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Original Article

Investigating the Growth Rate of *Tetraselmis* Algae and its Effect on Nitrogen Removal From Aquaculture Wastewater

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Abstract

Background: This research investigated the ability of *Tetraselmis* microalgae to remove nitrates from aquaculture centers' wastewater. Specifically, this study aimed to investigate the amount of nitrogen reduction by *Tetraselmis* microalgae under laboratory conditions. Therefore, algal density, chlorophyll a, specific growth rate, nitrate, and ammonia levels were investigated.

Methods: *Tetraselmis* microalgae was planted under laboratory conditions with constant density of 2000 mL including 1900 mL of distilled water and 100 mL of stock to investigate the refining effects for 15 days. Along to the test steps, the concentration of the mentioned factors was determined using standard methods and ultraviolet spectrophotometer.

Results: The results showed that algal density, chlorophyll concentration, and specific growth rate performed better than others in the pilot plants containing wastewater. Also, use of the studied algae caused a 50% reduction in ammonia and 80% in nitrate from aquaculture center wastewater. The data modeling showed that the above-mentioned microalgae were effective in reduction of nitrogen from aquaculture center wastewater as much as 75%.

Conclusion: The results showed that the species can reduce organic substances in the wastewater of aquaculture center and their discharge into the environment.

keywords: Wastewater treatment, *Tetraselmis* algae, Purification, Removal of nitrates

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Introduction

Nowadays, due to the increase in population and accordingly the need for healthy food such as fish, the food producers have been turned to breed fish to meet part of the food needs of the communities.[1] However, the breeders who want to invest in this sector usually face regulatory obstacles related to the wastewater from this industry. Wastewater from aquaculture farms which pollute the water resources include impurities such as nitrate, phosphate, ammonium, etc.. They enter into water resources and the food chain. The environmental effects of aquaculture centers are variable including changes in hydrological regimes, the introduction of non-native species, and pollution of water sources.[2,3] The pollution caused by aquaculture centers occurs through the metabolic products of fish feces and eaten food., However, due to their compatibility with the environment, the methods are preferred.[4]

There are various methods to reduce the amount of nitrate, ammonia, organic load, and other pollutants such as physical electrocoagulation, activated sludge, of which the biological methods are preferred due to their compatibility with the environment.[2]

One innovative approach for wastewater treatment and nitrate removal involves harnessing the capabilities of microalgae. This method entails cultivating microalgae and optimizing their compatibility with specific wastewater environments. In addition to providing the required nutrients, the wastewater treatment of the same section is also performed at the same time (e.g., *Scenedesmus*, *Nannochloropsis*).[5]

The primary anions in aquaculture systems are nitrogen compounds. In freshwater fish, the ultimate byproduct of excretion is ammonia, which undergoes transformation into compounds such as nitrite and subsequently nitrate through the activity of ammonia-decomposing bacteria. In this research, microalgae were used to remove nitrates from fish center wastewater. The utilization of algae as a remedial agent in sewage canals holds significance due to their ability to assimilate nitrates and phosphates for metabolic processes. Through photosynthesis, algae release oxygen, which, in turn, supports aerobic bacteria

in decomposing the raw materials present in wastewater.[6,7]

Numerous algae species exhibit resilience to various pollutants present in wastewater, showcasing rapid growth and effective assimilation of organic substances, particularly nitrogen. This capability allows them to efficiently remove nitrogen and other nutrient substances from wastewater. The microalgae breeding system emerges as a viable alternative for the secondary wastewater treatment process, demonstrating its effectiveness in nutrient removal.[8-11]

Removal of nutrients by microalgae is through their cell density and metabolic activity. Nitrogen removal by microalgae during metabolic activities is one of the main processes. Nitrogen is the second most important nutrient for microalgae.[12]

The researchers observed a parallel relationship between the rise in nitrogen content within cells and an increase in cellular protein. This study demonstrated that as cell growth increased, there was a corresponding elevation in cell protein. This growth was associated with the absorption of wastewater nitrate by the cells, leading to a reduction in nitrate concentration. Nitrate is generally preferred as a source of nitrogen by microalgae, and previous studies have shown that nitrate's absorption precedes nitrite's absorption.[13]

Inoculation of microalgae into a new culture medium (wastewater) is accompanied with stress of microalgae cells, and they usually need time to relieve the stress and regulate their metabolic activity as well as enzyme levels. The early-stage elevation in nitrate and nitrite concentrations can be attributed to stress-induced factors, potentially stemming from the death and disintegration of certain microalgae cells.[14-17]

This research aimed to investigate the ability of *Tetraselmis* microalgae to remove nitrogen from the wastewater of aquaculture centers for 15 days.

