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Abstract

Dunaliella salina is a green microalgae species industrially exploited for its capacity to produce important amounts of carotenoid pigments. However in low nitrogen conditions in which they produce these pigments, their concentration is low, which results in harvesting difficulties and high costs. In this work, we propose a new solution to efficiently harvest *D. salina* at the pre-industrial scale, using flocculation/flotation harvesting induced by NaOH addition in the medium. We first show, using numerical simulations and nanoscale atomic force spectroscopy experiments, that sweeping mechanism in formed magnesium hydroxide precipitate is only responsible for *D. salina* flocculation in hypersaline culture medium upon NaOH addition. Based on this understanding of the flocculation mechanism, we then evaluate the influence of several parameters related to NaOH mixing and magnesium hydroxide precipitation and show that NaOH concentration, mixing, and salinity of the medium can be optimized to achieve high flocculation/flotation harvesting efficiencies in laboratory-scale experiments. We finally successfully scale-up the data obtained at lab-scale to a continuous pre-industrial flotation pilot, and achieve up to 80% of cell recovery. This interdisciplinary study thus provides original results, from the nano- to the pre-industrial scale, which allow the successful development of an efficient large-scale *D. salina* harvesting process. We thus anticipate our results to be the starting point for further optimization and industrial use of this flocculation/flotation harvesting technique.

Keywords

Dunaliella salina, Harvesting, Flocculation, Flotation, Atomic force microscopy

1. Introduction

Microalgae are receiving increasing attention worldwide as an alternative and renewable source of energy because of their eminent oil producing capacity (Pragya et al., 2013). But the potential of microalgae is in fact even greater and they also represent an important source of biomass and of molecules of interest for the fields of food, feed or health. Indeed, microalgae are unique microorganisms which convert light energy, water and inorganic nutrients into biomass resource rich in value-added products such as lipids, carbohydrates, proteins and pigments (Minhas et al., 2016; Pragya et al., 2013). Among the wide diversity of microalgae (Metting, 1996), *Dunaliella salina,* a halotolerant green microalgae, is among the species currently industrially exploited, as it is one of the most important producer of natural *β*-carotene pigments (Pirwitz et al., 2015; Ambati et al., 2018). Because of this specificity, *D. salina* has been the subject of many studies dedicated to the optimization of its culture conditions (Prieto et al., 2011; Kim et al., 2012; Khadim et al., 2018; Béchet et al., 2018) and of its harvesting (Besson and Guiraud, 2013; Pirwitz et al., 2015; Xiong et al., 2015). Moreover, other studies have showed the utility of this microalgae species in genetic and metabolic engineering (Feng et al., 2014; Anila et al., 2016), and also recently in concrete biomedical applications, for example to reduce tumor growth in mice (Srinivasan et al., 2017).

However, under *β*-carotene production culture conditions (nitrogen deficiency conditions), *D. salina* reaches low cell concentrations which thus has consequences on the energy input and overall cost of their separation from water in industrial processes. In a general manner, several methods can be used for microalgae harvesting, including centrifugation, filtration, flocculation and flotation (Garg et al., 2012). However, most of these methods are synonymous with high costs and energy consumption, often for low efficiency rates. For example, centrifugation, the most commonly used method, consumes a large amount of energy and can cause damages to the cells because of high shear forces (Pragya et al., 2013). Filtration involves using membranes, which, in the case of microalgae separation, can get clogged because of the small size of the cells and/or of the exopolysaccharides they secrete, resulting in high operating costs (Uduman et al., 2010). As for

flocculation, it seems to be a promising low-cost approach for large-scale harvesting; however, contamination is a major issue in this technique, as the chemical flocculants classically used to induce flocculation end up in the harvested biomass, and can interfere with the final application of the biomass (food or feed) (Vandamme et al., 2013).

In this context, flotation is believed to be a promising harvesting technique that takes advantage of microalgae's natural low density and self-floating tendency (Garg et al., 2012). Assisted flotation consists in air or gas transformed into bubbles rising through a microalgae suspension. As a result, microalgae cells get attached to bubbles and are carried out and accumulated on the surface. Thus flotation allows for low-cost cell harvesting, without necessarily using flocculants that could damage the cells. In addition, it is a relatively rapid operation, which needs low space and has moderate operational costs. However, as both the surface of microalgae and bubbles present a negative charge in aqueous medium (Yang et al., 2001; Garg et al., 2015), and given the low hydrophobicity of microalgae cells, the interactions between cells and bubbles are repulsive, which prevents adhesion, and thus capture and flotation, resulting in the poor efficiency of this harvesting technique.

Among the possible strategies to enhance flotation efficiency, natural auto-flocculation is an interesting alternative. There are several known auto-flocculation mechanisms, among which one is based on the precipitation of magnesium ions into magnesium hydroxide at high pH (Sukenik and Shelef, 1984). Such increased pH in the culture medium can result either from the photosynthesis activity of the cells or from the direct addition of OH^-. Then the flocculation of the cells can occur through charge neutralization, *i.e.* through the interaction between the positively charged formed magnesium hydroxide precipitates and cells, through sweeping where cells are entrapped in the massive precipitation of the magnesium hydroxide in the medium, or both (Vandamme et al., 2013). Auto-flocculation of cells at high pH using NaOH addition has already been described for different microalgae species, such as *Chlorella vulgaris* (Wu et al., 2012), or *Phaeodactylum tricornutum* (Wu et al., 2012; Vandamme et al., 2015; Formosa-Dague et al., 2018) and has been showed to occur by

95 both sweeping flocculation and charge neutralization. In the case of *D. salina*, a previous study that
96 we conducted in 2013 showed that addition of NaOH was necessary to increase the pH in the
97 medium, and that further flocculation was caused by sweeping of the cells by forming magnesium
98 hydroxide precipitate (Besson and Guiraud, 2013). In the experimental conditions used in this study,
99 the more NaOH was added, the more magnesium hydroxide was precipitated and the more efficient
100 the flocculation was; a cell recovery up to 100% could be achieved using further flotation. The results
101 obtained also showed that the NaOH flow rate had no influence on the flocculation and further
102 flotation recovery; however they suggested that the type of injection of NaOH, and thus it's mixing in
103 the medium and the speed of magnesium hydroxide precipitation, could have an influence in the
104 final harvesting efficiency.

105 In the work presented here, we will first evidence the only contribution of sweeping
106 mechanism in the flocculation of cells induced by the precipitation of magnesium hydroxide at high
107 pH, and exclude the role of charge neutralization. For that we will first simulate the precipitation of
108 magnesium hydroxide in the medium as a function of the NaOH concentration to understand the
109 behavior of the culture medium during flocculation. Then, we will use recent developments made in
110 the team that consist in using atomic force microscopy (AFM) (Binnig et al., 1986) to probe the
111 interactions between microalgae cells and particles (Formosa-Dague et al., 2018). For that, AFM tips
112 functionalized with magnesium hydroxides particles will be used in force spectroscopy experiments
113 to measure directly, at the piconewton scale, the interactions between these particles and the cells.
114 Then, knowing precisely the flocculation mechanism at play, we will be able to optimize parameters
115 to harvest, in the most effective possible way, *D. salina* cells at laboratory scale but also at pre-
116 industrial scales (suspension flow rate 0.6 m^3/h). For that, we will complete our previous study and
117 determine the role of NaOH mixing in the medium by testing different injection types, agitation and
118 medium salinity, and by evaluating their effects on the flocculation/flotation recovery of the cells at
119 laboratory scale. Based on these results and their interpretation, we will adapt and optimize an
120 injection system to efficiently flocculate and harvest cells by flotation in a pre-industrial continuous

cultivation system. This way we will provide a cost-effective solution to efficiently harvest low-concentrated *D. salina* cells at industrial scales, thus contributing to the industrial valorization of this *β*-carotene rich biomass.

2. Material and methods

2.1. Strain and culture conditions

Dunaliella salina strain CCAP19/25 (Culture Collection of Algae and Protozoa) was cultivated in dechlorinated tap water containing the following: NaCl (107 g/L); $MgSO_4.7H_2O$ (4.8 g/L); $MgCl_2.6H_2O$ (4.0 g/L); $CaCl_2$ (1.1 g/L); KCl (0.1 g/L). A complete nutritive Conway medium (without silicates) (Walne, 1970) was also added. The salinity of this synthetic water is of 10%, the ionic strength of 2.3. This medium was used for all the experiments performed in laboratory. It mimics the medium resulting from the evaporation of see water in which *D. salina* can grow with a limited competition from other microorganisms, used in pre-industrial scale cultures. A *D. salina* strain isolated from saline ponds in Gruissan, Occitanie, France, was also used to evaluate the influence of the strain on the harvesting efficiencies.

Laboratory-scale cultures: For AFM experiments, cells were cultivated at 20°C, under agitation (120 rpm), in 75 mL non-coated culture flasks (15 mL of culture), 500 mL non-coated culture flasks (150 mL of culture). The incubator was equipped with white neon light tubes providing illumination of approximately 40 µmol photons m^{-2} s^{-1} with a photoperiod of 12h light : 12h dark. For flotation experiments, *D. salina* culture was achieved in 10 L glass photobioreactors. The culture was continuously agitated by the gentle bubbling of 0.2 µm filtered atmospheric air (2 L/min). Illumination was provided by daylight fluorescent tubes (OSRAM FQ 965 Biolux) with a photoperiod of 16h light : 8h dark. The temperature of the culture was regulated by air conditioning at 21 ± 3°C.

