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EFFECTS OF SODIUM SULFATE ON THE FRESHWATER MICROALGA *CHLAMYDOMONAS MOEWUSII*: IMPLICATIONS FOR THE OPTIMIZATION OF ALGAL CULTURE MEDIA[1]

Roi Mera, Enrique Torres[2] *and Julio Abalde*

Laboratorio de Microbiología, Facultad de Ciencias, Universidade da Coruña, Alejandro de La Sota, No. 1, La Coruña 15008, Spain

The study of the microalgal growth kinetics is an indispensable tool in all fields of phycology. Knowing the optimal nutrient concentration is an important issue that will help to develop efficient growth systems for these microorganisms. Although nitrogen and phosphorus are well studied for this purpose, sulfur seems to be less investigated. Sulfate is a primary sulfur source used by microalgae; moreover, the concentration of this compound is increasing in freshwater systems due to pollution. The aim of this study was to investigate the effects of different sodium sulfate concentrations in the culture medium on growth and growth kinetics of the freshwater microalga *Chlamydomonas moewusii*. Production of biomass, chl content, kinetic equations, and a mathematical model that describe the microalgal growth in relation with the concentration of sodium sulfate were obtained. The lowest concentration of sodium sulfate allowing optimal growth was 0.1 mM. Concentrations higher than 3 mM generated a toxic effect. This work demonstrates that this toxic effect was not directly due to the excess of sulfate ion but by the elevation of the ionic strength. An inhibition model was successfully used to simulate the relationship between specific growth rate and sodium sulfate in this microalga.

***Key index words:* *Chlamydomonas moewusii*; culture medium; growth; ionic strength; kinetics; sodium sulfate; substrate inhibition**

Currently, microalgal growth studies are gaining importance due to their ecological and commercial relevance. In fact, these microorganisms are used in the production of biodiesel (Schenk et al. 2008) since they have emerged as one of the most promising feedstock for the production of this fuel. Growth conditions and nutrient concentrations have an influence on the quantity and quality of lipids within the cells (Gao et al. 2013, Wahidin et al. 2013). Moreover, microalgal mass culture appears to be a feasible way to remove inorganic nutrients and, in some instances, to convert them into useful biomass (Mata et al. 2010). Therefore, the study of microalgal growth kinetics is an indispensable tool in all fields of phycology. Different physicochemical parameters such as pH, temperature, light, and medium composition influence the rates of growth and activities of microalgae. However, microalgae do not need the same amount of each nutrient. Knowing the best nutrient concentration is an important issue that will help to develop a more efficient growing process for these microorganisms and therefore, greater microalgal production. The artificial media are mostly used for experimental purposes, since they allow for the generation of constant and reproducible results under laboratory conditions. Many defined freshwater algal media have been designed (Brown et al. 1967, Guillard 1975, Vonshak 1986, Kilham et al. 1998, Ilavarasi et al. 2011). Although nitrogen and phosphorus, along with certain cations (iron, calcium, cobalt, etc.) are well studied for this purpose (Romero et al. 1999, Zhang et al. 2008, Gallardo Rodríguez et al. 2009, Chen et al. 2011, Ruiz et al. 2011), sulfur seems to be neglected, perhaps because sulfur is the least abundant of the six macronutrients required by these cells. However, it plays an important role in the growth of microalgae.

Sulfur is an essential macroelement because it is required for the biosynthesis of many molecules and cellular constituents. The major form of sulfur available in nature is sulfate. Plants, algae, yeast, and most prokaryotes are able to take up the sulfate anion and thus they satisfy their demand for reduced sulfur by reduction of this compound. This reduced form is then incorporated into organic compounds. For this reason, all culture media contain a sulfate salt. The first organic compounds in the assimilation of reduced sulfur are the amino acids cysteine and methionine (Wirtz and Droux 2005, Hell et al. 2008), which is well defined in autotrophic cells (Shibagaki and Grossman 2008). In addition to proteins, photosynthetic organisms synthesize a wide variety of sulfur compounds, using sulfate as a primary sulfur source (Leustek et al. 2000, Saito 2004). For example, it is contained in membrane sulfolipids, cell walls, and in vitamins and cofactors such as thiamine, biotin, and coenzyme A (Benning 1998, Popper et al. 2011). Sulfur is also necessary for the biosynthesis of reduced

[1]Received 21 May 2015. Accepted 20 October 2015.
[2]Author for correspondence: e-mail torres@udc.es.
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glutathione (GSH). This compound is the major reservoir of nonprotein reduced sulfur and is an important ubiquitous intracellular peptide with many biological roles that include detoxification, antioxidant defense, maintenance of thiol status, and modulation of cell proliferation (Lillig and Berndt 2013).

Recently, different molecular studies on acclimation of microalgae to sulfur deficiency (Aksoy et al. 2013, 2014) were developed, but there are few studies on the nutritional requirements of microalgae for this element in order to optimize culture media. In fact, most microalgal growth models are mainly based on optimizing the carbon, nitrogen, and phosphate levels, especially in heterotrophic cultures (Perez-Garcia et al. 2011); sulfur is considered less in such models. Therefore, the study of sulfur with respect to optimization of the composition of an algal culture medium requires more attention, especially since the sulfate content can vary widely between different culture recipes (0.1–5 mM). Indeed, it is important to optimize the levels of all components in the growth medium in order to maximize microalgal growth and understand how to manipulate the medium for the operation of microalgal-based production systems. In addition, the increase of sulfate in the natural environment, due to diverse anthropogenic sources, is a proven fact. Assessing the impact of this increase in freshwater systems can be an interesting strategy to establish a water quality guideline for sulfate in order to protect aquatic ecosystems.

Thus, the aim of the present work was to investigate the effect of different concentrations of sodium sulfate (the most common salt of sulfate) on growth, growth kinetics and chl content of a freshwater microalga, *Chlamydomonas moewusii*. In addition, a mathematical model that describes the microalgal growth in relation to the concentration of this compound was obtained. Kinetic equations, which describe the growth in relation to a nutrient, are important in understanding many phenomena in biotechnological and ecological processes. *C. moewusii* is a microalga widely used as a model organism for many studies in biology, for example, toxicological studies (Suárez et al. 2010, Mera et al. 2014), flagellar structure (Jones and Lewin 1960), studies with chloroplasts (Richard et al. 1994), biosynthesis of metabolites (Yang et al. 2013), biotransformation (Otto et al. 2015), and genetic studies (Bussieres et al. 1996).

MATERIALS AND METHODS

Test organism and culture conditions. The microalgal species chosen for this study was *C. moewusii* Gerloff (strain CCAP 11/5B) obtained from the Culture Collection of Algae and Protozoa (CCAP) of Freshwater Ecology Institute (Cumbria, UK). Cells of this freshwater microalga were grown and maintained in a photoautotrophic culture medium. Modified Bristol medium (BBM; Stein 1980) was chosen for the experiments because is a well-known growth medium for many freshwater algal cultures. This medium was sterilized at 121°C for 20 min and all compounds of sulfate were replaced by chlorides, preserving the original concentration of the cations. The composition of the culture medium is shown in Table 1. Different concentrations of sodium sulfate were added depending on the treatments. The pH was measured with an Orion 720A+ pH meter (Thermo Electron Corporation, Witchford, Cambridgeshire, UK). The initial pH of the cultures was 6.8 ± 0.2. Cultures were maintained at a stable temperature of 18 ± 1°C under a light intensity of 68 μmol photons · m^{-2} · s^{-1} using cool fluorescent light with a light/dark cycle of 12:12 h. Natural air sterilized by a 0.22 μm filter was constantly bubbled at a flow rate of 10 L · min^{-1}. Sterilized distilled water was added daily to the cultures to replenish that lost by evaporation.

Table 1. Composition of the culture medium for the experiments.