This research simultaneously investigated the nitrogen removal and chlorophyll levels, specifically focusing on the species employed, within the actual wastewater of the aquaculture center which is the novelty of this research.

Materials and Methods

Chemicals

All laboratory materials from Merck, Germany, and *Tetraselmis* algae stocks were obtained from the Algae Bank of Fars Algae Biological Reserves Development Company located in Shiraz. To conduct this research the following steps were taken:

Pilot Design and Construction

In this study, the dimensions of the reactors (4 bioreactors) under study were $20 \times 20 \times 40$ cm, which are made of glass, and their free space was 3 cm. The oxygen required by the algae is provided by the aeration pump that is supplied by the hoses that are installed at the bottom of the pilot (Figure 1). The light needed for algae photosynthesis was provided by LED lamps for algae growth. To control light interference and other disturbing side factors, the pilots were enclosed by aluminum foil.[18]

Algae Culture

The cultivation of algae was conducted in Conway culture medium, with volumes of 100 mL and 1000 mL, under controlled conditions within a germinator incubator. The temperature was maintained at 24 ± 1 units, illuminated by fluorescent lamps with a light intensity of 2500 lux. The pH of the medium was adjusted to the range of 5.7-6.8, and the salinity was set at 10 parts per thousand (PPT). Aeration was provided throughout the cultivation process.[18,19] After initial cultivation in 10-liter pots, the algae were subsequently mass-cultivated in 200 L. Light exposure was regulated using a luminometer device to optimize growth during the logarithmic phase. The algae were provided with aeration and the culture medium temperature was controlled for a period of 10 days. Under standard conditions, the optimal salinity for growth was maintained at 10 parts per thousand.[19,20] Seawater with a salinity of 12 parts per thousand was used as a base

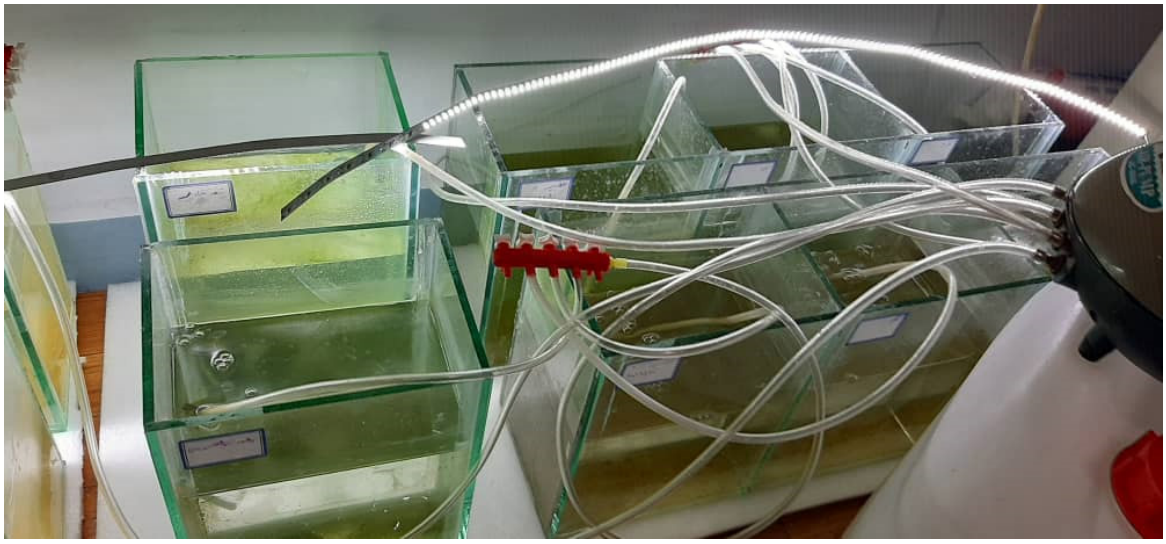

Figure 1. Pilot Design and Construction of the Study

solution for the culture of this microalgae. Sea salt was then added to achieve the required concentration. Also, water salinity was adjusted to 20 parts per thousand using a refractometer.