Pre-industrial scale cultures: The pre-industrial experimental site of the Salinalgue research project (2010-2014) was located in a salt marsh on the Mediterranean Sea coast, at Gruissan, Occitanie, France (Latitude: 43.094 N; Longitude: 3.084 E), as fully described in Béchet *et al*. (Béchet et al.,

2018). Briefly, cultures were achieved in 4 outdoor raceways of 250 m², containing between 30 and 100 m^3. See water concentrated by sun evaporation (salinity of 12%) and disinfected with bleach fed the raceways before being inoculated. The raceways were mixed by paddle wheels and by suspension pumping which was also used to limit the pH to 7.4 by a monitored addition of dissolved CO_2 in the recirculated suspension.

2.2. Precipitation model for hydroxide magnesium in the culture medium used

The concentration of magnesium hydroxide, calcium carbonate and dolomite were modeled as a function of the added NaOH concentration in the culture medium. To do so, we simulated the NaOH addition effects and developed a numerical model, using the software Phreeqc, based on the Pitzer equations to simulate the precipitate formation in our culture medium. Indeed, in order to assess the equilibrium state of hypersaline solutions in which *D. salina* is grown and understand the precipitation phenomena involved during NaOH addition, it is necessary to evaluate the activity of species in solution. This requires the evaluation of coefficients of activity, which take into account the various ionic interactions in these complex environments. The models for calculating these activity coefficients are generally based on the coupling of the Debye-Hückel (Debye and Hückel, 1923) theory translating electrostatic ionic interactions, and of the theory of the ionic association that takes into account ionic interactions at short distances. These models are well suited for solutions with ionic strength that does not exceed the one of regular seawater (ionic strength of 0.7). But since the culture of *D. salina* is carried out in a hypersaline environment, the ionic forces encountered in its culture medium exceed the areas of validity of the different ion association models (for a 10% salinity culture medium, the ionic strength is of 2.3). Therefore, the models obtained with these theoretical descriptions of saltwater thermodynamics are not satisfactory for concentrated saline water. In the case of *D. salina*, it was thus necessary to implement other types of models, known as specific ion interaction models, such as the one proposed by Pitzer. The Pitzer model is based on a different thermodynamic approach, and is well suited to evaluate the thermodynamic properties of

hypersaline solutions. The model used is based on the Pitzer equations, and allows to simulate the ionic equilibria during the injection of NaOH in our culture medium. The models developed by Pitzer (Pitzer, 1973; Pitzer and Mayorga, 1973, 1974; Pitzer and Kim, 1974; Pitzer, 1975) describes the specific ionic interactions of diverse species in complex media, at high ionic strength. For pure components, this model uses the parameters given by (Pitzer and Mayorga, 1973, 1974). The asymmetric mixing parameters come from Pitzer ~~et~~ and Kim (Pitzer and Kim, 1974; Pitzer, 1975). The whole set of parameters were validated and listed by Harvie *et al.* (Harvie et al., 1984), the relevant database being "pitzer.dat" This database accounts for the significant elements for Mediterranean see water (Na-K-Mg-Ca-H-Cl-S-O-C-Fe-Mn-Ba-Sr-B-Li-Br) with a large spectrum of possible precipitates. Before its application for the simulations presented here, the model has been successfully (Besson, 2013) compared to the experimental results of sea water evaporation proposed by Baseggio (Baseggio, 1974). The composition of the culture medium used for the simulations (Table 1) was entered using a temperature of 25°C and a total dissolved carbon concentration of 0.00021 mol/kg_water.

Table 1. Concentrations of the main ions in the culture medium used in simulations in g/L

Na^+	Cl^-	SO_4^{2-}	Mg^{2+}	Ca^{2+}	K^+
42.12	67.08	1.87	0.95	0.39	0.04

2.3. Flotation experiments

Due to the small size of the microalgae, Dissolved Air Flotation (DAF) was used to harvest *D. salina* after NaOH-induced flocculation. Figure 1 presents the DAF devices used in this study.

Laboratory scale: A detailed presentation of the laboratory scale flotation experiments can be found in Besson & Guiraud (Besson and Guiraud, 2013). DAF experiments were performed in a Multiplace Orchidis™ FTH3 Flottatest. Three flotation-test beakers were run simultaneously, in which 600 mL samples were collected from the algal culture and added to each beaker. Then NaOH was added: in the case of direct NaOH injection, x mL of NaOH at a concentration of 1M was added to the microalgae suspension, as well as 100 - x mL of distilled water. In the case of the diluted NaOH

198 injection, a unique solution containing x mL of NaOH at a concentration of 1M and 100 - x mL of
199 distilled water was added to the microalgae suspension. The volume of NaOH (x) added was
200 calculated depending on the final concentration wanted. This procedure is presented in figure 4a.
201 The depressurization at atmospheric pressure of 200 mL culture medium free of algae and saturated
202 by air at 6 bars for 15 min induced the formation of microbubbles. The recycle ratio (pressurized
203 culture medium volume/initial sample volume) was of 33%. For flocculation, the concentration of the
204 added NaOH solution was calculated taking into account the volume of the culture and the volume of
205 the added white water, so that the pH does not change upon addition of the white waters.
206 Pre-industrial scale: The pre-industrial DAF system was adapted from the CY1 flotation unit proposed
207 by Sérinol (Bram, Occitanie, France), and is presented in Figure 1. Built in 316L stainless steel to avoid
208 corrosion by suspensions at high salinity, this continuous cylindrical (0.6 m of diameter) DAF
209 separation equipment, with a conical bottom, works as an airlift. A cylindrical Clifford delimits the
210 ascending contact zone where the suspension to be treated and the white waters, containing
211 bubbles, mix. At the periphery, the descending annular separation zone is equipped with a vertical
212 lamellar packing to keep the flow as quiet as possible. The nominal descending flow velocity is of 4.0
213 m/h. The flotation tank volume is of 600 L and its maximum flow capacity is of 1 m^3/h. The floated
214 microalgae are mechanically removed from the tank surface by a tunable rotating scrapper. Salted
215 waters identical to the culture medium or recycled from the harvesting are continuously pressurized
216 at 6 bars by a centrifugal pump and saturated with air within a pressurization tank. White waters are
217 produced by passing this pressurized solution through a needle valve. The microbubble size
218 distribution was measured by Laser Diffraction Sizer (Malvern Spraytec): most of the bubbles
219 produced in these saline waters have a diameter smaller than 60 µm (Besson and Guiraud, 2012).
220 NaOH solutions at different concentrations in distilled water were added at different injection places
221 on the supply line at a controlled flow injection using a peristaltic pump. Given that the microalgae
222 suspension flow rate is of 300 L/h, and the final NaOH concentration is of 0.02 mol/L, if a NaOH
223 solution of 0.1 mol/L is injected, the flowrate is of 66 L/h, and if a NaOH solution of 0.2 mol/L is

injected, the flowrate is of 0.33 L/h. Flowrates were measured thanks to Khrone Optif lux – 4100 electromagnetic flowmeters. For the operating conditions, microalgae suspensions were extracted from the external raceways using a peristaltic pump, into the flotation unit. Simultaneously, the pressurization system was launched and the NaOH solutions were injected on the supply line. The scrapping of the microalgae started as soon as the pressurization system was launched. After one hour at constant operating parameters (three times the time of the continuous state settling), samples required to quantify the separation efficiency (treated suspension) were harvested: the harvesting efficiency was then measured as described below.

2.4. Harvesting efficiency quantification

Harvesting efficiency quantification is based on optical density measurements. The harvesting efficiency represents the quantity of algae floated compared to the quantity in the initial suspension: it was evaluated using the following equation:

$$\mathrm{E}(\%) = \left(1 - \frac{\mathrm{OD}aVa}{\mathrm{OD}iVi}\right) \times 100$$

Where OD*i* and V*i* are the initial optical density at 800 nm and the volume of algal suspension before NaOH addition and flotation, OD*a* and V*a* are the optical density at 800 nm of the aqueous phase and the volume of the aqueous phase after injection of pressurized water.

2.5. Zeta potential measurements

The global electrical properties of *D. salina* cell surface were assessed by measuring the electrophoretic mobility which corresponds to the velocity of suspended cells exposed to an electric field. To this end, microalgae were harvested by centrifugation (1500 rpm, 3 min), washed two times in sorbitol buffer 375 mM at pH = 10 and resuspended in the same buffer at a final concentration of 1.5×10^6 cell/mL. Using this procedure, electrolytes present in the culture medium do not interfere and only the surface charge of the cells is measured. The electrophoretic mobility was then

measured using an automated laser zetameter (Zetasizer NanoZS, Malvern Instruments). Cell suspensions coming from 2 independent cultures were analyzed.

2.6. AFM tip functionalization with hydroxides

To prepare functionalized AFM with $Mg(OH)_2$, MLCT AUWH tips were first dipped into a thin layer of UV-curable glue (NOA63, Norland Edmund Optics), then into a thin layer of $Mg(OH)_2$ particles (Sigma-Aldrich) deposited on a glass slide. Functionalized tips were then put under UV-light for 10 min to allow the glue to cure.

