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Production of Microalgal Biomass Using Aquaculture Wastewater as Growth Medium

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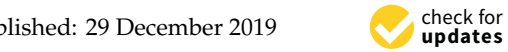

Abstract: Aquaculture wastewater contains a huge amount of substances that can cause environmental pollution. However, microalgae can absorb these compounds and convert them into useful biomass. In this study, *Chlorella minutissima* was grown in the wastewater resulting from saline aquaculture. The microalgae were found to effectively utilize nitrogen and phosphorus in the wastewater for its growth. During wastewater treatment, the cell density increased almost fivefold compared to the initial value (OD_{680} 0.502). Moreover, batch culture resulted in the maximum biomass concentration and productivity of 4.77 g/L and 0.55 g/L/day, respectively. The contents of total nitrogen and total phosphorus in wastewater decreased by 88% and over 99%, respectively. In addition, the content of N-NO_3 was reduced by 88.6%, N-NO_2 by 74.3%, and dissolved orthophosphates (V) by 99%. At the beginning and throughout the experiment, the content of N-NH_4 in wastewater remained below 0.05 mg/L. Furthermore, a high lipid content of 46.4% (w/w) was also obtained from the studied microalgae.

Keywords: microalgal biomass; aquaculture; wastewater treatment; nitrogen/phosphorus removal; lipids

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture is the fastest growing industrial sector, and currently, its products account for almost 50% of the world's food [1]. Water originating from the fish farming process generally contains feces, uneaten food, and possibly residues of medicines used in the production or added for cleaning [2]. It is estimated that only 20–30% of the nitrogen in fish feed is assimilated or used by fish, while the rest is released into the water [3]. Therefore, in addition to the suspended solids, a significant amount of nitrogen and phosphorus [4,5] is found in wastewater, which may adversely affect the environment [6]. Aquaculture is required to be carried out at a large scale in order to meet the growing demand for fish. Therefore, it is predicted that, in 2030, the global aquaculture production will expand to around 93.2 million tons, and beyond 2030, aquaculture will be the leading source of fish supply. Consequently, ensuring successful and sustainable development of aquaculture worldwide is an imperative agenda for improving the global economy [7]. However, along with the increase in fish farming, the amount of sewage generated also increases [8], which should be managed in various ways due to its chemical composition [3,9].

The high content of nutrients in wastewater stimulates the excessive production of phytoplankton, reducing water quality [10]. Thus, the need to protect the aquatic ecosystems against this type of

pollution leads to the use of effective technologies for the treatment of aquaculture wastewater [11], which are based on physical, chemical, and biological methods [12]. Plants and hydroponics, bacteria, and microalgae are successfully used in the purification or the treatment of postculture waters. Oxygen produced during photosynthesis by algae may reduce the biological oxygen demand in wastewater. Moreover, these organisms are highly capable of removing the nutrients present in wastewater, including nitrogen and phosphorus, which are responsible for the eutrophication processes [13]. The use of algae in wastewater treatment is beneficial because the process is not only cost-effective but also enables the recycling of nitrogen and phosphorus for the production of biomass or for using it as fertilizers. In addition, in the case of photoautotrophic organisms, it is not necessary to provide an additional source of carbon to remove these elements [14]. Phosphorus is a nonrenewable raw material, and hence, its recovery and reuse (e.g., in the production of algal biomass) [15] is highly advantageous.

Integrating the treatment of aquaculture wastewater with algal cultivation in the Recirculation Aquaculture System (RAS) can bring not only ecological but also economic benefits. Algae obtained in this process can be used in cosmetic and pharmaceutical industries and as a substrate for the production of food, animal feed, and biofuels [16,17], including those from the lipids accumulated in their cells [18]. In this study, we examined whether (1) *Chlorella minutissima* can grow and (2) promptly remove the nutrients from the wastewater originating from saline aquaculture. We also evaluated the efficiency of lipid accumulation in algae biomass as a potential source in the production of biodiesel.

