1 **Alkaline flocculation of *Phaeodactylum tricornutum* induced by brucite and**
2 **calcite**

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7 4 Dries Vandamme^{1*}, Philip I. Pohl², Annelies Beuckels¹, Imogen Foubert^{3,4}, Patrick V.
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9 5 Brady², John C. Hewson², Koenraad Muylaert¹

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13
14 7 ¹KU Leuven Kulak, Laboratory Aquatic Biology, E. Sabbelaan 53, 8500 Kortrijk,
15
16
17 8 Belgium

18
19 9 ²Sandia National Laboratories, Albuquerque, NM, USA.

20
21 10 ³KU Leuven Kulak, Research Unit Food & Lipids, Department of Molecular and
22
23 11 Microbial Systems Kulak, Etienne Sabbelaan 53, B-8500 Kortrijk, Belgium

24
25 12 ⁴Leuven Food Science and Nutrition Research Centre (LFoRCe), KU Leuven
26
27 13 Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium

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34 15 *Corresponding Author: dries.vandamme@kuleuven-kulak.be

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36 16 Tel: +32 56 24 60 41

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Abstract

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20 Alkaline flocculation holds great potential as a low-cost harvesting method for marine
21 microalgae biomass production. Alkaline flocculation is induced by an increase in pH
22 and is related to precipitation of calcium and magnesium salts. In this study, we used
23 the diatom *Phaeodactylum tricornutum* as model organism to study alkaline
24 flocculation of marine microalgae cultured in seawater medium. Flocculation started
25 when pH was increased to 10 and flocculation efficiency reached 90% when pH was
26 10.5, which was consistent with precipitation modeling for brucite or $\text{Mg}(\text{OH})_2$.
27 Compared to freshwater species, more magnesium is needed to achieve flocculation
28 (> 7.5 mM). Zeta potential measurements suggest that brucite precipitation caused
29 flocculation by charge neutralization. When calcium concentration was 12.5 mM,
30 flocculation was also observed at a pH of 10. Zeta potential remained negative up to
31 pH 11.5, suggesting that precipitated calcite caused flocculation by a sweeping
32 coagulation mechanism.

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34 **Keywords: coagulation, marine algae, enmeshment, biomass, autoflocculation,**
35 **ballasted flocculation**

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38 Introduction

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40 Microalgae are a promising new source of biomass for production of food, animal
41 feed, biofuels and feedstocks for the chemical industry (Draaisma et al., 2013; Foley
42 et al., 2011). Production of microalgal biomass is still very expensive and energy-
43 intensive and is therefore only economically feasible for high-value products such as
44 nutritional supplements or fine chemicals (Guedes et al., 2011). The high cost and
45 energy demand of microalgal biomass production is to a large extent due to the cost of
46 harvesting (Barros et al., 2015; Show et al., 2013).

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48 Flocculation is seen as a promising way to reduce the cost and energy inputs of
49 harvesting of microalgae as it may allow a first separation of the biomass from the
50 culture medium by simple gravity sedimentation (Benemann et al., 1980; Molina
51 Grima et al., 2003; Schlesinger et al., 2012). In recent years, several methods for
52 flocculating microalgae have been developed, including chemical flocculation using
53 metal coagulants or cationic polymer flocculants , electro-coagulation-flocculation
54 using aluminum electrodes, microbial flocculation using bacteria or fungi and the use
55 of magnetic nanoparticles (Vandamme et al., 2013).

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57 A simple and inexpensive way of inducing flocculation is by spontaneous flocculation
58 at high pH, which is referred to as autoflocculation or alkaline flocculation (Sukenic
59 and Shelef, 1984). Studies in the 1980's demonstrated that autoflocculation was
60 related to precipitation of calcium phosphate (Lavoie and Noüe, 1987; Sukenic et al.,
61 1985). Autoflocculation by calcium phosphate precipitation, however, only occurs

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when phosphate concentrations are high (> 0.35 mM), making this method only useful in phosphate-rich wastewaters (Beuckels et al., 2013).