2.7. AFM imaging and force spectroscopy experiments

Before AFM experiments, cells were harvested by centrifugation (3000 rpm, 10 min) and washed two times in sorbitol buffer 375 mM at pH = 10. This salt-free buffer is used for force spectroscopy experiments as it allows to accurately measure interactions between the cells and magnesium hydroxide particles functionalized on the AFM tips without introducing a bias from electrolytes present in *D. salina* culture medium. Moreover, the sorbitol present in this buffer keeps the cells from exploding because of the osmotic pressure. Finally, the pH of 10 allows to reproduce the conditions in which the cells are during flocculation induced by addition of NaOH. Cells were then immobilized on polyethylenimine (PEI, Sigma P3143) coated glass slide prepared as previously described (Francius et al., 2008). Briefly, freshly oxygen activated glass slides were covered by a 0.2% PEI solution in deionized water and left for incubation overnight. Then the glass slides were rinsed with deionized water and dried under nitrogen. A total of 1 mL of cell suspension was then deposited on the PEI slides, allowed to stand for 30 min at room temperature, and rinsed with sorbitol buffer 375 mM at pH = 10. For force spectroscopy experiments, MLCT AUWH cantilevers with a nominal spring constant of 0.01 N/m, functionalized with hydroxides or not, were used at a constant applied

force of 0.25 nN. The cantilevers spring constants were determined using the thermal noise method (Hutter and Bechhoefer, 1993) before each experiment.

3. Results and discussion

3.1. Precipitation modeling and AFM force spectroscopy confirm that *D. salina* is flocculated by sweeping in magnesium hydroxide precipitate at high pH

In our previous study in 2013 (Besson and Guiraud, 2013), our work evidenced the positive effect of NaOH addition on the flocculation of *D. salina*. Our main results showed indeed an increase in the harvesting efficiency with the addition of NaOH into the medium, up to 80% of cell recovery. While the hypothesis that the flocculation occurred through sweeping in magnesium hydroxide precipitate could be formulated, notably thanks to ions chromatography experiments performed on the suspension before and after flocculation/flotation, no proof of this mechanism were brought. The first part of this study thus focus on providing a full understanding of the NaOH-induced flocculation mechanism. For that we first assessed the equilibrium state of the hypersaline solutions in which *D. salina* is grown to understand the precipitation phenomena involved when flocculating this microalgae using NaOH addition. For that, we used the model described in section 2.2, elaborated from the Pitzer model, to successfully simulate the influence of the addition of NaOH on the ionic equilibria in our culture medium described in Table 1. Results are presented in Figure 2: on this graph, the precipitation of magnesium hydroxide $Mg(OH)_2$ is plotted as a function of the added NaOH concentration. The resulting pH as well as two other precipitate candidate concentrations are also presented. Even if the measure of pH in hypersaline solutions with a high ionic strength presents some problems, the simulated pH profile is similar to experimental measurements, as the one showed in our previous study (Besson and Guiraud, 2013). The pH sharply increases until the beginning of the precipitation of the magnesium hydroxide. Then, it continues to slightly increase

until the end of the $Mg(OH)_2$ precipitation, that is to say when all the Mg present in the solution is used. Thus this shows that upon addition of NaOH, magnesium hydroxide precipitation increases until reaching a maximum for a NaOH concentration between 0.08 and 0.1 mol/kg of water, while calcium carbonates precipitation remains non-significant. Thus we can conclude from this simulation that the hypothesis made in our previous study, stating that flocculation of *D. salina* at increased pH, under hypersaline conditions at least, results from the precipitation of only magnesium hydroxide at high pH, among a great number of other possible salts, is correct. While this precipitation creates a gel that entraps the cells and flocculate them through sweeping, the question is now to know if charge neutralization is also involved in this flocculation mechanism, thus to have a full understanding of it.

To answer this question we used AFM and performed force spectroscopy experiments using functionalized tips with magnesium hydroxides particles. In this type of experiments, the AFM tip is moved towards the surface until touching it, and then retracted. If an interaction takes place between the tip and the sample, upon retraction, the tip will bend until the force applied is higher than the force of the interaction and the interaction breaks. The tip then goes back to its initial position, which is materialized on AFM retract force curves as a peak, referred to as retract adhesion. The results of these experiments are presented in figure 3, they show in the case of bare AFM tips no adhesion between the tip and the cells, as seen on the retract force curves which present no retract adhesions (Figure 3a and b, n=2400 curves recorded on 6 cells from 2 independent cultures). In the case of tips functionalized by magnesium hydroxides (Figure 3C and d), force curves also show no retract adhesions, thus demonstrating that hydroxide particles do not interact with cells (n=3200 curves recorded on 8 cells coming from 2 independent cultures, with 7 different $Mg(OH)_2$ tips). Note that in order to avoid any interaction between electrolytes in the medium and the AFM tips, experiments were performed in a salt-free buffer at pH=10 to make sure only the interactions between the cells and the tips are probed. As no interactions were probed in this buffer, it is then evident that in the hypersaline waters in which *D. salina* grows, these interactions do not occur

neither given the high quantity of charged ions that can screen both cells and $Mg(OH)_2$ particles charges. To give an explanation to this absence of interactions between the cells and $Mg(OH)_2$ particles, we then performed zeta potential measurements of *D. salina* cells in the same salt-free buffer at high pH (pH = 10), and measured a surface charge of -15.4 ± 0.9 mV (n=8 measurements on cell suspensions coming from 2 independent cultures). Thus the cells do not present a sufficient negative surface charge in our conditions, and do not interact with the positive surface of magnesium hydroxide (Lin and Wang, 2009).

Therefore thanks to these simulations and AFM experiments, we are able to confirm the hypothesis we made in our previous study, and show that addition of NaOH in the culture medium precipitates only magnesium hydroxide. This precipitation results in the formation of a gel that is only responsible for entrapping the cells and flocculating them, as AFM experiments proved that charge neutralization is not involved in the case of *D. salina.* Moreover, in further experiments that we performed, $Mg(OH)_2$ already formed was directly added to *D. salina* cultures, which resulted in no flocculation of the cells, thus reinforcing our nanoscale conclusions. This is an interesting point as for all the microalgae species for which pH-induced flocculation has been described, charge neutralization mechanism is always involved (Sukenik and Shelef, 1984; Wu et al., 2012; Vandamme et al., 2012; Nguyen et al., 2014; Vandamme et al., 2015; Formosa-Dague et al., 2018; Branyikova et al., 2018). Indeed, microalgae often have a cell wall, composed of lipids, proteins and polysaccharides. These last ones have a pK_a of 11-12; when the pH increases in the medium, the hydroxyl functions of these polysaccharides are deprotonated, which can give the surface a more important negative charge. As microalgae are usually grown in waters containing calcium or magnesium ions, which precipitate into positively charged particles at high pH, then flocculation occur also thanks to the interaction between these particles and the cells. However in our case, *D. salina* cells do not interact with positively charged magnesium hydroxide. Indeed, *Dunaliella* genus is unique in the absence of a rigid polysaccharidic cell wall; cells only present a thin plasma membrane (Oren, 2005; Chen and Jiang, 2009). This thus explains why its surface charge is not more negative at

high pH, and thus why charge neutralization is not involved in its flocculation mechanisms in presence of magnesium hydroxide. Thus our results give a full understanding of the mechanism of *D. salina* flocculation at high pH; based on these information, we can now evaluate the influence of different parameters, such as NaOH concentration, agitation and salinity in order to determine the best possible separation conditions of the cells from the water, and provide a solution for high-scale harvesting.

3.2. Magnesium hydroxide precipitation phase is determinant for flocculating the cells

Because the flocculation of the cells occur only through sweeping, the precipitation of magnesium hydroxide is then determinant for the successful flocculation/flotation of the cells. Indeed, the precipitation phase must be carried out as homogeneously as possible, so that as much of the sample volume as possible is affected by the trapping of microalgae in the precipitate in formation. Therefore, the conditions for adding NaOH to the suspension should be adapted to optimize its fast mixing. To address this point, we performed flocculation/flotation experiments using two different injection ways (Figure 4a), reaching the same final NaOH concentration. In the first way (direct injection), NaOH (1 mol/L) and water are directly injected in a flotation unit containing the microalgae suspension. In the second way, called here diluted injection, water and NaOH (1 mol/L) are first mixed into one solution, which is further added to the microalgae suspension. Then, in the first case, concentrated NaOH is delivered locally into the medium and in the second case, the local instant concentration of the NaOH solution is reduced. Using these two injection scenarios, flocculation/flotation harvesting was performed: the results, presented in Figure 4b show a clear increase of the harvesting efficiency in the case of diluted injection, for the same final NaOH concentration in the microalgae suspension (black-filled symbols). For example at 0.02 mol/L of final NaOH concentration, the harvesting efficiency using direct injection is of almost 30% whereas using the diluted injection system, it reaches up to 90%. Indeed, in the case of direct injection, a high quantity of concentrated NaOH is locally delivered in the solution, thus the speed of precipitation is increased but only concerns a local area of the suspension. Therefore in this case, only the cells

present in this area can be entrapped during the precipitation of magnesium hydroxide. In the case of the diluted injection, the low local concentration allows for a slower precipitation, which then has time to occur in the entire, or at least a larger volume of the microalgae suspension, leading to the entrapment of more cells. This phenomena is further illustrated in Figure 4c; for a final NaOH concentration in the microalgae suspension of 0.02 mol/L, the more diluted the added NaOH solution is, the more efficient the harvesting is. Indeed, for an added NaOH solution of 0.1 mol/L, the flocculation/flotation cell recovery reaches 80% while for NaOH solutions higher than 0.5 mol/L, the harvesting efficiency is reduced to 20%.

The results of these experiments then prove that the local concentration of NaOH and the phase of the magnesium hydroxide precipitation is determinant for the harvesting efficiency. While diluted injection allows lowering this local NaOH concentration, to optimize the conditions to reach the best cell separation possible, other parameters such as the agitation and the salinity (quantity of magnesium ions available) of the medium should be taken into account as they will thus also influence the harvesting efficiency.