Prior to introducing the algae culture medium and algae stock, the water underwent a 30-minute exposure to UV rays for complete sterilization. Algae collection was performed using a high-speed centrifuge to separate suspended and dissolved compounds effectively. After the deposition and collection of algae in the inner part of the rotor of the device, the concentrated algae were collected and transferred to sterile containers.[21]

Wastewater

During the testing phase, samples were extracted from the wastewater of fish culture ponds in the Azadegan region, situated 20 km southeast of Ahvaz, during the summer season. The characteristics of wastewater samples has been provided in Table 1. Sampling was done according to the standard methods of water and sewage. The samples were transferred using isolated packs and the garbage particles or insects were separated from the environment by straining. One sample was sent to the laboratory for preliminary analysis, while the remaining wastewater was transferred to the pilot after passing through a sieve to remove insects and potential debris particles. All pilots and sampling containers were washed using distilled water.

Stock Preparation

The stock preparation from the microalgae is cultivated in a 2000 mL Erlenmeyer flask containing sterilized distilled water (1900 mL of distilled water and 100 mL of stock). The salinity of the environment is measured using sea salt by a digital electronic scale which is brought to 20 ppm salinity.[22] The culture medium used for *Tetraselmis* was TMRL medium, which includes potassium nitrate and Sod.[19] Microalgae cells were counted daily using the Nicobar slides and a binocular microscope with an XSZ-801 BN monitor until they reach their maximum growth (rate of 10^5).[23]

Setting Up Biological Reactors as Batch

Filling the four reactors with wastewater and inoculating the multiplied microalgae species into the reactors were done by controlling and recording the temperature, light, pH, and dissolved oxygen of the environment when the reactor started. The pH was between 7 and 8.5 during the experiment. The aeration system was installed in the reactors so that the system continuously worked and the algae rotated in the entire pilot. Sampling, analysis and measurement of necessary parameters were done in 5 stages over 15 days.[24]

Table 1. The initial characteristics of wastewater

Characteristics	Value
pH	7.5
DO (mg/L)	7.7
TDS (mg/L)	4200
BOD (mg/L)	115
COD (mg/L)	220
NO_3 (mg/L)	0.24
NO_2 (mg/L)	0.004
NH_3 (mg/L)	0.25
PO_4 (mg/L)	0.2

Measurement of Cell Density

After counting the slide, the cell density (OD) of the samples was measured using a spectrophotometer (UV-Visible Varian, Cary 50 scan) with a light intensity of 540 nm. A blank was employed for each sample, which consisted of water with salinity and culture medium. The cell density (OD) was adjusted to zero for the transparent sample, and subsequently, the OD of the targeted sample was measured with two separate repetitions.[25,26]

Measurement of chlorophyll (a)

To measure the amount of chlorophyll according to the instructions below, we read the absorbance at 664, 647, and 630 nm wave. We then put it in Equation 1 to calculate the amount of chlorophyll.[23]

$$\text{Chlorophyll a } (\mu g/L) = 11.85\ (OD\ 664) - 1.54\ (OD\ 647) - 0.08\ (OD\ 630) \quad (1)$$

Measurement of Specific Growth Rate

To determine the specific growth rate, Equation 2 was used[27]:

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \quad (2)$$

Where, μ is the growth rate, and X_2 and X_1 represent the dry weight value of the biomass at times t_2, and t_1, respectively.

Statistical Analysis

To optimize a process including several variables is sometimes complex and requires the use of statistical and mathematical techniques. One of the most valid and widely used optimization methods is the response surface method (RSM). The RSM comprises mathematical and statistical techniques applied to model and analyze problems influenced by multiple factors, with the objective of optimizing the response. In this study, to determine the optimal conditions, the center composite design (CCD) was used by Design Expert software.[27] Experiments were conducted at four levels for three variables: algae density, chlorophyll a, and specific growth rate. Each experiment was replicated at least twice.

Results and Discussion

According to the conducted experiments and the sampling method carried out by the pilots, the results were as follows. During the experiment, the temperature was constant as 25 ± 1°C and the TDS was in the range of 12000-18000

mg/L. Also, the EC ranged between 20,000 and 30,000 µS/cm, pH was between 7.5 and 8.5, and DO was between 5 and 10 mg/L.

