content, and soil texture (Barton and Northup, 2011, Saul-Tcherkas and Steinberger, 2009). In dry land and deserts, several factors such as dryness and salinity significantly affect soil microbial flora (Zhang et al., 2019; Šťovíček et al., 2017).

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Soil salinity is determined based on the concentration of soluble salts such as sodium (Na^+), potassium (K^+), chloride (Cl), and sulfate (SO_4^{-2}) (Qadir et al., 2007). Salinity and high concentration of salts are among the characteristic features of soil in dry desert areas. These factors mostly have adverse effects on the microflora of the growing medium of plants, as well as their growth efficiency and performance (Litalien and Zeeb, 2020). It should be noted that the high concentration of all salts does not necessarily reduce or stop the biological activity of plants and soil microorganisms. Some cations such as Na^+ are mainly responsible for creating salt marshes leading to biological damage to plants, while K^+ is considered an essential element for the growth of plants (Jafary, 2000; Qadir et al., 2007). Also, researchers believe K^+ is the most important essential element for plants after nitrogen (Chookalaii et al., 2020; Prajapati and Modi, 2012)

Another factor affecting the composition of soil biological communities is the presence of industrial heavy metal pollution. An increase in the concentration of heavy metals in the soil naturally causes an increase in the concentration of these metals in plant tissues. Consequently, it causes poisoning due to creating a disturbance in the absorption process and plant growth (Alam et al., 2020; Štofejová et al., 2021). Moreover, previous studies show that the high concentrations of heavy metals in the soil significantly affect soil microflora and the biodiversity of soil microorganisms (Ahlesaadat et al., 2017).

Some algae and cyanobacteria can live in habitats contaminated with heavy metals or saline soils (Hokmollahi et al. 2016; Heidari et al. 2017). These photosynthetic microorganisms grow and socialize well in damaged soils and polluted environments. These resistant taxa have received particular attention in recent years to improve the soil texture in affected and polluted soils (Rocha et al., 2020). Therefore, these microalgae and cyanobacteria are good candidates for environmental cleaning which is known as phytoremediation in biotechnology (Fawzy and Mohamed, 2017; Sarmah and Rout, 2020).

Yazd, a province located in central Iran and southeast Isfahan, is a hyperarid region with limited water resources. According to the reports, the cultivated lands of this province (agricultural lands, gardens, and tree-planted areas) occupy 40,781 hectares (Dashtakian and Baghestani, 2002). In recent years, a decrease in rainfall and an increase in evaporation caused more dryness, accumulation of salts on the surface of the soil, and the destruction of soil texture. These factors can lead to a decrease in crop yields and to the complete desertification of agricultural lands (Ghaeminia et al., 2019). Due to the limitation of water resources in this province, using wastewater or underground water sources in agriculture is one of the leading solutions for providing agricultural water. Studies show that the underground water in Yazd is contaminated with heavy metals due to the tile and ceramic industry (Rahmani, 2009). The results of research on the pollution load of the factories of this region show the presence of large amounts of lead, cadmium, chromium, and zinc in the industrial wastewater of these factories

(Morshedizadeh et al., 2009; Rahmani, 2009).

In the present work, a group of cyanobacteria was studied for their resistance to soil salinity and environmental pollutants. Our purpose was to investigate the cyanobacterial biodiversity in the wheat fields of Yazd province with an emphasis on some fields adjacent to the industrial areas. In addition, the abundance and diversity of these microalgae, which were influenced by the physico-chemical characteristics of the soil and the concentration of environmental pollutants such as lead and cadmium, were investigated.

Materials and Methods

Study stations and soil sampling

The study area is located in the Yazd-Ardakanplain, the northern part of Yazd province (latitudes: 32º 17′ to 32º 24′ N; longitudes: 53º 55′ to 54º 5′ E, average elevation: 1050 m above sea level). The average amount of rainfall in many parts of this province is less than 65 milliliters per year, and the mean annual rainfall is 61.9 milliliters (Ghahraman and Taghvaeian, 2008). The dominant soil texture is loam, silt loam, and sandy loam textures in this area (Rahimian et al., 2014).

About 81% of the industries and factories of Yazd province are located in the Yazd-Ardakanplain which makes the area one of the most contaminated places with heavy metals. Also, an increase in soil salinity as a result of drought has been reported in this area (Sharifi et al., 2020). Therefore, in this study, stations were selected from wheat fields adjacent to the industrial areas of the Yazd-Ardakan plain. Five study stations were considered for the biological evaluation of farm soils in the distance between Yazd city and Ardakan (Table 1). Soil samples were collected according to the Rangaswamy method (1966).

cultivation, purification, and identification of cyanobacteria

In order to cultivate cyanobacterial samples for the morphological identification of taxa, the soil culture method was used. For this purpose, 10 gr of soil from each station was transferred to sterile plates containing liquid nitrate-free BG11 culture medium (Andersen, 2005). Then the plates were placed in the conditions of 12 hours of light, and 12 hours of darkness, with a light intensity of 74 µmol photons $m^{-2}s^{-1}$, and at a temperature of 25±2 ºC. The morphology

Table 1. Geographical details of the sampling locations

Site	Location	Latitude/Longitude
1	Torkabad	32º 20′ N/54º 58′ E
2	Sadrabad	32º 18′ N/54º 00′ E
3	Shamsi	32º 05′ N/54º 06′ E
4	Kalantar farm	32º 11′ N/54º 05′ E
5	Tabas crossroad	32º 02′ N/54º 12′ E

of cyanobacteria was studied using a light microscope (Olympus, Japan). Samples were classified according to morphological characteristics such as the shape and color of the colony, the length, and width of trichomes, the shape and dimensions of vegetative cells, heterocyst, akinete, the position of the akinete in relation to the heterocyst, the presence or absence of heterocysts, the presence or absence of mucilaginous sheath, apoheterocytic or paraheterocytic form of trichomes. In this study, we used valid identification keys to identify the taxa, such as Komárek (2013), Komárek and Anagnostidis (2005).

Counting of cyanobacterial colonies

The colony counting method was used to determine the frequency of cyanobacterial taxa. For this purpose, after identifying microalgae, one percent of soil extracts were obtained by homogenizing one gram of each station's soil in 100 mL of distilled water. One milliliter of the resulting suspension was transferred to the surface of the solid BG11 medium. After spreading the soil suspension on the surface of the culture medium uniformly, the plates were placed in the conditions of 12 hours of light, and 12 hours of darkness, with a light intensity of 74 µmol photons $m^{-2}s^{-1}$, and at a temperature of 25±2 ºC. The number of colonies was counted and calculated after 21 days.

Soil analysis

To evaluate the degree of correlation between the environmental factors and the diversity and abundance of taxa, the quantitative analysis of the physical and chemical factors of the soils were analyzed, including pH, EC, the concentration of Na^+, K^+, phosphorus, total nitrogen, salts, and the concentration of lead and cadmium. Analysis was performed by Arian FanAzma Company, Tehran, Iran. The method of measuring the factors is presented in Table 2. Soil texture was another factor evaluated in this study by the particle size analysis (PSA) method. For this purpose, the type of soil texture was determined based on the ratios of the amounts and percentages of sand, silt, and clay.

Results

In this study, a total of 32 species of cyanobacteria were identified, including four orders, 10 families, and 18 genera. Among the identified taxa, the order Oscillatoriales (filamentous and non-heterocystous cyanobacteria) had the most diversity with 8 genera and 13 species while the members of the order Chroococcales (unicellular or colonial taxa), only had three genera and four species among the identified taxa. In the reported microflora, *Jaaginema* and *Oscillatoria* showed the highest species diversity with 18% and 15%, respectively (Figure 1).

Although Turkabad station had less cyanobacterial diversity than Tabas crossroad and Kalantar farm stations (Figure 2), the dominant taxa present in the soil of this station, especially members of the *Pseudanabaena*, can develop their communities under environmental stress conditions. Moreover, some members of this genus can fix nitrogen. Among the study stations, Sadrabad station also showed less diversity and abundance of cyanobacteria than other stations (Figure 2).

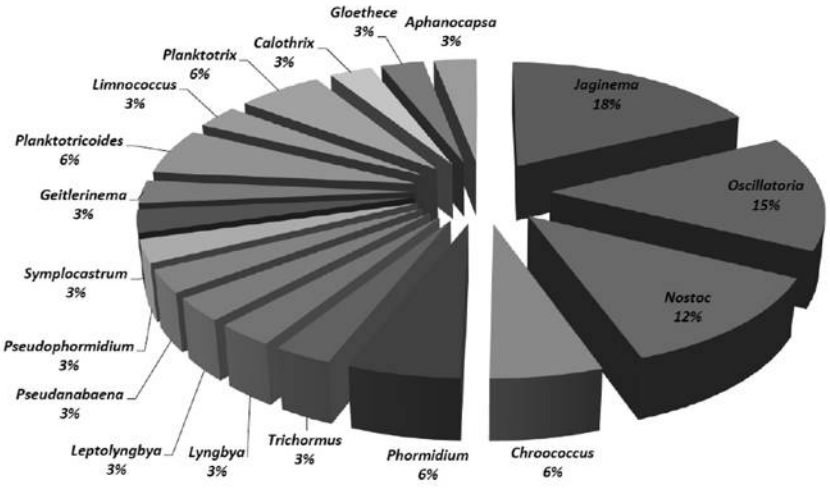

Fig. 1. Frequency percentage of cyanobacterial taxa in soil of studied stations

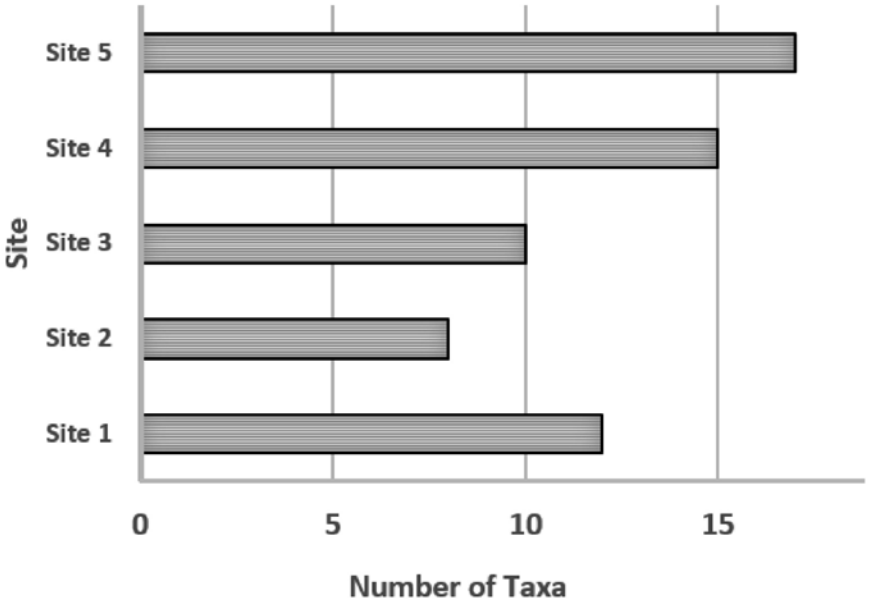

Fig. 2. Genera diversity of cyanobacteria in the studied sites (Site 1. Torkabad, Site 2. Sadrabad, Site 3. Shamsi, Site 4. Kalantar farm, Site 5. Tabas crossroad)

According to the results, the pH of the soil in the studied sites is in the optimal range (between 6.5 and 7) (Figure 3), and only the Kalantar farm station has a pH greater than 7 (Table 2). Tabas crossroad station also showed the lowest pH among the study stations. Based on the results of the present study, the abundance of taxa showed a high correlation with the salinity of the stations (R^2=0.87). In this case, the cyanobacteria abundance increased in the EC of 3.03 to 8.85 dSm^{-1}. In the station 1, a higher EC (12.96 dSm^{-1}), a decrease in the diversity and abundance of taxa was observed (Figure 3). An increase in the diversity and abundance of cyanobacterial taxa was also observed in a specific range of sodium concentrations similar to the effect of EC (except site 1).

The soil of station 1 showed a decrease in abundance and diversity of cyanobacteria taxa in a high concentration of sodium 89.54 $mEqL^{-1}$ (Figure 4).

According to the results, the amount of cadmium in the soil of all the studied stations was significantly lower than 0.1 ppm, but the concentration of lead metal varied between 1.602 ppm and 4.044 ppm depending on the study station (Table 2). In our studied, lead concentration up to 2.68 ppm accompanied by an increase in the diversity of cyanobacteria. According to Figure 5, growth in diversity and abundance of cyanobacteria are observed by increasing lead concentration in a specific range.

Phosphorus and nitrogen are essential nutrients, which have a significant effect on soil microflora and impact on the development of biological communities in the soil. In this study, the amount of soil nitrogen did not show a significant correlation with the abundance and diversity of cyanobacteria. Besides, based on the results, an increase in soil phosphorus up to 30 ppm increased the cyanobacterial abundance. However, in the Shamsi station an increase in phosphorus (54.48 ppm) was associated with a decrease in the abundance and diversity of cyanobacteria (Table 2). Our results show that the soil's percentage of sand and silt correlates with the species diversity of the investigated stations. The correlation was particularly high concerning the percentage of sand, and the diversity and abundance of cyanobacteria decreased in most stations with the increasing amount of sand. In contrary to the negative correlation between the diversity and abundance of cyanobacterial taxa with the percentage of sand in the soil, the diversity of cyanobacteria species increased with the increase in the silt

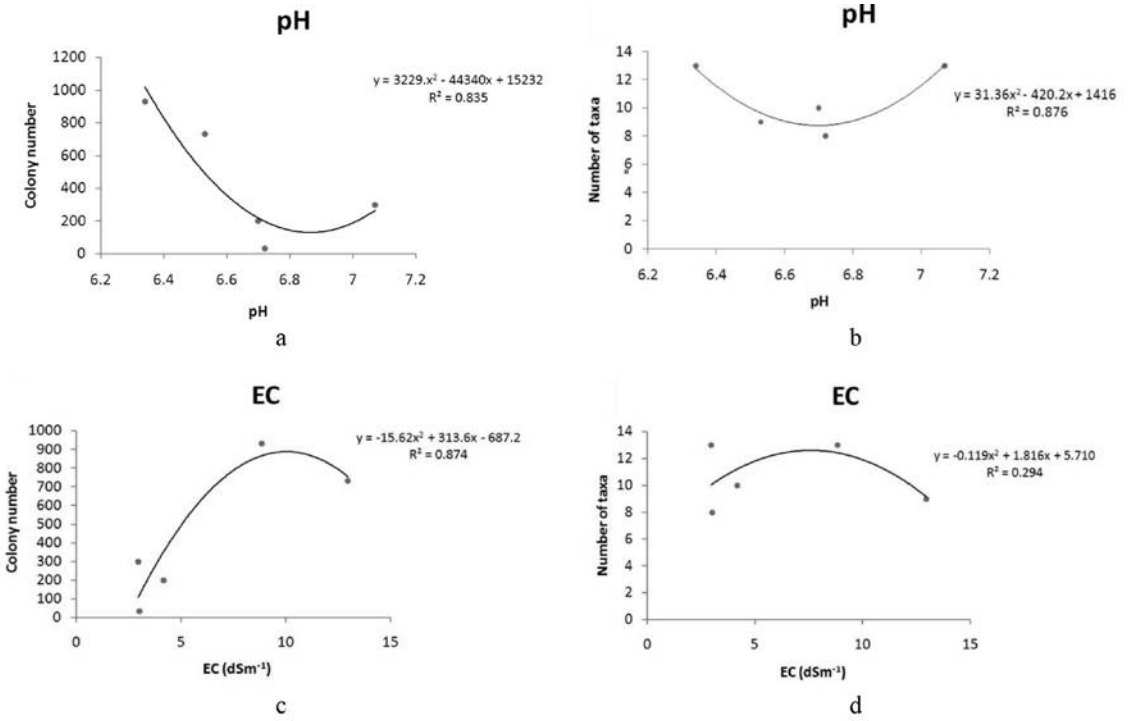

Fig. 3. a, b. The correlation curve between pH and colony number and number of taxa; c, d. The correlation curve between EC and colony number and number of taxa

