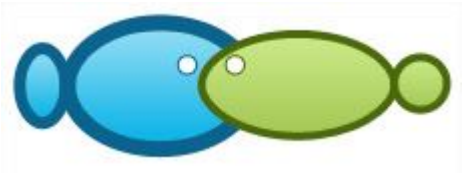

Performance of microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* in wastewater treatment of Gomishan (Golestan-Iran) shrimp farms

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Abstract. The effects of *Chlorella vulgaris* and *Scenedesmus obliquus* in reducing pollution of waste water coming from Gomishan shrimp farms were examined. First, each of the micro-algae gathered from Gomishan shrimp farms were separated and purified in the laboratory. The physicochemical factors including pH, oxygen, temperature, phosphate, nitrate, from waste water before and after exposure to microalgae, were measured every 24 hours during 10 days. In addition to these factors, biological parameters and the production of algae density such as biomass, specific growth rate and chlorophyll *a* were measured. Treatments include control (without any algae), *C. vulgaris* ($10.3\times10^6\pm0.13\times10^6$), *S. obliquus* ($2.7\times10^6\pm0.16\times10^6$) and Mix ($8.6\times10^6\pm0.12\times10^6$) from both of these algae. The results showed that in different treatments, dry matter content and chlorophyll *a* have significantly increased during the period ($p < 0.01$) and the phosphate-P and PO_4 show a significant reduction in the duration of experiments ($p < 0.05$). But no significant effects on nitrate N and NO_3 were observed ($p > 0.05$). On the other hand, the number of algae cells and the specific growth rate during the period had a significant change, which means that these factors at the beginning were increased and then significantly decreased ($p < 0.05$). The results showed that among the various treatments, mix treatment shows the best result in the removal of organic and inorganic compounds from Gomishan shrimp farms. These results can be used as a model to improve the water quality of these farms before discharge into the sea or reused in the culture system.

Key Words: *Chlorella vulgaris*, *Scenedesmus obliquus*, shrimp farms, nitrogen, phosphate.

Introduction. Microalgae is the term used to name all microscopic algae, both prokaryotic and eukaryotic algae which distinguishes them from macroscopic algae.

Chlorella vulgaris is one of the important species of green algae in fish farms, due to the fact that rotifers, larvae and some types of fish (such as silver carp) feed these type of algae (Falahi et al 2003). Also, green algae *Scenedesmus obliquus* is one of Chlorophyta that lives in freshwater and is a biological indicator of these environments. The algal cells are immobile and without flagella and sometimes forms colonies (Riahi 2002).

Aquaculture activities associated with the use of chemical fertilizers, foods with different combinations, each of them having different destructive effects on aquatic organisms, humans and the environment (Esmaeili Sary 2004). To overcome some of the mentioned problems, one efficient way is using the biological processes in water treatment (Campbell 1999).

Micro-algae such as *Chlorella* and *Scenedesmus*, due to high growth rate and resistance to manipulation technologies in cultivation systems, as well as simple and inexpensive producing process, can be useful in wastewater treatment (Chevalier & De la Noue 1985). So, *Scenedesmus* and *Chlorella* algae have been used in many studies to isolate nitrogen and phosphorus and positive results were obtained.

Research of Lau et al (1996) show the ability of *C. vulgaris* to remove nutrients. Their report shows that these algae can remove 86% inorganic nitrogen and 78% inorganic phosphorus from water. Gonzales et al (1997) also stated that *C. vulgaris* and *Scenedesmus dimorphus* can attract 55% phosphorus of the total phosphorus concentration (111 ppm) from agricultural-industrial wastewater in 216-hour.

Lee & Lee (2001) stated that *Chlorella kessleri* in a 12-hour photoperiod, can absorb between 8-20% of phosphates from environment.

Aslan & Kapdan (2006) examined the role of *C. vulgaris* in separating of nitrogen and phosphorus from sewage and found that this species shows a higher capacity in nitrogen isolation comparing to phosphorus. In a study conducted by Kothari et al (2012) *Chlorella pyrenoidosa* grown in wastewater can daily eliminate 80-85% of phosphorus and 60-80% of nitrogen from wastewater. In a study by Han et al (2015) about municipal wastewater treatment by *Scenedesmus quadricauda* it was found that phosphorus and nitrogen separation rate is almost 100% and 70% respectively during the first 5 days of the study. Abolhasani et al (2016) expressed that *S. obliquus* can be used for the removal of phosphate and nitrate and also for algae production in urban wastewater systems. Hence, the aim of this study is to compare the efficiency of micro-algae *C. vulgaris* and *S. obliquus* in removing phosphate and nitrogen from Gomishan shrimp farms and estimate the use of wastewater as a suitable medium for cultivation of these algae for biomass production.

Material and Method. To evaluate the effect of different algal species (*Chlorella vulgaris* and *Scenedesmus obliquus*) on waste water treatment of Gomishan shrimp farms, experiments were done in 4 treatments and repeated 4 times, which includes the following treatments: 1. *Chlorella vulgaris* algae; 2. *Scenedesmus obliquus* algae; 3. mixed *Chlorella vulgaris* and *Scenedesmus obliquus*; 4. waste-water without algae (control).

