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Influence of Nutrient-Stress Conditions on *Chlorella vulgaris* Biomass Production and Lipid Content

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Abstract: Microalgal biomass and its cellular components are used as substrates for the production of fuels. A valuable group among the components of microalgal biomass is lipids, which act as a precursor for the production of biodiesel in the transesterification process. Some methods, including the creation of stressful conditions, are applied to increase the accumulation of lipids. This study aimed to determine the effect of limited nutrient access on the growth and development of the microalga *Chlorella vulgaris* and the amount of lipids stored in its cells. Aquaculture wastewater (AWW) was used in the study as a source of nutrients at doses of 20%, 40%, 60%, 80% and 100%. The amount of microalgal biomass, optical density, lipid content after extraction of the biomass in Soxhlet apparatus and chlorophyll a content were determined. It was observed that the microalgae efficiently used the nutrients contained in the AWW. The largest amount of biomass was obtained in AWW_{80} (727 ± 19.64 mg·L^{-1}). The OD_{680} (0.492 ± 0.00) determined under the same conditions was almost five times higher in AWW than in the synthetic medium. Under nutrient-stress conditions, the content of lipids in biomass ranged from 5.75% (AWW_{80}) to 11.81% (AWW_{20}). The highest content of chlorophyll a in microalgal cells was obtained in AWW_{20} (206 ± 11.33 mg·m^{-3}).

Keywords: nutrient limitation; stress conditions; microalgal biomass; lipid content; aquaculture wastewater

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1. Introduction

The use of conventional energy sources leads to a number of problems including global warming, environmental degradation and energy crises [1]. Due to these threats, there is an urgent need to develop sustainable and novel environmentally friendly energy sources. Microalgae seem to be an ideal raw material for the production of biofuels. Compared to the biomass of terrestrial plants used for energy purposes, microalgal biomass is characterized by a lower water footprint, higher photosynthetic efficiency, the ability to grow on marginal soils and the possibility of using waste streams, including carbon dioxide, as a source of nutrients for cell growth and development [2]. Moreover, a lack of competition with edible plants for the cultivation area means improved food safety [3].

Despite many advantages, the use of microalgae for energy purposes is associated with several limitations, including economic competitiveness and the feasibility of large-scale biofuel production, as well as high investment costs which constitute a significant barrier to potential investment [4,5]. Therefore, it is necessary to improve the production of biofuels from algae and increase economic profitability. This can be achieved by developing an appropriate technology through the optimization of growing conditions [6], taking into account the nutrient availability, temperature changes, salinity, supplementation with plant hormones and differentiation of light intensity [7–9].

Under optimal conditions of cultivation, microalgae show high biomass growth but do not accumulate a large amount of reserve materials [10,11] such as carbohydrates or lipids which are useful for biofuel production [12]. The typical abiotic stress factors that significantly affect the biochemical composition of algal cells include lipid production, extreme temperatures, lighting, the amount of carbon dioxide, UV exposure, salt content and

nutrient starvation [13–18]. An unfavorable culture condition may change the metabolism of fatty acids toward the biosynthesis and accumulation of triacylglycerols, which may constitute up to 80% of the total lipid content in the cell. The main factor that influences fatty acid metabolism is the limitation of nitrogen availability [19]. Under limited nitrogen access, many microalgal species produce and accumulate a large amounts of lipids [20–22]. However, the lack of nitrogen, which is responsible for increasing the content of proteins and assimilation pigments in cells, causes a reduction in the productivity of biomass. The chlorophyll content in microalgal cells is an indicator of the intensity of photosynthesis and photochemical processes during which the energy accumulated in ATP is generated [23]. Thus, an important element in the development of a technology for biofuel production using microalgae is determining the appropriate doses of nutrients, which would enable achieving a high production of lipids as well as a high efficiency of biomass growth [24]. Additionally, it is necessary to ensure optimal culture conditions, including temperature, pH, mixing [25,26] and lighting, which should be evenly distributed to allow the photons to reach the entire volume of the photobioreactor [27].

The supply of nutrients for the production of microalgae biomass [28] can be associated with the purification of the aquatic environment [29] and the removal of nutrients, including nitrogen and phosphorus, which cause water eutrophication [30]. Microalgae can be potentially used in the treatment of municipal industrial wastewaters, agricultural wastewaters and post-cultivation waters. Moreover, the use of microalgae in disposal processes has been economically justified [31].

This study assessed the growth and development of the microalga *Chlorella vulgaris* in saline aquaculture wastewater (AWW) used in different doses. It was hypothesized that diluting the wastewater and thus limiting the nitrogen content in the culture medium would have a positive effect on increasing the content of lipids in microalgal cells. Additionally, the content of chlorophyll a was determined in the microalgal biomass.