Algae Density Chart

As seen in the Figures 2-4, we saw the growth of algae. In the initial phase, the species exhibited a slow rate of growth and demonstrated limited adaptation to the environment. Then, it started to grow rapidly and reached its maximum population on the ninth day. The peak of species population growth occurred on the 9th to 10th day, with both pilot cultures, including types of culture medium and wastewater. A notable increase was observed in the wastewater treatment, reaching over 3 million cells per milliliters, while the pilot containing the culture medium exhibited approximately 2 million cells per milliliters. The elevated microalgae population in the wastewater can be attributed to the presence of numerous organic substances serving as nutrients. However, after the ninth day, the algae entered a phase of decline and eventual death. Zheng et al found that the hydraulic retention time increased when the concentration of total nitrogen and phosphorus remained constant which was affected by the water depth of algae growth. The average density of algae in the lake was increasing until eighth day. The authors also found that nutrients and light intensity caused the growth of algae,[28] which is consistent with our findings.

Chlorophyll Diagram

As shown in Figures 5-7, the concentration of chlorophyll reached the maximum between the 8 and ninth days, which was result of the algae density at the peak of growth. The concentration of chlorophyll a in the pilot containing the wastewater was approximately 6 mg/L, showing a slight variation compared to the concentration of chlorophyll a in the pilot containing the culture medium. Following the tenth day, there was a significant decrease

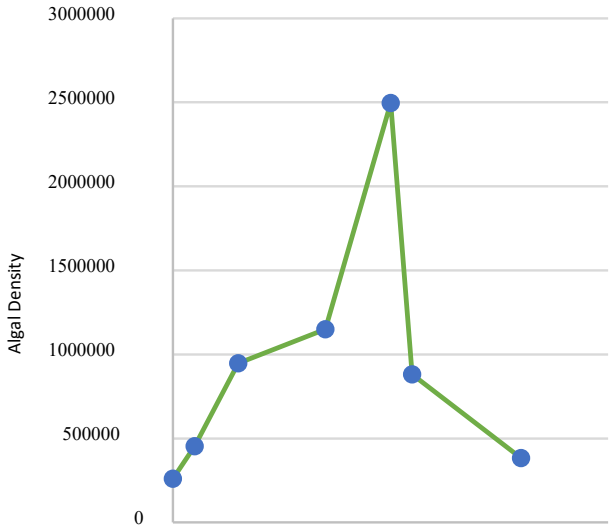

Figure 2. Algae Density in Wastewater (pilot 1, 2, 3)

