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ESCALONAMENTO DO CULTIVO E DA

FLOCULAÇÃO DA MICROALGA MARINHA

Nannochloropsis oculata

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AGRADECIMENTOS

1. RESUMO GERAL

As microalgas estão atraindo o interesse dos pesquisadores para a produção de alimentos, rações, produtos químicos e biocombustíveis. *Nannochloropsis oculata* é uma espécie marinha com alta taxa de crescimento, tolera amplas condições ambientais e pode produzir mais de 50% do seu peso seco na forma de lipídios. Atualmente, o cultivo comercial das microalgas é realizado em sistemas abertos pois sistemas fechados têm elevado custo de produção. No entanto, em sistemas abertos não é possível controlar os parâmetros ambientais, o que reduz a sua produtividade. No Capítulo 1 desta Tese, um sistema semifechado foi comparado com sistemas abertos, em escala piloto (1.200 L). O sistema semifechado consistiu em tanques circulares instalados em uma estufa agrícola, o que proporcionou melhores condições para o cultivo de *N. oculata*, principalmente nas estações de baixa temperatura e alta pluviosidade. No entanto, apesar de ser relativamente fácil cultivar microalgas, a coleta da biomassa é um dos principais gargalos para a sua produção em larga escala, responsável por até 30% do custo total. A floculação é uma tecnologia de baixo custo proposta para a concentração de microalgas. Desta forma, a coleta de *N. oculata* por floculação também foi estudada. No Capítulo 2 foram analisados vinte e cinco polímeros naturais e sintéticos, de baixo e alto peso molecular e com diferente densidade de carga. Comparando os resultados com *Chlorella vulgaris*, uma espécie de água doce, observou-se que apenas os polímeros naturais foram eficientes para ambas as espécies, enquanto que os polímeros sintéticos apresentaram baixa eficiência para *N. oculata*. De uma forma geral, aumentando a densidade de carga dos polímeros resultou no incremento da eficiência. Comparando o custo e a performance, os polímeros naturais apresentaram os melhores resultados. No Capítulo 3, os melhores polímeros sintéticos e naturais foram selecionados e os efeitos de diferentes fatores foram avaliados. De forma geral, a presença de matéria orgânica afetou a eficiência de todos, enquanto que salinidade e pH afetaram os polímeros sintéticos e os naturais, respectivamente. O efeito da dose foi observado apenas nos polímeros sintéticos, onde o aumento resultou na queda da eficiência. Nenhum dos polímeros testados apresentaram toxicidade para *N. oculata*. No entanto, por não serem afetados pela salinidade, apenas os polímeros naturais foram recomendados para a espécie. No Capítulo 4, foi realizado o escalonamento da floculação de *N. oculata* utilizando um polímero natural. Não houve diferença entre os resultados dos

experimentos em bancada (300 mL) e em escala piloto (250 L). No entanto, apesar do excelente resultado obtidos anteriormente com água sintética, o polímero natural apresentou queda na eficiência quando água natural foi utilizada. Reduzindo a salinidade de 30 para 10, a eficiência do polímero aumentou de 50% para 98%. Os resultados obtidos indicam que o escalonamento do cultivo e da floculação de *N. oculata* foi atingido. No entanto, estudos futuros devem ser realizados para otimizar a eficiência da floculação de *N. oculata* utilizando água marinha natural.

2. ABSTRACT

Microalgae are attracting the interest of researchers for the production of food, feed, chemicals and biofuels. *Nannochloropsis oculata* is a marine species with high growth rate, tolerates a broad range of environmental conditions and can accumulate more than 50% of its dry weight as lipid. Currently, the commercial cultivation of microalgae is carried out mainly in open-air systems as enclosed systems have high production costs. However, the environmental parameters are difficult to control in open-air systems, which reduces their productivity. In Chapter 1 of this Thesis, a semi-enclosed system was compared with an open-air system, both at pilot-scale (1,200 L). The semi-enclosed system consisted of circular tanks installed inside a greenhouse, which provided better conditions for the cultivation of *N. oculata*, especially during the colder and rainy seasons. However, although it is relatively easy to cultivate microalgae, harvesting is one of the major bottlenecks for its large-scale development, representing up to 30% of the total cost. Flocculation is a low-cost technology that has been proposed for harvesting microalgae. Thus, harvesting of *N. oculata* by flocculation was also studied in this Thesis. In Chapter 2, twenty-five natural and synthetic polymers, of low and high molecular weight, and with different charge density were compared between *N. oculata* and *Chlorella vulgaris*, a freshwater species. It was observed that only the natural polymers were efficient for both species, whereas the synthetic polymers presented low efficiency for *N. oculata*. In general, increasing the charge density of the polymer resulted in increased efficiency. Comparing the cost and performance, natural polymers obtained the best results. In Chapter 3, the best synthetic and natural polymers were selected and the effects of different factors were evaluated. In general, the presence of organic matter affected the efficiency of all polymers, whereas salinity and pH affected synthetic and natural polymers, respectively. The effect of dosage was only observed in synthetic polymers, resulting in efficiency loss when overdosed. None of the tested polymers exhibited toxicity to *N. oculata*. However, the natural polymers were not affected by salinity and are recommended for further studies. In Chapter 4, *N. oculata* flocculation was scaled-up using a natural polymer. No difference was observed between bench (300 mL) and pilot scale (250 L) experiments. However, despite the excellent results obtained previously with synthetic water, the natural polymer presented a reduction in efficiency when natural water was employed. Reducing the salinity from

30 to 10 increased polymer efficiency from 50% to 98%. The results from this Thesis indicate that up scaling the cultivation and flocculation of *N. oculata* was successfully achieved. However, future studies should be performed to optimize the efficiency of *N. oculata* flocculation using natural seawater.

3. INTRODUÇÃO GERAL

3.1. Definição de microalgas

O termo microalga refere-se a um agrupamento polifilético de microrganismos autótrofos, fotossintetizantes, unicelulares, coloniais ou multicelulares simples (Guiry, 2012). As microalgas podem ser encontradas em ambientes dulciaquícolas ou marinhos. Além de água, as microalgas necessitam de CO_2, fosfato, nitrato e elementos traços como zinco e cobre para o seu desenvolvimento. Devido às suas estruturas simples (ausência de raízes, caules e folhas), elas são capazes de captar nutrientes de forma eficiente, apresentando altas taxas fotossintéticas e crescimento exponencial quando as condições são ótimas (Brennan e Owende, 2010). A biodiversidade das microalgas é enorme e elas representam um recurso quase inexplorado. Estima-se que existam cerca de 72.500 espécies, das quais 44.000 já foram descritas, distribuídas nos reinos *Bacteria* (cianobactérias), *Plantae* (algas verdes), *Chromista* (diatomáceas) e *Protozoa* (dinoflagelados) (Guiry, 2012).

3.2. Importância comercial das microalgas

As microalgas possuem um enorme potencial comercial como fonte de biomassa para a produção de alimentos, rações, produtos químicos ou biocombustíveis (Borowitzka, 2013). Por exemplo, a composição química das microalgas é comparável com a da soja, contendo altos níveis de proteínas e lipídios (Becker, 2007; Brown et al., 1998). O teor médio de lipídios varia entre 1% e 70% do peso seco (Metting, 1996). Entre os ácidos graxos produzidos pelas microalgas, os poliinsaturados das famílias ω3 e ω6 são de particular interesse (Borowitzka, 2013). Os ácidos eicosapentaenóico (EPA; C20:5) e docosahexaenóico (DHA; C22:6), por exemplo, possuem efeitos benéficos no combate à doenças (Fraeye et al., 2012). Além disto, as microalgas produzem carboidratos como amido, glucose e outros polissacárides de alta digestibilidade, além de vitaminas (A, B_1, B_2, B_6, B_{12}, C, E, biotina, ácido fólico e ácido pantotênico), sendo indicadas para alimentação de animais e seres humanos (Becker, 2007). As microalgas também são ricas em pigmentos como clorofila, carotenoides (astaxantina e fucoxantina) e ficobiliproteínas (ficocianina e ficoeritrina), empregados na coloração de alimentos e como fármacos (Metting, 1996; Borowitzka, 2013). Recentemente, as microalgas têm

sido utilizadas como matéria prima para a produção de compostos como metano, biodiesel e biohidrogênio (Chisti, 2007).

3.3. Principais microalgas comercialmente cultivadas

A produção comercial de microalgas está restrita a apenas poucas espécies (Fig. 1), sendo a maioria extremófilas, crescendo em ambientes altamente seletivos (Spolaore et al., 2006). Por exemplo, o gênero *Chlorella* (*Trebouxiophyceae*), composto principalmente por espécies de água doce e empregado na alimentação humana, aquicultura e indústria cosmética, é cultivado em ambientes ricos em nutrientes. *Arthrospira* (*Cyanophyceae*), outro gênero de água doce que é empregado na nutrição humana e animal, na produção de ficobiliproteínas e na indústria cosmética, cresce em ambientes com elevadas concentrações de bicarbonato e pH. *Dunaliella* (*Chlorophyceae*), empregada na alimentação humana, na produção de β-carotenos e na indústria cosmética, necessita de alta salinidade (Borowitzka e Moheinami, 2013). Estas características auxiliam na manutenção de cultivos monoespecíficos uma vez que estas microalgas apresentam vantagens competitivas nestas condições. Além destes, outros gêneros cultivados comercialmente são *Aphanizomenon* (*Cyanophyceae*) e *Haematococcus* (*Chlorophyceae*), ambos de água doce e empregados na alimentação humana e na produção de astaxantina, respectivamente (Milledge, 2010).
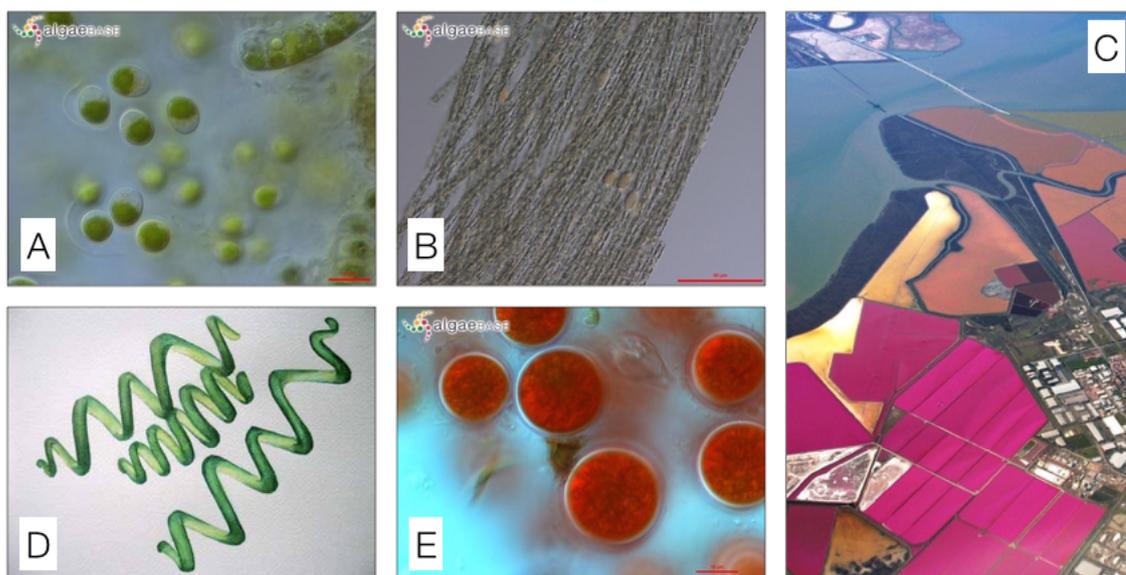
Fig. 1: Principais gêneros cultivados. a) *Chlorella*, b) *Aphanizomenon*, c) *Dunaliella*, d) *Arthrospira*, e) *Haematococcus*. (a,b,e: www.algaebase.com, c: www.flickr.com, d: media.paperblog.fr)

Porém, a produção de microalgas para fins energéticos necessita de grandes quantidades de água, tanto para o crescimento das próprias microalgas quanto para a conversão da sua biomassa em combustível (Dominguez-Faus et al., 2009). Devido à escassez de água doce no planeta, não é recomendável que espécies de água doce sejam empregadas (Schlesinger et al., 2012). Assim, a produção de microalgas em larga escala deve focar em espécies capazes de crescer em água salina (Borowitzka e Moheinami, 2010).