When phosphate concentrations are low, autoflocculation can still be induced by precipitation of calcite (calcium carbonate) and/or brucite (magnesium hydroxide). In wastewater treatment, this method of flocculation is induced by addition of slaked lime and is known as lime softening (Ayoub et al., 1986; Elmaleh, 1996). In a previous study, we elucidated the relative contribution of calcium and magnesium precipitation to alkaline flocculation for the freshwater microalgae *Chlorella vulgaris* (Vandamme et al., 2012). Although both calcium and magnesium precipitated during alkaline flocculation, flocculation could be induced in media containing only magnesium but not in media containing only calcium. This suggested that, in freshwater media, alkaline flocculation is induced by precipitation of magnesium hydroxide or brucite but not by precipitation of calcite.

Autoflocculation has also been observed in seawater. In seawater, concentrations of calcium and magnesium are much higher than in freshwater. Therefore, the relative importance of calcite and brucite in inducing flocculation may be different. The fact that flocculation starts at a lower pH in seawater than in freshwater suggest that the underlying mechanism may indeed be different (Sales and Abreu, 2014; Spilling et al., 2011). Experiments with *Dunaliella* suggested that flocculation at high pH was mainly related to brucite precipitation (Besson and Guiraud, 2013). Because *Dunaliella* is grown in hypersaline media, the alkaline flocculation mechanism may be different in regular seawater media.

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87 The aim of this study was to understand the role of calcium and magnesium salts in
88 alkaline flocculation of microalgae in seawater media. Therefore, we combined
89 precipitation and surface speciation modeling with jar test flocculation experiments
90 for the model marine microalgae *Phaeodactylum tricornutum*. We elucidated the
91 relative importance of calcite and brucite by investigating flocculation as a function of
92 pH in artificial seawater medium containing only calcium, only magnesium or both
93 cations. Additionally, zeta potential measurements were conducted to study alkaline
94 flocculation in medium with variable concentrations of calcium and magnesium.
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97 **Materials and methods**

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99 ***Precipitation model and surface speciation for brucite and calcite in seawater***

100 The concentrations of magnesium (hydroxide) and calcium (carbonate) were
101 modelled as function of pH in seawater to understand the calcium and magnesium
102 speciation during alkaline flocculation. The concentrations of total dissolved
103 magnesium, total dissolved calcium, (bi)carbonate, calcite as $\text{CaCO}_3 \cdot x\text{H}_2\text{O}(\text{s})$ and
104 brucite as $\text{Mg}(\text{OH})_2$ (active) were modelled as functions of pH using Visual Minteq
105 3.0 (KTH, Department of Sustainable Development, Environmental Science and
106 Engineering, Stockholm, Sweden). The nominal composition of synthetic seawater
107 medium (Table 1) was entered using a temperature of 25°C.
108 The surface speciation of brucite and calcite was modeled through simple protonation
109 and deprotonation reactions described in Brady et al. (2014). Calculations were
110 carried out using the speciation code PHREEQC (Parkhurst and Appelo, 1999). The
111 surface site was assumed to be 10 sites nm^{-2} (Pokrovsky, 2004). The surface area was
112 set to $10^4 \text{ m}^2 \text{ mol}^{-1}$.

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114 ***Culturing of microalgae***

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116 *Phaeodactylum tricornutum* (Pt 86, UGent Belgium) was cultivated in dechlorinated
117 deionized water to which 30 g L^{-1} synthetic sea salt (Homarsel, Zoutman, Belgium)
118 was added. This synthetic seawater (Table 1) was enriched with inorganic nutrients
119 according to the concentrations of the Wright's Cryptophyte medium (Guillard and
120 Lorenzen, 1972). *Chlorella vulgaris* was cultivated in freshwater medium (Table 1).
121 Both species were cultured in 30 L bubble column photobioreactors that were mixed

by sparging with 0.2 μm -filtered air (5 L min^{-1}). The pH was controlled at 8.5 by addition of CO_2 -enriched air (2–3%) using a pH-stat system. Growth of the microalgae in the photobioreactor was monitored by measuring the absorbance at 750 nm. Flocculation experiments were conducted at the end of the exponential growth phase when microalgal density was approximately 0.4 g L^{-1} dry weight.

