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# Magnesium Uptake by the Green Microalga *Chlorella vulgaris* in Batch Cultures

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The accumulation (internal and superficial distribution) of magnesium ions ($Mg^{2+}$) by the green freshwater microalga *Chlorella vulgaris* (*C. vulgaris*) was investigated under autotrophic culture in a stirred photobioreactor. The concentrations of the three forms of $Mg^{2+}$ (dissolved, extracellular, and intracellular) were determined with atomic absorption spectroscopy during the course of *C. vulgaris* growth. The proportions of adsorbed (extracellular) and absorbed (intracellular) $Mg^{2+}$ were quantified. The concentration of the most important pigment in algal cells, chlorophyll *a*, increased over time in proportion to the increase in the biomass concentration, indicating a constant chlorophyll/biomass ratio during the linear growth phase. The mean-average rate of $Mg^{2+}$ uptake by *C. vulgaris* grown in a culture medium starting with 16 mg/l of $Mg^{2+}$ concentration was measured. A clear relationship between the biomass concentration and the proportion of the $Mg^{2+}$ removal from the medium was observed. Of the total $Mg^{2+}$ present in the culture medium, 18% was adsorbed on the cell wall and 51% was absorbed by the biomass by the end of the experiment (765 h). Overall, 69% of the initial $Mg^{2+}$ were found to be removed from the medium. This study supported the kinetic model based on a reversible first-order reaction for $Mg^{2+}$ bioaccumulation in *C. vulgaris*, which was consistent with the experimental data.

**Keywords:** *Chlorella vulgaris*, metal ions uptake, adsorbed $Mg^{2+}$ (extracellular), absorbed $Mg^{2+}$ (intracellular), photobioreactor

## Introduction

Different culture systems can be used to grow microalgae. The photobioreactors used for this purpose can be open or closed. The open systems [9] tend to be less costly, but the closed systems allow better control of the culture conditions [10]. For the present study, the BIOSTAT Bplus stirred cylindrical photobioreactor, with additional LED light fitted to its exterior, was used.

Microalgae have received much attention in recent years because of their potential for metal removal [15]. The biosorption of metal ions depends on the specific surface properties of the biomass, the concentration of these ions, and the physicochemical parameters of the solution (temperature, pH, *etc.*) [12, 23, 27].

The metal ion removal by microorganisms has been suggested to consist of two stages. The first stage seems to be a rapid physical and/or chemical adsorption to the extracellular cell wall in contact with the metal ions in solution. The second stage consists of an absorption into the cell that is related to metabolic activity. Each step has its own kinetics. As a result, the metal ions could exist in three forms in the system; namely, free in solution, adsorbed extracellularly, and absorbed intracellularly [5, 13].

Magnesium, unlike toxic heavy metals, is essential for the growth and development of microalgae; it occupies a central position in the chlorophyll molecule and influences the activity of the photosynthetic enzymes. In addition, magnesium acts with other chlorophyll compounds as antennae to capture the electrons (light energy) necessary

for photosynthesis reactions [2]. This metal ion also influences other enzymes responsible for different cell activities [22]. Additionally, the chlorophyll content of the microalgae depends on the growth conditions such as temperature [24], light intensity [11], and $CO_2$ [4]. Accordingly, magnesium involves a crucial interest for the cells, and its behavior is then interesting to investigate. Furthermore, a biomass enriched in magnesium could have potential cosmetic applications.

Consequently, this work intended to study the bioaccumulation of magnesium in *Chlorella vulgaris* in order to understand the different processes in action and produce an enriched biomass for cosmetic tests. The removal of $Mg^{2+}$ from the culture medium by *C. vulgaris* was investigated in a batch photobioreactor. The adsorbed and absorbed $Mg^{2+}$ concentrations by biomass were measured during the culture process. In addition, the model of bioaccumulation of this metal previously published [1] was confirmed by these new experimental data.

## Materials and Methods

### Strain and Growth Conditions

The strain *Chlorella vulgaris* CCAP211/e 11B (Trebouxiophycae) was obtained from the Culture Collection of Algae and Protozoa (CCAP, UK). The medium used was the modified 3N-Bristol medium (MBM) [4, 26]. The basic $Mg^{2+}$ concentration of this medium is theoretically 7.4 mg/l, but its concentration was always measured precisely prior to experiments.