3.4. Importância de Nannochloropsis oculata

Nannochloropsis oculata (Eustigmatophyceae) é uma das cinco espécies que compõem o gênero *Nannochloropsis*. Esta espécie é caracterizada por possuir células esféricas de pequeno tamanho (2-4 µm) e por viver em habitats de água salgada (Fig. 2, Andersen et al., 1998). Além disto, apresenta alta taxa de crescimento, tolera uma ampla faixa de condições ambientais como temperatura e salinidade, podendo acumular mais de 50% do seu peso seco na forma de lipídio (Molina Grima et al., 2003; Gouveia e Oliveira, 2008; Mata et al., 2010; Moazami et al., 2012). Esta espécie é geralmente cultivada em pequena escala para uso na aquicultura, como alimento para *Artemia* e rotíferos, que são consumidos por larvas de peixes e crustáceos (Benemann, 1992). No entanto, recentemente *N. oculata* tornou-se amplamente reconhecida como uma potencial fonte de lipídios para a produção de biodiesel (Gouveia e Oliveira, 2008; Moazami et al., 2012), além de também produzir carotenoides como astaxantina, cantaxantina e zeaxantina (Lubián et al., 2000), com aplicação em nutracêuticos e antioxidantes (Borowitzka, 2013).
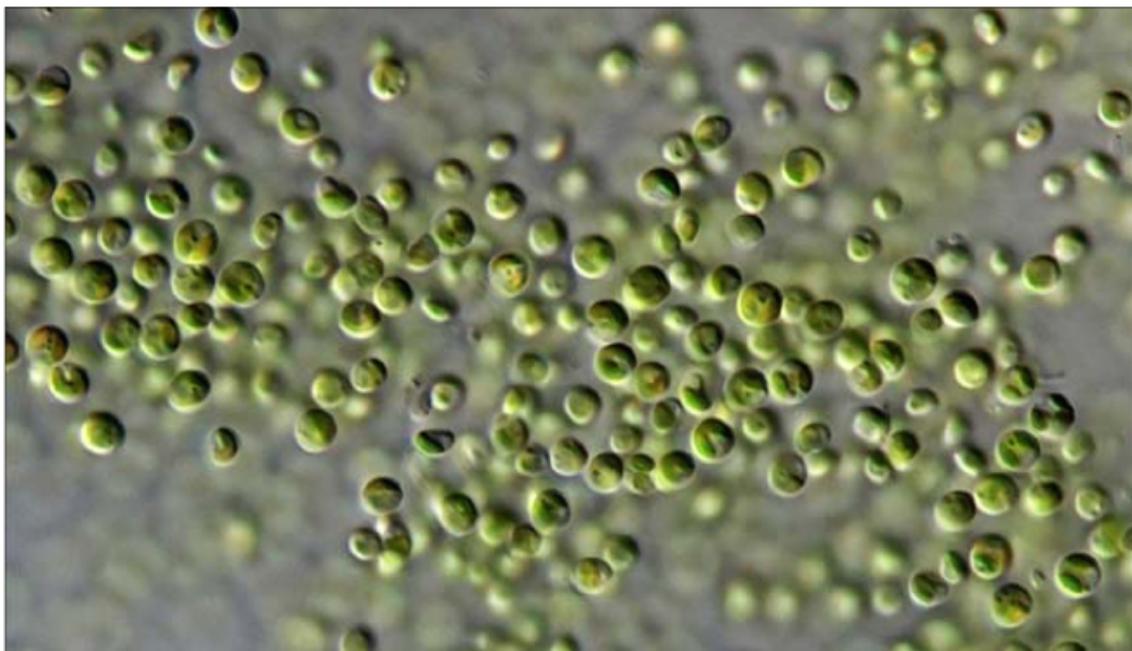
Fig. 2: Células de *Nannochloropsis oculata*. (www.sbae-industries.com)

Vários estudos foram realizados com a microalga *N. oculata*. Por exemplo, Alves Sobrinho et al. (2015) recentemente estudaram o perfil e a produção de ácidos graxos para a produção de biodiesel, a partir da biomassa úmida. Os autores concluíram que, para biomassas com até 50% de água, os processos de hidrólise e esterificação resultaram em maiores rendimentos de ácidos graxos. Beacham et al. (2014) estudaram a relação entre a parede celular e a extração dos lipídios. Como *N. oculata* possui parede celular muito grossa (112 nm), é necessário o uso de métodos mais intensos para obter a lise celular. Wei et al. (2013) otimizaram a acumulação de lipídios, sendo que a combinação de meio de cultivo contendo 0,44 mmol N L^{-1}, 1,2 x 10^{-1} mmol Fe L^{-1} e 20ºC permitiu a maior produção de lipídios (60%). Olofsson et al. (2012) estudaram a variação sazonal de lipídios e ácidos graxos em *N. oculata* cultivada em fotobioreatores. A produção variou de 11% no inverno a 30% no outono, sendo que 50% da variação foi explicada por luz e temperatura. Gu et al. (2012) avaliaram o efeito da salinidade no crescimento e na produtividade lipídica. Quando cultivada em salinidade 35, *N. oculata* apresentou os melhores resultados de crescimento e de produtividade lipídica (64 mg L^{-1} d^{-1}). No entanto, a produção de EPA (US\$ 100,00 g^{-1}) foi maior em valores de salinidade menor. Nobre et al. (2013) avaliaram o potencial de biorefinaria, com extração de óleos, pigmentos e produção de biohidrogênio com a biomassa restante e

extraíram 45% de lipídios, 70% dos pigmentos disponíveis e produzir 60 mg g^{-1} de biohidrogênio. Em suma, estes estudos destacaram o potencial de *N. oculata* como matéria-prima para a produção de diversos compostos, tanto de baixo quanto de alto valor agregado.

3.5. Cultivo comercial de microalgas

O cultivo comercial de microalgas em larga escala começou no início dos anos 60 no Japão, com o cultivo de *Chlorella* sp. pela empresa *Nihon Chlorella*. Nos anos 70 a empresa Sosa Texcoco SA (México) iniciou o cultivo de *Arthrospira maxima* no lago Texcoco. Na década de 80, existiam 46 empresas com uma produção mensal de 5 toneladas, principalmente de *Chlorella* sp.. Na década de 90, várias empresas em Israel, Estudos Unidos e Índia iniciaram o cultivo de *Haematococcus pluvialis* para a produção de astaxantina (Borowitzka, 1999). Atualmente, a produção anual total de microalgas é de cerca de 10.000 toneladas, empregada na alimentação humana e animal, nas indústrias cosmética, farmacêutica e química (Borowitzka e Moheinami, 2013).

Para que o cultivo comercial de microalgas possa atender a crescente demanda de bioprodutos, é necessária a realização de cultivos em larga escala (Borowitzka, 1999). No entanto, não há um consenso em relação à qual tecnologia de produção seria mais promissora para adoção em larga escala (Norsker et al., 2011). Tem-se afirmado que sistemas fechados (fotobiorreatores) são inadequados devido seu alto custo de produção e difícil escalonamento (Chisti, 2007; Waltz, 2009), enquanto que os sistemas abertos (tanques raceways e circulares) apresentam baixa produtividade, baixo controle de parâmetros ambientais e alta susceptibilidade a microrganismos invasores (Posten, 2009; Bartley et al., 2013). Apesar disto, os sistemas abertos são os mais utilizados para a produção comercial de microalgas devido ao baixo custo de produção (Borowitzka e Moheinami, 2013).

Fig. 3: Sistemas empregados para o cultivo de microalgas. a) Sistema aberto (tanques circulares), b) Sistema fechado (fotobioreator tubular horizontal). (a: www.sunchlorella.com, b: www.algaeparc.com)

A produtividade das microalgas varia de acordo com as condições geográficas e meteorológicas (Ugwu et al., 2008). No entanto, a maioria das pesquisas sobre produção ao ar livre são realizadas em regiões com regimes ideais de irradiância e temperatura (López-Elías et al., 2005). Portanto, além do desenvolvimento de sistemas de cultivo em larga escala que tenham baixo custo de produção e alta produtividade, é de extrema importância que o potencial de regiões climáticas menos favoráveis sejam também explorados (Roleda et al., 2013).

3.6. Coleta de microalgas

Independente do sistema de cultivo empregado, a coleta das microalgas é reconhecida como uma das principais restrições para o desenvolvimento da sua produção comercial em larga escala (Vandamme et al., 2013). De acordo com Gudin e Thepenier (1986), a coleta pode representar até 30% do orçamento total da produção. Como as microalgas são organismos unicelulares, microscópicos (3–30 μm) e atingem concentrações relativamente baixas (0,5% do volume), uma grande quantidade de água deve ser manipulada para concentrar a biomassa (Wileman et al., 2012). Além disso, as microalgas apresentam cargas superficiais negativas e baixa taxa de sedimentação, formando suspensões estáveis e dificultando a sua concentração (Rawat et al., 2013; Vandamme et al., 2013).

Atualmente, a coleta de microalgas é realizada principalmente por centrifugação (Wijffels e Barbosa, 2010), no entanto, esta tecnologia é muito dispendiosa devido ao alto consumo de energia (Rawat et al., 2013), e somente é aceitável para a produção de bioprodutos de alto valor comercial como pigmentos (e.g. β-caroteno, astaxantina), ácidos graxos (e.g. DHA, EPA) e extratos para uso em cosméticos, cujos preços podem ultrapassar US$ 1.000,00 g^{-1} (Borowitzka, 2013). Para tornar a produção de bioprodutos de baixo valor comercialmente viável, seja para a produção de alimentos ou de biocombustíveis, os custos devem diminuir drasticamente. Para o mercado energético, por exemplo, os custos de produção precisam ser inferiores a US$ 1 kg^{-1} de biomassa para que sejam competitivas (Wijffels e Barbosa, 2010).

A floculação é uma tecnologia de baixo custo comumente empregada em tratamento de águas para a retirada de partículas suspensas em grandes volumes de líquido, seja por flotação ou por sedimentação. Basicamente, a floculação é um processo onde as partículas são desestabilizadas, induzindo a coagulação e, consequentemente, a formação de aglomerados maiores. Quatro mecanismos de coagulação podem ser empregados, separadamente ou em conjunto (Gregory, 2013). (1) Neutralização de carga é o fenômeno no qual íons, polímeros ou coloides eletricamente carregados se adsorvem na superfície de partículas de cargas opostas, seguida por desestabilização, coagulação e floculação (Fig. 4a). (2) Interação eletrostática "patch" é o fenômeno no qual um polímero eletricamente carregado se liga a uma partícula de carga oposta. O polímero reverte localmente a carga superficial da partícula, resultando em regiões de carga oposta. Assim, as partículas se conectam umas as outras por regiões de carga superficial oposta, causando floculação (Fig. 4b). (3) O mecanismo de pontes é o fenômeno no qual polímeros ou coloides eletricamente carregados se adsorvem em duas partículas diferentes, formando uma ponte. Esta ponte aproxima as partículas e causa floculação (Fig. 4c). (4) Floculação por arraste é o processo no qual partículas são capturadas por uma precipitação massiva de minerais, causando floculação (Fig. 4d).
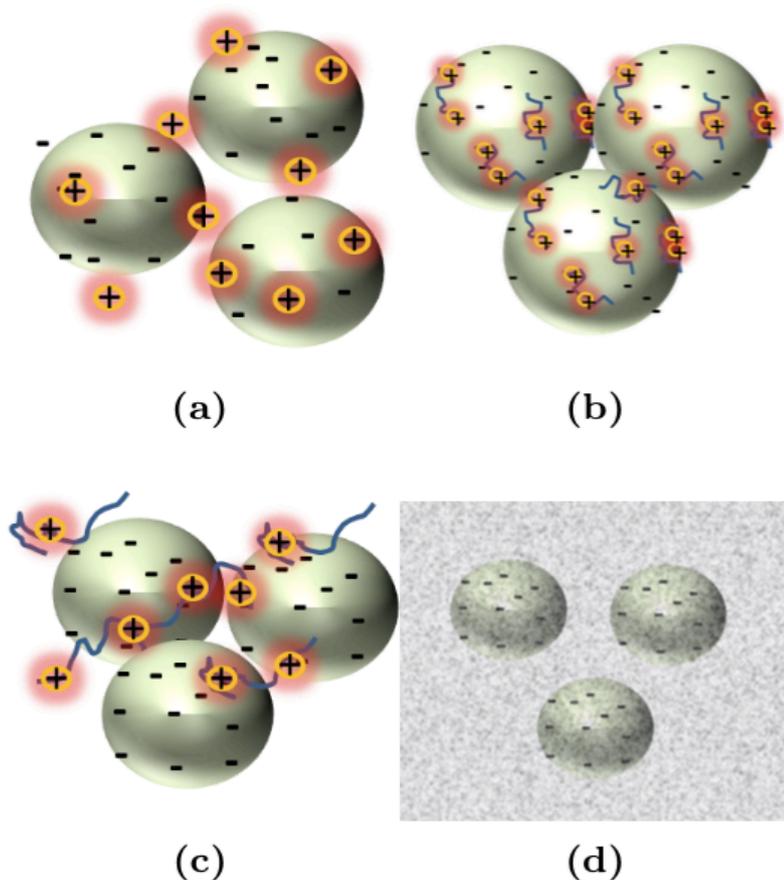
Fig. 4: Mecanismos de coagulação (a) neutralização de carga, (b) interação eletrostática "patch", (c) formação de pontes e (d) floculação por arraste (Vandamme, 2013).

3.7. Tipos de floculantes

Uma classe importante de produtos utilizados na floculação é a dos sais metálicos, tais como cloreto férrico ou sulfato de alumínio (Gregory, 2013). Quando dissolvidos na água, estes sais metálicos formam hidróxidos positivamente carregados que causam floculação por neutralização da carga ou por arraste. Os sais metálicos foram aplicados com sucesso para a floculação de microalgas (Sukenik et al., 1988; Vandamme et al., 2012; Garzon-Sanabria et al., 2012). No entanto, eles têm a desvantagem de requerer uma dosagem relativamente elevada e contaminar a biomassa com elevada concentração de metais, limitando a utilização devido à sua toxicidade (Farooq et al., 2015).

Outra classe de produtos amplamente utilizados para a floculação, é a dos polímeros orgânicos. Eles podem induzir floculação tanto por neutralização da carga como por formação de pontes. A eficácia de tais polímeros depende do seu tamanho e da sua

estrutura secundária, bem como da sua densidade de carga. Os polímeros orgânicos são preferidos porque a dosagem necessária é muito mais baixa que a dos sais metálicos. A maioria dos polímeros orgânicos comercialmente disponíveis são sintéticos baseados em poliacrilamida (Gregory, 2013), e foram aplicados com sucesso na floculação de microalgas (Ebeling et al., 2005; Knuckey et al., 2006; Uduman et al., 2010; Granados et al., 2012; Lam et al., 2014).

Embora os polímeros sintéticos de poliacrilamida não sejam tóxicos, eles podem conter monômeros de acrilamida, estes sim tóxicos para os organismos aquáticos (Bolto e Gregory, 2007). Portanto, é preferível usar polímeros naturais, principalmente se a biomassa for utilizada para alimentação humana ou animal. Um polímero natural bem conhecido é a quitosana, um derivado da quitina obtida a partir de cascas de camarão e bastante eficaz para a floculação de microalgas (Renault et al., 2009; Rashid et al., 2013).

