Flocculation of *Chlorella vulgaris* induced by high pH: role of magnesium and calcium and practical implications.

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pH-induced flocculation of *Chlorella*

Abstract

Microalgae hold great potential as a feedstock for biofuels or bulk protein or treatment of wastewater or flue gas. Realizing these applications will require the development of a cost-efficient harvesting technology. Here, we explore the potential of flocculation induced by high pH for harvesting *Chlorella vulgaris*. Our results demonstrate that flocculation can be induced by increasing medium pH to 11. Although both calcium and magnesium precipitated when pH was increased, only magnesium (≥ 0.15 mM) proved to be essential to induce flocculation. The costs of four different bases (sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide and sodium carbonate) were calculated and evaluated and the use of lime appeared to be the most cost-efficient. Flocculation induced by high pH is therefore a potentially useful method to preconcentrate freshwater microalgal biomass during harvesting.

Keywords:

dewatering; autoflocculation; magnesium hydroxide; microalgae; harvesting, calcium carbonate

Introduction

Because microalgae can achieve much higher areal productivities than agricultural crops, they are considered to be an attractive alternative source of biomass (Lee, 2011; Tredici, 2010; Wijffels and Barbosa, 2010). Microalgal biomass is poor in lignocellulose and rich in lipids and proteins, making it a valuable feedstock for biofuels or animal feed production (Skrede et al., 2011). By supplying inorganic nutrients through wastewater and CO_2 through flue gas, production of microalgal biomass can be coupled to wastewater treatment and CO_2 abatement (Chen et al., 2010; Feng et al., 2011; Putt et al., 2011). So far, however, the production cost of microalgae is still high and only economically feasible for high-value applications such as food supplements, natural pigments or poly-unsaturated fatty acids. To be able to use microalgae for low-value applications such as feed or fuel production or wastewater and flue gas treatment, the production cost has to be reduced by at least an order of magnitude (Fon Sing et al., 2011; Greenwell et al., 2010; Park et al., 2011).

A significant reduction in the cost of microalgal biomass production will require cost-efficient methods for harvesting microalgae (Molina Grima et al., 2003; Shelef et al., 1984). Due to the small size of microalgae (a few µm) and their low concentration in the medium (0.5 – 5 g L^{-1}), this is a major challenge. In commercial systems, microalgae are currently harvested using centrifugation. Centrifugation is, however, too expensive for low-value applications because of the high investment costs and high energy demand (Uduman et al., 2010). If the microalgae could be pre-concentrated by flocculation prior to centrifugation, the energy demand of centrifugation for final dewatering could be significantly reduced. Microalgae can easily be flocculated using metal coagulants such as alum and iron chloride. However, this requires large amounts of coagulants and results in contamination of the harvested biomass with metals (Shelef et al., 1984). Recently, we demonstrated that electro-coagulation can be used as an alternative to metal coagulants, resulting in lower metal contents in the microalgal biomass as well as in the medium (Vandamme et al., 2011). Moreover, electro-coagulation has a low energy demand, particularly when used in seawater medium. Alternatively, the natural biopolymer chitosan can be used, but the cost of this flocculant is relatively high and availability is limited (Ravi Kumar, 2000). Cationic starch can be used as a cheaper and more widely available alternative to chitosan (Vandamme et al., 2010).

Several studies have demonstrated that flocculation of microalgae can also be induced by increasing the medium pH, a phenomenon that is often referred to as ‘autoflocculation’. Golueke and Oswald (1970) observed that microalgae in waste stabilisation ponds flocculated on warm and sunny days, when CO_2 was depleted and the pH increased. Microalgal suspensions are generally stabilised by a negative surface charge of the cells which is generated by carboxyl and/or sulphate groups. The fact that flocculation of microalgae occurs at a high pH is therefore surprising, since the surface charge of microalgal cells is expected to become more negative at a high pH and flocculation is thus inhibited (Lavoie and De la Noüe, 1987). It has been suggested that flocculation at high pH is caused by chemical precipitation of calcium and/or magnesium salts at a high pH (Shelef et al.,1984). Indeed, Nurdogan and Oswald (1995) noted that autoflocculation did not occur in waters poor in calcium and magnesium. They demonstrated that flocculation in such waters could be induced by addition of lime. Sukenik and Shelef (1984), on the other hand, suggested that flocculation at high pH was caused by precipitation of calcium phosphate. High phosphate concentrations (0.1 -0.2 mM) are required for this process to be effective. Lavoie and De la Noüe (1987) could not induce flocculation of *Scenedesmus* in a medium that was low in phosphate, which supports the role of calcium phosphate.