The strain was maintained in liquid culture (50 ml in a 250 ml flask), at 25°C, in a lit incubator under continuous orbital agitation (50 cm rotation diameter; 100 rpm, light intensity at the surface of the culture of 20 µmol/$m^2$/s, and air enriched with 0.7–2.1% (v/v) of $CO_2$). The pre-culture for each experiment was prepared by inoculation in a fresh medium at 1% (v culture/v medium) and incubation under the above conditions for 48–72 h. The flasks of pre-cultures were stoppered with foam bungs (11901935-X100; Fisher Scientific, France) to allow sterile gaseous exchanges between the culture and the atmosphere.

### Stirred Photobioreactor Batch Culture Conditions

A baffled BIOSTAT Bplus bioreactor (5 L working volume; Sartorius, Germany) was used; it was equipped with 3-bladed segment impellers (UniVessel 5l, Germany), each inclined at 45° from the horizontal axis. The planar diameter of the impellers was 65 mm. The lower impeller was at the bottom of the rotating axis, just above the sparger ring. A rotation of 750 rpm was used in order to obtain a homogeneous culture. The temperature was controlled at 25°C with the aid of an external water jacket. Continuous aeration with sterile air (0.2 µm Sartorius gas filter; Germany) at 500 ml air/min (1 atm, 25°C) was used. This meant that the culture was fed with the atmospheric concentration of $CO_2$ (0.035 % (v/v)), approximately equivalent to $8.6 \times 10^{-5}$ g C per minute. The airflow rates into and out of the bioreactor were measured daily. The culture was continuously illuminated from the outside by six LED lamps (Ledare 130 lumen, 2700 Kelvin, 27° dispersion angle; IKEA, France). The total light intensity at the inner surface of the bioreactor was measured at 2,900 µmol/$m^2$/s. This measurement was performed when the bioreactor was empty. All light intensities were measured with a photometer (LI250A; LI-COR, USA).

The culture pH was not controlled with the addition of chemicals, but the pH reading from the internal probe (EF-Set 12/325/2 K8-HM-UniVessel 5/10l; Hamilton, Switzerland) was recorded and verified daily against a freshly calibrated bench-top pH meter (Consort C 864; Fischer Bioblock Scientific, France). Both pH meters were calibrated in the range of 4.01–7.00 at the beginning of the experiment, and the bench-top pH meter was calibrated in the range of 7.00–9.21 towards the end of the experiment. A polarographic oxygen probe (12/325/2-l-HM-Univessel 5-10l; Hamilton) was used to monitor the dissolved oxygen concentration in the culture. This was set to indicate 100% just before inoculation of the continuously stirred and aerated medium, with 100% indicating equilibrium with air; the scale 0 to 100% referred to the degree of equilibrium reached, and was directly related to the concentration of oxygen dissolved in the liquid. The inoculation of the bioreactor with *C. vulgaris* was carried out with 1% of pre-culture prepared as described above, which corresponded to $1.6 \times 10^5$ cells/ml.

### Analytical Methods

**Growth parameters.** The cell concentrations were determined by microscopy (Axioplan Imaging 2; Carl Zeiss, Germany) using a Thomas counting chamber of 0.1 mm depth and were expressed in cells/ml. The culture was diluted with deionized water to yield a final cell concentration of 200–300 cells over the entire volume of the counting chamber consisting of 256 cubes of 0.1 $mm^3$. The cells present in all of the 256 "cubes" were counted, and the cell concentration was calculated using the dilution factor.

Absorbance measurements were also used in order to follow growth. The absorbance ($A_{800}$) of the algal cultures was measured (Spectrophotometer Cary 300 Scan UV-visible; Varian Inc., Netherlands) at 800 nm after dilution; this quantity is known to be proportional to the cell concentration.

The dry weight concentration of the biomass DW (g/l) was determined by subjecting 10 ml of culture to centrifugation (10 min, 1,800 ×*g*). The pellet was washed through resuspension in an equal volume of deionized water, subjected to centrifugation again (10 min, 1,800 ×*g*), and the final pellet was transferred into a dry pre-weight cup. The pellet was dried for 24 h at 105°C and cooled in a desiccator containing dry silica gel prior to weighing.

The cell viability was obtained using a GUAVA easyCyte flow cytometer (Merck Millipore, France) with the ViaCount (14-0155) method, and the cell size was measured using a Mastersizer laser

granulometer (Malvern, France).