3.8. Oportunidades de pesquisa com N. oculata

Tendo em vista o exposto acima, fica evidente que pesquisas devem ser realizadas no desenvolvimento de sistemas de cultivo em larga escala empregando água salgada, que tenham baixo custo de produção e alta produtividade. Também é de extrema importância que o potencial de regiões climáticas menos favoráveis para o cultivo de microalgas seja explorado. Da mesma forma, métodos de coleta eficientes e de baixo custo devem ser desenvolvidos a fim de viabilizar economicamente a produção de microalgas, principalmente para bioprodutos de baixo valor comercial como biocombustíveis. Finalmente, diversas pesquisas demonstraram que *N. oculata* é uma espécie robusta, de rápido crescimento e capaz de produzir diversos tipos de bioprodutos. seja realizada em regiões geográficas menos propícias como o sul do Brasil, que apresenta períodos de baixa temperatura e alta pluviosidade ao longo do ano.

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4. OBJETIVOS GERAIS

- Cultivar e coletar a microalga marinha *Nannochloropsis oculata* em escala piloto.

5. OBJETIVOS ESPECÍFICOS

- Comparar dois sistemas de cultivo, aberto e semifechado, para a produção em escala piloto (1.200 L) da microalga marinha *N. oculata* em regiões subtropical e temperada (Capítulo 1).
- Avaliar a eficiência de vinte e cinco polímeros comerciais sintéticos e naturais, de baixo e alto peso molecular e diferentes densidades de carga, para a floculação da microalga *N. oculata* em escala de bancada com água marinha sintética, usando a microalga de água doce *Chlorella vulgaris* como controle (Capítulo 2).
- Avaliar os efeitos de pH, matéria orgânica dissolvida, salinidade, concentração de biomassa e dose na eficiência de quatro polímeros sintéticos e naturais na floculação de *N. oculata* em escala de bancada com água sintética, usando a microalga de água doce *C. vulgaris* como controle (Capítulo 3).
- Selecionar o melhor polímero e determinar as melhores condições de salinidade, pH, concentração de biomassa e dose para a floculação de *N. oculata* em escala piloto (250 L) com água marinha natural (Capítulo 4).

Comparison of open-air and semi-enclosed cultivation system for massive microalgae production in sub-tropical and temperate latitudes

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Abstract

This study compared open-air and semi-enclosed production system of the marine microalgae *Nannochloropsis oculata* in a sub-tropical region (32ºS; 52ºW) under uncontrolled environmental conditions. The semi-enclosed system was composed of 1.2 m^3 circular tanks installed inside of a greenhouse. Water temperature was 4ºC higher in the indoor treatment than in the outdoor, mainly in winter although no difference was observed in warmer seasons. Moreover, variation in salinity was observed in the outdoor treatment due to rainfall (austral winter) and evaporation (austral spring), whereas indoor treatment experienced an increase (up to 100 PSU) due to evaporation only in warmer seasons. Light transmission was approximately 20% lower in the indoor treatment although cell densities and biomass yields were higher indoor during winter. As the temperature increased (austral spring) no differences were observed among treatments. In summary, partial control of temperature and salinity in the semi-enclosed system, especially during the colder and rainy season, allowed higher microalgae biomass production. Further experiments must be conducted with CO_2 addition, larger pH range and salinity control.

Keywords: Circular tank; Agricultural greenhouse; Abiotic control; Biodiesel feedstock; Massive cultivation.

1. Introduction

Due to the rapid increase in the price of petroleum, the projected exhaustion of supplies and awareness of environmental damage resulting from the historical use of fossil fuels, there has been increased interest in developing alternative technologies for biofuel production [1,2]. One of the most prominent biofuel is biodiesel, produced after the transesterification of lipids from various feedstocks, such as seed oil and animal fat [3]. In recent years, the cultivation of microalgae has been pointed out as a viable alternative for the production of biodiesel on a large scale, as they present some advantages when compared to traditional biodiesel feedstocks [4,5]. Specifically, microalgae do not occupy fertile lands and can be grown using seawater supplemented with commercial fertilizers, or with domestic or industrial effluents [4-7].

According to several authors [6-9], the only practicable microalgae large-scale cultivation systems used commercially are open (raceways and circular tanks) and enclosed (photobioreactors) systems. Nevertheless there is still an intense debate concerning the best culture system since both present advantages and limitations [9-12]. Open systems are mainly used in very large commercial productions and are characterized by lower installation, operational and maintenance costs but are subject to lower control of environmental parameters (i.e. temperature, salinity, irradiance). By contrast, enclosed systems although more efficient have higher costs and are difficult to scale up to attend commercial production [7,9,13].

Microalgal productivity varies with geographical and meteorological conditions [14] and most research on outdoor production has been performed in tropical regions with optimal irradiance and temperature regimes [15]. However, according to Roleda *et al.* [16] at lower latitudes high irradiance and temperature may interactively depress photosynthetic rate and cause cell death increasing the production costs. Therefore there is also a need to investigate microalgal performance in sub-tropical and temperate regions under lower light and temperature regimes [16,17]. Thus, in order to meet the large and growing demand and to establish a sustainable production in the long-term is of utmost importance the development of massive cultivation systems characterized by low production cost, high biomass production, ease of handling and exploring the potential of large-scale biomass production in higher latitudes [3,7,16,18-20].

Nannochloropsis oculata, a marine microalgae, is widely recognized as a good candidate for biodiesel production as it is robust with high growth rates in response to a broad range of environmental conditions and can accumulate up to 53% lipid content by weight under nutrient stress with a lipid productivity of 37.6-90.0 g m^{-3} day^{-1} [3,16,21,22].

The aim of this study was to compare the growth and biomass production of *N. oculata* when cultured in indoor (semi-enclosed) and outdoor (open-air) pilot-scale circular tanks, exposed to a wide range of culture and environmental conditions in a sub-tropical region, in different light and temperature conditions from austral autumn to spring.

2. Materials and methods

2.1. Experimental design

Indoor and outdoor experiments were conducted at the Marine Aquaculture Station of the Institute of Oceanography from the Federal University of Rio Grande (FURG), in Southern Brazil (32º12'15" S, 52º10'40" W). The massive cultivation system was evaluated in three different austral seasons, winter (June 21st – July 30th 2010), spring (November 11th - December 18th 2010) and autumn (March 15th – April 22nd 2011), each experiment lasting for 40 days.

All experiments were run in 1.2 m³ circular tanks (in triplicate) maintained in indoor and outdoor conditions. The circular tanks (2.9 x 0.3 m) consisted of metallic structures covered with 1 mm thick white PVC geomembrane. For the indoor treatment tanks were placed inside a metallic structured greenhouse (9.0 x 12.5 m), covered with transparent LDPE UV stabilized film with a light transmission of 89%. To avoid critical temperatures (> 30ºC) inside the greenhouse a thermostat-controlled fan was placed. Cultures were stirred by continuous air injection (1.2 m³ h^{-1}) through a PVC aeration system and both treatments were maintained under natural light and day-night photoperiod.

2.2. Culture conditions

Nannochloropsis oculata (NANN OCUL-1) was obtained from the collection of the Marine Phytoplankton and Microorganism's Laboratory from FURG. For the culture medium, seawater (32 PSU) was filtered through 1 µm filter, treated with 0.2 ml L^{-1} of 5% sodium hypochlorite and neutralized with 6 mg L^{-1} of sodium thiosulphate after 8 hours. The culture medium employed consisted of inexpensive commercial fertilizers, containing ammonium sulphate, urea, calcium superphosphate, ferric chloride and vitamins B1, B6 and B12 as proposed by Yamashita & Magalhães [23]. Cultures were inoculated with stock algae so that the initial *N. oculata* abundance in all experiments was approximately 2.1x10^7 cm^{-3}. Atmospheric CO_2 was supplied into the cultures through atmospheric air bubbling. Experiments were carried out in uncontrolled

conditions in order to compare the effect of dynamic environmental changes in both indoor and outdoor treatments.

2.3. Biotic parameters

Samples were collected three times a week to determine the microalgae biomass yield, as dry weight, according to Strickland & Parsons [24]. Cell abundances were also conducted three times a week counting at least 400 cells within a Neubauer haemacytometer [25].

2.4. Abiotic parameters

Culture salinity (± 0.01 PSU), temperature (± 0.1°C) and pH (± 0.01 unit) readings were taken daily in all cultures with an YSI 556 Handheld Multiparameter (Yellow Springs Instrument, OH, USA). To establish the light transmission difference between indoor and outdoor treatments, light intensity (W m^{-2}) was measured twice a day using a LD-240 light meter (Instrutherm, SP, Brazil). Daily meteorological data were obtained from the Brazil's National Meteorology Institute by conventional (WMO 83995) and automatic (A802) meteorological stations located at the campus of FURG (32º04'43'' S, 52º10'03'' W and 2.46 m). Meteorological data consisted of maximum and minimum air temperature (ºC), rainfall (mm), evaporation (mm), humidity (%) and radiation (kJ m^{-2} s^{-1}).

2.5. Statistical analysis

Data normality and homoscedasticity were verified for each data set using Shapiro-Wilk and Bartlett's test. Comparison inside seasons was performed using Student's unpaired t test (α = 0.05) whereas treatments were compared for each season using ordinary one-way ANOVA (α = 0.05) followed by Tukey's multiple comparisons test [26]. The Spearmen correlation coefficient (ρ) was used to evaluate the association between biotic and abiotic parameters. Statistical analyses were performed using Prism 6 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Water Temperature

Water temperature showed a significant difference (P<0.05) between indoor and outdoor treatments throughout all the experiments (Table 1). Indoor treatments were, on average, 3-4ºC warmer than outdoor, varying from 15ºC in winter (Figure 1A) to 36ºC in spring (Figure 2A) while outdoor temperatures varied from 9ºC to 34ºC in the same period. A maximum difference between indoor and outdoor temperatures were 7.8, 8.9 and 5.5 in winter (Figure 1A), spring (Figure 2A) and autumn (Figure 3A), respectively. The comparison of water temperatures throughout seasons showed that data from winter statistically differed (P<0.05) from autumn and spring, independent of treatments (Table 1). There were no differences in temperatures between autumn and spring. Table 2 presents mean air temperatures (minimum and maximum) for all the experiments.

3.2. pH

In general pH showed similar decreasing behavior throughout the experiments. In the winter minimum and maximum values were around 5.6 and 8.0 (Figure 1B), showing no statistical differences (Table 1). In the spring pH showed a marked increase at the end of experiment due to medium addition (Figure 2B). Outdoor tanks had both highest (8.77) and lowest (5.87) values although not differing from indoor tanks (Table 1). In overall, during autumn pH was higher in indoor (9.59) than in outdoor (9.19) which presented the lowest pH value (6.85, Figure 3B). Treatments were significantly different (P< 0.05, Table 1).

3.3. Salinity

Salinity of cultures showed great oscillations throughout all the experiments. In general, outdoor treatments had great salinity changes due to the effect of precipitation and evaporation. In winter, as observed in figures 1C and 1D, outdoor treatments showed a marked decrease (from 30 to 17) due to precipitation while salinities increased (from 31 to 35) in indoor treatments. These differences in winter were statistically significant (P<0.05, Table 1). Decrease in salinity observed indoor on day 25 was due to freshwater addition. Spring season did not show as much precipitation as winter and autumn and, therefore, was mainly subjected to evaporation (Figures 1D, 2D and 3D). Outdoor and

indoor treatments showed no significant differences, reaching salinities of 142 and 96, respectively (Figure 2C). Decreases observed on days 32 (indoor) and 37 (outdoor) were also due to freshwater addition. Autumn was the rainiest season of all, with precipitations reaching 110 mm (Figure 3D, Table 2). Despite of that, outdoor salinities varied from 31 to 43 while in indoor salinities varied from 34 to 47, treatments being statistically different (P<0.05, Table 1). Salinity decrease observed on day 18 (indoor) was due to freshwater addition. Table 2 presents mean precipitation and evaporation values for the three experiments.

3.4. Light transmission difference between treatments

Indoor treatment differed from outdoor treatment by being placed inside a greenhouse covered with a transparent LDPE film. According to the manufacturer the film has a light transmission of 89% although, in practice, measures taken during the experiments revealed a mean light transmission of 80%.

3.5. Cell abundance and biomass yields

Cell numbers and biomass yields were greatly influenced by precipitation and evaporation, thus data correction was performed in order to compensate volume dilution or concentration. Indoor treatment achieved the highest cell abundance (3.4×10^7 cm^{-3}) during winter than in any other seasons. A positive correlation for temperature ($\rho = 0.53$, P = 0.025) was observed in the outdoor treatment and for salinity ($\rho = 0.79$, P = 0.0001), whereas pH was negatively correlated ($\rho = -0.61$, P = 0.008). Outdoor treatment in spring followed the same pattern as indoor tanks, achieving 2.5×10^7 cm^{-3} (P<0.05, Table 3). Salinity was negatively correlated in both indoor ($\rho = -0.55$, P = 0.017) and outdoor ($\rho = -0.56$, P = 0.015) treatments. Radiation had a negative correlation ($\rho = -0.64$, P = 0.004) with cell abundance and biomass in indoor treatments. No differences were observed among indoor and outdoor treatments in autumn although temperature presented a negative correlation ($\rho = -0.52$, P = 0.034) and salinity was positively correlated ($\rho = 0.62$, P = 0.010) in outdoor treatments.

Winter biomass yields were greater indoor (300 g m^{-3}) than outdoor (200 g m^{-3}), being statistically different (P<0.05, Table 1). There was a positive correlation between

biomass and salinity (ρ = 0.75, P = 0.0004) and a negative correlation with pH (ρ = -0.80, P = <0.0001) in indoor treatments. During spring, indoor and outdoor treatments achieved values around 600 g m^{-3} and showed no difference. Both indoor (ρ = 0.73, P = 0.0006) and outdoor (ρ = 0.66, P = 0.003) treatments were positively correlated with salinity whereas evaporation (ρ = 0.69, P = 0.002) and radiation (ρ = 0.48, P = 0.046) were positively correlated only in outdoor treatments. Autumn, although producing less biomass (200 g m^{-3}) than spring, also showed no difference among treatments. Salinity showed a positive correlation (ρ = 0.56, P = 0.021) while evaporation was negatively correlated (ρ = -0.48, P = 0.049) with biomass yields in outdoor. In general, outdoor treatments showed significant differences among seasons with spring producing more biomass than winter and autumn. Indoor treatments produced more biomass in spring that in winter and autumn, respectively (P<0.05, Table 3).