3.3. Agitation and salinity are parameters that influence harvesting efficiency

While diluted injection allows the magnesium hydroxide precipitate to form slowly in the medium, agitation of the medium allows for the precipitation phase to sweep the entire microalgae suspension. In Figure 5a, the effects of different agitation speed on the flocculation/flotation harvesting efficiency were measured. It is clear on this figure that an increase from 20 to 40 rpm allows increasing by approximately a two-fold the cell separation whatever the final NaOH concentration in the microalgae suspension. However, increasing again the agitation speed from 40 to 80 rpm do not show the same effect, as the harvesting efficiency in the case of 80 rpm agitation is slightly lower than in the case of 40 rpm. This can be explained by the fact that a too fast agitation can change the structure of the flocs formed and thus have consequences on their capture by the bubbles during flotation. As for the salinity of the medium, to assess its effects on the harvesting

404 efficiency, we performed experiments with *D. salina* cells grown in natural seawaters presenting
405 salinities of 7.5, 12 and 15.2%, using a dilute injection system and an agitation of 40 rpm. The results
406 obtained, presented in Figure 5b, show that the more saline the medium is, the less efficient the
407 harvesting is, whatever the NaOH final concentration. Indeed, while high magnesium concentrations
408 found in highly saline media can be thought, in the case of sweeping flocculation, to enhance
409 harvesting efficiency, it is the opposite effect. In high saline media, the NaOH added directly meets
410 high concentrations of magnesium ions, which induces a fast precipitation of magnesium hydroxide.
411 OH^- has thus no time to reach the entire volume of the suspension. Then a high salinity has similar
412 effects than injection of concentrated NaOH, where only the cells present in the area where fast
413 precipitation of $Mg(OH)_2$ occurs can be entrapped in the precipitate, leading to lower separation
414 rates.

415 Therefore thanks to these experiments, the parameters to reach the best separation rate
416 possible can be optimized: while a dilute injection is needed, the agitation of the medium must be
417 optimized to allow an efficient mixing of NaOH with cells and a $Mg(OH)_2$ precipitation that reaches
418 the entire volume, without having negative effects on the flocs formed. As for the salinity of the
419 culture medium, it needs to be low enough to avoid supersaturation of magnesium hydroxide
420 precipitation, the adequate NaOH concentration depending on the salinity. In order to make sure
421 that only parameters related to the magnesium hydroxide precipitation have an influence on the
422 harvesting efficiency, we also evaluated the influence of the calcium concentration, the *D. salina*
423 strain used, and the nutritive conditions in which the cells are grown. Regarding the influence of the
424 calcium concentration in the medium, Sukenik *et al*. in 1984 have established that the precipitation
425 of calcium and phosphate ions at high pH could induce the flocculation of microalgae cells (Sukenik
426 and Shelef, 1984). The Conway medium used here to cultivate the cells containing phosphate, we
427 thus used waters of different calcium concentrations to evaluate the potential effect of calcium
428 phosphate precipitate on the flocculation of *D. salina*. Our results (Supplementary Data 1) showed no
429 difference in the harvesting efficiencies, thus reinforcing our conclusions on the role of only

magnesium hydroxide precipitate in sweeping the cells. The influence of the *D. salina* strain and of its nutritive conditions have then also been evaluated, and showed that for the two strains we tested (the CCAP 19/25 and one other *D. salina* strains isolated from saline ponds in France), the same flocculation/flotation conditions resulted in the same harvesting efficiencies (Supplementary Data 2). Regarding the nutritive conditions in which cells are grown, we chose to focus on nitrogen deficiency conditions. Indeed, the overproduction of *β*-carotene after nitrogen starvation (*i.e.* in conditions of unbalanced growth in response to lack of nitrogen) is a well-documented biological process in *D. salina* (Lamers et al., 2012; Bonnefond et al., 2017). We thus chose to address this question as the harvesting method we propose here is intended to be used for industrial use. Our results, with cells grown in nitrogen deficient conditions, showed no difference in the harvesting efficiencies obtained. Thus these two last points show that the flocculation/flotation method that we focus on in this study, when used with the good injection system, agitation and salinity of the medium, is efficient for different *D. salina* strains, in different relevant culture conditions. This demonstrates the robustness of this harvesting method, and thus the parameters identified (NaOH injection, agitation and salinity) can now further be used to develop and adapt a NaOH injection system to efficiently flocculate and harvest cells by flotation in a pre-industrial continuous cultivation/harvesting system.

3.4. Development of a pre-industrial harvesting process of *D. salina* by flocculation/flotation.

In this part of the work, the knowledge previously acquired at the laboratory scale is used to harvest *D. salina* cells at high-scales. For that, raceways of 250 m² were built in Gruissan (Occitanie, France) and used to cultivate *D. salina*; an industrial continuous flotation unit (600 L) was adapted and installed on the raceway site to harvest cells using NaOH-induced flocculation/flotation. A network of pumps with controlled flow rates were used to collect the cells from the raceway and bring pressurized water into the flotation unit. For adapting the flotation unit to the specific conditions of *D. salina*, we first faced a technical challenge regarding the size of the bubbles produced in the pilot. Indeed, using a method based on laser light diffraction, we evaluated the size

of the bubbles produced by DAF in *D. salina* culture medium (Supplementary Data 3a). These experiments show that in this medium (NaCl concentration of 107 g/L), bubbles have a size of approximately 40 µm, compared to 60-100 µm in freshwater (Edzwald, 1995). Thus the salinity reduces the size of the bubbles, which results in the reduction of their ascending velocity. While this is not a problem at small-scale, because the flotation units consist of one small cylinder (1 L of maximum volume) with only one entry for the pressurized water at the bottom (Figure 1a), at high-scale in a high-dimensioned flotation unit, presenting descending flow zones, it is a problem. For instance, this flotation unit presents holes at the base of the Clifford in order to accentuate the airlift effect and obtain, in nominal functioning conditions, descending speeds of 4 m/h (Figure 1b). We thus roughly calculated the speed of our bubbles, considering them as rigid particles obeying the Stokes law corrected by Oseen (Oseen, 1910; Clift et al., 1978) and found in our conditions an average bubble ascending velocity comprised between 3.6 and 5.4 m/h depending on the contamination degree of their surface (1 and 1.5 mm/s, Supplementary Data 3b). Thus some of the bubbles may not reach the surface and can be aspirated in the flow descending zones of the flotation unit. Thus adaptations directly on the flotation unit were realized, and consisted in decreasing the surface of the holes present at the base of the Clifford to decrease the airlift effect and decrease the velocity in these descending flow zones. This way the rising velocity of the microbubbles is higher than the velocity in the descending flow zones, and thus a functioning similar to what is obtained at the laboratory-scale can be provided.

These adaptations made, we then scaled-up the NaOH-induced flocculation/flotation process optimized in batch mode at the laboratory scale, to a continuous pre-industrial scale mode. For that, given the specific flocculation mechanism of *D. salina* by sweeping, it is needed to adapt an injection system that will ensure a good mixing between the cells and the added NaOH, as it is determinant to efficiently flocculate the cells. Given the previous results concerning the salinity of the medium, these experiments were performed in natural seawater at a salinity of 12% that ensured the growth of the cells in the raceway without presenting precipitation supersaturation problems. For the

experiments, microalgae cultures were injected into the industrial flotation unit, as well as pressurized waters, through supply lines following the injection system represented in Figure 6a. Note that in this system, the waters used for pressurization are recycled from the flotation unit after microalgae recovery, and may contain also some microalgae. NaOH mixing with microalgae and microbubbles in this system takes place directly in the injection system, and is different depending on its location on this injection system. For instance, in positions 3 and 6 (Figure 6a), mixing will be more efficient than in position 1 and 4 because there the flow rate is the most important and the fluid is already a tri-phase fluid (culture medium, cells and bubbles). Thus to find the best mixing conditions, we chose to inject NaOH at of 0.2 mol/L (final concentration of 0.02 mol/L in the microalgae suspension) at the different places in the injection system represented in red on the schematic representation in Figure 6a. After flocculation/flotation, the harvesting efficiencies were measured: results are presented in Figure 6b. They show that the best cell separation rates, of approximately 60%, are reached when NaOH is injected in the locations 3 and 6, where mixing is the most efficient. If NaOH is injected into the microalgae supply line, the harvesting performance is slightly higher if this injection is made at the outlet of the elbow (position 1), where recirculation phenomena occur because of the 90° bend located just upstream of position 1. However, these performances do not reach those achieved for a NaOH injection in the positions 3 and 6. On the pressurized water supply line, the further away the injection is from the confluence with the suspension feed, the lower the harvesting efficiency is. For instance, the harvesting efficiency is significantly reduced when NaOH is injected in the position 4; this can be explained by the low presence of microalgae in this pipe. The magnesium precipitate needs to be formed in the presence of the cells to best entrap them, thus explaining the lower separation efficiencies reached in the pressurized water supply line.

However in these experiments, the maximum harvesting efficiency obtained is of 60%. They were realized with a NaOH solution at a concentration of 0.2 mol/L: as showed before, best separation rates are obtained for diluted NaOH solutions, the best efficiencies being achieved in the

case of NaOH solutions of 0.1 mol/L (Figure 4c, 80% of cell separated from water). Thus, in order to optimize the NaOH concentration, using a NaOH injection in the position 3 in the injection system, the experiments were repeated with injected NaOH solutions of different concentrations (final NaOH concentration of 0.02 mol/L). The results are showed in Figure 7, where both the harvesting efficiencies obtained in batch-mode at laboratory scale and in continuous mode at pre-industrial scale are represented. It is clear on this graph that indeed, for a solution of NaOH of 0.1 mol/L, the separation rate reaches 80%, and decreases as the NaOH concentration increases. The interesting point is that for both harvesting modes (batch or continuous), the harvesting efficiencies are the same, thus showing the successful scale-up of our NaOH-induced flocculation/flotation process. Therefore, we could adapt an efficient injection system, allowing to separate in a single pass up to 80% of the cells from their culture medium at high scale, and concentrate them by a factor of 230, compared to approximately 20 in laboratory-scale experiments. This difference in the concentration factor is explained by the fact that in the continuous DAF system, the rotation velocity of the scrapper, which removes floated microalgae, and its vertical position in the tank can be tuned in order to adapt the residence time of the foam containing the microalgae at the tank surface. During this residence, the microalgae concentration increases in the foam, due to the liquid drainage by gravity, resulting in higher concentration factors compared to lab-scaled experiments.