Daily samples were generally removed from the bioreactor, except during the exponential growth phase (0–70 h), in which two samples were analyzed daily. The specific growth rate (μ) was determined using the absorbance data. A semi-log relationship (ln absorbance vs. time) was used to determine the μ value during the exponential phase (0 to 70 h; 6 points and $R^2$ of above 0.948).

**Measurement of chlorophyll concentration.** In order to determine the chlorophyll *a* concentration, a modified extraction method of Porra [20] was used. Three samples (in triplicate) per day were removed from the bioreactor and subjected to centrifugation (5 min; 6,400 ×*g*). An aqueous solution of 85% methanol containing 1.5 mmol/l of sodium dithionite was added to the pellet. The samples were extracted at 40°C for 32 min and then recentrifuged (5 min; 6,400 ×*g*). The absorbance of the supernatant was measured at 664 and 650 nm (Spectrophotometer Cary 300 Scan UV-visible). The chlorophyll *a* content was calculated according to Porra's formula [20].

**Estimation of nitrate ($NO_3^-$) concentration.** Samples were first filtered through C18 and 0.2 μm filters prior to $NO_3^-$ analyses in order to retain all proteins and macromolecules that could interfere with the analysis.

The $NO_3^-$ analyses were carried out with ionic chromatography using a Dionex AS11-HC column. The run conditions were as follows: system Dionex DX-500, conductimetric detector CD20, column (CR-ATC, P/N 060477; Fisher Sientific, France) (4 mm) with a guard column AG11-HC. The eluent was KOH (30 mmol/l at 1.5 ml/min). This eluent concentration was lower than the one recommended by the column manufacturer, leading to slightly higher retention times than those that would have been expected. The total pressure applied was approx. 2,300 psi at 30°C. Each injection was made with a sampling loop of 20 μl. Chromatograms were recorded with the Peaknet software (Dionex, France) on a Pentium PC. The system was installed in a Class 10,000 clean room, and all samples were handled under a Class 100 laminar flow hood.

**Measurement of magnesium ion concentration.** In order to measure the $Mg^{2+}$ concentration, an atomic absorption spectrophotometer (AAS) (Z-2300; Hitachi, Japan) was used: air-acetylene flame; measurement of the absorbance was performed at 202.6 nm with the appropriate magnesium lamp (Agilent Technologies, France). The standard deviations for the calibration scale (0.15 to 20.0 mg/l) were in the range of 1% to 2%.

The full set of glassware was soaked for at least 24 h in $HNO_3$ (10% (w/v); Merck Suprapur, France) and thoroughly rinsed with ultrapure water (Milli-Q; Millipore, France) before use, and all samples were diluted with acidified (less than pH 3 adjusted with hydrochloridric acid) Milli-Q water to prevent metal precipitation. Each acidified calibration solution was prepared from a 1,000 mg/l magnesium stock solution (Fisher Scientific, France) and measured five times in order to acquire the precision and the accuracy of the analysis.

**Determination of the intracellular and extracellular magnesium concentrations.** The concentrations of extra- and intracellular magnesium were estimated using adapted published methods [6, 14], with minor modifications as published in our previous work [1].

$Mg^{2+}$ uptake by algae between times 0 and t, Q (mg), was calculated as follows:

$$Q = (C_0 - C)\,V \tag{1}$$

and compared with the initial magnesium mass in the liquid: $m_0$ (mg) = $V\,C_0$. The removal efficiency was then expressed by $Q / m_0$.

In the previous equation, $C_0$ and C are the magnesium concentrations in the algal culture at times 0 and t, expressed in mg/l, and V is the total volume of culture expressed in l (assumed to be constant throughout the experiment).

**Model for Magnesium Uptake**

The model for $Mg^{2+}$ uptake by algae in a photobioreactor culture assumed a reversible reaction of first order with respect to the magnesium concentration in the liquid. This model has been already published with all details [1]. Accordingly, the model is represented by the following equation:

$$\frac{C(t)-C_{eq}}{C_0-C_{eq}} = e^{-kat} \tag{2}$$

where $C_{eq}$ is the $Mg^{2+}$ ion concentration at equilibrium and ka the kinetic constant.

## Results and Discussion

### Algal Growth and Nutrient Consumption in a Stirred Photobioreactor

Two independent experiments were performed in the photobioreactor, each lasting 32 days (765 h). The initial $Mg^{2+}$ concentration was measured at 16 mg/l in the inoculated Bristol medium. The cell concentrations in g/l and cells/ml as well as the absorbance of the culture at 800 nm ($A_{800}$) were measured for each sample (Fig. 1).