Although it appears from these previous studies that calcium and/or magnesium play a role in flocculation of microalgae at high pH, there is still uncertainty about the general underlying mechanism. Moreover, the practical implications and the potential for reducing the cost of harvesting microalgae have not been fully explored. The goal of this study was therefore (1) to investigate the role of calcium and magnesium in the flocculation process, and (2) to evaluate the practical implications for flocculation induced by high pH for harvesting microalgae.

Materials and methods

Culturing of microalgae

We used *Chlorella vulgaris* (211-11b SAG, Germany) as a model species for investigating the mechanism of and practical implications for the use of pH induced flocculation in freshwater medium. *Chlorella vulgaris* is a promising species for the production of microalgal biomass for food, feed or fuel, and is currently intensively studied (Feng et al. 2011). *Chlorella vulgaris* as cultured in dechlorinated tap water enriched with inorganic nutrients according to the concentrations of the WC medium (Guillard and Lorenzen, 1972). Table I shows the concentrations of the main ions in this medium . The microalgae were cultured in 30 L bubble column photobioreactors that were mixed by sparging with 0.2 µm-filtered air (5 L min^{-1}). Growth of the microalgae was monitored by measuring the absorbance at 550 nm.

Flocculation experiments were conducted at a microalgal density of approximately 0.5g dry weight per liter.

General setup of flocculation experiments

Flocculation of the microalgal suspensions induced by high pH was investigated using jar test experiments (n=2). These experiments were carried out in 100 mL beakers that were stirred using a magnetic stirrer. pH was adjusted by addition of 0.5 M sodium hydroxide. The microalgal suspension was mixed intensively (1,000 rpm) for 10 min during and just after pH adjustment. Then, the suspensions were mixed gently (250 rpm) for another 20 min, after which they were allowed to settle for 30 min. The flocculation efficiency η_a was estimated by comparing absorbance at 550 nm between the pH-adjusted treatment and a control treatment. Samples (3.5 ml) were collected in the middle of the clarified zone. The flocculation efficiency η_a was calculated as:

Microalgal recovery efficiency $\eta_a = \frac{OD_i - OD_f}{OD_i}$ (Eq. 1)

where OD_i is the optical density of the suspension after 30 min sedimentation without pH adjustment, and OD_f is the optical density of the suspension after the complete treatment.

The role of magnesium and calcium

Flocculation induced by high pH was tested at different pH levels between 9 and 12. To unequivocally demonstrate the role of bivalent cations in the flocculation process, we tested if flocculation induced by high pH could be inhibited by addition of EDTA (10 mM) , a chelating agent that can sequester polyvalent cations. This was done at the three pH levels where flocculation occurred (pH 11, 11.5 and 12).

To investigate the fate of calcium and magnesium during pH increase, we monitored concentrations of these cations in the dissolved and particulate phase before and after pH induced flocculation at pH 11. Concentrations of cations in the dissolved phase were measured after removal of algal cells by centrifugation followed by filtration over Whatman GF/C glass microfibre filters. Concentrations in the particulate phase were measured in the pellet obtained during centrifugation. The pellet was first dried at 100°C for 24 h, then incinerated at 450°C for 24 h and the ashes were dissolved quantitatively in HNO_3:HCl 1:1. Calcium and magnesium concentrations were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Jobin-Yvon Ultima, Horiba Scientific) and a mass balance was constructed. In addition to these measurements, we calculated the saturation index (SI) of $Ca_3(PO4)_2$, $CaCO_3$, $CaMg(CO3)_2$, $CaSO_4$ and $Mg(OH)_2$ at different pH levels between 10 and 11.5 using PHREEQC version 2 (USGS, USA) to estimate the influence of pH on precipitation of different calcium and magnesium salts.

To further investigate the relative importance of calcium and magnesium in the flocculation process, we tested whether flocculation at high pH could be induced in a medium lacking calcium or magnesium. To do so, *Chlorella* was separated from the medium using centrifugation and resuspended in fresh medium lacking calcium and magnesium. Preliminary experiments demonstrated that concentration of *Chlorella* using centrifugation and subsequent resuspension in the original medium had no influence on flocculation induced by high pH. In a first series, *Chlorella* cells were resuspended in a medium lacking magnesium and containing different calcium concentrations ranging between 0 and 2.5 mM. In a second series, calcium was omitted and magnesium was added in concentrations ranging between 0 and 1.5 mM. The range of calcium and magnesium concentrations used encompassed the range of calcium and magnesium observed in typical surface waters (Bezonik and Arnold, 2011).

