A comprehensive analysis of an effective flocculation method for high quality microalgal biomass harvesting

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Abstract

Flocculation is a low-cost harvesting technique for microalgae biomass production, but flocculation efficiency is species dependent. In this study, we investigated the efficacy of two synthetic (polyacrylamide) and one natural (chitosan) flocculants against three algal species: the cyanobacterium *Synechocystis sp.*, the freshwater *Chlorella vulgaris*, and the marine *Phaeodactylum tricornutum* at laboratory and pilot scales to evaluate harvesting efficiency, biomass integrity and media recycling. Growth phase affected the harvesting efficiency of the eukaryotic microalgae. The flocculation was optimal at stationary phase with high flocculation efficiency achieved using polyacrylamides at 24 – 36 mg/g dry weight. The effect of the flocculants on the harvested biomass was investigated. The flocculated *Synechocystis sp.* showed a higher proportion of compromised cells compared to *C. vulgaris* and *P. tricornutum* likely due to differences in cell walls composition. Compromised cells could lead to the release of valuable products into the surrounding growth media during flocculation. The residual culture media was recycled once with no impact on cell growth for all treatments and algal species. The flocculation technique was demonstrated at pilot-scale using 350 L microalgal suspension, showing an efficiency of 82 – 90% at a polyacrylamide dosage of 6.5 – 10 mg/L. This efficiency and the biomass quality are comparable to the laboratory-scale results. Overall, results indicate that polyacrylamide flocculants work on a wide range of species without the need for pre-treatment. The information generated in this study can contribute to making the microalgae industry more competitive.

Keywords: Microalgae; Flocculation; Polyacrylamide; Chitosan; Biomass recovery; Biomass harvesting.

1 Introduction

Microalgal biotechnology applications are increasing in popularity, due to the demand for aquaculture, value-added products (e.g. astaxanthin and DHA) (Borowitzka, 2013; Fabris et al., 2020), their potential for biofuels (Kumar et al., 2020; Milledge, 2011; Su et al., 2017), and wastewater treatment (Gupta et al., 2019; Nie et al., 2020; Vo et al., 2019). Microalgae are a sustainable source of proteins, carbohydrates, lipids, pigments, and in some cases high-value secondary metabolites. Algae are phototrophs, meaning that they do not rely on external organic carbon addition and can grow rapidly in large-scale systems at relatively low costs (Fabris et al., 2020). However, there are still technological barriers for wide scale production of this feedstock. Harvesting in particular is an issue since algal cells are small (1 – 100 μm) and tend to grow in relatively dilute suspensions of up to 0.05% solids. The harvesting step currently can contribute up to 30% of the final production cost (Milledge and Heaven, 2013). There are various options for harvesting, including mechanical methods (e.g. centrifugation, filtration), sedimentation (e.g. gravity sedimentation), biological methods (e.g. bioflocculation or inducing autoflocculation), chemical methods (e.g. flocculation), or a combination of these (e.g. coagulation with electroflotation) (Gao et al., 2010; Milledge and Heaven, 2013; Ummalyma et al., 2017). The development of these methods to full scale application are hindered by a number of factors. For example, centrifugation requires high energy cost (Milledge and Heaven, 2013). Flocculation is a low-cost method that is commonly used in wastewater treatment. Flocculation is often combined with other steps, such as filtration or dissolved air flotation for microalgal biomass harvesting (Branyikova et al., 2018; Nguyen et al., 2019; Wang et al., 2019).

Ideally, a flocculant should be non-toxic, inexpensive, allow for media recycling, and be effective at low concentration across a wide range of algal species (Milledge and Heaven, 2013). Flocculation works by the addition of chemicals or alteration of the environment in

order to overcome the natural repulsion of the algae, allowing them to coalesce into aggregates, and thus fall out of solution (Vandamme et al., 2013). Flocculants can work through several mechanisms (e.g. charge neutralization, bridging) (Vandamme et al., 2013), and be highly versatile for a number of different algal species in various environmental and media conditions. There are several classes of flocculants currently in use, such as: inorganics (e.g. aluminium sulphate); synthetic polyacrylamide polymers; and bio-agent flocculants which come from a biological origin, such as chitosan (Vandamme et al., 2013). There is a possibility of combining biopolymers with metal salts to get increased synergistic effects compared each by itself (Vu et al., 2020). However, metal salts typically have potential detrimental effects on the product (e.g. cause cells to change colour) (Papazi et al., 2010), can linger in the harvested biomass, or may impact the recycled media (Rwehumbiza et al., 2012). Biological biopolymers, like chitosan, typically requires a certain pH level as well as being inefficient with some algal species (Divakaran and Pillai, 2002). There is significant interest in synthetic polymers, which can work at low dosages and with high efficiency, although there are still questions remaining as to how they function in different environmental conditions. The charge of the polymer can also impact the effectiveness of the flocculation (e.g. higher cationic degree leads to better flocculation) (Wang et al., 2019). Changes in operating conditions can have an effect on the optimal dosage or final flocculation efficiency of synthetic flocculants, which in turn can alter the overall cost savings that might be achieved (Fasaei et al., 2018).

The efficacy of any flocculant is subject to environmental conditions, such as salinity, pH, culture conditions and released organic matter by the algae. A lot of the flocculation studies have been done on freshwater species, and often marine systems are not as effective with the same flocculant (Uduman et al., 2010). The level of salinity has been shown to be one of the largest factors in flocculation efficiency (Pérez et al., 2016; Roselet et al., 2017, 2015). pH has also been shown to affect the efficiency of various flocculants, especially chitosan (Şirin et al.,

2012). This would increase the overall cost and complexity of using certain flocculants, as it would require pre-treatment of the culture to adjust the pH. Algal cell surfaces, as well as the type and amount of released organic matter, may alter during course of the algal growth cycle which can impact flocculation (Barofsky et al., 2009; Lee et al., 1998; Loftus and Johnson, 2019). This organic matter is predominantly hydrophilic with a negative zeta potential which can then interact with cationic polymers (Henderson et al., 2008). The levels of algal organic matter released by the algae have been shown to reduce the efficacy of both biopolymers and synthetic polymers, necessitating a larger flocculant dosage (Roselet et al., 2017; Vandamme et al., 2012). As such, the media and growth phase of the algae can affect the overall effectiveness and dosage required of any flocculant.

The type of flocculant used and the potential for media recycling can reduce the overall cost of production. For example, chitosan is almost five times more expensive (per kg) than polyacrylamides (Wu et al., 2015). Recycling the used growth media can save up to 80% of the water requirements of a system, in addition to reducing the required nutrient inputs (de Carvalho et al., 2019; Fret et al., 2017). To date, there is a rising interest on the impact of flocculants on the biomass quality and culture media recycling (Farooq et al., 2015; Granados et al., 2012; Rwehumbiza et al., 2012). Flocculants added to the algal suspension, can be difficult to remove from the growth media and be carried over to next culture cycle. In particular, polyacrylamides may leave traces of toxic acrylamide (Vandamme et al., 2013), with cationic polymers having the most long-term toxic effect on ecosystems (Beim and Beim, 1994). A previous study showed that neither chitosan nor a polyacrylamide impacted the regrowth of flocculated *Scenedesmus* cells, nor did the reused media have any negative effect on algal groups (Wu et al., 2015). However, this study only evaluated a single genus of green algae.

