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Sodium chloride and nitrogen effects on *Chlorella vulgaris* growth and biocommodities production

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ABSTRACT

Environmental conditions can affect the growth and biomass production of microalgae. Microalgae species react differently to changes in growing conditions. The aim of the present study was to investigate the effect of sodium chloride on *Chlorella vulgaris* cultured in 1/3 N BG-11 medium supplemented with different NaCl concentrations (0 to 35 g/L) in 2 L glass flasks. The algal growth was similar in cultures with 1 and 3.5 g/L NaCl and was 5 to 15 times higher compared to cultures in 15 and 35 g NaCl/L. Nitrates consumption by *C. vulgaris* was increased by the increase of salinity, while the opposite was observed for soluble phosphorus. The increase of salinity resulted in higher content of lipid, proteins and sugars. The combination of salinity and nitrates stress was fully examined in this study. During nitrate starvation, a remarkable increase of soluble phosphorous was observed and the culture with the highest NaCl concentration (35 g/L) exhibited the highest soluble phosphorus concentration approaching 50 mg/L. Nitrates starvation resulted in the increase of lipid (22%–57%), protein (13%–27%) and sugars (12%–56%) content and the higher the NaCl the higher protein and sugar content.

Keywords: Microalgae; Sodium chloride; Salinity; Biomass characteristics; Nitrates stress

1. Introduction

Microalgae can grow in different environments from fresh to seawater even in sewage. The use of microalgae in wastewater treatment plants reduces nutrient concentration while at the same time leads to biomass production [1,2]. The environmental conditions affect algal growth and the characteristics of the produced biomass. Not all species have the ability to survive in their natural environment and produce high-quality biomass [3]. There is a growing interest in the cultivation of microalgae for biofuel production [4]. Like plants, microalgae require solar energy to grow. Unlike plant species, microalgae grow rapidly and are extremely rich in oils and therefore lipids. The lipid yield of microalgae can exceed 80% by dry mass [5–7]. Depending on species type, microalgae can produce many different types of lipids (in particular methyl esters), and therefore hydrocarbons. In addition to oils, microalgae also contain significant amounts of protein and sugars and other compounds [8,9]. The production of biofuels by microalgae found in fresh and saline water has attracted the interest of researchers as non-potable water is used, CO_2 emissions are reduced, and a high amount of biomass is produced [10].

A typical example is *Chlorella vulgaris*, which belongs to the genus of eukaryotic Chlorophyta, which is usually found in freshwater, soil, and sewage [11,12]. Cells have a spherical or ellipsoid shape with a size ranging from 2 to 10 μm, and often form aggregates that are more intense in high salinity media. The cells are surrounded by a thin cell wall that encompasses a pearl-shaped chloroplast and has a centrally located nucleus [13]. *C. vulgaris* is a green microalgae used both in human nutrition and biofuel

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feedstock [14], is rapidly growing and has been reported for substantial lipid production ability [15]. Kakarla et al. [16] and Yun et al. [17] examined the effect of NaCl presence on the growth and biomass production of *C. vulgaris* and *Chlorella sorokiniana*, respectively. In cultures with different salinity, after a period of time, an additional quantity of NaCl was added to increase the salinity level (2nd stage). The increase of NaCl resulted in a lower algal cells number [16]. The content of algal biomass in lipid, proteins and sugars was increased by increasing the salinity [17]. Many researchers have already examined the effect of salinity on *C. vulgaris* combining carbon type [7], temperature [18] and salt type [19]. Even though *C. vulgaris* has been investigated in different culture conditions, the combination of salinity and nitrates stress hasn't been investigated so far.

Wastewater particularly in coastal areas may have high electric conductivity due to seawater intrusion into the sewer network. The aim of this work was to investigate the effect of sodium chloride salinity on the behavior of a freshwater culture, which could be exposed to elevated salinity levels. For this reason, the cultivation of the microalgae *C. vulgaris* was investigated at three different salinity levels (0, 1, 3.5 g/L NaCl) to simulate fresh and brackish water. Nitrates starvation was investigated in order to examine the response *C. vulgaris* cells cultured in different salinities, which can be found in coastal areas of water. In addition, the behavior of *C. vulgaris* was also examined at 15 and 35 g/L NaCl to study the response of the freshwater alga to higher salinity levels under nitrates stress conditions. Prior to the experiments, *C. vulgaris* was acclimated at each NaCl concentration. *C. vulgaris* growth, nutrient removal and biomass characteristics were evaluated during the cultivation period. Operational parameters such as mixing and CO_2 supply were also examined to assess their effect on algal growth.

2. Materials and methods

2.1. Microalgae

C. vulgaris SAG 22.83 is a freshwater species and was obtained from the bank SAG Culture Collection of the University of Göttingen and was used as model autotrophic microalgae. *C. vulgaris* was selected due to its presence in municipal wastewater and for its high lipid content.