4. Conclusions

We provide here an interdisciplinary multi-scale study to propose an efficient harvesting method for *D. salina* cells using flocculation/flotation, which can be used at industrial scales. Experiments at the nanoscale as well as simulations allowed first to precisely understand the complete mechanism of flocculation by addition of NaOH in the complex hypersaline medium in which *D. salina* grows. We thus brought strong scientific arguments proving that addition of NaOH in the medium creates a magnesium hydroxide precipitate that entraps the cell and flocculate them through sweeping. Because no other mechanism is involved, the formation of this precipitate, at low

speed, in the entire microalgae suspension, is determinant for the harvesting efficiency. Understanding this then led us to evaluate the influence of pertinent parameters to achieve high-efficiency harvesting, all related to the precipitation of $Mg(OH)_2$, that are the NaOH concentration in the medium, the agitation and the salinity of the medium. Our results, in laboratory-scale flocculation/flotation experiments, allowed us to show that the added NaOH solution had to be diluted, the agitation had to be optimal to bring the NaOH in the entire volume without breaking the forming flocs, and that too high salinities were resulting in magnesium hydroxide supersaturation phenomena. It is based on this understanding of the separation mechanisms and on the identification of the influence of different parameters on the harvesting performances that the transition of the process to the pre-industrial scale could be addressed from in an efficient way. For that we focused on the mixing efficiency at the injection site and the NaOH concentration injected to provide optimal parameters and achieve efficient microalgae harvesting at high-scale. Now further studies needs to be done to evaluate with precision the cost and energy consumption of this process at high-scale, and optimize better flocculation conditions to achieve effective harvesting at lower costs. Experiments have already been performed in this way, using slaked lime as a base to induce the precipitation of magnesium hydroxide. Our results so far show promising harvesting efficiencies, achieved at lower cost as calcite is less expensive than NaOH.

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Author contribution

P. G. conceived the project. A. B. and C. F.-D. conceived and performed the experiments. P. G., A. B. and C. F.-D. discussed and interpreted the results. C. F.-D. and A. B. wrote the manuscript. P. G., A. B., and C. F.-D. reviewed and contributed to the manuscript. All authors approved the final manuscript.

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689

690 **Figure captions**

691 **Figure 1. Schematic representation of the dissolved air flotation devices used.** (a) DAF device used
692 at the laboratory-scale and (b) at the pre-industrial scale (with the courtesy of Serinol). In (b), the
693 position of the NaOH line and needle valve depend on the injection site chosen to perform the
694 experiment.

695

696 **Figure 2. Simulating the influence of the addition of NaOH on the ionic equilibria in *D. salina***
697 **culture medium.** Phreeqc simulation of the pH and precipitates formation upon addition of NaOH in
698 the culture medium.

699

700 **Figure 3. Probing the interactions between *D. salina* cells and magnesium hydroxides.** (a) Histogram
701 representing the adhesion force distribution recorded on cells in sorbitol buffer at pH = 10 with bare
702 AFM tips, and (b) histogram representing the rupture distance distributions in these conditions. The
703 inset in (a) is an optical image of a *D. salina* cell and of the AFM probe; the inset in (b) shows
704 representative retract force curves obtained. (c) Histogram representing the adhesion force
705 distribution recorded on cells in sorbitol buffer at pH = 10 with $Mg(OH)_2$ functionalized AFM tips, and
706 (d) histogram representing the rupture distance distributions in these conditions. Inset in (d) shows
707 representative retract force curves obtained.

708

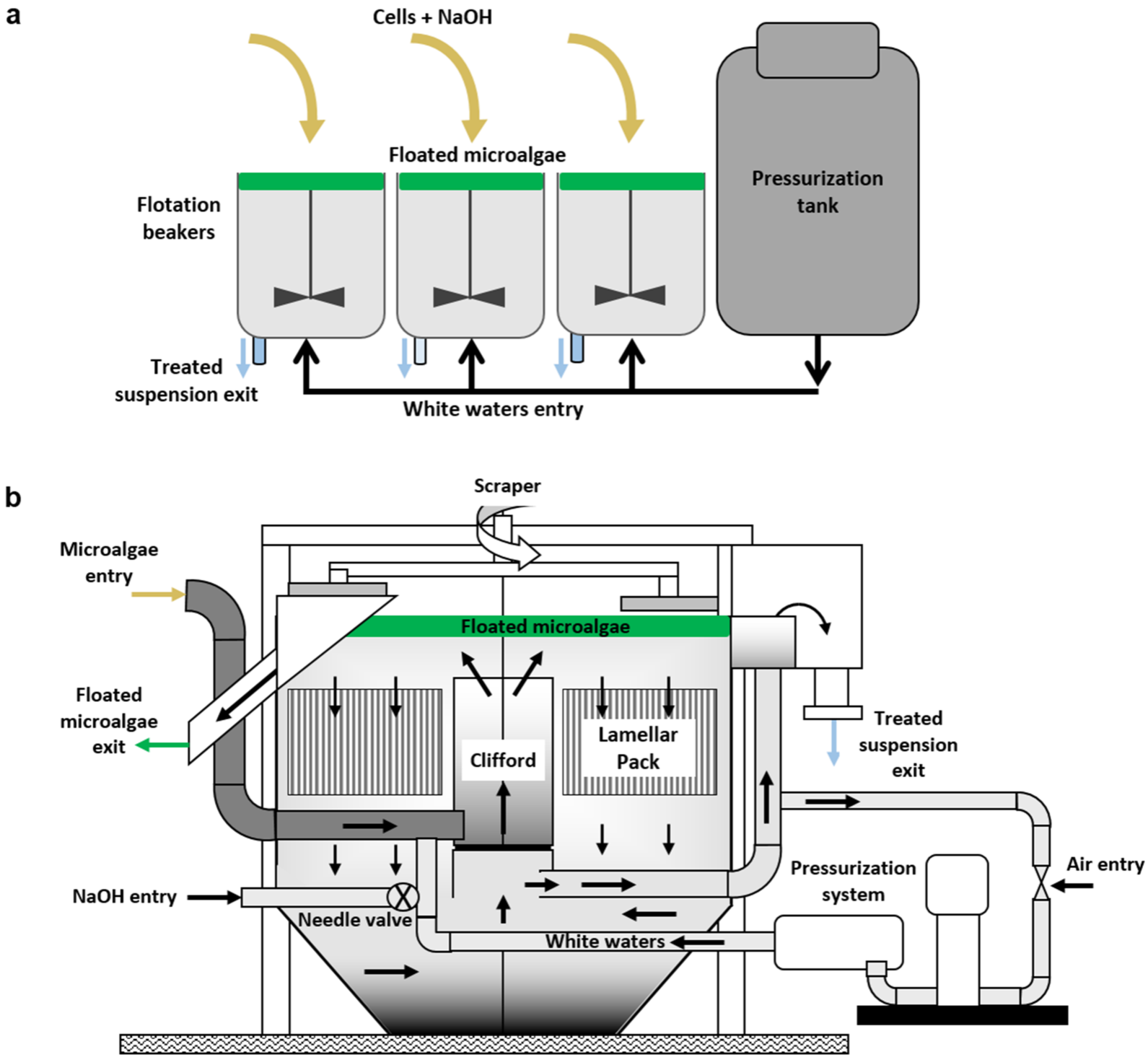
709 **Figure 4. Influence of the NaOH injection way on the harvesting efficiencies.** (a) Scheme
710 representing the principle of the two injection systems used. In each case, the same quantity of
711 NaOH is added, and the same final NaOH concentration in the microalgae suspension is reached. (b)
712 Flocculation/flotation harvesting efficiencies obtained for a final NaOH concentration of 0.02 mol/L
713 using the direct injection system (open symbols) or the diluted injection system (black-filled symbols)
714 and in both case a NaOH solution of 1 mol/L. (c) Flocculation/flotation harvesting efficiencies