General setup of flocculation experiments

Alkaline flocculation was assessed using jar test experiments. 100 mL jars were stirred using a magnetic stirrer and pH was adjusted between 9 and 12 by addition of 0.5 M sodium hydroxide. The microalgal suspension was mixed intensively (1000 rpm) for 10 min during and just after pH adjustment, followed by a gentle mixing (250 rpm) for another 20 min. The suspension was subsequently allowed to settle for 30 min. The flocculation efficiency was estimated by comparing absorbance at 750 nm between the pH-adjusted treatment and a control treatment. Samples for analysis of optical density were collected in the middle of the clarified zone. The flocculation efficiency η_a was calculated as:

$$\eta_a = \frac{OD_i - OD_f}{OD_i} \text{ (Eq. 1)}$$

where OD_i is the optical density of the control suspension after 30 min sedimentation without pH adjustment, and OD_f is the optical density of the suspension after pH adjustment. Each test was carried out in duplicate.

The role of magnesium and calcium in flocculation

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146 The relative importance of calcium and magnesium in alkaline flocculation was
147 investigated by testing whether alkaline flocculation could be induced in media with
148 varying concentrations of calcium or magnesium. *Phaeodactylum* was separated from
149 the medium using centrifugation at 4,000 g and resuspended in fresh prepared
150 synthetic seawater medium lacking calcium chloride and magnesium sulphate.
151 Preliminary tests had shown that *Phaeodactylum* formed a stable suspension after
152 resuspension in its original medium and that centrifugation and resuspension had no
153 effect on alkaline flocculation. To investigate the role of calcium chloride in alkaline
154 flocculation, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added in a concentration between 0 and 12.5 mM. To
155 investigate the role of magnesium, magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) was added in
156 concentration between 0 and 80 mM. The maximum concentrations used are in the
157 order of magnitude of the concentrations of these cations occurring in seawater. The
158 flocculation efficiency of *Phaeodactylum* cells in these synthetic media was assessed
159 at three pH levels (9.5; 10; 10.5) using jar tests as described above.

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161 To evaluate whether magnesium or calcium had precipitated during flocculation, the
162 concentrations of magnesium and calcium were analyzed after flocculation in the
163 supernatant and in the biomass pellet using ICP-MS (Agilent 7700x ICP-MS) based
164 on a method previously used in (Vandamme et al., 2015). In short, supernatant and
165 biomass pellet were separated using centrifugation at 3,005g and both fractions were
166 stored at -20°C . Prior to analysis, samples from the supernatant were diluted and
167 acidified using nitric acid (70%) to a final concentration of 0.1M. The samples from
168 the biomass pellet were acidified with 10 ml concentrated HNO_3 (70 %) and after 48
169 h the clear solution was diluted 20 times with deionized water.

171 Because precipitation of calcite is relatively slow compared to precipitation of
172 magnesium hydroxide, the influence of additional mixing time was tested for
173 *Phaeodactylum* resuspended as previously described in synthetic seawater medium
174 containing 5 mM of bicarbonate and lacking sulfates and magnesium. Jar tests were
175 conducted as previously described and mixing time was varied from 35 till 95 min in
176 triplicate. Flocculation efficiency was determined as previous described.

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178 ***Zeta potential measurements***

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180 The surface charge *Phaeodactylum* cells was assessed by the zeta potential based on
181 electrophoretic mobility using a Malvern Zetasizer Nano. Zeta potential was
182 calculated using the Smoluchowski equation. Zeta potential was measured at different
183 pH levels in media containing either calcium bicarbonate or magnesium sulphate at
184 concentrations of 7.5 mM. The pH was increased stepwise from 8 to 11.5 using
185 sodium hydroxide (0.5 M). The zeta potential was evaluated at room temperature in
186 the electrophoresis cell. For each data point, triplicate cultures were taken for
187 measurements and for each data, approximately, 25 readings were done. The average
188 values were then reported.

Results and discussion

Modeling of brucite and calcite precipitation and surface complexation

Precipitation of magnesium as brucite (magnesium hydroxide) is predicted to start when pH exceeds 10. At pH 10.5, residual concentration of Mg^{2+} in the seawater is below 40 mM (Fig 1A). As brucite precipitation is quadratic in OH^- , most of the available Mg^{2+} is predicted to be precipitated as brucite at $\text{pH} \geq 11$. The surface charge of brucite crystals is determined by the pH of the medium. As the isoelectric point of brucite is 11, the surface charge of brucite precipitates is positive up to a pH of 11 (Fig 1C). As a result, brucite precipitation should be capable of causing coagulation of microalgae through charge neutralisation in the pH range 10 to 11.