2.2. Experimental system

The preculture was conducted in 1 L Erlenmeyer flasks at an initial cell concentration of 7.6×10^5 cells/mL. Microalgae were cultivated in 1/3N BG-11 (BlueGreen-11 enriched with one-third time the nitrates concentration), which is often used for the cultivation of freshwater microalgae, at different NaCl concentrations (0, 1, 3.5, 15 and 35 g/L). The ingredients of 1/3N BG-11 are Na_2CO_3 (20 mg/L), $NaNO_3$ (500 mg/L), Na_2Mg EDTA (1 mg/L), ferric ammonium citrate (6 mg/L), citric acid $1H_2O$ (6 mg/L), $CaCl_2{\cdot}2H_2O$ (36 mg/L), $MgSO_4{\cdot}7H_2O$ (75 mg/L), K_2HPO_4 (30.5 mg/L), H_3BO_3 (2.86 mg/L), $MnCl_2{\cdot}4H_2O$ (1.81 mg/L), $ZnSO_4{\cdot}7H_2O$ (0.222 mg/L), $CuSO_4{\cdot}5H_2O$ (0.079 mg/L), $CoCl_2{\cdot}6H_2O$ (0.050 mg/L), $NaMoO_4{\cdot}2H_2O$ (0.391 mg/L). The preculture lasted for 51 d and was periodically fed with 1/3N BG-11. The cell concentration increased according to the NaCl concentration, and the cultures' cell concentration on Day 51 was 1.7×10^8, 1.4×10^8, 1.4×10^8, 9.9×10^7 and 3.8×10^6 cells/mL for the 0, 1, 3.5, 15 and 35 g/L culture, respectively. The purpose of the preculture was to allow the acclimation of microalgae in different NaCl concentrations.

An amount of algal preculture was transferred in a 2-L Erlenmeyer flask, and all cultures had an initial cell concentration of 10^5 cells/mL in 1/3 N BG-11. The first period lasted for 16 d and was followed by a second period (8 d) where the aeration stopped, to examine the effect of not providing external CO_2 on algae growth. At the end of the second period, the content of the 2-L flask was centrifuged at 5,000 rpm for 5 min, and the separated biomass was transferred into the 2-L Erlenmeyer flask. The flask was filled up to 2 L with BG-11 without $NaNO_3$. The nitrates starvation lasted 4 d, higher than other studies [10,11], and samples were taken at regular time intervals in order to evaluate nutrient removal, algal growth rate, and biomass content in terms of lipids, sugars and proteins.

The experimental system was placed in a walk-in incubator room at a temperature of 21°C ± 2°C. The photosynthetic radiation intensity was 95 μmol/m^2 s. A continuous air supply of 3.5 L/min was provided by an air pump (air pump, HP-400, Sunsun, Zhejiang, China), and the air was filtered through a 0.22 μm syringe filter.

2.3. Analytical methods

The effect of NaCl on microalgae metabolic reactions was evaluated by the systematic determination of algal biomass, nutrients, pH, optical density, and turbidity. The algal biomass was also characterized in terms of lipids, proteins, and sugars. Samples were taken every four days during the culture and every day during nitrates stress to determine the algal growth, nitrates, phosphorus, Chlorophyll-a, total suspended solids (TSS) concentration and the content of biomass in lipids, proteins, and sugars.

Cell number was employed for the direct observation, according to [20] by an optical microscope (model DMLB, Leica Microsystems GmbH). Microalgal biomass was determined by the measurement of TSS and volatile suspended solids using a gravimetric method according to Standard Methods [21]. Nitrates were determined by 2,6-dimethylphenol (Nitrate Test, 1.09713.00, Spectroquant, Merck). Total and soluble phosphorus (STotal-P) was measured by the persulfate method and ascorbic colorimetric technique [21].

Chlorophyll-a (Chl-a) was measured via a spectrophotometric method [21]). A volume of the culture was filtered through a glass fiber filter, which was placed in a screw-cap centrifuge tube containing 10 mL of 90% acetone. The filter was pulverized and shaken in the acetone solution in order to detach algae from the filter and kept at 4°C in the dark, at least for 2 h and less than 24 h. Then the sample was centrifuged, and 3 mL of the supernatant was transferred in a cuvette and measured in a spectrophotometer at 750, 665 and 664 nm.

Total lipids were extracted from algal suspensions using the Folch method by gravimetric analysis [1]. A quantity of dry algal biomass (approximately 100 mg) was

homogenized and extracted three times with a chloroform: methanol (2:1) mixture. The biomass was removed by filtration through a filter paper and the extracted lipids were transferred quantitatively into a tared Erlenmeyer flask. The procedure was repeated three times. Weight measurements were made by a precision analytical balance (AE200, Mettler Instrument AG, Zurich, Switzerland). The flask was placed in an oven at 37°C until all reagents were removed. The flask was allowed to cool to ambient temperature in a desiccator and then was weighed. The weight difference corresponded to intracellular lipids.