2. Materials and Methods

2.1. Culture of Chlorella Minutissima

Chlorella minutissima algae (Figure 1) were obtained from the Culture Collection of Baltic Algae (CCBA). The algae were inoculated in F/2 medium (pH 8.0) and cultured at 23 ± 1 °C under a 16-h light/8-h dark cycle. Light-emitting diodes (LEDs) with an intensity of 8.4 W/m^2 were used as the light source.

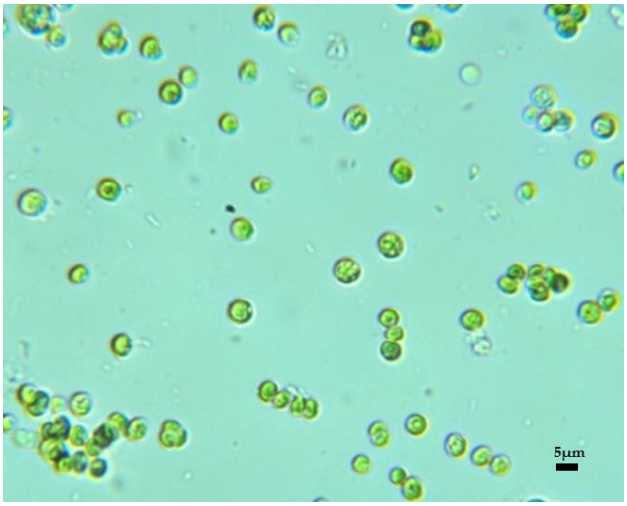

Figure 1. Light microscope image of *Chlorella minutissima* obtained during the cultivation process.

2.2. Aquaculture Wastewater

The wastewater used in the study was obtained from the RAS used for salmon farming in Jurassic Salmon company (Karnice, Poland, 54°2′12.903″ N, 14°58′50.2996″ E). It is the world's first fully ecological farm that cultures Atlantic salmon. The fish production process is carried out with low-temperature saline geothermal water obtained from a well at a depth of over 1220 m below the sea level. Breeding is carried out in accordance to the standards of the ASC (Aquaculture Stewardship Council). After mechanical and biological treatment, wastewater from the aquaculture returns to the

environment to a salty-fresh water lake. For this study, the wastewater with an average salinity of 10‰ was collected from the storage reservoir at the production facility.

2.3. Experimental Design and Analytical Methods

Aquaculture wastewater taken from the storage tank was filtered twice, initially to remove the suspended impurities and then through filters having pores with an average diameter of 1.2 µm. The experiment was conducted in 14 L tubular photobioreactors (PBRs) with 10.8 L of postproduction water or synthetic F/2 medium.

The F/2 nutrient medium consisted of the following (pH 8.0): $NaNO_3$—0.075 g; $NaH_2PO_4{\cdot}2H_2O$—0.00565 g; stock solution of trace elements—1 mL/L (Na_2EDTA 4.16 g, $FeCl_3{\cdot}6H_2O$ 3.15 g, $CuSO_4{\cdot}5H_2O$ 0.01 g, $ZnSO_4{\cdot}7H_2O$ 0.022 g, $CoCl_2{\cdot}6H_2O$ 0.01 g, $MnCl_2{\cdot}4H_2O$ 0.18 g, $NaMoO_4{\cdot}2H_2O$ 0.18 g); and stock solution of vitamin mix—1 mL/L [cyanocobalamin (vitamin B12) 0.0005 g, thiamine HCl (vitamin B1) 0.1 g, biotin 0.0005 g [19]. This standard F/2 medium with optimal nutrient content was used to compare the growth of *C. minutissima* and the lipid content in biomass obtained in wastewater.