Our previous laboratory-scale study has evaluated the use of a cationic polyacrylamide, commonly used in wastewater, on the recovery of stationary phase freshwater green alga *Chlorella vulgaris* and the marine diatom *Phaeodactylum tricornutum.* Microalgal biomass recovery at ~98% was achievable at dosages as low as 18.9 and 13.7 mg per gram of dry *C. vulgaris* and *P. tricornutum* respectively, and that algae recovery was independent of pH. Charge neutralization was determined to be the primary mechanism, with a 64% and 86% change in the zeta potential for *C. vulgaris* and *P. tricornutum* observed at the optimal dosage (Nguyen et al., 2019). However, recovery of media and the effects of harvesting at different phases of the growth on the flocculation efficiency were not evaluated, nor was the impact on the quality of the biomass. Expanding on previous work, this study examines the effect of two high-molecular weight cationic polyacrylamide flocculants from SNF, as well as the low-molecular weight biopolymer chitosan, on the eukaryotic *C. vulgaris, P. tricornutum*, as well as the freshwater cyanobacterium *Synechocystis sp.* A dose-response experiment was performed to evaluate the optimal flocculation efficiency at various points in the growth cycle of the algae (early exponential, late exponential, and mid stationary). The impact of the flocculants on the algal cell membrane integrity (i.e. the harvested biomass quality), the effect of the flocculants on the recycled media used to regrow the same algae, as well as the ability to scale up to a large microalgal suspension (350 L) were investigated.

2. Materials and methods

2.1 Microalgae strains and growth conditions

Three strains were used in this study from different sources. The freshwater cyanobacteria *Synechocystis sp.* PCC6803 was obtained from the Pasteur Culture collection (Paris, France). The freshwater green alga *C. vulgaris* CS-41 was obtained from the Australian National Algae Culture Collection at CSIRO Microalgae Research (Hobart, TAS, Australia). The marine diatom *P. tricornutum* CCMP 632 was obtained from the National Centre for Marine Algae

and Microbiota (NCMA) (East Boothbay, ME, USA). *Synechocystis sp.* PCC6803 and *C. vulgaris* CS-41 were maintained in 0.22 µm filtered autoclaved freshwater MLA media (Algaboost; Wallaroo, SA, Australia), while *P. tricornutum* CCMP 632 was kept in marine f/2 media (Guillard, 1975) using 0.22 µm filtered autoclaved seawater collected from Sydney Harbour (salinity of 33-35 g/L). Stock cultures were maintained at the Climate Change Cluster (C3, University of Technology Sydney).

The microalgal cultures used for flocculation assay were prepared in three steps from 1 L Schott's bottles to pilot scale 350 L photobioreactor, as outlined in Figure 1. In step one, the stocks were sub-cultured to an early stationary phase in Schott bottles (1 L volume). In step 2, the resultant culture from step 1 was transferred to a 10 L photobioreactor. The photobioreactor was maintained in standard conditions for the algae at ~25 °C and ~100 µmol photons/m^2/s light in a 16:8 hour light:dark cycle and bubbled with air to an early stationary phase. In step 3, the culture in 10 L photobioreactor was used to inoculate a 350 L photobioreactor. The photobioreactor has a diameter of 65 cm, total liquid height of 135 cm. It was sparged with air through air lines on either side of the bioreactor (1 mm needle). It was illuminated with 6 cool white LED strips running the height of the bag, equidistantly placed surrounding the bag. The marine or fresh water for the large-scale bioreactor was first sterilized by addition of 100 mL of 12% sodium hypochlorite, followed by 100 mL of 2 M sodium thiosulphate. Filter sterilized stock solutions of MLA media for freshwater or f/2 media for marine water was then added, and the algal bioreactors were maintained in a temperature controlled room of ~23 °C and given ~400 µmol photons/m^2/s light in a 16:8 hour light:dark cycle. The bioreactors were sparged daily with CO_2 for up to 3 min / day to provide carbon and maintain a pH below 9.3. The growth of the algae was monitored daily by measuring the OD at 750 nm in a spectrophotometer (Agilent Cary 60; Santa Clara, CA, USA). The cells were grown in 350 L photobioreactor and

146 regularly sampled for the different analyses described below (technical replicates per bag with
147 one bag for each species).

148 Early exponential growth was defined as the period when cells have adapted to the new
149 environment and are beginning to grow and multiply. Late exponential or linear phase is
150 defined as the time when the growth of cells is no longer increasing exponentially, as they
151 become limited in light, carbon, or nutrients. Stationary phase is defined as the time when the
152 culture is no longer growing (e.g. substrates exhausted) (Richmond and Hu, 2013).

153 *2.2 Flocculation experiments*

154 Two high charge, high molecular weight cationic polyacrylamide flocculants FO 3801 and
155 FO 4808 (SNF Pty Ltd; Geelong, VIC, Australia) and one natural flocculant, chitosan (Sigma-
156 Aldrich; St. Louis, MO, USA) were used in this study. Flocculant solutions (0.4% v/v) were
157 prepared one hour before each experiment in Milli-Q water. The algal growth in 350 L
158 photobioreactors was monitored, and 200 mL of cells were harvested at early exponential, late
159 exponential and stationary growth phases (Table 1). Three samples of culture for each dosage
160 (200 mL) was immediately used for the flocculation assay without any adjustments. Different
161 polymer doses (1-200 mg flocculant/L culture), chosen based on previous work (Nguyen et al.,
162 2019) were added to the algae, and then mixed using a magnetic stirrer for 30 s. This was then
163 followed by a settling time to allow for stable flocs, which varied from 2 min for *C. vulgaris*
164 and *Synechocystis*, and 10 min for *P. tricornutum*. The polymer doses were subsequently
165 normalized against dry cell weight. After flocculation, an aliquot from half the height of the
166 200 mL bottle was taken for the evaluation of the flocculation effect using a UV
167 spectrophotometer at OD 750 nm.

The flocculation efficiency was calculated based on previously described methods (Nguyen et al., 2019; Roselet et al., 2015). Briefly, the change in the UV absorbance (i.e. optical density) was measured before and after each polymer addition, as shown in the following equation.

$$Flocculation\ efficiency\ (\%) = \left(\frac{Cell\ density_{\ i} - Cell\ density_{\ f}}{Cell\ density_{\ i}}\right) \times 100 \qquad \text{Eq. 1}$$

Where; Cell density $_{i}$ and Cell density $_{f}$ is the optical density of the culture before and after flocculant addition.

The biomass concentration was measured following the standard method. A 100 mL aliquot algal culture was filtered through a 0.22 µm pre-weighed glass fibre filter paper. The retained biomass on the filter paper was dried at 60 °C degree overnight. The weight of the final filter paper was used to calculate the dry microalgal biomass.

2.3 Flocculated biomass characterisation

2.3.1 Cell membrane integrity analysis

Cell membrane integrity of the flocculated biomass was tested in an endpoint assay using Celltox Green (Promega; Madison, WI, USA), as per the manufacturer's guidelines. In brief, 20 µL of the thawed 5x concentrated reagent was added to 80 µL of algal culture, incubated in the dark for 15 min, then 15 µL measured on a flow cytometer (FACS Cytoflex S, Becton, Dickinson and Company; Franklin Lakes, NJ, USA) using an excitation of 485 nm and emission at 520–530 nm. The flocculated biomass at stationary phase was used to test the cell membrane integrity. The microalgal cultures (500 mL) from the 350 L photobioreactor were collected. The optimal dosage of flocculants was used to flocculate the biomass. Only flocculants that achieved >75% flocculation efficiency were evaluated. For the two polyacrylamides, the dosages were the same for each species to compare and to remove any biases that may occur due to dose-dependent effect on cell membranes. The flocculated cells were then collected and stained along with two controls (i.e. live cells and "dead cells" after 3

min boiling). These controls were used to gate the populations according to the healthy cells and compromised cells using CytExpert v2.3 (Becton, Dickinson and Company).