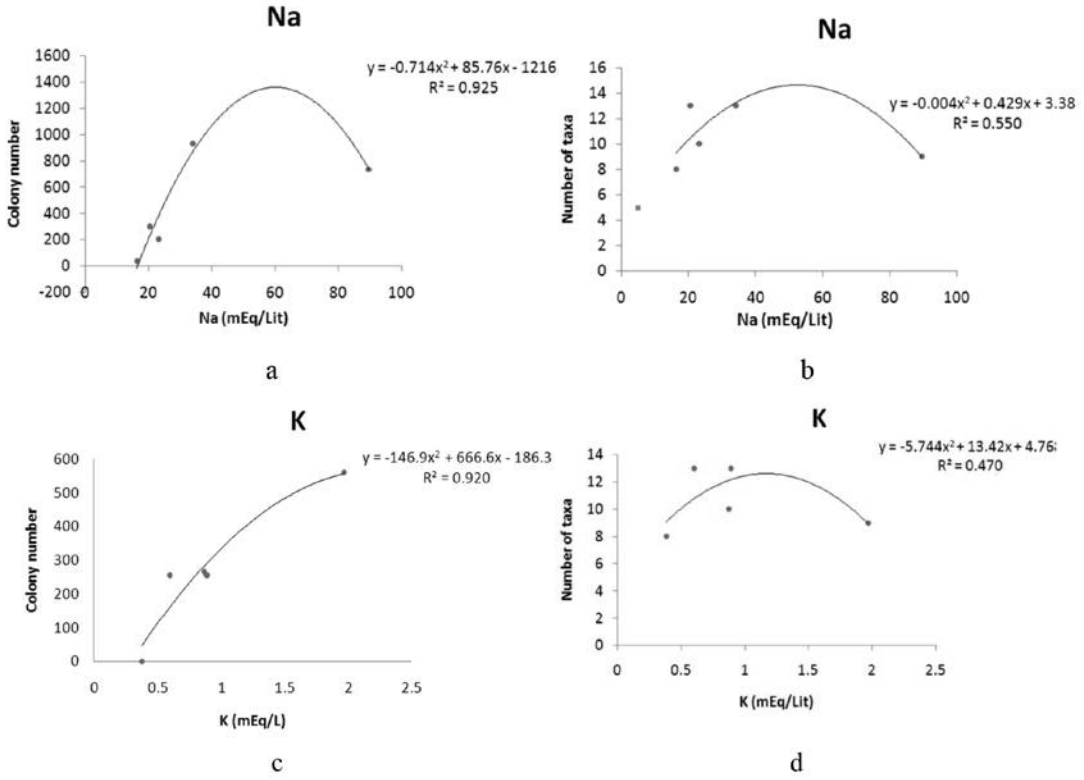

Fig. 4. a, b. The correlation curve between Na^+ concentration and colony number and number of taxa; c, d. The correlation curve between K^+ concentration and colony number and number of taxa

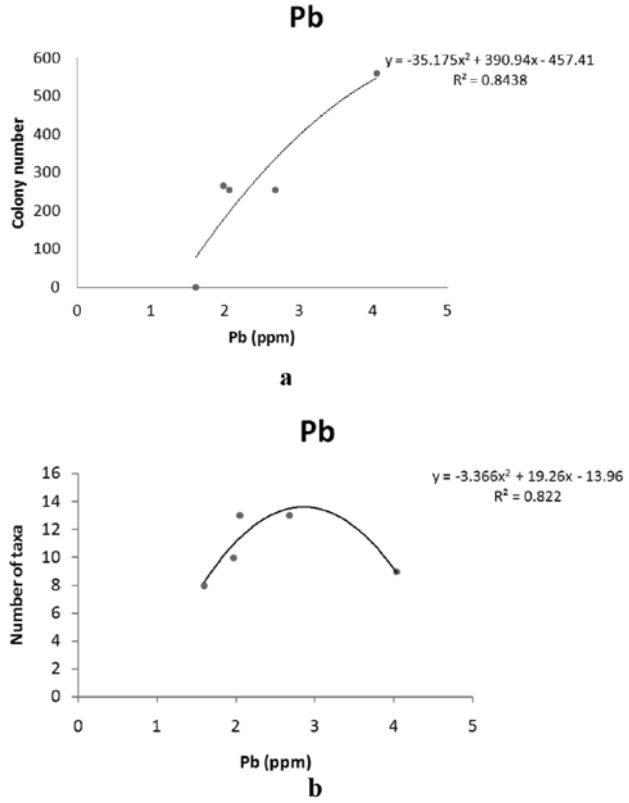

Fig. 5. a, b. The correlation curve between Pb concentration and colony number and number of taxa

Table 2. Physical and chemical data of soils collected from five stations

Parameters	Method	Sites				
		Site 1	Site 2	Site 3	Site 4	Site 5
pH	Electrometric	6.53	6.72	6.70	7.07	6.34
EC (dSm^{-1})	Platinum Electrode	12.96	3.03	4.18	2.98	8.85
Cd (ppm)	Atomic absorption	0.1>	0.1>	0.1>	0.1>	0.1>
Pb (ppm)	Atomic absorption	4.04	1.60	1.97	2.68	2.05
Na^+ ($mEqL^{-1}$)	Flame Emission Photometric	89.54	16.18	22.98	20.30	33.95
K^+ ($mEqL^{-1}$)	Flame Emission Photometric	1.97	0.38	0.87	0.60	0.89
Total nitrogen (mgL^{-1})	Macro kjeldahl	0.05	0.04	0.08	0.07	0.02
phosphorus(ppm)	Vanadomolybdophosphoric	14.50	4.96	54.48	10.76	35.40
Sand (%)	-	42.16	58.16	48.16	38.16	78.16
Silt (%)	-	34.00	26.40	26.00	40.40	10.40
Clay (%)	-	23.84	15.44	25.84	21.44	11.44
Texture	PSA method	L	SL	SCL	L	SL

Site 1. Torkabad, Site 2. Sadrabad, Site 3. Shamsi, Site 4. Kalantar farm, Site 5. Tabas cross road, L:Loam, SL:Sandy Loam, SCL:Sandy Clay Loam

in most of the studied stations, except for station 5. However, the abundance of taxa did not show a high correlation with the amount of silt in the soil (Figure 6).

Discussion

Microalgae, especially cyanobacteria, are an important part of soil microbial communities. The high adaptability of these microorganisms allows them to be present in many habitats such as the soil of terrestrial ecosystems. Soil is a complete ecosystem affected by the interaction of various elements, including biological, physical, and chemical factors. This tripartite interaction creates many vital relationships in nature. In other words, just as the microorganisms in the soil are able to influence the structure of the soil and its compounds, the physical and chemical properties of the soil, including soil texture, pH, EC, and minerals can also influence the formation of biological communities (Sneha et al., 2021; Santra, 1993).

Among the physical properties of soil, pH is the most critical factor affecting the diversity and growth of cyanobacterial communities (Nayak and Prasanna, 2007). The neutral to slightly alkaline pH range is the most suitable pH for the optimal growth of these microalgae (Shariatmadari et al., 2013). On the other hand, the acidic pH range (between 4 and 5) is considered a stressful environment for cyanobacteria. Based on the results of the present study, the investigated soil samples did not show any noticeable difference in pH, and the pH of the soil of the studied sites is in the optimal range (between 6.5 and 7) (Figure 3). Therefore in our study, pH cannot be considered a determining factor in relation to species abundance and diversity.

Salinity is another environmental factor that can affect the diversity and development of cyanobacterial communities in different

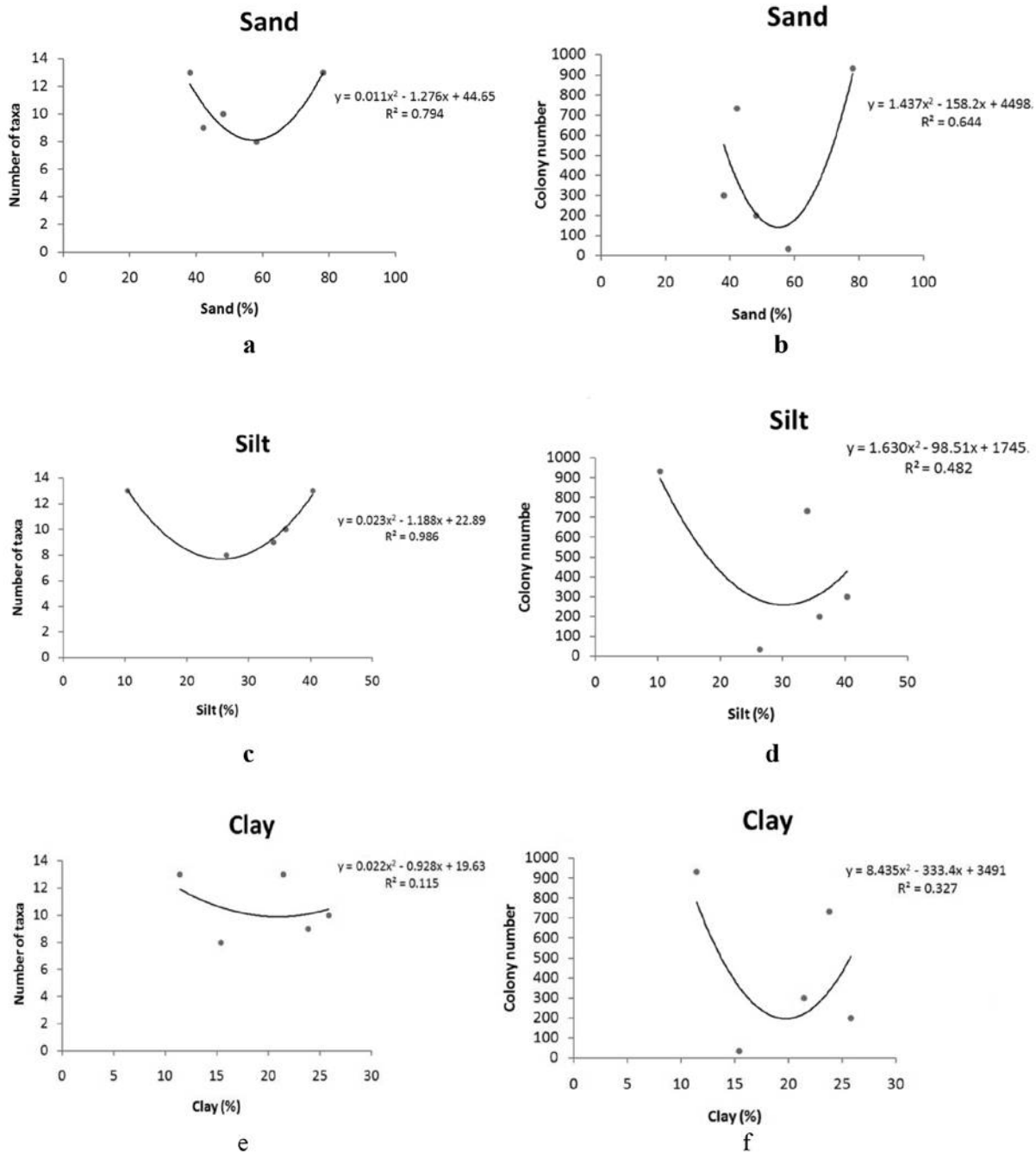

Fig. 6. a-f. a, b. The correlation curve between sand (%) and number of taxa and colony number; **c, d.** The correlation curve between silt (%) and number of taxa and colony number; e, f. The correlation curve between clay (%) and number of taxa and colony number

habitats by influencing the physiological activities of cyanobacteria, such as photosynthesis, growth, and nitrogen fixation (Srivastava et al., 2009). In the present study, the soil salinity of the studied farms is moderate to non-saline (Table 2). Although a decrease in the diversity and abundance of algae was expected with increasing salinity levels, the reduction was not necessarily observed.

According to some reports, non-heterocystous cyanobacteria grow better in saline soils (Srivastava et al., 2009). In the present study, the frequency of two

genera *Jaaginema* and *Oscillatoria* were 18% and 15%, respectively. The estimation showed that these non-heterocystous taxa were the most diverse genera in the examined soil (Figure 1). In other respects, it has been shown that heterocyst-forming cyanobacteria, such as *Anabaena* and *Nostoc* species, are also resistant to salinity and play an important role in maintaining the fertility and productivity of saline soils (Zahran et al., 1992). In the present study, the members of the genus *Nostoc* had relatively high diversity and accounted for about 12% of the total cyanobacterial diversity of the study station's soil (Figure 1).

Based on the results, the abundance of taxa showed a high correlation with the salinity of the stations (R^2=0.87). The concentration of different salts, like sodium, affects the EC value. This study observed an increase in the diversity and abundance of cyanobacterial taxa in a specific range of sodium concentrations (Figure 4). Therefore, it can be said that the cyanobacterial flora of the study area is generally halophilic, and the increase in salinity level is tolerated by some native taxa in the studied sites.

Physical characteristics of soil (soil texture) and their effect on the diversity and abundance of cyanobacteria have been mentioned less in previous studies. In this research, besides studying the effect of other physical and chemical soil factors on species diversity, the effect of soil texture on the diversity and abundance of cyanobacteria was also investigated.

According to the results of our study, the percentage of sand and silt in the soil shows a correlation with the cyanobacterial species diversity. In other words, the diversity and abundance of cyanobacteria decreased in most stations with the increasing amount of sand. Some previous studies also show that the diversity of cyanobacteria in sandy soils is much lower than non-sandy soils (Bhatnagar et al., 2008). One of the reasons for the negative correlation between the percentage of soil sand and the cyanobacterial species diversity is that an increase in the percentage of sand reduces the amount of the active part of the soil and causes a decrease in the ability to retain soil moisture. Obviously, with the decrease in the humidity level, there will be the possibility of establishing more limited communities of these moisture-loving algae. Notably, the results of studies conducted in recent years show that the establishment of resistant cyanobacteria in sandy soils could gradually improve the texture and performance of the soil (Issa et al., 2001). Thus, soil inoculation with cyanobacteria has been proposed as a sustainable biotechnological technique for rehabilitating degraded areas and dry lands (Issa et al., 2001).

Other factors examined in this study include the concentration of lead and cadmium in the soil. Pinchasov et al. (2006) believed that lead toxicity is not only dependent on its concentration in the environment, but the resistance of living organisms present in that environment, the chemical form of lead and its solubility and ease of entry into the cell are the factors that matter to the subject. According to the results of our study, with the increase of lead concentration in a certain range, an increase in the diversity and abundance of algae is observed.

Although, this can be affected by other environmental factors, what is clear is that the lead concentration up to 2.68 ppm has not caused a reduction in soil cyanobacterial diversity and has not had a limiting effect on the native taxa of the region. However, a decrease in diversity has been observed at a concentration higher than this value, which can indicate inappropriate concentration range of lead for native cyanobacteria in this region.

Based on the results, despite the high lead concentration in the soil, the Turkabad station has a relatively high abundance of cyanobacteria. Unlike the abundance of algae observed in this site, the results showed low species diversity in this station compared to the Tabas crossroad and the Kalantar farm stations. The higher abundance of cyanobacteria, despite their low diversity of them in the Turkabad station, can be due to the higher resistance of the dominant taxon, *Pseudanabaena* sp. against high concentrations of heavy metals. In other words, considering the high concentration of lead in the soil of the Turkabad station, it seems that the cyanobacterial taxa present in this station are heavy metal-resistant. Despite their low diversity, they can form communities in extreme environments.

The diversity and abundance of cyanobacteria in the soil of the studied wheat fields depend on the sum of the evaluated items, including the physical and chemical factors of the soil, and the resistance level of the taxa to various environmental factors. In the studied stations, the abundance and diversity of cyanobacteria showed a high correlation with the EC, pH, as well as sodium and lead concentration of soil.

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References

Ahlesaadat M, Riahi H, Shariatmadari Z, HakimiMeybodi MH. (2017). A taxonomic study of cyanobacteria in wheat fields adjacent industrial areas in Yazd Province. Rostaniha. 18 (2): 107-121.

Alam R, Ahmed Z, Howladar MF. (2020). Evaluation of heavy metal contamination in water, soil and plant around the open landfill site Mogla Bazar in Sylhet, Bangladesh. Ground Water for Sustainable Development. 10: 100311.

Andersen RA. (2005). Algal culturing techniques. Elsevier academic Press. 578 pp.

Barton LL, Northup DE. (2011). Microbial Ecology. Wiley-Blackwell Press. 440 pp.

Bhatnagar A, Basha M, Garg MK, Bhatnagar M. (2008). Community structure and diversity of cyanobacteria and green algae in the soils of Thar Desert (India). Journal of Arid Environments. 72 (2): 73-83.

Chookalaii H, Riahi H, ShariatmadariZ, Mazarei Z, SeyedHashtroudi M. (2020). Enhancement of total flavonoid and phenolic contents in *Plantago major* L. with plant growth promoting

cyanobacteria. Journal of Agricultural Science and Technology. 22 (2): 505-518.

Dashtakian K, Baghestani N. (2002). Vegetation Types of Yazd Area. Research Institute of Forests and Rangelands. 125 pp.

Fawzy MA and Mohamed AK.(2017). Bioremediation of heavy metals from municipal sewage by cyanobacteria and its effects on growth and some metabolites of Beta vulgaris. Journal of Plant Nutrition. 40 (18): 2550-2561.

Ghaeminia M, HakimzadehArdakani MA, Taghizadeh-Mehrjardi R, Dehghani F. (2019). Mapping soil salinity of the Northern region of Yazd-Ardakan Plain using EM38 instrument and ESAP modeling software. Iranian Journal of Soil Research. 33 (2): 241-252.

Ghahraman B, Taghvaeian S. (2008). Investigation of annual rainfall trends in Iran. Journal of Agricultural Science and Technology. 10(1): 93-97.

Heidari F, Riahi H, Aghamiri MR, ShariatmadariZ, Zakeri F. (2017). Isolation of an efficient biosorbent of radionuclides (226Ra, 238U): green algae from high-background radiation areas in Iran. Journal of Applied Phycology. 29 (6): 2887-2898.

Hokmollahi F, Riahi H, Soltani N, Shariatmadari Z, HakimiMeybodi MH. (2016). A taxonomic study of blue-green algae based on morphological, physiological and molecular characterization in Yazd province terrestrial ecosystems (Iran). Rostaniha. 16 (2): 152-163.