Practical implications of using flocculation induced by high pH for harvesting microalgae

The efficiency of several widely available bases (sodium hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide and sodium carbonate) was compared for flocculation induced by high pH. We gradually added a 0.5 M base solution to 100 mL beakers filled with a *Chlorella* suspension and monitored the increase in pH until coagulation could be visually observed. We estimated the ratio of base over microalgal biomass required for flocculation based on the quantity of base added and the biomass concentration of *Chlorella* in the medium.

We also investigated the influence of the pH increase on the viability of the cells. First, cell numbers were compared before and after flocculation . Flocculation tests were done by increasing the pH to 12, resulting in a flocculation efficiency of 99%. The medium was removed, the particulate phase was resuspended in destilled water, neutralized (pH 6.5) and stirred at 550 rpm for 60 min, resulting in a complete dissolution of the flocs and resuspension of the algal cells. Cell numbers were determined by counting intact cells using a Bürker count chamber. A minimum of 200 algal cells were counted in duplicate giving a counting error of maximum 5% (Andersen and Trondsen, 2003). Average cell numbers before and after flocculation and resuspension were compared using a t-test with equal variances (as checked with an F-test; Sigmaplot 11, Systat Software, Inc.). Secondly the quantum yield of photosystem II of the microalgal cells was compared before and after flocculation by pH increase (pH 10.5; 11; 12). The quantum yield was measured after 20 min of dark adaptation of the cells using a PSI AquaPEN PAM fluorometer (sample size = 3.5 ml; n = 3). The quantum yield is a sensitive indicator for stress in microalgae (Cid et al., 1995). Finally microscopical observations were made on the flocculated microalgae (pH 12) versus the untreated microalgae using light microscopy (Olympus BX 51 upright microscope + Infinity 2 digital camera, Luminera Corporation, Canada). These images are provided as supplementary data.

Results and discussion

The role of magnesium and calcium

The influence of pH on flocculation efficiency was tested in a pH range from 9 to 12 (Fig. 1 – pH treatment). No flocculation occurred up to pH 10.5. At pH 11, a flocculation efficiency of 75 % was observed. At pH 11.5 and 12, the flocculation efficiency exceeded 95 %. This indicates that *Chlorella* can be flocculated efficiently by increasing the pH of the culture to 11. This observation is in agreement with previous studies (e.g. Blanchemain et al., 1994; Yahi et al., 1994).

Several studies have suggested that bivalent cations such as calcium and magnesium play a role in the flocculation process at high pH (Nurdogan and Oswald, 1995; Shelef et al., 1984). We wanted to unequivocally confirm the role of calcium and/or magnesium in pH-induced flocculation by addition of EDTA to remove bivalent cations from solution at pH levels 10.5, 11 and 12 (Fig. 1 – pH treatment + EDTA). Addition of EDTA resulted in a strong and significant decrease in the flocculation efficiency η_a at the three pH levels tested, confirming that bivalent cations such as calcium and/or magnesium are indeed involved in flocculation at high pH.

To evaluate whether flocculation at high pH was related to precipitation of calcium and/or magnesium, we monitored both cations in the dissolved (medium) and particulate (biomass) phase before and after flocculation at pH 11 (Fig. 2). Before flocculation, 97% of magnesium and 41 % of calcium was in the dissolved phase. After flocculation, only 41 % of magnesium and 6 % of the calcium was in the dissolved phase. This indicates that during flocculation, precipitation of both calcium and magnesium occurred. The fact that a substantial fraction of the calcium was already found in the particulate phase before the increase of pH to 11 suggest that some precipitation may already have occurred, probably due to increases in pH as a result of photosynthetic depletion of carbon dioxide. This observed precipitation of calcium and magnesium is in agreement with predictions of the PHREEQC model, which indicates that at pH 11 both calcium and magnesium are expected to precipitate as calcium carbonate, calcium magnesium carbonate, calcium phosphate and magnesium hydroxide.