To perform the experiments, samples from the effluent of Gomishan shrimp farms (lagoon with an area of 24 hectares - 37°15'18 N, and 58°00'10 E) were prepared using this water to set treatments. Gomishan shrimp farms are 17 kilometers far from north of Gomishan city (Golestan province, Iran) and established in southeast of the Caspian Sea (Figure 1).

Figure 1. Gomishan lagoon (southeast of the Caspian Sea - Iran).

The medium for culturing these microalgae is Z-8 (Esmaeili Sary 2000). To identify the microalgae, the keys of Bellinger (1992) were used. Afterwards, larger particles were taken from water samples by filtering through a microfiber filter and then were autoclaved for 20 minutes at 110°C. Several 500 mL Erlenmeyers glasses were used for the experiments. Each Erlenmeyer glass was filled with 475 mL of water from the shrimp

farms. Then to each flask in treatments 1 and 2, 25 mL of *C. vulgaris* and *S. obliquus* algae were added respectively, and in treatment 3 that should contain the same amount of both algae, 12.5 mL of *C. vulgaris* and 12.5 mL of *S. obliquus* were added. For providing the needed light, oxygen and temperature, we have used a culture room equipped with culture desk with 20 fluorescent lamps, a cooler and each Erlenmeyer flask has been aerated through an air pump. All equipments and additional tools have been disinfected with UV before the experiments. Also all the dishes were autoclaved in order to be disinfected for 20 minutes either (110^oC).

During the 10-days of experiments (during October 2016), all the samplings were taken every 24 hours and for this purpose, 50 mL of algal culture medium were separated and then centrifuged at 4000 rpm for 10 minutes at 10°C. After this process, the supernatant obtained was used to measure the nitrate and phosphate levels (Han et al 2015).

The separation rate was calculated according to the following formula:

$$\text{Removal efficiency} = (C_i - C_0) / C_0 \times 100\%$$

where C_i represents the concentration at the *i* time and C_0 represents the initial concentration (Han et al 2015).

To calculate algae biomass we used the method proposed by Lavens & Sorgeloos (1996). The specific growth rate (SGR) was calculated according to the following formula:

$$SGR = (\ln N2 - \ln N1)/\Delta t$$

where N2 was the number of algal cells at the end of experiment and N1 was the number of algal cells in the initiation of the experiment and Δt was the duration of the experiment (Omori & Ikeda 1984). Nitrate measurement was done using the method of APHA (1992), the phosphate was measured by method of Healey (1978). To measure the oxygen, pH and temperature we used an oxygen meter (DO meter az-8403 Taiwan), pH meter (orp meter pH - 206 Taiwan) and thermometer (Hydro thermometer- LM-81HT- Taiwan), respectively. Microalgae cells counting was made on a daily basis by haemocytometer slide, using the method of Martinez et al (2000). The amount of chlorophyll a was calculated according to Parsons et al (1984) formula.

Data analysis This experiment was completely randomized design in split plot in time (in 11 levels of 0, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 hours) by a factor (type of algae, including *C. vulgaris*; *S. obliquus*; the mix of *C. vulgaris* and *S. obliquus;* and no algae) in four replications. It also reviews the trends in each of the measured parameters at the time must be regression testing used. Statistical analysis of data using software SPSS17 and ANOVA and mean comparison with Duncan's test was conducted at a confidence level of 5% (Zar 1984).

Results and Discussion. Physical and chemical factors during the experiments are shown in Table 1. As this chart shows, it has been tried to keep level of temperature, light and oxygen stable during the experiments. Growth of algae and extraction of nutrients by algae is not only the effect of nutrients in the water but is related to some physical factors such as pH (Azov & Shelef 1987), light intensity, temperature and biological factors (Talbot & De la Noue 1993). As Table 1 shows, in the same and different treatments no significant difference between the parameters of temperature, pH and dissolved oxygen was observed with control ($p > 0.05$). Also, in most of the days, there was no significant difference between treatments ($p > 0.05$). The temperature and dissolved oxygen changed during the experiments, increased and then decreased ($p < 0.05$), but pH was unchanged ($p > 0.05$).

In terms of dry matter in different treatments, there was no difference between days, indicating all treatments had the same exposure to new culture medium and the presence of two algae species together in the third treatment did not show any significant adverse effect on the dry matter content. The number of algal cells was different between treatments, but there was no significant difference in the amount of dry matter between the treatments in the same day which can be attributed to the characteristics of algae appearance. *Chlorella* algae are usually in single and round forms, but *Scenedesmus* algae are generally multiple, forming colonies and apparently several times larger than

Chlorella. Therefore, according to different shape and size of *Chlorella*, fewer cells of *Scenedesmus* in compare to *Chlorella* were observed ($p > 0.05$).