Precipitation of calcium as calcite (calcium carbonate) is predicted to start when pH exceeds 8.5. Ca^{2+} concentration in seawater is approximately 9 to 12 mM while carbonate concentration is variable but rarely exceeds 5 mM in seawaters (Feely et al., 2004). Therefore, calcite precipitation in seawater is limited by carbonate availability. As a result, only a fraction of the calcium present in seawater will precipitate as calcite. Calcite precipitation increases with increasing pH and reaches a maximum at pH 11 (Fig 1B). Over this pH range, the concentration of carbonate increases as a result of conversion of bicarbonate to carbonate by increasing pH ($\text{pK}_{\text{a}2} = 8.95$ in seawater 25°C). The surface charge of calcite precipitates is determined by adsorption of calcium, magnesium, carbonate and sulphates on the surface of calcite crystals (Brady et al., 2014). The calcite surface in seawater is dominated by sorbed Mg , $>\text{CO}_3\text{Mg}^+$, below pH 11, and $>\text{CO}_3^-$ groups at $\text{pH} > 11$ (Fig 1D). When seawater

concentrations of calcium and magnesium are present, the overall surface charge is predicted to be positive at pH < 11.

Flocculation of Phaeodactylum tricornutum in synthetic seawater medium as a function of pH, magnesium and calcium concentrations.

When alkaline flocculation was induced in synthetic seawater containing both calcium and magnesium, flocculation started as soon as pH was 10 and reached over 90% when pH was 10.5 (Fig 2). This was at a lower pH than in freshwater, where flocculation occurred at a pH of 11 (Vandamme et al., 2012). Similar results have been reported in recent studies in experiments with the marine microalgae *Nannochloropsis*, where flocculation was initiated at a pH of 10 to 10.5 (Sales and Abreu, 2014; Şirin et al., 2013). At a pH of 10.5, the total dissolved magnesium concentration remaining in the medium was 70 mM and the total dissolved calcium concentration was 8.5 mM, which indicates that only 10.5 mM magnesium and 0.6 mM calcium had precipitated during flocculation. The amount of precipitation of both magnesium and calcium is significantly less than the equilibrium expected (cf. Fig 1). In general, brucite precipitation is expected to be relatively rapid so that the fact that equilibrium levels are not reached will motivated further investigation of the effects of mixing time below (cf. Fig. 5).

We then evaluated whether alkaline flocculation could be induced in synthetic seawater lacking either calcium, or magnesium or both (Fig 3). In these experiments, we also monitored zeta potential to evaluate whether flocculation might be associated with changes in the surface charge of microalgal cells. No flocculation occurred at

any of the pH levels tested when both calcium and magnesium were lacking in the medium. Also, zeta potential remained constant when pH was increased (Fig 4A).

In medium lacking calcium, flocculation could be induced when magnesium was added to the medium. Flocculation efficiencies higher than 80% were observed at the same pH as in the medium containing both calcium and magnesium (pH 10.5; Fig 3A). When magnesium concentration in the medium was 0.15 mM or higher, moderate flocculation occurred at pH 10.5 (flocculation efficiency of about 40%). When magnesium concentration was 7.5 mM or higher, the flocculation efficiency increased to about 90% at pH 10.5. In medium with 7.5 mM magnesium, the zeta potential increased from negative to positive values when pH was increased above pH 10.5 (Fig 4B). As predicted by precipitation and surface complexation modeling, our experimental observations confirm that alkaline flocculation can be induced in medium containing only magnesium, and that flocculation is at least partly mediated by charge neutralization. In a previous study, we demonstrated that magnesium hydroxide precipitation can cause flocculation of the freshwater microalgae *Chlorella vulgaris* (Vandamme et al., 2012). For *Chlorella*, however, a lower magnesium concentration was required to induce alkaline flocculation than for *Phaeodactylum*. It is unlikely that this difference is due to differences in biomass concentrations, as biomass concentrations were comparable for both species (0.4 mg L⁻¹ in *Chlorella* versus 0.3 mg L⁻¹ in *Phaeodactylum*). The observed difference may be due to differences in cell properties between *Chlorella* and *Phaeodactylum* and/or due to an effect of the ionic strength of the medium (freshwater versus seawater) (Ayoub et al., 1986).