2.3.2 Biochemical properties

In the similar fashion to Section 2.3.1, biomass was collected from 500 mL of flocculated culture and was used to characterise biochemical content in the cell. Gas chromatography–mass spectrometry (GC-MS) analysis was carried out to generate a chemical finger print mainly targeting primary metabolites such as sugars, organic acids, amino acids, fatty acids, sterols, etc. Extraction and analysis method were performed as reported by Lohr et al. (Lohr et al., 2019). Briefly, freeze dried biomass were extracted sequentially using 100% methanol and 50% methanol, pooled and derivatised using methoxylamine in pyridine followed by N-methyl-N-(trimethylsilyl) trifluoroacetamide. The derivatised samples (1 µl) were injected on a Shimadzu GCMS-QP2020 (Shimadzu Corporation; Kyoto, Japan) equipped with an AOC-20is autosampler (Shimadzu Corporation) and SH-Rxi-5Sil MS fused silica capillary column (30.0 m×0.25 mm×0.25 µm) operating in electron impact mode at 70 eV. Helium was used as the carrier gas at a constant flowrate of 1.0 mL/min and an injection volume of 1 µl, with an injector temperature of 280°C and an ion source temperature of 230°C. The temperature gradient of the oven was 70°C for 1 min, then increased by 7°C per minute to 325°C. The scan range was 50-600 *m/z*. Raw GC-MS data were pre-processed using XCMS for non-linear retention time alignment, matched filtration, peak detection and peak matching. The output peak list from XCMS comprising features (ions, retention time, and intensity) was further processed for area normalization using the total peak area of internal standards. Statistical analysis was performed using the normalized features with a unique m/z and retention time. The chemical profiles were compared by using principal component analysis using Mass Profiler Professional (Agilent).

2.4 Preparation of recycled media

The residual culture media after removing flocculated biomass in Section 2.3 was used as "recycled media" for subsequent microalgal culture. Only flocculants that achieved >75% flocculation efficiency were evaluated. The recycled media was filtered through a 0.22 µm filter. Triplicates of 100 mL were then transferred to flasks. The flasks were supplemented with stock culture media used for cultivation in Section 2.1. Microalgal stock culture was inoculated at 1/10 dilution in the flask (biological triplicates). Growth was monitored daily for 14 days by measuring OD 750 nm. The optical density was converted to cell density through the calibrated standard curve (Eqs 2-4).

$$Synechocystis\ sp.\ cell\ density\ \left(\frac{cells}{mL}\right) = 29595643 * OD\ 750\ nm \quad (R^2 = 0.99) \quad \text{Eq. 2}$$

$$C.\ vulgaris\ cell\ density\ \left(\frac{cells}{mL}\right) = 3316827 * OD\ 750\ nm \quad (R^2 = 0.98) \quad \text{Eq. 3}$$

$$P.\ tricornutum\ cell\ density\ \left(\frac{cells}{mL}\right) = 280888 * OD\ 750\ nm \quad (R^2 = 0.97) \quad \text{Eq. 4}$$

On day 14, half of the algal samples (50 mL) were centrifuged at 4000 rpm for 10 min, and the biomass collected for the cell membrane integrity assay. The other half of the algal cultures were filtered through pre-weighed 0.22 µm filters, dried in a 60 ºC oven overnight, and the dry cell weight determined. In addition, spent medium collected (centrifuged at 4000 rpm for 10 min) from a "non-flocculated" culture was used to grow the control algal sample. Data were processed using Prism v8.0 (GraphPad; San Diego, CA, USA). Statistical significance was determined using a two-way ANOVA and Tukey test.

2.5 Pilot-scale demonstration of microalgal harvesting

The best flocculant was identified from the above methods and used to evaluate its performance in a pilot-scale 350 L photobioreactor. The flocculant was added and mixed through heavy sparging of air for 5 min and settled in 10 min for *Synechocystis sp.* and *C.*

vulgaris, and 30 min for *P. tricornutum*. The OD 750 nm was then measured from a sample taken midway up the photobioreactor. To test the stability of the flocs, the bag was allowed to sit for 24 hrs, and the OD measured again at that point.

3. Results and discussion

3.1 Flocculation efficiency

The effectiveness of the flocculants was dependent on growth stage, which was particularly evident for the eukaryotic algae. The dose-dependent responses of the flocculants indicated that at stationary phase the polyacrylamide flocculants could achieve high flocculation efficiencies (>90%) at relatively low dosages (25-35 mg/g algae) for the three tested microalgae and cyanobacterium, while chitosan was only effective with the cyanobacterium, and at much higher dosage (250 mg/g algae) (Figure 2).

The flocculant FO 3801 was most effective at concentrations between 22.7 – 31.25 mg/g (which correlates to 0.5 – 5 mg/L culture depending on growth phase) for the cyanobacterium *Synechocystis sp*., regardless of growth stage, with the highest flocculation efficiency of 98.9% achieved during stationary (Figure 2A). In contrast, for the eukaryotic algae, the flocculant was less effective during early exponential phase. *C. vulgaris* required 277.8 mg/g to achieve a maximum 59.2% flocculation at early exponential. 92.3% flocculation was achieved at late exponential for the green alga, but required 96.9 mg/g of flocculant, compared to only 34.5 mg/g to achieve similar levels of flocculation at stationary (corresponding to 14 mg/L at late exponential compared to only 8 mg/L during stationary) (Figure 2B). This effect was most pronounced for *P. tricornutum*, where the flocculant could not flocculate the algae during its early exponential phase, and only achieved a maximum of 82.3% during late exponential with

a dosage of 24.7 – 37.0 mg/g (or 4 – 6 mg/L). Only at stationary was an optimal flocculation achieved above 90%, with a dosage of 35.7 mg/g algae (or 6 mg/L) (Figure 2C).

A similar effect was seen for the flocculant FO 4808, which differs only by having a differing R group to FO 3801. A similar range of effective dosages, independent of growth stage, was noted for the cyanobacterium *Synechocystis sp.*, with maximum dosages ranging from 22.7 – 31.3 mg/g (or 0.5 – 5 mg/L) for a maximum flocculation efficiency of 98.5% at stationary (Figure 2D). For *C. vulgaris* during early exponential phase, a maximum flocculation efficiency of 64.2% was achieved with a dosage of 833.3 mg/g algae (or 30 mg/L), while late exponential achieved at least 90% but with a dosage of 110.7 mg/g algae (or 20 mg/L). It was only when the culture was at stationary phase that it achieved 98.4% flocculation with a lower dosage of 25.9 mg/g (or 6 mg/L) (Figure 2E). *P. tricornutum* was unable to flocculate above 50% at early exponential growth phase (with tested range up to 200 mg/L), and only achieved a maximum of 77.5% flocculation at late exponential, at a dosage of 12.4 mg/g (or 2 mg/L). It was only during stationary growth phase that >90% flocculation was achieved, at a dosage of 23.8 mg/g (or 4 mg/L) (Figure 2F).

Chitosan was able to effectively flocculate the cyanobacterium *Synechocystis sp.*, but unlike the two polyacrylamide flocculants, the efficiency was linked to the growth phase and/or pH (Figure 2G). In early and late exponential growth, the optimal flocculation of up to 98.8% was achieved with dosage of 833.3 and 826.5 mg/g algae (or 50 and 100 mg/L for early and late exponential respectively). However, in stationary phase, chitosan effectively flocculated above 90% at 250 mg/g (corresponding to 40 mg/L). Chitosan could not effectively flocculate *P. tricornutum* at all, and could not flocculate *C. vulgaris* during exponential phase, only achieving a maximum of 74.0% with 258.6 mg/g (60 mg/L) during stationary phase (Figure 2H& I).