Fig. 1 shows a proportionality with a good accuracy between growth parameters, absorbance, cell concentration, and dry biomass weight concentration, as previously reported by Rocha *et al*. [21]. We found the following correlations: $A_{800} = 4.2 \times 10^{-8}$ N and DW (g/l) = $1.0 \times 10^{-8}$ N with N cell concentration in cells/ml. The average dry weight of one cell was then calculated and found to remain constant at $1.1 \times 10^{-11}$ g throughout the experiments, which corresponds to published values for this organism [25]. Chlorophyll *a* is the major photosynthetic pigment in *C. vulgaris* cells [2], and its concentration in the culture increased in step with the increase in the biomass, indicating a constant chlorophyll/cell ratio throughout the

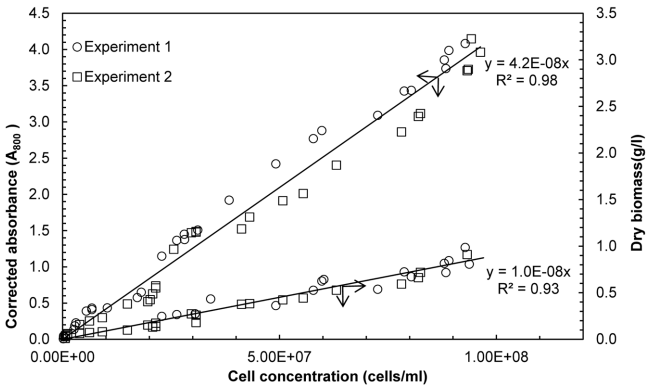


**Fig. 1.** Relationship between different biomass measurements: cell number concentration (cells/ml), corrected absorbance, and dry-weight biomass concentration (g DW/l).

The corrected absorbance ($A_{800}$) was calculated by taking into account the dilution factor.

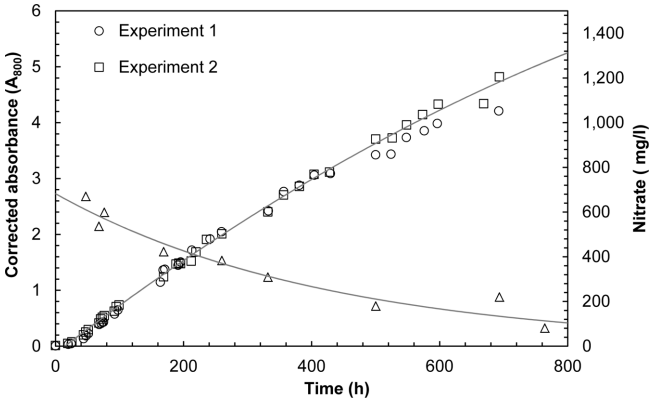


**Fig. 3.** Growth curve of *C. vulgaris* grown in a stirred photobioreactor and consumption of nitrate during the cultivation.

Absorbance measured at 800 nm of wavelength. The nitrate concentration in the medium was measured with ionic chromatography during the experiment.

experiment (Fig. 2). The constant ratio of (chlorophyll *a)*/(g DW) suggests the possibility that in autorotrophic cultures, the chlorophyll concentration could be used as an indication of the photosynthetic biomass [18]. Further studies are required to confirm these results.

The microalgal culture grew exponentially for the first 70 h. This phase was then followed by an apparently linear growth phase that lasted until the end of the experiment (765 h). During the entire experiment, the viability remained at 96%, and the average cell size was constant at 4.4 μm diameter. The concentration of nitrate, the sole nitrogen source, decreased with the increasing biomass, but this nutrient was never completely exhausted (Fig. 3).

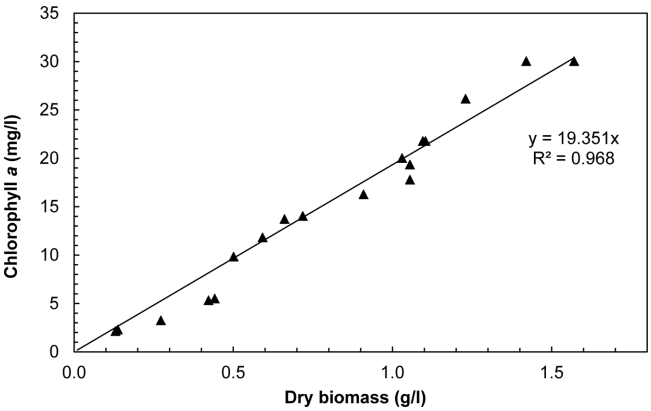


**Fig. 2.** Relationship between chlorophyll content and dry biomass during the cultivation.

The chlorophyll *a* was extracted in triplicate samples during the experiment (765 h); the results presented here are the average of the three values.