Issa OM, Bissonnais YL, Défarge C, Trichet J. (2001). Role of a cyanobacterial cover on structural stability of sandy soils in the Sahelian part of western Niger. Geoderma. 101 (3-4): 15-30.

Jafary M. (2000). Saline Soils in Natural Resources (Diagnosis and Reclamation). University of Tehran Press. 195 pp.

Komárek J. (2013). Cyanoprokaryota 3. Teil / 3rd part: Heterocytous Genera. 1st ed. Germany: Springer spectrum.

Komárek J and Anagnostidis K. (2005). Cyanoprokaryota 2. Oscillatoriales. – In: Büdel, B., KrienitzL.,Gärtner G. & Schagerl M. (eds): Süsswasserflora von Mitteleuropa. 19/2. -759 p., Elsevier/ Spektrum, Heidelberg.

Litalien A and Zeeb B. (2020). Curing the Earth: A R eview of Anthropogenic Soil Salinization and Plant-Based Strategies for Sustainable Mitigation. Science of the Total Environment. 698: 134235.

Morshedizadeh M, Afkhami M, Zarei H. (2009). Determination of heavy metals in ground water resources near tile and ceramic industry of Mayboud. First congress of Hydrogeology, Behaban. Azad University of Behbahan.

Nayak S and Prasanna R. (2007). Soil pH and its role in cyanobacterial abundance and diversity in rice field soils. Ecology and Environmental Research. 5 (2): 103-118.

Pinchasov Y, Berner T, Dubinsky Z. (2006). The effect of lead on photosynthesis, as determined by photoacoustics in Synechococcus leopoliensis (Cyanobacteria). Water, Air, and Soil Pollution. 175 (1-4): 117-125.

Prajapati K and Modi HA. (2012). The

importance of potassium in plant growth - A review. Indian Journal of Plant Sciences. 1 (2-3): 177-186.

Qadir M, Oster JD, Schubert S, Noble AD, Sahrawat KL. (2007). Phytoremediation of sodic and saline-sodic soils. Advances in Agronomy 96:197-247.

Rahimian MH, Taghvaeian S, Asce AM, Nouri MR, Tabatabaei SH, Mokhtari MH, Hasheminejhad Y, Neshat E. (2014). Estimating Pistachio evaporanspiration using MODIS imagery: a case study from ardekan, Iran. World Environmental and Water Resources Congress, ASCE. 1784-1794.

Rahmani HR. (2009). Study the most important source of industrial pollutant soil, water and plant in Yazd Province. Journal of Environmental Studies. 35 (51): 10-12.

Rangaswamy G. (1966). Agricultural Microbiology. Asia Publishing House, Bombay.

Rocha F, Lucas-Borja ME, Pereira P, Muñoz-Rojas M. (2020). Cyanobacteria as a nature-based biotechnological tool for restoring salt-affected soils. Agronomy. 10: 1321.

Santra SC. (1993). Biology of rice fields blue-green algae. Daya Publishing House. 184 pp.

Sarmah P and Rout J. (2020). Chapter 22 - Role of algae and cyanobacteria in bioremediation: prospects in polyethylene biodegradation, Editor(s): Prashant Kumar Singh, Ajay Kumar, Vipin Kumar Singh, Alok Kumar Shrivastava,. Advances in Cyanobacterial Biology, Academic Press.

Saul-TcherkasV and Steinberger Y. (2009). Substrate utilization patterns of desert soil microbial communities in response to xeric and mesic conditions.Soil Biology and Biochemistry. 41 (9): 1882-1893.

Shariatmadari Z, Riahi H, SeyedHashtroudi M, Ghassempour AR, Aghashariatmadary Z. (2013). Plant growth promoting cyanobacteria and their distribution in terrestrial habitats of Iran. Soil Science and Plant Nutrition. 59: 535-547.

Sharifi A, SoltaniGorfaramarzi S, TaghiZadeh R, Yarmi N. (2020). Investigating the spatial variations of drought indices and their effect on soil salinity in Yazd-Ardakan Plain. The Desert Ecosystem Engineering Journal. 9 (27): 1-12.

Sneha GR, Yadav RK, Chatrath A, Gerard M, Tripathi K, Govindsamy V, Abraham G. (2021). Perspectives on the potential application of cyanobacteria in the alleviation of drought and salinity stress in crop plants. Journal of Applied Phycology. 33: 3761-3778.

Srivastava AK, Bhargava P, Kumar A, Rai LC, Neilan BA. (2009). Molecular characterization and the effect of salinity on cyanobacterial diversity in the rice fields of Eastern Uttar Pradesh, India. Saline Systems. 5 (1): 4.

Šťovíček A, Kim M, Or D, Gillor O. (2017). Microbial community response to hydration-desiccation cycles in desert soil. Scientific Reports.7: 45735.

Štofejová L, Fazekaš J, Fazekašová D. (2021). Analysis of heavy metal content in soil and plants in the dumping ground of magnesite mining factory Jelšava-

Lubeník (Slovakia). Sustainability. 13: 4508.

Zahran HH, Moharram AM, Mohammad HA. (1992). Some ecological and physiological studies on bacteria isolated from salt-affected soils of Egypt.Journal of Basic Microbiology. 32: 405-413.

Zhang WW, Wang C, Xue R, Wang LJ. (2019). Effects of salinity on the soil microbial community and soil fertility. Journal of Integrative Agriculture. 18 (6): 1360-1368.

Response of Antioxidant Enzymes to Colchicine and Phytohormones Treatments in *Dunaliella salina*

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Abstract

In this experiment, we compared the antioxidative response and biomass production of three ploidy levels induced by three colchicine concentrations (0, 0.1, and 0.5%) and the effects of different concentrations of two phytohormones (auxin and gibberellin) on *D. salina*. The fresh and dry weight of algae treated with colchicine significantly increased. Auxin (1 µM) and gibberellin (10 µM) caused a further increase in biomass in colchicine-treated algae. Colchicine treatment induced catalase and superoxide dismutase activity, but peroxidase activity showed a decrease under this condition. Auxin only increased the superoxide dismutase activity at concentrations of 10 and 100 µM. The catalase activity decreased in the treated algae with 1 and 10 µM auxin. Different auxin concentrations caused an induction in the mentioned enzymes in the colchicine-treated algae. The most increase was observed in catalase activity by adding 1 µM auxin to the colchicine-treated algae. Gibberellin at concentrations of 1 and 10 µM induced peroxidase, catalase, and superoxide dismutase activity. Gibberellin caused a considerable increase in enzyme activity in colchicine-treated algae. The results show that polyploidy along with phytohormones increases the activity of antioxidant enzymes and thus gives the algae the potential for better stress resistance.

Key words: Auxin; Gibberellin; Biomass, Polyploidy; Antioxidant enzyme

Introduction

Dunaliella salina is a green microalga that has many benefits as promising sources for diverse applications, including food for humans, animal feed, and cosmetics due to its provitamin and antioxidant functions. It has been proven that *Dunaliella* is a significant natural source of antioxidants (β-carotene and lutein), to protect against the harsh condition, and glycerol to protect against osmotic pressure (Lamers et al. 2008; Raja et al. 2007).

This alga can be easily cultured in laboratory conditions with a relatively high growth rate and an ability to survive in various environmental conditions compared to other algae. *D. salina* is present at various locations worldwide. The halotolerant

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strains can survive even at 5 M NaCl by maintaining a gradually low intracellular ion concentration, and also by forming compatible solutes such as glycerol; the cells strive to maintain the structure and volume of the cell (Ahmed et al. 2017). This offers an opportunity for commercial biological production of these substances.

Polyploid organisms contain more than two complete sets of chromosomes in the nucleus. Polyploids have naturally induced in different organisms (Baatout 1999). Polyploids can also be experimentally induced in both animals and plants. Colchicine is an alkaloid extracted from seeds or corms of *Colchicum autumnale* L. that can induce polyploidy by tubulin disruption. Polyploid plants may have a wide variety of uses including overcoming hybridization barriers, improving stress tolerance, improving pest resistance, and restoring fertility in wide hybrids (Levin 1983). There are few types of research about the induction of polyploidy in algae and its effects on the physiology and biochemistry of these microorganisms.

It has been proved that phytohormones are present in algae and have regulating roles in metabolism. Phytohormones are widely used as an effective and economical way to achieve high cell density in algal cultures. Gibberellins and auxins are two types of these regulators that involve in numerous aspects of cell growth and development. Positive effects of auxin and gibberellin on the growth and biosynthesis of bioactive compounds have been reported (Lu et al. 2010; Dao et al. 2018; Mansouri and Talebizadeh 2016; Mansouri and Talebizadeh 2017; Mansouri and Nezhad 2020).

In this study, we investigated the effect of colchicine and phytohormone treatments and also their interaction effects on the activity of antioxidant enzymes in *D. salina*.

Materials and methods

Growth conditions

Dunaliella salina was isolated (Sharma et al. 2012) from the Salt River of Shahdad (30° 24′ 16.164″ N 57° 40′ 57.828″ E) in autumn 2017, in Kerman, Iran, and identified based on physiological and morphological descriptions in the references cited in (Massyuk 1973) and Borowitzka and Siva (Borowitzka and Siva 2007; Joseph and Roe 1955). EC and pH of river water were 33.5 ds cm^{-1} and 7.75 respectively. Algae were cultured on the agar plate. After 2–3 weeks, each colony was transferred to a 20 ml liquid growth medium (Artificial Seawater, ASW) with NaCl added to obtain the required salinity medium at 2 M (pH 7.5) (Raja et al. 2007).

The cultures were incubated in a growth chamber under a 16/8 h light-dark provided by cool white fluorescent lamps at an intensity of 49 μmol photons $m^{-2}s^{-1}$ at 25 ± 2°C and were shaken manually twice a day to ensure uniform illumination of the cells.

Colchicine and phytohormones treatments

Haploid cells of *D. salina* were treated with concentrations of 0.1 and 0.5% colchicine (Sigma-Aldrich) and made in culture solution for 36 h. Centrifugation was used

to separate cells from culture media and pellets were washed completely two times with culture solution to free algae from colchicine traces and then were finally transferred to a sterile fresh medium for 21 days. For phytohormone treatment, the fresh culture mediums containing 0, 1, 10, and 100 µM auxin (indol-3-butyric acid) and gibberellic acid (GA_3) (Merk, Hamburg, Germany) in 3 replications were provided, and inoculated with 5×10^6 cells from stock cultures of every colchicine treatment (0, 0.1 and 0.5%). After 3 weeks, all samples were centrifuged and the pellets obtained were then frozen and stored at -70°C before analysis.

Biomass measurement

Biomass was determined by filtering 20 ml of algal culture through a pre-weighed Whatman GF/C filter. The filter with algae was dried overnight at 60°C in a hot air oven and weighed again to estimate the final dry weight. To obtain fresh weight, the Whatman filter was wetted with culture medium and then weighed. After filtering 20 ml algal culture by vacuum pump, the wetted filter with fresh biomass was weighed again.

Enzyme assay

Enzymes were extracted by grinding 0.15 g of fresh algae in a porcelain mortar containing 1.5 ml phosphate buffer containing 50 mM (pH 7.5) ethylene diamine tetraacetic acid (EDTA), 1 mM phenyl methane sulfonyl fluoride (PMSF) and polyvinylpyrrolidone (PVP) 1%. The extract was centrifuged for 15 min at 4°C at 14,000 g and the supernatant was assayed for enzymatic activity and quantification of protein by the Bradford method (Bradford 1976). All operations were performed at 4 °C. The activity of superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC1.11.1.6), and guaiacol peroxidase (GPX) (EC 1.11.1.7) was determined according to the methods used by Giannopolitis and Ries (Giannopolitis and Ries 1977), Azevedo et al. (Azevedo et al. 1998) and Urbanek et al. (Urbanek et al. 1991), respectively.

Statistical analysis

The experiment was arranged in a completely randomized design with three replicates. SPSS software was used for statistical analysis, and graphs were plotted by Excel software. Means were compared using Duncan's multiple range tests at $P < 0.05$.

Results

Changes in fresh and dry weight in colchicine and phytohormones treatments

Effects of auxin on the fresh and dry weight of treated algae were shown in Fig. 1. Fresh and dry weight of algae treated with 1 and 10 µM auxin significantly increased (15 and 42.8% respectively) in comparison to the control. Also in treated algae with colchicine, the fresh and dry weight increased. In colchicine-treated algae, adding 1 µM auxin improved growth, and the highest fresh and dry weights were obtained in the combined treatment of colchicine (0.1 and 0.5%) and 1 µM auxin with 45.89 and 61% increase in comparison to control.

According to the results, the fresh and dry weights increased with increasing

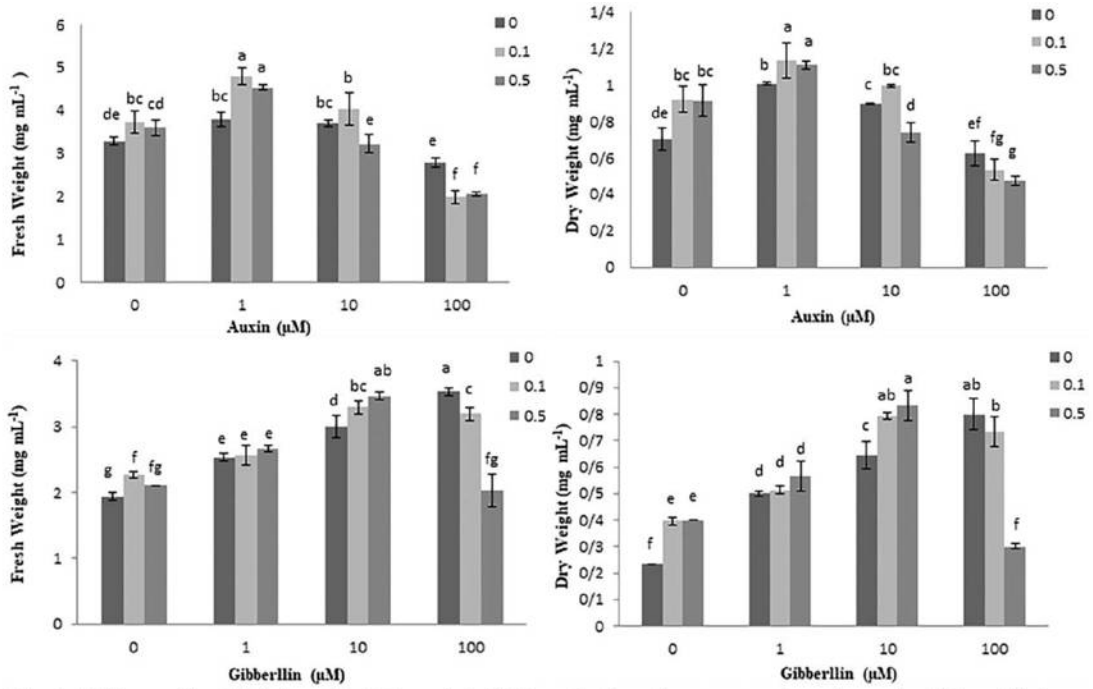

Fig.1. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on fresh and dry weight in *D. salina*. Values are means of three-replications±standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

gibberellin concentration to 100 µM (Fig. 1). The enhancement effect of gibberellin was dependent on its concentration. At a concentration of 100 µM Gibberellin, the fresh and dry weights of algae increased by 1.8 and 3.47 times respectively in comparison with the control. Colchicine treatment on both levels significantly increased the fresh and dry weights of algae. The effect of colchicine on dry weight gain was greater than the fresh weight. In algae treated with colchicine, 10 µM gibberellin increased the fresh and dry weights of algae. At 100 µM of gibberellin, fresh weights decreased significantly at both polyploid levels (0.1% and 0.5%). In this regard, 0.5% colchicine had a greater effect on fresh weight loss. A 0.5% colchicine treatment significantly reduced dry weight in treated cultures with 100 µM gibberellin.

Peroxidase activity in colchicine and phytohormones treatments

Treatment with 1 and 10 µM auxin reduced the activity of peroxidase in the *Dunaliella* algae relative to the control (Fig. 2). Both concentrations of colchicine also reduced enzyme activity. The highest peroxidase activity was observed in the treatment of 100 µM auxin and 0.1% colchicine. The 10 µM auxin in the algae treated with both levels of colchicine (0.1 and 0.5%) increased the activity of peroxidase compared to the treatment without colchicine and indicated the effect of polyploidy on increasing the activity of this enzyme.

Results of Figure 2 indicate that 1 and 10 µM gibberellin treatments significantly increased peroxidase activity in comparison

with the control. Colchicine treatment at both levels decreased the enzyme activity. In the simultaneous treatments, the use of 10 and 100 µM gibberellin increased the activity of peroxidase in polyploid algae induced almost 2 times by 0.1 and 0.5% colchicine. The algae treated with both levels of colchicine at a concentration of 1 µM gibberellin showed less enzyme activity than non-polyploidy cultures.

Catalase activity in colchicine and phytohormones treatments

Only 10 µM of auxin increased the catalase activity in comparison with the control (Fig. 3). Algae treated with 0.1% and 0.5% colchicine indicated higher enzyme activity than the control, but there was no significant difference between the two levels of colchicine. Adding 1 µM of auxin to treated algae with 0.1% and 0.5% of colchicine increased the catalase activity by almost 2 times. Furthermore, 100 µM auxin treatment also increased the catalase activity by 34% in algae treated with 0.5% colchicine in comparison to control samples.