We then evaluated whether precipitation of calcium and magnesium salts separately were capable of inducing flocculation of the *Chlorella* suspension. We therefore isolated *Chlorella* cells from the growth medium and resuspended them in fresh medium lacking either calcium or magnesium or both and quantified the flocculation efficiency η_a at pH 10.5, 11 and 12. When *Chlorella* was resuspended in medium lacking both calcium and magnesium η_a was low at all pH levels (< 20 %) (Fig. 3, control). This confirms the results of the EDTA addition experiment that calcium and/or magnesium are essential for the occurrence of flocculation at high pH. When calcium was added to the medium at concentrations ranging between 0.025 and 2.5 mM (Fig. 3A), η_a remained low (< 20 %) at all pH levels. This indicates that, although calcium precipitated, it did not induce flocculation. In a similar experiment, magnesium was added to the medium at concentrations between 0.015 and 1.5 mM. At a magnesium concentration of 0.015 mM, the flocculation efficiency was below 20 %

at the three pH levels. At a concentration of 0.075 mM, the flocculation efficiency increased with pH, being about 25% at pH 10.5 and close to 85% at pH 12. At magnesium levels of 0.15 mM or higher, the flocculation efficiency was between 90 and 100% at the three pH levels tested. This indicates that in contrast to calcium, magnesium precipitation was capable of inducing flocculation of *Chlorella*.

The DLVO theory states that suspensions are stabilised by surface charges of the particles (Bratby, 2006). In the case of microalgal cells, the surface charges are negative. The balance between the electrostatic repulsion and the Van der Waals attraction can be shifted towards attraction, causing coagulation and flocculation through several mechanisms. First of all, flocculation can result from an increase in medium ionic strength, which causes double layer compression. It is unlikely that this mechanism was involved in our experiments, as the change in ionic strength caused by pH increase is limited. Furthermore, this mechanism cannot explain the observed differences in flocculation behaviour upon addition of calcium *versus* magnesium. Another possible mechanism can be a reduction in surface charge of the microalgae. However, as microalgae generally carry a negative surface charge, an increase in pH will cause an increase in surface charge rather than a decrease, excluding this mechanism as a possible cause for flocculation induced by high pH.

In contrast, we propose that flocculation in our experiments was caused by a third mechanism, being charge neutralisation. During pH increase, magnesium hydroxide or brucite is formed. In brucite, some of the bivalent magnesium cations in the crystal structure are replaced by trivalent cations such as iron or aluminium, resulting in a layered double hydroxide crystal that carries positive charges (Alexeev, 1980). These positive charges probably neutralize the negative surface charge of the microalgal cells, reducing the energy barrier between cells and thus causing destabilisation and subsequent flocculation of the microalgal suspension. In wastewater treatment, precipitation of magnesium hydroxides at high pH is indeed sometimes used as an alternative for metal coagulants or synthetic polymeric flocculants to remove pollutants from wastewater (Leentvaar and Rebhun, 1982; Semerjian and Ayoub, 2003).

Although calcium also precipitated when pH was increased, this precipitation did not appear to induce flocculation of *Chlorella*. Calcium may have precipitated as calcium carbonate or calcium phosphate. In contrast to magnesium hydroxide, calcium carbonate crystals are not charged and are thus unlikely to induce coagulation by charge neutralisation. On the other hand, Sukenik and Shelef (1984) demonstrated that flocculation of microalgae at high pH was related to precipitation of calcium phosphate. They found that the calcium phosphate precipitate had a positive surface charge, inducing flocculation through charge neutralisation, similar to the mechanism proposed here for magnesium hydroxide. A concentration above 0.1 mM phosphate was required to induce flocculation at high pH. It should be noted that in our experiments, the phosphate concentration before the flocculation treatment was only 0.006 mM. Therefore, calcium phosphate precipitation was probably insufficient to induce flocculation. Possibly, at higher phosphate concentrations, calcium phosphate precipitation plays a more important role in flocculation induced by high pH.

Practical implications of using flocculation induced by high pH for harvesting microalgae

Because flocculation of microalgae induced by high pH is correlated to precipitation of magnesium, the mechanism is dependent on the presence of magnesium in the growth medium. As magnesium is removed from the medium during flocculation, this may also have implications for the recycling of process water, which is required for sustainable cultivation of microalgae and their products. When *Chlorella* was flocculated induced by high pH, the magnesium concentration in the medium was reduced from an initial concentration of 1 mM to a final concentration of 0.35 mM. As a we showed that a minimum concentration of about 0.15 mM of magnesium is required to induce flocculation at high pH, this means that repeated re-use of the water may lead to gradual depletion of magnesium, resulting in a failure of pH induced flocculation. Consequently, magnesium addition will be requested from time to time.

Based on the mass balance in Fig. 2, per 100 ml of microalgal culture, 1.2 mg of magnesium is transferred to the particulate phase after flocculation, which results in an increase of 30 mg Mg g^{-1} biomass dry weight. However, if the magnesium concentration in the medium is higher, the concentration of magnesium hydroxide precipitates is also likely to be higher. Further research is required to evaluate whether this may interfere with specific applications of the microalgal biomass (e.g. as animal feed) or with further processing of the biomass.