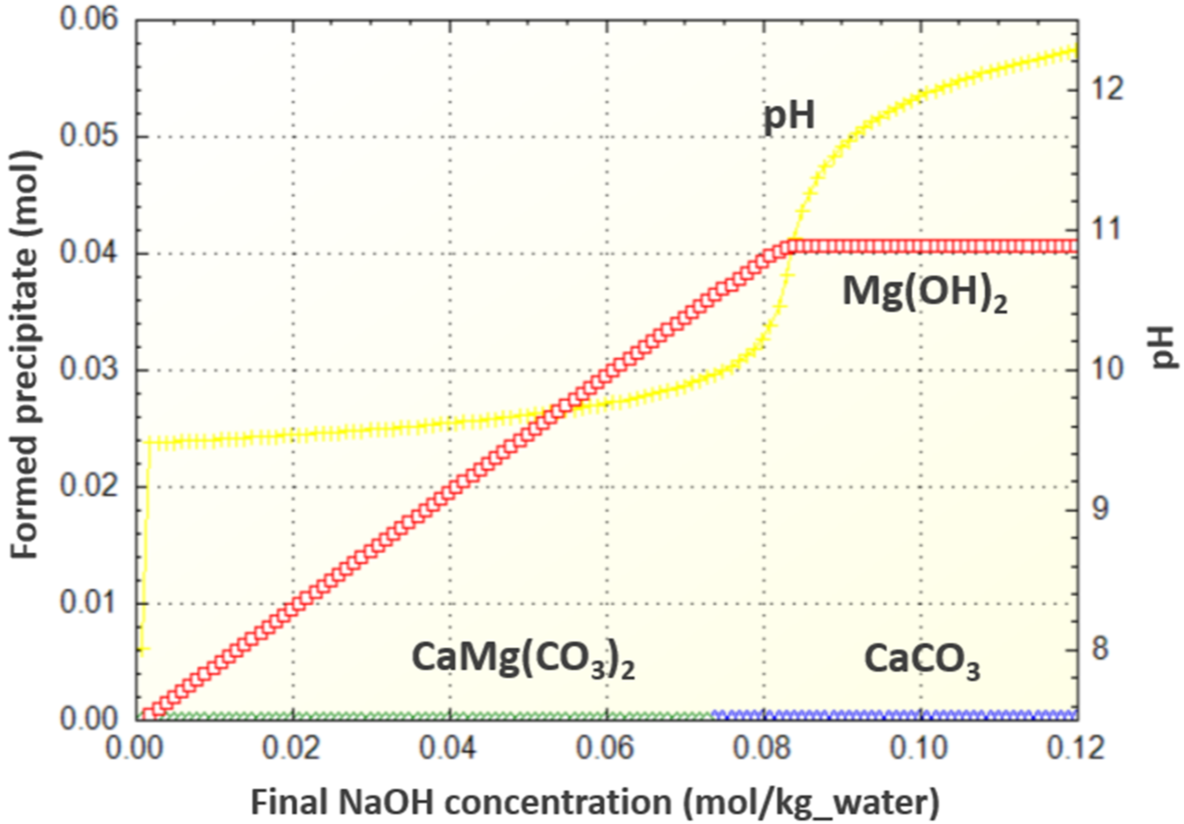
obtained for a final NaOH concentration of 0.02 mol/L using the diluted injection system and injected NaOH solutions of different concentrations.

Figure 5. Influence of agitation and salinity on the harvesting efficiencies. (a) Influence of the agitation speed on the harvesting efficiency obtained for a final NaOH concentration of 0.02 mol/L using the diluted injection system with a NaOH solution of 1 mol/L. Open diamonds symbols correspond to an agitation speed of 20 rpm, black-filled circles correspond to 40 rpm, and black-filled diamonds correspond to 80 rpm. (b) Influence of the salinity of the culture medium on the harvesting efficiency obtained for a final NaOH concentration of 0.02 mol/L using the diluted injection system with a NaOH solution of 1 mol/L. Cross symbols correspond to a salinity of 7.5%, open-squares correspond to a salinity of 12% and open-circles correspond to a salinity of 15.2%.

Figure 6. Adaptation of the NaOH injection system for high-scale harvesting. (a) Schematic and simplified representation of the injection system of the microalgae suspension and of the pressurized water into the industrial flotation unit. Red crosses represents the positions on the injection system where NaOH can be injected. The waters sued for pressurization are recycled from the flotation unit after cell recovery and may contain some microalgae. (b) Influence of the NaOH injection position on the flocculation/flotation harvesting efficiency obtained for a final NaOH concentration of 0.02 mol/L and a NaOH solution of 0.2 mol/L.

Figure 7. Influence of the injected NaOH concentration on the harvesting efficiency. Flocculation/flotation harvesting efficiencies obtained for a final NaOH concentration of 0.02 mol/L injected NaOH solutions of different concentrations. Results obtained in batch mode using diluted injection (open diamond symbols) or in continuous mode with NaOH injection in position 3 represented in Figure 5a (black-filled diamond symbols).


a
Cells + NaOH
Floated microalgae
Flotation beakers
Pressurization tank
Treated suspension exit
White waters entry
b
Scraper
Microalgae entry
Floated microalgae
Floated microalgae exit
Clifford
Lamellar Pack
Treated suspension exit
Pressurization system
Air entry
NaOH entry
Needle valve
White waters