*C. vulgaris* consumed nitrate ions available in the medium during the autotrophic growth phase; however, it was not completely consumed even by the end of the experiment.

The maximum specific growth rate (μ) was determined from the absorbance data during the exponential growth phase, at 0.060 $h^{-1}$ (Fig. 3), according to a published method [26]. This value of μ was relatively higher than that (0.041 $h^{-1}$) found by Clément-Larosière *et al.* [4] for *C. vulgaris* grown in a batch bubble-column photobioreactor, but was close to that reported by Perner Nochta *et al.* [19] at 0.058 $h^{-1}$ for the same strain in a batch tubular bioreactor equipped with helical static mixers and plates. The cell concentration reached 2 × $10^7$ cell/ml by the end of the exponential growth phase and 1 × $10^8$ cell/ml by the end of the experiment (765 h). No stationary phase was observed in either experiment.

The linear growth phase described in this study was considerably longer than previously reported [4, 16, 17] and could be related to the specific growth conditions used here, the bioreactor geometry, and the mode and intensity of illumination. Under the conditions used in this study, light limitation is strongly suspected.

The pH change was followed during *C. vulgaris* growth (Fig. 4). The pH increased initially from 6 to 8 during the exponential growth phase. After exponential growth (70 h) and during the linear growth phase, the pH remained constant at 8.5 and increased slightly towards a value of 9. The initial increase in pH has been reported [3] to be linked to a decrease in the $CO_2$ concentration in the medium, although it could also be linked to changes in the ionic

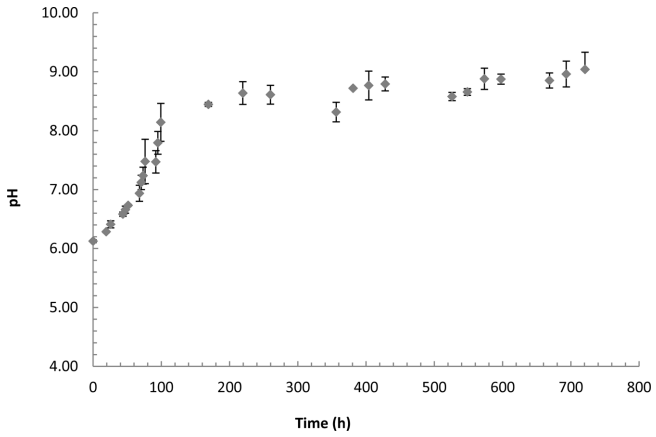


**Fig. 4.** Kinetic profile of pH during *C. vulgaris* culture.
Each pH value represents the mean of the pH measurement performed in both experiments.

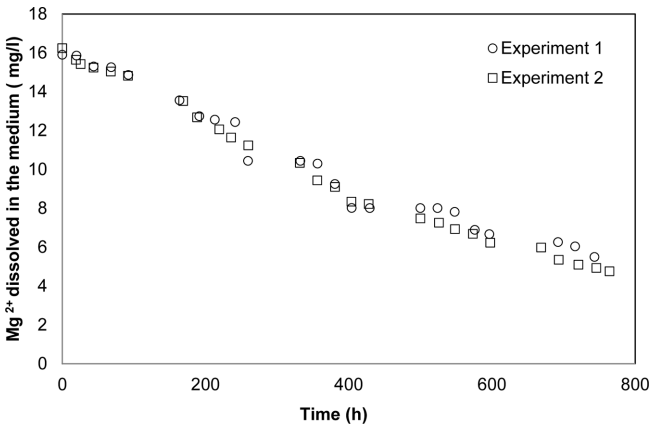


**Fig. 5.** Kinetic profile of dissolved $Mg^{2+}$ in the growth medium.
Dissolved $Mg^{2+}$ concentration in the medium measured daily during the experiment with AAS and plotted versus time of culture.

concentration of other medium components. With a decrease in the photosynthetic activity during the linear growth phase, a balance between $CO_2$ dissolution and utilization must have been achieved that could suggest an explanation for the stability of the pH during this growth phase.