Harvesting of microalgae induced by high pH requires the addition of a base to raise the pH. Although we used sodium hydroxide to increase pH in our experiments, other bases may be cheaper or safer to use in an industrial environment. We thus tested whether other bases could be used to induce flocculation (Table II). Sodium carbonate failed to induce flocculation, even when pH was increased to well above 11 (data not shown). Potassium hydroxide and calcium hydroxide induced flocculation at the same pH as sodium hydroxide (pH ≈ 10.8). The lowest quantity of base was required for sodium hydroxide (9 mg g^{-1} biomass), followed by calcium hydroxide (12 mg g^{-1} biomass) and potassium hydroxide (18 mg g^{-1} biomass). Magnesium hydroxide induced flocculation at a lower pH than the other bases (pH ≈ 9.7) but a relatively large quantity of base was required (27 mg g^{-1} biomass). In industrial applications, both the low cost and the low risk would favour calcium hydroxide or slaked lime over the other bases tested. Assuming a cost of 150 \$ ton^{-1} slaked lime, flocculating microalgae induced by high pH would cost approximately 18 \$ ton^{-1} biomass.

Obviously, the sludge obtained after flocculation should be dewatered further using centrifugation. In our experiments with *Chlorella*, flocculation induced by high pH could be used to concentrate microalgal suspensions by a factor of about 50, which implies a large reduction in the electricity consumption during centrifugation.

An alternative to the use of lime may be to induce an increase in pH by intense microalgal photosynthesis. In cultures of *Phaeodactylum*, Spilling et al. (2010) observed that flocculation occurred without addition of base when CO_2 supply was interrupted and pH increased to 9.5 as a result of photosynthesis. Nurdogan and Oswald (1995) also noted that flocculation of microalgae in high rate algal ponds occurred during warm and sunny days, when low solubility of CO_2 and high photosynthetic uptake of CO_2 resulted in a high pH. More research is required to investigate what conditions favour a natural increase in pH in microalgal cultures up to a point that precipitation of magnesium hydroxides occurs.

The use of flocculation induced by high pH for harvesting microalgae may have as advantage that the high pH effectively sterilizes the microalgal biomass as well as the process water. This may be advantageous when microalgae are used in wastewater treatment, as the high pH may kill pathogenic microorganisms (Semerjian and Ayoub, 2003).

Care should however be taken that the high pH does not destroy the microalgal cells, as this may result in loss of useful bioproducts from the biomass. To evaluate whether the high pH causes cell lysis of *Chlorella*, the cell numbers were compared before and after flocculation by pH increase to pH 12 (Table III). Although a significant decrease in cell numbers was detected upon flocculation ($p=0.034$; $\alpha = 0.05$), it is clear that the major part of the microalgal cells was still intact (> 85 %). Secondly, we measured the maximum quantum yield of photosystem II in *Chlorella* biomass after flocculation induced by high pH using lime. We did not notice a significant decrease in the maximum quantum yield as long as pH remained below 12. The results of both methods suggest that the loss of useful bioproducts will most likely be minimal. Knuckey et al. (2006) also did not observe any apparent deterioration of microalgal biomass harvested using pH increase but an earlier study by Blanchemain et al. (1994) noted that cell lysis occurred after 1 hour. These findings suggest that operation time will be an important process parameter to take into account in the development of a pH induced harvesting process for microalgae.

Conclusions

Our experiments demonstrate that the terminology ‘autoflocculation’ is misleading because flocculation induced by high pH is not related to changes in properties of the microalgal cells, but is a kind of chemical flocculation in which precipitation of magnesium is involved. Flocculation induced by high pH is a potentially useful method to preconcentrate microalgal biomass. However, the method depends on sufficiently high magnesium (> 0.1 mM) concentrations. From a cost as well as safety perspective, pH is best increased using calcium hydroxide. Further research regarding the impact of pH induced flocculation on cell viability and recycling of process water is needed.

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Figures

Fig. 1 Flocculation efficiency η_a of *Chlorella vulgaris* as a function of pH in treatments with and without EDTA (0.5 M) .

Fig. 2 Mass balance of calcium (A) and magnesium (B) before treatment (BF) and after flocculation treatment (AF) at pH 11 for the dissolved phase and the particulate phase.

Fig. 3 Flocculation efficiency η_a of *Chlorella vulgaris* at three pH levels as a function of the calcium concentration (A) and the magnesium concentration (B) in the medium. Control treatment is without addition of calcium or magnesium.