After sterilizing the medium with UV light, 1.2 L of *C. minutissima* inoculum was added to the PBRs. The average initial concentration of biomass used to inoculate PBRs was 1.3 g/L. Carbon dioxide together with air was introduced into the PBRs using a membrane pump at a constant flow rate of 14 L/min. The fluid flow and mixing were driven by bubbles released by an air sparger at the bottom. The mixing process carried out with air did not cause foam in PBRs. In addition, the working capacity of PBRs was maintained lower than their total volume. To limit the possibility of contamination of the culture during aeration, polytetrafluoroethylene (PTFE) membrane filters were used. The wastewater treatment was carried out at 23 ± 1 °C.

During the experiment, samples for chemical analysis were taken from each of the photobioreactors on the day of setting up and then after 2, 4, 6, 8, and 10 days. The pH, total nitrogen (TN), nitrite nitrogen (NO_2), nitrate nitrogen (NO_3), ammonium nitrogen, total phosphorus (TP), and orthophosphate ion were determined. Orthophosphate (PN EN 1189:2000) [20], nitrogen nitrate (PN–C-04576-08) [21], nitrogen nitrite (PN-C-04576-06) [22], and nitrogen ammonium (PN ISO7150-1: 2002) [23] were measured by the spectrophotometric method according to the Polish Standards. The pH of the tested water was measured potentiometrically using a microcomputer pH-meter CI-316.

Microalgae growth was monitored by measuring optical density (OD). Algae suspension samples (2.5 mL) were taken from the culture and analyzed using a SEMCO S91E spectrophotometer at 680 nm (OD_{680}). The biomass content (g/L) in the culture was also determined. The determination was carried out by gravimetric method after centrifugation of samples taken from photobioreactors from the culture (15 min, 4000 rpm) and drying them at 105 °C. Productivity (g/L/d) was determined based on the biomass yield using the following formula:

$$\text{Biomass productivity (BP)} = \frac{B_f - B_0}{d}, \quad (1)$$

where B_f is the final amount of biomass (g), B_0 is the initial amount of biomass (g), and d is the cultivation time (day).

The lipid content of the biomass was determined by Soxhlet extraction. After sedimentation, the algae biomass obtained from individual photobioreactors was dried at 80 °C, and then 1 g samples were weighed, and each of them was placed in a cellulose casing. Hexane was used for extraction. The process was carried out for 6 h at the frequency of 20 cycles/h. After removal of the solvent, lipids were determined gravimetrically and presented as a percentage relative to biomass, substituting the data for the following Equation:

$$\textit{Lipid content (LC)} = \left(\frac{m_L}{m_{DAB}}\right) \times 100, \quad (2)$$

where m_L is the lipid mass (g) and m_{DAB} is the dry algal biomass (g).

All the determinations were carried out in triplicate. The parameters related to aquaculture wastewater with algae were analyzed using the analysis of variance. The significance of differences was evaluated using Tukey's honestly significant difference test at a level of $\alpha = 0.001$. Pearson's coefficient of correlation (r) between the biomass content and the optical density was also determined ($\alpha = 0.001$). All the statistical analyses were carried out using the statistical software package for Windows (Dell Statistica (data analysis software system) version 13.3 (2016); Dell Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Removal of Nitrogen and Phosphorus from Aquaculture Wastewater

Nitrogen and phosphorus present in wastewater contribute to eutrophication. Therefore, the wastewater must be properly treated to remove these compounds before it is discharged into the water reservoirs [24]. The content of total nitrogen and total phosphorus determined in the culture wastewater tested in the present study is shown in Table 1. The initial concentration of total nitrogen in the wastewater was 31.83 mg/L, whereas after 10 days of treatment with *C. minutissima,* it decreased by 87.9%. The initial concentration of total phosphorus was 1.1 mg/L, and the content decreased with the microalgal treatment by 99.1%. In the study conducted by Gao et al. [25], the average reductions of total nitrogen and total phosphorus in aquaculture wastewater achieved in the presence of *Chlorella vulgaris* were 86.1% and 82.7%, respectively. Van Den Hende et al. [26] used a mixed culture of microalgae and bacteria for the removal of these nutrients and noted reductions of 57.9% and 88.6%, respectively. The high treatment efficiency achieved with the culturing of *C. minutissima* was also confirmed by the study carried out by Singh et al. [27] on other types of sewage wastewater, in which the concentration of nitrogen was decreased by 99.19% and phosphorus by 96%.