4. Discussion

The indoor treatment presented a series of advantages in comparison to the tanks placed outdoor, regarding salinity and temperature variation. In general, it allowed the cultures to reach higher temperatures (about 4ºC more) especially during autumn and winter. Because of that, indoor treatment resulted in higher biomass especially in the winter experiment, when water temperature reached the lowest values. However in warmer seasons no differences were observed as clearly demonstrated in the spring and autumn experiments. Several authors [16,27-29] determined the optimum temperature for *N. oculata* being 21-26ºC although some strains seem to grow in lower (15ºC) [30] or higher temperatures (33ºC) [28].

Likewise, during the rainy seasons the indoor system avoided a decrease in salinity due to rainwater input and, hence, the decrease in productivity due to the cells dilution. Results obtained in autumn and winter experiments clearly demonstrated a drop in salinity values in the outdoor system. However, in the spring experiment, where temperatures were higher, cultures maintained indoor showed a steady rise in salinity due to evaporation, which required the addition of fresh water.

According to Renaud & Parry [31], *N. oculata* has a wide salinity tolerance. Abu-Rezq *et al.* [27] verified optimal range between 20 and 40 for this species whereas Pal *et al.* [32], on the other hand, observed that *Nannochloropsis sp.* presented a wide tolerance to high salinity, and that combined with high light incidence, increased biomass and lipid productivity. In the spring experiment, the salinity in outdoor and indoor systems reached values above 140 PSU. This fact is extremely important because cultivation in salinities that high can prevent invasive species in monospecific cultures. pH decreased during experiments probably due to nutrient impoverishment [33] or microorganisms respiration whereas addition of fresh medium resulted in pH increase.

In general, cell densities and biomass yields were higher indoor during winter although as the temperature increased (spring) no differences were observed. The biomass production obtained in this study is close to those of massive cultures of *N. oculata* in photobioreactors. For instance, Olofsson *et al.* [34] obtained dry weight concentrations of $1,100$ g m^{-3} in closed vertical flat panel flow-through photobioreactors. In the spring experiment we got similar biomass production (830 g m^{-3}) but certainly at lower costs. Regarding cell abundance, Huang *et al.* [35] obtained 5.2×10^7 cm^{-3} culturing *N. oculata* in photobioreactors. Indoor cultures in the winter reached similar results, around 4.6×10^7 cm^{-3}.

5. Conclusion

The use of open tanks inside greenhouses represents an improvement in the *N. oculata* production under colder seasons in subtropical regions as southern Brazil. This improvement is mainly due to higher temperatures within the greenhouse and better control of salinity, avoiding culture dilution due to precipitation. However further experiments must be made under controlled environmental conditions, CO_2 addition, pH range, salinity control due to evaporation, nutrients and initial cell density in order to maximize *N. oculata* production in the proposed semi-enclosed system.

Acknowledgments

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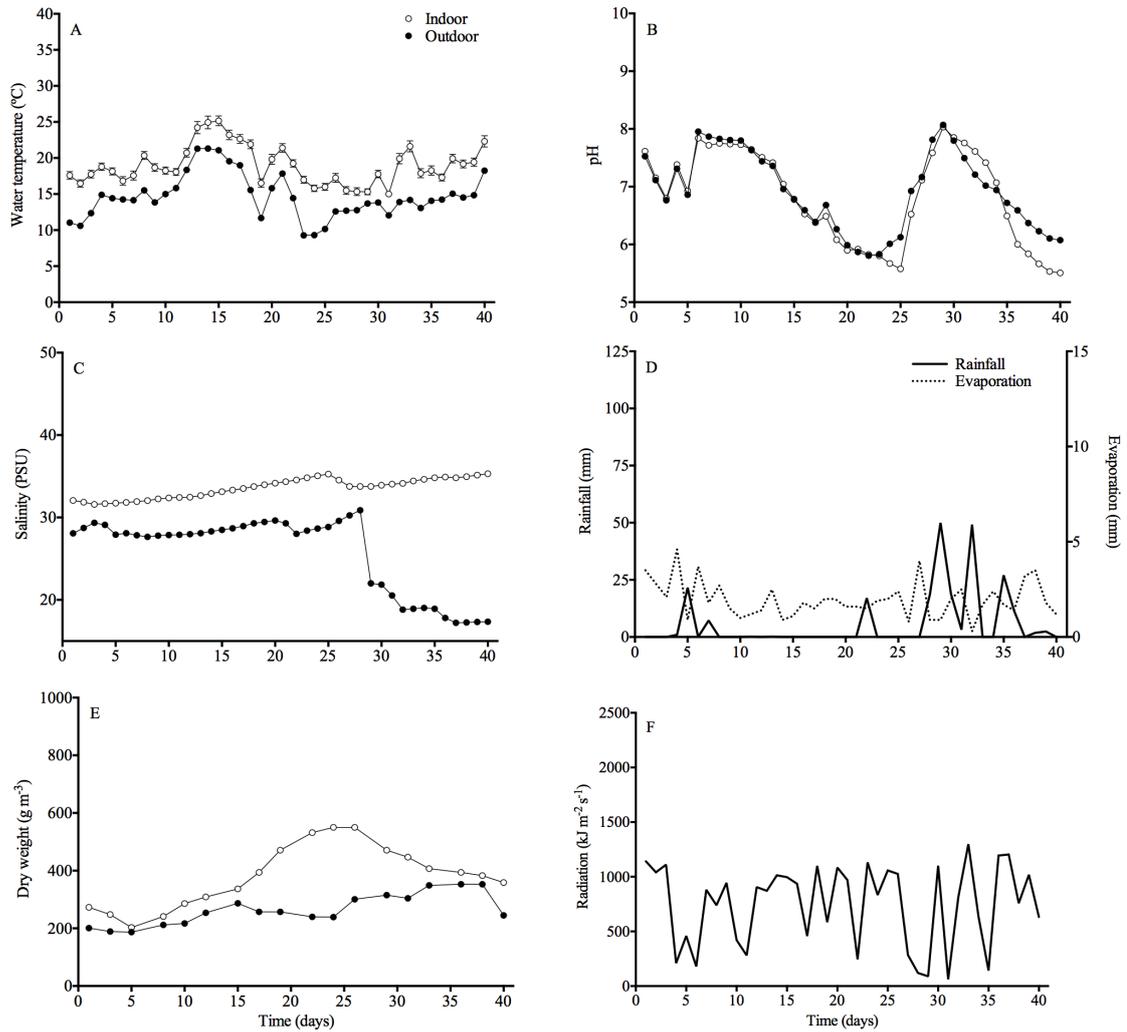
Fig. 1: a) Water temperature (ºC), b) pH, c) salinity (PSU), d) rainfall and evaporation (mm), e) dry biomass yields (g m^{-3}) and f) radiation (kJ m^{-3} s^{-1}) in indoor and outdoor cultures of *Nannochloropsis oculata* in winter experiment. Data are represented with mean values ± standard error (SE) (n=3) except for meteorological data.
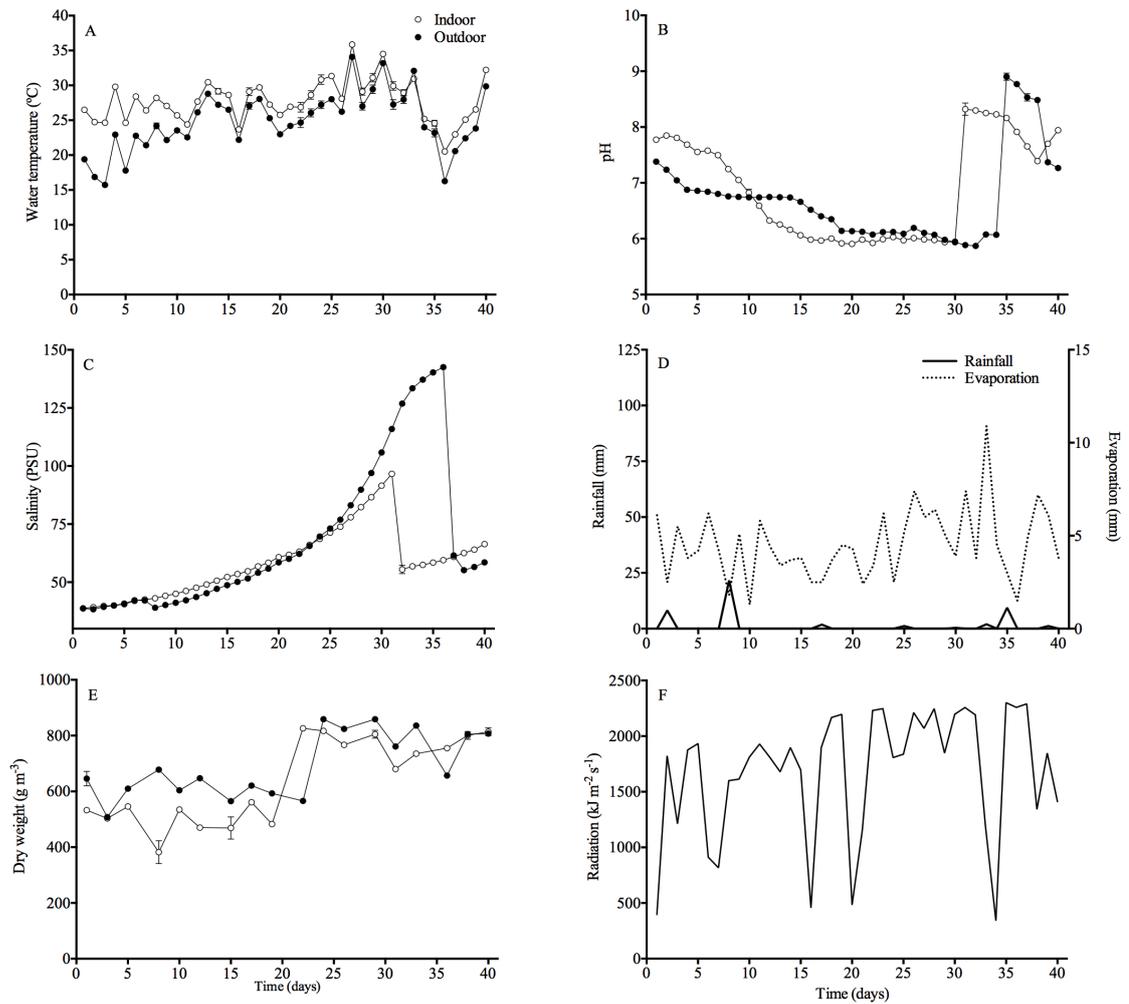
Fig. 2: a) Water temperature (℃), b) pH, c) salinity (PSU), d) rainfall and evaporation (mm), e) dry biomass yields (g m^{-3}) and f) radiation (kJ m^{-3} s^{-1}) in indoor and outdoor cultures of *Nannochloropsis oculata* in spring experiment. Data are represented with mean values ± standard error (SE) (n=3) except for meteorological data.
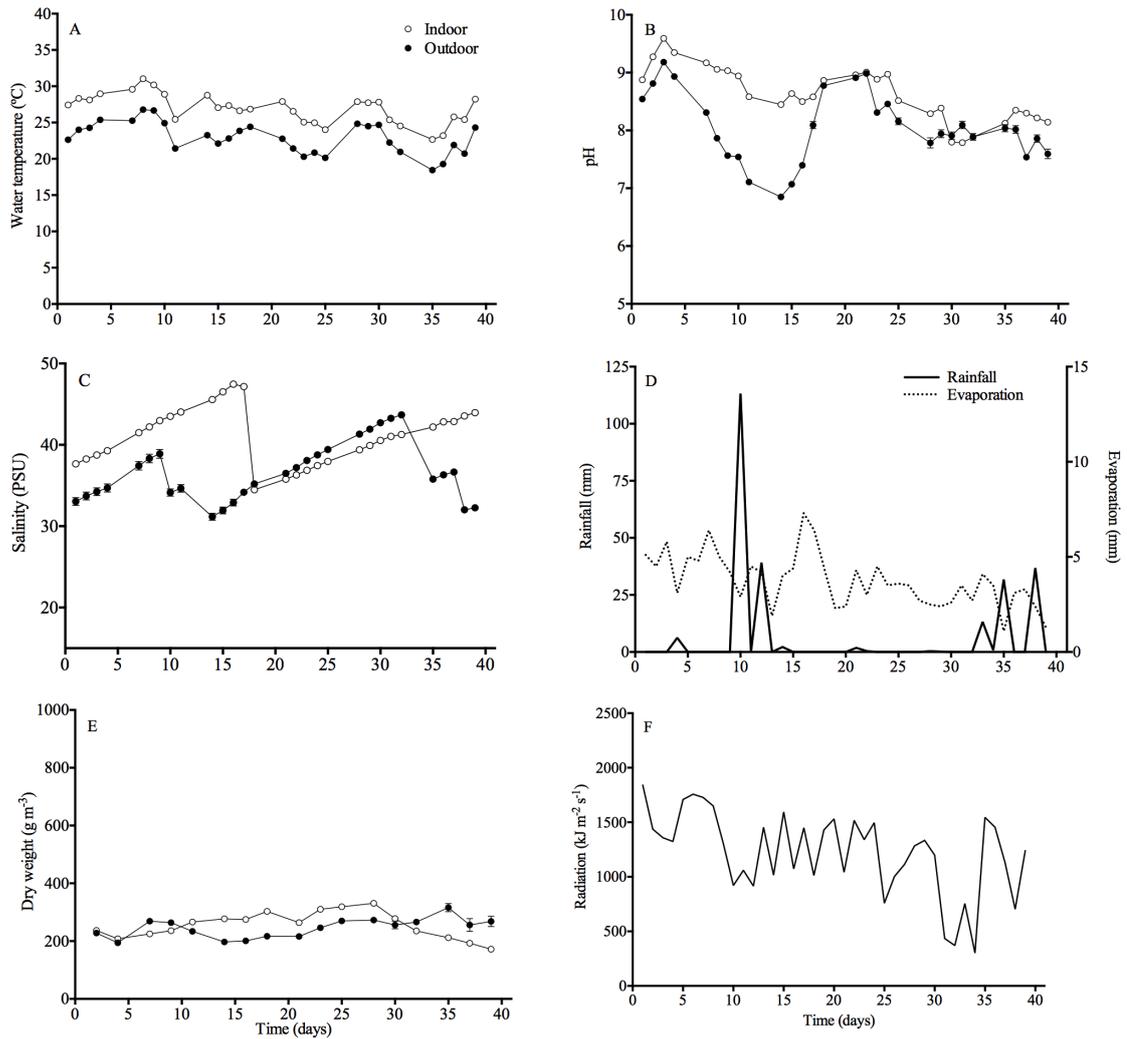
Fig. 3: a) Water temperature (ºC), b) pH, c) salinity (PSU), d) rainfall and evaporation (mm), e) dry biomass yields (g m^{-3}) and f) radiation (kJ m^{-3} s^{-1}) in indoor and outdoor cultures of *Nannochloropsis oculata* in autumn experiment. Data are represented with mean values ± standard error (SE) (n=3) except for meteorological data.