Compound	(g · L^{-1})
$NaNO_3$	0.250
KH_2PO_4	0.175
K_2HPO_4	0.075
Na_2SO_4	Variable
$MgCl_2$	0.029
$CaCl_2{\cdot}2H_2O$	0.029
NaCl	0.025
$CoCl_2{\cdot}6H_2O$	4.0×10^{-3}
$MnCl_2{\cdot}4H_2O$	1.8×10^{-3}
$FeCl_3{\cdot}6H_2O$	5.1×10^{-4}
$MoO_4Na_2{\cdot}2H_2O$	3.9×10^{-4}
H_3BO_3	2.0×10^{-4}
$ZnCl_2$	1.1×10^{-4}
$CuCl_2$	4.3×10^{-5}
EDTA	0.050
KOH	0.031
pH = 6.8 ± 0.2	

Chemicals. All chemicals used were of the highest purity available. Reagents for the culture media, sodium sulfate anhydrous (Na_2SO_4), sodium chloride (NaCl), acetone (C_3H_6O), and Lugol's iodine solution (I_2-KI) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Filters were obtained from Millipore (Millipore Ibérica, Madrid, Spain). The different chemicals were prepared with Milli-Q® water obtained from a Milli Q Plus system (Millipore Ibérica).

Sodium chloride stock was prepared by dilution of sodium chloride in Milli-Q water to obtain a concentration of 2.5 M. This solution were filtered through a 0.22 μm Millipore filter and sterilized at 121°C for 20 min.

Experimental design and sodium sulfate treatments. *C. moewusii* was grown in sterilized 500 mL Pyrex glass bottles and cultured for 11 d under the conditions listed above. An appropriated amount of sodium sulfate was added to the culture medium to obtain the concentrations 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 5, 10, 15, 20, 25, 50, 100, 150, 200, 250, and 300 mM in each experiment. Inoculum for all experiments was taken from a culture maintained for 21 d on sulfate-free medium in order to decrease the cellular pool of organic sulfur. Initial cell density in the assays was 40×10^4 cells · mL^{-1}. Cultures without sodium sulfate were also included. There were 27 treatments, with each treatment carried out in triplicate (81 treatments in total).

Growth measurement. Growth of microalgal cultures was measured by counting culture aliquots in an improved Neubauer hemocytometer chamber (Marienfeld-Superior,

Lauda-Königshofen, Germany) after fixation with Lugol's iodine solution and using a phase contrast light microscope Nikon Labophot (Nikon, Tokyo, Japan). The *C. moewusii* growth was recorded daily over a period of 11 d and cell densities were expressed as $\times 10^4$ cells $\cdot$ mL^{-1} $\pm$ SE for all replicates of each treatment. Growth parameters were only calculated for days 4 and 11. The specific growth rate (μ), expressed as d^{-1}, was calculated from the following equation:

$$\mu = \left[\frac{\ln(N_t) - \ln(N_0)}{\ln(2)(t - t_0)}\right] \quad (1)$$

where N_t and N_0 are the mean number of cells $\cdot$ mL^{-1} ($n = 3$) at final and initial time, and t and t_0 are the final and initial times, respectively, of this period, expressed as days.

Chl determination. *C. moewusii* cells were harvested by centrifugation (4,000 *g* for 10 min) after 4 and 11 d of growth. The chl concentration was determined following extraction of the pigments in cold 90% (v/v) acetone. To improve extraction, the cells suspended in 90% acetone were homogenized with an ultrasonic cell disruptor in an ice bath at 4°C for 1 min (steps of 30 s) and at 126 µm, using a Labsonic® P ultrasonic homogenizer (Sartorius AG, Göttingen, Germany). The extracted material was kept at 4°C in the dark for 24 h and then centrifuged at 4,000 *g* for 10 min to remove cell debris prior to measuring the absorbance of the supernatants at 630, 645, and 660 nm in an UV/Vis spectrophotometer PharmaSpec UV-1700 (Shimadzu Corporation, Kyoto, Japan). A 90% acetone solution was used as blank. The chl *a* and *b* concentrations were determined by the equations of Jeffrey and Humphrey (Jeffrey and Humphrey 1975).

Growth kinetics studies. This study analyzed different unsegregated kinetics models for microalgal growth in relation to the sodium sulfate concentration in order to obtain a mathematical model that best fits the experimental data of *C. moewusii* growth with respect to this nutrient. Two classes of models are usually distinguished:

Monotonic kinetics: the growth rate increases with the increase in the nutrient concentration. This growth corresponds to the Monod growth model (eq. 2).

$$\mu = \frac{\mu_{max} S}{K_S + S} \quad (2)$$

where μ is the specific growth rate of the microalga, μ_{max} the maximum specific growth rate (d^{-1}), S is the sodium sulfate concentration (mM) and K_s is the half-saturation constant (mM).

Nonmonotonic kinetics: where there is no linearity between growth and nutrient concentration. This class includes the substrate inhibition models. Three substrate inhibition models, competitive, uncompetitive, and noncompetitive inhibition (eqs 3–5, respectively) were evaluated with respect to their fit to the experimental data:

$$\mu = \frac{\mu_m S}{K_S\left(1 + \frac{S}{K_I}\right) + S} \quad (3)$$

$$\mu = \frac{\mu_m S}{K_S + S + \frac{S^2}{K_I}} \quad (4)$$

$$\mu = \frac{\mu_m S}{(K_S + S)\left(1 + \frac{S}{K_I}\right)} \quad (5)$$

where μ_m is the specific growth constant (d^{-1}) and K_I is the inhibition constant, which numerically equals the highest substrate concentration (mM) at which the specific growth rate is equal to one half of the maximum specific growth rate. The equations were fitted to the obtained growth data by means of a nonlinear regression. The parameters μ_m, K_s and K_I were estimated from this regression.

In competitive models, the asymptote that determines the maximum growth rate (μ_{max}) is given by the eq. 6:

$$\mu_{max} = \frac{\mu_m K_I}{K_S + K_I} \quad (6)$$

whereas in uncompetitive and noncompetitive inhibition models the maximum growth rate is given by (7) and (8), respectively, both at the various sodium sulfate concentrations of (9):

$$\mu_{max} = \frac{\mu_m}{1 + 2(K_S/K_I)^{1/2}} \quad (7)$$

$$\mu_{max} = \frac{\mu_m}{\left(1 + (K_S/K_I)^{1/2}\right)^2} \quad (8)$$

$$[S] = (K_S K_I)^{1/2} \quad (9)$$

Finally, μ_{max} in the Monod equation was obtained directly from the regression analysis.

Curve fitting and data analysis were performed using SigmaPlot for Windows 12.5 (Systat Software, Inc., Chicago, IL, USA).

Effect of different concentrations of sulfate ion at the same initial ionic strength. To determine the effect of the sulfate ion, an experiment was conducted with different concentrations of sulfate at the same initial ionic strength. The ionic strength of the culture media was calculated by means of Visual MINTEQ software (Version 3.1) for Windows (Gustafsson 2013), assuming ideal behavior and molarity concentrations. The theoretical ionic strength used as reference for these experiments was calculated from culture medium containing a concentration of 20 mM sodium sulfate; this value was 63.2 mM. The sulfate concentrations tested were 0, 0.0001, 0.00025, 0.0005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 3, 5, 10, 15, and 20 mM. The ionic strength of each of these media was previously calculated with Visual MINTEQ. The difference in the theoretical ionic strength with respect to the reference culture was adjusted by adding sodium chloride to the simulation with Visual MINTEQ to achieve the same ionic strength as the reference culture. These amounts of sodium chloride were added to the respective culture media. The pH was experimentally measured to verify that all of the cultures had the same pH; the average value obtained was 6.9 $\pm$ 0.3. Each treatment was carried out in triplicate.

Statistical analysis. Data were expressed as means $\pm$ SE and analyzed using the statistical program SPSS statistical package (IBM SPSS Statistics for Windows, Version 22.0; IBM Corp, Armonk, NY, USA). A one-factor analysis of variance (one-way ANOVA) was used to test for differences among treatments. The treatments means were statistically compared with a *post-hoc* analysis by means of Tukey test to a level of 5% ($P < 0.05$).