Table 1. The chemical composition of wastewater from aquaculture.

Time (Days)	TN (mg/L)	TP (mg/L)
0 (initial stage)	31.83 ± 0.3	1.1 ± 0.0
10 (final stage)	3.83 ± 0.2	0.01 ± 0.0

Nitrogen is the second most important nutrient for the growth of algae besides carbon [28]. The best assimilated forms of nitrogen are ammonium nitrogen (NH_4^+) and nitrate (NO_3^-) [29]. Before beginning the treatment process with *C. minutissima* algae, the concentration of N-NO_3 in the wastewater was 27.5 mg/L, N-NO_2 was 8.63 mg/L, and PO_4 was 0.987 mg/L (Figures 2–4). However, the concentration of N-NH_4 remained constant, both at the beginning and throughout the experiment, and was at a level below the measuring range of the analytical method used (i.e., <0.05 mg/L). A significant change in concentration was noted for all the compounds as early as 2 days after culture, but the course of reduction varied. Among the analyzed nutrients, orthophosphates were consumed at the fastest rate by the tested microalgal species (Figure 4), and after 6 days of the experiment, the concentration of orthophosphates was lower than 0.05 mg/L. Interestingly, Beuckels et al. [30] emphasized that the removal of P by microalgae was influenced by the concentration of N in the wastewater. In the present study, the low amount of orthophosphates appeared to affect the intensity of the biological removal of the oxidized forms of nitrogen from the 6th day of the experiment. Thus, a significantly reduced availability of nitrate forms was observed until the end of the experiment (Figure 2). Ultimately, the reduction of nitrites observed during the experiment was 77.46%, nitrates was 88.64%, and orthophosphates was 100%, and the concentration of these components observed in the wastewater after 10 days of treatment was 0.435 mg/L, 3.1 mg/L, and <0.05 mg/L, respectively. Liu et al. [31] assessed the potential of five species of microalgae (*C. vulgaris*, *Chlorococcum* sp. GD, *Parachlorella kessleri* TY, *Scenedesmus quadricauda*, and *Scenedesmus obliquus*) in the treatment of aquaculture wastewater and found that, after 5 days, nitrates, nitrites, total ammonium, and phosphorus were removed

by 85.7–97.1%, 94.3–99.8%, 97.9–98.9%, and 90.2–98.9%, respectively. In the study conducted by Ansari et al. [32], the reduction of ammonium ions achieved with *S. obliquus*, *Chlorella sorokiniana*, and *Ankistrodesmus falcatus* was in the range of 86.45–98.21%, nitrates in the range of 75.76–80.85%, and phosphates in the range of 98.52–100%.