264 In medium lacking magnesium, flocculation occurred when calcium was added to the
265 medium. When calcium concentration was 2.5 and 5 mM, flocculation only occurred
266 at pH 10.5 (Fig 3B). When calcium concentration was 12.5 mM (the normal
267 concentration in seawater medium) flocculation was also observed at a pH of 10, but
268 the flocculation efficiency was lower than when pH was 10.5. This is in agreement
269 with the 50% flocculation efficiency observed at pH 10 when calcium is present in
270 sufficient concentration in artificial seawater medium (Fig 2). In medium containing
271 7.5 mM calcium, the zeta potential remained negative up to pH 11.5 (Fig 4C).
272 Calculations using the results of Brady et al. (2014) as in Fig. 1 suggest that in the
273 absence of magnesium, the calcite surface remains negative, although the magnitude
274 of the negative charge varies significantly with the $\text{Ca}^+/\text{CO}_3^{-2}$ ratio. For the solutions
275 studied here where calcium concentrations go from 1.25 to 12.5 the reduction in the
276 calcite surface charge is reduced by more than an order of magnitude, making
277 interaction between precipitated calcite and the anionic algal surface more favorable.
278 Our experimental results showed that alkaline flocculation can also be induced in
279 water containing calcium but lacking magnesium. Flocculation must have been due to
280 calcite precipitation and not calcium phosphate precipitation as phosphate
281 concentrations in the medium were very low (<0.05 mM). While our model predicted
282 that calcite precipitation is initiated at pH 8.5, our experiments showed that
283 flocculation only occurred at a pH of 10, or an even higher pH when calcium
284 concentrations were lower than those typically occurring in seawater. This
285 discrepancy could be related to kinetic aspects of calcite formation, which is not
286 included in our model (Shaojun and Mucci, 1993). Therefore we additionally tested
287 the influence of mixing time on flocculation efficiency as a function of pH in
288 synthetic seawater containing 5 mM of bicarbonate and lacking magnesium and

sulfates (Fig 5). At pH 8, no flocculation was observed even for 95 min of mixing. At pH 9, flocculation efficiency was 34% for 35 min of mixing and increased up to 70% for 50 min of mixing. This increase in flocculation efficiency at pH 9 is now in correspondence with the predictions for calcite precipitations (Fig 1B). This additional experiment showed that in contrast to brucite, calcite mediated flocculation requires longer mixing than 30 minutes, which is traditionally used in jar tests (Bratby, 2006).

Relative importance of calcite and brucite

When pH of *Phaeodactylum* cultures in synthetic seawater medium is increased, alkaline flocculation occurred at a pH of 10 -10.5 (Fig 2). In this pH window, precipitation of both calcite and brucite may contribute to flocculation. Theoretically, calcite could precipitate at a lower pH of 8.5 but it was demonstrated that longer mixing was needed to obtain satisfactory flocculation in relation to precipitation model (Fig 5). In outdoor microalgal cultures, pH increases gradually due to photosynthetic activity and pH variation is stabilized by supply of carbon dioxide. Due to exchange with the atmosphere and conversion of carbon dioxide to carbonates, calcite concentrations may slowly increase and induce flocculation. Measurements of zeta potential indicated that alkaline flocculation of *Phaeodactylum* in medium lacking magnesium was not associated with a reversal of the surface charge (Fig 4C). This suggests that calcite precipitates were either uncharged or negatively charged and flocculation occurred through a sweeping mechanism rather than charge neutralization. The surface charge of calcite crystals, however, depends on the presence of both calcium and magnesium in solution (Brady et al. 2014). The medium

that was used in our experiment for Figs. 4C and 5 lacked magnesium, and in the absence of magnesium the calcite surface charge is expected to be negative; similar behavior is seen in Fig. 1D for pH values where magnesium precipitates as brucite. As discussed around Fig. 1D, the calcite surface charge is a balance between sorption of cations as $> \text{CO}_3\text{Ca}^+$ and $> \text{CO}_3\text{Mg}^+$ versus $> \text{CO}_3^-$ surface groups, and the lack of Mg shifts the balance to negative.