RESULTS

Effect of sodium sulfate on C. moewusii *growth.* The biomass of *C. moewusii* obtained after 4 and 11 d of culture and depending on the sodium sulfate concentration in the medium can been seen in

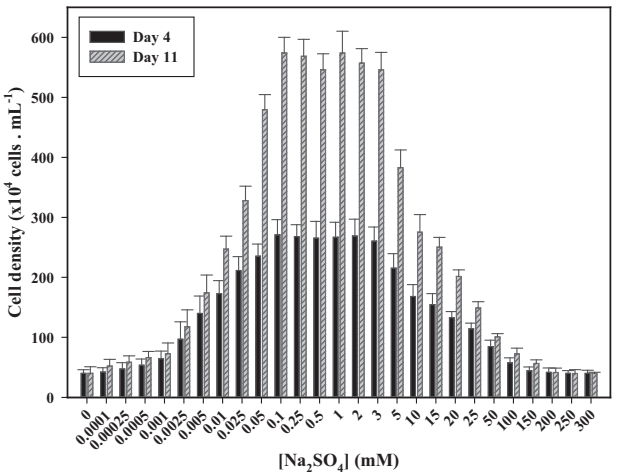

FIG. 1. Cell densities of *Chlamydomonas moewusii* after 4 and 11 d of culture with the different sodium sulfate concentrations assayed. Each point represents mean ± SE ($n = 3$). The *x*-axis is not to scale.

Figure 1. After 4 d, the range of sodium sulfate concentrations from 0.0001 to 0.1 mM had a favorable effect on the cell density of *C. moewusii* ($F_{10,22} = 57.29$, $P < 0.001$). This effect was directly proportional to the concentration of this compound during this time of culture. Sodium sulfate exerted a beneficial effect on the biomass production of this microalga. The highest cell density was obtained when the sodium sulfate was in the range of 0.1–3 mM. This cell density was around 6.8 times that of the control without sodium sulfate, where no growth was observed. Sulfur limitation clearly stopped cell division. Therefore, 0.1 mM was the lowest concentration of sodium sulfate in which optimal growth was achieved. This optimal growth was maintained until a sodium sulfate concentration of 3 mM. No significant differences were observed in this range of concentrations ($F_{5,12} = 0.061$, $P = 0.997$). However, once the concentration exceeded 3 mM, the density of the cells gradually declined in proportion to the level of sulfate in the medium ($F_{11,24} = 84.78$, $P < 0.001$) indicating that there was substrate inhibition at concentrations higher than the optimal range.

This behavior remained unchanged after 11 d of growth (Fig. 1). The cultures that had the highest growth reached stationary phase on this day. At the optimal concentrations (0.1–3 mM), the reached final cell density was around 14.35 times that of the control. From this optimal concentration, the cell density also decreased as the sodium sulfate concentration increased ($F_{11,24} = 275.3$, $P < 0.001$).

Growth rate of *C. moewusii* was affected by the concentration of sodium sulfate after 4 d of culture. Sodium sulfate reduction in the culture medium reduced the microalgal growth to zero in the cultures without sodium sulfate and the growth rate was restored as sodium sulfate was incorporated into the medium, reaching a maximum value in the range of concentrations of 0.1–3 mM (Fig. 2). However, from the concentration of 3 mM, a decrease in the specific growth rate was obtained with the increase in the sodium sulfate concentration. This result clearly showed the occurrence of substrate inhibition.

Growth rate after 11 d of culture was calculated (Fig. 3) and a similar result was seen as in cultures after 4 d, and both results showed substrate inhibition.

Kinetic parameters of C. moewusii *growth in relation to sodium sulfate.* The kinetic parameters obtained by fitting the data to the four growth models studied are summarized in Table 2. Uncompetitive and noncompetitive models generated higher values of the determination coefficients. The lowest coefficients were obtained with the Monod and competitive models. The lines drawn in Figure 2 correspond to the prediction obtained by nonlinear regression of the experimental data (solid circles) according to the four models studied. The uncompetitive and noncompetitive models were able to predict the experimental results fairly well. Thus, the dependence of the growth rate to the sodium sulfate concentration ranging from 0 to 300 mM was expressed by an equation that corresponds to inhibition models. Taking into account these models, the sodium sulfate concentration obtained using the eq. 9 that would yield the maximum growth rate would be 0.29 mM after 4 d of culture, and the maximum growth rate achieved in these conditions would be 0.699 d^{-1} (eqs 7 and 8). The half-saturation constant (K_s) was 3.2 ± 0.4 μM and with the data obtained from these models, the 50% inhibition occurred at a concentration of 26.3 ± 2.4 mM of sodium sulfate (K_I). This result was maintained after 11 d of culture, the uncompetitive and noncompetitive models also generated the best determination coefficients with a higher K_s and a lower K_I compared with those obtained on day 4 (Table 2). According to these models, the maximum growth rate was obtained with a sodium sulfate concentration of 0.32 mM (eqs 7–9).

Effect of sodium sulfate on chl content. There were significant differences in the chl *a* concentration per unit volume ($F_{26,54} = 140.8$, $P < 0.001$; $F_{26,54} = 482.81$, $P < 0.001$) and in the chl *b* ($F_{26,54} = 19.54$, $P < 0.001$; $F_{26,54} = 69.49$, $P < 0.001$) between the different treatments after 4 and 11 d of culture (Fig. 4). The increase in sodium sulfate allowed that the cultures had a higher content of both chls · mL^{-1}, reaching a maximum value in the optimal concentrations (0.1–3 mM). However, the content of chls · mL^{-1} decreased from the concentration of 5 mM sodium sulfate, showing an inhibitory effect. In contrast, the content of chls per cell showed no significant differences (even in the limiting sulfate concentrations) up to the concentration