Amount of chlorophyll *a* in the *Scenedesmus* treatments is more than other treatments, maybe because of higher density of chlorophyll due to its larger size compared with the *Chlorella* ($p < 0.05$). This process continued until the final days but after study the trends in each treatment during the 10-day concluded that between the first and second days, significant changes were observed in chlorophyll *a* content and dry matter in experimental treatments that the reason for this can be attributed to the Lag phase in which algae cultivation adapt themselves to the new environment. Significant increase was observed in all treatments ($p < 0.05$) from the second to the fourth day because of growth phase of algae and full compatibility with the medium. In the fifth and sixth days, almost constant cycle of algae was seen which shows that the reproduction rate and mortality rate are the same and from the sixth day until the eighth day in all treatments confront a significant reduction in the biomass and chlorophyll *a* which indicated that the algae enter the stage of deaths and the number of algae cells fell sharply and from ninth day onwards, the dry matter and chlorophyll *a* fell to zero, which indicated that all algae were removed. Since there was no algae in the control group, the amount of dry matter and chlorophyll in them were zero, so had a significant difference from all treatments in terms of 8 days ($p < 0.05$) but on ninth and tenth day, given that the biomass and chlorophyll *a* contents were also zero and amount of them was similar to the control group.

Research has shown that increasing and decreasing biomass is due primarily to the amount of nutrients in the environment (especially nitrogen) and then related to light (Samori et al 2013) because the light intensity in the environment has not changed during the experiments, so reduction of biomass in the environment can be attributed to low nutrient environment. Given that until the sixth day, nitrogen compounds were present but not zero and phosphate concentration was very close to zero; so it can be concluded that phosphorus is the limiting factor in growth of algae and results in cell death and thus reduces amount of dry matter. Similar to our results, Tam & Wong (1989) assessed the growth and biomass of *Chlorella* and *Scenedesmus* within 10-days and showed that the biomass of both algae species were similar during the initial days but the same as Table 2, then biomass will increase in *Scenedesmus*. Their investigators believed that this single-cell structure of *Chlorella* and colonial structure of *Scenedesmus* is leading to increase of *Scenedesmus* biomass in compare to *Chlorella*. Tang et al (2011) reported that algal biomass range of *S. obliquus* was between 0.155-0.083 g L^{-1} per day, which is lower than our foundation. In fact, they used this factor as a limiting factor and prevent the growth of algae *S. obliquus* without restrictions through controlling the amount of CO_2.

Another measured factor in this study and similar studies in evaluation of different algae performance to improve effluent quality is the amount of phosphorus in water. According to Table 3 the amount of P and PO_4 at the beginning of experiments and in the control were 0.06±0.01 and 0.14±0.02 mg L^{-1}, respectively; and over time its value decreased significantly ($p < 0.05$) and in sixth days of rest to its minimum levels (0.01 g L^{-1}). From the seventh to tenth day the amount of P and PO_4 increased significantly with gentle slopes and at the end of the tenth day, these factors were 0.02 and 0.04 mg L^{-1}, respectively ($p < 0.05$). Among different treatments on absorption of phosphate, performance of Mix treatment shows significantly better than *Chlorella* and *Scenedesmus* and had a higher retention rate ($p < 0.05$). Although Mix treatment had a higher intake of phosphate from the environment, but at the end of the experiment secreted a greater amount of phosphate into the medium. Study of Valderrama et al (2002) on *C. vulgaris* showed that when the initial concentration of phosphate was 1.5-3.5 g L^{-1}, the algae released only 28% PO_4 of wastewater that this is lower than our results. Similar to our results, research of Aslan & Kapdan (2006) showed that when the initial concentration of PO_4 was 7.7 mg L^{-1}, *C. vulgaris* can remove up to 78% of phosphate from the water and this percentage rose to 80% in our research. Ruiz-Marin et al (2010) after an investigation on *Chlorella* and *Scenedesmus* concluded that *Scenedesmus* algae had a higher ability to absorb PO_4 of water. According to their results, *Chlorella* and

Scenedesmus algae are reduced in phosphate water up to 70% and 85%, respectively; that is highly in agreement with our results. Similar to our investigation, Ahmad et al (2013) also examined the trend of change of *Chlorella* in attracting municipal sewage and showed that it can remove PO_4 from waste water up to 95.5%, while in our experiments *Chlorella*, *Scenedesmus* algae and mix treatment, were able to absorb 92.8%, 85.8% and 92.8% of PO_4, respectively. However, at the end of 10 days, this percent changed to 71.4% in all treatments.