Total proteins in algal biomass were determined by the Lowry method [22]. A quantity of 5 mg dry algal biomass was transferred into a glass vial and 3 mL of 20% (w/w) NaOH, and 0.75 mL 1/15 M KH_2PO_4 were added. After stirring, the vials were placed in an autoclave for 30 min. The vials were allowed to cool to ambient temperature and 0.125 mL 20% (w/w) $CuSO_4{\cdot}5H_2O$ were added. The sample was periodically stirred for 10 min and centrifuged at 4,000 rpm for 20 min. The absorbance of a 3 mL volume of the supernatant was measured at 540 nm in a spectrophotometer. A reference curve with a known concentration of bovine serum albumin (BSA) solutions (0 to 10 mg/mL) was prepared for the determination of protein concentration. The percentage of proteins content (w/w) of algal biomass is estimated by the following equation:

$$\text{Proteins}(\%) = \frac{P_a}{P_b} \times 100 \quad (1)$$

where P_a mass of algal proteins (mg), P_b mass of extracted microalgae (mg)

The content of biomass in sugars were determined by the Dubois method [23]. A quantity of 5–10 mg dry algal biomass was transferred into a glass vial and 5 mL of H_2SO_4 (96% v/v) were added. The sample was allowed to stand for 1 h, and a volume of 0.05 mL of phenol (87%) was added, and the absorbance was measured after 30 min at 495 nm. A reference curve with a known concentration of glucose ($C_6H_{12}O_6$) solutions (0 to 100 mg/L) was prepared for the determination of sugars. The percentage of sugars content (w/w) of algal biomass is estimated by the following equation:

$$\text{Sugars}(\%) = \frac{S_a}{S_b} \times 100 \quad (2)$$

where S_a mass of algal sugars (mg), S_b mass of extracted microalgae (mg).

2.4. Data analysis

The specific growth rate (μ) was determined from the growth phase by the following equation:

$$\ln C_t = \mu_t + \ln C_0 \quad (3)$$

where C_t is the concentration of biomass (g/L) at time t (d), C_0 is the initial concentration of biomass (g/L).

The value of the specific growth rate (μ) was determined by the slope of the plot of $\ln C_t$ vs. time [1].

3. Results and discussion

3.1. Effect of salinity on algal growth

C. vulgaris was cultured in BG-11 (1/3 N) at five different NaCl levels (0, 1, 3.5, 15 and 35 g/L). Although *C. vulgaris* is a freshwater alga, the two highest NaCl concentrations were tested to examine its behavior at extreme salinity levels. The algal biomass concentration in terms of cell number, turbidity, optical density, TSS and Chl-a is shown in Fig. 1. The cell number in cultures with low salinity (1 and 3.5 g/L NaCl) was higher in the first and second periods compared to the culture without NaCl. This indicates the need for sodium ions to facilitate photosynthesis in microalgae through intracellular processes [24]. Yun et al. [17] examined the effect of NaCl concentration (0–600 mM) on the behavior of *C. vulgaris* YH703. At NaCl concentrations of 15–30 mM (0.9 and 1.8 g/L) they found higher cell number, compared to the culture without NaCl. A similar observation was also reported by Alyabyev et al. [25] who examined the effect of NaCl (0–35 g/L) on the growth of *C. vulgaris*. The cell number of *C. vulgaris* cultured in 15 and 35 g/L NaCl showed a similar trend and was quite lower compared to cultures in lower NaCl concentrations. The increase of NaCl concentration resulted to the decrease of *C. vulgaris* growth [26]. Alyabyev et al. [25] reported that *C. vulgaris* was unable to adapt at NaCl concentrations of 0.5 M; in the present work, the exposure of *C. vulgaris* to 15 g/L NaCl (0.26 M) exhibited significant inhibition.

Even from the first four days of the experiment, the growth rate of the cultures with 0 to 3.5 g/L NaCl was close to 1 d^{-1} (Table 1). At higher NaCl concentrations of 15 and 35 g/L the growth rate was quite lower reaching values of 0.693 and 0.402 d^{-1}, respectively, indicating the non-adaptation of *C. vulgaris* to the saline environment, as was expected. During the first period, the growth rate in cultures at low NaCl concentration from 0 to 3.5 g/L was similar (0.346–0.366 d^{-1}) but the increased NaCl concentrations resulted in a lower growth rate of 0.265 and 0.192 d^{-1} in 15 and 35 g/L NaCl cultures, respectively. During the second period, aeration was not provided and the growth of cultures was significantly affected (Table 1). During the period from 0 to 24 d, the growth rates were similar (around 0.25 d^{-1}) in cultures up to 3.5 g/L NaCl, while the increase of NaCl concentration to 15 and 35 g/L resulted in lower values by 25% and 29%, respectively compared to the low salinity cultures. Ebrahimi and Salarzadeh [18] mentioned that the growth rate of *Chlorella capsulata* decreased by 16% when the salinity increased from 25‰ to 30‰. The growth of microalgae can be expressed both by the number of cells, the turbidity and optical density of the culture. The increase of the algal population is associated with the increase in turbidity and optical density. The turbidity and optical density (Fig. 1b and d) showed a different mode. The relationship between NTU and OD of all cultures is shown in Table S1. A higher correlation between turbidity and OD was observed in cultures at 0, 15 and 35 g NaCl/L. The concentration of TSS was affected