Table 1: Mean seasonal (±standard deviation) values of water temperature (ºC), salinity (PSU) and pH of *Nannochloropsis oculata* in indoor and outdoor treatments.

	Water Temperature		Salinity		pH	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Autumn	26.96 (±2.04)[Aa]	22.94 (±2.15)[Ba]	40.87 (±3.27)[b]	36.56 (±3.59)[Bb]	8.64 (±0.47)[Aa]	8.05 (±0.60)[Ba]
Winter	18.98 (±2.69)[Ab]	14.66 (±3.04)[Bb]	33.57 (±1.19)[Ac]	25.76 (±4.67)[Bb]	6.83 (±0.82)[Ab]	6.93 (±0.69)[Ab]
Spring	27.80 (±3.10)[Aa]	24.78 (±4.26)[Ba]	58.74 (±15.04)[Aa]	66.88 (±30.39)[Aa]	6.89 (±0.91)[Ab]	6.71 (±0.79)[Ab]

[ABC] Different uppercase letters indicate significant differences between indoor and outdoor (rows), for each season ($P < 0.05$).

[abc] Different lowercase letters indicate significant differences between seasons (columns), for indoors and outdoors ($P < 0.05$).

Table 2: Mean seasonal (±standard deviation) values of precipitation (mm), evaporation (mm), humidity (%), radiation (kJ m^{-2} s^{-1}), maximum and minimum air temperatures during the experiments.

	Precipitation	Evaporation	Humidity	Air Temperature		Radiation
				Minimum	Maximum	
Autumn	6.31 (±20.12)	3.75 (±1.38)	77.64 (±7.47)	16.84 (±2.86)	24.91 (±2.08)	1220 (±901)
Winter	5.74 (±12.39)	1.96 (±0.96)	82.59 (±10.09)	9.28 (±3.58)	17.35 (±3.73)	749 (±525)
Spring	1.15 (±3.86)	4.52 (±1.90)	71.83 (±10.65)	16.58 (±3.81)	24.54 (±3.29)	1686 (±1069)

Table 3: Mean seasonal (±standard deviation) cell densities (x10^7 cm^{-3}) and dry biomass yields (g m^{-3}) of *Nannochloropsis oculata* in indoor and outdoor cultures.

	Cell Density		Dry Weight	
	Indoor	Outdoor	Indoor	Outdoor
Autumn	2.15 (±1.04)^{Ab}	1.52 (±0.72)^{Ab}	250 (±50)^{Ac}	240 (±30)^{Ab}
Winter	3.44 (±0.85)^{Aa}	1.98 (±0.70)^{Bb}	360 (±100)^{Ab}	260 (±60)^{Bb}
Spring	1.62 (±0.63)^{Bb}	2.58 (±0.47)^{Aa}	630 (±150)^{Aa}	690 (±110)^{Aa}

^{ABC} Different uppercase letters indicate significant differences between indoor and outdoor (rows), for each season ($P < 0.05$).

^{abc} Different lowercase letters indicate significant differences between seasons (columns), for indoors and outdoors ($P < 0.05$).

Screening of commercial natural and synthetic cationic polymers for flocculation of freshwater and marine microalgae and effects of molecular weight and charge density

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Abstract

Twenty-five natural and synthetic cationic polymers of different molecular weights and charge densities were evaluated for microalgae flocculation. Tanfloc is a natural low molecular weight tannin polymer whereas Zetag and Flopam are both synthetic high molecular weight polyacrylamide polymers. Five exponential concentrations (0.55, 1.66, 5, 15 and 45 mg L^{-1}) were tested for freshwater *Chlorella vulgaris* and marine *Nannochloropsis oculata*. All polymers were efficient (>90% at $\geq$ 1.66 mg L^{-1}) for *C. vulgaris*. However, for *N. oculata,* only Tanfloc was effective. Charge density positively influenced flocculation decreasing the required polymer dosage. Restabilisation was observed only for synthetic polymers when overdosed. Natural polymers performed similarly for both species. In overall, Tanfloc SL and Flopam FO 4990 SH were the most efficient polymers for microalgae flocculation though Tanfloc is a more economic option (US\$ 37 ton^{-1} of biomass) and environmentally friendly than Flopam (US\$ 171 ton^{-1} of biomass).

Keywords: Microalgae; Coagulation; Biopolymer; Harvesting; Dewatering

1. Introduction

Microalgae are attracting a lot of interest as a new source of biomass for production of food, feed, bulk chemicals, or biofuels [1]. Harvesting is currently one of the major bottlenecks to large-scale production of microalgae [2]. Because of their small size (3 to 30 μm) and low biomass concentration (< 5 g L^{-1}), harvesting using centrifuges is too energy-intensive and costly, being only justified for high value bioproducts such as carotenoids or poly-unsaturated fatty acids [3-5]. For bulk production of biomass for commodities, a low-cost harvesting method is needed that can process large volumes of microalgae culture at a minimal cost.

Spontaneous flocculation of microalgae in suspension is prevented by electrostatic repulsion caused by the negative surface charge of the cells [6]. This negative charge is related to the presence of carboxyl, sulfate or phosphate groups on the microalgae cell surface. Hence, positively charged chemicals that interact with those negative surface charges can induce flocculation. In flocculation, small particles are combined into larger

aggregates. These large aggregates can be much more easily separated from the liquid medium than the individual cells [2]. Thus, flocculation has a lot of potential to be used as a low-cost and high-throughput method for harvesting microalgae.

An important class of chemicals used in flocculation is metal salts, such as ferric chloride or aluminum sulfate [7]. When dissolved in water, these metal salts form positively charged hydroxides that cause flocculation by neutralizing the negative charge of the microalgae cells or by causing a positively charged precipitate that enmeshes the microalgae cells and removes them from suspension ('sweep flocculation'). Metal salts have been successfully applied for flocculating microalgae [8-10]. However, these elements have the disadvantage that they require a relatively high dosage and that the biomass is contaminated with high concentrations of metals, limiting the application of the biomass due to metal toxicity [11].

Another class of chemicals that are widely used for microalgae flocculation is organic polymers. They can induce flocculation by neutralizing the negative surface charge, similar as for metal salts, and by forming bridges between the microalgae cells. The effectiveness of such polymers depends on their size, secondary structure in solution as well as on their charge density [7]. Organic polymers are generally preferred over metal salts because they require a much lower dosage. The majority of organic polymers that are commercially available are synthetic based on polyacrylamide [7]. Some studies have successfully applied synthetic polyacrylamide polymers for flocculating microalgae (e.g. [12-16]). Nevertheless, these studies have made clear that there are often large disparities in the effectiveness of different polymers when applied to microalgae (e.g. [12, 16]). It is not clear, however, which properties of polymers (e.g. charge density, polymer size, secondary structure) determine this variation in effectiveness.

Although synthetic polyacrylamide polymers as such are non-toxic, they may contain acrylamide residues that are presumable carcinogenic or display a high toxicity towards aquatic organisms [17]. Therefore, it is preferable to use natural based polymers, particularly when fractions of the microalgae biomass are to be used for animal feed,

which may be economically attractive in a biorefinery context [1]. A well-known natural cationic polymer is chitosan, a derivative of chitin obtained from shrimp shells. Several studies have shown that chitosan is quite effective for flocculating microalgae (e.g. [18, 19]). Other natural based polymers include derivatives of cassia gum [20] or starch [21]. Tanfloc is a relatively recently developed commercial biopolymer that is based on tannin [22]. It differs from other natural polymers in that it is not based on a polysaccharide but on a phenolic polymer. Tannins are branched polymers and thus have a different secondary structure than linear polymers such as chitosan or polyacrylamide. While Tanfloc has been used for removal of chemical contaminants [23] and turbidity in wastewater treatment [24], its potential for flocculating microalgae has not been thoroughly evaluated, although Roselet et al., [25] have recently analyzed the effect of pH, salinity, polymers dose and biomass concentration on Tanfloc efficiency in concentrating the marine microalga *N. oculata*, with good results.

A disadvantage of both synthetic and natural polymers is that they often undergo coiling when used in high ionic strength medium such as seawater (e.g. [8, 26]). Coiling changes the secondary structure of the polymer and this generally results in a decrease in the flocculation efficiency [27]. Many species of microalgae, including those that have a lot of potential for biodiesel production, are marine species. Therefore, it is important to evaluate whether synthetic and natural polymers have potential for harvesting of marine microalgae species.

The main objective of this study was to evaluate the potential of 25 different commercially available cationic polymers for flocculating microalgae. These polymers included different charge density variants of a low molecular weight natural tannin polymer (Tanfloc) and two high molecular weight synthetic polyacrylamide polymers (Flopam and Zetag). To evaluate the potential of these polymers for harvesting marine as well as freshwater microalgae, screening was performed on two model species, the freshwater *Chlorella vulgaris* and the marine *Nannochloropsis oculata*. The effects of molecular weight and charge density on the microalgae flocculation were evaluated and cost analysis was conducted for all tested polymers and compared with hydrolyzing metal salts and chitosan.

2. Materials and methods

2.1. Microalgae cultivation

The two microalgae model species used in this study were freshwater *Chlorella vulgaris* (SAG 211-11b) and marine *Nannochloropsis oculata* (SAG 38.85), obtained from the Culture Collection of Algae at Göttingen University (SAG, Germany). The microalgae were cultured in Wright's Cryptophyte medium prepared from pure salts and deionized water. For *N. oculata*, synthetic sea salt (Homarsel, Zoutman, Belgium) was added at a final concentration of 30 g L^{-1}. Both species were cultured for 6 days in 30 liters plexiglass bubble column photobioreactors mixed by sparging with 0.2 μm filtered air (5 L min^{-1}) in a temperature-controlled room (20ºC) [9]. The pH was maintained at 8 by addition of CO_2 (2-3%) using a pH-controller system. Each photobioreactor was continuously irradiated with daylight fluorescent tubes (100 μmol photons m^{-2} s^{-1}).

Microalgae biomass concentration was monitored daily by measuring the absorbance at 750 nm. Optical density measurements were calibrated against dry weight measured gravimetrically on pre-weighed GF/F glass fiber filters (R^2 = 0.998). The marine microalga was washed with 0.5 M ammonium formate, prior to filtration to remove salts absorbed on the cell surface. The final biomass concentrations after 6 days were 260 mg L^{-1} and 290 mg L^{-1} for *C. vulgaris* and *N. oculata*, respectively. The final concentrations were later confirmed by dry weight measurements.

2.2. Flocculation experiments

After day 6, the microalgae cultures were collected from the photobioreactors to be used in the flocculation experiments. All 25 polymers were simultaneously screened and flocculation experiments lasted approximately 4 hours. Microalgae may excrete large amounts of dissolved organic matter (DOM) into the culture medium and this may interfere with flocculation [9]. To avoid DOM interference in the flocculation experiments, the microalgae was centrifuged from the medium and resuspended in the same volume of fresh medium. This treatment reduced carbohydrate concentrations in the medium from 10 and 58 mg L^{-1} to 2 and 10 mg L^{-1} of glucose equivalent for *C. vulgaris* and *N. oculata*, respectively. Previous experiments had demonstrated that

centrifugation and subsequent resuspension in fresh medium had no significant effect on flocculation [9].

Twenty-five cationic polymers were compared. Table 1 lists the properties of the polymers used. Tanfloc is a natural low molecular weight quaternary ammonium polymer based on tannins extracted from the black wattle tree (*Acacia mearnsii*) and manufactured by TANAC (Brazil). Flopam and Zetag are both synthetic copolymers of acrylamide and quaternized cationic monomer polymers manufactured by SNF Floerger (France) and BASF (Germany), respectively. For Flopam, a series of polymers with similar molecular weight ($4.1 - 8.6 \times 10^6$ Da) but increasing charge densities ($2.5 - 100$ mol%) was used. For Zetag, we compared polymers with high (8125, 8160, 8180) and very high (7652, 8165, 8185) molecular weight and variable charge densities. For each polymer a 1 g L^{-1} stock solution was prepared by adding 50 mg of polymers to 50 mL of deionized water and mixed for 1 hour. Zetag was initially moistened with 3% acetone as indicated by the manufacturer. For each polymer, five exponential concentrations (0.55, 1.66, 5, 15 and 45 mg L^{-1}) were selected to determine the order of magnitude of the dosage required to induce flocculation. All polymers used in this study were kindly provided by the manufacturers.