Table 4 shows that the effect of different treatments on the amount of nitrate N and NO_3 did not show proven process and suggested that these algae had no ability to absorb water nitrate and it seems that other factors are effective in increasing and decreasing nitrate ($p > 0.05$) in the values listed. Investigation of Ruiz-Marin et al (2010) on algae *Chlorella* and *Scenedesmus* corresponds with our results. They suggested that nitrification was limited and *Chlorella* and *Scenedesmus* algae tendency to absorb ammonia compared to other forms of nitrogen in water (Garcia et al 2006) were able to reduce 43% of NO_3 from municipal waste water by *Chlorella* algae. Lack of algae's ability to absorb some of nutrients or incomplete absorption of some nutrients could be attributed to excessive algal density. The excess density might reduce the ability of algae to remove nutrients because it reduces the intensity of light. Despite our results, Wang et al (2010) expressed that *Chlorella* in 9 days can absorb up to 62.5% NO_3 and 50.8%-82.8% of total nitrogen from municipal wastewater. Mousavi et al (2010) in their research on urban waste water concluded that *Chlorella* could reduce more than 80% of NO_3 within 14 days. Also, Ahmad et al (2013) could remove up to 97% NO_3 from urban wastewater by *Chlorella*.

In the first treatment containing only *Chlorella*, the number of cells in the initial of experiments was $10.6\times10^6\pm0.13\times10^6$ which significantly reduced during the first day which is likely due to inability of some of *Chlorella* cells to adapt to a new culture medium. In the following days, a significant increase in the number of cells was performed until fifth day ($p < 0.05$). In the sixth day also the number of cells increase that this increasing was not significant ($p>0.05$). From seventh days the number of cells decreased significantly sharply to the extent that the number of cells on day tenth was zero ($p < 0.05$). This increase and decrease represented that the algae showed maximum of their performance to removal organic material until day 6 and from this point onwards due to lack of nutrients or other factors could not survive and declined sharply. By comparing this treatment with the control group, it can be concluded that in all days except the second day number of cells was significantly different from the control group ($p < 0.05$). Research of Ahmad et al (2013) on the potential of *C. vulgaris* in waste water treatment in transparency and dark pools is quite similar to our results in the growth process of algae. They found that *Chlorella* algae in the first two days held in Lag phase and then the significant growth of algae cell occurred until the sixth and seventh days and at this point the stationary phase happened. Wang et al (2010) investigated the growth of *Chlorella* in 10 days in four different effluents and observed that the Lag phase increased until the third day and algae grew in 6 remaining days. It can be concluded from Table 5 that Lag phase did not happen in *Scenedesmus* algae, which indicates better implementation of *Scenedesmus* cells in to a new environment compared to other treatments.

Trend of changes of *Scenedesmus* algae cells was similar to the trend observed in the *Chlorella* algae cells in reports of Wang et al (2010). They found that Lag phase did not exist in these algae and it is clear that the process of cell growth is associated with cell types and nutrient environment. Some researchers have also reported that *Scenedesmus* species has better compatibility features than other species in entrance to the new medium (Martinez et al 2000; Ruiz-Marin et al 2010). Ruiz-Marin et al (2010) confirmed our results, they examined growth and nutrient removal of municipal sewage by algae *C. vulgaris* and *S. obliquus* and showed lag phase before the proliferation of the algae *Chlorella* and *Scenedesmus* was 20 and 8 hours, respectively.

As discussed in previous issues, Mix treatment contained the same composition in terms of volume (12.5 cc *Chlorella vulgaris* algae+12.5 cc *Scenedesmus obliquus* algae) by the initial amount of $7.0\times10^6\pm0.16\times10^6$ and $1.6\times10^6\pm0.06\times10^6$ *Chlorella* and

Scenedesmus cells, respectively; and a total of $8.6\times10^6\pm0.1\times10^6$ cells. The trend of this Mix treatment indicated that each of these algae showed similar property to the treatments 1 and 2. In other words, in the Mix treatment, amount of *Chlorella* cells significantly decreased in the first day, and then increased, but amount of *Scenedesmus* increased from the first day and reproduced and growth was completed in both species in six days and then started to decline and on the ninth day fell down to zero ($p < 0.05$). Then on the sixth day this trend stopped and on the seventh day decreasing trend started, number of algae fell to zero in tenth days ($p < 0.05$). It seems that the reason was the higher number of *Chlorella* in comparison with *Scenedesmus*. The control group in all days showed a significant difference from Mix treatment ($p < 0.05$).

As shown in Table 6, comparing different treatments on the same day showed that generally specific growth rate in most days were not significantly different between treatments ($p > 0.05$) and the growth trend and decline between *Chlorella* and *Scenedesmus* cells was roughly equal to Mix treatment. Only on the third day, *Chlorella* treatment had higher growth compared with other treatments and also at the end of experiment was lower than two other treatments ($p < 0.05$). Daily comparison of trends in each treatment separately showed until the third day of growth rate has been about 0.5 grams per day indicating that in each day 0.5 grams was added until days when cells and algae were on growth phase. On the fourth and fifth days, the number decreased to near zero which indicated the stationary phase that the number of deaths was against the proliferation of algae and from the sixth day, especially the growth rate was negative in all treatments that specifically represented the decaying algae. Given that all the algae died on the tenth day, the specific growth rate for the ninth and tenth days was not easy to calculate. Wang et al (2010) over a period of 9 days, investigated growth of *Chlorella* in four different treatments of municipal sewage and their results are the same as the present results. They did not find significant growth until the third day and the range of specific growth rate in their treatments were between 0.343 and 0.948 grams per day that is consistent with our results. Ruiz-Marin et al (2010) calculated the specific growth rate for *C. vulgaris* and *S. obliquus* 0.377 and 0.401 grams per day, respectively; that was consistent with our results. Samori et al (2013) reported that specific growth rate of algae *Desmodesmus communis* was 0.48 grams per day which is in agreement with our results. The researchers expressed that the low specific growth rate was related to insufficient concentrations of nutrients (N and P) which is considered inadequate for algae growth.