Figure 3 shows the results of the effects of gibberellin and colchicine treatments on catalase activity. 1 and 10 µM gibberellin treatments significantly increased the catalase activity. The enzyme showed more

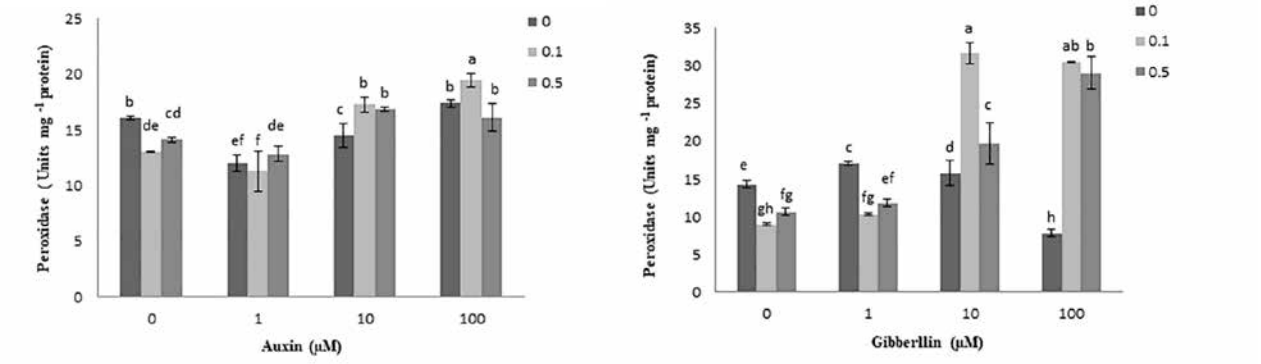

Fig. 2. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on peroxidase activity in *D. salina*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

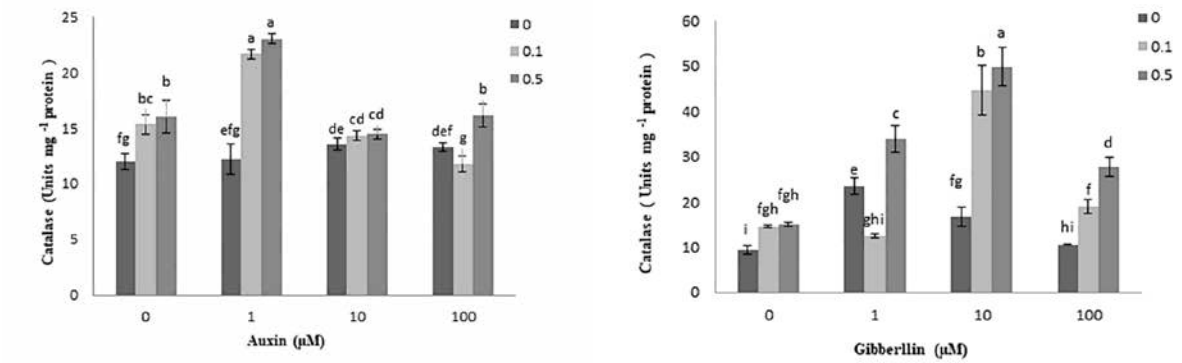

Fig. 3. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on catalase activity in *D. salina*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

activity in algae treated with colchicine. All concentrations of gibberellin (except 1 µM) caused an increase in the catalase activity in colchicine-treated algae. The enzyme activity was 5.3 times higher than the control in the 10 µM gibberellin and colchicine 0.5% treatment.

Superoxide dismutase (SOD) activity in colchicine and phytohormones treatments

The highest activity of superoxide dismutase was seen in the treatment of 100 µM auxin with a 44% increase compared to the control (Fig. 4). The enzyme activity in algae treated with 0.5% colchicine was 14% higher than the control. Auxin with 1 and 10 µM concentrations, significantly increased the superoxide dismutase activity in algae treated with 0.5% and 0.1% colchicine, but samples, which were previously exposed to 0.1% colchicine in the 100 µM auxin treatment, showed less enzyme activity in comparison to control samples.

Gibberellin treatment alone at concentrations of 10 µM and 100 µM significantly increased and decreased the activity of dismutase superoxide (Fig. 4). Colchicine treatment increased enzyme activity only at a level of 0.5%. Adding gibberellin, especially at a concentration of 10 µM, to cultures treated with colchicine significantly increased enzyme activity; the increase in colchicine 0.1% and 0.5% was respectively 3% and 2.7% higher than the control.

Discussion

The present study found the positive effects of all three-treatment groups: auxin, colchicine, and auxin and colchicine on fresh and dry weights of *Dunaliella* algae. The results indicated an increasing effect of auxin on polyploidy cultures. Auxin-induced mitosis significantly increased the number of cells in *Chlorella vulgaris* (Piotrowska-Niczyporuk and Bajguz, 2014). Reports indicated that the biomass of *Chlorella sorokinian* increased in the presence of 1-naphthaleneacetic acid (NAA) compared with the control (Hunt et al., 2010). It has been reported that auxin might increase

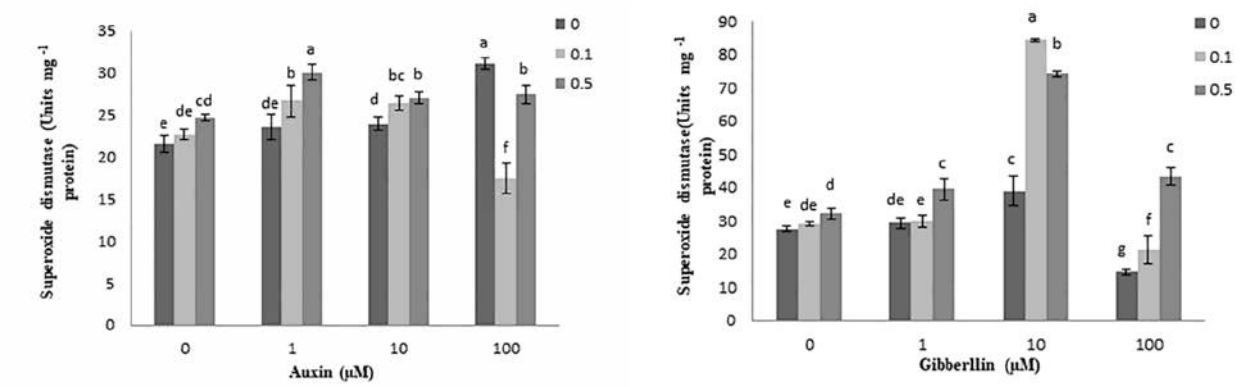

Fig. 4. Effect of colchicine (0, 0.1 and 0.5%) and phytohormones (auxin and gibberellin) on superoxide dismutase activity in *D. salina*. Values are means of three-replications ± standard deviation. Different letters show the significant difference with $p < 0.05$ in one-way ANOVA and Duncan's tests

cell growth through the production of ROS. Some evidence indicates that ROS can be an essential component of the biochemical mechanism involved in loosening during IAA-induced extension growth. It has been indicated that natural auxins (IAA, IBA, and PAA) and synthetic auxins (NAA) decreased the accumulation of ROSs such as H_2O_2 in *C. vulgaris* cells after 48 hours of culture. The finding was according to the antagonistic effects of auxins and ROS on physiological processes. Low levels of ROS have been reported to enhance many cellular processes, including the progression of the cell cycle and the initiation of secondary cell wall differentiation. However, the results indicate that auxin affects algae growth and metabolism through the regulation of ROS levels (Piotrowska-Niczyporuk and Bajguz, 2014). Also, auxin treatment caused an increase in the fresh weight of *Nostoc linckia* algae; and the result was consistent with the results of the present study (Mansouri and Talebizadeh, 2017). As seen in the cultures treated with colchicine 0.5%, the cell division rate decreased in the culture which could be the reason for the reduction of fresh and dry weights of algae (Soltani Nezhad and Mansouri, 2019). Given that the effects of auxin on cell division have been identified, the use of this plant hormone can improve conditions of polyploidy growth. The results of this research indicate the correctness of this hypothesis.

Gibberellins are plant hormones that play important roles in plant growth (Tuna et al., 2008). There are reports of the protective roles of gibberellins in adapting plants to non-biological stresses (Siddiqui et al., 2011). The growth and cell size increase in response to gibberellins have been documented in some algae (Gonai et al., 2004). Results of algae treatment with gibberellin indicated an increase in fresh and dry weights by this hormone so a direct relationship was observed between fresh and dry weights and gibberellin concentration. According to the results of the present study, there was an increase in the number of cells, and thus an increase in the fresh weight of *Chlorella vulgaris* algae treated with gibberellin (Falkowska et al., 2011). These results are similar to those obtained in previous research on blue-green algae, *Nostoc linckia* (Mansouri and Talebizadeh 2016). Also, it was reported that the number of cells and thus dry weight increased in *Microcystis aeruginosa* by GA_3 treatment (Pan et al., 2008).

Treatment with 1 µM auxin reduced the peroxidase activity in Dunaliella algae compared to the control. On the other hand, the highest growth rate was observed in the same treatment. It led to the conclusion that auxin increases growth by reducing oxygen free radicals. In *C. vulgaris*, auxin treatment affected the activity of enzymes involved in ROS scavenging, and levels of H_2O_2 decreased in response to the external application of all synthetic and natural auxins (Piotrowska-Niczyporuk and Bajguz, 2014). In this study, auxin increased the activity of peroxidase in cultures treated with colchicine, indicating the effect of

polyploidy on increasing enzyme activity. The highest activity of superoxide dismutase was seen in the 100 µM auxin treatment, but 1 µM and 10 µM auxin just increased the enzyme activity in polyploid cultures. These results indicated the higher sensitivity of enzymes in polyploidy cells to auxin.

Low concentrations of gibberellin significantly increased catalase and peroxidase activity. Gibberellin treatment increased the catalase and peroxidase activity in all parts of *Catharanthus roseus* in comparison with the control (Jaleel et al., 2010). Gibberellin increased the enzyme activity in cultures treated with colchicine. Treatment with GA3 increased the activity of superoxide dismutase in *C. roseus*. H_2O_2 scavenging systems, which are provided by ascorbate peroxidase and catalase, are more important than superoxide dismutase in coping with oxidative stress (Jaleel et al., 2007). The enzyme activity increased in algae treated with colchicine 0.1% at concentrations of 10 µM and 100 µM gibberellin. The increase was very significant at a concentration of 10 µM.

According to reports, levels of superoxide anhydride and hydrogen peroxide anions were lower in tetraploids than in diploid plants, but the activity of antioxidant enzymes such as superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, and glutathione reductase increased, and antioxidant compounds such as ascorbic acid and glutathione were maintained at high concentrations. Overall results indicated that polyploidy plants had a stronger antioxidant system and were more resistant (Gill and Tuteja, 2010). On the other hand, it is suggested that phytohormones can regulate the synthesis of basic antioxidant enzymes (Szechyńska-Hebda et al., 2007). Auxins may delay protein loss because they stimulate the synthesis of protease inhibitors. In this way, they can increase the number of enzyme molecules and thus increase activity.

Our results showed that treatment with auxin and gibberellin affects the activity of antioxidant enzymes, especially in polyploidy conditions. This feature can give algal cells the ability to cope with stress. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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References

Ahmed RA, He M, Aftab RA, Zheng S, Nagi M, Bakri R, Wang C. (2017). Bioenergy application of *Dunaliella salina* SA 134 grown at various salinity levels for lipid production. Scientific Reports. 7 (1): 1-10.

Azevedo R, Alas R, Smith R, Lea P. (1998).

Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiologia Plantarum. 104 (2): 280-292

Baatout S. (1999). Molecular basis to understand polyploidy. Hematology and cell Therapy. 41 (4): 169-170.

Borowitzka MA and Siva CJ. (2007). The taxonomy of the genus Dunaliella (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. Journal of Applied Phycology. 19 (5): 567-590.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72 (1-2): 248-254.

Dao G-H, Wu G-X, Wang X-X, Zhuang L-L, Zhang T-Y, Hu H-Y. (2018). Enhanced growth and fatty acid accumulation of microalgae Scenedesmus sp. LX1 by two types of auxin. Bioresource Technology. 247:561-567.

Falkowska M, Pietryczuk A, Piotrowska A, Bajguz A, Grygoruk A, Czerpak R (2011) The effect of gibberellic acid (GA3) on growth, metal biosorption and metabolism of the green algae *Chlorella vulgaris* (Chlorophyceae) Beijerinck exposed to cadmium and lead stress. Polish Journal of Environmental Studies. 20 (1): 53-59.

Giannopolitis CN and Ries SK. (1977). Superoxide dismutases: I. Occurrence in higher plants Plant Physiology. 59 (2): 309-314.

Gill SS and Tuteja N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry. 48 (12): 909-930.

Gonai T, Kawahara S, Tougou M, Satoh S, Hashiba T, Hirai N, Kawaide H, Kamiya Y, Yoshioka T. (2004). Abscisic acid in the thermoinhibition of lettuce seed germination and enhancement of its catabolism by gibberellin. Journal of Experimental Botany. 55 (394): 111-118.

Hunt RW, Chinnasamy S, Bhatnagar A, Das K. (2010). Effect of biochemical stimulants on biomass productivity and metabolite content of the microalga, *Chlorella sorokiniana*. Applied Biochemistry and Biotechnology. 162 (8): 2400-2414.

Jaleel CA, Gopi R, Manivannan P, Sankar B, Kishorekumar A, Panneerselvam R. (2007). Antioxidant potentials and ajmalicine accumulation in *Catharanthus roseus* after treatment with giberellic acid. Colloids and surfaces B: Biointerfaces. 60 (2): 195-200.

Jaleel CA, Salem MA, Hasanuzzaman M, Nahar K. (2010). Plant growth regulator interactions results enhancement of antioxidant enzymes in *Catharanthus roseus*. Journal of Plant Interactions. 5 (2): 135-145

Joseph H and Roe H. (1955). The

determination of sugar in blood and spinal fluid with anthrone reagent. Journal of Biological Chemistry. 212: 335-343.

Lamers PP, Janssen M, De Vos RC, Bino RJ, Wijffels RH. (2008). Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. Trends in Biotechnology. 26 (11): 631-638

Levin DA. (1983). Polyploidy and novelty in flowering plants. The American Naturalist. 122 (1): 1-25.

Lu Y, Jiang P, Liu S, Gan Q, Cui H, Qin S. (2010). Methyl jasmonate-or gibberellins A3-induced astaxanthin accumulation is associated with up-regulation of transcription of β-carotene ketolase genes (bkts) in microalga *Haematococcus pluvialis*. Bioresource Technology. 101 (16): 6468-6474.

Mansouri H and Nezhad SE. (2020). Improvement in biochemical parameters and changes in lipid profile of *Scenedesmus obliquus* by plant growth regulators under mixotrophic condition. Biomass and Bioenergy. 140: 105708.

Mansouri H and Talebizadeh B. (2016). Effect of gibberellic acid on the cyanobacterium *Nostoc linckia*. Journal of Applied Phycology. 28 (4): 2187-2193.

Mansouri H and Talebizadeh R. (2017). Effects of indole-3-butyric acid on growth, pigments and UV-screening compounds in *Nostoc linckia*. Phycological Research. 65 (3): 212-216.

Massyuk N. (1973). Morphology, taxonomy, ecology and geographic distribution of the genus Dunaliella Teod. and prospects for its potential utilization. Kiev, Naukova Dumka Massyuk: 312.

Pan X, Chang F, Kang L, Liu Y, Li G, Li D (2008) Effects of gibberellin A3 on growth and microcystin production in *Microcystis aeruginosa* (cyanophyta). Journal of Plant Physiology. 165 (16): 1691-1697.

Piotrowska-Niczyporuk A and Bajguz A. (2014). The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Growth Regulation. 73 (1): 57-66.

Raja R, Hemaiswarya S, Rengasamy R. (2007). Exploitation of Dunaliella for β-carotene production. Applied Microbiology and Biotechnology 74 (3): 517-523.

Sharma P, Agarwal V, Mohan MK, Kachhwaha S, Kothari S. (2012). Isolation and characterization of Dunaliella species from Sambhar Lake (India) and its phylogenetic position in the genus Dunaliella using 18S rDNA. National Academy Science Letters. 35 (3): 207-213.

Siddiqui MH, Al-Whaibi MH, Basalah MO. (2011). Interactive effect of calcium and gibberellin on nickel tolerance in relation to antioxidant systems in *Triticum aestivum* L. Protoplasma 248 (3): 503-511.

Soltani Nezhad F and Mansouri H. (2019). Induction of polyploidy by colchicine

on the green algae *Dunaliella salina*. Russian Journal of Marine Biology. 45 (2): 106-112

Szechyńska-Hebda M, Skrzypek E, Dąbrowska G, Biesaga-Kościelniak J, Filek M, Wędzony M. (2007). The role of oxidative stress induced by growth regulators in the regeneration process of wheat. Acta Physiologiae Plantarum. 29 (4): 327-337.

Tuna AL, Kaya C, Dikilitas M, Higgs D. (2008). The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environmental and Experimental Botany. 62 (1): 1-9.