0.06
0.05
0.04
0.03
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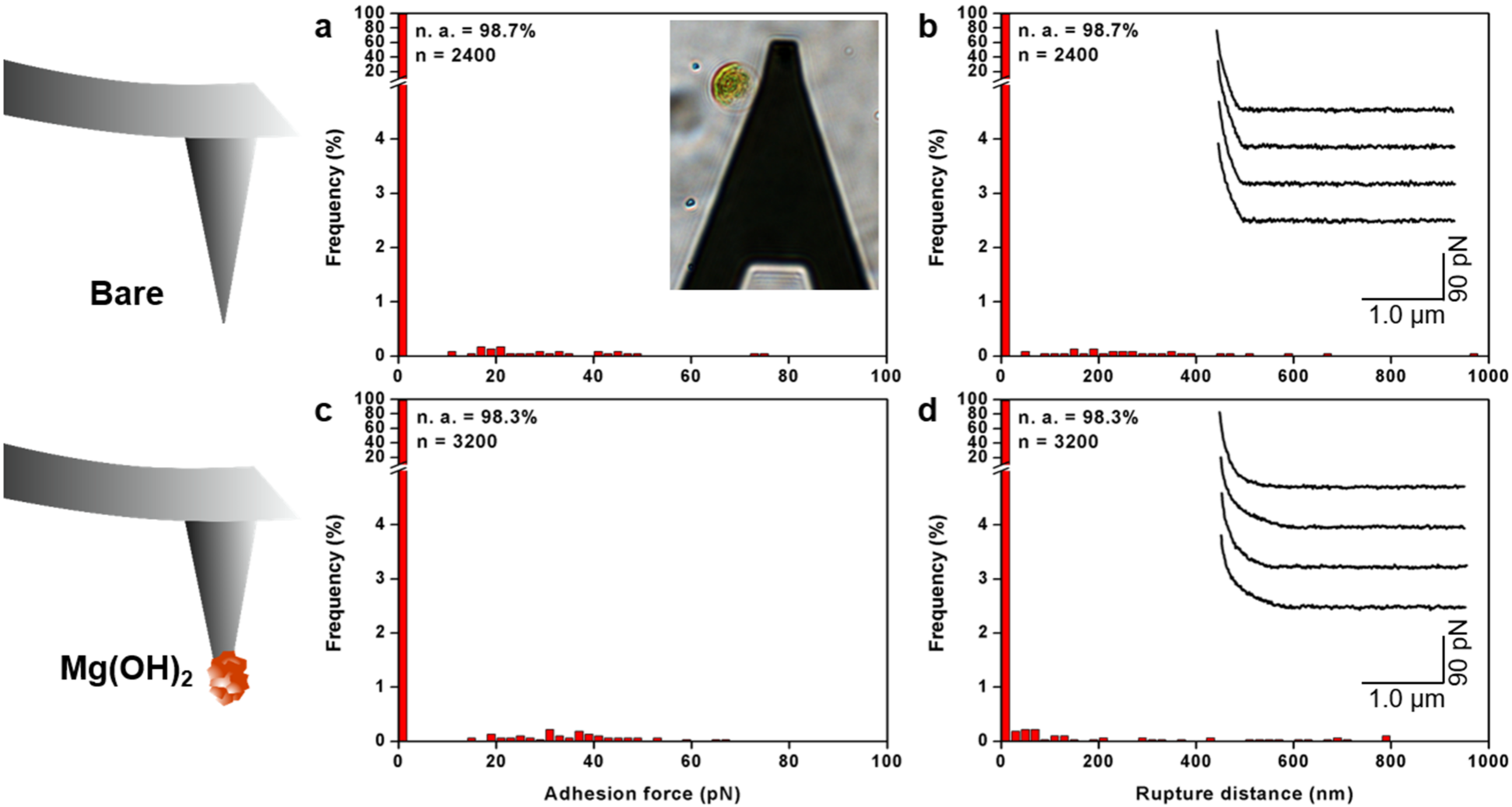
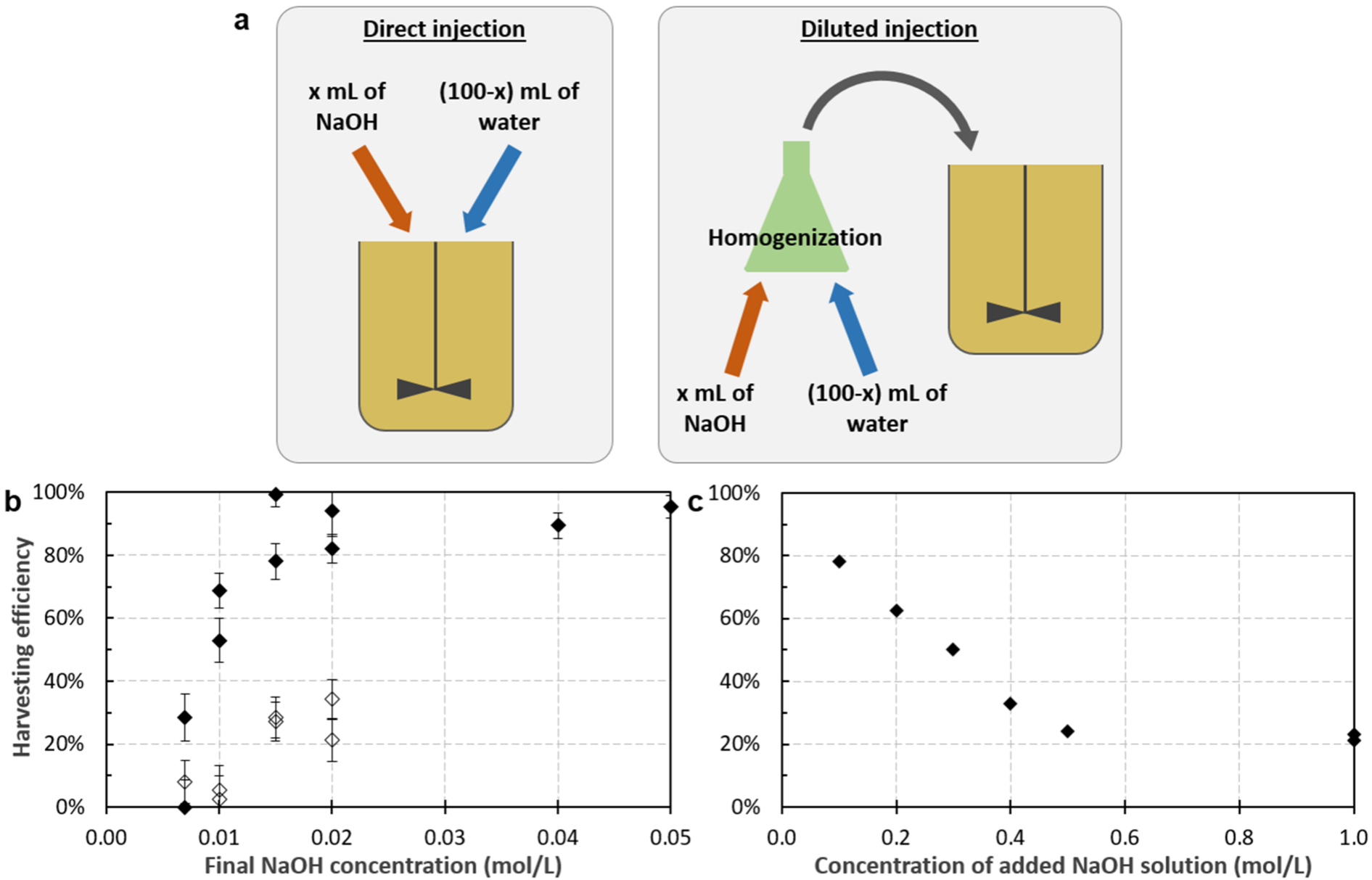
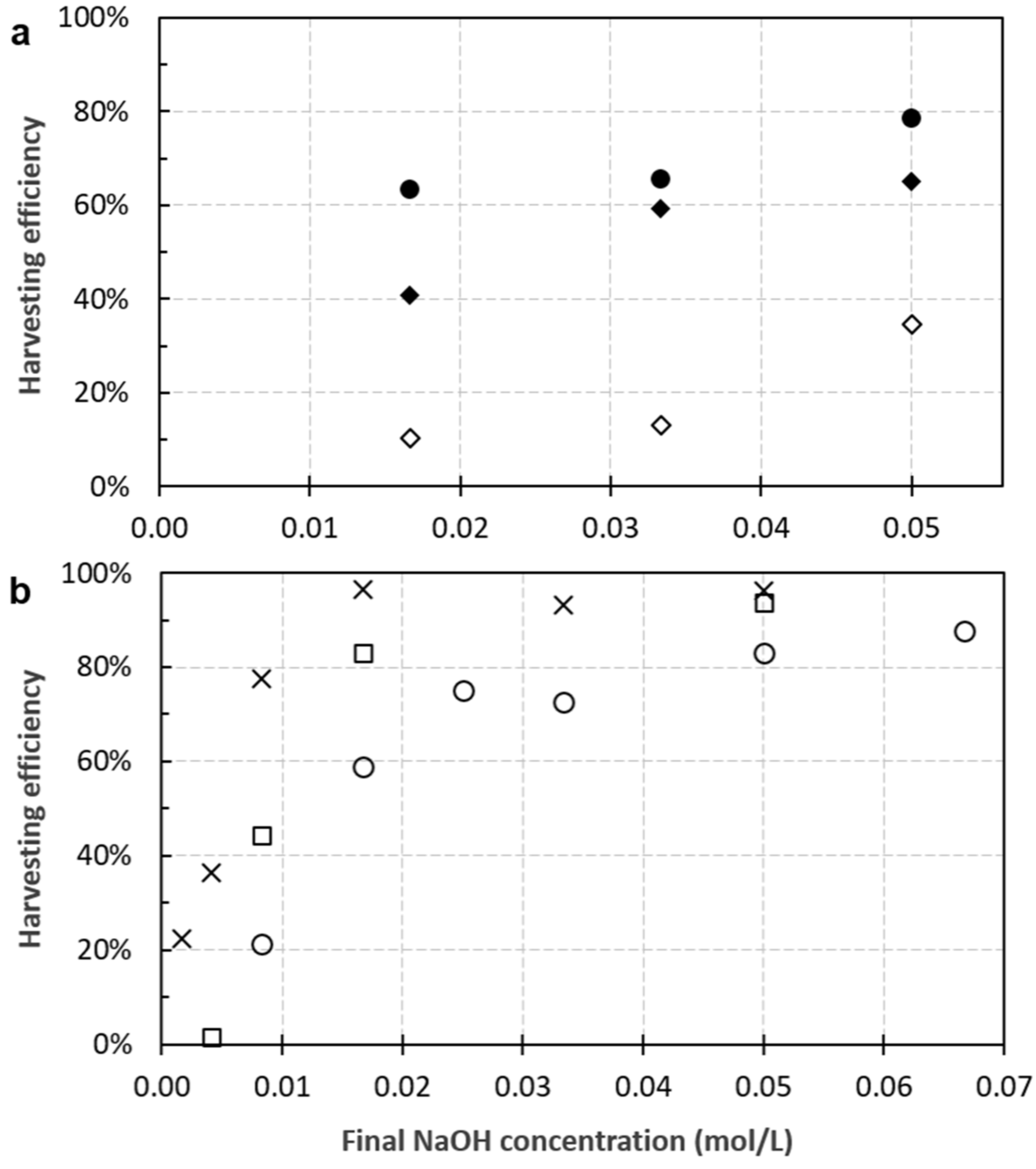
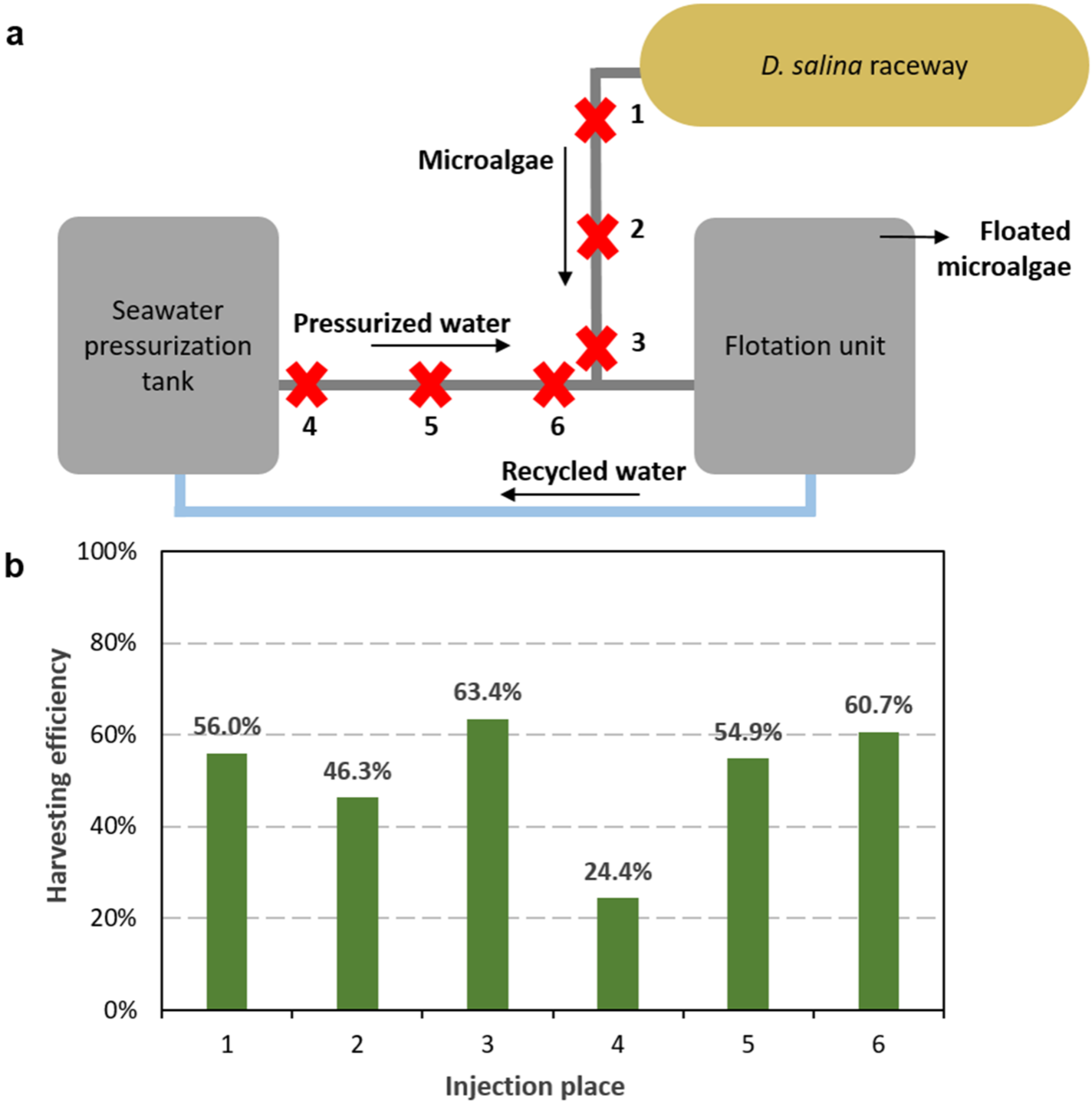
0.01
0.00
Formed precipitate (mol)
12
11
10
9
8
pH
pH
Mg(OH)2
CaMg(CO3)2
CaCO3
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Final NaOH concentration (mol/kg_water)