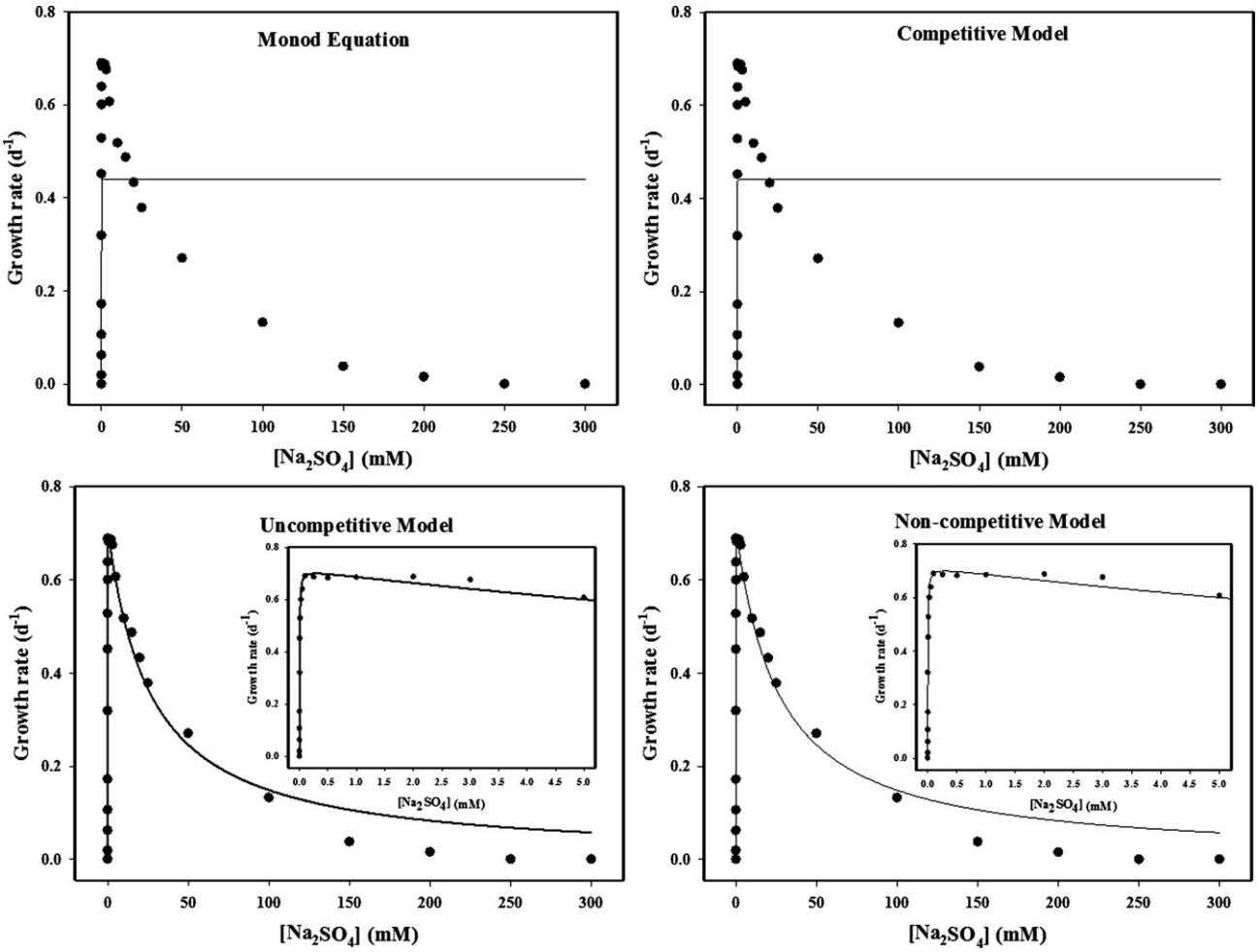

FIG. 2. Growth rate (d^{-1}) of *Chlamydomonas moewusii* cells after 4 d of exposure to the different sodium sulfate concentrations assayed (experimental data and model fitting). Each inner graph is an enlargement of the sodium sulfate concentration from 0 to 5 mM.

of 10 mM, from which there was an increase, reaching a maximum value in the concentration of 25 mM. From this concentration, the amount of chls per cell decreased sharply. As expected, chl *a* predominated versus chl *b* because chl *a* is the primary photosynthetic pigment in microalgae. The chl *a*/*b* ratio remained constant in all sodium sulfate concentrations tested.

Effect of different concentrations of sulfate ion at the same initial ionic strength. Cell density reached after 4 and 11 d of culture with different concentrations of sulfate ion, but with the same initial ionic strength can be seen in Figure 5. This figure shows the increase in the number of cells (up to a maximum value) with the increase of sulfate concentration. As was obtained in cultures with different initial ionic strength (Fig. 1), the minimum sulfate concentration required to achieve the optimal value was 0.1 mM. The differences in the cell density of the cultures with <0.1 mM after 4 and 11 d and with the same initial ionic strength were significant ($F_{10,22} = 24.84$, $P < 0.001$; $F_{10,22} = 74.96$, $P < 0.001$), however, these statistical analyses also revealed that the differences in the biomass production obtained from the concentration of 0.1 mM were not significant ($F_{8,18} = 0.14$, $P = 0.996$; $F_{8,18} = 0.293$, $P = 0.959$). It is noteworthy that unlike the cultures with different initial ionic strength, high sulfate concentrations (>3 mM) were not toxic to this microalga. No substrate inhibition was observed, even at 20 mM of sulfate ion. Therefore, the inhibition observed in cultures with a sodium sulfate concentration higher than 3 mM was not due to the increase in the concentration of sulfate ion, but by the increase in the initial ionic strength of the culture medium.

Figures 6 and 7 show the experimental and predicted growth rates of the cultures exposed to different concentrations of sulfate ion with the same initial ionic strength. The kinetic parameters obtained by fitting the data to the four growth models (Table 3) indicated that all the models generated higher values of the determination coefficients. However, as there was no inhibition at higher

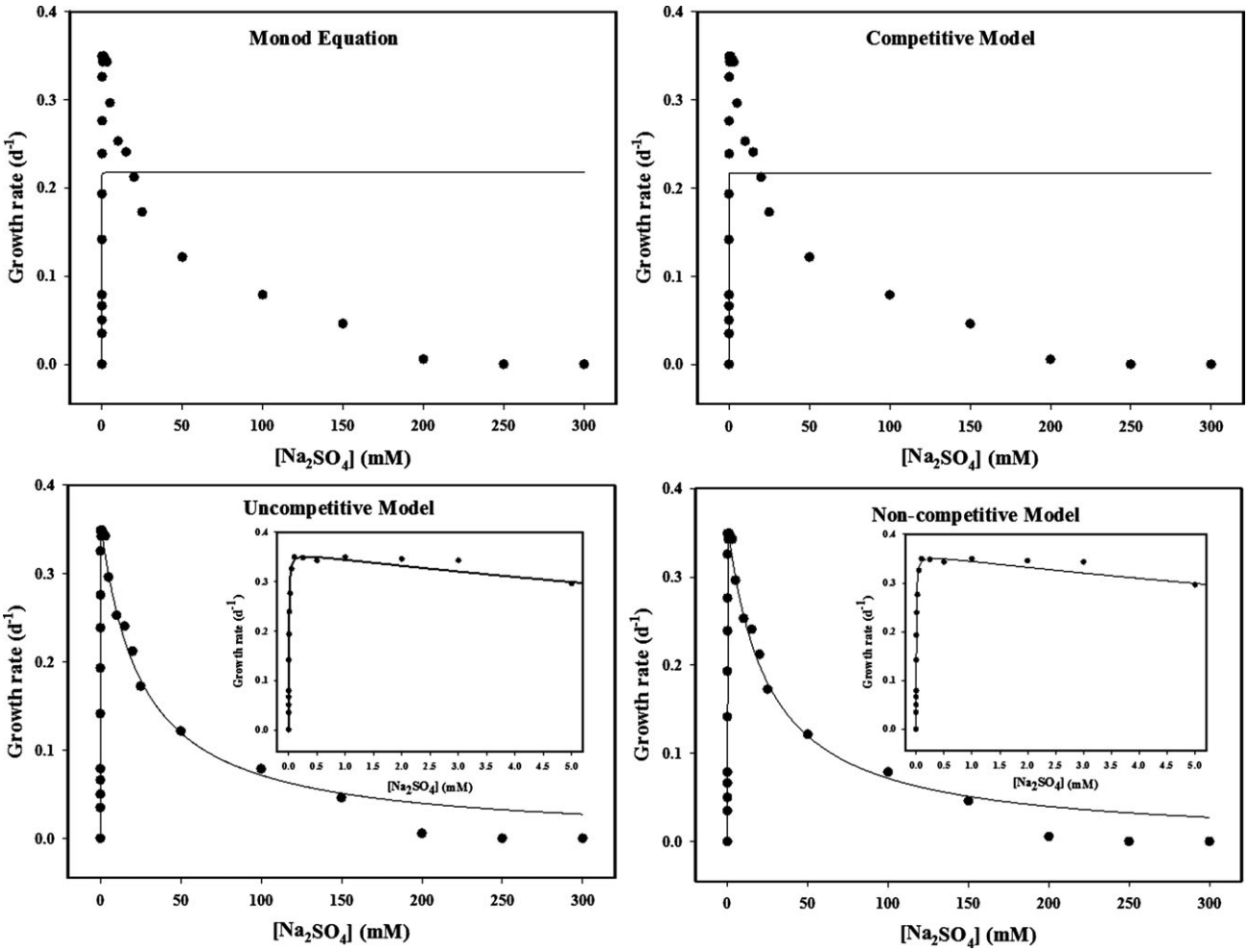

FIG. 3. Growth rate (d^{-1}) of *Chlamydomonas moewusii* cells after 11 d of exposure to the different sodium sulfate concentrations assayed (experimental data and model fitting). Each inner graph is an enlargement of the sodium sulfate concentration from 0 to 5 mM.

TABLE 2. Kinetic parameters (± SE) of *Chlamydomonas moewusii* cells after 4 and 11 d of exposure to different sodium sulfate concentrations.