Jar test experiments were used to quantify the efficiency of *C. vulgaris* and *N. oculata* flocculation. During addition of polymers, the microalgae suspensions were intensively mixed (350 rpm) for 10 minutes, to allow uniform polymer dispersal, followed by gentler mixing (250 rpm) for 20 minutes to allow floc formation. Subsequently, the microalgae suspensions were allowed to settle for 30 minutes and then samples were collected in the middle of the clarified zone. Optical density at 750 nm was measured prior to polymer addition (OD_i) and after settling (OD_f) and the flocculation efficiency (η_a) was calculated as:

$$\eta_a = \frac{OD_i - OD_f}{OD_i} \times 100$$

Only flocculation efficiencies higher than 90% were considered effective.

2.3. Statistical analysis

Polymers doses and flocculation efficiencies were log transformed and a nonlinear regression analysis with least square iteration was performed to describe the polymers effectiveness. Each dose-response curve was compared by extra sum-of-squares F test ($P < 0.05$) and D'Agostino-Pearson omnibus test was performed to verify dataset normality.

2.4. Cost analysis

Analysis was conducted to quantify the cost of flocculating *C. vulgaris* and *N. oculata* using hydrolyzing metal salts ($Al_2(SO_4)_3$ and $AlCl_3$), synthetic (Flopam and Zetag) and natural (chitosan and Tanfloc) flocculants. Initial biomass concentration, flocculant dose and efficiency for hydrolyzing metal salts and chitosan were obtained from previous studies for both species [9, 10] and are presented in Table 3. Costs of Tanfloc, Flopam and Zetag were provided by the manufacturers whereas costs of hydrolyzing metal salts and chitosan were obtained from bulk vendors of industrial chemicals (Alibaba). All flocculant costs were calculated in US$ per metric ton of dried microalgae. Costs related to harvesting apparatus or energy consumption were not considered.

3. Results and discussion

3.1. Screening results

The polyacrylamide polymers Flopam and Zetag were very effective at flocculating the freshwater *C. vulgaris* and no differences were observed within each polymer series as the dose-response curves did not differ ($P > 0.05$). However, Flopam and Zetag were not capable of flocculating the marine *N. oculata* and performance within polymer series varied significantly ($P < 0.05$) due to differences in charge density. The tannin polymers, on the other hand, were effective at flocculating both *C. vulgaris* and *N. oculata* and no differences ($P > 0.05$) were observed within Tanfloc variants (Table 1). The poor performance of Flopam and Zetag polymers in marine medium is not surprising, as it is well known that polymers often undergo coiling because of the high ionic strength of saltwater. Bilanovic et al. [26] employed Zetag to harvest the marine *Chlorella stigmatophora* and reported that reducing the medium salinity significantly improved flocculation. König et al. [28] employed Flopam to harvest the marine

microalga *Conticribra weissflogii*, reporting that salinity negatively impacted flocculation. A poor performance in marine medium has also been observed for polymers based on natural polysaccharides such as chitosan [8] and cationic starch [21].

Flopam and Zetag generally had high flocculation efficiency at a dosage of 1.66 mg L^{-1} while a dosage of 5 mg L^{-1} was required for effective flocculation with Tanfloc. At the highest dosages, the flocculation efficiency of the polyacrylamide polymers declined. This is an indication of restabilisation, caused by charge reversal of the microalgae cell surface. Restabilisation has also been observed for other natural polymers, such as chitosan [29] or cationic starch [21]. However, no such restabilisation was observed when using Tanfloc.

To date, hydrolyzing metal salts, synthetic and natural polymers were reported for flocculating freshwater and marine microalgae (Table 2). For example, Vandamme et al. [9] employed $Al_2(SO_4)_3$ to harvest *C. vulgaris* whereas Garzon-Sanabria et al. [10] used $AlCl_3$ for *N. salina*. However, the required dosage for such flocculants is higher than the dosage needed for synthetic or natural polymers (20-50 mg L^{-1}). In this study, several Flopam polymers were evaluated. For *N. oculata*, efficiency ranged from 8 to 90% at 0.55 mg L^{-1} polymer concentration (Table 1). Garzon-Sanabria et al. [10], working with *N. salina*, also employed four Flopam polymers (4550, 4650, 4800 and 4990), reporting efficiencies ranging from 73% to 94% at 3 mg L^{-1} dose, similar with the present study (Table 2). The higher biomass concentration (700 mg L^{-1}) employed in the Garzon-Sanabria et al. [10] experiment may explain the increased optimal dosage used by the authors. In the present study, *C. vulgaris* was readily harvested (100%) with 5 mg L^{-1} of Zetag 8185, a very high molecular weight and high charge density polymer. For *N. oculata* the same polymer resulted in 75% removal at 0.55 mg L^{-1}. Udom et al. [30] employed Zetag polymers of high and very high molecular weight (8846, 8848, 8814, 8816 and 8819), ranging from medium to very high charge densities, to concentrate *Chlorella sp.* grown on wastewater. Zetag 8819, according to the authors, presented the highest efficiency (98%) at the lowest optimal dosage (34 mg L^{-1}). However, Eldridge et al. [31] reported Zetag 7570 (of high molecular weight and charge density) as being ineffective for *N. salina* at doses up to 20 mg L^{-1}. Both studies

reported higher dosages, which may be explained by the higher biomass concentration employed and by the presence of DOM in the medium, which may have inhibited flocculation [9] (Table 2).

The present work tested Tanfloc, a tannin polymer, for harvesting *C. vulgaris* and *N. oculata*. Flocculation was achieved at 5 mg L^{-1} for both species, resulting in more than 97% removal. These results are in accordance with Roselet et al. [25], who achieved 95-98% removal for *N. oculata* employing Tanfloc doses between 1 and 10 mg L^{-1}. Wang et al. [32] recently tested a quaternized-modified tannin to harvest *Microcystis aeruginosa*. Applying a dose of 10 mg L^{-1} also resulted in 97% removal efficiency, though in a medium containing DOM. Comparing with chitosan, Vandamme et al. [9] and Garzon-Sanabria et al. [10] required 8 mg L^{-1} and 3 mg L^{-1} to flocculate *C. vulgaris* and *N. salina* achieving 85% and 98% efficiency, respectively (Table 2). This study confirms that Tanfloc works well in marine medium and therefore has potential to be used for harvesting other marine microalgae species. The fact that the flocculation efficiency of Tanfloc does not differ between freshwater and marine medium may be due to different secondary structure of tannin in comparison to polyacrylamide or polysaccharides, being Tanfloc a branched rather than a linear polymer. As a result, it may be less affected by coiling than polyacrylamide polymers.

3.2. Effect of molecular weight and charge density

The Tanfloc series is only composed of low molecular weight polymers with low-medium charge densities. Considering the aggregation mechanism, low molecular weight polymers act mostly by charge neutralization [12] and require higher dosages than high molecular weight polymers [33]. However Tanfloc dosages were much lower than other low molecular weight flocculants like $AlCl_3$ and $Al_2(SO_4)_3$ and similar to high molecular weight Flopam and Zetag (Tables 1 and 2). Regarding charge neutralization, molecular weight has little importance, thus increasing charge density should prove most effective [33]. Therefore, the different flocculation efficiencies observed for Tanfloc may be related to differences in charge density though no significant ($P > 0.05$) differences were observed within variants (Table 1).

On the other hand, the Flopam series is composed of high molecular weight polymers $(4.1 - 8.6 \times 10^6$ Da) with charge densities ranging from very low (2.5 mol%) to very high (100 mol%). It is acknowledged that high molecular weight polymers act better as bridging agents [3]. Interestingly, results demonstrate that increasing the molecular weight negatively affected the flocculation efficiency (Figure 1). From the Flopam series, we notice that those polymers with the highest molecular weight presented lower charge densities. This can be explained as, for high molecular weight polymers, size depends on the interaction between polymer segments. Thus, increasing the charge density, the polymer adopts a more expanded configuration [7]. Figure 1 exemplifies that effect for *C. vulgaris* and *N. oculata*. For 0.55 mg L^{-1}, increasing the charge density improved the flocculation efficiency from 1% to 80% and from 8% to 90% for *C. vulgaris* and *N. oculata*, respectively. Despite having high molecular weights, those with lower charge densities were unable to expand the polymer segments or to neutralize the cell surface charge.

For *N. oculata*, however, we can distinguish four statistically different (P < 0.05) regions relating Flopam efficiency and charge density (Table 1). For very low charge density polymers (≤ 10 mol%), efficiency improves as charge increases, with an optimal dosage exceeding 45 mg L^{-1}. Therefore, very low charge density polymers require larger dosages than polymers with higher charges. Low (≤ 25 mol%) and medium charge densities polymers (≤ 45 mol%) attained maximal efficiency between 1.66 and 5 mg L^{-1} whereas restabilisation was evident to occur at higher doses. However, low and medium charge density polymers composed two different groups (P < 0.05). At last, for high (≤ 70 mol%) and very high charge densities polymers (≥ 80 mol%), the optimal dosage seems to be under 0.55 mg L^{-1} and increasing dosages induced restabilisation.

Similarly, the Zetag series is constituted of high molecular weight (8125, 8160, 8180) and very high molecular weight (7652, 8165, 8185) polymers, with charge densities ranging from low to high. The effects of charge density are comparable to those described for Flopam. In general, three statistically different (P < 0.05) regions were observed, mostly related to charge density than to molecular weight (Table 1). Region 1, with lower efficiencies, was composed of Zetag 8125 and 7652. Region 2, with

medium efficiency, was composed of polymers 8160 and 8165. At last, Region 3 was composed of high charge densities Zetag 8180 and 8185 polymers.

Garzon-Sanabria et al. [10] evaluated the effect of polymer molecular weight and charge density on harvesting of *N. salina* comparing a low molecular weight polyamine polymer (Floquat FL 2949) with four high molecular weight polyacrylamide polymers from the Flopam series (4550, 4650, 4800 and 4990). The authors concluded that Floquat did not resulted in a substantial flocculation even at concentrations up to 100 mg L^{-1} whereas Flopam achieved >90% at concentrations between 20-30 mg L^{-1}. Regarding charge density, flocculation was most efficient when using FO 4990 SH, the highest charge density polymer. Udom et al. [30] compared several Zetag polymers (8846, 8848, 8814, 8816 and 8819), with molecular weight ranging from high to very high. Zetag 8819 was selected for further study because it provided the highest harvesting efficiency (98%) at the lowest optimal dose (34 mg L^{-1}).

3.3. Cost analysis

Polymer cost is an important factor to be considered as biomass recovery can contribute 20-30% to the total budget of the produced biomass [34]. Thus, a cost analysis based on dose and efficiency among hydrolyzing metal salts ($Al_2(SO_4)_3$ and $AlCl_3$), synthetic (Flopam and Zetag) and natural polymers (Chitosan and Tanfloc) can be found in Table 3. Hydrolyzing metal salts were the least expensive, costing ~US\$ 34 metric ton^{-1} of biomass harvested, thought the quantity needed was higher (~86 kg metric ton^{-1} of biomass) comparing to polymers (~21 kg metric ton^{-1} of biomass). Furthermore, hydrolyzing metal salts are not recommended for harvesting microalgae due to biomass contamination with residual metal [11]. On the other hand, synthetic polymers, like Zetag and Flopam, were highly efficient at a very low dosage although they were much more expensive than metal salts, at ~US\$ 171 metric ton^{-1}. Moreover, dispersion of toxic acrylamide oligomers to the environment may happen, which may also present a health hazard [18]. Regarding Zetag, the manufacturer recommends it to be moistened with 3% acetone prior to dissolving with water, what may increase not only costs but also environmental risks. For these reasons, alternative natural polymers like chitosan have been considered for environmental applications [18]. However, the costs for

employing chitosan vary greatly, depending on the studies. For example, in table 3, the calculated cost for harvesting *N. salina* was only US$ 44 metric ton^{-1} whereas for *C. vulgaris* it increased to US$ 376 employing concentrations lower than 10 mg L^{-1}. Nonetheless, Rashid et al. [19] reported 120 mg L^{-1} as being the optimal dosage for chitosan, removing 92% of *C. vulgaris*, what would cost prohibitive US$ 1,860 metric ton^{-1} of biomass. In addition, the bulk price for chitosan varies between US$ 10,000 and 100,000 metric ton^{-1}. Instead, Tanfloc presented both performance and cost advantages, costing about US$ 37 for harvesting one ton of *C. vulgaris* and *N. oculata* in the present study. Sánchez-Martín et al. [35] also employed Tanfloc though to reduce turbidity in surface waters. Applying a dose of 10 mg L^{-1} resulted in 99% removal what, using the same calculations from Table 3, would cost ~US$ 73 metric ton^{-1} of biomass produced. More recently, Wang et al. [32] employed 10 mg L^{-1} of tannin to harvest 97% of *M. aeruginosa* which would cost ~US$ 75 metric ton^{-1}. Even at this high costs, having in mind that Tanfloc is a natural biopolymer, it is not only a much more economical but also a more ecological option for flocculating microalgae than potentially toxic hydrolyzing metal salts or synthetic polymers.

4. Conclusions

The result of this screening of a broad range of synthetic and natural polymers showed that flocculation of *N. oculata* and *C. vulgaris* was readily achieved using Tanfloc. On the other hand, Flopam and Zetag were most effective in freshwater. In addition, for synthetic polymers, data indicates that flocculation is largely influenced by charge density. Contrarily to synthetic polymers, restabilisation was not observed for Tanfloc. In overall, Tanfloc is a promising low cost and environmentally friendly polymer for both freshwater and marine flocculation.

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Table 1: Summary of screened polymers (0.55, 1.66, 5, 15 and 45 mg L⁻¹), molecular weight (10^6 Da), charge density (mole %) and flocculation efficiency (%) for *C. vulgaris* (260 mg L⁻¹) and *N. oculata* (290 mg L⁻¹). Efficiencies above 90% threshold are highlighted in bold.