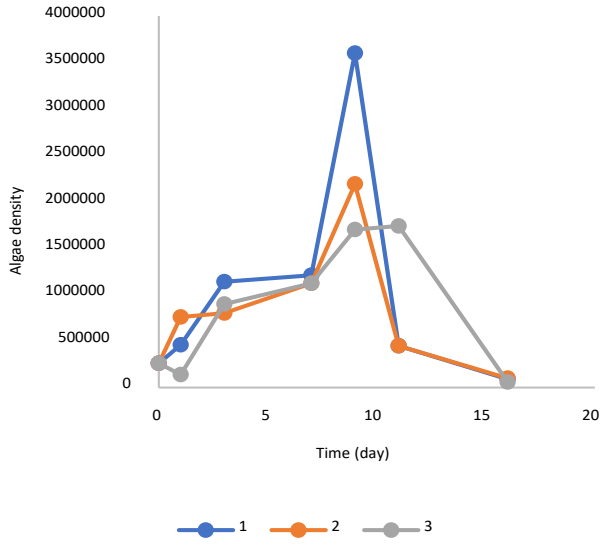

Figure 3. Algae Density in The Culture Medium

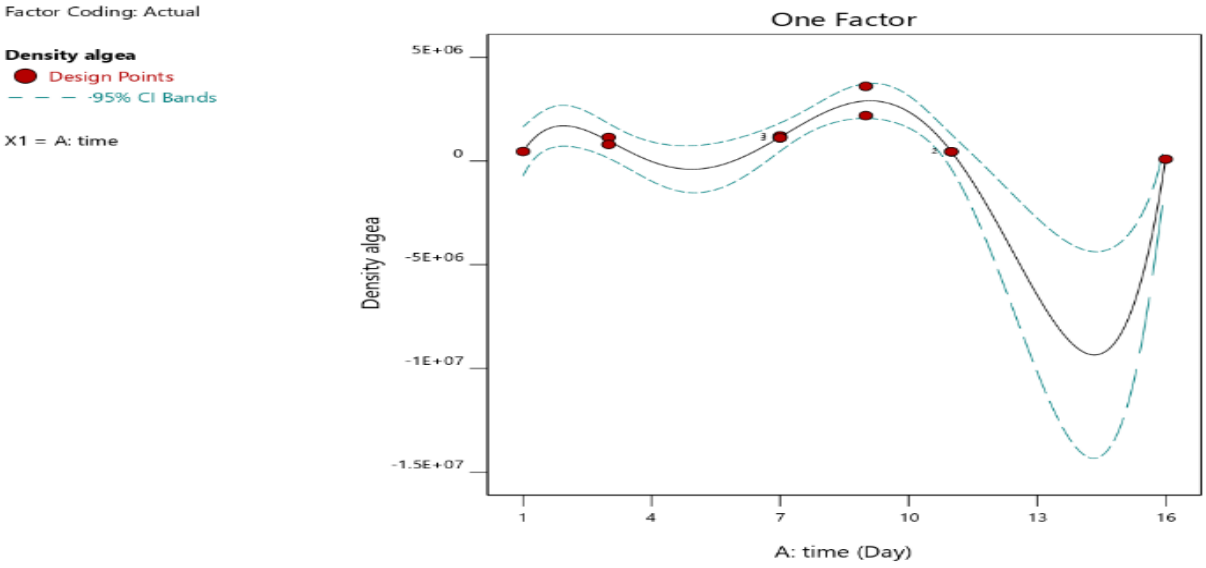

Figure 4. Density of Algae in Wastewater

in the concentration of chlorophyll. This decline can be attributed to the completion of the algae life cycle and the onset of the declining phase. In a study by Patrinou and colleagues, the total chlorophyll produced by *Tetraselmis* under different pH conditions was reported as 4.6 and 5.1 mg/L.[29]

Specific Growth Rate of Algae

The amount of specific growth rate was dependent on the amount of algae density; with the increase of algae density, the specific growth rate increased, as well. Also, on the 9th day, it reached its maximum value of 5 d^{-1}, which had better results than the pilot containing the culture medium (Figures 8-10). Hu et al studied the relationship between the algae diversity and *Hyriopsis schlegelii* growth in mixed fish-mussel aquaculture. Their results showed that the diversity and richness of the species increased in summer and autumn, while they decreased in spring and winter. However, there was a few differences throughout the year. In the study, correlation analysis showed that the factors related to water quality including ammonia nitrogen, dissolved oxygen and temperature had great effect on the algae growth.[30]

Diagram of the Relationship Between Nitrogen and Chlorophyll

According to the results, the nitrogen, ammonia, nitrate and culture media in wastewater were reduced by the *Tetraselmis* as 50, 36, 41,6 and 80% respectively. Given that nitrogen is one of the nutrients required for the growth of the species and given the optimal conditions such as aeration and light for the growth of the species, the decreasing trend can be attributed to the growth of the species. As seen in the Figures 11-13, with the start of algae growth, the amount of nitrogen in the pilot plants decreased. Also, there was nitrogen removal until the ninth day, when the microalgae reached their maximum growth. The conversion of ammonia to nitrate in wastewater is probably associated with the findings. These findings are consistent with those of Chaboki et al They showed