Tables

Table I. Concentrations of the main ions in the medium used to culture *Chlorella*.

Table II
Comparison of the use of four bases to induce coagulation at high pH.

Table III

Cell numbers for untreated microalgae (control) and microalgae after flocculation at pH 12 (AF pH12).

Supplementary Data

Fig. 4 Microscopical analysis of *Chlorella vulgaris*: untreated (400 times magnified) (A) and flocculated at pH 12(400 times magnified) (B).

Table

Table I

	Freshwater (mM)
Cl	1.7
Na	1.9
Mg	1.2
Ca	2.0
K	0.3
P	0.06
SO_4	1.3
Conductivity (mS cm^{-1})	0.8

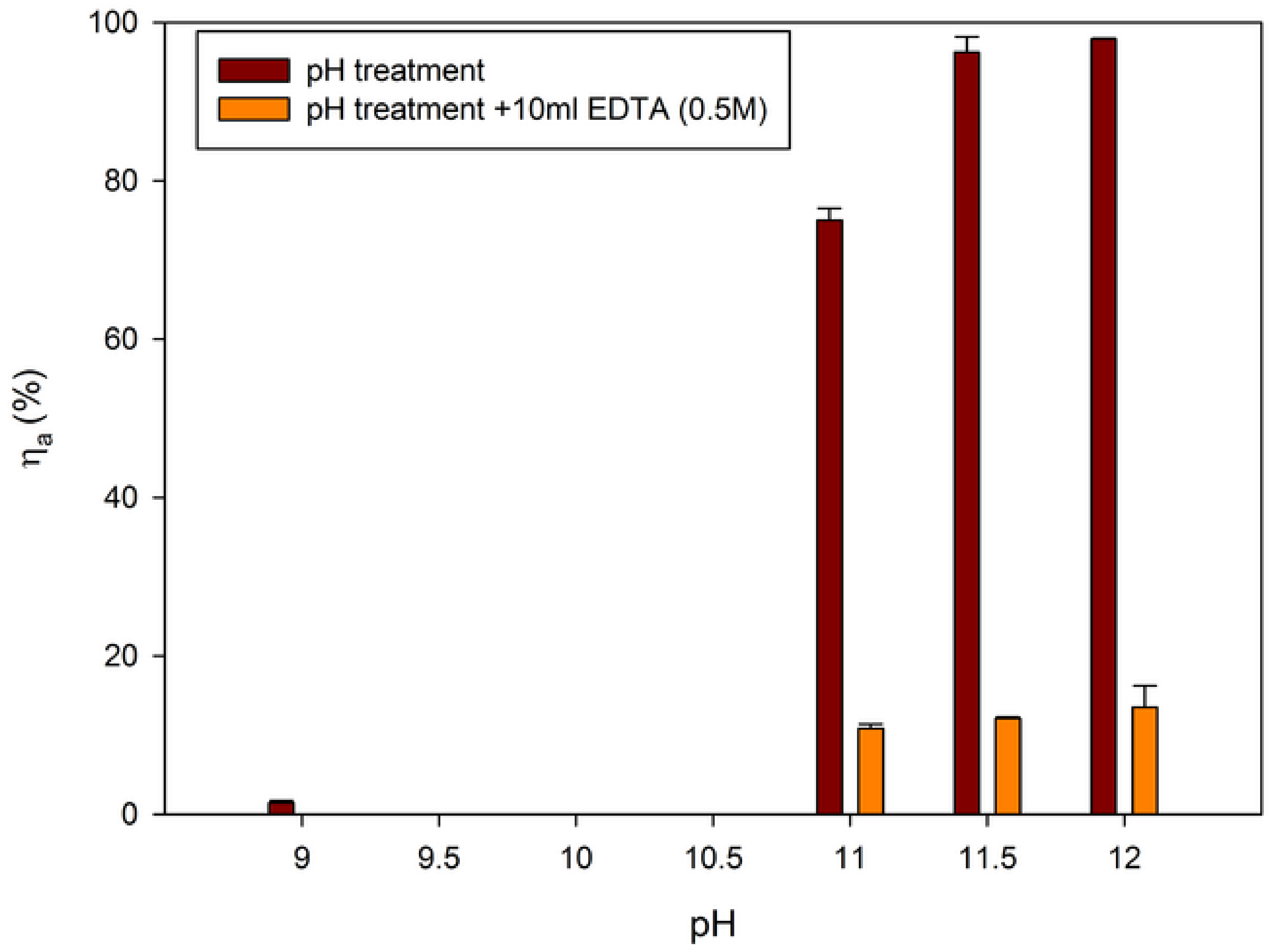
Table II

	NaOH	KOH	$Ca(OH)_2$	$Mg(OH)_2$
$pH_{coagulation}$	10.8	10.8	10.8	9.7
Final base concentration (mM)	5.75	8.00	4.00	11.5
Required amount of base (mg g^{-1} biomass)	9	12	18	27