Chitosan has previously been shown to be effective for several algae, including marine diatoms and freshwater green algae (such as *Chlorella*), but at high concentrations (>40 mg/L), low pH, and low salinity. In fact, there is a direct correlation between efficacy and pH, with the optimal pH around 7 – 8 (Gerchman et al., 2017; Morales et al., 1985; Pérez et al., 2016; Zhu et al., 2018). While the algal cultures were sparged daily with CO_2 to provide the necessary carbon for growth and keep the pH at an acceptable range, as expected the exponentially growing algae had a higher pH (>9) as they more rapidly consumed the carbon and raised the pH. The stationary phase algal cultures all had pH below 9 (Table 1). This could account for the difference in flocculation efficiency between the different growth phases, as chitosan efficiency rapidly drops off after pH 9. However, no difference was observed in efficacy with differing pH using the polymer FO 3801 in a previous study with *C. vulgaris* and *P. tricornutum* (Nguyen et al., 2019). Autoflocculation of certain algal species can occur with a substantial increase in pH, which has been observed with *C. vulgaris* and *P. tricornutum*; however, that required a pH > 10 (Wu et al., 2012). As such, it is unlikely to be the major factor accounting for the differences in flocculation efficiencies observed for the polyacrylamides.

Different species have different metabolite secretions or cell surface properties, which can impact their susceptibility to various flocculants (Barros et al., 2015). Algal cells secrete organic matter as they grow, including polysaccharides and proteins, which can impact the efficacy of the flocculation (Henderson et al., 2008). However, secreted organic matter can often decrease the overall flocculation efficiency and increase the dosage demand up to 7 to 9 fold for marine and freshwater microalgae (Chen et al., 2009; Garzon-Sanabria et al., 2013; Vandamme et al., 2012). Instead, in this study a decrease in dosage demand was noted with more dense, stationary phase cultures for *C. vulgaris* and *P. tricornutum*. Cell density is certainly a factor in lowering dosage, as it can indicate a higher collision rate (Gerde et al., 2014; Schlesinger et al., 2012). Diluting a dense culture has been shown to have limited to no

311 effect on biomass flocculation using the same flocculant (de Godos et al., 2011). Cell size could
312 also play a role, as that may impact the cell surface area available to the flocculant (Branyikova
313 et al., 2018). *Synechocystis sp*. is a small prokaryote (~ 2 µm), while *C. vulgaris* is a larger
314 eukaryote (2 – 10 µm), with *P. tricornutum* the largest among the three (~10 µm). In addition,
315 cell surface properties could change depending on the growth stage. The zeta potential was
316 shown to decrease in stationary phase compared to exponential phase for the green algae
317 *Botryococcus sp.*, *Chlorella* spp., and *Desmodesmus bijugatus* (Xia et al., 2017; Zhang et al.,
318 2012), along with reduced cell surface functional groups (Zhang et al., 2012). This led to
319 similar results in those studies, whereby a lower dosage was required at stationary phase algae
320 compared to exponential phase. Generally, the metabolites of interest, including lipids, are
321 produced in late exponential/stationary phase (Salim et al., 2013; Tonon et al., 2002), which
322 correspond to the time when a lower dosage of flocculant per gram of algae is required. One
323 aspect that would need to be reviewed however is the effect in continuous culture. Escapa et
324 al. (2015) found that the flocculation efficiency during steady state of a semi-continuous culture
325 was lower than for batch cultures in stationary phase of *Chlorella sorokiniana* with a polymer.

326 It is interesting to note that there is less difference in optimal dosage and efficiency rates
327 between the different media types than expected for both the polyacrylamide treatments. The
328 range of optimal dosages required, and the final flocculation efficiencies achieved was
329 comparable between the different algal species (varying by 10 mg/g algae, rather than by orders
330 of magnitude). Previous studies have indicated the saline systems require a higher dosage of
331 flocculant (e.g. 5 – 10x higher) compared to the freshwater algae (’t Lam et al., 2016; Bilanovic
332 et al., 1988; Pérez et al., 2016; Roselet et al., 2017; Sukenik et al., 1988). A previous study
333 looking at 25 different natural and synthetic cationic polymers found that while *C. vulgaris*
334 was efficiently flocculated by a wide range of flocculants, only a low molecular weight natural
335 polymer (Tanfloc) was most effective with the marine eustimatophyte *Nannochloropsis*

oculata (Roselet et al., 2015). One difference worth noting that for all flocculants was that *P. tricornutum* took a longer settling time of 10 min compared to 2 min for the other algal species tested. This could be due to the salinity of the media, difference in cell morphology (the diatom has a silica cell wall that could impact flocculation), or some other factor, that could warrant further research. However, there is promise in the use of the polyacrylamide polymers tested here, which were effective for different algal groups in different media at relatively similar dosages.

3.2 Quality of the flocculated biomass

3.2.1. Cell membrane integrity

The polyacrylamide flocculants negatively altered the cell membrane integrity of the cyanobacterium, while only having a minor effect on the eukaryotic microalgae, when compared to control biomass (i.e. without flocculation) (Figure 3A). The obtained data suggested that polyacrylamide flocculants can be used to efficiently harvest *P. tricornutum* and *C. vulgaris* with minimal impact on cell membrane and thus biomass quality. Flocculation methods are developed to harvest microalgal biomass without impacting the cells, as the product of interest is often intracellular (e.g. proteins and lipids). Preventing cell membrane damage during harvesting avoids the leakage of the compounds of interest into the growth medium, assuring overall yield and quality of the biomass harvested. Surprisingly, the percentage of compromised cells seemed inversely proportional to the change in biomass composition. For instance, *C. vulgaris* showed the lowest proportion of cells with compromised membranes, but the highest changes in biomass composition compared to the control (Figure 3). This does not necessary infer that compounds are being released into the growth medium, further studies should investigate the composition of the medium before and after harvesting.

Cationic polyacrylamide and chitosan altered the cell membrane integrity differently across three species. For example, cell membrane of the *Synechocystis sp*., was significantly impacted by polyacrylamide (between 60 – 70% at dosages of 31.3 mg/g), while chitosan (at 375 mg/g) compromised the membrane of 23.6% of the cells in the total population. The eukaryotic microalgae *C. vulgaris, and P. tricornutum* had between 14.0 – 19.3% compromised cells in the presence of the polyacrylamides (at dosages of 31.3 – 34.5 mg/g algae). It is also worth noting that the control biomass (i.e. without flocculation) showed 2.0 to 5.6% compromised cells in the total population, which correspond to the background dying cells. The percentage of compromised cells after flocculation include the background dying cells, suggesting that the actual percentage of compromised cells due to flocculation is slightly lower.

The difference in cell surface property could account for the differences in membrane integrity observed. Cyanobacteria have a cell membrane with peptidoglycan architecture, while eukaryotic microalgae have carbohydrate rich cell walls with a large diversity of cell wall components, which can affect their resilience to various stressors (Popper et al., 2011; Popper and Tuohy, 2010). The polyacrylamides in this study did result in increased cell membrane damage to the cyanobacterium. Cyanobacteria can contain toxins, which affect wastewater, drinking water and, in this case, the recycled media if released from the cells. Hence, previous studies looking at the effect of flocculants on algal cell integrity tend to focus on cyanobacteria. These studies have generally found that using alum did not damage the cells (Sun et al., 2013, 2012), while other common water treatments such as copper sulphate, chlorine, and ozone decreased cell membrane integrity, although without an accompanying release of toxins (Fan et al., 2014). Further testing is required to determine the potential effect of this on any value-added products in cyanobacteria, or its further use in wastewater systems. A previous study on various eukaryotic microalgae and a non-ionic polymer showed similar effects, whereby the flocculated cells were indistinguishable from control cells (Knuckey et al., 2006). A green alga,

Scenedesmus, was able to be recultivated in fresh media after flocculation with the polyacrylamide, indicating limited effect on the cells themselves by the flocculant (Wu et al., 2015). Neither of these studies directly assessed the membrane integrity, nor did a direct comparison of the same flocculant on different species. As such, the analysis here provides unique insight about the effects of the different flocculants on different types of algae.

3.2.2 Composition of biomass

The flocculants affected the chemical profile (mainly primary metabolites) of the cells differently, with *C. vulgaris* being the most affected while *P. tricornutum* composition stayed mostly unchanged compare to the control (Figure 3B-D). Although the two cationic polyacrylamide flocculants have similar chemical structure, they seem to affect cell composition differently.