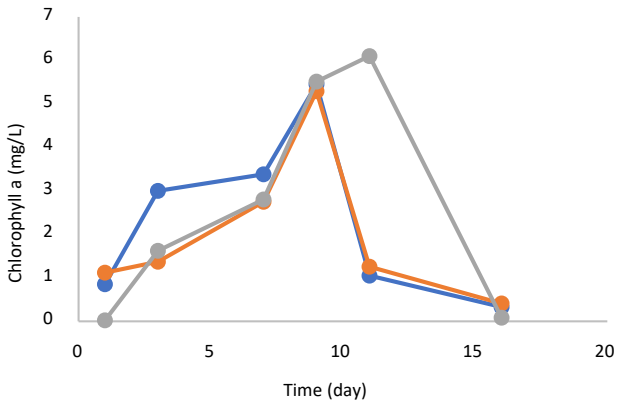

Figure 5. Chlorophyll in Wastewater

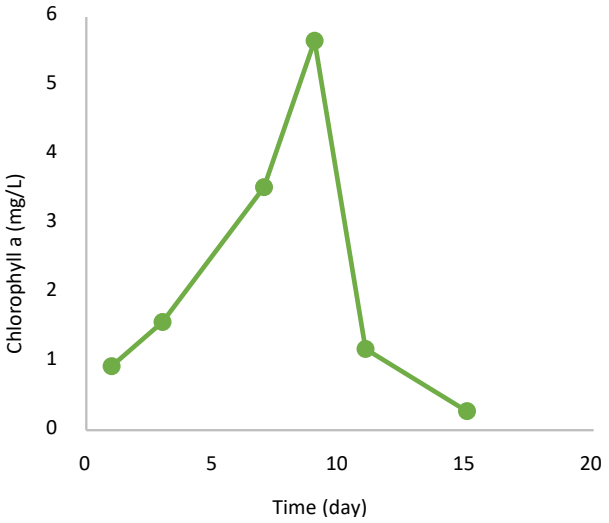

Figure 6. Chlorophyll in Culture Medium

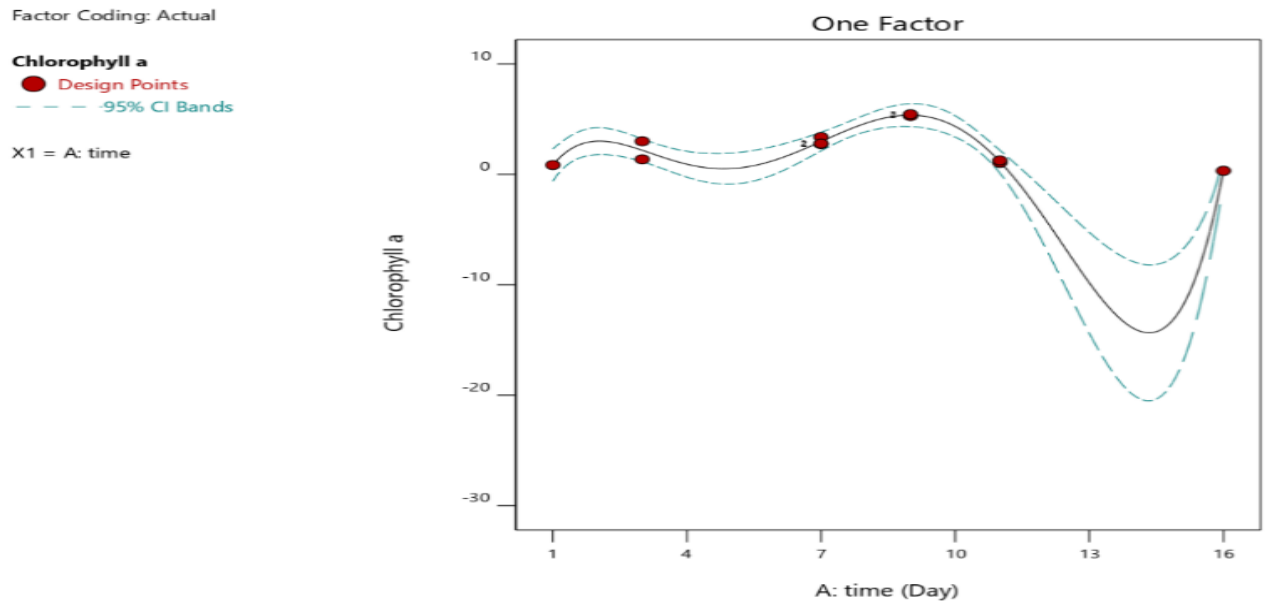

Figure 7. Chlorophyll in Wastewater

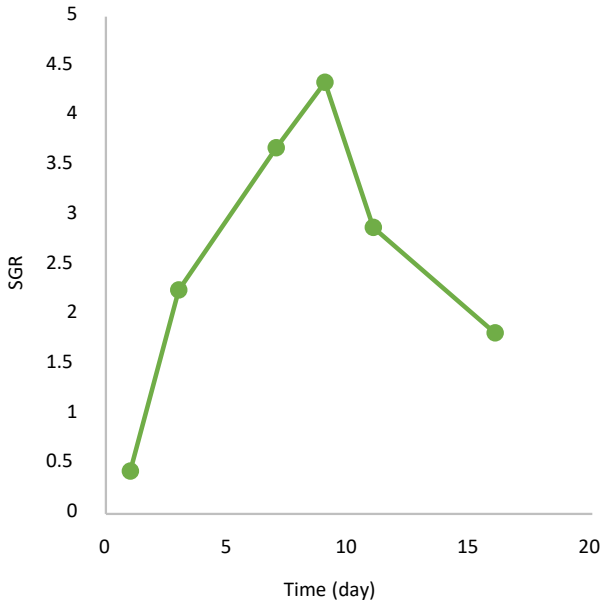

Figure 8. Specific Growth Rate in Wastewater

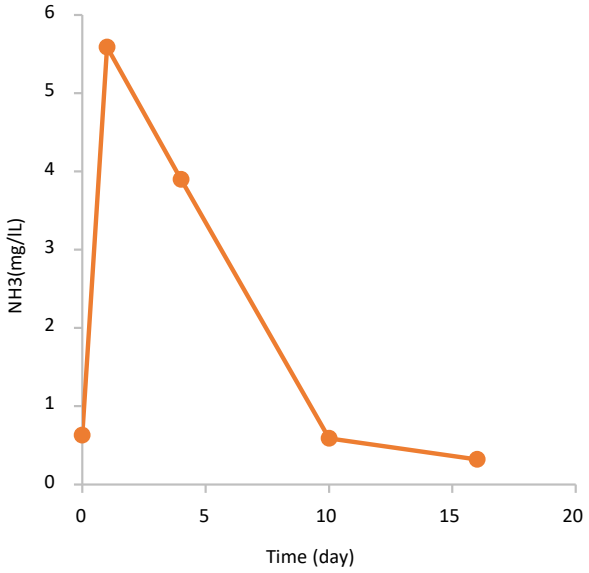

Figure 11. Ammonia Concentration

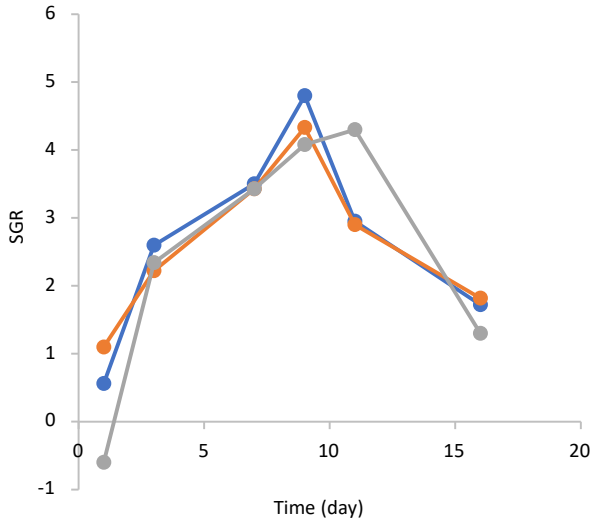

Figure 9. Specific Growth Rate in Culture Medium

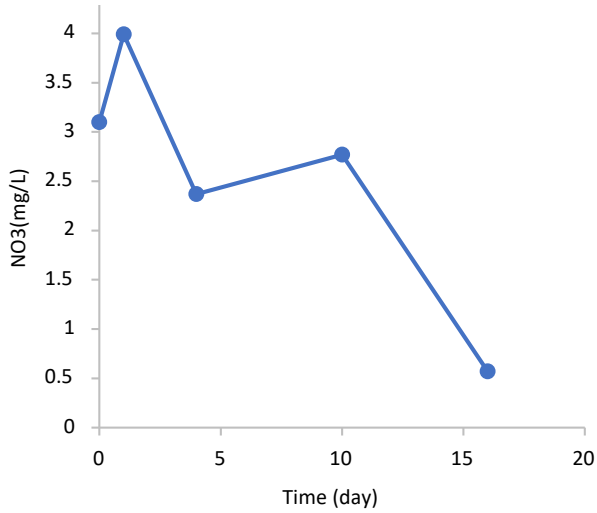

Figure 12. Nitrate Concentration

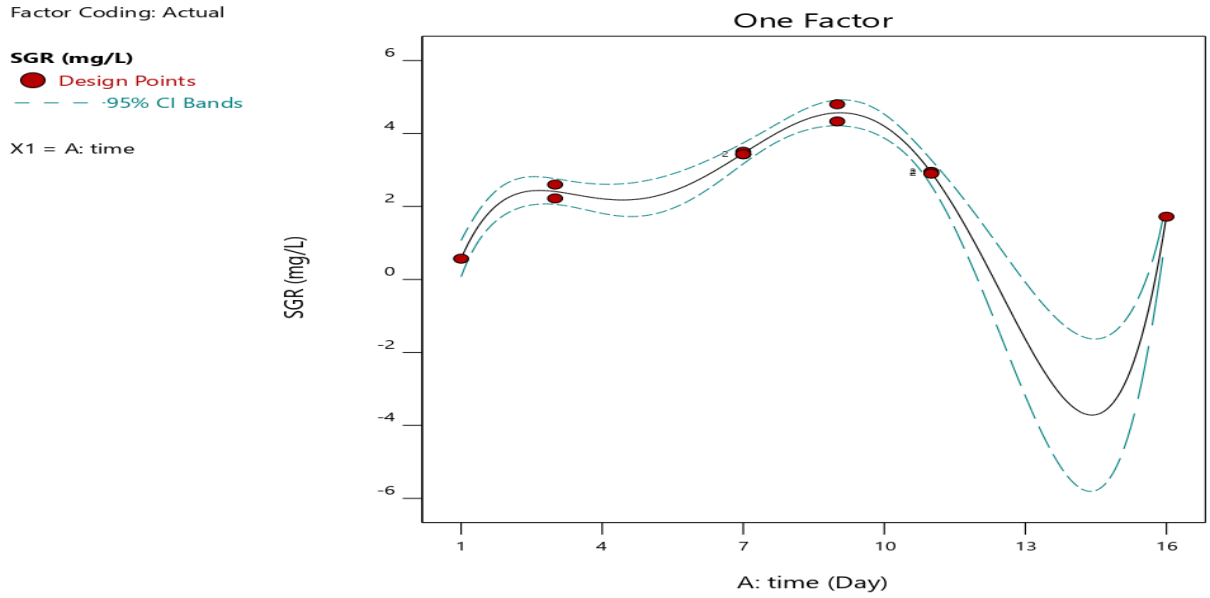

Figure 10. The Specific Growth Rate in Wastewater

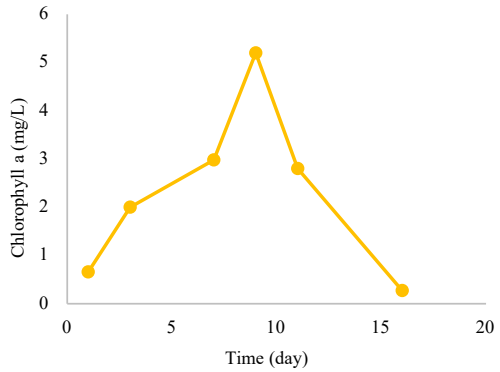

Figure 13. Chlorophylla concentration

that removal efficiency of total nitrogen in industrial wastewater by Chlorella vulgaris was 13.79%.[31]

Conclusion

Based on the results, we can conclude that the density of algae, the concentration of chlorophyll a, and the specific growth rate in the pilots containing wastewater had better outputs. We also saw 50% removal of ammonia and 80% removal of nitrate from the wastewater of the aquaculture center. Based on the findings of this research, future studies may explore the implications of varying salinity conditions, different microalgae species, and the population ratio of microalgae species to wastewater quantity.

Authors' Contribution

Conceptualization: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh.
Data curation: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh, Narges Javadzadeh, Azita Koushafar.
Formal analysis: Mahsa Ghadamzadeh.
Investigation: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh, Narges Javadzadeh.
Methodology: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh, Narges Javadzadeh, Azita Koushafar.
Project administration: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh, Narges Javadzadeh.
Software: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh, Sina Attarroshan.
Supervision: Reza Jalilzadeh Yengejeh.
Validation: Reza Jalilzadeh Yengejeh, Narges Javadzadeh.
Visualization: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh.
Writing–original draft: Mahsa Ghadamzadeh, Reza Jalilzadeh Yengejeh.
Writing–review & editing: Reza Jalilzadeh.

Competing Interests

The authors declared no conflict of interest.

Ethical Approval

There were no ethical considerations to be considered in this research.

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