Conclusions. According to the results, the algae *Chlorella vulgaris* and *Scenedesmus obliquus* had high adaptability with wastewater of Gomishan shrimp farms and its unique efficacy in reducing the organic and inorganic materials of effluents but when they were mixed together, additional positive effects than other treatments of individual that led to better results. Many physical and chemical water parameters decreased during the sixth day and it seems phosphate is the limiting factor in this study and when there is phosphate in the media, absorption process happened and finally on the sixth day when phosphate was much closer to zero, the growth of microalgae cells declined and from the sixth day until the end of experiments (the tenth day) all treatments lost ability to absorb organic and mineral materials of water and some of the absorbed phosphate compounds were released into the the environment after their death, on the sixth day algae again was injected into the environment in order to refine the process.

Table 1

Temperature, pH and dissolved oxygen during testing in experimental treatments

Day	Parameter								
	Temperature (^oC) (control = 24.7±0.0)			pH (control = 5.7±0.0)			Dissolved oxygen (mg L^{-1}) (control = 3.21±0.08)		
	CH	SE	MIX	CH	SE	MIX	CH	SE	MIX
1	24.8 ± 0.06^{ABCa}	24.6 ± 0.17^{BCDb}	24.7 ± 0.06^{ABab}	5.7±0.06	5.7±0.06	5.7±0.11	3.2 ± 0.04^{AB}	3.2 ± 0.01^{AB}	3.3 ± 0.09^{AB}
2	24.6 ± 0.12^{ABCb}	24.5 ± 0.3^{BCDb}	25.0 ± 0.06^{Aa}	5.9±0.15	5.7±0.06	5.8±0.23	3.2 ± 0.11^{AB}	3.2 ± 0.16^{AB}	3.3 ± 0.03^{ABC}
3	25.8 ± 1.65^{A}	24.8 ± 0.15^{ABC}	24.7 ± 0.06^{AB}	5.9±0.21	5.9±0.17	5.9±0.35	3.2 ± 0.15^{AB}	3.2 ± 0.08^{AB}	3.2 ± 0.06^{ABC}
4	24.5 ± 0.11^{BCb}	25.0 ± 0.25^{ABa}	25.1 ± 0.2^{Aa}	6.0±0.35	5.8±0.15	5.7±0.32	3.2 ± 0.08^{ABb}	3.5 ± 0.12^{Aa}	3.4 ± 0.22^{Aab}
5	25.1 ± 0.21^{AB}	25.2 ± 0.32^{A}	25.0 ± 0.3^{A}	5.9±0.29	5.9±0.11	5.9±0.4	3.0 ± 0.01^{Bb}	3.2 ± 0.18^{ABa}	3.3 ± 0.08^{ABa}
6	24.8 ± 0.15^{ABC}	24.7 ± 0.15^{ABC}	24.8 ± 0.21^{AB}	5.9±0.4	5.7±0.36	5.9±0.44	3.4 ± 0.36^{A}	3.3 ± 0.21^{AB}	3.14 ± 0.17^{A}
7	24.5 ± 0.49^{BC}	24.3 ± 0.42^{CDE}	24.8 ± 0.15^{AB}	6.0±0.44	6.0±0.21	5.9±0.32	3.0 ± 0.15^{B}	2.9 ± 0.23^{B}	3.0 ± 0.15^{C}
8	23.9 ± 0.6^{C}	23.9 ± 0.5^{EF}	24.1 ± 0.57^{C}	5.9±0.21	5.9±0.06	5.9±0.15	3.2 ± 0.29^{AB}	3.1 ± 0.37^{AB}	3.1 ± 0.15^{BC}
9	23.8 ± 0.7^{C}	23.7 ± 0.52^{F}	23.8 ± 0.5^{C}	5.9±0.29	6.0±0.32	6.0±0.27	3.1 ± 0.18^{AB}	3.3 ± 0.26^{AB}	3.2 ± 0.21^{ABC}
10	24.2 ± 0.35^{BC}	24.1 ± 0.26^{DEF}	24.3 ± 0.3^{BC}	5.9±0.68	5.9±0.46	6.1±0.71	3.3 ± 0.23^{AB}	3.3 ± 0.24^{AB}	3.1 ± 0.13^{BC}

Different small letters indicate significant differences in an array and large letters comparison in column. CH: *Chlorella*; SE: *Scenedesmus*; MIX: *Chlorella* and *Scenedesmus*. Comparing vertically and horizontally with one-way ANOVA and Duncan's test.