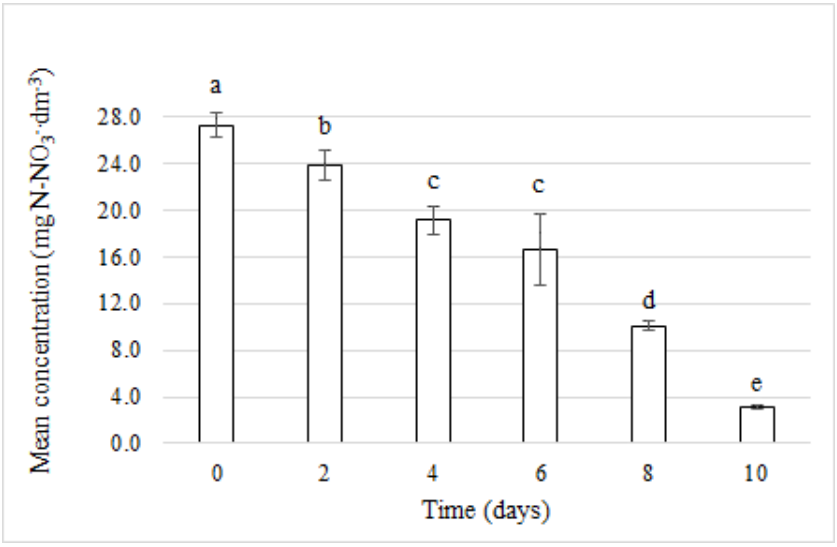

Figure 2. Changes in N-NO_3^- concentration during cultures of *Chlorella minutissima* in wastewater (mean ± SD; ANOVA F (5, 48) = 327; $p < 0.001$; mean over each columns not marked with the same letter is significantly different at $p < 0.001$).

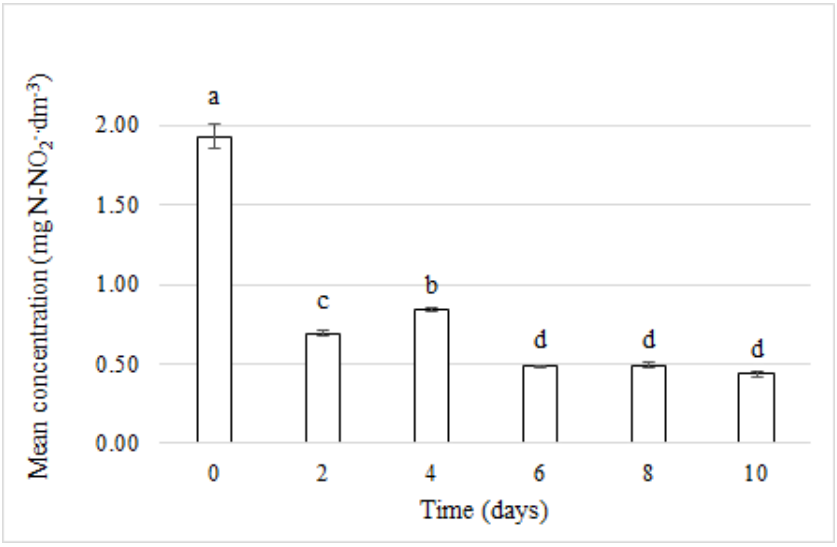

Figure 3. Changes in N-NO_2^- concentration during cultures of *Chlorella minutissima* in wastewater (mean ± SD; ANOVA F (5, 48) = 2501; $p < 0.001$; mean over each columns not marked with the same letter is significantly different at $p < 0.001$).

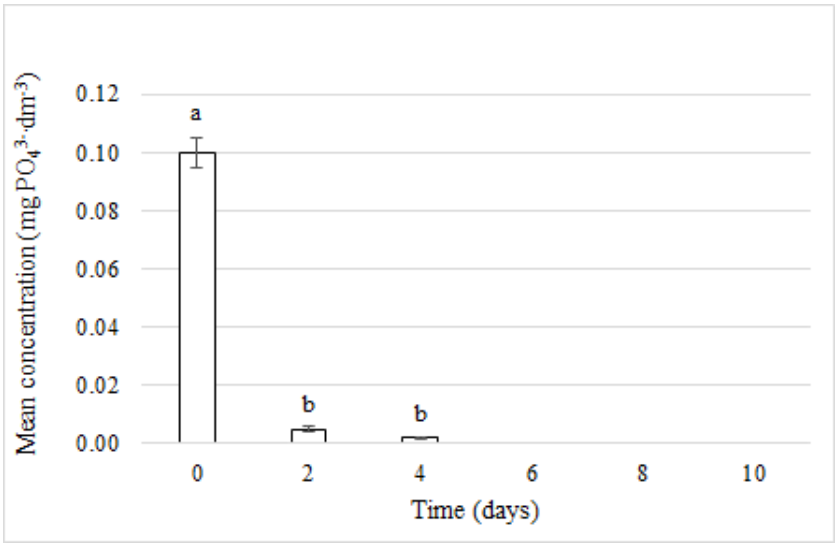

Figure 4. Changes in PO_4^{3-} concentration during cultures of *Chlorella minutissima* in wastewater (mean ± SD; ANOVA F (2, 24) = 2938; $p < 0.001$; mean over each columns not marked with the same letter is significantly different at $p < 0.001$).