In one experiment, the dissolved-oxygen concentration was followed with a dissolved-oxygen ($dO_2$) electrode. The $dO_2$ concentration was constant at 100% until 90 h (corresponding approximately to the exponential phase) and then decreased to 60% by the end of the experiment. This indicates a consumption of $O_2$ from 90 h onwards, probably due to respiration occurring as photosynthesis decreased. This observation could suggest light limitation of the cultures.

This hypothesis of light limitation was tested by the addition of 50% more LED light. A slight increase in $dO_2$ was observed (data not shown), supporting the hypothesis that growth was limited by light. The extra source of light was removed after 10 min, upon which the $dO_2$ trace returned to its original position. This observation strengthens the case in favor of light limitation from 90 h onwards under the conditions used in this experiment; however, other supporting data would be necessary to prove this definitively.

With respect to carbon, it is more difficult to state whether it was limiting growth or not. Gaseous $CO_2$ was continuously supplied into the reactor at a rate of $8.6 \times 10^{-5}$ g C/min, giving a total calculated input of 3.93 g C during the experiment (765 h). Assuming that the biomass contains 50% C by weight, it is possible to calculate the mass of carbon absorbed by the cells over the experiment; with the final DW concentration being 1 g/l and the volume 5 L, the corresponding carbon mass was then 2.5 g. The necessary amount of carbon for *C. vulgaris* growth was accordingly $5.45 \times 10^{-5}$ g of C/min. The carbon supply into the bioreactor was higher than necessary. At first glance, it could seem improbable that the culture was limited in carbon, but the limitation strongly depends on the transfer rate of $CO_2$ from gas to liquid, which is difficult to measure in the absence of a dissolved $CO_2$ probe. It is not possible to conclude whether the culture was carbon-limited or not. Further experiments would be necessary, for example with air enriched with different concentrations of $CO_2$. All indications are that the culture was probably light (energy) limited. After light limitation was reached, the constant supply of the limiting "nutrient," energy in this case, would have resulted in a linear growth phase, as it would be under carbon limitation with a carbon supply at a constant rate.

A high degree of reproducibility was observed between the results of the duplicate experiments for their entire duration (765 h).

## Magnesium Concentrations

**Dissolved $Mg^{2+}$ concentration.** The concentration of $Mg^{2+}$ decreased during the course of the experiment but was never completely exhausted (Fig. 5). The final dissolved $Mg^{2+}$ concentration in the medium was approximately 5 mg/l at the end of the experiment.

**Extracellular (adsorbed) and intracellular (absorbed) $Mg^{2+}$ concentrations.** The dissolved (residual in the culture medium, not removed by algae) and biomass-associated

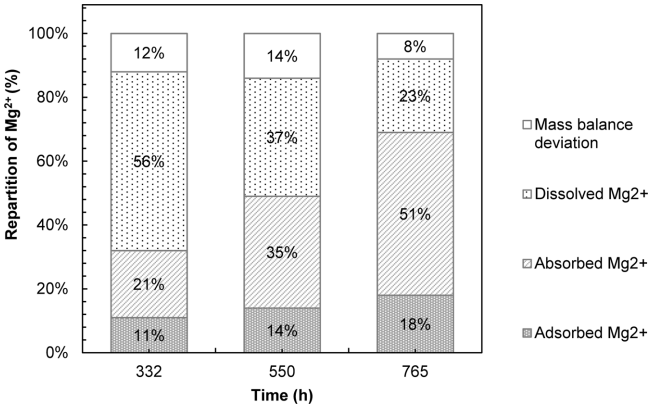


**Fig. 6.** Distribution of $Mg^{2+}$ in the culture of *C. vulgaris* during growth.

"Dissolved $Mg^{2+}$" indicates the residual quantity of these ions in the growth medium during the course of the *C. vulgaris* growth. The partition of $Mg^{2+}$ within the biomass was determined at three time points during growth. Each stack represents the mean of the analysis performed in triplicate samples (mean ± SD, $n$ = 3). The mass balance deviation refers to the amount of $Mg^{2+}$ that could not be accounted for, between the amount of $Mg^{2+}$ added at the start of the experiment and that found in sampling. The difference in the results between experiments 1 and 2 was 4%.