Table 2

Changes in dry matter and chlorophyll *a* during testing in experimental treatments

Day	Parameter					
	Dry matter (mg L^{-1}) (control = 0.00±0.00)			Chlorophyll a (mg) (control = 0.000±0.00)		
	CH	SE	MIX	CH	SE	MIX
1	$1.27\pm0.11^{CD**}$	$1.17\pm0.15^{BC**}$	$1.17\pm0.06^{C**}$	$0.242\pm0.06^{Eb**}$	$0.490\pm0.050^{CDa**}$	$0.212\pm0.039^{DEb**}$
2	$1.23\pm0.15^{D**}$	$1.2\pm0.12^{BC**}$	$1.1\pm0.00^{CD**}$	$0.420\pm0.076^{Db**}$	$0.662\pm0.060^{Ca**}$	$0.288\pm0.044^{Dc**}$
3	$1.67\pm0.11^{AB**}$	$1.6\pm0.26^{AB**}$	$1.47\pm0.15^{B**}$	$0.927\pm0.400^{Ca**}$	$0.996\pm0.101^{Ba**}$	$0.689\pm0.128^{Cb**}$
4	$1.87\pm0.21^{A**}$	$1.87\pm0.67^{A**}$	$1.77\pm0.25^{A**}$	$1.255\pm0.145^{Bab**}$	$1.613\pm0.475^{Aa**}$	$0.878\pm0.244^{BCb**}$
5	$1.63\pm0.58^{B**}$	$1.57\pm0.42^{AB**}$	$1.63\pm0.3^{AB**}$	$1.694\pm0.144^{Aa**}$	$1.819\pm0.223^{Aa**}$	$1.091\pm0.264^{ABb**}$
6	$1.47\pm0.23^{BC**}$	$1.63\pm0.11^{AB**}$	$1.63\pm0.06^{AB**}$	$1.642\pm0.114^{Aa**}$	$1.790\pm0.245^{Aa**}$	$1.246\pm0.178^{Ab**}$
7	$0.87\pm0.06^{E**}$	$0.87\pm0.06^{CD**}$	$0.9\pm0.1^{D**}$	$0.223\pm0.015^{Eb**}$	$0.273\pm0.024^{DEa**}$	$0.240\pm0.030^{DEb**}$
8	$0.37\pm0.06^{F**}$	$0.43\pm0.1^{D**}$	$0.37\pm0.06^{E**}$	$0.155\pm0.063^{EF**}$	$0.100\pm0.027^{E**}$	$0.115\pm0.076^{DE**}$
9	0.00 ± 0.00^{G}	0.00 ± 0.00^{D}	0.00 ± 0.00^{F}	0.000 ± 0.000^{G}	0.000 ± 0.000^{E}	0.000 ± 0.000^{E}
10	0.00 ± 0.00^{G}	0.00 ± 0.00^{D}	0.00 ± 0.00^{F}	0.000 ± 0.000^{G}	0.000 ± 0.000^{E}	0.000 ± 0.000^{E}

Different small letters indicate significant differences in an array and large letters comparison in column. CH: *Chlorella*; SE: *Scenedesmus*; MIX: *Chlorella* and *Scenedesmus*. Comparing vertically and horizontally with one-way ANOVA and Duncan's test; *Compared with the control Dunnett test (** = $p < 0.01$).

Table 3

Amount of phosphate P and PO_4 during testing in experimental treatments

Day	Parameter					
	Phosphorus P (mg L^{-1}) (control = 0.06±0.01)			Phosphate PO_4 (mg L^{-1}) (control = 0.14±0.02)		
	CH	SE	MIX	CH	SE	MIX
1	0.05 ± 0.01^{Aa}	0.05 ± 0.01^{Aa}	$0.04\pm0.01^{Ab**}$	0.14 ± 0.01^{Aa}	0.14 ± 0.01^{Aa}	$0.09\pm0.01^{Ab**}$
2	$0.04\pm0.01^{B**}$	$0.04\pm0.01^{AB*}$	$0.03\pm0.01^{AB**}$	0.13 ± 0.01^{Aa}	0.12 ± 0.01^{Ba}	$0.04\pm0.01^{Bb**}$
3	$0.03\pm0.00^{BC**}$	$0.04\pm0.01^{BC**}$	$0.03\pm0.00^{ABC**}$	0.10 ± 0.01^{Ab}	$0.09\pm0.01^{Ca**}$	$0.02\pm0.00^{Cb**}$
4	$0.02\pm0.01^{CD**}$	$0.03\pm0.01^{CD**}$	$0.02\pm0.01^{CDE**}$	$0.08\pm0.01^{Ba**}$	$0.08\pm0.02^{Ca**}$	$0.02\pm0.01^{Cb**}$
5	$0.01\pm0.00^{E**}$	$0.02\pm0.01^{D**}$	$0.01\pm0.01^{DE**}$	$0.05\pm0.02^{Ca**}$	$0.04\pm0.01^{Dab**}$	$0.02\pm0.01^{Cb**}$
6	$0.01\pm0.00^{E**}$	$0.01\pm0.01^{D**}$	$0.01\pm0.00^{E**}$	$0.01\pm0.00^{E**}$	$0.02\pm0.01^{E**}$	$0.01\pm0.00^{Ca**}$
7	$0.02\pm0.01^{DE**}$	$0.02\pm0.01^{D**}$	$0.02\pm0.01^{DE**}$	$0.03\pm0.01^{D**}$	$0.04\pm0.01^{D**}$	$0.04\pm0.01^{B**}$
8	$0.02\pm0.01^{CD**}$	$0.020.01^{D**}$	$0.02\pm0.01^{BCD**}$	$0.04\pm0.01^{CD**}$	$0.04\pm0.00^{D**}$	$0.04\pm0.00^{B**}$
9	$0.02\pm0.01^{CD**}$	$0.02\pm0.01^{D**}$	$0.02\pm0.01^{BCD**}$	$0.04\pm0.01^{CD**}$	$0.04\pm0.01^{DE**}$	$0.04\pm0.01^{B**}$
10	$0.02\pm0.01^{CD**}$	$0.02\pm0.01^{D**}$	$0.02\pm0.01^{BCD**}$	$0.04\pm0.01^{CD**}$	$0.04\pm0.01^{DE**}$	$0.04\pm0.01^{B**}$