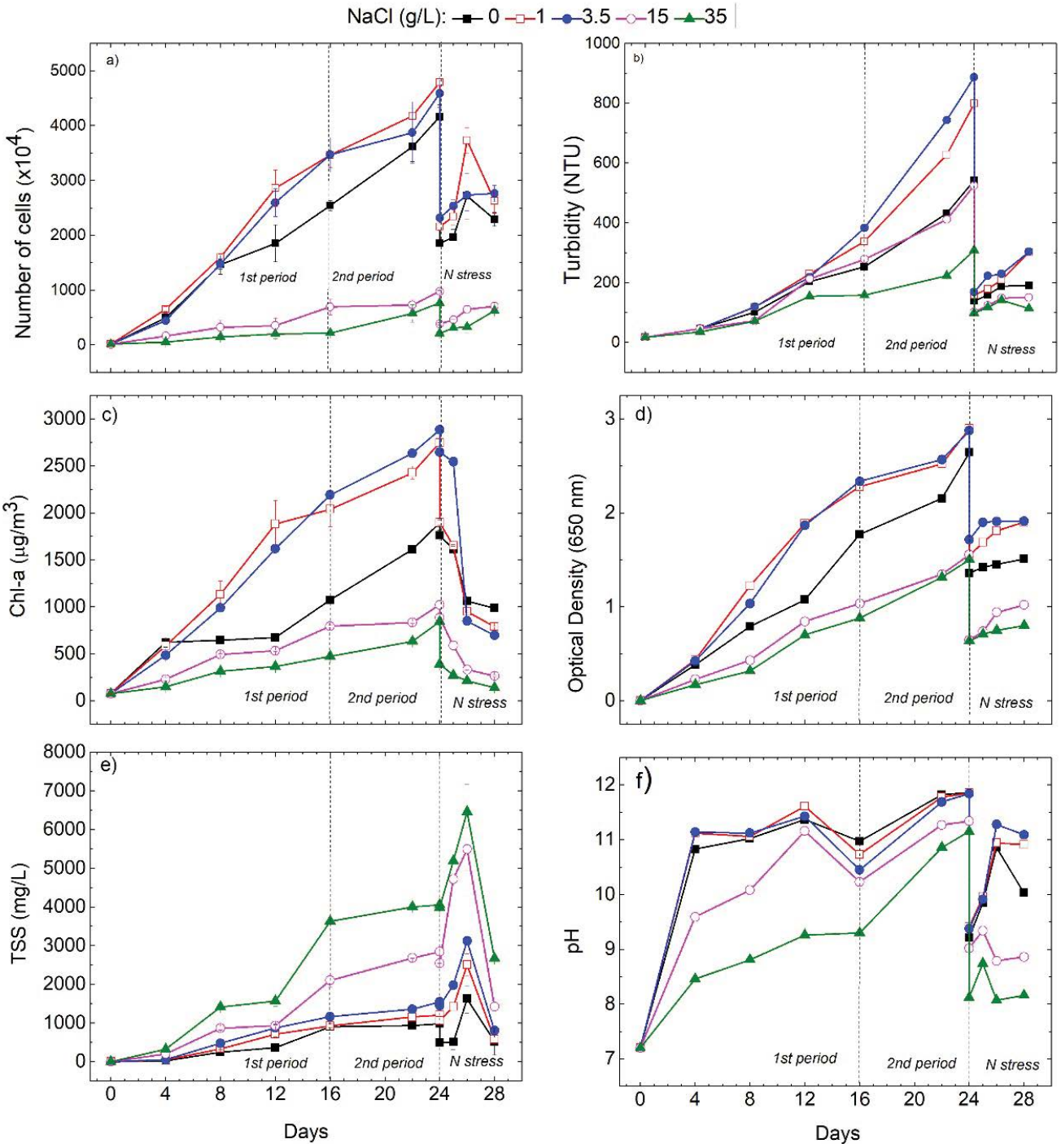

Fig. 1. Variation of (a) cells number, (b) turbidity, (c) Chlorophyll-a, (d) optical density, (e) TSS and (f) pH during cultivation of *Chlorella vulgaris*. The values represent mean ± standard deviation (SD) (n = 3), except for pH.

by the presence of NaCl in the culture, and the higher the salinity, the higher the concentration of TSS. Therefore, TSS is not a representative parameter for the determination of algal biomass at high NaCl concentrations.

pH is an indicator of algal growth, and at the beginning of the experiment in all cultures was 7. During the first period, pH was raised up to 11 in cultures with 0, 1 and 3.5 g/L even from the fourth day of operation. In cultures with 15 and 35 g/L NaCl, pH was more slowly increased, reaching values of 10 and 9, respectively at the end of the first period, while at the end of the second period was around 11. Kalla and Khan [27] also noticed that in cultures with high pH values the growth was faster, and the biomass concentration higher.

The concentration of Chl-a was affected by the salinity of the culture, and in cultures with low NaCl concentration

Table 1
Growth rate of *Chlorella vulgaris* cultures

Period	μ (d^{-1})				
	NaCl (g/L)				
d	0	1	3.5	15	35
0–4	0.973	1.044	0.946	0.693	0.402
0–16	0.346	0.365	0.366	0.265	0.192
0–24	0.251	0.257	0.255	0.191	0.181
25–29*	0.0533	0.0497	0.0446	0.154	0.274

*Nitrates stress period

(1 and 3.5 g/L) was higher compared to the cultures with higher NaCl levels (15 and 35 g/L), indicating inhibition of algal growth. Hiremath and Mathad [28] examined the cultivation of *C. vulgaris* Beijerinck at NaCl concentrations from 0 to 0.4 M and reported low Chl-a content at higher salinities, due to the decrease of photosynthetic activity caused by osmotic and toxic ionic stress. The cultures with 0.1 and 0.2 M NaCl had higher Chl-a concentrations, 24% and 40%, respectively compared to the control. In the present study, the absence of NaCl resulted also in a lower Chl-a concentration.