In this study, the pH of the growth environment of *C. minutissima* was slightly alkaline and ranged from 7.98 to 8.54 (Table 2). The pH changes may have been a result of CO_2 consumption from the medium or the absorption of nitrate ions by the microalgae [33].

Table 2. The reaction pH of wastewater with algae.

Time (Days)	0	2	4	6	8	10
pH	7.98 ^{e*} ± 0.01	8.47 ^{c} ± 0.02	8.52 ^{b} ± 0.02	8.54 ^{a} ± 0.01	8.47 ^{c} ± 0.02	8.09 ^{d} ± 0.01

(ANOVA F (5, 48) = 2718; $p < 0.05$; *mean over each columns not marked with the same letter is significantly different at $p < 0.001$).

3.2. Microalgal Growth in Aquaculture Wastewater

The effect of nutrients in aquaculture wastewater on the growth of *C. minutissima* is shown in Figure 5. In the growth curve plotted for the microalgal growth in wastewater, no phase lag was found, which indicates that *C. minutissima* adapts well to saline waters. Similar findings were reported by Gao et al. [26] for *C. vulgaris* and *S. obliquus*. The presence of the nitrogenous form of nitrogen in sewage, a macronutrient that is necessary for the synthesis of amino acids, proteins, nucleic acids, and other nitrogen-containing compounds [34], was reported to favor the production of microalgal biomass. This is in line with the present study, in which the amount of biomass increased by approximately 30% in both F/2 medium and the tested wastewater after two days of breeding. Statistically significant differences between the objects were recorded after six days, the time at which the microalgae reached a stationary growth phase. The time needed for the microalgae to enter the stationary growth phase is known to depend on the initial nitrate concentration in the culture. The relationship between the biomass growth of *C. minutissima*, *Tetraselmis suecica*, and *Monoraphidium contortum* and the concentration of nitrates was also confirmed by Sánchez-García et al. [34].

In the present study, the maximum biomass concentration of *C. minutissima* was obtained after eight days of culture. In the postculture wastewater, the concentration increased to 4.69 g/L, while in the F/2 medium, it increased to 3.61 g/L. In the studies carried out by Halfhide et al. [35] a maximum mean biomass concentration for *Scenedesmus* (0.41 g/L) in the tilapia RAS wastewater was achieved after 36 h. The high contents of nitrogen and phosphorus in wastewater are reported to promote the production of microalgal biomass, with the proportion of these components playing an important

role [30]. In the studies conducted by Tossavainen et al. [36], the largest content of mixed biomass of *Euglena gracilis* and *Selenastrum* (1.5 g/L) was observed in the wastewater obtained from pikeperch aquaculture, in which the contents of nitrogen and phosphorus were 34.4 and 6.1 mg/L, respectively. In the sewage wastewater obtained from catfish aquaculture, where the content of biogen was much lower (23.7 mg/L of N and 3.6 mg/L of P), the amount of biomass determined was only 0.08 g/L, and the N/P value of the algal biomass was N15/P1. The optimal internal molar ratio for C:N:P in algal biomass (the Redfield Ratio) has been established as 106:16:1 [37]. Beuckels et al. [30] emphasized that sufficiently high concentrations of N are needed to ensure effective removal of P from wastewater due to the positive effect of N on the accumulation of P. In the present study, the N/P value in wastewater at the beginning of the experiment was over 1.8 times higher than the optimal value in algal biomass, but it quickly decreased during the experiment.