Different small letters indicate significant differences in an array and large letters comparison in column. CH: *Chlorella*; SE: *Scenedesmus*; MIX: *Chlorella* and *Scenedesmus*. Comparing vertically and horizontally with one-way ANOVA and Duncan's test; *Compared with the control Dunnett test (* = $p < 0.05$, ** = $p < 0.01$).

Table 4

Amount of Nitrate N and NO_3 during testing in experimental treatments

Day	Parameter					
	Nitrogen N (mg L^{-1}) (control = 1.36±0.12)			Nitrate NO_3 (mg L^{-1}) (control = 1.65±0.29)		
	CH	SE	MIX	CH	SE	MIX
1	1.38 ± 0.15^{ab}	1.19 ± 0.10^{C}	1.39 ± 0.30^{a}	1.243±0.011	1.453±0.174	1.480 ± 0.193^{AB}
2	1.72 ± 0.17^{ab}	1.41 ± 0.19^{CDb}	$1.79\pm0.13^{a*}$	1.489±0.209	1.655±0.298	1.483 ± 0.234^{AB}
3	1.57±0.37	1.68 ± 0.18^{AB}	1.66±0.30	1.392±0.236	1.288±0.084	1.412 ± 0.260^{AB}
4	1.64 ± 0.27^{a}	1.21 ± 0.17^{Cb}	1.54 ± 0.15^{ab}	1.533±0.232	1.594±0.303	1.309 ± 0.276^{B}
5	1.58±0.40	1.52 ± 0.18^{ABC}	1.56±0.18	1.448±0.477	1.485±0.424	1.369 ± 0.356^{AB}
6	1.50±0.26	1.51 ± 0.16^{ABC}	1.46±0.19	1.415±0.204	1.460±0.294	1.389 ± 0.139^{AB}
7	1.44±0.34	1.63 ± 0.28^{AB}	1.44±0.21	1.618±0.378	1.373±0.072	1.317 ± 0.045^{B}
8	1.56±0.25	1.70 ± 0.12^{AB}	1.49±0.23	1.378±0.276	1.378±0.210	1.783 ± 0.105^{A}
9	1.61±0.32	$1.86\pm0.20^{A*}$	1.63±0.04	1.465±0.281	1.739±0.120	1.715 ± 0.240^{AB}
10	1.39±0.23	1.54 ± 0.35^{ABC}	1.61±0.20	1.315±0.120	1.483±0.242	1.450 ± 1.67^{AB}

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Table 5

The trend of changing number of algal cells during testing in experimental treatments