2. Results and Discussion

2.1. Production of *Chlorella vulgaris* Biomass in Aquaculture Wastewater

The biomass of *C. vulgaris* can serve as a source of valuable chemical components, and as an additive to animal feed and human food. Its production can also be utilized for the treatment of wastewaters [32]. Statistical analysis of the results obtained in this study showed that the AWW doses had a significant effect on biomass production, and also that the amount of biomass produced in the AWW was greater compared to that produced in the F/2 synthetic medium (Figure 1A). This is in line with results of other authors confirming the relationship between the content of nutrients in wastewater and the increase in microalgal biomass [33,34].

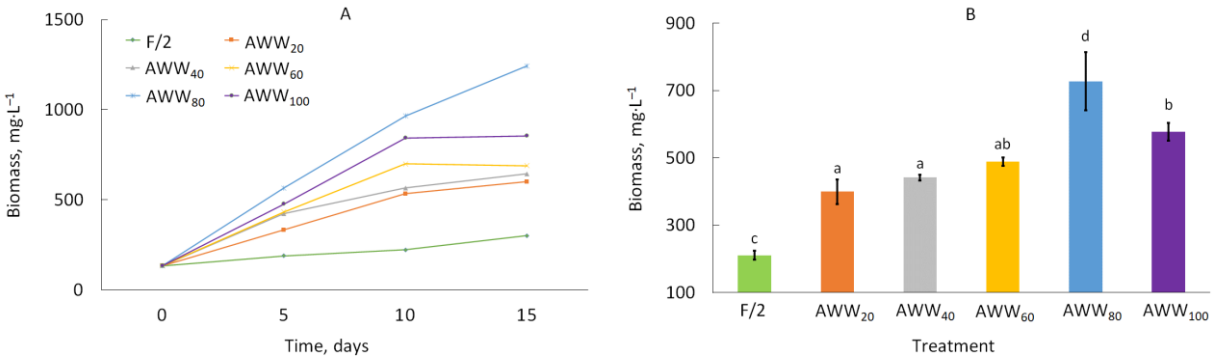

Figure 1. Dynamics of changes in biomass content (**A**) and the average biomass in culture (**B**).

The content of microalgal biomass is one of the most important parameters for evaluating the growth of microalgae. From the biomass content, it is possible to determine

the time required to reach the maximum concentration of biomass as well as the growth phase [35]. At the beginning of the experiment, the biomass content was 133 ± 9.43 mg·L^{-1}. In the following days, a linear growth with no growth inhibition phase was observed, which indicates that *C. vulgaris* can adapt and grow well in saline effluent from fish farming. Similar results were obtained in a study by Liu et al. [36], in which *C. vulgaris* was cultivated in AWW from a fishery and an increase in biomass was observed from the first days of measurement. In the present study, the largest amount of biomass after 15 days was determined in AWW_{80} (1244 ± 62.85 mg·L^{-1}), which indicates an increase of over 300% compared to the control treatment (F/2). Similar positive results of microalgal cultivation and *C. vulgaris* biomass growth in pre-treated, nutrient-rich municipal sewage were also presented by Li et al. [37].

The average amount of biomass in this study ranged from 211 ± 10.39 mg·L^{-1} (F/2) to 727 ± 19.64 mg·L^{-1} (AWW_{80}), which is 345% of the values determined in the control treatment (Figure 1B). Hawrot-Paw et al. [38] cultivated *Monoraphidium* and achieved a significantly higher biomass content in AWW compared to the synthetic substrate F/2. Fernández-Linares et al. [39] cultivated *C. vulgaris* in pre-treated municipal sewage and obtained an amount of biomass of 1575 mg·L^{-1}. Such a high biomass content was achieved due to the ability of the strain to adapt to the cultivation conditions (*C. vulgaris* strain was isolated from the environment of treated municipal wastewater). However, Guldhe et al. [40] presented different results on the use of AWW for the cultivation of *Chlorella minutissima*. The authors observed lower biomass productivity in AWW (0.35 g·L·day^{-1}) than in synthetic BG11 medium (0.57 g·L·day^{-1}). Mtaki et al. [41] added NPK fertilizer to AWW at a maximum dose of 1.0 g·L^{-1}, which resulted in a significantly increased amount of *C. vulgaris* biomass compared to that obtained in synthetic bold basal medium (BBM).