Table III

	control	**AF (pH 12)**	**p (α=0.05)**
Cell number (#cells ml^{-1})	6.10E+07 ± 2.12E+06	5.15E+07± 1.41E+06	0.034

Figure 1



pH treatment
pH treatment +10ml EDTA (0.5M)
100
80
60
40
20
0
η_a (%)
9
9.5
10
10.5
11
11.5
12
pH

Figure 2

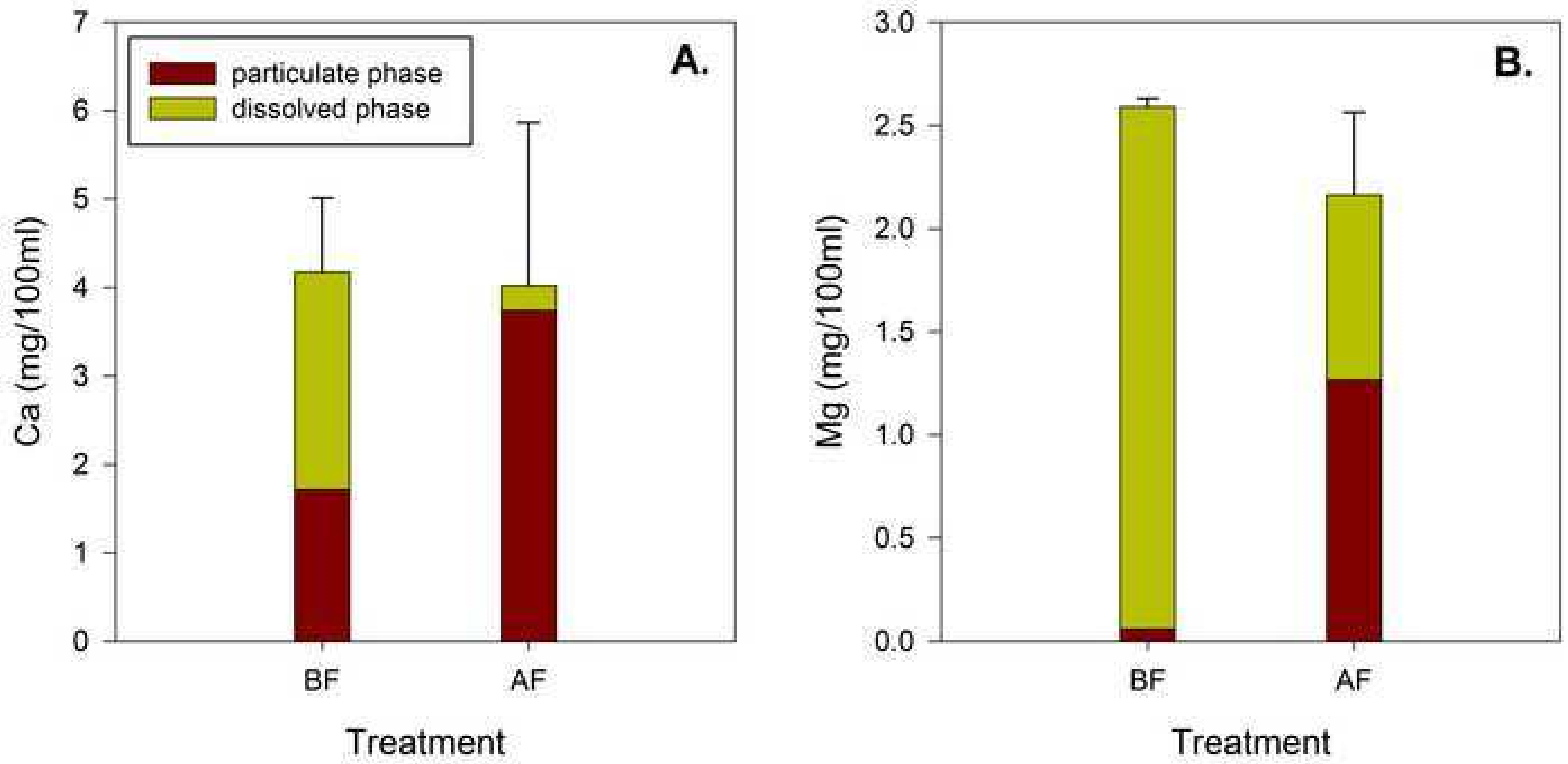

A.
particulate phase
dissolved phase
Ca (mg/100ml)
0
1
2
3
4
5
6
7
BF
AF
Treatment
B.
Mg (mg/100ml)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
BF
AF
Treatment