Polymer	Molecular weight	Charge density	*Chlorella vulgaris* (260 mg L⁻¹)					*Nannochloropsis oculata* (290 mg L⁻¹)				
			0.55 mg L⁻¹	1.66 mg L⁻¹	5 mg L⁻¹	15 mg L⁻¹	45 mg L⁻¹	0.55 mg L⁻¹	1.66 mg L⁻¹	5 mg L⁻¹	15 mg L⁻¹	45 mg L⁻¹
TANFLOC												
SG 1500	Low	Low-medium	2	5	81	**98**	**99**[a]	8	37	**90**	**95**	**98**[a]
POP	Low	Low-medium	4	11	**99**	**100**	**100**[a]	14	71	**92**	**96**	**99**[a]
SG	Low	Low-medium	4	12	**99**	**100**	**100**[a]	22	76	**90**	**97**	**99**[a]
SL	Low	Low-medium	8	**90**	**100**	**100**	**100**[a]	70	**94**	**97**	**98**	**99**[a]
FLOPAM												
FO 4115 SH	5.9–7.7	2.5	1	7	57	**90**	**94**[a]	10	13	16	27	45[d]
FO 4125 SH	5.9–7.7	4	8	13	**98**	**96**	86[a]	8	8	12	15	38[d]
FO 4140 SH	5.9–7.7	5	5	84	**98**	**99**	**95**[a]	10	10	11	13	36[d]
FO 4190 SH	6.3–8.1	10	28	**99**	**99**	**99**	**97**[a]	12	12	27	33	54[c]
FO 4240 SH	6.3–8.1	15	38	**99**	**100**	**100**	**98**[a]	16	16	41	38	32[c]
FO 4290 SH	5.9–8.5	20	38	**99**	**99**	**99**	**98**[a]	22	23	50	41	36[c]
FO 4350 SH	5.5–8.5	25	35	**98**	**98**	**100**	**98**[a]	30	28	56	42	38[c]
FO 4400 SH	4.9–7.4	30	38	**99**	**100**	**100**	**97**[a]	42	37	63	52	47[b]
FO 4440 SH	4.8–7.1	35	45	**99**	**99**	**100**	**97**[a]	47	42	66	54	47[b]
FO 4490 SH	4.6–7.1	40	62	**99**	**98**	**100**	**98**[a]	67	66	66	56	47[b]
FO 4550 SH	4.1–7.1	45	77	**100**	**98**	**99**	**95**[a]	67	66	72	68	53[b]
FO 4650 SH	4.5–7.1	55	58	**99**	**99**	**99**	**94**[a]	81	72	75	61	48[a]
FO 4700 SH	4.9–7.1	70	70	**99**	**99**	**98**	**93**[a]	88	72	78	70	50[a]
FO 4800 SH	4.9–7.1	80	80	**99**	**99**	**98**	87[a]	87	72	73	63	45[a]
FO 4990 SH	4.9–7.1	100	77	**99**	**98**	**98**	**92**[a]	90	79	74	60	44[a]
ZETAG												
8125	High	Low	5	**99**	**99**	**99**	**96**[a]	10	15	25	27	44[c]
8160	High	Medium-high	30	**94**	**99**	**99**	**96**[a]	47	48	47	39	33[b]
8180	High	High	94	**95**	**99**	**98**	**91**[a]	70	60	48	41	35[a]
7652	Very High	Medium	3	63	**99**	**99**	**96**[a]	10	10	27	25	25[c]
8165	Very High	Medium-high	6	75	**99**	**99**	**97**[a]	43	33	51	43	35[b]
8185	Very High	High	12	**90**	**100**	**99**	**95**[a]	75	60	64	53	42[a]

[a] Different letters indicate significant differences between dose-response curves within each polymer series ($P < 0.05$).

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Table 2: Summary of different flocculants (hydrolyzing metal salts, synthetic and natural polymers) reported for harvesting *Chlorella* and *Nannochloropsis* species.

Flocculant	Microalgae species	Biomass (mg L⁻¹)	DOM ᵃ	Efficiency (%)	Dosage (mg L⁻¹)	Reference
Hydrolyzing metal salts						
AlCl₃	*N. salina*	700	–	90	50	[10]
Al₂(SO₄)₃	*C. vulgaris*	250	–	85	20	[9]
Synthetic						
Flopam FO 4550 SH	*N. salina*	700	–	73	3	[10]
	N. oculata	290	–	67	0.55	This study
	C. vulgaris	260	–	100	1.66	This study
Flopam FO 4650 SH	*N. salina*	700	–	73	3	[10]
	N. oculata	290	–	81	0.55	This study
	C. vulgaris	260	–	99	1.66	This study
Flopam FO 4800 SH	*N. salina*	700	–	88	3	[10]
	N. oculata	290	–	87	0.55	This study
	C. vulgaris	260	–	99	1.66	This study
Flopam FO 4990 SH	*N. salina*	700	–	94	3	[10]
	N. oculata	290	–	90	0.55	This study
	C. vulgaris	260	–	99	1.66	This study
Zetag 7570	*N. salina*	414	+	10	20	[31]
Zetag 8819	*Chlorella sp.*	700	+	98	34	[30]
Zetag 8185	*N. oculata*	290	–	75	0.55	This study
	C. vulgaris	260	–	100	5	This study
Natural						
Chitosan	*C. vulgaris*	250	–	85	8	[9]
Chitosan	*N. salina*	700	–	98	3	[10]
Tannin	*M. aeruginosa*	n/a	+	97	10	[32]
Tanfloc SL	*N. oculata*	290	–	97	5	This study
	C. vulgaris	260	–	100	5	This study

ᵃ – medium without DOM, + medium with DOM

Table 3: Cost analysis of harvesting *C. vulgaris* and *N. oculata* with hydrolyzing metal salts (Al$_2$(SO$_4$)$_3$ and AlCl$_3$), synthetic (Flopam and Zetag) and natural (chitosan and Tanfloc) polymers. All costs are in US$. Hydrolyzing metal salts and chitosan data were obtained from [9, 10].

	Chlorella vulgaris					*Nannochloropsis oculata*				
	Al$_2$(SO$_4$)$_3$	Flopam	Zetag	Chitosan	Tanfloc	AlCl$_3$	Flopam	Zetag	Chitosan	Tanfloc
Initial biomass (mg L^{-1})	250[a]	260	260	250[a]	260	700[b]	290	290	700[b]	290
Flocculant dosage (mg L^{-1})	20[a]	5	5	8[a]	5	50[b]	5	5	3[b]	5
Flocculant efficiency (%)	85[a]	98	100	85[a]	100	90[b]	74	64	98[b]	97
Biomass harvested (mg L^{-1})	213	255	260	213	260	630	215	186	686	281
Flocculant needed per ton of biomass harvested (ton)	0.094	0.020	0.019	0.038	0.019	0.079	0.023	0.027	0.004	0.018
Flocculant cost (US$ ton^{-1})	300	8,000	8,000	10,000	2,000	500	8,000	8,000	10,000	2,000
Flocculant cost per ton of biomass harvested (US$)	28	157	154[c]	376	38	40	186	216[c]	44	36

[a] [9]

[b] [10]

[c] Cost of wetting with 3% acetone not included
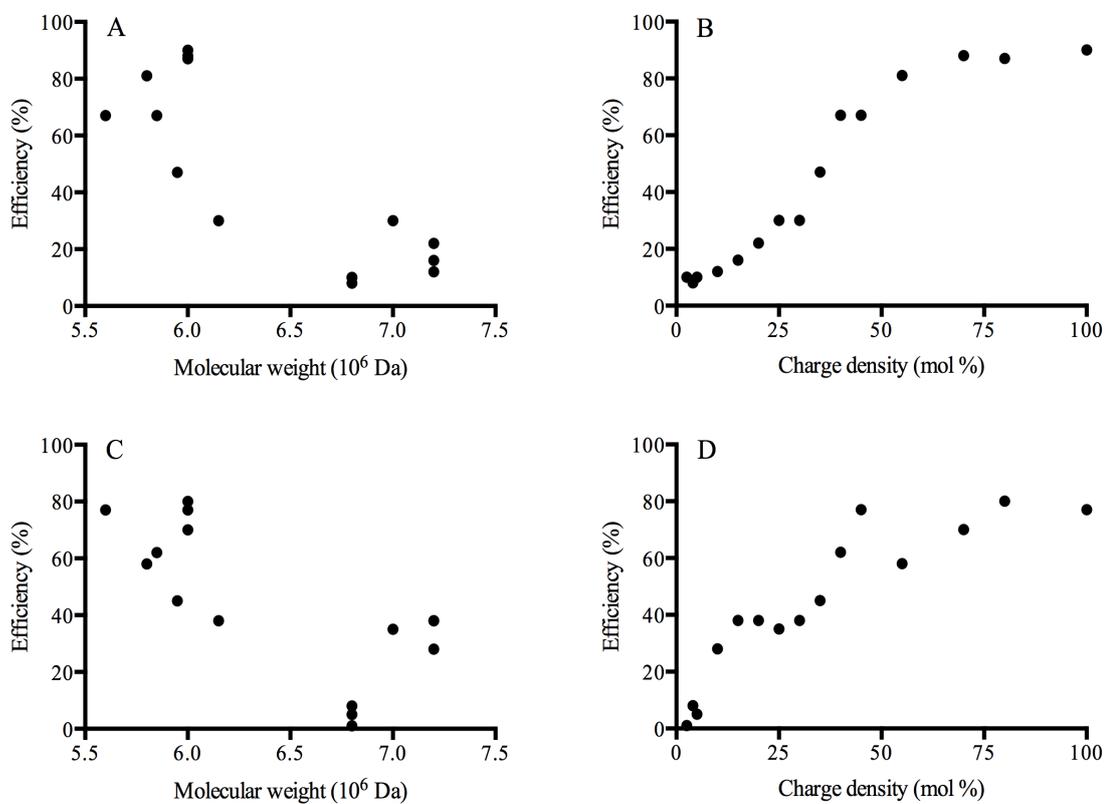
Fig. 1: Effect of mean molecular weight (10^6 Da) and charge density (mol%) on flocculation efficiency of *N. oculata* (A, B) and *C. vulgaris* (C, D). All polymers from the Flopam series were dosed at 0.55 mg L^{-1}.

CAPÍTULO III

Effects of pH, organic matter, salinity, biomass concentration and dosage on natural and synthetic polymers for flocculation of freshwater and marine microalgae

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Abstract

Commercial cationic polymers were evaluated for freshwater *Chlorella vulgaris* and marine *Nannochloropsis oculata* flocculation. Tanfloc SG and SL are natural low molecular weight tannin polymers whereas Flopam FO 4800 SH and FO 4990 SH are synthetic high molecular weight polyacrylamide polymers. Effects of pH, salinity, algal organic matter (AOM), and biomass concentrations were evaluated. The potential toxicity of polymers was evaluated by the maximum quantum yield of photosystem II. All polymers were efficient (>90%), however, flocculation was strongly regulated by the studied factors. In general, AOM and biomass concentrations regulated all polymers. Salinity and pH regulated Flopam and Tanfloc, respectively. Restabilisation was observed only for Flopam whereas results for Tanfloc indicate that the aggregation mechanism may be other than charge neutralization alone. Decrease in the maximum quantum yields was observed only for *C. vulgaris* flocculated with Flopam, indicating potential toxicity. Tanfloc was not affected by salinity, being recommended for flocculating *N. oculata*.

Keywords: Microalgae, Coagulation, Biopolymer, Harvesting, Dewatering

1. Introduction

Harvesting is one of the major bottlenecks to microalgae large-scale production for biofuels and other low value bioproducts (Vandamme et al., 2013) and can contribute 20-30% to the total budget of the produced biomass (Gudin and Thepenier, 1986). This cost is due to the small size of the microalgae cells (3 – 30 μm) and the relatively low biomass concentration (<0.5% dry weight) achieved in high productivity systems such as closed photobioreactors (Molina-Grima et al., 2003). Therefore, a large amount of water needs to be removed to concentrate the microalgae biomass (>90%). Currently, harvesting is mainly achieved by centrifugation (Wijffels and Barbosa, 2010), however this technology is expensive due to the high energy consumption (Rawat et al., 2013). Thus, centrifugation is only acceptable for highly valued bioproducts for use in food, cosmetics or pharmaceuticals (Borowitzka, 2013).

Contrarily, flocculation is a promising low-cost harvesting technology to harvest small amounts of suspended particles from large volumes of liquid. In addition, flocculation can be combined as a concentration step prior to centrifugation, thus reducing the volume to be manipulated and the production costs (Vandamme et al., 2013). In general, flocculation is achieved by addition of coagulants-flocculants that destabilize and aggregate small particles in suspension, by processes of charge neutralization or by establishment of bridges between the polymer and the particle. Traditionally, two broad classes of coagulants-flocculants are employed for flocculation, being hydrolyzing metal salts and organic polymers, based on synthetic polyacrylamide or on natural products (Gregory, 2013). Hydrolyzing metal salts are not recommended for microalgae harvesting as the presence of residual metal in the final biomass can interfere in downstream processing or cause toxicity (Farooq et al., 2015).

Currently, synthetic polyacrylamide polymers form the majority of coagulants-flocculants in commercial use (Gregory, 2013) and have successfully been applied for flocculating microalgae (Ebeling et al., 2005; Garzon-Sanabria et al., 2013). Synthetic polyacrylamide polymers as such are non-toxic, but they may contain monomer residues that are presumable toxic (Bolto and Gregory, 2007). Instead, natural based polymers are biodegradable and non-toxic which may be attractive if the microalgae biomass is to be used for animal feed, especially in a biorefinery context (Wijffels and Barbosa, 2010). A well-known natural cationic polymer is chitosan, a derivative of chitin obtained from shrimp shells, which is effective for flocculating microalgae (Vandamme et al., 2012; Garzon-Sanabria et al., 2013).