The optical density of microalgal culture varied with the AWW dose (Figure 2A). Tossavainen et al. [42] and Hawrot-Paw et al. [38] observed a decrease in the content of microalgal biomass along with a decrease in nutrient contents in the culture medium. In the present study, the optical density, with the exception of AWW_{60}, increased throughout the experiment. The mean optical density ranged from 0.246 ± 0.00 in AWW_{20} to 0.492 ± 0.00 in AWW_{60} (Figure 2B). The correlation coefficient between the OD680 values and the amount of biomass was r = 0.941, which indicates a strong positive linear relationship.

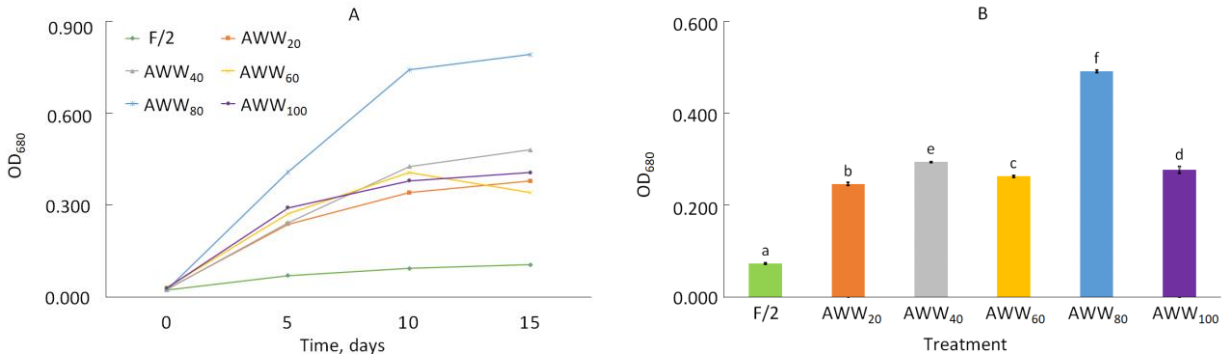

Figure 2. The dynamics of changes in optical density (**A**) and the average optical density in culture (**B**).

2.2. Effect of Nitrogen Content on the Biomass and Lipid Content

2.2.1. Production of Biomass under Nutrient-Stress Condition

Culture medium is one of the key factors influencing the production of microalgal biomass [43]. In industrial-scale production, the supply of an appropriate amount of nutrients constitutes an economic problem [44]. Therefore, the present study analyzed the possibility of using AWW rich in basic nutrients for biomass production and the impact of limiting the access to nutrients by AWW. It was observed that there was no phase delay in growth (Figure 3A), which clearly indicated that the strain adapted well to growth

under conditions of limited nutrient access and salinity. In the initial phase of growth, on the fifth day of the experiment, the largest amount of biomass was recorded in AWW_{80} (878 ± 15.71 mg·L^{-1}). This could be related to the method of inoculum preparation and the lack of the need to adapt to cultivation conditions. Similar results were obtained by Gao et al. [33] for the cultivation of *C. vulgaris* in photobioreactors using AWW in a batch culture. They obtained the maximum amount of biomass on the sixth day of the experiment (0.07 g·L^{-1}). Tang et al. [45] reported that inhibition of microalgal growth could be related to a low content of nitrogen in the medium, which was fully taken up by algal cells in the initial growth phase. In the presented study, the start of the stationary growth phase, except in the case of AWW_{80}, was observed after 10 days of cultivation. This could be related to the higher content of nutrients and their accumulation in algal cells. Algae can absorb excess nutrients and use them for growth in the later stages of cultivation [46]. In this study, the largest amount of biomass (1045 ± 31.43 mg·L^{-1}) was determined in AWW_{60}. The efficiency of microalgal biomass production is influenced by the presence of nitrogen [47]. The average amount of biomass ranged from 748 ± 0.00 mg·L^{-1} (AWW_{20}) to 842 ± 25.76 mg·L^{-1} (AWW_{60}) (Figure 3B). Ansari et al. [47] cultivated *Scenedesmus obliquus*, *Chlorella sorokiniana* and *Ankistrodesmus falcatus* in two media—BG11 and AWW—with lower nutrient content and observed a small amount of biomass in AWW. A similar dependence for *C. minutissima* was presented by Sánchez-García et al. [48], who found that a higher nitrogen dose in synthetic culture medium (BBM) caused an increase in biomass productivity. A higher content of microalgal biomass compared to the present study was observed by Tossavainen et al. [42]. The authors used pike perch AWW with a nitrogen content of 34 mg·L^{-1} for the cultivation of *Euglena gracilis* and *Selenastrum* and obtained a maximum amount of algal biomass (1.5 g·L^{-1}). This suggests that the biomass content may depend on the type of microalga, culture conditions, culture medium used and nutrient availability [49,50].