Urbanek H, Kuzniak-Gebarowska E, Herka K. (1991). Elicitation of defence responses in bean leaves by *Botrytis cinerea* polygalacturonase. Acta Physiologiae Plantarum. 13: 43-50.

Primary Productivity and Phytoplankton Diversity of the Golestan Dam Reservoir, Golestan Province, Iran

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Abstract

Golestan Dam Lake is located 12 kilometers east of Gonbad City and was constructed in 1379. The volume of the Golestan Dam reservoir is 52 million cubic meters and its area is 1500 hectares, which was built for the purposes of agricultural and aquaculture operations.. The present study on phytoplankton biodiversity and its relationship with the primary productivity of the Golestan Dam reservoir was conducted from April to July 2019. Furthermore, water quality parameters such as transparency, pH, alkalinity, free carbon dioxide, air and water temperature, dissolved oxygen, primary productivity, conductivity, nitrate-nitrogen, and orthophosphate were analyzed along with qualitative and quantitative estimation of phytoplankton. The results indicated that the average primary productivity or gross primary production (GPP) was 0.399 ± 0.081 gcm^3h^{-1}, Net Primary Production (NPP) (0.307±0.061 gcm^3h^{-1}) and Community Respiration (CR) 0.094±0.024 gcm^3h^{-1} in the surface. Algae in freshwater have numerous environmental functions and are based upon the recycling of nutrients. Totally 73 species of phytoplankton belonging to different taxonomic group were identified which belonges to 32 genera. Among these 32 genera,12 belongs to Cyanophyceae,8 belongs to Chlorophyceae, 10 belongs to Bacillariophyceae and 2 genera belonges to Euglenophyta. On the basis of mean primary productivity, the fish production potential of reservoir Golestan Dam showed considerable scope for the enhancement of current average production.

Keywords: Golestan Dam, Phytoplankton Diversity, Primary Productivity

Introduction

Iran is a status as a mega-diverse country and a major centre of biodiversity. The aquatic ecosystems of Iran have been subjected to various forms of environmental stress during the past few decades. Most of such environmental problems are manufactured and thus increased human activities in the catchment area of various aquatic systems have affected the natural processes of these systems adversely thereby threatening the survival and growth of biotic communities

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(Khanna et al., 2012).

Golestan Province is also endowed with varied surface freshwater resources like reservoirs, seasonal and a couple of perennial rivers, canals, small tanks and ponds. Golestan Province has having around 21000-sqkm area. Furthermore, eleven reservoirs behind the dam are in operation with a volume of 183 million cubic meters which three tanks ready for operation with a volume of 132 million cubic meters and two investigated reservoirs with a volume of 30.5 million cubic meters (Javid Imanpour et al., 2013).

Golestan Dam is a shallow lake with a catchment area of 5000 sqkm, maximum depth (Zm) of 6 m, and an area of 1500 ha. The lake is totally rain-fed and retains water throughout the year.

Phytoplankton is the main primary producers in water bodies and influence structure and density of consumers and characteristics of water. Moreover, phytoplankton organisms are sensitive indicators, as their structure and metabolism change quickly in response to environmental changes (Mishra et al., 2012). Growth rate and variability of phytoplankton are subject to cyclic changes of fluctuation and succession. Phytoplanktons constitute a major part of aquatic vegetation, they being primary producers, which support the growth of aquatic fauna and produce oxygen by the photosynthetic process (Chinnaiah et al., 2010). Biological parameters such as temperature, transparency, pH, alkalinity, free carbon dioxide, dissolved oxygen, electrical conductivity, nitrate-nitrogen, orthophosphate of any water body grossly determine the trophy status of the water body. Such parameters influence the primary productivity and in turn the growth of the fish. The primary productivity of different water bodies has been widely investigated to assess the fish production potentialities of a water body to formulate appropriate fishery management policies (Khanna et al., 2012). Considering the local conditions in the account, an attempt was made to find out the phytoplankton biodiversity in relation to primary productivity of the Golestan Dam.

Materials and methods

Golestan Dam Lake is located 12 kilometers east of Gonbad city and was opened in 1379. The volume of this dam reservoir is 52 million cubic meters and its area is 1500 hectares, and it was built with the purpose of agriculture and aquaculture. The study of the biological conditions of the this reservoir was carried out based on the initial production of the lake for three months from April 2019 to the end of June of the same year are illustrated in Figure 1.

In the present study, five stations were selected for collection in the Golestan Dam Lake which are illustrated in Figure 1 for collection and weekly analysis. Three stations are located in south eastern, two station in eastern and two stations is in the Western side. The geographical distribution of the selected stations is given in Table 1.

Primary productivity analysis

Primary productivity was measured at all the three stations following light and dark bottles method. For this purpose, glass stopper black and white BOD bottles of 250 ml were used. The bottles were suspended about 15 cm below the water line. The incubation period

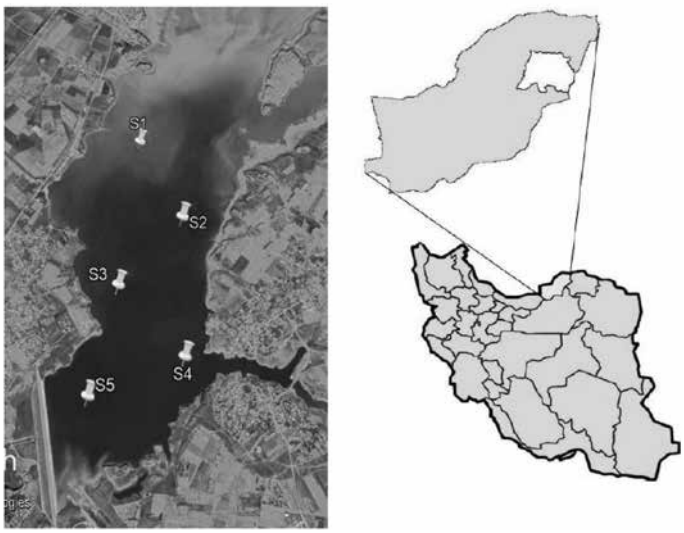

Fig. 1. Location map of Golestan Dam

Table 1. Geographical distribution of stations

	S1	S2	S3	S4	S5
Latitude	37°20'40.86"N	37°20'12.17"N	37°19'55.67"N	37°19'30.44"N	37°19'26.22"N
Longitude	55°17'49.58"E	55°18'0.77"E	55°17'32.40"E	55°17'52.36"E	55°17'16.06"E

was kept three hours. Then, Oxygen (O_2) estimations in the BOD bottles were made following usual Winkler's method (APHA, 2005). The calculation was done as follow.

Gross Oxygen Production (GOP) mg l^{-1} = LB-DB,

Net Oxygen Production (NOP) mg l^{-1} = LB-IB,

Community Respiration (CR) mg l^{-1} = IB-DB.

The values of GPP and NPP were calculated as follow.

Gross Primary Productivity (gcm^3h^{-1}) = GOP × 0.375/1.2 ×h

Net Primary Productivity (gcm^3h^{-1}) = NOP × 0.375/1.2 × h

Where;

LB = Dissolved oxygen in light bottle, DB = Dissolved oxygen in dark bottle

IB = Dissolved oxygen in initial bottle, h = Duration of incubation or exposure

1.2 = A constant, 0.375 A factor value (1 g of oxygen is equal to 0.375 g carbon).

Phytoplankton identification

Firstly, 50 liters of water was filtered through bolting cloth No. 25 (mesh size 60 µm) and obtained planktons was preserved in Lugol's solution for further quantitative and qualitative analyses. Then, quantitative and qualitative analysis was performed according to APHA, 1989. After that, the mass density of phytoplankton was calculated (Boney, 1989; Sourina, 1978). Lastly, samples were identified using valid identification keys (Edmondson, 1959; Presscot, 1970; Maranon, 2015; Sourina, 1978; Maosen, 1983). (Senthilkumar and Sivakumar, 2008). The identification of phytoplankton was limited up to Cynophyceae, Chlorophyceae, Bacillariophyceae and Euglenophyceae.

Resuts

The results of GPP and NPP analysis of

Golestan Dam during the study period (April to July) are presented in Table 2. In general, the GPP ranged between 0.26 to 0.50, 0.23 to 0.47, 0.25 to 0.53, 0.25 to 0.47 and 0.25 to 0.50 gcm^3h^{-1} at stations S1, S2, S3, S4, and S5, respectively. The average values of GPP were 0.25, 0.49 gcm^3h^{-1} and 0.40 gcm^3h^{-1}.

The statistical correlation of GPP was positive with NPP, community respiration (CR) and phytoplankton community structure. The value of net primary productivity (NPP) at stations S1, S2, S3, S4, and S5 ranged from 0.21-0.40, 0.18-0.37, 0.10-0.43, 0.2-0.37 and 0.20-0.40 gcm^3h^{-1}. Furthermore, the average values of NPP were 0.18, 0.39 $gcm^3h^{-1}$and 0.31 gcm^3h^{-1} (Table 2).

The statistical relationship of NPP was positive with GPP and total phytoplankton. The respective values of community respiration (CR) at stations S1, S2, S3, S4 and S5 ranged from 0.05 to 0.13, 0.05 to 0.12, 0.10 to 0.15, 0.05 to 0.13 and 0.05 to 0.15 gcm^3h^{-1}. The corresponding average values of CR were 0.06, 0.14 gcm^3h^{-1} and 0.09 gcm^3h^{-1} (Table 2).

The statistical correlation of CR was positive GPP. However, there was a

Table 2. Weekly observation of Physico-chemical and biological characteristics of surface water at the selected station of Golestan Dam

Gross primary productivity gcm^3h^{-1}					
Gpp	S1	S2	S3	S4	S5
Avg.	0.39	0.37	0.43	0.39	0.42
SD	0.081	0.083	0.09	0.076	0.085
max	0.5	0.47	0.53	0.47	0.5
min	0.26	0.23	0.25	0.25	0.25
CV	0.21	0.23	0.21	0.19	0.20
Net primary productivity gcm^3h^{-1}					
Npp.	S1	S2	S3	S4	S5
Avg.	0.31	0.29	0.31	0.30	0.32
SD	0.055	0.060	0.10	0.052	0.059
max	0.4	0.37	0.43	0.37	0.4
min	0.21	0.18	0.10	0.2	0.2
CV	0.18	0.21	0.32	0.17	0.18
Community Respiration gcm^3h^{-1}					
CR.	S1	S2	S3	S4	S5
Avg.	0.09	0.08	0.12	0.09	0.095
S.D	0.032	0.028	0.02	0.032	0.036
max	0.13	0.12	0.15	0.13	0.15
min	0.05	0.05	0.10	0.05	0.05
CV	0.38	0.37	0.15	0.35	0.38

negative relationship between NPP and total phytoplankton. The results of Means analyses and resulting Weekly variations in Gross Primary Productivity (GPP), Net Primary Productivity (NPP), and Community Respiration (CR) in Sampling stations of Golestan Dam reservoir is shown in Figure 2 and Figure 3.

Phytoplankton community

The phytoplankton community constitutes bulk of primary producers and is the base of food chains in any water bodies. The phytoplankton community of Golestan Dam during the present was represented by four major group's viz., Cyanophyta, Chlorophyta, Bacillariophyta and Euglenophyta. Overall, 73 species of algae were identified (Table 3). Overall, 73 species, belonged to 32 genera Among these 32 genera, 12 were from Cyanophyceae (blue green algae), 8 from

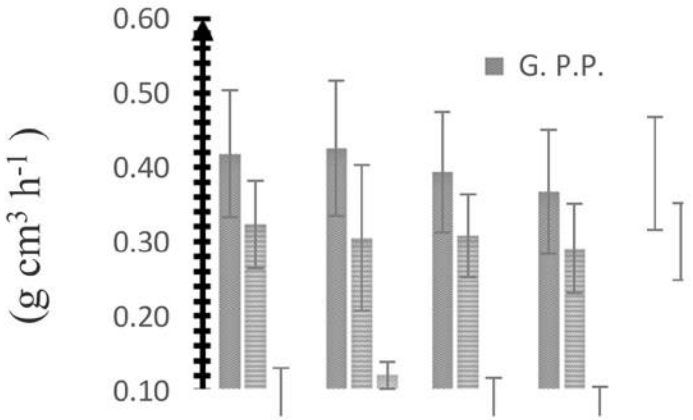

Fig. 2. Mean variations in Gross Primary Productivity (GPP), Net Primary Productivity (NPP), and Community Respiration (CR) in Sampling stations of Golestan Dam reservoir

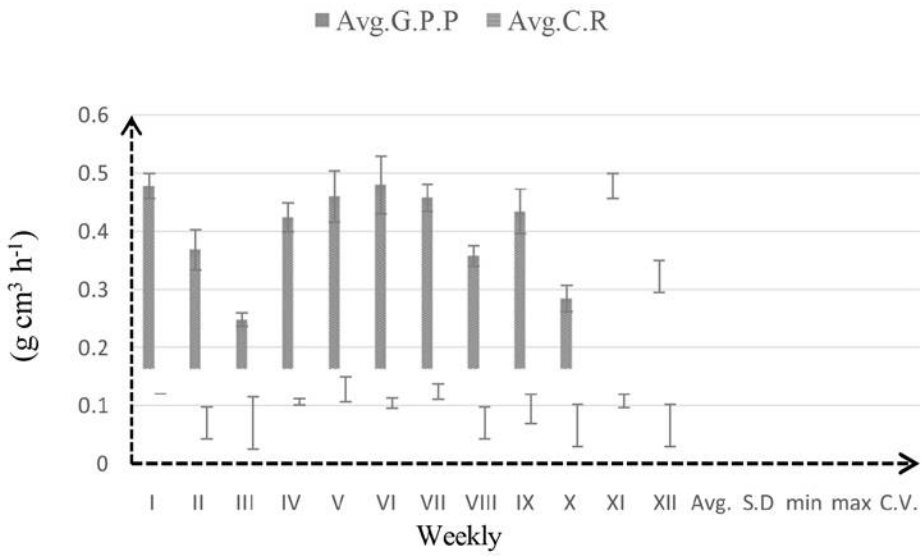

Fig. 3. Weekly variations in Gross Primary Productivity (GPP) and Community Respiration (CR) in Golestan Dam reservoir

Chlorophyceae (green algae), 10 from Bacillariophyceae (diatoms) and 2 belonged to Euglenophyta (Table 4).

Discussion

The most prominent phytoplanktons in the present study were *Microcystis aeruginosa*, *Anabeanaposis* sp., *Lyngbya* sp., *Oscillatoria* sp., and *Merismopedia* sp. It was from Cyanophyceae group and *Ankistrodesmus* sp., *Chlamydomonas* sp., *Oocystis* sp., *Scendesmus* sp., and *Chlorogonium* sp. from Chlorophyceae. As a result, Cyanophyceae was the most dominant group. Some other researches on this reservoir also reported four phytoplankton phyla including Bacillariophyceae, Chlorophyceae, Cyanophyceae, Euglenophyceae. They observed that blue green algae and diatoms are the most abundant phytoplankton in this reservoir. (Imanpour et al., 2022; Azizi et al., 2022; Ghorbani et al., 2016).