Chitosan did not cause any significant difference in the chemical profile of *Synechocystis sp*. when compared to the control (Figure 3B). Chitosan also caused significantly less damage to the cell membrane of the cyanobacterium (Figure 3A), which may be a factor in this. FO 3801 affected the metabolite composition of both *Synechocystis sp.* and *C. vulgaris*, while FO 4808 affected only *C. vulgaris* (Figure 3B-C). In comparison, the flocculants did not have a significant effect on the chemical profile of *P. tricornutum* (Figure 3D). This indicates that the type of flocculant, even those relatively similar (i.e. two cationic polyacrylamides) can cause different changes to the cell composition, which may affect downstream processing. Additionally, *C. vulgaris* showed the lowest amount of compromised cells but the highest shift in cell composition after flocculation (Figure 3A, C). A decrease in cell membrane integrity may lead to metabolites leaching out of the cell into the supernatant, resulting in overall change in cell composition. Both eukaryotic species had similar levels of cells with compromised membranes in their population (Figure 3A). However, cell membrane integrity and cell

composition data did not seem to correlate. For instance, the cell membrane integrity of *Synechocystis* sp. was most affected by FO 4808, while it only slightly affected the cell composition compare to the control (Figure 3A-B). However, differences in the cell wall of the microalgae (the diatom has a silica cell wall) may account for differences in the chemical profiles. Further analysis is required to see if the difference is most likely due to 'leakage' of products during flocculation, or other factors.

Some studies have indicated that the choice of flocculant can impact the quality of the product, such as final lipids recovered or suitability as aquaculture feeds (Borges et al., 2011; Knuckey et al., 2006; Lee et al., 1998), which in turn can affect the overall production cost. A polyacrylamide flocculant had limited effect on *Scenedesmus* total lipid, carbohydrate, and protein content (Wu et al., 2015). Chitosan did not affect the lipid content of *C. vulgaris* (although aluminium sulphate did decrease the lipid content) (Zhu et al., 2018). No difference in lipid amount was found for *Nannochloropsis oculata* or the marine diatom *Thalassiosira weisflogii* when flocculated with a cationic polyacrylamide. However, the fatty acid profile was affected (e.g. higher C20:5 compared to the control), with a larger change noted in the diatom. This was hypothesized to be due to the flocculant interacting with lipids and secreted algal matter (e.g. attracting the secreted matter), thereby altering the profile of the harvested biomass (Borges et al., 2011). These studies investigated specific areas of interest (e.g. lipids), compared to the untargeted approach used in this study, which indicates an overall shift in the profile. Further analysis is required for detailed understanding of what has shifted, and what, if anything, the impact is on downstream processing and final product.

3.3 Algal growth in recycled media

3.3.1 Algal growth rates in recycled media

One caveat with using polyacrylamides is their potential for leaving a residual toxic acrylamide residue in the waste stream (Beim and Beim, 1994; Vandamme et al., 2013). However, the residual flocculants in the recycled media did not affect the growth rate of freshwater algae and only slightly altered the growth of the marine diatom, *P. tricornutum* (Figure 4).

In general, there was no detrimental effect noted on the growth rates of the different algal species using the different flocculants (Figure 4A-C). *P. tricornutum* only had two days where a significant difference (post-hoc Tukey's HSD test, $P < 0.05$) was observed against the control: day 3 with FO4808 and day 12 with FO3801. *Synechocystis sp.* and *C. vulgaris* showed no significant difference in growth levels between the different recycled media used, nor was there any difference in the final amount of biomass harvested (data not shown).

The lack of any deleterious effect on algal cells cultivated on recycled media is promising for any economic analysis of a system using these flocculants, as recycling water can reduce the overall cost of production (Fasaei et al., 2018; White and Ryan, 2015). Other systems have investigated the use of pH change for flocculation (which is highly dependent on species), and found limited effect of the recycled media on subsequent growth (Wu et al., 2012), or the use of $FeCl_3$ which seemed to have a slight boosting effect on biomass grown on recycled media (Farooq et al., 2015). A polyacrylamide tested against two species of the green algae *Scenedesmus* did show that one species was slightly more affected than the other (with slight reduction in growth) (Wu et al., 2015). The results from this study would indicate that the polyacrylamides tested (and chitosan where applicable) do not leave behind enough of a residue to impact recycled media, with no toxic effect noticed on subsequent batches of culture.

453 3.3.2 Cell membrane integrity of algae grown on recycled media

454 Since the flocculants had an effect on the cell membrane integrity (Figure 3A), the stationary
455 phase cells were harvested to determine if the recycled media had any lingering effect on the
456 cell membrane (Figure 4D). There was no significant difference between treatments for
457 *Synechocystis sp*., with compromised cells accounting for 19.5 – 20.4% of the population,
458 regardless of media. However, an interesting trend was observed for the eukaryotic algae. The
459 number of compromised cells in the media that had been flocculated with polyacrylamides was
460 4 times lower than the control media for *C. vulgaris*, and halved for *P. tricornutum*, indicating
461 that there was an overall reduction in the number of compromised cells in those systems. This
462 could be due to the fact that initial lingering trace amounts of the flocculant during early growth
463 caused some cells to floc and fall out of solution (before day 10), and as such removed
464 unhealthy cells from the system. Alternatively, cells could sense the remaining flocculants and
465 adapt their metabolism to limit damage to their cell membranes. This could potentially explain
466 the longer lag phase and the overall reduced percentage of cells with compromised membranes
467 when compare to Figure 3A, however, further investigation is needed to explain this
468 phenomenon.

469 *3.4 Performance of flocculation at the pilot-scale*

470 The assessment of flocculant FO 3801 at pilot-scale showed an efficiency of 82.7 – 87.5%
471 for the three species at dosages below 10 mg/L (Table 2, Suppl. Figure S1-3, Suppl. video). FO
472 3801 was chosen because of its high effectiveness and low impact on final cell membrane
473 integrity. The dose-dependent responses of the flocculants have been tested at a small scale
474 (200 mL with >94% flocculation efficiency) where homogeneity within the culture can be
475 easily achieved. Large scale flocculation systems generally have between 70 – 90% efficiency
476 (Branyikova et al., 2018), but often require over 10 mg/L of flocculant (Pugazhendhi et al.,
477 2019). The relative speed and subsequent stability of the flocs is noteworthy, as previous

studies have indicated that it could take up to 60 min for flocs to stabilize in other large sized system (Chen et al., 2013) compared to the 15-35 min observed in this study for the 350 L system.

To evaluate whether the flocs would break up over time, the flocculation efficiency was measured again after 24 hrs. At this time, the large flocs had either risen to the surface or sunk to the bottom, thereby increasing the flocculation efficiency in the middle of the bag, with the exception of *C. vulgaris*, where smaller flocs seemed to have broken off from the large aggregates, thereby decreasing the flocculation efficiency slightly.

3.5 Environmental implications

There are still several questions to be answered before selecting the optimal flocculant, such as potential accumulation and long-term effects. However, these polyacrylamides have been demonstrated to form stable flocs, with limited impacts on the cell membrane and media recycling (for the eukaryotic cells). The dosage is also significantly lower compared to chitosan (40 – 60 mg/L culture for chitosan compared to 6.5 – 10 mg/L for polyacrylamides). Assuming chitosan costs A$20/kg, compared to A$4/kg for polyacrylamides (Australia and China pricing) (Wu et al., 2015), flocculating a 1000 L system would cost under A$0.04 with polyacrylamides, compared to over A$0.80 with chitosan – resulting in savings of at least 20x folds. Low dosages are required for the polyacrylamide, meaning there is less residue left in reused water, affecting overall water usage. Up to 6.4 m^3 / m^2 / year water is required for open pond microalgal cultivation (Guieysse et al., 2013). Recycling water would decrease the water demand, thus lowering ecological impact of microalgal cultivation and cost. Utilizing new water for each culture could make up to 2% of the final production cost depending on the geographical location of the microalgal facility (Panis and Carreon, 2016). The polyacrylamides would therefore lead to cost savings in production of microalgae, without sacrificing biomass quality.