Model	R^2	μ_{max}	μ_m	K_s	K_I	$[S]_{\mu max}$
Day 4						
Monod	0.252	0.440	–	0.90 ± 1.10	–	–
Competitive	0.221	0.405	2.553 ± 1.865E+08	5.30 ± 3.901E+08	0.001 ± 0.001	–
Uncompetitive	0.986	0.699	0.714 ± 0.013	3.20 ± 0.40	26.297 ± 2.402	0.29
Noncompetitive	0.986	0.699	0.714 ± 0.013	3.20 ± 0.40	26.294 ± 2.402	0.29
Day 11						
Monod	0.234	0.218	–	1.00 ± 1.10	–	–
Competitive	0.202	0.202	1.816 ± 1.585E+07	8.00 ± 6.947E+07	0.001 ± 0.001	–
Uncompetitive	0.980	0.351	0.360 ± 0.008	4.20 ± 0.50	24.955 ± 2.605	0.32
Noncompetitive	0.980	0.351	0.360 ± 0.008	4.20 ± 0.50	24.951 ± 2.605	0.32

R^2 = determination coefficient, μ_{max} = maximum growth rate (d^{-1}), μ_m = specific growth constant (d^{-1}), K_S = half-saturation constant (μM), K_I = inhibition constant (mM) and $[S]_{\mu max}$ = sodium sulfate concentration (mM) corresponding to μ_{max}.

concentrations, the Monod model better explained the obtained results.

DISCUSSION

Microalgae require a number of macro and micronutrients for growth. Due to the current prominence of these photosynthetic microorganisms, the optimization of the culture conditions is a key aspect to obtain good yields and better economic feasibility. In this sense, sulfate has been less studied despite being regarded as an important nutrient for microalgae. The results of the present work clearly showed the influence of this nutrient

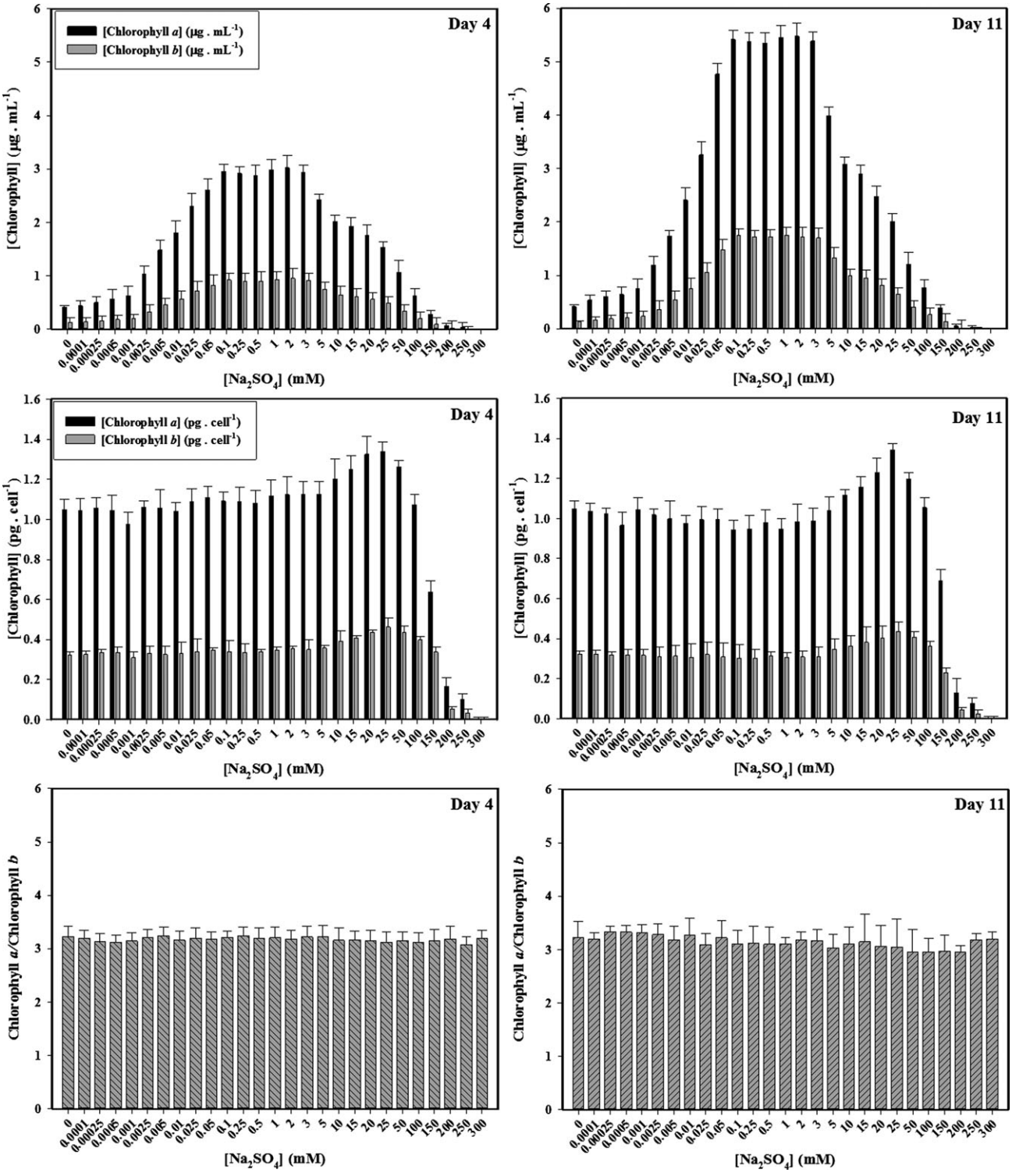

FIG. 4. Amount of chl in *Chlamydomonas moewusii* cells cultured with different sodium sulfate concentrations after 4 and 11 d.

on microalgal growth, using the freshwater microalga *C. moewusii* (Fig. 1). Although magnesium sulfate is the usual way of adding sulfate to microalgal culture media, in the present work and in order to avoid other possible effects due to magnesium, sulfate was added as sodium sulfate; however, an experiment with magnesium sulfate was conducted to justify this change. The result of this experiment showed that the amount of biomass obtained in the cultures with different concentrations of magnesium

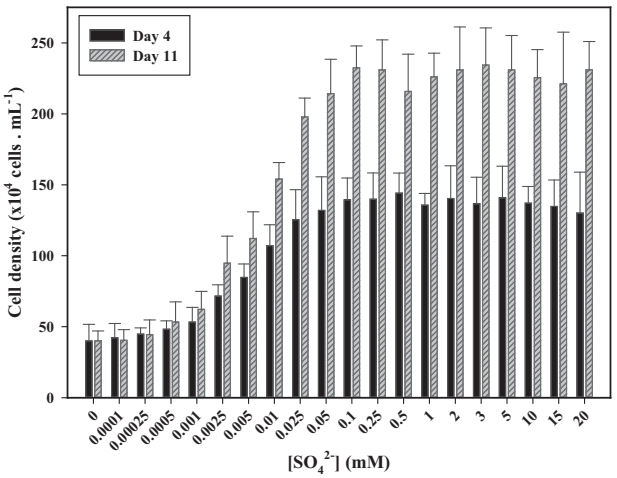

FIG. 5. Cell densities of *Chlamydomonas moewusii* cells cultured with different sulfate ion concentrations but at the same initial ionic strength after 4 and 11 d. Each point represents mean ± SE (n = 3). The x-axis is not to scale.

sulfate was always lower than the obtained in the cultures with sodium sulfate (Fig. 8). Magnesium is also an important nutrient for microalgae; the use of magnesium sulfate instead of sodium sulfate reduced the amount of two essential nutrients in the suboptimal concentrations, causing a higher decrease in the growth parameters. When sulfate was added in form of sodium sulfate, the reduction in growth was lower because this effect was only due to the variation of this anion since all the cultures had the same initial concentration of magnesium ($7.4\ mg \cdot L^{-1}$, the usual in the BBM medium), therefore, there was no variation in the concentration of this cation in the cultures with different sodium sulfate concentrations. However, the optimal value of biomass was obtained at the same concentrations of both salts. With this in mind, the experimental design with sodium sulfate was the most suitable to study the effects of the sulfate anion.