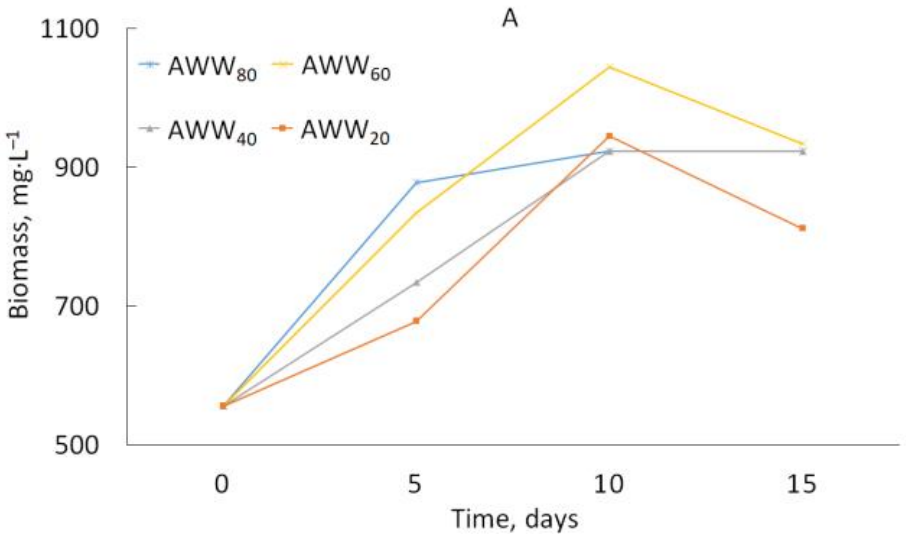

Figure 3. *Cont.*

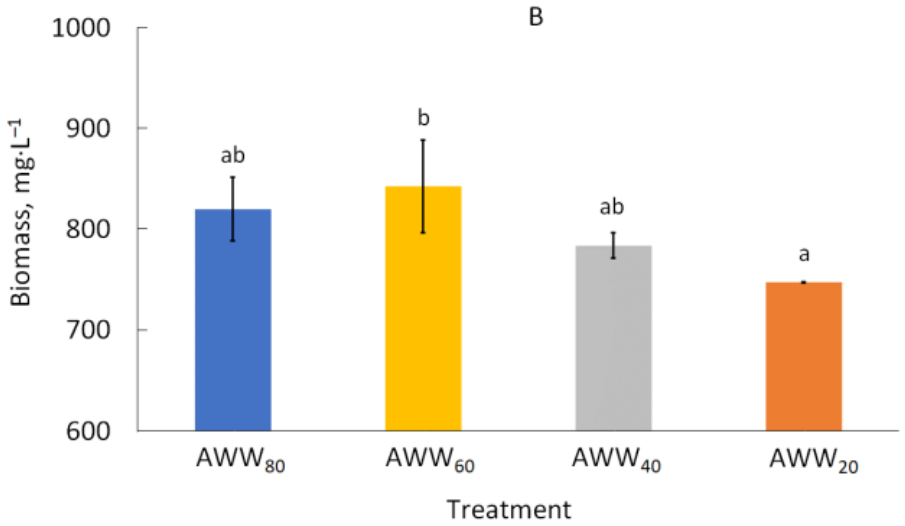

Figure 3. Dynamics of the growth of microalgal biomass (**A**) and the average amount of microalgal biomass (**B**).

2.2.2. Optical Density of Microalgal Cultures

In the present study, it was observed that the amount of biomass correlated with the optical density (r = 0.841). The largest amount was obtained on the 15th day in AWW_{60} (0.333 ± 0.01), which is over 550% higher compared to the first day of measurement (Figure 4A). The average optical density ranged from 0.140 ± 0.00 (AWW_{40}) to 0.201 ± 0.00 (AWW_{60}) (Figure 4B). Similar values of optical density (OD 0.304) were obtained by Hawrot-Paw et al. [34] during the cultivation of *C. minutissima* in the same type of salted AWW. This may be a result of different nutritional requirements of microalgae [51,52]. The increase in optical density with higher doses of AWW indicates that the high content of nutrients in the medium promotes the growth and development of algal cells. This was also confirmed by Bhatnagar et al. [53] when they cultivated microalgae in an organic enriched medium. Mtaki et al. [41] cultivated *C. vulgaris* and determined a much higher optical density value (OD 4.872), but in their study, AWW was supplemented with NPK.