Several classes including Cyanophyceae, Bacillariophyceae and Chlorophyceae was observed as the dominant phytoplankton in respect of the total species and density in station 1, which confirms the result obtained by Imanpour et al. (2022). Indeed, this results are in agreement with Naz Türkmen et al. (2005),

Table 3. Composition of phytoplankton population in the stations selected of Golestan Dam reservoir

Class	Order	Familly	Genus	Species
Bacillariophyceae	Chaetocerotanae	Chaetocerotaceae	Chaetoceros	Chaetoceros convolutus *Chaetoceros peruvianus* *Chaetoceros throndsenii* *Chaetoceros simplex* *Chaetoceros mirabilis* *Chetoserus mueelleri* *Chetoserus rigidus* *Chaetoceros socialis* *Chaetoceros subtilis*
	Thalassiosirales	Stephanodiscaceae	*Cyclotella*	*Cyclotella caspica* *Cyclotella menenghiniana*
	Fragilariales	Fragilariaceae	*Diatoma* Synedra	*Diatoma vulgar* *Diatoma ochki* *Synedra amphirhynchus*
	Naviculales	Naviculaceae	*Navicula*	*Navicula bombus* *Navicula cryptocephala* *Navicula* sp.
	Bacillariales	*Bacillariaceae*	*Nitzschia*	*Nitzschia* sp. *Nitzschia* SP.2 *Nitzschia acicularis* *Nitzschia parva* *Nitzschia reversa* *Nitzschia sigma* *Nitzschia sigmoidea* *Nitzschia* sp.1 *Nitzschia tenirustris* *Nitzschia sublinearis*

				Nitzschia closterium
				Nitzschia sp.
				Nitzschia sp.2
				Nitzschia tennuis
				Nitzschia longgisma
				Scletonema costata
	Rhabditida	*Allantonematidae*	*Scatonema*	*Scletonema costatum*
				Scletonema subsalsum
	Thalassiosirales	*Thalassiosiraceae*	*Thalassiosira*	*Thalassiosira variabilis*
	Nostocales	*Nostocaceae*	*Anabaena*	*Anabaenabergii*
				Anabaena aphanizomenoides
				Anabaena spiroides
				Anabaena hisselevii
			Aphanizomenon	*Aphanizominon flos-aqua*
				Aphanizominon sp.
cyanophyceae			*Cylindrospermopsis*	*Cylindrospermopsis racib orskii*
	chroococcales	*chroococcaceae*	*Chroccoccus*	*chroococcus* sp.
	oscillatoriales	*Oscillatoriaceae*	*Lyngbya*	*Lyngbya limnetica*
				Lyngbya SP.
			Oscillatoria	*Oscillatoria limosa*
				Oscillatoria agardhii
				Oscillatoria sp.
				Oscillatoria tennuis
	Oscillatoriophycideae	*Spirulinaceae*	*Spirulina*	*Spirulina* sp.
Trebouxiophyceae	*Chlorellales*	*Chlorellaceae*	*Actinastrum*	*Spirulina laxissma*
			Chlorella	*Actinastrum hantzschii*
		Oocystaceae	*Oocystis*	*Chlorella SP.*
				Oocystis borgi
				Oocystis solitaria
Chlorophyceae	*Sphaeropleales*	*Selenastraceae*	*Ankistrodesmus*	*Oocystis parva*
		Neochloridaceae	*Golenkinia*	*Ankistrodesmus* SP.
		Scenedesmaceae	*Scendesmus*	*Golenkinia Paucispina*
				Scenedesmus bijuga
	Chlamydomonadales	*Chlamydomonadaceae*	*Chlamydomonas*	*Scenedesmus quadricauda*
		Volvocaceae	*Pandorina*	*Clamydomonas* SP.
	Volvocales	*Haematococcaceae*	*Chlorogonium*	*Pandorina morum*
Conjugatophyceae	*Desmidiales*	*Desmidiaceae*	*Cosmarium*	*Chlorogonium* SP.
Malacostraca	*Amphipoda*	*Stegocephalidae*	*Tetradeion*	*Cosmarium* SP.
Trebouxiophyceae	*Chlorellales*	*Chlorellaceae*	*Actinastrum*	*Tetradeion* SP.
	Euglenida	*Euglenaceae*	*Euglena*	*Euglena* SP.
				Euglena acus
				Euglena caudata
Euglenoidea			*Trachelomonas*	*Trachelomonas* SP.1
				Trachelomonas spiculifera
				Trachelomonas verrucosa

Table 4. contribution of different planktonic groups in the selected stations of Golestan Dam reservoir

stations	S1	S2	S3	S4	S5
	(Cells ml^{-1})				
Cyanophyta	26.6	14.2	37.4	40.4	23.2
Chlorophyta	10.6	14.8	12.6	10.2	7.2
Bacillariophyceae	13.6	11.8	11	6.6	13.4
Euglenophyta	2	0.8	1.4	2.4	1.6
Sum	52.8	41.6	62.4	59.6	45.4

Fathi and Ebrahimi (2016), Abolhasani et al., (2019), that reported a higher abundance of Cyanophyceae and Bacillariophyceae in an Anatolian Dam Lake compared to other phytoplankton groupsresults are in agreement. In addition, this results are in agreement with Abolhasani et al. (2018) who reported of Cyanophyceae and Bacillariophyceae as the most dominance classes in the international Gavkhooni Wetland, Iran.

Javid Imanpour et al. (2022) found that the average phytoplankton count in Golestan Dam was 32.31 $Noml^{-1}$ compose of 39 genera belongs to Chlorophyceae, Bacillariophyceae, Cyanophyceae and Desmidiaceae, respectively. Moreover, there is a significant decline in the biodiversity of phytoplankton compared to the present study Therefore, The monthly average values of all five stations of overall mean phytoplankton density were higher at station S3 i.e. (62.4 Cells ml^{-1}), S4 (59.6 Cells ml^{-1}), S1 (52.8 Cells ml^{-1}), S5 (45.4 Cells ml^{-1}), and S2 (41.60 Cells ml^{-1}), respectively. The trend of dominance among the five phytoplankton groups at station S1 was Cyanophyceae> Bacillariophyceae> Chlorophyceae> Euglenophyta, station S2 was Chlorophyceae> Cyanophyceae> Bacillariophyceae > Euglenophyta, at station S3 was Cyanophyceae> Chlorophyceae > Bacillariophyceae> Euglenophyta and at station S4 was Cyanophyceae> Chlorophyceae> Bacillariophyceae> Euglenophyta. However, at station S5 the relative dominance of four algal groups was Cyanophyceae> Bacillariophyceae> Chlorophyceae> Euglenophyta. Overall, the dominance of phytoplankton is similar to the trend found at station S1 (Table 4).

Kumar et al. (2015) were found six groups namely Chlorophyceae, Bacillariophyceae, Desmidiaceae, Xanthophyceae, Myxophyceae, and Dinophyceae represented the phytoplankton community of water body. Total 58 species were identified which 28 belonged to Chlorophyceae, 11 to Bacillariophyceae, 9 to Myxophyceae, 4 to Dinophyceae, 3 to Desmidiaceae, and 3 to Xanthophyceae (Mishra et al. 2016).

Whereas, the average phytoplankton density was 52.36± 8.9 Cells ml^{-1} (Table 4) while the highest phytoplankton density (62.4 Cells ml^{-1}) was observed on Spring 2019 at station S3 and the lowest (41.6 Cells ml^{-1}) during spring in station S2 (Tables 4). Considering the average phytoplankton biomass, Cyanophyceae was the most dominant (26.6, 14.2, 37.4, 40.4, 23.2 Cells ml^{-1}) followed

by Bacillariophyceae (13.6, 11.8, 11.00, 6.6, 13.4 Cells ml^{-1}), Chlorophyceae (10.6, 14.8, 12.8, 10.2, 7.2 Cells ml^{-1}), Euglenophyceae (2.00, 0.80, 1.4, 2.4, 1.6 Cells ml^{-1}) in station S1, S2, S3, S4 and S5, respectively (Table 4). In conclusion, Cyanophyceae and Bacillariophyceae were dominant, followed by Chlorophyceae and Euglenophyta (Table 3). The statistical analysis of phytoplankton density shows a positive correlation with GPP and NPP. However, there was a negative correlation with community respiration (CR).

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Reference

Abolhasani MH, Pirestani N, Ghasemi S. (2018). Assessment of the primary production statues of the international Gavkhooni Wetland, Iran. Aquatic Biology. 6 (5): 248-253. DOI: org/10.22034/ijab. v6i5.446.

Abolhasani MH, Pirestani N, Ghasemi S. (2019). Effects of nutrients on the primary production and determination of the restricting factors in primary production in the international wetland of Choghakhor (Iran). Journal of Advances in Environmental Health Research. 7(2): 94-100. DOI: 10.22102/jaehr.2019.140617.1092.

APHA (2005). Standard Methods for the Examination of Water and Wastewater. 21st Edition, American Public Health Association, American Water Works Association, Water Environment Federation, Washington.

Azizi F, savari Aa Faedeh Amini, Asgari HM, Doustshenas B. (2022). Evaluation of flood period effects on nutrient and gross primary productivity changes in Shadegan wetland. Science of the Total Environment. 346: 256-273. DOI: .org/10.21203/rs.3.rs-2005277/ v1.

Boney AD. (1989). Phytoplankton. Edward Annoid. British Library Cataloguing Publication Data. 118 p.

Chinnaiah B, Madhu V. (2010). Primary productivity of Darmasagar Lake in Adilabad, Andhra Pradesh, India. International Journal of Pharmacy and Life Sciences. 1 (8): 437-439.

Demir N, Kirkagac MU, Süleyman Bekca SP. (2001). Influence of trout cage culture on water quality, plankton and benthos in an Anatolian Dam lake. The Israeli Journal of Aquaculture, Bamidgeh. 53: 115-127. DOI: org/10.46989/001c.20304

Dabiri F, Nassiri P, Ahan Robaee N. (2016). A Comparative Analysis of the Legal Status of Noise Pollution in Iran and Some Asian Countries. Human and Environment. 8 (3): 11-17.

Edmonson WT. (1959). Fresh Water Biology. John Wiley and Sons Inc. New York. 1248 p.

Fathi P, Ebrahimi E, Mirghafari N, Esmaeili A. (2016). The study spatial and temporal changes of water quality in Choghakhor wetland using water quality index (WQI). Journal of Aquatic Ecology. 5 (341-50):.

Ghorbani R, Hosseini SA, Hedayati SAA, Hashemi SAR, Abolhasani MH. (2016). Evaluation of effects of physico-chemical factors on chlorophyll-a in Shadegan international Wetland-Khouzestan

province-Iran. Iranian Journal of Fisheries Sciences. 15 (1): 360-368.

Imanpour Namin J, Safarbibi K, Allaf Noveirian H, Amini K. (2022). Effects of cage culture of rainbow trout, *Oncorhynchus mykiss* on phytoplankton and zooplankton communities (Case study: Golestan Reservoir 1, Gorgan, Iran). Caspian Journal of Environmental Sciences. 20 (1): 1-15. DOI: 10.22124/CJES.2022.5387

Khanna DR, Bhutiani R, Matta G, Singh V, Bhadauriya G. (2012). Study of planktonic diversity of river Ganga from Devprayag to Roorkee, Uttarakhand (India). Environment Conservation Journal. 13 (1-2): 211–217.

Kobayashi T, Ralph TJ, Ryder DS, Hunter SJ, Shiel RJ, Segers H. (2015). Spatial dissimilarities in plankton structure and function during flood pulses in a semi-arid flood plain wetland system. Hydrobiologia. 747 (1): 19-31. DOI:.org/10.3390/ w14010093.

Kumar S, Sharma BK, Sharma SK, Upadhyay B. (2015). Primary productivity and phytoplankton diversity in relation to fisheries potential of the Lake Udai Sagar, Udaipur. International Journal of Fauna and Biological Studies. 5: 09-12. DOI: org/10.23910/M/2019.10.1.1942.

Malekmohammadi B and Jahanishakib F. (2017). Vulnerability assessment of wetland landscape ecosystem services using driver-pressure-state impact-response (DPSIR) model. Ecological Indicators. 82: 293-303. DOI: 10.1016/J.ECOLIND.2017.06.060.

Maosen, H. (1983). Freshwater plankton illustration. Agriculture publishing house. 85 p.

Maranon E. (2015). Cell size as a key determinant of phytoplankton metabolism and community structure. Annual Review Marin Sciences. 7: 241-64.

Mishra V, Sharma SK, Sharma BK, Upadhyay B, Choubey S. (2012). Phytoplankton, primary productivity and certain physico-chemical parameters of Goverdhan Sagar Lake of Udaipur, Rajasthan. Universal Journal of Environmental Research and Technology. 2 (6): 569-574.

Mishra V, Surnar SR, Sharma SK. (2016). Some limnological aspects of Goverdhan Sagar Lake of Udaipur, Rajasthan to suggest its fisheries management. International Journal of Science, Environment and Technology. 5: 2943-2948.

Naz M and Türkmen M. (2005). Phytoplankton biomass and species composition of Lake Gölbaşı (Hatay-Turkey). Turkish Journal of Biology. 29 (1): 49-56. DOI: 10.12714/ egejfas.2006.23.1.5000156798.

Presscot GW. (1970). The freshwater algae. W.M.C. Brown Company publishing. Iowa. U.S.A. 348 P.

Senthilkumar R and Sivakumar K. (2008). Studies on phytoplankton diversity in response to abiotic factors in Veeranam Lake in the Cuddalore district of Tamil Nadu. Journal of Environmental Biology. 29 (5): 747-752. PMid:19295076.

Sourina A. (1978). Phytoplankton manual. United nations educational, scientific and culture organization. 337 p.

Tahami FS, Mazlan B, Negarestan H, Lotfi B. (2011). Abundance and biomass of phytoplanktons in different seasons in southern caspian sea before and after mnemiopsis leidyi. International Congress on Applied Biology, Mashhad, IRAN. PP. 31.

The Effect of Linoleic Acid and Nanoparticle Treatments as Inducers on Biomass and Fatty Acid Content in the Microalga, *Haematococcus lacustris*

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Abstract

Increased biomass density of microalgae is a critical factor in the enhancement of the algal metabolites. In this study, the effects of linoleic acid, TiO_2, and SiO_2 nanoparticles were investigated as elicitors on the production of biomass, and fatty acids in the microalga, *Haematococcus lacustris*. Several treatments of TiO_2 and SiO_2 nanoparticles were analyzed as well as linoleic acid on *Haematococcus lacustris* in two separate designs. Microalgal biomass in nanoparticles was investigated using the Neobar chamber and in linoleic acid using the freeze-dryer methods. Fatty acids compositions were tested by gas chromatography method and five of them named Palmitic, Palmitoleic, Stearic, Oleic, and Linoleic acids (LA) were measured. The results showed that the biomass significantly increased by LA (30 µM) and TiO_2NPs (40 mg/L) treatments, and consequently, these treatments increased the biomass density by 2 and 1.3 times more than the control treatment, respectively. Palmitic and linoleic acids were the most frequent fatty acids produced by 60 and 30µM of LA treatments with 1.4 (53.26 % w/w) and 1.5 (32.51 % w/w) folds, respectively. To conclude, the different concentrations of LA and TiO_2NP boosted the production of algal biomass, and some fatty acids in *Haematococcus lacustris*. Moreover, LA may be used as an effective inducer to increase biomass production in the valuable microalga *Haematococcus lacustris*.

Keywords: *Haematococcus lacustris*, Oxylipin, Salicylic acid, TiO_2NP, SiO_2NP

Introduction

In recent decades, with the increasing population and shortages of food, fuel, and by-products, researchers are making greater efforts to find new alternative sources such as high-potential microalgae for the production of the considerable amounts of proteins, lipids, vitamins, and by-products (Khalili et al., 2019a). To increase the production of this useful substance, it is necessary to increase the production of microalgal cells (biomass). *H. lacustris*, as a high-potential natural producer, is a unicellular microalga living in temperate freshwaters (Khalili et al., 2020).

Scientists have tried to increase biomass production in microalgae via several methods. Using environmental conditions

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and different materials as stresses and inducers are two common strategies, respectively (Hu et al., 2020). Most researchers have shown that some environmental stresses such as high light, salt increase, nitrogen deficiency, and some inducers such as linoleic acid (Khalili et al., 2020), methyl jasmonate (MJ), salicylic acid (SA), and nanoparticles (NPs) affect the production of biomass in microalgae (Hong, 2016). Similar experiments showed that the addition of SA and MJ (500 µM) as inducers in H. lacustris medium culture reduced cell growth (biomass) and carotenoid production (Raman and Ravi, 2011). Linoleic acid is a bio acid (fatty acid), a small molecule in the first stage of oxylipin and arachidonic acid (ARA, C20: 4Δ5), (Khozin-Goldberg et al., 2011) pathways in the plants and microalgae, respectively (Shanab et al., 2018). Two main pathways containing two sidetracks each are derived from Linoleic acid named 13-LOX (lipoxygenase), 9-LOX in plant and macroalgae, as well as ω-6 and ω-3 in microalgae (Shanab et al., 2018).

MJ is produced by some stresses and inducers such as salt, light, wound, LA, and nanoparticles. LA is a precursor of Jasmonic acid (JA) and traumatic acid (TA) phytohormones (Khalili et al., 2019a, Pathak et al., 2018), it is also an allelochemical and a part of its behaviors such as growth inhibition in microalgae is related to this feature. For example, in a resurch which was condected on 32 µM LA increased biomass in *C. sorokiniana* and they displayed that LA disrupted cell membranes and changed cellular functions in *Auxenochlorella pyrenoidosa* (Qian et al., 2018), also inhibited the photosynthetic efficiency in *Chlorella vulgaris* (Qian et al., 2009).

Another inducer of biomass production in microalgae is the nanoparticle. Nanoparticles and other inducers using reactive oxygen species (ROS) stimulate cellular defense mechanisms in the microalgae. ROS in combination with cellular compounds such as DNA, enzymes, and lipids causes algal cell damage or death (Chekanov et al., 2019), thus, the cellular defense mechanism is activated for ROS naturalization.

Accordingly, astaxanthin and other metabolites are produced as the main or bypass products of the defense mechanism for ROS naturalization in microalgae, macroalgae, and plants (Barati et al., 2019; Howe and Schilmiller, 2002). In addition, nanoparticles (NPs) have recently been used by scientists as inducers to produce biomass in microalgae. For instance, the addition of Cadmium (Cd) NP decreased the cell growth rate in *H. lacustris.* Also, Ag, Cd, Fe, and Zn NPs decreased biomass and increased astaxanthin contents by high concentrations in different microalgae. In general, nanoparticles in low concentrations have a positive effect on biomass production (Hong, 2016).

It is important to investigate the type and concentrations of fatty acids in microalgal cells due to their nutritional and fuel value, and find a way to increase them through different treatments. Fatty acids in the endoplasmic reticulum (ER) of microalgae esterify natural astaxanthin (Hempel et al., 2012). It was found by a test that fatty acid accounts for 33% of *H. lacustris*, while its main part was composed of polyunsaturated

fatty acids (PUFAs) that are very helpful for humans, fatty acids have important roles in microalgae cells, for example, the permeability of cell walls and environmental relationships. (Tan et al., 2020) In a study, linoleic, palmitic, and oleic acids were identified as the highest fatty acids among 38 screened-microalga strains in *H. lacustris* respectively (Kabir et al., 2020). Based on the results, oleic, palmitic, and linoleic acids were the most abundant fatty acids that esterify astaxanthin in *Haematococcus pluvialis*. (Zhekisheva et al., 2002). It found that in the microalga, *Bracteacoccus aggregatus* BM5/15 palmitic (C16:0), oleic (C18:1Δ9), and linoleic (C18:2Δ9, 12) acids were the most, respectively (Chekanov et al., 2021).