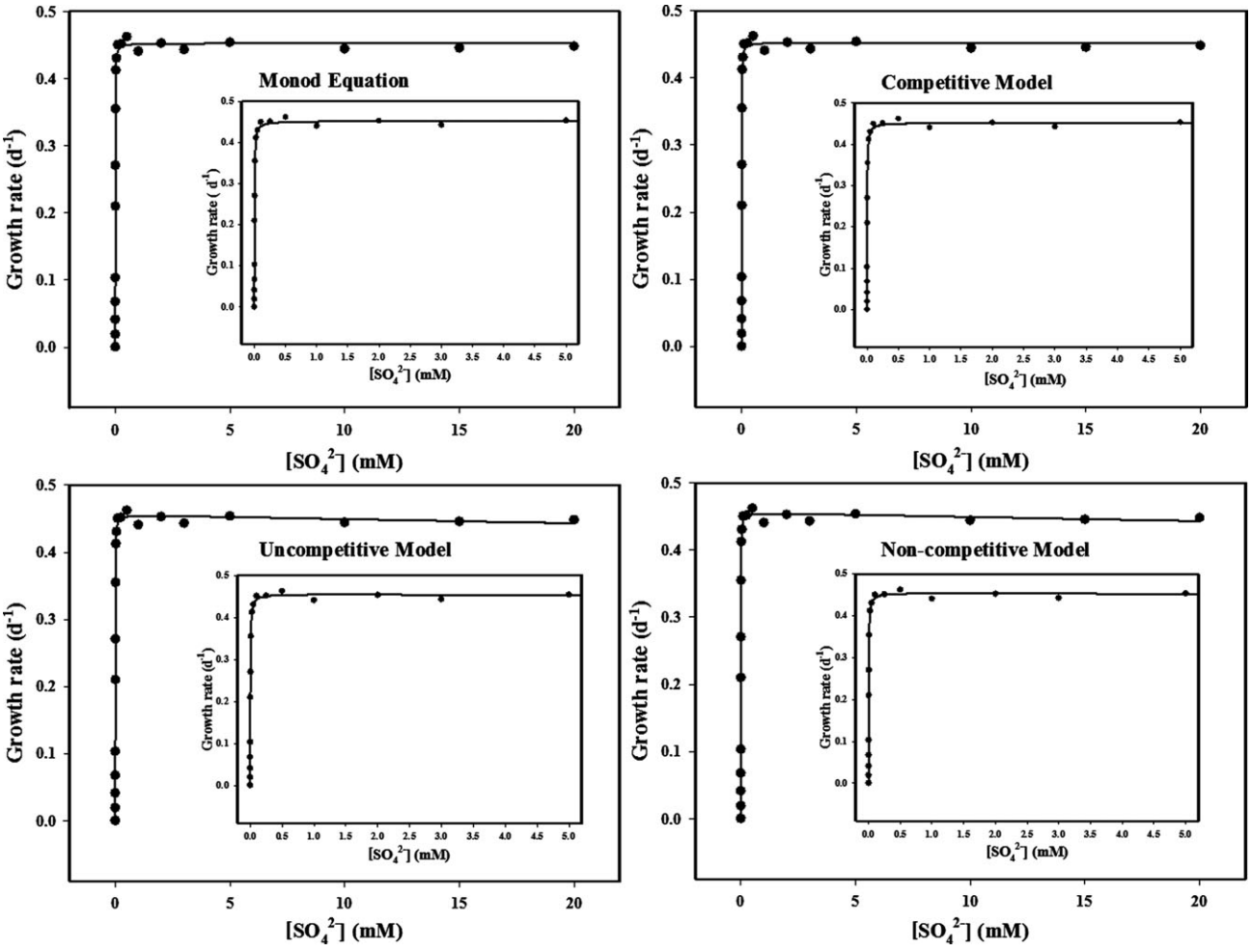

FIG. 6. Growth rate (d^{-1}) of *Chlamydomonas moewusii* cells after 4 d of exposure to different concentrations of sulfate ion at the same initial ionic strength (experimental data and model fitting). Each inner graph is an enlargement of the sulfate ion concentration from 0 to 5 mM.

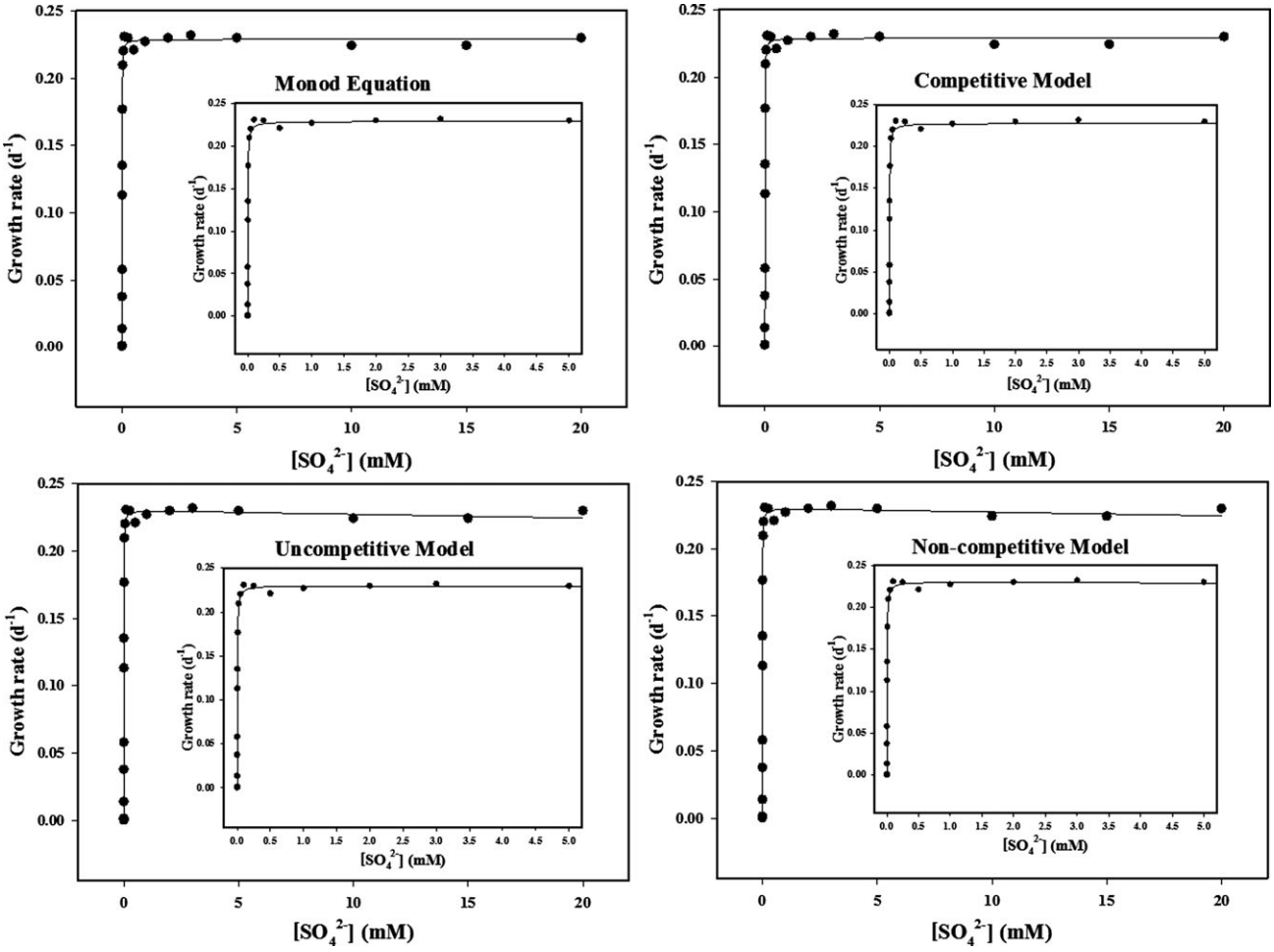

FIG. 7. Growth rate (d^{-1}) of *Chlamydomonas moewusii* cells after 11 d of exposure to different concentrations of sulfate ion at the same initial ionic strength (experimental data and model fitting). Each inner graph is an enlargement of the sulfate ion concentration from 0 to 5 mM.

TABLE 3. Kinetic parameters (± SE) of *Chlamydomonas moewusii* cells cultured with different sodium sulfate concentrations at the same initial ionic strength after 4 and 11 d.