The consumption of nutrients differs according to the NaCl level. More specifically, the increase of NaCl results in higher consumption of nitrates, while the opposite is observed with phosphorus consumption (Fig. 2). The consumption of nitrates continued in all cultures from day 12 up to the end of the experiment. From day 0 to 24 the first order constant for nitrates consumption was 0.0395, 0.0533, 0.0678, 0.0751 and 0.111 d^{-1} in cultures with 0, 1, 3.5, 15 and 35 g/L NaCl, respectively. As it is observed, the higher NaCl concentration the higher nitrates consumption. Hyper salinity increases the production of metabolites [29]. Further investigation is needed to clarify the role of salinity on nitrates consumption by microalgae. The cease of aeration and mixing during the second period, did not affect the consumption of phosphorus in the 35 g/L NaCl culture, while a drop was observed in the culture with 15 g/L NaCl. In conclusion, the stop of aeration did not affect the algal growth nor the nutrients consumption as well as the lipid and protein content of biomass.

3.2. Effect of salinity on algal biomass characteristics

The characteristics of biomass in terms of lipids, sugars, and proteins were investigated (Fig. 3). The biomass had different content in terms of lipids at the beginning of the experiment. This may be attributed to the exposure of algae to different NaCl concentration during preculture period. Salinity as a stressor induces a wide range of detrimental effects on algal cell. If the stress conditions persist long enough, they may also cause permanent damage in the genome following the initial acclimation to these new conditions. During the first period, the lipid content was increased (Fig. 3a). The culture with the highest NaCl concentration reached the highest content (55%), while the culture without NaCl the smallest (13%). In the

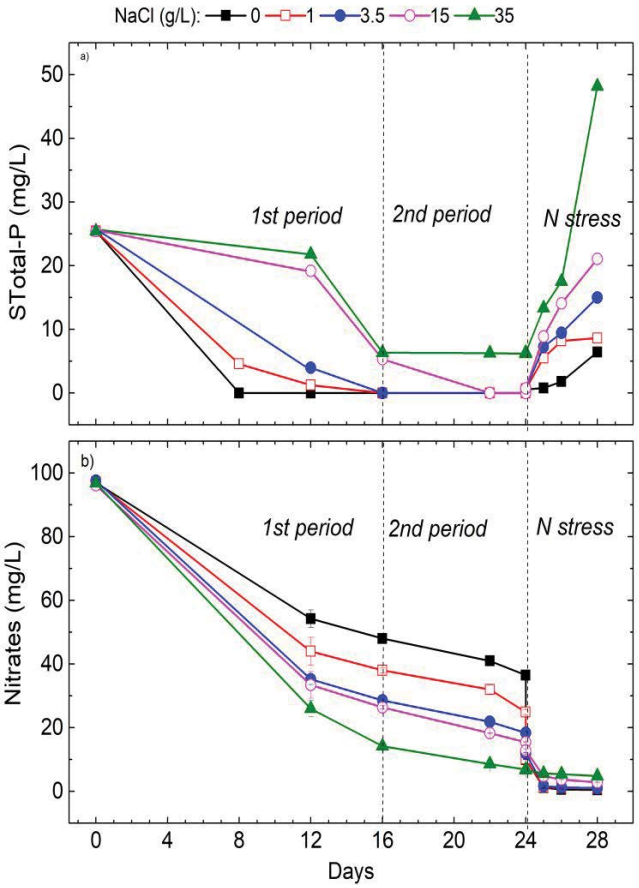

Fig. 2. Variation of nutrients (a) nitrates and (b) STotal-P. The values represent mean ± standard deviation (SD) (n = 3).

2nd period the content of algal lipids was generally decreased (11%–24%) probably due to CO_2 limitation, [30] with the culture in 1 g/L NaCl having the highest drop compared to other studies (Table S2), it is observed that under normal growth conditions *C. vulgaris* showed the highest lipid content (55%) after 16 d of operation with 35 g/L NaCl. A high lipid content (44%) was also observed by Kakarla et al. [16] for *Chlorella sorokiniana* cultured 60 g/L at NaCl. Pandit et al. [29] reported that the increase of NaCl concentration from 0.06 to 0.4 M NaCl (3.5 and 23.38 g/L) resulted in the improved content of lipid from 31.5% to 49.5% and from 23.4% to 43.4% in *C. vulgaris* and *Acutodesmus obliguus*, respectively.