Materials and methods

The total salts and chemicals required for microalgae cultivation in this experiment were obtained from Merck (Germany) or Sigma (USA) in an analytical grade. Silicon dioxide Nanoparticles (SiO_2NPs) were amorphous and powdery with a size of 10-15 nm and purity of 99.999%, which were purchased from TECNAN Inc (Tecnología Navarra de Nanoproductos S.L., Navarra Spain). Titanium dioxide Nanoparticles (TiO_2NPs) had ~25 nm size, 55-m^2g^{-1} external surface, a combination of anatase and rutile with more ratio of anatase (89.2%), purity of 99.9%, and provided from Degussa Inc (Frankfurt, Germany).

Microalgae culture condition

The unicellular freshwater microalga, *Haematococcus lacostris* CCAP34/7, was obtained from the Microalgae Laboratory of Alzahra University, Tehran, Iran. It was kept at 25 °C until use. In the cultural stage, *H. lacustris* cells with a concentration of 20×10^4 cells ml^{-1} were incubated in 250 ml of Erlenmeyer flasks containing 150 ml Bold's Basal Medium (BBM) culture (Sorokina et al., 2020). Then, the cultures was exposed to 139.35 foot-candle fluorescent light in a growth chamber at 25 ± 2 °C at PH of 6.5 -7 without aeration (shaking), and kept for 12:12 h light-dark cycles. Furthermore, Samples were exposed to 325.16 foot-candle fluorescent light from the seventh until the end of the experiment (19^{th} day). NaOH regulated the pH of the medium culture before autoclaving (Khalili et al., 2019b).

Linoleic acid and Nanoparticles treatments

The treatments were selected at three concentrations of linoleic acid (0, 30, and 60 µM) and two concentrations of TiO_2 and SiO_2 NPs (0 and 40 mg/L) each with three repetitions, which were added to the BBM culture in two separate tests in three days after inoculation. The linoleic acid was added to BBM culture by Tween 20 and NaOH method (Grosch and Schwarz, 1971). TiO_2 and SiO_2NPs stocks were freshly prepared in deionized water, and then autoclaved) It was performed at 120° C for 15 to 20 minutes (and placed in a water bath sonicator at the frequency of 50 Hertz for 15 min. They were kept at 4° C until use (Kahila et al., 2018).

Growth and biomass measurement

The number of cells per day in the experimental section of the effect of nanoparticles on the characteristics of microalgae (NPs TiO_2 and SiO_2) was counted using a hemocytometer and Neobar lam from the first to the 19^{th} day due to the

escape of light absorption and weight gain of NPs. According to the specific method (Ahmed et al., 2015) biomass was dried by the Freeze Dryer (model: FDB-5503 Model, company: Operon, Seoul, Korea) and then measured by a digital scale with an accuracy of 10^{-3} g in LA treatment test.

Fatty acids Measurement

To extract and measure fatty acids at ten to twelve days after inoculation, 0.3 g of each algal sample (measured with an accuracy of 0.0001 g) was transferred to 10 ml tubes. 3 ml of methanol and acetyl chloride solution (20 by 1 volume/volume) was added to each. The tubes were placed in Ben Marie at 85° C for 50 minutes. During this time, the tubes were shaken and after cooling, 1 ml of distilled water and 3 ml of hexane were added, the mixture was centrifuged in tubes (4000 rpm for 15 minutes). Then, the upper phase (hexane with fatty acids) was separated. Identification of fatty acids using Gas chromatography was performed with the following conditions. (Nazeri et al., 2017).

The carrier gas was Argon, column RTX-2230 RESTK, Pennsylvania, USA, Inlet: Heater of 240° C, Pressure of 27.939 psi, Septum purge Flow of 2 ml/m, Split of 1:20, Detector: Heater of 240° C, H_2 Flow of 27 ml/ m, Air Flow of 270 ml/m, and N_2 Flow of 27 ml/ m.

Statistics and data analysis

All data were analyzed in a Randomized Complete Design (RCD). Mean± SD of three replications for Nanoparticles and LA treatments, compared with the post-hoc Tukey test. The one-way *analysis* of variance *(*ANOVA*)* was used to determine any significant differences using SPSS software (v16, USA). The statistical probability level less than 5% ($p< 5\%$) was statistically significant (without LA and NPs).

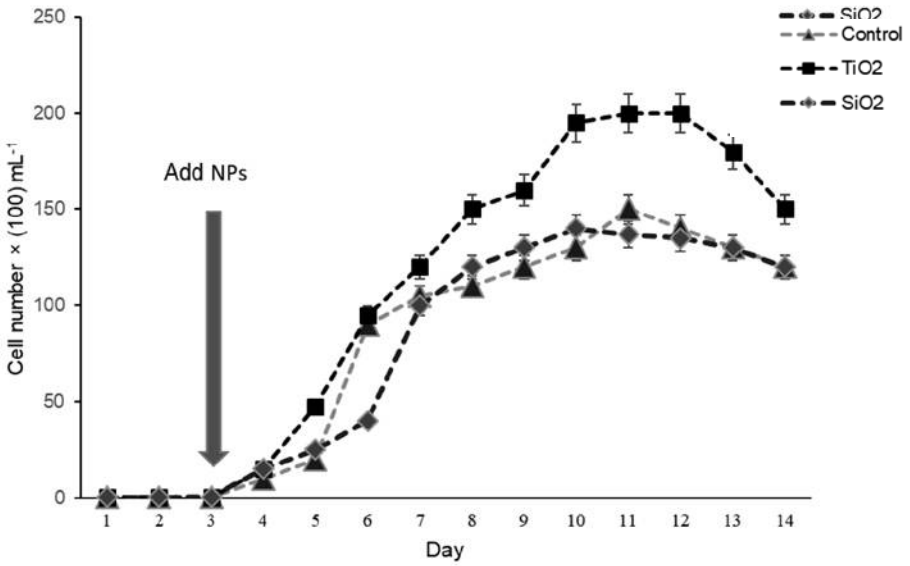

Fig. 1. Effect of nanoparticles on the vegetative growth of *Haematococcus lacustris*. Vertical red arrow: Nanoparticle addition. Average values ± standard deviation are shown (n = 3)

Results

Effect of TiO_2 and SiO_2NPs on biomass accumulation

The results showed that the cellular growth under control and two nanoparticles treatments (TiO_2 and SiO_2) increased rapidly after three days of inoculation, but TiO_2NP (40 mg/L) had a higher effect on the cellular growth on the 11th day and an average of 1.3 (20000 cells/ml) and 1.3 times that of the control, respectively ($p < 0.05$) (Fig. 1).

SiO_2NP (40 mg/L) showed a slightly less cell growth rate than that of the control and, in turn, biomass production for the 11th day and the entire growth cycle with 13700 and ~6400 (cells/ml) were 0.91 and 0.86 times that of the control, respectively. TiO_2NP (40 mg/L) effects on SiO_2NP (40 mg/L) on average and the 11th day (maximum point) were 1.56 and ~1.46 times, respectively.

Biomass production under NPs treatments showed an overall superiority of TiO_2 compared to the control, but SiO_2NPs were less than TiO_2NPs, generally. These results showed that SiO_2NP at a concentration of 40 mg/L could not stimulate the growth of microalgae cells and may damage the cells. Briefly, the order of biomass accumulation was as follows: TiO_2NP (40 mg/L)> control> SiO_2NP (40 mg/L) (Table 1).

Linoleic acid effects on biomass accumulation

Biomass measurement showed that 30 µM LA had the highest effect on the biomass increase on average and twelfth day (maximum biomass) by 2 and 1.8 times that of the control (1.4 and 2.2 mg/ml), respectively. There is a slight difference in the biomass production of 60 µM LA concentration with the control treatment on the 4th and 11th days and the mean, which is 0.2, 1.3 and 0.7 mg/ml biomass production respectively (Figure 2). 30 µM of LA, significantly outperformed the control and 60 µM of LA treatments in biomass production. The effect of 30 µM LA on biomass accumulation was 1.9 times more than that of 60 µM LA concentration, on average (average of 19 days). The order of biomass accumulation was briefly as follows: 30 µM LA>60 µM LA> control (Table 1).

Fatty acid composition

The results of HPLC for astaxanthin measurements agreed well with the spectrophotometry method. Gas chromatography results showed that the contents of palmitic and oleic acids in 60 µM LA and control treatments were the highest and the least fatty acids with 53 and 2.3% w/w, i.e., 1.4 and 0.84 times that of the control, respectively. Also, after palmitic acid, oleic acid in control, oleic acid in SiO_2NPs (40 mg/L), and Palmitoleic acid in TiO_2NPs (40 mg/L) treatments were the highest fatty acids by 1.56, 0.49, and 3.33 times compared to the controls respectively, (Fig. 3). The quantity of palmitic (53.3% w/w)> linoleic (32.5% w/w)> oleic (17.7% w/w)> Palmitoleic (9.7% w/w)> stearic (2.3%w/w) acids had the highest concentrations in one treatment sequentially, also on average, palmitic (44.8% w/w)> linoleic (29.7% w/w)> oleic (15.4%, w/w)> stearic (5.5% w/w)> Palmitoleic (4.7%w/w) were the uppermost fatty acids. The concentration order of palmitic acid in treatments was 60µM LA (53.3% w/w)> 30 µM LA (47.8 % w/w)> SiO_2NP (42.8% w/w)> TiO_2NP

Table 1. The effects of LA and NP treatments on Biomass and Fatty acids in *H. lacustris*

Treatments / Traits	Control	30 µM LA	60 µM LA	TiO_2NPs (40 mg/L)	SiO_2 NPs (40 mg/L)
*Biomass (Cell number×100)/ ml, the average of 19 days	7398.7			9966.4	6383.6
*Biomass mg/ml, the average of 19 days	0.68	1.4	0 .7		
Biomass to the control		2	1.02	1.3	0.86
	Max	Palmitic	Palmitic	Palmitic	Palmitic
	Min	Stearic acid	Stearic acid	Palmitoleic acid	Stearic acid

(35.3% w/w). In addition, for linoleic acid, it was 30 µM LA (32.5 %w/w)> TiO_2NP (30.4 %w/w)> SiO_2NP (28.7 %w/w)> 60µM LA (~27.3%w/w).

These results revealed that the maximum production of two main fatty acids, palmitic and linoleic acids, occurred at two LA treatments (60 and 30µM) while the ultimate production of three other fatty acids, namely Oleic, Stearic, and Palmitoleic was achieved

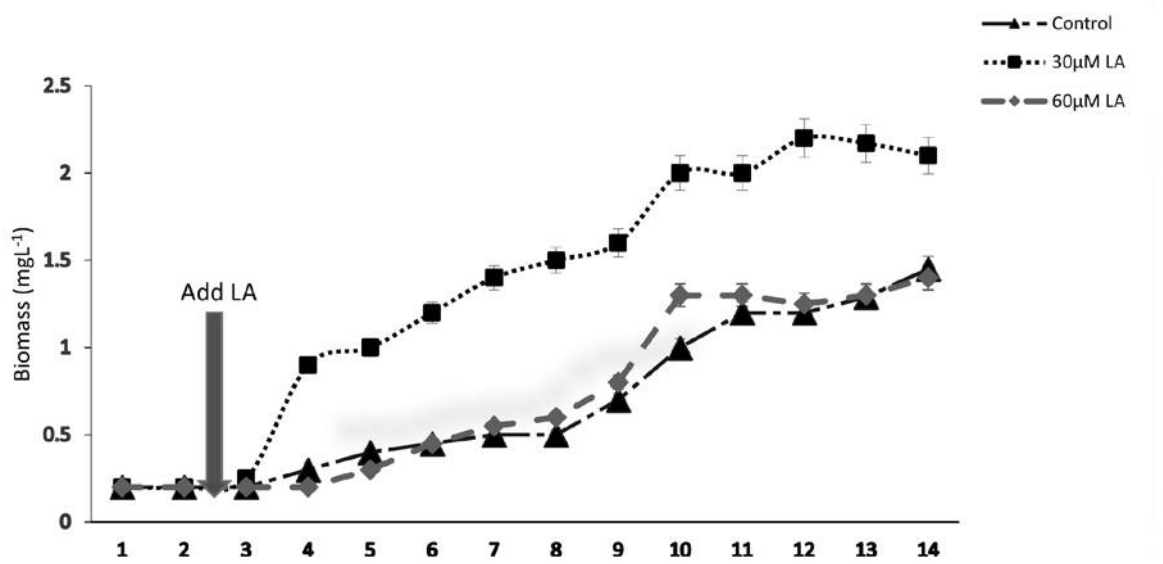

Fig. 2. Effects of linoleic acid on vegetative growth of *Haematococcus lacustris*. Vertical red arrow: Linoleic acid addition, Average values ± standard deviation are shown (n = 3)

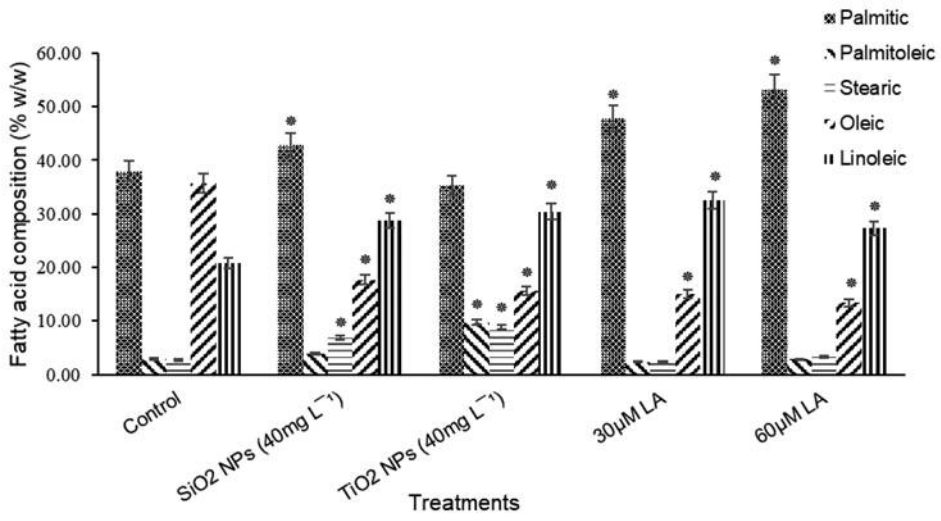

Fig. 3. The results of fatty acid analysis by gas chromatography (*) on the error bars shows the significant difference with control ($p< 0.05$, Duncan's test). Median values± standard deviation (SD) are shown (n = 3).

in NPs (TiO_2 and SiO_2) treatments. In addition, there was a direct relationship between linoleic acid (fatty acid) production, and biomass, as all two were maximized by LA treatment of 30 µM (Table 1).

Discussion

Due to the importance and value of microalgae biomass, extensive research is being done to increase its production from inexpensive materials and methods. The effect of linoleic acid on Biomass production is related to its precursor of two Phytohormones (Khalili et al., 2019a). The starter of oxylipin and arachidonic acid pathways (Shanab et al., 2018), the acidic nature of this substance (Wu et al., 2006), as well as allelochemical properties (Qian et al., 2018), and the effects of Nanoparticles to the microalga are related to their ability to create ROS. TiO_2NPs are more motivating than SiO_2NPs in the biomass production of microalgae (Manzo et al., 2015).

Effect of TiO_2 and SiO_2NPs on biomass accumulation

The produced biomass under the influence of TiO_2 NP (40 mg/L) in the stationary phase was higher than the other two treatments (SiO_2NP and control). Usually, different NPs limit the growth of microalgae and have toxic effects on cells, but some of them have various effects on cell growth at low concentrations (Adams et al., 2006). In general, TiO_2NP has a greater effect than SiO_2NP on the growth and division of microalgal cells. In this regard, some researchers reported that TiO_2NP neutralizes the toxic effect of Cadmium (Cd) on microalga *Chlamydomonas reinhardtii* at lower concentrations than SiO_2Np (A quarter of the concentration) (Yu et al., 2018). The effect of TiO_2NP on *Dunaliella tertiolecta* microalga is much greater and faster than that of SiO_2NP due to the higher accumulation of TiO_2NP in algal medium culture (Manzo et al., 2015).

that of 60µM LA and their difference was significant. When linoleic acid was added to the medium culture from the third day onwards, a significant increase in biomass production was observed in 30µM LA treatment compared to the other two treatments (Fig. 2). It shows the positive effect of the level of this hormone on the growth of microalgae cells, which is consistent with the results of some researchers (Khalili et al., 2019a). For example, 32 µM LA increased biomass in *C. sorokiniana* the reduction of biomass at LA concentrations higher than 30µM is under debate, where some researchers believe that LA is an allelochemical substance with an allelopathy effect on microalgae. According to some other reports, the cellular growth of *C. sorokiniana* is decreased at high concentrations of LA (100 µM) (Qian et al. 2018) . Some materials and phytohormones, such as IAA, IBA, NAA, and PAA increased and decreased cell growth rate in *Chlorella vulgaris* at low and high concentrations, respectively (Piotrowska-Niczyporuk and Bajguz, 2014).