Model	R^2	μ_{max}	μ_m	K_s	K_I	$[S]_{\mu max}$
Day 4						
Monod	0.998	0.453	–	3.00 ± 0.10	–	–
Competitive	0.998	0.447	1.709 ± 1.727E+06	11.30 ± 1.145E+07	0.004 ± 1.487E+03	–
Uncompetitive	0.998	0.454	0.456 ± 0.003	3.10 ± 0.10	698.698 ± 378.071	1.472
Noncompetitive	0.998	0.454	0.456 ± 0.003	3.10 ± 0.10	698.695 ± 378.071	1.472
Day 11						
Monod	0.996	0.229	–	2.90 ± 0.20	–	–
Competitive	0.996	0.254	1.288 ± 6.088E+05	16.30 ± 7.701E+06	0.004 ± 3.597E+02	–
Uncompetitive	0.996	0.229	0.230 ± 0.002	3.00 ± 0.20	766.110 ± 656.553	1.516
Noncompetitive	0.996	0.229	0.230 ± 0.002	3.00 ± 0.20	766.107 ± 656.553	1.516

R^2 = determination coefficient, μ_{max} = maximum growth rate (d^{-1}), μ_m = specific growth constant (d^{-1}), K_S = half-saturation constant (μM), K_I = inhibition constant (mM) and $[S]_{\mu max}$ = sodium sulfate concentration (mM) corresponding to μ_{max}.

Moreover, this microalga, prior to conducting the experiments, was cultured for 21 d in BBM medium without sulfur (renewed every week) in order to effectively remobilize its S-reserves and thus be able to study with better clarity the effect of sulfate as a sulfur source for this microorganism. During this time, cultures remained viable (99.7 ± 0.2% by fluorescein diacetate test, data not shown). When the sulfate levels were restored during the experiments, the growth of this microalga increased and its final amount of biomass was proportional to the concentration of sodium sulfate until the optimal value was reached (Fig. 1). Obviously, as with other macronutrients, sulfate should be taken into

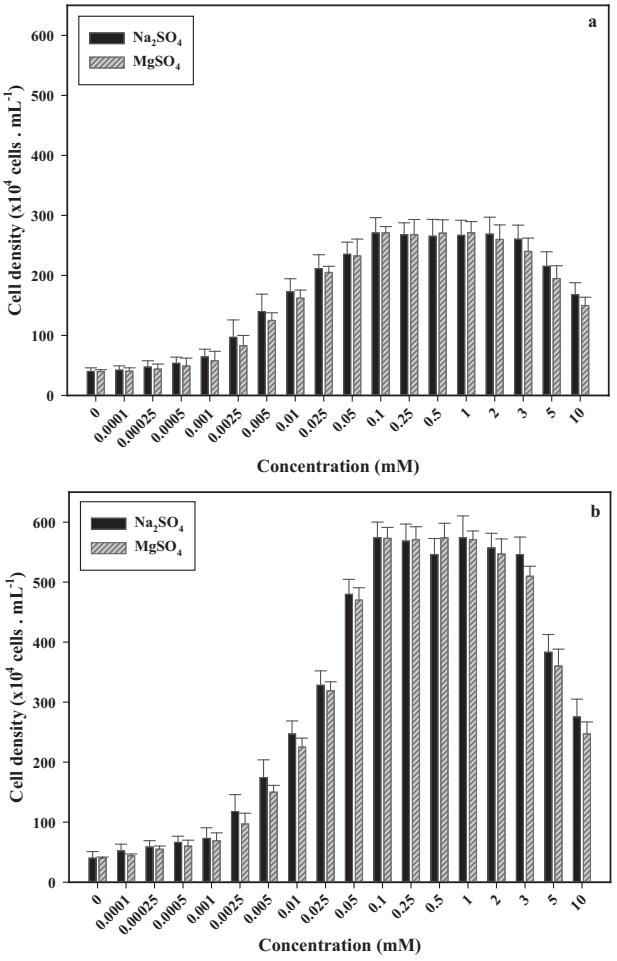

FIG. 8. Comparison of cell densities of *Chlamydomonas moewusii* after 4 (a) and 11 (b) d of culture with sodium sulfate and with magnesium sulfate. Each point represents mean ± SE (n = 3). The x-axis is not to scale.

consideration to optimize a culture medium and achieve a high productivity. In fact, it has been shown that microalgae are very sensitive to low levels of sulfur in the environment, exhibiting rapid acclimation responses that can be observed at different cellular levels, even with modifications in cellular structures and proteins with fewer sulfur-containing amino acids (Shibagaki and Grossman 2008, González-Ballester et al. 2010). The sulfate limitation in the marine microalga *Emiliana huxleyi* also affected the expression of 1,718 genes (Bochenek et al. 2013). An adaptive response of microalgae to sulfur-deprivation was their ability to reduce the photosystem II activity in the presence of light, this fact was correlated with a decline of both Rubisco protein and chl *a*/*b*-binding proteins, indicating that both light and dark reactions of photosynthesis were affected by sulfur deficiency (Wykoff et al. 1998, Giordano et al. 2005). Moreover, it has been reported that when the microalga *Chlorella sorokiniana* was subjected to sulfate deficiency conditions, the cells exhibited a series of responses relationship with sulfur metabolism, which included a decrease in the growth rate, a reduction in the photosynthetic O_2 evolution, an increase in starch content and free amino acids (Di Martino Rigano et al. 2000), and a decrease in the Cys and GSH intracellular concentrations (Carfagna et al. 2011). However, unlike what happens in nitrogen-deficient cultures, in which a reduction in chl content per cell was observed (Siaut et al. 2011), that decline did not occur in sulfur-deficient cultures, in which the amount of chl per cell remained constant (Fig. 4). Perhaps because the chl molecule does not contain sulfur and therefore, there would be no limitation for its biosynthesis despite the low bioavailability of sulfate.

Another response caused by sulfur deficiency was obtained by Mosulén et al. (Mosulén et al. 2003), these authors observed in cells of *C. reinhardtii* submitted to S-starvation an increase in the sulfate uptake rate, in the enzyme activities involved in sulfate assimilation and in the biosynthesis of cysteine. In fact, sulfate uptake is highly regulated in plants (Hawkesford and Wray 2000, Davies and Grossman 2004). The first step in sulfate utilization involves its transport into the cell and is well characterized at the physiological and functional level (Saito 2000, Hawkesford 2003, Buchner et al. 2004).

Therefore, the above results support the importance of sulfate in the microalgal nutrition and the need to properly optimize this nutrient. In the present work with *C. moewusii*, an optimal range of sulfate concentrations was obtained, 0.1–3 mM. Jo et al. (2006) optimized the growth of the microalga *C. reinhardtii* and obtained a value of 0.79 mM of sulfate in TAP medium for photoheterotrophic growth. A later review of the mineral supplement for *C. reinhardtii* showed that a decrease in the sulfate content up to 0.4 mM did not cause symptoms of deficiency (Kropat et al. 2011). However, in the present work with *C. moewusii*, with only a concentration of 0.1 mM, an optimal biomass production was reached. Moreover, it should be noted that above the concentration of 3 mM sodium sulfate, the opposite effect was observed, this salt generated a negative effect (Fig. 1). In very few works with nutrients is considered an effect of this type because the growth rate data are usually modeled with monotonic kinetics when non-monotonic models would provide more accurate results from the physiological point of view. In fact, the Monod equation has been used to describe saturation kinetics for nutrient-limited phytoplankton growth in many field and laboratory studies, and not without criticism (Morel 1987, Flynn 2003, 2005). Monod-type models are often incorporated into growth simulations where complexity must be kept to a minimum; however, the data obtained in the present work show that the growth rate of these microalgal cells in relation with the sodium sulfate

concentration was governed by uncompetitive or noncompetitive inhibition models (Figs. 2 and 3). In a Monod model, the growth rate is related to the concentration of a single growth-limiting substrate, microorganisms grow at the maximum possible rate under the limitation of noninhibitory substrates. However, this model becomes unsatisfactory for explaining inhibition of growth of microorganisms at higher substrate concentrations. As shown in Figures 2 and 3, a rectangular hyperbola was not suitable to describe the change from sulfate limitation to inhibition. Concerning inhibition due to negative effects of excess of substrate, inhibition models are more useful and provide a better fit to experimental data. In fact, the experimental data for sodium sulfate obtained with this microalga were fitted very well to this type of models. With *C. moewusii* and with the conditions used in the experiments, the mean value of the kinetic constants obtained in relation with sodium sulfate were, $K_s = 3.7$ μM and $K_I = 25$ mM (with slight variations depending on the time of culture).