Bare
Mg(OH)2
a
n. a. = 98.7%
n = 2400
b
n. a. = 98.7%
n = 2400
90 pN
1.0 μm
c
n. a. = 98.3%
n = 3200
d
n. a. = 98.3%
n = 3200
90 pN
1.0 μm
Frequency (%)
Adhesion force (pN)
Rupture distance (nm)


a
Direct injection
x mL of NaOH
(100-x) mL of water
Diluted injection
Homogenization
x mL of NaOH
(100-x) mL of water
b
Harvesting efficiency
100%
80%
60%
40%
20%
0%
0.00
0.01
0.02
0.03
0.04
0.05
Final NaOH concentration (mol/L)
c
100%
80%
60%
40%
20%
0%
0.0
0.2
0.4
0.6
0.8
1.0
Concentration of added NaOH solution (mol/L)


a
100%
80%
60%
40%
20%
0%
Harvesting efficiency
0.00
0.01
0.02
0.03
0.04
0.05
b
100%
80%
60%
40%
20%
0%
Harvesting efficiency
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
Final NaOH concentration (mol/L)


a
D. salina raceway
1
Microalgae
2
Floated
microalgae
Seawater
pressurization
tank
Pressurized water
3
Flotation unit
4
5
6
Recycled water
b
100%
80%
60%
40%
20%
0%
Harvesting efficiency
56.0%
46.3%
63.4%
24.4%
54.9%
60.7%
1
2
3
4
5
6
Injection place

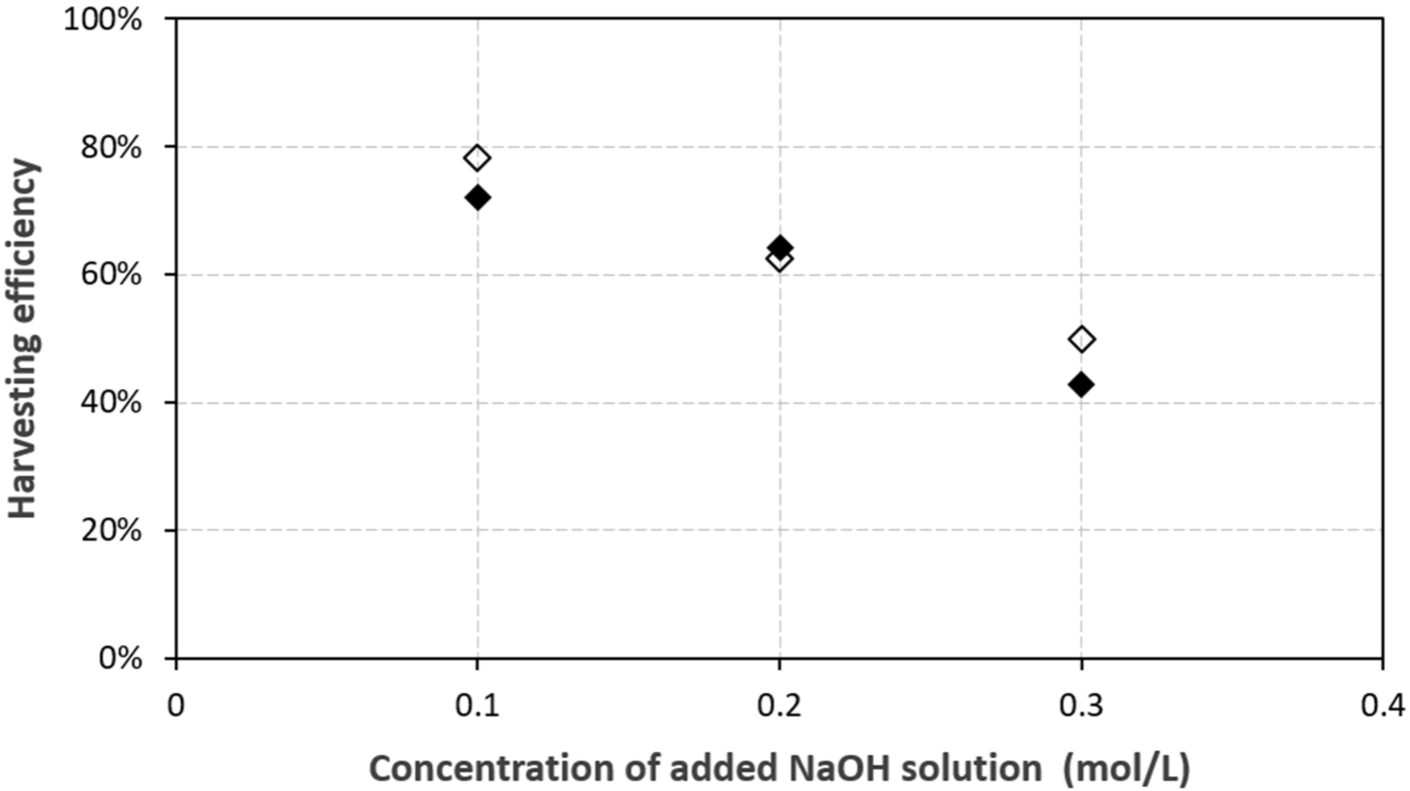

100%
80%
60%
40%
20%
0%
Harvesting efficiency
0
0.1
0.2
0.3
0.4
Concentration of added NaOH solution (mol/L)

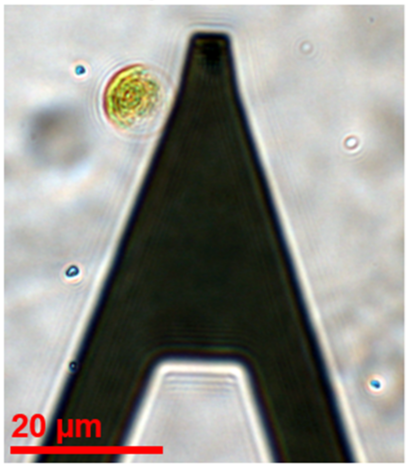
Table 1. Concentrations of the main ions in the culture medium used in g/L

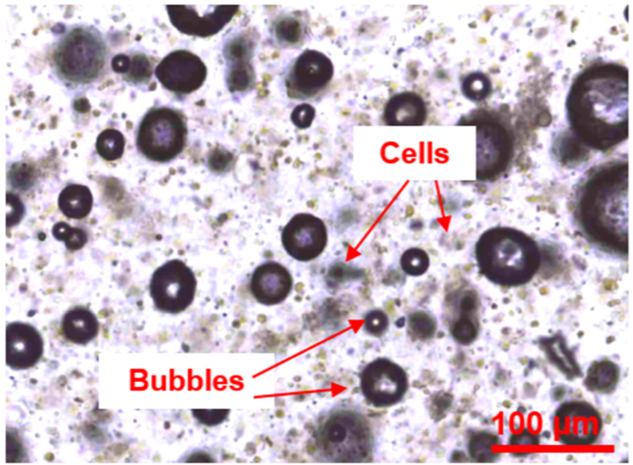
Na^+	Cl^-	SO_4^{2-}	Mg^{2+}	Ca^{2+}	K^+
42.12	67.08	1.87	0.95	0.39	0.04

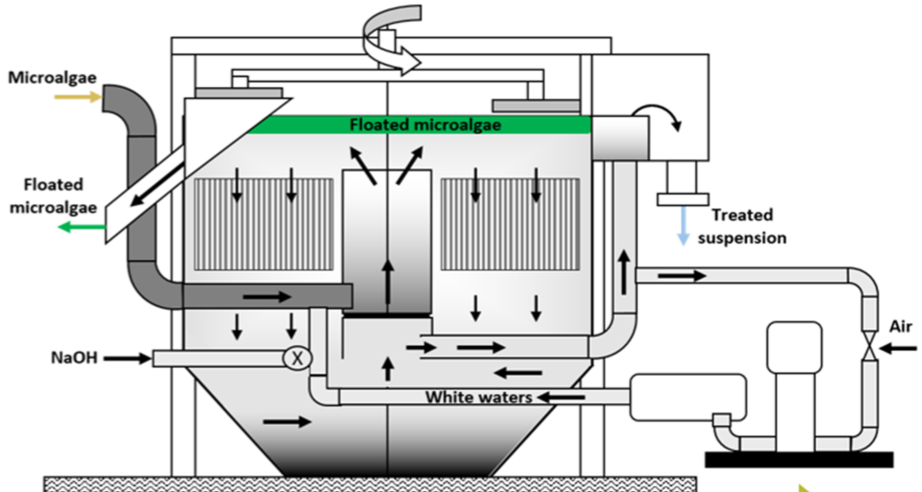
AFM to probe *D. salina* interactions with $Mg(OH)_2$ particles

Sweeping of cells and microbubbles during $Mg(OH)_2$ precipitation

Industrial harvesting of *D. salina* by flocculation/flotation







Molecular scale

Pre-industrial scale