The average biomass concentration in the F/2 medium was 2.61 g/L, while in saline sewage obtained from the aquaculture process, it was 2.67 g/L. The maximum biomass productivity of *C. minutissima* in wastewater (0.55 g/L/day), which was determined after six days of cultivation, was over 150% higher compared to the maximum values obtained at the same time in the F/2 medium. Guldhe et al. [38] cultivated *Chlorella sorokiniana* in tilapia aquaculture wastewater and observed lower biomass productivity (0.35 g/L/day) than in synthetic BG11 medium (0.57 g/L/day). In the studies conducted by Sánchez-García et al. [34], the productivity of *C. minutissima* biomass in the synthetic Bold's Basal Medium (BBM) ranged from 0.01 to 0.04 g/L/day depending on the initial nitrate concentration in the medium. The availability of nitrogen affects the growth of microalgae; however, the most important factor influencing the microalgal growth is carbon. Microalgae can be grown using an inorganic or an organic carbon source. For the formation of chemical energy during the photosynthesis process, the most commonly used carbon source is carbon dioxide [39]. The productivity of *C. minutissima* biomass determined in the saline wastewater obtained from the aquaculture process was higher than that determined in other types of wastewater (e.g., municipal wastewater or wastewater resulting from animal production) [40,41], which may have been due to, among other factors, the varied content of nutrients in wastewater, cultivation conditions, and most importantly, the type of microalgae used in the purification process. It should be noted that the postculture sewage must be free from toxic components, including heavy metals, which are commonly found in industrial effluent, municipal waste stream, and mining wastewater [42], so that the obtained microalgal biomass can be used for the production of feed in aquaculture, among other applications.

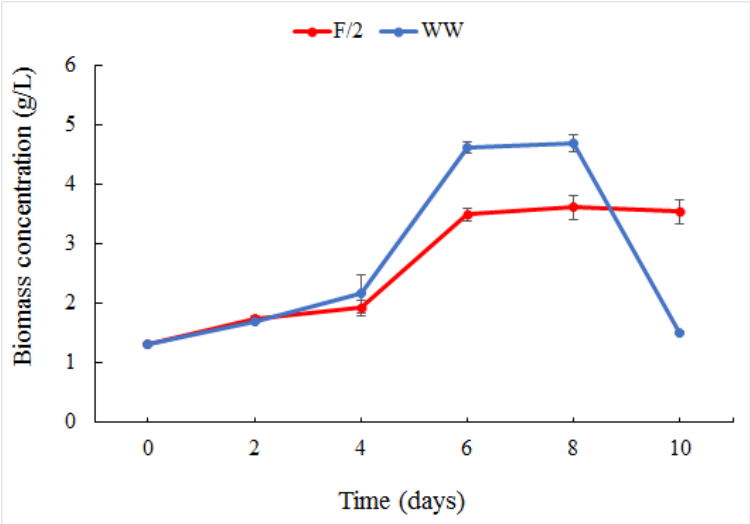

Figure 5. Changes in biomass concentration during cultures of *Chlorella minutissima* (mean ± SD; F/2—synthetic medium, WW—wastewater).

Tossavainen et al. [36] suggested that the growth of microalgae stopped a few days after the nutrients were removed, indicating a limitation of bioavailable nutrients. Similarly, in our study, after 10 days of breeding *C. minutissima* in wastewater, a significant reduction in biomass was noted. A factor limiting the further growth of microalgae could be phosphorus, which is taken from the substrate and converted into biomass. This element is also necessary for cellular metabolism, production of phospholipids and nucleic acids, and energy transfer [42,43]. Similar findings were reported by Dziosa and Makowska in their study [44]. The decrease in the content of phosphorus resulting in complete exhaustion could cause a reduction in nitrogen assimilation and a significant reduction in optical density (Figure 6). According to Aussant et al. [45], the changes in the values of optical density do not always correspond to changes in biomass concentration, but in the present study, these parameters correlated positively and significantly (r = 0.81). In the F/2 medium, the OD_{680} value gradually increased to 0.304 after eight days of incubation, and a slightly lower value was recorded after 10 days (0.292). Similar changes were observed during the cultivation of microalgae in wastewater, in which the highest optical density was observed after eight days, but this value was over 160% higher compared to the F/2 medium.