According to our experiments, flocculation by precipitation of brucite occurred in a very narrow pH interval of 10 to 10.5. This is a consequence of the strong dependence of brucite precipitation on OH^- as well as the relatively rapid kinetics associated with brucite precipitation. As a pH of 10.5 is near the limit of the process of bicarbonate utilization in photosynthesis (Prins and Elzenga, 1989), addition of base may be required to induce flocculation by brucite precipitation; however, this might be mitigated by the buffering associated with brucite precipitation. The magnesium hydroxide that is formed is positively charged and will cause immediate flocculation through charge neutralization and possibly also through a sweeping mechanism. Although the high ionic strength of seawater results in higher magnesium requirements to induce flocculation when compared to freshwater, this is not a limiting factor due to the high magnesium concentration in seawater. This makes brucite mediated flocculation an easier process to control compared to calcite mediated flocculation.

Ayoub et al. 1986 concluded that seawater induced microalgal flocculation was independent of microalgal biomass concentration, while in freshwater conditions there was a linear behavior reported between magnesium and biomass concentration

for alkaline flocculation associated with brucite precipitation (García-Pérez et al., 2014). However, present results in marine conditions showed that brucite-mediated flocculation was mainly caused by charge neutralization. By this, one could expect a linear behavior of Mg dosage with biomass concentration which is in line with the observation made by García-Pérez et al. (2014). While for calcite, one could expect an independent behavior between calcium dosage and biomass concentration because calcite-mediated flocculation was mainly caused by sweeping, which is in line with the conclusions made by Ayoub et al. 1986. This suggests that flocculation conditions and process strategy could be adapted based on the initial biomass concentration. The effect of microalgae biomass concentration on the flocculation efficiency, pH, calcium and magnesium concentration in marine conditions deserves therefore further detailed study.

Conclusions

This study demonstrates that alkaline flocculation can be induced in a narrow pH range of 10-10.5 for the marine diatom *Phaeodactylum tricornutum* by the precipitation of brucite. A higher dosage of magnesium was needed to obtain similar flocculation efficiency compared to freshwater conditions. Alkaline flocculation was also induced in medium lacking magnesium. The precipitation of calcite was slower compared to brucite but it caused alkaline flocculation as well. This process was based on a sweeping mechanism, while brucite mainly caused alkaline flocculation based on a charge neutralization mechanism.

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Figure Captions

Fig 1(A) Concentration of total dissolved magnesium (as Mg^{2+}) and precipitated brucite (as $\text{Mg}(\text{OH})_2$ (active); (B) total dissolved calcium (as Ca^{2+}), bicarbonate (as HCO_3^-) carbonate (as CO_3^{2-}) and precipitated calcite (as $\text{CaCO}_3 \cdot \text{xH}_2\text{O}$ (s) modelled as function of pH in artificial seawater. (C) Brucite and (D) calcite surface speciation modelled as function of pH in artificial seawater.

Fig 2 Flocculation efficiency of *Phaeodactylum tricornutum* in synthetic media as function of pH (n = 2, 1σ)

Fig 3 Flocculation efficiency of *Phaeodactylum tricornutum* cells resuspended in (A) synthetic seawater lacking calcium with varying concentrations of magnesium and (B) lacking magnesium with varying concentrations of calcium at pH levels 9.5, 10 and 10.5 (n = 2, 1σ)

Fig 4 Zeta potential as function of pH for resuspended *Phaeodactylum tricornutum* (A) lacking calcium and magnesium, (B) with 7.5 mM magnesium sulphate and (C) with 7.5 mM calcium bicarbonate (n = 3, 1σ)

Fig 5 Influence of mixing time on flocculation efficiency for *Phaeodactylum* resuspended in synthetic seawater containing 5mM of bicarbonate lacking magnesium and sulphates at pH 8,9,10 and 11 (n = 2, 1σ)

Table 1. Concentrations of the main ions in the medium used to culture *Phaeodactylum tricornutum* (synthetic seawater) in comparison with Wright’s Cryptophyte freshwater medium used for *Chlorella vulgaris*

	Synthetic seawater (mM)	Freshwater (mM)
Cl ⁻	442.1	1.7
Na ⁺	338.6	1.9
Mg ²⁺	80.5	1.0
Ca ²⁺	9.1	2.7
K ⁺	6.4	0.3
HCO ₃ ⁻	5.0	0.15
SO ₄ ²⁻	40.2	1.3
Conductivity (mS cm ⁻¹)	43.0	0.8

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5