Few research studies tend to express the content of biomass in lipids according to the content of proteins in the biomass, since lipids contain an amount of proteins [31]. At the beginning of the experiment the protein content of *C. vulgaris* was 0.35% to 0.7%, and during the first period increased to 10.1%, 8.7%, 7.3%, 6.4% and 8.2% in cultures with 0, 1, 3.5, 15 and 35 g/L NaCl, respectively. A sharp increase in protein content was observed during the second period for all cultures (48% to 66%) while the lipids were decreased, and the highest increase was associated with the highest NaCl concentration. Maybe some lipids (lipoproteins etc) are recognized as proteins during the limitation of CO_2. The algal content in sugars

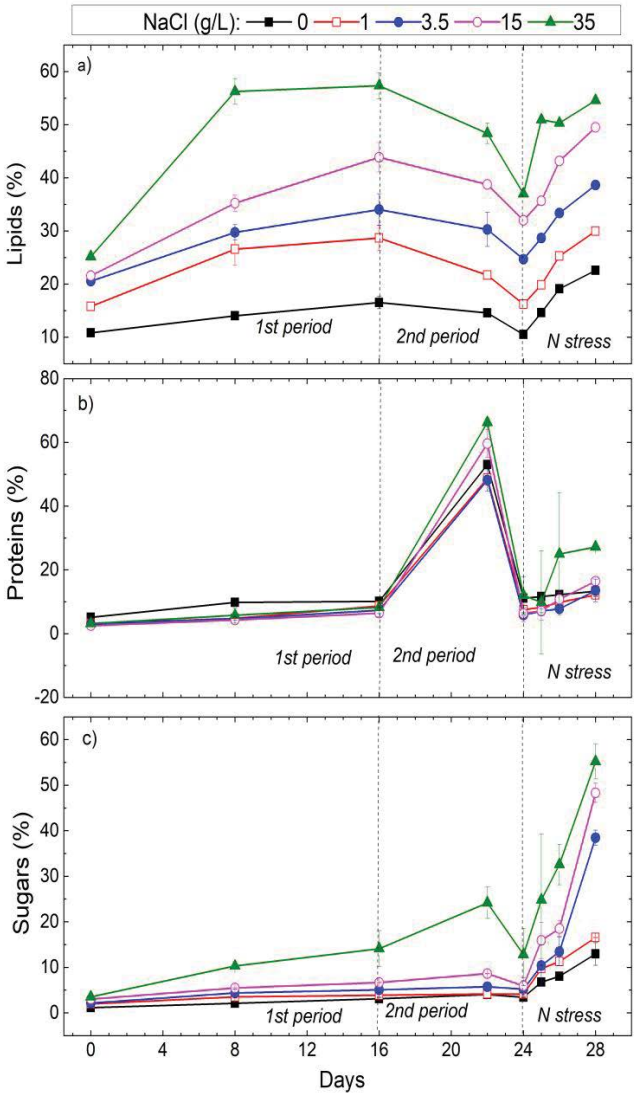

Fig. 3. Variation of (a) lipids, (b) proteins and (c) sugars. The values represent mean ± standard deviation (SD) (n = 3).

was increased during the first period to 3.1%, 3.9%, 5.1%, 6.6% and 14% in cultures with 0, 1, 3.5, 15 and 35 g/L NaCl, respectively. During the second period the increase of sugars was more intense in the 35 g/L culture. Under normal growth conditions (first period), although the algal protein and sugars content increased, it was lower compared to other studies in the literature (Table S2). The increase of NaCl concentration resulted in lower protein and higher sugars yield of *C. vulgaris* and *Actodesmus obliquus* [30].

Generally, the results revealed that the presence of NaCl above 15 g/L inhibited the photosynthetic activity of *C. vulgaris* and at the same time increased the cell diameter. Based on microscope observations, even from the 8th day of cultivation the diameter of *C. vulgaris* was increased about 50% and 64% at 15 and 35 g/L NaCl, respectively compared to the control cells. Church et al. [19] similarly reported an increase of 48% of *C. vulgaris* diameter cultured in 30 g/L NaCl after 5.4 d of exposure. Ahmad and Hellebust [33], reported that the cell size of *Chlorella autotrophica* in 600% (161.3 g NaCl/L) artificial sea water (ASW) was about twice as large as the cells at 10% ASW (2.69 g NaCl/L).

3.3. Nitrates starvation

At the end of the second period, the content of each 2 L flask was centrifuged, and the settled biomass was transferred again into the 2 L Erlenmeyer flask, which was filled up to 2 L with BG-11 without $NaNO_3$. Centrifugation, although is an intense energy process, was selected for the complete separation of biomass. In full-scale facilities, alternative low-cost harvesting processes may be examined. At the beginning of the starvation period, the cells number was lower compared to the population on the 24th day since only the settled biomass was transferred into the new substrate. Nitrate's starvation causes a decrease in population in low salinity cultures; however, the population was significantly increased in high salinity cultures. During the nitrates starvation the growth rate in the 0, 1 and 3.5 g/L NaCl cultures was quite low 0.0455 to 0.0533 d^{-1}, while in the higher salinity cultures with 15 and 35 g/L NaCl was quite higher 0.154 and 0.274 d^{-1}, respectively. The cells of the microalgae at high salinity cultures became durable at stress conditions, so they can further grow during nitrate starvation. As it observed in Fig. 1a the cells number was slightly increased after 4 days of starvation for all salinity levels. Nitrate starvation resulted in the decrease of Chl-a concentration in all cultures and this decrease was higher in cultures with 0, 1 and 3.5 g/L NaCl (Fig. 1c).