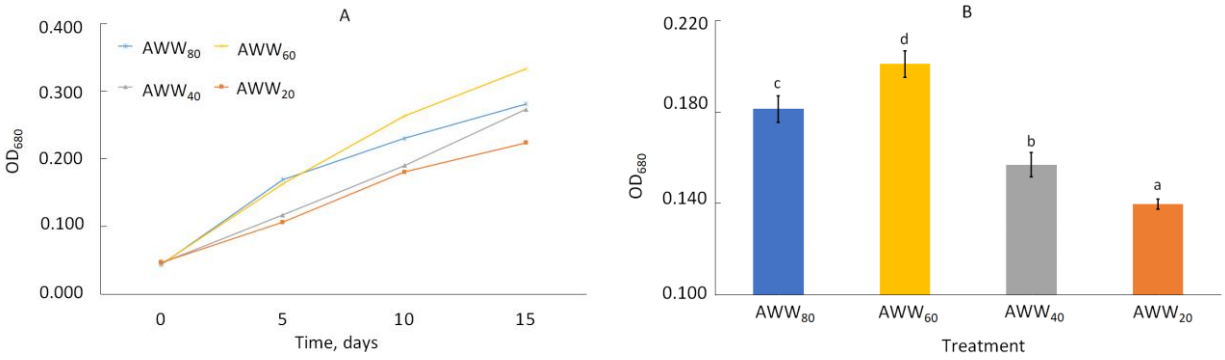

Figure 4. The dynamics of changes in optical density (**A**) and the average values of OD_{680} in culture (**B**).

2.2.3. Lipid Accumulation under Nitrogen Limitation

The mean lipid content of *C. vugaris* strains ranges from 14 to 22% [54,55], however, depending on the environment, it may vary from 5% to 58% [56]. In this study, the lipid content in biomass ranged from 5.75% (AWW_{80}) to 11.81% (AWW_{20}) (Figure 5). The effect on lipid accumulation in microalgal cells depends on the microalga species as well as the composition of the culture medium [57]. Wong et al. [58] analyzed the effect

of the composition of 13 different culture media on the growth of *C. vulgaris* and lipid production. The authors observed that the maximum values for both these parameters were obtained with BBM. Synthetic culture media are expensive and can be replaced by AWW [41], which is not only cost-effective but also environmentally sustainable. The two-fold increase in lipid content achieved in the present study was associated with nutrient stress caused by dilution of AWW. The nitrogen content in the culture medium ranged from 6.67 ± 0.06 mg·L^{-1} (AWW_{20}) to 24.73 ± 1.10 mg·L^{-1} (AWW_{80}). Under nitrogen-deficient conditions, the cellular metabolism of algae changes, resulting in the accumulation of lipids and starch in the biomass [59]. In a study by Pribyl et al. [60], the lipid productivity by *C. vulgaris* was higher at lower nitrate concentrations. Similar results were presented by Mutlu et al. [61] and Adenan et al. [62]. The authors noted that, under the nutritional stress caused by nitrogen limitation, lipid production significantly increased in *C. vulgaris* cells. The relationship between the increase in lipid content and nitrogen limitation was also reported by Ansari et al. [47]. The authors cultivated *S. obliquus*, *C. sorokiniana* and *A. falcatus* in aquaculture effluent with lower nitrogen content compared to the synthetic substrate and recorded increased lipid content for all algal species.

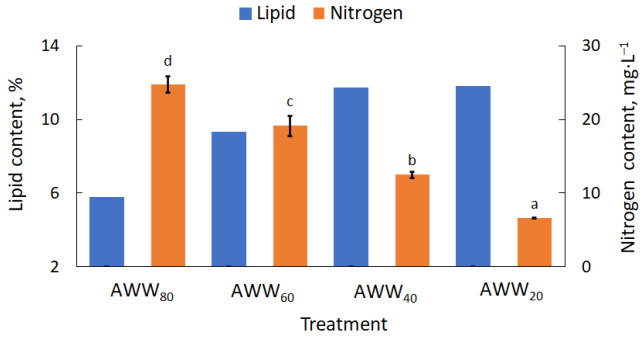

Figure 5. Nitrogen content in AWW and lipid content in microalgal biomass.