In a study, the effect of 60µM LA on biomass accumulation was approximately equal to that of the control and their difference was insignificant (Khalili et al., 2019). According to previous study by Khalili et al. (2019), the effect of LA on biomass production was higher than this concentration (> 60 µM) that was gradually decreased or become negative. However, this concentration is different for each species in microalgae (specific species). Interestingly, LA is the precursor to MJ and traumatic acid (TA), both of which act similarly to LA (de Los Reyes

There are three hypotheses for this result. Maybe TiO_2NP kills more bacteria and other prokaryotes, so that microalga cells can grow well in the medium culture without any nutritional competition (poor possibility). TiO_2NP is activated by the UV spectrum so that it produces ROS and consequently, the ROS breaks down medium culture components. TiO_2NP covers the whole surface of the microalgae cell and increases optical absorption which enhances chlorophyll content and biomass (Kulacki and Cardinale, 2012; Vargas-Estrada et al., 2020).

Indeed, the accumulation of TiO_2NPs in the medium culture is higher than SiO_2NPs So TiO_2NPs can penetrate cellular organ and genomes more and faster than SiO_2Nps (Manzo et al., 2015).

In addition, SiO_2NP (40 mg/L) had a slightly greater (non-significant) inhibitory effect on cell growth (biomass) compared to the control group (Pikula et al., 2020. Therefore, SiO_2NP in comparison with TiO_2NP and control treatments at this concentration (40 mg/L) could not stimulate cell growth but reduced it, but why cannot SiO_2NPs stimulate biomass production? It seems that aggregation of SiO_2NPs (40 mg l^{-1}) in medium culture is not enough to penetrate (Manzo et al., 2015) in the genome and affect it but can only damage cellular walls and kill or weak microalgal cell so biomass production becomes less than control treatment in this concentration.

Linoleic acid effects on biomass accumulation

The general effect of 30µM LA on biomass production was approximately two times

et al., 2014). For example, it was shown that 1 µM methyl jasmonate increased the cell number in *Chlorella vulgaris*, while further concentrations diminished it, this result was obtained for TA as well (Pietryczuk et al., 2014), and our results are consistent with mentioned results. Finally, LA is an organic acid and in high concentrations inhibits the cellular growth of microorganisms such as microalgae, but this prevention is different for each microorganism (Wu et al., 2006).

Fatty acid composition

In general, Palmitic> Linoleic> Oleic> Stearic> Palmitoleic were the most abundant fatty acids in microalgae, respectively. The astaxanthin molecule in the microalgal cell is covered with different fatty acids, due to its greater stability, the percentage of which varies from one microalga to another and even at different stages of cell growth. The results of some experiments indicate that fatty acids esterify 90% of astaxanthin molecules in *H. lacustris*, whereas the highest fatty acid that covers and attaches to the astaxanthin molecules is oleic acid (Holtin et al. 2009), which does not confirm our results. In some investigations linoleic and oleic acids (Doan et al., 2019) while in others palmitic acid were the predominant fatty acids in the *H. lacustris* (Boonnoun et al., 2014), which almost confirm our results. Here the question arises, why does the type of fatty acids change? Why are palmitic and linoleic acids the major primary fatty acids in microalgae cells? It can be concluded that linoleic acid is the major fatty acid in both pathways GƆ-3 and GƆ-6 (defense pathways of microalgae), but it is the least fatty acid in the cell walls of microalgae. In contrast, palmitic (structural and saturated fatty acid) is the major fatty acid in the cell walls or cell organelle walls (Sharathchandra and Rajashekhar, 2011; Tan et al., 2020). LA is not produced when cells live in the green motile stage (the first stage of the life cycle of *H. lacustris*), while palmitic acid is the first fatty acid in this stage. However, when the microalga enters later stages of growth, such as the late red stage and aplanospore (haematocyst), linoleic acid and its derivatives (α-linolenic, oleic) are produced and become the first fatty acid in them (Butler et al., 2018), which confirms our findings. In our study, palmitic acid was the predominant fatty acid in all treatments due to the GC assay time (10 to 12 days after inoculation), which was at the end of the logarithmic phase which time that the cells were in the transition from green to red. Most likely, if sampling were done in the last days of the experiment, for example, on the seventeenth to nineteenth days, linoleic acid and its derivatives would be more than palmitic acid. Interestingly, linoleic acid in 30µM LA and TiO_2NP (40 mg/L) treatments was the most produced fatty acid after palmitic acid, while these treatments produced the highest amount of biomass, which means 30 µM LA and TiO_2NP (40 mg/L) were directly related to the accumulation of LA (fatty acid), and biomass in *H. lacustris*.

Briefly, the effects of linoleic acid and nanoparticles (TiO_2 and SiO_2NP_s) on the biomass content and fatty acid composition in *H.lacustris* were investigated. To our knowledge, this is the first time that the effects of LA and NPs (TiO_2 and SiO_2) have been investigated simultaneously

on biomass, and fatty acid in *H. lacustris*. In biomass production, the treatments of 30 μM LA and TiO_2NP (40 mg/L) had the highest production, respectively. GC study showed that palmitic and stearic in 60 and 30 μM LA was maximum and minimum produced fatty acids, respectively. There was a direct relationship between 30 μM LA and biomass production.

It was shown that LA could stimulate biomass production in *H. lacustris*. Since this type of microalgae, *H. lacustris*, which is closely similar to *Haematococcus pluvialis*, has received less attention from researchers around the world. This study was a step towards demonstrating the importance and economic potential of *H. lacustris* in the production of fatty acids. We feel the importance of this work would substantially increase with the performance of additional experiments, such as identifying the mechanism on pathways that nanoparticles induced in *Haematococcus lacustris*. Because of microalgae biomass and fatty acid importance, we hope that in the future, more research about the effects of elicitors and stressors on biomass and fatty acid production take place to produce easier and faster than now in different microalgae.

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References

Adams LK., Lyon DY, Mcintosh A, Alvarez PJ. (2006). Comparative toxicity of nano-scale TiO_2, SiO_2 and ZnO water suspensions. Water Science and Technology. 54: 327-334. DOI: 10.2166/wst.2006.891.

Ahmed FLY, Fanning K, Netzel M, Schenk PM. (2015). Effect of drying, storage temperature and air exposure on astaxanthin stability from *Haematococcus pluvialis*. Food Research International. 74: 231-236. DOI: 10.1016/j.foodres.2015.05.021.

Barati B, Gan SY, Lim PE, Beardall J, Phang SM. (2019). Green algal molecular responses to temperature stress. Acta Physiologiae Plantarum. 41: 26. DOI: 10.1007/s11738-019-2813-1.

Boonnoun P, Kurita Y, Kamo Y, Machmudah S, Okita Y, Ohashi E, Kanda H, Goto M. (2014). Wet extraction of lipids and astaxanthin from *Haematococcus pluvialis* by liquefied dimethyl ether. Journal of Nutrition and Food Sciences. 4: 1000305. DOI: 10.4172/2155-9600.1000305.

Butler T, Mcdougall G, Campbell R, Stanley M. Day J. (2018). Media screening for obtaining *Haematococcus pluvialis* red motile macrozooids rich in astaxanthin and fatty acids. Biology. 7: 2. DOI: 10.3390/biology7010002.

Chekanov K, Litvinov D, Fedorenko T, Chivkunova O, Lobakova E. (2021). Combined production of astaxanthin and β-carotene in a new strain of the microalga *Bracteacoccus aggregatus* BM5/15 (IPPAS C-2045) cultivated in photobioreactor. Biology. 10**:** 643. DOI: 10.3390/biology10070643.

Chekanov K, Schastnaya E, Neverov K, Leu

S, Boussiba S, Zarka A, Solovchenko A. (2019). Non-photochemical quenching in the cells of the carotenogenic chlorophyte *Haematococcus lacustris* under favorable conditions and under stress. Biochimicaet Biophysica Acta (BBA) -General Subjects. 1863: 1429-1442. DOI: 10.1016/j.bbagen.2019.05.002.

De Los Reyes C, Ávila-Román J, Ortega MJ, Jara A, García-Mauriño S, Motilva V, Zubía E. (2014). Oxylipins from the microalgae *Chlamydomonas debaryana* and *Nannochloropsis gaditana* and their activity as TNF- inhibitors. Phytochemistry. 102**:** 152-161. DOI: 10.1016/j.phytochem.2014.03.011.

Doan LP, Nguyen TT, Pham M Q, Tran QT, Pham Q L, Tran DQ, Than VT, Bach LG. (2019). Extraction process, identification of fatty acids, tocopherols, sterols and phenolic constituents, and antioxidant evaluation of seed oils from five Fabaceae species. Processes. 7: 456. DOI: 10.3390/pr7070456.

Grosch W and Schwarz JM. (1971). Linoleic and linolenic acid as precursors of the cucumber flavor. Lipids. 6: 351-352. DOI: 10.1007/BF02531828.

Hempel N, Petrick I, Behrendt F. (2012). Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. Journal of Applied Phycology. 24: 1407-1418. DOI: 10.1007/s10811-012-9795-3.

Holtin K, Kuehnle M, Rehbein J, Schuler P, Nicholson G, Albert K. (2009). Determination of astaxanthin and astaxanthin esters in the microalgae Haematococcus pluvialis by LC-(APCI) MS and characterization of predominant carotenoid isomers by NMR spectroscopy. Analytical and bioanalytical chemistry. 395**:** 1613. DOI: 10.1007/s00216-009-2837-2.

Hong Y Z, (2016). Effect of different Concentrations of Cadmium Nanoparticle, PH and Salinity on Production of Astaxanthin in *Haematococcus Pluvialis.* INTI International University.

Howe GA and Schilmiller AL. (2002). Oxylipin metabolism in response to stress. Current Opinion in Plant Biology. 5: 230-6. DOI: 10.1016/s1369-5266(02)00250-9.

Hu C, Cui D, Sun X, Shi J, Xu N. (2020). Primary metabolism is associated with the astaxanthin biosynthesis in the green algae *Haematococcus pluvialis* under light stress. Algal Research. 46: 101768. DOI: 10.1016/j.algal.2019.101768.

Kabir F, Gulfraz M, Raja GK, Inam-Ul-Haq M, Awais M, Mustafa M. S, Khan SU, Tlili I, Shadloo MS. (2020). Screening of native hyper-lipid producing microalgae strains for biomass and lipid production. Renewable Energy. 160: 1295-1307. DOI: 10.1016/j.renene.2020.07.004.

Kahila M, Najy AM, Rahaie M, Mir-Derikvand M. (2018). Effect of nanoparticle treatment on expression of a key gene involved in thymoquinone biosynthetic pathway in *Nigella sativa* L. Natural Product Research. 32**:** 1858-1862. DOI: 10.1080/14786419.2017.1405398.

Khalili Z, Jalili H, Noroozi M, Amrane A. (2019a). Effect of linoleic acid and methyl jasmonate on astaxanthin content

of *Scenedesmus acutus* and *Chlorella sorokiniana* under heterotrophic cultivation and salt shock conditions. Journal of Applied Phycology. 1:12. DOI:10.1007/s10811-019-01782-0.

Khalili Z, Jalili H, Noroozi M, Amrane A. (2019b). Effect of linoleic acid and methyl jasmonate on astaxanthin content of Scenedesmus acutus and Chlorella sorokiniana under heterotrophic cultivation and salt shock conditions. Journal of Applied Phycology. 31: 2811-2822. DOI: 10.1007/s10811-019-01782-0.

Khalili Z, Jalili H, Noroozi M, Amrane A, Ashtiani FR. (2020). Linoleic-acid-enhanced astaxanthin content of *Chlorella sorokiniana* (Chlorophyta) under normal and light shock conditions. Phycologia. 59: 54-62. DOI: 10.1080/00318884.2019.1670012.

Khozin-Goldberg I, Iskandarov U, Cohen Z. (2011). LC-PUFA from photosynthetic microalgae: occurrence, biosynthesis, and prospects in biotechnology. Applied Microbiology and Biotechnology. 91: 905-915. DOI: 10.1007/s00253-011-3441-x.

Kulacki K J and Cardinale BJ. (2012). Effects of nano-titanium dioxide on freshwater algal population dynamics. Plos One. 7: 47130. DOI: 10.1371/journal.pone.0047130.

Manzo S, Buono S, Rametta G, Miglietta ML, Schiavo SD, Francia G. (2015). The diverse toxic effect of SiO_2 and TiO_2 nanoparticles toward the marine microalgae *Dunaliella tertiolecta.* DOI: 10.1007/s11356-015-4790-2.

Nazeri V, Kiani R, Rezaei K, Kalvandi R. (2017). Diversity study of some ecological, morphological and fatty acid profile of Linum album Ky. ex Boiss. Iranian Journal of Medicinal and Aromatic Plants. 33: 168-183. DOI: 10.22092/ijmapr.2017.109721.

Pathak J, Maurya PK., Singh SP, Häder DP, Sinha RP. (2018). Cyanobacterial farming for environment friendly sustainable agriculture practices: innovations and perspectives. Frontiers in Environmental Sciences. 6: 7. DOI: 10.3389/fenvs.2018.00007.

Pietryczuk A, Biziewska I, Imierska M, Czerpak R. (2014). Influence of traumatic acid on growth and metabolism of *Chlorella vulgaris* under conditions of salt stress. Plant Growth Regulation. 73: 103-110. DOI: 10.1007/s10725-013-9872-x.

Pikula K, Chaika V, Zakharenko A, Markina Z, Vedyagin A, Kuznetsov V, Gusev A, Park S, Golokhvast K. (2020). Comparison of the level and mechanisms of toxicity of carbon nanotubes, carbon nanofibers, and silicon nanotubes in bioassay with four marine microalgae. Nanomaterials. 10: 485. DOI: 10.20944/preprints202002.0168.v1.

Piotrowska-Niczyporuk A and Bajguz A. (2014). The effect of natural and synthetic auxins on the growth, metabolite content and antioxidant response of green alga *Chlorella vulgaris* (Trebouxiophyceae). Plant Growth Regulation. 73: 57-66. DOI: 10.1007/s10725-013-9867-7.

Qian H, Xu J, Lu T, Zhang Q, Qu Q, Yang Z, Pan X. (2018). Responses of

unicellular alga *Chlorella pyrenoidosa* to allelochemical linoleic acid. Science of the Total Environment. 625: 1415-1422. DOI: 10.1016/j.scitotenv.2018.01.053.

Qian H, Xu X, Chen W, Jiang H, Jin Y, Liu W, Fu Z. (2009). Allelochemical stress causes oxidative damage and inhibition of photosynthesis in *Chlorella vulgaris*. Chemosphere. 75: 368-375. DOI: 10.1016/j.chemosphere.2008.12.040.

Raman V, Ravi S.(2011). Effect of salicylic acid and methyl jasmonate on antioxidant systems of *Haematococcus pluvialis*. Acta Physiologiae Plantarum. 33: 1043-1049. DOI: 10.1007/s11738-010-0623-6.

Shanab S, Hafez RM, Fouad AS. (2018). A review on algae and plants as potential source of arachidonic acid. Journal of Advanced Research, 11: 3-13. DOI: 10.1016/j.jare.2018.03.004.

Sharathchandra K and Rajashekhar M. (2011). Total lipid and fatty acid composition in some freshwater cyanobacteria. Journal of Algal Biomass Utilization. Environmental Science and Pollution Research. 2 (2): 83-97. DOI: 10.1007/s11356-015-4790-2.

Sorokina KN, Samoylova YV, Parmon VN. (2020). Comparative analysis of microalgae metabolism on BBM and municipal wastewater during salt induced lipid accumulation. Bioresource Technology Reports. 11: 100548. DOI: 10.1016/j.biteb.2020.100548.

Tan JS, Lee SY, Chew KW, Lam MK, Lim JW, Ho S-H, Show PL. (2020). A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids. Bioengineered. 11: 116-129. DOI: 10.1080/21655979.2020.1711626.

Vargas-Estrada L, Torres-Arellano S, Longoria A, Arias DM, Okoye PU, Sebastian P. (2020). Role of nanoparticles on microalgal cultivation. Fuel (a review). 280: 118598. DOI: 10.1016/j.fuel.2020.118598.

Wu JT, Chiang YR, Huang W Y, Jane W N. (2006). Cytotoxic effects of free fatty acids on phytoplankton algae and cyanobacteria. Aquatic Toxicology. 80: 338-45. DOI: 10.1016/j.aquatox.2006.09.011.

Yu Z, Hao R, Zhang L, Zhu Y. (2018). Effects of TiO2, SiO2, Ag and CdTe/CdS quantum dots nanoparticles on toxicity of cadmium towards *Chlamydomonas reinhardtii*. Ecotoxicology and Environmental Safety. 156: 75-86. DOI: 10.1016/j.ecoenv.2018.03.007.

Zhekisheva M, Boussiba S, Khozin-Goldberg I, Zarka A, Cohen Z. (2002). Accumulation of oleic acid in *Haematococcuspluvialis*(chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters 1. Journal of phycology. 38: 325-33. DOI: 10.1046/j.1529-8817.2002.01107.x.