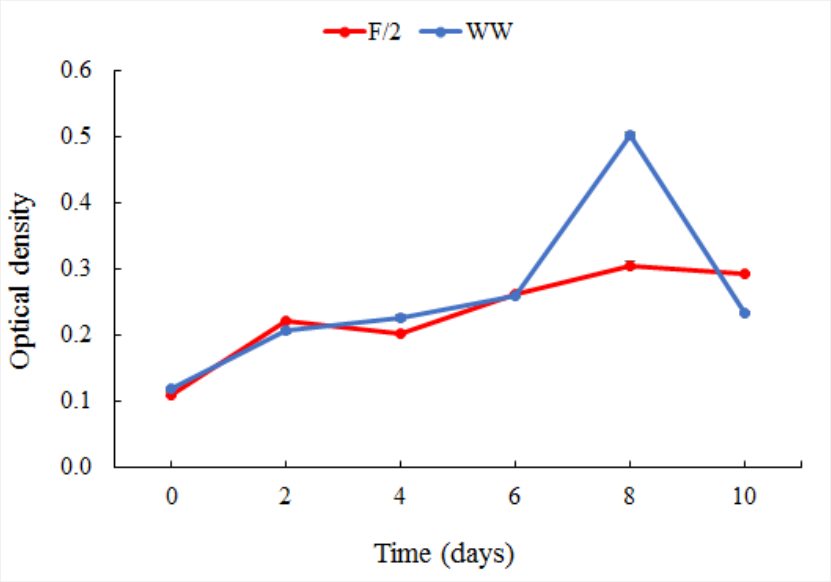

Figure 6. Changes in optical density during cultures of *Chlorella minutissima* (mean ± SD; F/2—synthetic medium, WW—wastewater).

3.3. Lipid Content

The lipid content determined in the *C. minutissima* biomass in the present study is shown in Table 3. Oil from algal biomass is commonly used to produce biodiesel [46]. The advantage of lipids obtained from *C. minutissima* cells is their composition and appropriate fatty acid profile, mainly high content of triacylglycerols, which makes them extremely useful in the production of this biofuel [47]. However, not all the lipids can be converted to biodiesel, and the most desirable ones are those that are rich in oleic acid [34].

Microalgae mainly produce lipids as an energy reserve during the stationary growth phase [48], which was observed to be relatively short in the postculture wastewater in the present study. The nitrogen or the phosphorus deficiency could increase the lipid content in algal cells [49,50]. The study showed a significant reduction in phosphorus content in wastewater after just two days of the experiment. However, the lipid concentration determined in the aquaculture effluent culture was 53 mg/L lower compared to that in F/2 medium in which the content of the nutrients and their relative proportion were optimal.

Table 3. Dry cell weights and lipid concentrations under culturing.

Objects	Dry Cell Weight (g/L)	Lipid Content (% Dry Weight Biomass)
F/2	1	51.67 ± 0.01
WW	1	46.37 ± 0.01

4. Conclusions

To our knowledge, this is the first time wastewaters from RAS with saline geothermal waters from salmon farming were used for algal cultivation. Research confirms that *C. minutissima* microalgae can be used to remove nutrients from aquaculture wastewater, and the cleaning process allows a considerable reduction in the load of compounds discharged into surface waters. Due to the high amount of nitrogen and phosphorus, salted sewage can be used as a growth substrate for the production of algal biomass and for obtaining lipids accumulated in their cells. Aquaculture wastewater resulted in biomass concentration of 4.77 g/L. The maximum biomass productivity for *C. minutissima* was 0.55 g/L/day. The biomass generated showed lipid content of 46.37%. The nutrient removal efficiencies during the cultivation of *C. minutissima* were 88% for total content of nitrogen, 74.3% for N-NO_2, 88.6% for N-NO_3, and over 99% for total phosphorus and orthophosphates (PO_4). The results of the present study can be used as the basis for creating an integrated system for the purification of saline postculture water and the simultaneous production of microalgal biomass.

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