Half-saturation constant (K_s) is a measure of the ability of a cell to use low concentrations of a certain nutrient. This constant is regarded as the affinity of microalgae in terms of nutrients (Smayda 1997). Although the sulfate utilization strategies may vary among microalgal species, the obtained data with this microalga may be fairly representative of freshwater microalgae. In these environments, the bioavailability of sulfate usually is not very restrictive, which would explain the value of K_s (3.2 μM) and the value of the ratio $\mu_{max}/K_s = 218$ (day 4 of culture). Some K_s values obtained, but for nitrate and phosphate, were 0.98–2.26 and 1.03–0.67 μM for *C. gracilis* (Sanem Sunlu et al. 2010), 1.4 and 0.73 μM for *Dunaliella tertiolecta*, or 29 and 0.18–0.31 μM for *Peridinium cinctum* (Smayda 1997).

On the other hand, the inhibitory effect can be quantified in terms of the inhibition constant (K_I). Low values of K_I show that this inhibitory effect can be observed at low concentrations of a compound. In fact, when K_I is very high, the inhibition models simplifies to the Monod equation. In the present work, *C. moewusii* showed a K_I value for sodium sulfate of 25 mM. This value indicates that the severe inhibitory effect could be only observed in high concentrations of this salt. It is rare to find these concentrations in culture media or in freshwater environments (even in eutrophic environments). However, it is necessary to note that the growth inhibition already occurred with only exceed the optimal values (>3 mM), resulting in a decrease in the final amount of biomass of this microalga. This result is interesting because most of culture media supply nutrients vastly in excess of the concentrations normally found in order to support high-biomass cultures, but this procedure can lead to the opposite effect. Even from an environmental point of view, in recent times, a large fraction of sodium sulfate comes from discharges of wastewater, acid mine drainages, salt water intrusions, or fertilizers, increasing the concentration of this salt in freshwater environments. This increase leads to negative consequences for these ecosystems due to the reduction in the growth of phytoplankton. However, in eutrophic environments may have certain advantages in preventing the overgrowth of these organisms, which would occur if the sodium sulfate concentration was lower.

At this point, it is necessary to answer the following question, is the sulfate anion by itself responsible for this inhibitory effect or is a consequence of the increase in salt (sodium sulfate) that alters the osmotic and ionic characteristics of the culture? High sulfate concentrations may have effect on the bioavailability of certain essential elements acquired as oxyanions, which are structurally similar to sulfate and therefore compete for the same uptake sites. Selenite (Raven et al. 1999) and molybdate (Marino et al. 2003) are examples of such competitive interactions with sulfate. In fact, these anions compete with sulfate for uptake, via common transporters, and when sulfate is in excess, the ability of the organisms to acquire these anions can be reduced (Ramaiah and Shanmugasundaram 1962, Tweedie and Segel 1970). This mechanism could explain the inhibition model observed in this work. However, there is another possible explanation, this same inhibitory response that the obtained with *C. moewusii* was also found in studies with the aquatic moss, *Fontinalis antipyretica*, when it was exposed to high sulfate concentrations (up to 1500 $mg \cdot L^{-1}$), resulting in significant reductions in several biological parameters (Davies 2007). The negative effects of this compound seemed to be due to the creation of an unsustainable osmotic imbalance between the aquatic organism and its surrounding environment. In the case of the microalga *C. moewusii,* such effects were already observed when the sodium sulfate concentration in the medium was higher than 3 mM (Fig. 1). In order to demonstrate which of the two mechanisms acted in *C. moewusii*, an experiment with different concentrations of sulfate, but at the same initial ionic strength, was performed. Ionic strength is considered the "effective salt content" of a solution. It can be seen in Figure 5 that with the same initial ionic strength, the concentrations higher than the optimum not generated toxicity, even in the range of concentrations from 5 to 20 mM, where toxicity was observed when the ionic strength was not adjusted (Figs. 1 and 2). Table 4 shows the initial ionic strength calculated with Visual MINTEQ for cultures of *C. moewusii* with different concentrations of sodium sulfate. The reduction in growth was obtained with only reach an ionic strength of 21.7 mM. Therefore, the elevation of the ionic strength of the culture medium due to the excess of sodium sulfate was responsible for the observed

TABLE 4. Initial ionic strength of the cultures with different concentration of sodium sulfate and its effect on the growth of *Chlamydomonas moewusii*.

$[Na_2SO_4]$ (mM)	Initial ionic strength (mM)	Effect on growth
0	7.51	D
0.0001	7.51	D
0.00025	7.51	D
0.0005	7.51	D
0.001	7.52	D
0.0025	7.52	D
0.005	7.53	D
0.01	7.54	D
0.025	7.58	D
0.05	7.65	D
0.1	7.80	O
0.25	8.22	O
0.5	8.94	O
1	10.40	O
2	13.20	O
3	16.10	O
5	21.70	I
10	35.80	I
15	49.60	I
20	63.20	I
25	76.60	I
50	141.30	I
100	260.60	I
150	368.70	I
200	467.20	I
250	557.20	I
300	639.90	I

D, deficiency; O, optimum; I, inhibition.

TABLE 5. Initial ionic strength and sulfate concentration in the main media used for algae culture.

Algal culture medium	Ionic strength (mM)	$[SO_4^{2-}]$ (mM)
Half strength Chu 10 Medium (HC10)	77.20	5.07
Modified Allen Medium	22.90	0.15
Blue–Green Medium (BG-11)	20.20	0.31
Acidified Bold's Basal Medium (ABB)	19.20	2.20
TAP Medium	12.70	0.41
Modified Chu 13 Medium	9.89	0.81
Bold's Basal Medium (BBM)	8.19	0.38
Z8 Medium	7.78	0.12
C Medium, Modified	4.33	0.16
Modified AF-6 Medium	2.78	0.12
COMBO	2.59	0.15
Jaworski's Medium (JM)	2.52	0.20
Diatom Medium (DM)	1.10	0.10

inhibitory effect. Although sulfate was not directly responsible for this inhibition, at least up to a concentration of 20 mM, the obtained inhibition models are still appropriate for modeling the microalgal growth relative to sodium sulfate.

The observed increase in the amount of chl per cell obtained in the cultures with sodium sulfate concentrations higher than the optimal range (Fig. 4) is a further evidence that the inhibitory effect was due to the increase in the ionic strength. Thus, it is known that the microalga *Dunaliella* responds to high salinity by enhancement of photosynthetic CO_2 assimilation (Liska et al. 2004), this response could also lead to an increase in the content of photosynthetic pigments (as observed in *C. moewusii*), all this in order to increase the biosynthesis of organic osmolytes, which can be used to tolerate this situation of osmotic stress(Meijer et al. 2001, Gustavs et al. 2010).

Finally, although an excess of sulfate ion not caused a direct toxicity, it did not generated any apparent benefit to the cultures in terms of biomass production. In fact, Bohutskyi et al. (2014) found that ~89% of added sulfur remained in the culture medium after allowing the growth of the microalga *A. protothecoides*. Moreover, if the resulting ionic strength is not adequately evaluated in a culture media, any nutrient/nutrients excess (in this case was sodium sulfate) to achieve higher biomass production would have negative effects. Therefore, ionic strength is an important parameter to be considered for the culture of freshwater microalgae. Taking as reference the results obtained with *C. moewusii* and as can be seen in Table 5, some typical recipes use sulfate in excess and/or an excessive ionic strength to achieve an optimal growth. Although each freshwater microalga may have its optimal range of ionic strength and needs of sulfur, the data obtained in this study with this microalga indicate that it is necessary to pay more attention to these two parameters when the objective is to optimize the growth of a microalga for certain purposes.

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