4. Conclusion

Two cationic polyacrylamide flocculants and one natural bioflocculant were investigated for harvesting different microalgae and cyanobacterium species, during different phases of their growth. Stationary phase showed to be the best time in the growth cycle for efficient flocculation. The dose-response relationship indicated that between 24-36 mg of cationic polyacrylamide flocculants per g of algae was required at this growth phase and led to 83-90% flocculation in the pilot-scale. The flocculants had differing effects on the cell membrane integrity, affecting the cyanobacterium significantly (causing 60-70% compromised cells), while having limited effect on the cell membrane of the eukaryotic microalgae (14-20% compromised cells). In addition, the flocculants caused a discernible shift in the biochemical composition of the algae, and further investigation is recommended to determine to the impact on the final product quality or processing requirements. Flocculation did not affect media recycling, which can help reduce production costs by 2% depending on the geographical location. Thus, these polyacrylamide flocculants show great potential as a low-cost, widely adaptable flocculant for microalgal harvesting.

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Tables

Table 1: Microalgal culture at time of flocculation assays

Species	Growth phase	Day	OD 750 nm	Biomass (g/L)	pH
Synechocystis sp.	Early exponential	12	3.52E+06	0.022	9.30
	Late exponential	25	1.88E+07	0.121	9.14
	Stationary	32	2.18E+07	0.160	8.80
Chlorella vulgaris	Early exponential	7	3.95E+05	0.036	9.38
	Late exponential	18	2.52E+06	0.145	8.84
	Stationary	28	3.29E+06	0.232	8.26
Phaeodactylum tricornutum	Early exponential	4	3.12E+04	0.028	9.25
	Late exponential	19	1.18E+05	0.162	8.84
	Stationary	31	1.74E+05	0.168	7.48

Table 2: Flocculation efficacy of 350 L bag reactor using FO 3801

Species	Dosage		Flocculation efficiency (%)	
	mg/g algae	mg/L culture	10 min*	24 hrs
Synechocystis sp.	44.6	6.5	87.5	98. 6
C. vulgaris	43.1	10	82.7	75.2
P. tricornutum	35.7	6.5	87.2	90.3

*With the exception of *P. tricornutum* which was tested after 30 min

Figures

Figure 1: Cultivation methodology of the microalgae. Scale up from stock culture to 350 L bag.

Figure 2: Dose dependent flocculation efficiency of flocculants indicated by optical density removal at OD 750 nm. Each row represents a different flocculant, with panels: (A, B, C) FO 3801, (D, E, F) FO 4808, and (G, H, I) Chitosan. Each column represents a different algal species, with panels: (A, D, G) *Synechocystis sp*., (B, E, H) *C. vulgaris*, and (C, F, I) *P. tricornutum*. Data are mean ± SE (n = 3, technical replicates).

Figure 3: Cell quality at stationary phase, after flocculation. (A) Cell membrane integrity of the flocculated and control biomass (i.e. without flocculants) of three species. Principal components analysis (PCA) was performed on the chemical profile, with ellipses showing 95% confidence interval, for: (B) *Synechocystis sp.*, (C) *C. vulgaris*, and (D) *P. tricornutum*. The optimal flocculant dosage was added for the different species, with: *Synechocystis sp.*, 31.3 mg/g for FO 3801 and FO 4808, and 375.0 mg/g for chitosan; *C. vulgaris*, 34.5 mg/g for FO 3801 and FO 4808; and *P. tricornutum*, at 35.7 mg/g for FO 3801 and FO 4808. The flocculation efficiency of chitosan for *P. tricornutum* and *C. vulgaris* was lower than 75%, therefore its effect on cells integrity and composition was not tested (showed as “n.a.” in panel A).

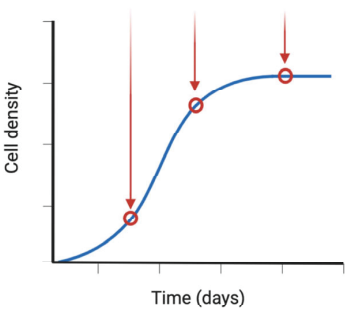
Figure 4: Growth of cells grown in recycled media. Cell growth (as OD 750 nm) for: (A) *Synechocystis sp*., (B*) C. vulgaris*, and (C) *P. tricornutum*. (D) Shows the cell integrity of the species at day 14. The optimal flocculant dosage was added for the different species, with: *Synechocystis sp.*, 31.3 mg/g for FO 3801 and FO 4808, and 375.0 mg/g for chitosan; *C. vulgaris*, 34.5 mg/g for FO 3801 and FO 4808; and *P. tricornutum*, at 35.7 mg/g for FO 3801 and FO 4808. The flocculation efficiency of chitosan for *P. tricornutum* and *C. vulgaris* was lower than 75%, therefore its effect on medium recycling was not tested ("n.a."). Control were grown on un-flocculated spent medium. * Indicates significant difference to the control (Dunnett's, $P < 0.05$). Data are mean ± SE ($n = 3$, biological replicates).

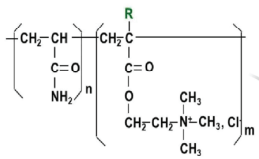
Phaeodactylum tricornutum
(diatom)

Chlorella vulgaris
(green algae)

Synechocystis sp.
(cyanobacteria)

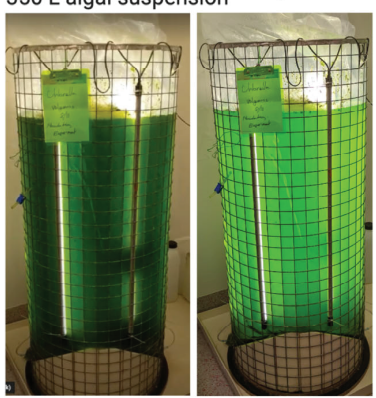
Evaluation of flocculation at different growth phases