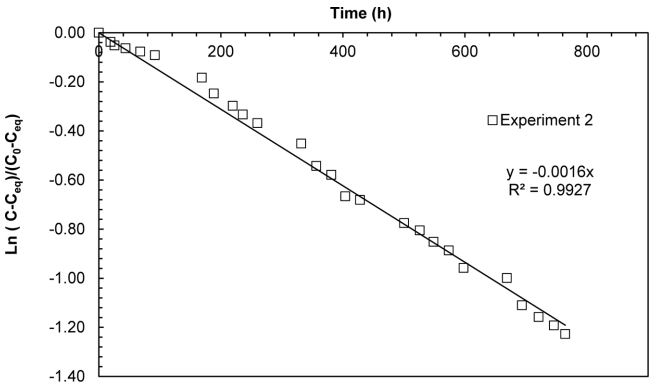


**Fig. 7.** Modeling $Mg^{2+}$ uptake by *C. vulgaris*.

Semi-logarithmic plot of the magnesium removal ratio $\frac{C(t)-Ceq}{C0-Ceq}$ versus time. At initial $Mg^{2+}$ concentration ($C_0$) of 16.0 mg/l, $C_{eq}$ is close to zero. The difference in the results between experiments 1 and 2 was 1%.

$Mg^{2+}$ concentrations (adsorbed and absorbed) were determined early (330 h), in the middle (550 h), and at the end (765 h) of the experiment. Each sample was treated in triplicate for $Mg^{2+}$ analysis, and then each measurement was performed in triplicate. The amount of $Mg^{2+}$ removed through daily sampling was taken into consideration when calculating the magnesium mass balance.

As the cells grew, the dissolved $Mg^{2+}$ concentration decreased and the biomass-associated $Mg^{2+}$ increased. The absorbed and adsorbed ion amounts increased with time and cell concentration (Fig. 6), with the absorbed (intracellular) $Mg^{2+}$ amount always being greater than the adsorbed (extracellular) one. $Mg^{2+}$ mass balances accounted for 90 ± 2.0% of the initial mass in the culture, the shortage being related to analytical imprecisions.

At the end of the experiment, 69% of $Mg^{2+}$ was associated with the microalgal biomass. Of this, 18% was adsorbed and 51% was absorbed. $Mg^{2+}$ removal from the growth medium was directly related to cell growth. $Mg^{2+}$ bound to the cell surface increased over time as $Mg^{2+}$ was absorbed into *Chlorella* cells. Adsorption was always less significant than absorption, probably due to differing kinetics. This result is in good agreement with our previous study [1].

The method first published by Franklin *et al*. [6] was found to be convenient for the measurement of $Mg^{2+}$ uptake in *C. vulgaris* and for following its profile. When the starting concentration of this ion was 16 mg/l, *C. vulgaris* was able to remove the majority of $Mg^{2+}$ from the growth medium by 765 h. A relatively high efficiency (69%) was obtained, but this was measured over a long period, representing a removal rate of 0.034 mg $Mg^{2+}$/ l/h.

### Model for $Mg^{2+}$ Uptake by *C. vulgaris*

The experiments conducted at the initial $Mg^{2+}$ concentration of 16.0 mg/l confirmed the validity of the exponential model (Fig. 7).

The modeling results are in good agreement with our previously published work [1] and with another published work [7] that presented an exponential model for the sorption (adsorption and absorption) of cadmium and copper by *Chlorococcum oleofaciens* at initial concentrations of 2.0 mg/l of each metal, and *Oocystis pusilla* at initial concentrations of 2.0, 5.0, 8.0, and 10 mg/l of cadmium.

The work presented here re-inforces the application of this model for metal ion sorption in microalgae cultures, and this modeling work will be further extended in future studies.

In the present study, we confirmed the possibility of magnesium bioaccumulation by *C. vulgaris* in a batch photobioreactor and determined the repartition of the metal ions between the cell wall and the cell interior; however, the rate of accumulation must still be improved before possible industrial applications. For this purpose, further experiments are needed in order to identify the

optimal set of conditions for a quicker accumulation. The global model previously developed [1] that describes $Mg^{2+}$ transfer from the liquid medium to the cells was confirmed with these data. In future experiments, we will apply similar modeling to different growth conditions and develop a phenomenological model describing all of the steps of the global process (external transfer, adsorption, internal transfer, *etc*.).

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