During starvation, the concentration of nitrates was almost the same (Fig. 2b). On the other hand, the soluble phosphorus concentration was increased in all cultures, (Fig. 2a), and the culture with the highest NaCl concentration (35 g/L) exhibited the highest soluble phosphorus concentration (50 mg/L). The release of phosphorus is probably due to osmotic stress and the collapse of algal cells. Therefore, the absence of nitrates in cultures leads to phosphorus release, which is more intense at high NaCl concentrations. Liu and Vyverman [33] reported that phosphorus content in the culture depends on the availability of nitrogen. As the N/P ratio increases, the phosphorus content of the benthic filamentous algae *Cladophora* sp., *Klebsormidium* sp. and *Pseudanabaena* sp. decreases. Huang et al. [31] mentioned that the phosphorous removal rate increased with the increase $NaNO_3$ concentration. So, the absence of nitrogen increased the concentration of released phosphorous at the high salinity cultures, where the cells were wider maybe due to osmotic stress.

Nitrates starvation mainly increases the percentage of lipids and sugars. The presence of NaCl affects the characteristics of the produced biomass (Fig. 3). In all cases, the culture with the higher NaCl concentration (35 g/L) has the higher lipid, protein and sugars content, and the culture without NaCl the less. Even though the algal content of lipids was different at the end of the second period, the increase during the starvation period tends to be the same, at about 10% compared to their initial values.

The lipid content of the control culture at the end of starvation was 23%, but higher values have been reported in the literature (77%) by Mujtaba et al. [11] with *C. vulgaris*. Under stress conditions, microalgae tend to increase the lipid content.

Borowitzka [29] mentioned that when an algal cell is exposed to osmotic stress different types of proteins are produced. Additionally, plant protein synthesis is positively correlated with stress tolerance. The proteins content of the biomass was almost the same at the beginning of the starvation in all cultures. Although that cultures at 0, 1, 3.5 and 15 g/L NaCl tend to have the same protein content (13%–16%), the culture with 35 g/L doubled its content after 4 days, reaching 27%. Despite the nitrate starvation, there was a slight increase in proteins content of *C. vulgaris* because photosynthesis continued [36]. This increase in lipid content may be achieved against other components, mainly proteins or from newly fixed carbon, as a reserve of energy for the cell after the stress [37]. Environment stress conditions to microalgae tend to diminish chlorophyll concentration and increase protein quantity [38]. The protein content at the end of starvation in the control culture of *Chlamydomonas* sp. JSC4 was 18% [10].

Finally, the biomass content in sugars increases during nitrate's starvation. Cultures with higher salinity tend to have higher content during starvation. In cultures with 3.5, 15 and 35 g/L NaCl the sugar content increased up to 30%, while in 0 and 1 g/L NaCl cultures increased 8%.

4. Conclusions

C. vulgaris was cultured in 1/3N BG-11 medium at five different NaCl concentrations (0, 1, 3.5, 15 and 35 g/L). *C. vulgaris* grows faster at low NaCl concentrations (1 and 3.5 g/L) compared to cultures in absence of NaCl. As was expected algal growth inhibition was observed at higher concentrations (15 and 35 g/L). During nitrate starvation, higher algal growth rates were observed in cultures at 15 and 35 g/L NaCl (0.154 and 0.274 d^{-1}, respectively), compared to lower NaCI concentrations (about 0.05 d^{-1}). The content of algal in lipid, proteins, and sugars was high, especially in cultures with elevated NaCl concentrations, and during nitrate's starvation had an increasing trend. Although *C. vulgaris* was grown faster in cultures with low salinity, the biomass content in lipids, proteins, and sugars was richer in high salinity.

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Supporting information

Table S1
Effect of salinity on *Chlorella vulgaris* turbidity and optical density

NaCl (g/L)	Equation	R^2
0	NTU = 200.39 × OD – 24.904	0.9539
1	NTU = 248.3 × OD – 88.671	0.8158
3.5	NTU = 279.16 × OD – 98.145	0.8063
15	NTU = 328.63 × OD – 32.799	0.9654
35	NTU = 181.87 × OD + 10.816	0.9729

Table S2
Characteristics of microalgae produced biomass cultured in different salinity medium