The results of this study thus showed a relationship between the nitrogen dose and lipid content in the microalgal biomass. With nitrogen deficiency, the metabolic pathway of carbon fixation changes from protein synthesis to lipid production [63]. It was found that the lower the initial content of nitrogen in the culture medium, the greater the amount of lipids in the biomass. The low lipid content observed in AWW_{80} could be caused by the intense growth of microalgae. A distinct stationary growth phase was not noted in the study, although it is in this phase that microalgae form lipids as an energy reserve [64]. This has been confirmed by other authors [65,66] for both marine and freshwater strains. A much higher lipid accumulation was determined by Rai et al. [67] during the cultivation of *C. vulgaris* in saline medium. While maintaining the nitrogen content at a level of <5 mg·L^{-1}, these authors recorded a lipid content of 40%. An additional factor stimulating lipid accumulation in microalgal cells may be the salinity level. This was supported by the results of Mirizadeh et al. [68], who analyzed the effect of introducing NaCl into the culture medium at doses ranging from 5 to 30 g L^{-1}. The authors found an increase in lipid content in the biomass of *C. vulgaris* with increasing concentration of NaCl up to 25 g L^{-1} (about 1.5-fold higher than without salt). In the study by Hawrot-Paw et al. [34], the lipid content in the biomass of *C. minutissima* cultured on saline salmon AWW decreased while the biomass content increased compared to the synthetic F/2 medium. The differences between the nutrient content of the medium and the amount of lipids in the cells are related to the type of microalga. The division of stress into three categories, from low to high, is conceptually adopted, where the increase in lipids exceeds the decrease in biomass content, the decrease in growth exceeds the increase in lipids, and the increase in lipid content is compensated by the decrease in growth [11].

2.2.4. Changes in PH during Microalga Cultivation

The pH of the culture medium affects the proper cell development, availability of nutrients and ability of algal cells to carry out photosynthesis [69]. In the present study, *C. vulgaris* was cultivated under uncontrolled pH conditions. At the start of the experiment, the pH of the medium ranged from 8.3 (AWW_{80}) to 8.5 (AWW_{20}) (Figure 6). After five days of cultivation, an increase to a value of 9 was recorded, which was related to CO_2 fixation [70] or the passage of air through the microalgal cultures and dissipation of carbon dioxide by agitation [71]. After 15 days, the pH of the medium was in the range of 8.6–8.9. This is considered as the optimal range for algae, which is also supported by Rai and Rajashekhar [67], who indicated that the optimal pH for algae ranges from 6 to 10. Scherholz and Curtis [72] described that the pH of the culture medium may be related to the influence of ammonium on the transport and reduction of nitrates.

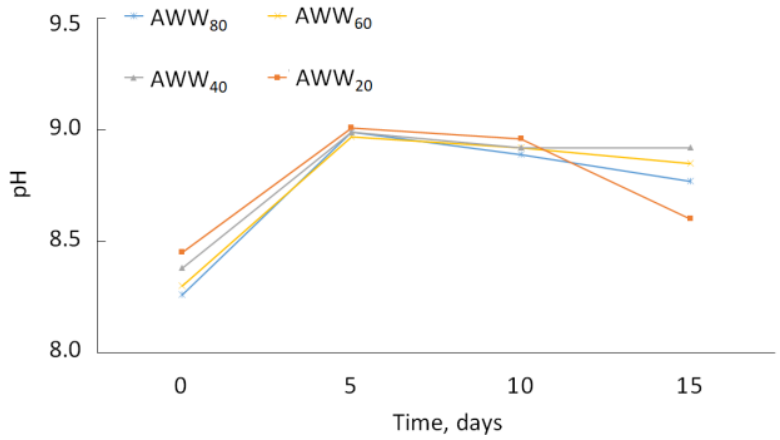

Figure 6. The dynamics of pH changes in culture medium.

2.2.5. Chlorophyll A Content in Microalgal Biomass

The initial content of chlorophyll a in microalgal cells was determined to be 50 ± 5.03 mg·m^{-3} (Figure 7A). The highest content of pigment (328 ± 21.95 mg·m^{-3}) was observed in AWW_{20} after 10 days of cultivation. Depending on the dose of AWW, the mean chlorophyll a content ranged from 160 ± 17.84 mg·m^{-3} (AWW_{80}) to 206 ± 11.33 mg·m^{-3} (AWW_{20}) (Figure 7B). The content of chlorophyll a in microalgal cells decreased with an increase in the amount of nitrogen in the medium. Contrasting results were reported by Ördög et al. [73] in their study on *Chlorella minutissima*, in which the content of chlorophyll increased after the addition of nitrogen. Similarly, Li et al. [74] observed a decrease in chlorophyll content in *Neochloris oleoabundans* cells cultivated in medium with lower nitrogen content and suggested that this may be related to the utilization of nitrogen compounds in chlorophyll by microalgae. The availability of nutrients affects the concentration of chlorophyll in microalgal cells, but it varies depending on the species [75]. The increase in pigment content in diluted AWW could be due to a higher intensity of photosynthesis caused by better light penetration into cells with lower biomass. The relationship between the increase in biomass and the decrease in chlorophyll in *C. vulgaris* cells was also presented by Mohsenpour et al. [76].