Figure 3

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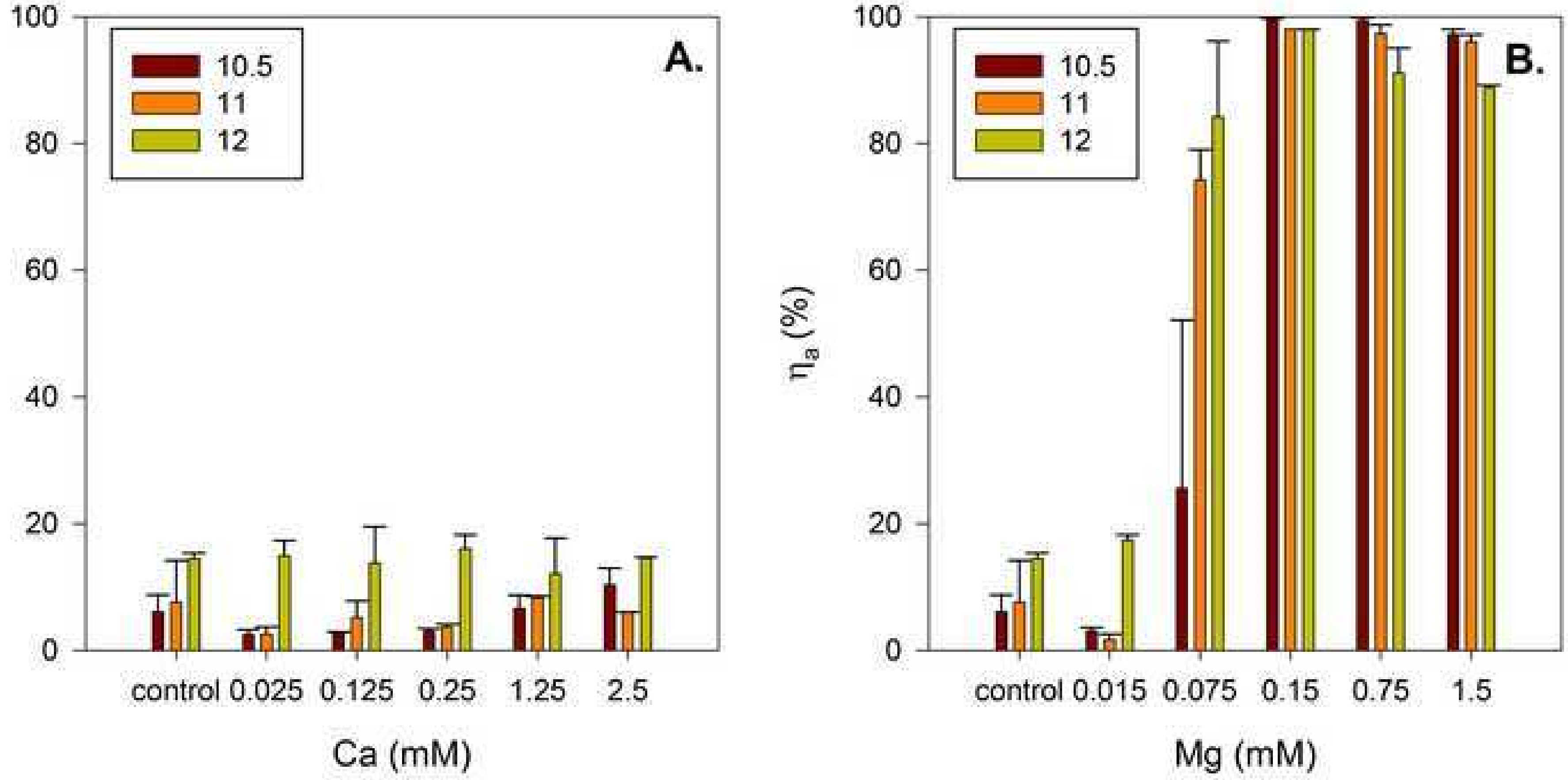

Supplementary data: Fig 4

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