Source	Microalgae	Medium	Normal growth conditions				Effect of salinity					Nitrate deprivation		
			Duration	Lipids	Proteins	Sugars	C NaCl	Duration	Lipids	Proteins	Sugars	Duration	Lipids	Proteins	Sugars
			(d)		(%)		g/L	(d)		(%)		(d)		(%)	
Present study	*Chlorella vulgaris*	BG-11	16	16.5	10	0.95						4	22.6	13.3	12.92
	Chlorella vulgaris	BG-11(N/3)+NaCl (1 g/L)	16	28.7	8.7	1.2						4	30	12.13	16.55
	Chlorella vulgaris	BG-11(N/3)+NaCl (3.5 g/L)	16	36	6.9	1.6						4	42.5	13.5	35.46
	Chlorella vulgaris	BG-11(N/3)+NaCl (15 g/L)	16	43.9	5	2.1						4	50	16.4	48.31
	Chlorella vulgaris	BG-11(N/3)+NaCl (35 g/L)	16	55	3.8	4.4						4	57.3	27	55.19
Aravantinou et al. [1]	*Chlorococcum*	BG-11+NaCl (26 g/L)	20	8								30	3		
	Neoclhoris vigensis	BG-11+NaCl (26 g/L)	20	18								30	12		
	Scenedesmus rubescens	BG-11+NaCl (26 g/L)	20	15								30	5		
Aravantinou et al. [2]	*Chlorococcum*	BG-11(N/3)	40	5								20	10		
Aravantinou et al. [2]	*Chlorococcum*	BG-11	7	22											
Aravantinou et al. [3]	Algae	BG-11	30	15											
Cheng et al. [36]	*Chlorella vulgaris*	ESP-6	6	31	28	8									
Ho et al. [10]	*Chlamydomonas* sp. JSC4	Modified Bold 3 N+NaCl (0.2 g/L)	6	15	45	30						7	41	18	33
Kakarla et al. [16]	*Chlorella sorokiniana*	BG-11	9	25			60	2	25	38	32				
	Chlorella sorokiniana	BG-11+NaCl (60 g/L)	9	44			61	2	41	38	24				
Mujtaba et al. [11]	*Chlorella vulgaris*	BG-11	8	31								1	77		

(Continued)

Table S2 Continued

Source	Microalgae	Medium	Normal growth conditions				Effect of salinity					Nitrate deprivation			
			Duration	Lipids	Proteins	Sugars	C NaCl	Duration	Lipids	Proteins	Sugars	Duration	Lipids	Proteins	Sugars
Ordog et al. [39]	*Chlorella* MACC 438	Tamiya (N/100)	6	20	28	24									
	Chlorella MACC 452	Tamiya (3N/100)	6	18	26	25									
	Chlorella MACC 728	Tamiya (N/10)	6	18	29	26									
Pandit et al. [29]	*Acutodesmus obliquus*	BG-11(N/2)	15	11.3	20	28									
	Acutodesmus obliquus	BG-11 (N/2)+NaCl (3.5 g/L)	15	22	25	27									
	Acutodesmus obliquus	BG-11 (N/2)+NaCl (4.34 g/L)	15	23	22	38									
	Acutodesmus obliquus	BG-11 (N/2)+NaCl (5.84 g/L)	15	23.8	18	41									
	Acutodesmus obliquus	BG-11 (N/2)+NaCl (17.5 g/L)	15	22.33	17	48									
	Acutodesmus obliquus	BG-11 (N/2)+NaCl (23.4 g/L)	15	11.7	15	52									
	Chlorella vulgaris	BG-11(N/2)	15	11.4	33	30									
	Chlorella vulgaris	BG-11 (N/2)+NaCl (3.5 g/L)	15	18.5	23	26									
	Chlorella vulgaris	BG-11 (N/2)+NaCl (4.34 g/L)	15	18.1	25	37									
	Chlorella vulgaris	BG-11 (N/2)+NaCl (5.84 g/L)	15	18.88	27	38									
	Chlorella vulgaris	BG-11 (N/2)+NaCl (17.5 g/L)	15	10.33	25	42									
	Chlorella vulgaris	BG-11 (N/2)+NaCl (23.4 g/L)	15	8.6	18	48									

Table S2 Continued

Source	Microalgae	Medium	Normal growth conditions				Effect of salinity					Nitrate deprivation			
			Duration	Lipids	Proteins	Sugars	C NaCl	Duration	Lipids	Proteins	Sugars	Duration	Lipids	Proteins	Sugars
Praveenkumar et al. [40]	*Chlorella* sp.	Chu10	20	30								2	42		
Ra et al. (2015)	*Dunaliella tertiolecta*	f/2+NaCl (30 psu)	10	23			0	2	28						
	Dunaliella salina	f/2+NaCl (30 psu)	10	24			10 (psu)	1	42						
	Isochrysis galbana	f/2+NaCl (30 psu)	12	18			10 (psu)	3	43						
	Nannochloropsis oculata	f/2+NaCl (30 psu)	14	26			0	2	28						
Tsavatopoulou et al. [4]	*Chlorococcum* sp.	Wastewater (enluent)	14	2.6											
	Scenedesmus sp.	BG-11 (N/3)	14	3.3											
Yun et al. [17]	*Chlorella vulgaris* YH703	BG-11	15	12			0	3	11	52	18				
	Chlorella vulgaris YH704	BG-11+NaCl (0.88 g/L)	15	12.5			58.4	3	12	48	21				
	Chlorella vulgaris YH705	BG-11+NaCl (17.5 g/L)	15	13			117	3	13	45	25				
	Chlorella vulgaris YH706	BG-11+NaCl (26.3 g/L)	15	14			175	3	17	44	26				
	Chlorella vulgaris YH707	BG-11+NaCl (35 g/L)	15	14.5			234	3	22	43	30				
	Chlorella vulgaris YH708	BG-11+NaCl (58.4 g/L)	15	15.5			292	3	25	32	40				
	Chlorella vulgaris YH703	BG-11+NaCl (117 g/L)	15	16			350	3	26	30	42				