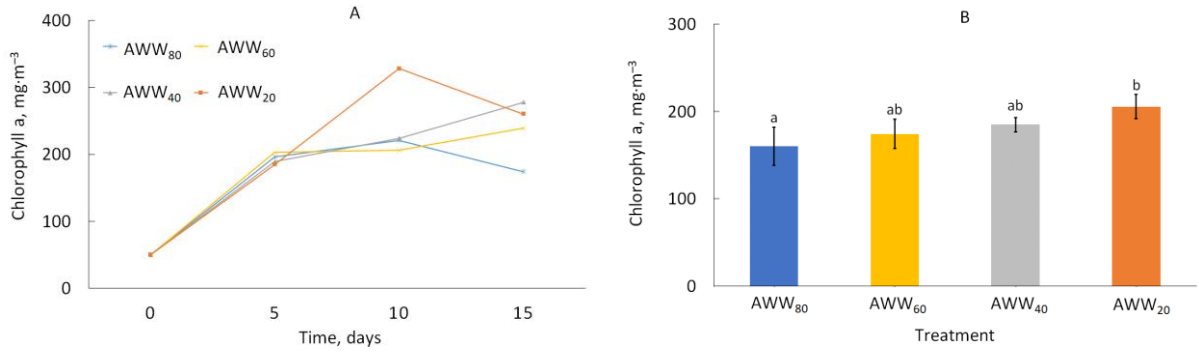

Figure 7. The dynamics of changes in the amount of chlorophyll a (**A**) and the average values of chlorophyll in culture (**B**).

3. Materials and Methods

3.1. Microalgal Culture

Green algae (*C. vulgaris* (BA 002)) were obtained from the Culture Collection of Baltic Algae (University of Gdansk, Gdańsk, Poland). The microalgae were cultured in Guillard's (F/2) Marine Enrichment liquid medium [77] at a temperature of 4 °C under lighting with a light-emitting diode (LED; 12/12 h light:dark cycle). The liquid medium had the following composition ($g \cdot L^{-1}$): $NaNO_3$—0.075 g; $NaH_2PO_4 \cdot 2H_2O$—0.00565 g; stock solution of trace elements—1 $mL \cdot L^{-1}$ (Na_2EDTA 4.16 g, $FeCl_3$ $6H_2O$ 3.15 g, $CuSO_4$ $5H_2O$ 0.01 g, $ZnSO_4$ $7H_2O$ 0.022 g, $CoCl_2$ $6H_2O$ 0.01 g, $MnCl_2$ $4H_2O$ 0.18 g and $NaMoO_4$ $2H_2O$ 0.18 g) and stock solution of vitamin mix—1 $mL \cdot L^{-1}$ (cyanocobalamin (vitamin B12) 0.0005 g, thiamine HCl (vitamin B1) 0.1 g, biotin 0.0005 g).

3.2. Aquaculture Wastewater

AWW was obtained from Jurassic Salmon (Karnice, Poland), an ecological closed-circuit salmon farm. The average content of nitrogen in the wastewater was 31.8 $mg \cdot L^{-1}$, while the content of phosphorus was 1.1 $mg \cdot L^{-1}$ at a salinity level of 10‰. AWW was collected from a storage tank and stored in a refrigerator at a temperature of 4 °C. Before use, the wastewater was filtered twice through membrane filters to remove solid impurities, and then filters with a pore diameter of 1.2 µm. Finally, the wastewater was sterilized with UV-C (13 W waterproof lamp, 15 min).

3.3. Experimental Setup

The experiment was divided into two stages. In the first stage, the influence of the AWW dose on the content of microalgal biomass was determined. Vertical tubular photobioreactors with a capacity of 2.5 dm^3 (Aqua Medic, Bissendorf, Germany) were used for the experiment. In these photobioreactors, 2 dm^3 of medium and 200 cm^3 of a 10-day inoculum of microalgae (*C. vulgaris*) were added. AWW was used at doses of 20%, 40%, 60%, 80% and 100%. Commercial F/2 culture medium was used as a control treatment in the experiment. All the photobioreactors were aerated with a 25 W pump (Aqua Medic, Bissendorf, Germany) at a capacity of 0.9 $m^{-3} \cdot h^{-1}$, which allowed us to introduce carbon dioxide and prevent biomass sedimentation. The pH of the medium was adjusted to 7 by adding 1N NaOH. The cultures were illuminated with LEDs: white, red (wavelength 600–700 nm, light intensity of 9.45 µmol s^{-1}) and blue (wavelength 400–500 nm, light intensity of 2.25 $\mu mol \cdot s^{-1}$). Lighting was maintained on an 18/6 h light:dark cycle. The total LED intensity was 13.5 µmol s^{-1} (HOLDBOX, Żabia Wola, Poland). The experiment was carried out for 15 days, and then the amount of biomass and optical density of cultures were determined.