Day	Number of algae cells				
	CH	SE	MIX		
			CH	SE	SUM
1	$6.7\times10^{6}\pm0.76\times10^{6DA**}$	$5\times10^{6}\pm0.84\times10^{6BCb**}$	$4.3\times10^{6}\pm0.34\times10^{6F**}$	$3.3\times10^{6}\pm0.46\times10^{6D**}$	$7.7\times10^{6}\pm0.76\times10^{6Fa*}$
2	$10.9\times10^{6}\pm0.92\times10^{6Ca*}$	$7.6\times10^{6}\pm0.72\times10^{6Bb*}$	$7.0\times10^{6}\pm0.53\times10^{6E}$	$5.1\times10^{6}\pm0.94\times10^{6C**}$	$12.1\times10^{6}\pm1.13\times10^{6Ea*}$
3	$19.2\times10^{6}\pm1.6\times10^{6Ba**}$	$14.6\times10^{6}\pm3.4\times10^{6Bb*}$	$13.5\times10^{6}\pm1.0\times10^{6D**}$	$7.7\times10^{6}\pm0.83\times10^{6AB**}$	$21.2\times10^{6}\pm0.89\times10^{6Da**}$
4	$29.0\times10^{6}\pm1.2\times10^{6Aa**}$	$15.2\times10^{6}\pm2.2\times10^{6Ab**}$	$19.7\times10^{6}\pm1.3\times10^{6C**}$	$7.4\times10^{6}\pm0.83\times10^{6B**}$	$27.1\times10^{6}\pm2.12\times10^{6Ca**}$
5	$29.6\times10^{6}\pm0.87\times10^{6A**}$	$16.3\times10^{6}\pm1.6\times10^{6Ab**}$	$23.2\times10^{6}\pm1.1\times10^{6B**}$	$7.9\times10^{6}\pm0.5\times10^{6AB**}$	$31.3\times10^{6}\pm0.94\times10^{6Ba**}$
6	$30.5\times10^{6}\pm0.7\times10^{6Ab**}$	$16.1\times10^{6}\pm1.4\times10^{6AC**}$	$24.8\times10^{6}\pm1.9\times10^{6A**}$	$8.9\times10^{6}\pm1.80\times10^{6A**}$	$33.4\times10^{6}\pm0.83\times10^{6Aa**}$
7	$7.9\times10^{6}\pm1.62\times10^{6Da**}$	$4.3\times10^{6}\pm1.3\times10^{6CDb**}$	$4.0\times10^{6}\pm0.53\times10^{6F**}$	$2.2\times10^{6}\pm0.72\times10^{6D*}$	$6.2\times10^{6}\pm1.27\times10^{6Fab*}$
8	$3.3\times10^{6}\pm0.91\times10^{6E**}$	$1.9\times10^{6}\pm1.02\times10^{6DE**}$	$1.5\times10^{6}\pm0.11\times10^{6E**}$	$0.7\times10^{6}\pm0.11\times10^{6E**}$	$2.3\times10^{6}\pm0.11\times10^{6G**}$
9	$0.9\times10^{6}\pm0.11\times10^{6Fa**}$	$0.1\times10^{6}\pm0.11\times10^{6Eb**}$	$0.1\times10^{6}\pm0.06\times10^{6G**}$	$0.0\pm0.0^{E**}$	$0.1\times10^{6}\pm0.06\times10^{6Hb**}$
10	$0.0\pm0.0^{H**}$	$0.0\pm0.0^{E**}$	$0.0\pm0.0^{G**}$	$0.0\pm0.0^{E**}$	$0.0\pm0.0^{F**}$
Control	$10.3\times10^{6}\pm0.13\times10^{6}$	$2.7\times10^{6}\pm0.16\times10^{6}$	$7.0\times10^{6}\pm0.16\times10^{6}$	$1.6\times10^{6}\pm0.06\times10^{6}$	$8.6\times10^{6}\pm0.12\times10^{6}$

Different small letters indicate significant differences in an array and large letters comparison in column. CH: *Chlorella*; SE: *Scenedesmus*; MIX: *Chlorella* and *Scenedesmus*. Comparing vertically and horizontally with one-way ANOVA and Duncan's test; *Compared with the control Dunnett test (* = $p < 0.05$, ** = $p < 0.01$).

Table 6

Amount of specific growth rate during testing in experimental treatments

Day	CH (control = -0.455±0.098)	SE (control = 0.619±0.131)	MIX (control = -0.357±0.073)
1	$0.480\pm0.112^{A**}$	0.433 ± 0.216^{AB}	$0.454\pm0.165^{AB**}$
2	$0.596\pm0.069^{A**}$	0.628 ± 0.204^{A}	$0.566\pm0.074^{A**}$
3	$0.414\pm0.072^{Aa**}$	0.191 ± 0.030^{ABb}	$0.245\pm0.071^{BCab**}$
4	$0.021\pm0.014^{B*}$	0.067 ± 0.065^{B}	$0.139\pm0.092^{C**}$
5	$0.031\pm0.016^{B**}$	$0.007\pm0.007^{B*}$	$0.070\pm0.034^{C**}$
6	$-1.361\pm0.191^{D**}$	$-1.339\pm0.0361^{D**}$	$-1.697\pm0.022^{E**}$
7	$-0.879\pm0.227^{C*}$	$-0.916\pm0.366^{C**}$	$-0.664\pm0.242^{D**}$
8	$-1.252\pm0.392^{Da**}$	$-2.294\pm0.444^{Eb**}$	$-3.091\pm0.00^{Fb**}$
9	Im	Im	Im
10	Im	Im	Im

Different small letters indicate significant differences in an array and large letters comparison in column. CH: *Chlorella*; SE: *Scenedesmus*; MIX: *Chlorella* and *Scenedesmus*. Im = immeasurable.
Comparing vertically and horizontally with one-way ANOVA and Duncan's test; *Compared with the control Dunnett test (* = $p < 0.05$, ** = $p < 0.01$).

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