Cationic polyacrylamides flocculant **(20-40 mg per g of algae)**

350 L algal suspension



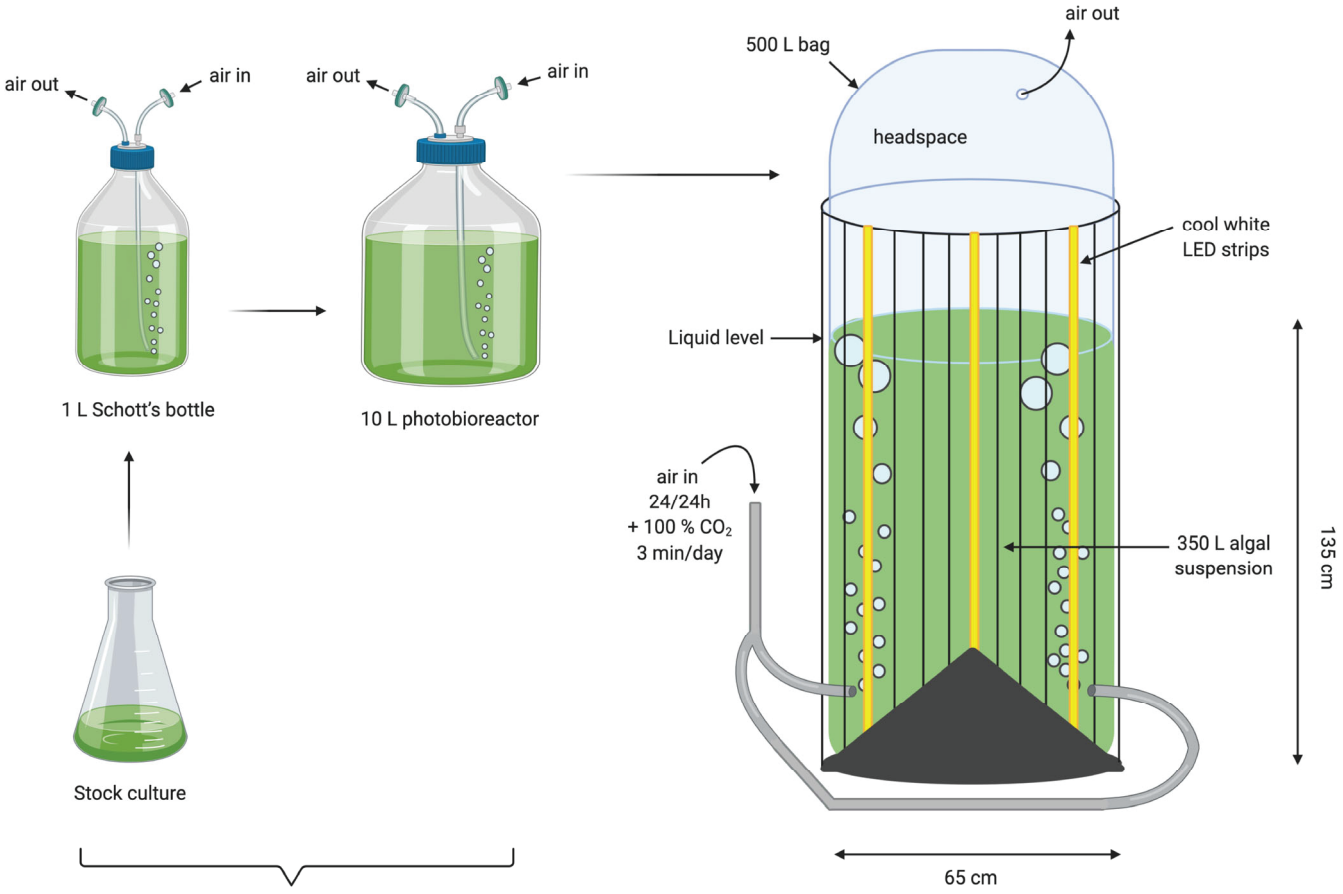
before / after flocculation

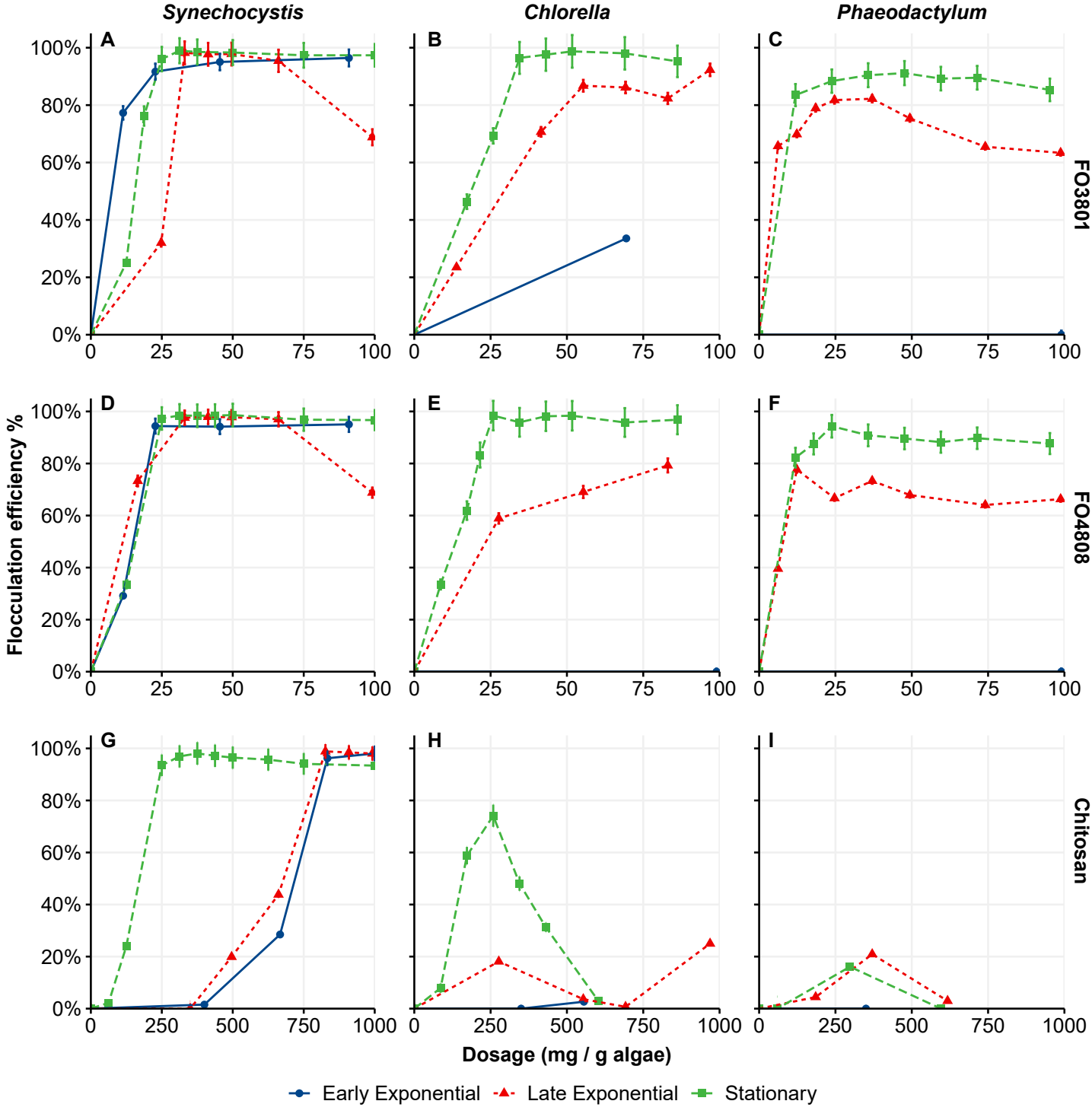
Floculation efficiency:
>20% by chitosan
>82% by cationic polyacrylamides