In the second stage of the experiment, the influence of nutrient stress on the amount of *C. vulgaris* biomass and on the content of lipids in biomass was assessed. Additionally, optical density and chlorophyll a content were calculated. At this stage, the biological

material that was obtained under optimal growth conditions for algal biomass was used as an inoculum (for the AWW dose). The experiment was carried out in vertical tubular photobioreactors with a capacity of 14 dm^3, with 10.8 dm^3 of AWW (doses of 20%, 40%, 60% and 80%) and 1.2 dm^3 of *C. vulgaris* inoculum. The cultures were illuminated with LED light, and aerated using a pump with a power of 12 W at a capacity of 14 $L \cdot min^{-1}$. The experiment was carried out as a batch culture for 15 days.

3.4. Analytical Methods

3.4.1. Biomass Quantification

The dry weight of microalgae was determined by a gravimetric method described by Ratha et al. [78] with modification [79]. The amount of biomass was estimated using a moisture analyzer (AXIS ATS, Gdańsk, Poland). The results are presented in $mg \cdot L^{-1}$.

The optical density of microalgal cultures was measured using a spectrophotometer (EMCO, Warszawa, Poland) at a wavelength of $\lambda = 680$.

3.4.2. Pigment Extraction and Analysis

The content of chlorophyll a in microalgal cells was measured according to PN-86 C-05560/02 standards [80]. Briefly, the algal biomass was concentrated by vacuum filtration on a glass fiber filter, and then the chlorophyll pigment was extracted with a solution of 90% acetone. Absorbance was measured at a wavelength of $\lambda = 665$ nm using a spectrophotometer (EMCO, Warszawa, Poland). The results are presented in $mg\ m^{-3}$.

3.4.3. Determination of PH

The pH of the culture medium was measured using a CI-316 microcomputer pH-meter (Conrad Electronic SE, Hirschau, Germany).

3.4.4. Lipids Extraction and Determination

The lipid content of the biomass was determined by Soxhlet extraction with hexane using the modified method of Shin et al. [81]. The analyses were carried out after 15 days of cultivation. Following sedimentation, the biomass was dried in a laboratory dryer (WAMED, Warszawa, Poland) at 70 °C for 24 h. Then, 0.5 g of the dried sample were transferred to a cellulose casing and extracted in a Soxhlet apparatus (Labmed HK, Łódź, Poland) with hexane for 4 h (20 cycles per hour). The lipid content of the sample was determined using the following formula (1):

$$LC = \left(\frac{mL}{mDAB}\right) \cdot 100 \quad (1)$$

where LC is the lipid content, mL is the mass of lipids (g) and mDAB is the mass of dry microalgal biomass (g).

3.4.5. Determination of Total Nitrogen Content

The total nitrogen in the culture medium was determined using the spectrophotometric method according to PN-EN ISO 11905-1: 2001 standards [82]. The obtained results were converted into $mg \cdot L^{-1}$. Nitrogen analyses were also performed once at the beginning of the study.

3.5. Statistical Analysis

All analyses, except for lipid content, were performed in triplicate. The results were statistically analyzed using a computer program for Windows (Statistica version 13.3, 2016; Dell Inc., Tulsa, OK, USA). An analysis of variance was performed, followed by Tukey's significance tests at a level of $p \leq 0.05$. In addition, Pearson's linear correlation coefficient (r) and standard deviations were determined.

4. Conclusions

This study confirmed the possibility of using saline wastewater from salmon farming for the production of *C. vulgaris* biomass, which will allow for the simultaneous removal of nutrients from AWW. The content of nutrients in the wastewater originating from fisheries was identified as an important parameter influencing the amount of biomass produced. The largest amount of biomass (727 ± 19.64 mg·L^{-1}) and the highest values of optical density (0.492 ± 0.00) were obtained at the higher doses of AWW (80%). On the other hand, the highest content of chlorophyll was determined in AWW_{20}. Due to the limited access to nitrogen, the lipid content in the biomass increased. The highest lipid content (11.81%) was determined in the most diluted culture medium (AWW_{20}), in which the initial nitrogen content was 6.67 ± 0.06 mg·L^{-1}. Considering that algal biomass can be used for energy purposes, the cost of the growth medium, which accounts for a significant part of the total production costs, is important for advanced biofuel production.

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