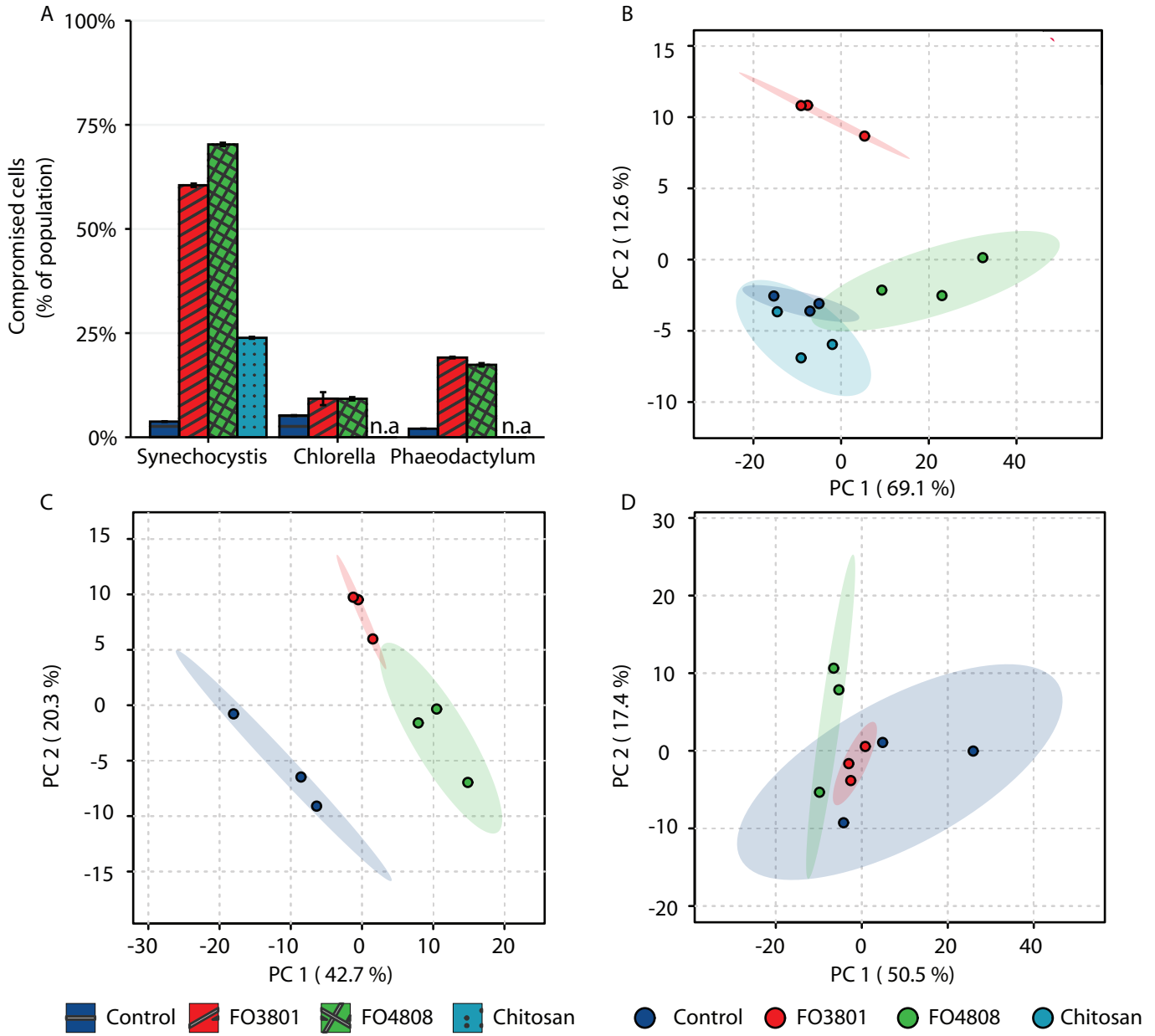
Flocculation efficiency higher at stationary phase ✔

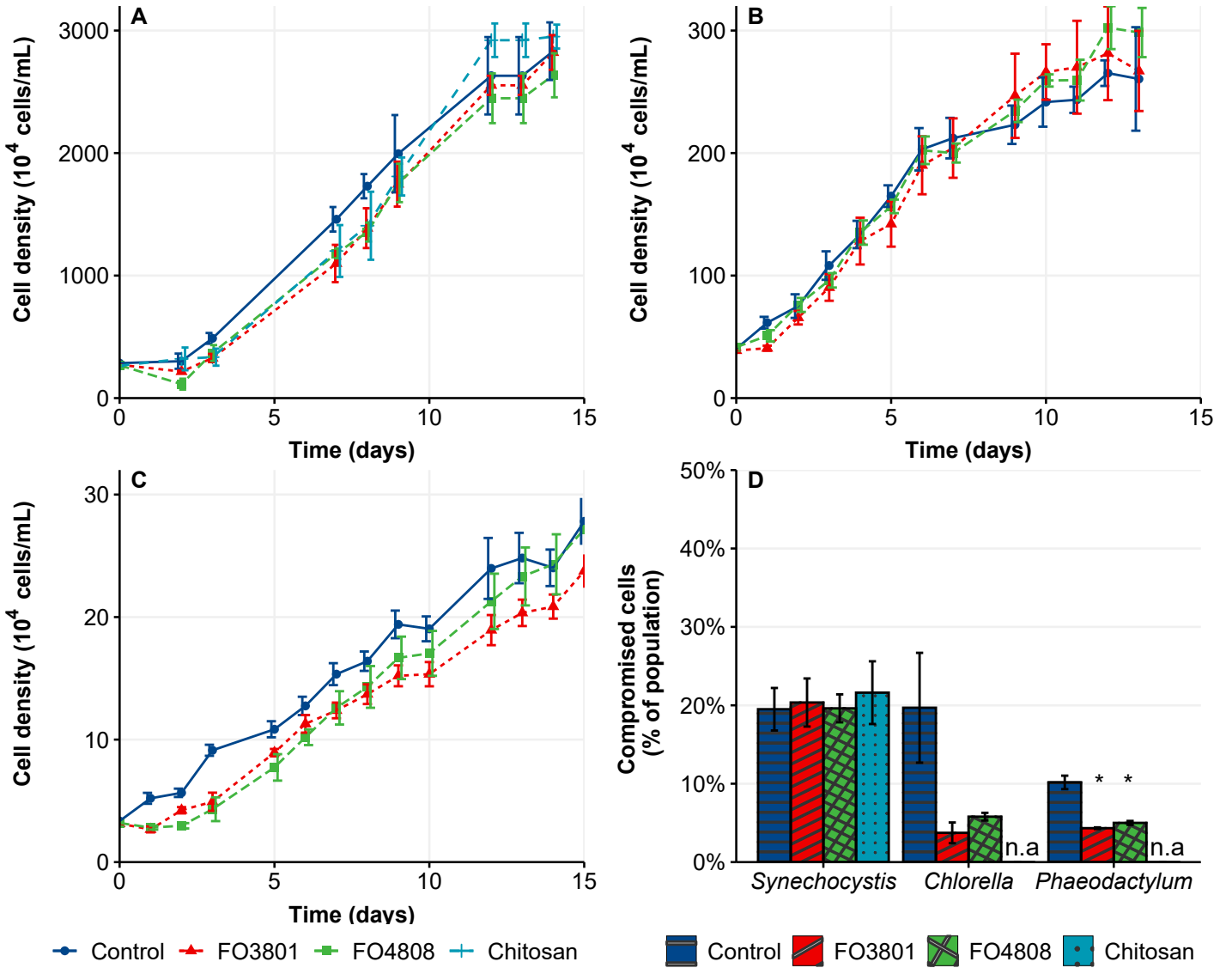
Biomass quality ✔

Medium recycling ✔


air out
air in
1 L Schott's bottle
air out
air in
10 L photobioreactor
Stock culture
Cultures grown at ~25 °C and ~100 µmol photons/m²/s light in a 16:8 hour light:dark cycle to an early stationary phase
500 L bag
air out
headspace
cool white LED strips
Liquid level
air in 24/24h + 100 % CO2 3 min/day
350 L algal suspension
135 cm
65 cm
Cells grown at ~23 °C and ~400 µmol photons/m²/s light in a 16:8 hour light:dark cycle, pH < 9.3


Synechocystis
Chlorella
Phaeodactylum
FO3801
FO4808
Chitosan
A
B
C
D
E
F
G
H
I
Flocculation efficiency %
100%
80%
60%
40%
20%
0%
0
25
50
75
100
250
500
750
1000
Dosage (mg / g algae)
Early Exponential
Late Exponential
Stationary


A
100%
75%
50%
25%
0%
Compromised cells
(% of population)
n.a
n.a
Synechocystis
Chlorella
Phaeodactylum
B
PC 2 (12.6 %)
PC 1 (69.1 %)
C
PC 2 (20.3 %)
PC 1 (42.7 %)
D
PC 2 (17.4 %)
PC 1 (50.5 %)
Control
FO3801
FO4808
Chitosan
Control
FO3801
FO4808
Chitosan


A
B
C
D
Cell density (10^4 cells/mL)
Time (days)
Compromised cells (% of population)
n.a
n.a
*
*
Synechocystis
Chlorella
Phaeodactylum
Control
FO3801
FO4808
Chitosan