In a previous study, Roselet et al. (2015b) screened twenty-five commercial natural (Tanfloc) and synthetic (Flopam and Zetag) polymers of varying degrees of molecular weight and charge density for freshwater *Chlorella vulgaris* and marine *Nannochloropsis oculata* flocculation. The authors reported that flocculation was readily achieved for both species with Tanfloc whereas Flopam and Zetag were most effective in freshwater. In addition, the flocculation efficiency of Flopam and Zetag was largely influenced by charge density, with high charge density polymers performing

better. In overall, Tanfloc SL and Flopam FO 4990 SH were considered the most efficient polymers for microalgae flocculation.

However, the flocculation efficiency of synthetic and natural polymer is regulated by several factors. For example, culture pH regulates flocculation due to changes in the surface charges of the microalgae cells, the extent of coiling and the degree of ionization of polymers (Tenney et al., 1969; Lavoie and de la Noüe, 1987). Moreover, salinity reduces the chemical activity of polymers, masking their functional sites and changing the molecular structure (Bilanovic et al., 1988). Negatively charged algal organic matter (AOM) has been reported of interacting with cationic polymers, resulting in increased polymer requirement (Henderson et al., 2008; Vandamme et al., 2012). Finally, the polymer dosage and biomass concentration ratio should not be too high, otherwise the microalgae surface will become so highly covered that charge reversal will occur, resulting in suspension restabilisation (Bolto and Gregory, 2007).

Therefore, the main objective of this study was to evaluate the effects of pH, salinity, AOM, biomass concentration and polymer dosage on microalgae flocculation with synthetic and natural polymers. These included two low molecular weight natural tannin polymers (Tanfloc SG and SL) and two high molecular weight synthetic polyacrylamide polymers (Flopam FO 4800 SH and FO 4990 SH). Experiments were performed on two microalgae model species, the freshwater *Chlorella vulgaris* and the marine *Nannochloropsis oculata*. In addition, the maximum quantum yield of photosystem II of *C. vulgaris* and *N. oculata* was quantified in different polymer concentrations to evaluate the potential toxicity.

2. Materials and methods

2.1. Microalgae cultivation

Two microalgae species were used as models: the freshwater *Chlorella vulgaris* (SAG 211-11b) and the marine *Nannochloropsis oculata* (SAG 38.85), obtained from the Culture Collection of Algae at Göttingen University (SAG, Germany). The microalgae were cultured in Wright's Cryptophyte medium, which was prepared from pure salts and deionized water. For *Nannochloropsis*, synthetic sea salt (Homarsel, Zoutman,

Belgium) was added at a final concentration of 30 g L^{-1}. The microalgae were cultured for 6 days in 30 liters plexiglass bubble column photobioreactors mixed by bubbling with 0.2 μm filtered air (5 L min^{-1}) in a temperature-controlled room (20ºC). The pH was maintained at 8.5 by addition of CO_2 (2-3%) using a pH-controller system. Each photobioreactor was continuously irradiated with daylight fluorescent tubes (100 μmol photons m^{-2} s^{-1}). Microalgae biomass was monitored daily by measuring the absorbance at 750 nm. These measurements were calibrated against dry weight measured gravimetrically on pre-weighed GF/F glass fiber filters. The marine microalgae was washed with 0.5 M ammonium formate prior to filtration to remove salts absorbed on the cell surface.

2.2. Flocculation procedure

Four commercial cationic polymers were used in the experiments. Tanfloc SG and SL are natural low molecular weight quaternary ammonium polymers based on tannins extracted from the black wattle tree (*Acacia mearnsii*) and manufactured by Tanac (Brazil). Flopam FO 4800 SH and FO 4990 SH are high molecular weight synthetic copolymer of acrylamide and quaternized cationic monomer polymers manufactured by SNF Floerger (France) with charge densities of 80 and 100 mol%, respectively. All polymers were kindly provided by the manufacturers. For each polymer a 1 g L^{-1} stock solution was prepared by adding 50 mg of polymers to 50 mL of deionized water and mixed for 1 h. Thirteen concentrations (0, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg L^{-1}) were selected to evaluate the flocculation behavior.

Standardized 100 ml jar test were used to evaluate flocculation of *C. vulgaris* and *N. oculata*. During addition of the polymers, the microalgae suspensions were intensively mixed (350 rpm) for 10 min, to allow uniform polymer dispersion, then followed by gentler mixing (250 rpm) for 20 min to allow floc formation. Subsequently, the microalgae suspensions were allowed to settle for 30 min and then samples were collected in the middle of the clarified zone. Optical density at 750 nm was measured prior to polymer addition (OD_i) and after settling (OD_f) and the flocculation efficiency (η_a) was calculated as:

$$\eta_a = \frac{OD_i - OD_f}{OD_i} \times 100$$

2.3. Effect of pH, AOM, salinity and biomass

After 6 days of culturing, the microalgae were collected from the photobioreactors to be used in the flocculation experiments. To investigate the influence of pH on flocculation, three pH values (5, 7 and 9) were adjusted using 0.5 N HCl or 0.5 N NaOH solutions. The importance of AOM on flocculation of *C. vulgaris* and *N. oculata* was compared in medium with and without AOM. To remove AOM, the microalgae were centrifuged from the original medium and resuspended in fresh medium. AOM in both mediums was estimated through measurement of the total carbohydrates, which comprises the major fraction of the AOM (Myklestad, 1995), using the phenol-sulfuric acid method. In overall, the carbohydrate content in the fresh medium was reduced approximately five times in comparison with the original medium. Previous experiments had demonstrated that centrifugation and subsequent resuspension in fresh medium had no significant effect on flocculation (Vandamme et al., 2012). Two concentrations of synthetic sea salt were added (15 mg L^{-1} and 30 mg L^{-1}) to fresh medium to evaluate the effect on flocculation of *N. oculata* and compared with flocculation of *C. vulgaris*. The importance of biomass concentration on flocculation was also investigated. Three biomass concentrations (1, 2 and 4x) were prepared by resuspending different volumes of centrifuged microalgae in fresh medium. The final concentrations were further confirmed by dry weight measurements.

2.4. Polymer toxicity

The potential toxicity of polymers was evaluated by measuring the photosynthetic performance (i.e. quantum yield of photosystem II). The quantum yield of photosystem II is responsible for producing ATP so any compound that affects photosynthesis would alter the intracellular ATP content, therefore being a sensitive indicator of physiological stress (Cid et al., 1995). The quantum yield of photosystem II was measured (n=3) 1 h after addition of cationic polymer and 30 min of dark adaptation of microalgae, using an AquaPen-C fluorometer (Photon Systems Instruments, Czech Republic). Cells treated with 15% H_2O_2 for 30 min were used as negative control whereas cells without addition of polymer (no treatment) were used as positive controls.

2.5. Statistical analysis

For each treatment polymer dosage was log transformed and a dose-response nonlinear regression analysis with least square iteration described the relationship among measured variables. Each dosage-response curve was compared by extra sum-of-squares F test ($P < 0.05$) and D'Agostino-Pearson omnibus test was performed to verify dataset normality. Toxicity was analyzed by ordinary one-way ANOVA followed by Dunnett's multiple comparison tests.

3. Results and discussion

3.1. Effect of pH

In general, no significant effect of pH was observed on Flopam efficiency for *C. vulgaris* and *N. oculata* (Fig. 1A-D) as the dosage-response curves did not differed among treatments ($P > 0.05$). These results are in agreement with previous studies with Flopam (e.g. Garzon-Sanabria et al., 2013). According to Kam et al. (1999), cationic polymers with quaternized groups are salts of a strong base and therefore are not subjected to loss of charge density, regardless of pH. Additionally, Graham et al. (2008) reported that polyDADMAC, which is 90% quaternized, was permanently charged and insensitive to pH changes. Therefore, as Flopam FO 4800 SH and FO 4990 SH are both quaternary ammonium compounds with 80% and 100% quaternization, respectively, it is expected that pH does not interfere with its charge density.

However, higher Flopam dosages resulted in better flocculation as pH increased (Fig. 1A and B). As the surface charge of microalgae cells becomes more negative in highest pH (Lavoie and de la Noüe, 1987), the optimal dosage also increases whereas at lower pH the required dosage is reduced due to a less negative surface charge. This conjecture is confirmed when observing the flocculation efficiency at low dosages. For example (Fig. 1C), at pH 5 the microalgae cell surface charge is less negative requiring only 5 mg L^{-1} to result in ~57% efficiency whereas, at pH 9 where the cell surface charge is more negative a similar efficiency is attained only at 20 mg L^{-1}.

In contrast, Tanfloc was significantly ($P < 0.05$) affected by pH as results demonstrate that efficiency decreased at pH 9 (Fig. 1E-H) and each treatment presented individual

dosage-response curves. Considering the aggregation mechanism, low molecular weight polymers act mostly by charge neutralization (Ebeling et al., 2005). According to the manufacturer, the point of zero charge of Tanfloc is at pH 8.17. Consequently, above that point the zeta potential of Tanfloc becomes negative, loosing its ability to neutralize the negative charge of the microalgae cells. These results are in accordance with previous studies with Tanfloc for anionic surfactant removal (e.g. Beltrán-Heredia et al., 2009). In addition, Roselet et al. (2015a) employed Tanfloc at pilot scale and reported that reducing pH from 8 to 6 improved *N. oculata* flocculation from 33% to 95%. Graham et al. (2008) observed that the deprotonation of the primary amine due to pH increase resulted in loss of charge and efficiency of Tanfloc. Moreover, Sánchez-Martín et al. (2009) employed Tanfloc for surface water treatment, and reported that lowering pH enhanced turbidity removal. Recently, Gutiérrez et al. (2015) employed Tanfloc for harvesting microalgae from wastewater treatment reporting 90.2% recovery at pH 7.9. Wang et al. (2013) modified a tannin extract by quaternization increasing the point of zero charge to 8.9, which improved flocculation and confirmed the charge neutralization mechanism. Therefore, results from the present work corroborate that Tanfloc is affected by pH.

It is interesting that at pH 9, therefore above the point of zero charge, Tanfloc resulted in more than 90% efficiency for both freshwater and marine species (Fig. 1E-H). The same trend was observed by Graham et al. (2008) who reported that increasing pH from 4 to 9 resulted in higher optimal dosage whereas the charge density was reduced from 3.07 to 0.21 mequiv g^{-1}. Therefore, due to the small charge density the authors suggested that the aggregation mechanism was principally by adsorption or enmeshment by precipitated Tanfloc (i.e. sweep flocculation).

3.2. Effect of AOM

The presence of AOM negatively (P < 0.05) affected both Flopam and Tanfloc efficiencies regardless of species, increasing the required dosage (Fig. 2). All treatments presented different dosage-response curves. It is well acknowledged that AOM has negative impacts on flocculation (Henderson et al., 2008; Vandamme et al., 2012; Garzon-Sanabria et al., 2013). According to Henderson et al. (2008), AOM has a

negative zeta potential in pH ranging from 2 to 10, consequently interacting with the cationic polymers. This interaction results in fewer polymers available for charge neutralization or bridging with microalgae, thus increasing the required dosage. For example, in the case of *C. vulgaris* 1.5 mg L^{-1} of AOM accounted for 84% of the charge (Henderson et al., 2010). In the present work, AOM concentrations in medium were 10.6 and 58.5 mg L^{-1} whereas in medium without AOM, concentrations were reduced to 2.3 and 15.6 mg L^{-1} for *C. vulgaris* and *N. oculata*, respectively. The difference in efficiencies obtained for both mediums reflects the interaction of AOM with the cationic polymers.

In general, flocculation was reduced in medium with AOM increasing dosages up to 10 fold to achieve efficiencies comparable to medium without AOM. Similarly, Vandamme et al. (2012) also studied *C. vulgaris* and reported that AOM affected five different flocculation methods increasing the dosage up to 9 fold. Flopam required optimal dosages up to ~20 mg L^{-1} in medium with AOM (Fig. 2A-D). These findings are similar with those by Garzon-Sanabria et al. (2013), who also employed Flopam to flocculate *N. salina* and observed that 20 mg L^{-1} (a 7 fold increase) in polymer dosage was required in medium with AOM. Comparing with Flopam, Tanfloc required higher dosages (~60 mg L^{-1}) to achieve more than 90% efficiency in medium with AOM (Fig. 2E-H), meaning that is more affected by AOM than Flopam.

In medium without AOM, dosages and efficiencies were similar within Flopam and Tanfloc (Fig. 2). In overall, those differences were mostly related to variations in polymers charge densities. For example, Flopam FO 4800 SH and FO 4990 SH have charge densities of 80 and 100 mol % respectively, which explains why FO 4990 SH performed better. No information is available for Tanfloc but SL performed better suggesting that it may have a higher charge density than SG. The optimal dosage for Flopam was 5-10 mg L^{-1} and 2 mg L^{-1} whereas Tanfloc had an optimal dosage of 5-10 mg L^{-1} and 20 mg L^{-1} for *C. vulgaris* and *N. oculata*, respectively.

The results from the present work indicate that AOM removal is essential to successful and economical flocculate the microalgae. In wastewater treatment, several

technologies, such as chlorine and ozonation, have been proposed to reduce the load of AOM (Henderson et al., 2008). However, these technologies can generate by-products such as trihalomethanes, which have been associated with adverse health effects (Krasner et al., 2006). In finfish and shellfish aquaculture, skimmers are often used to reduce the presence of proteins and polysaccharides in water (Barrut et al., 2013), which are the main constituents of AOM. Therefore, further studies must verify if skimmers can be successfully employed for AOM reduction and flocculation improvement.

3.3. Effect of salinity

Flopam efficiency was significantly ($P < 0.05$) affected by salinity as an increase from 15 g L^{-1} to 30 g L^{-1} of synthetic sea salt decreased flocculation (Fig. 3B and D). Besides, flocculation of freshwater *C. vulgaris* was more efficient than for the marine *N. oculata* clearly showing that flocculation was hindered in marine medium (Fig. 2A and C). The optimum dosages for Flopam were 2 mg L^{-1} for *N. oculata* and 5-10 mg L^{-1} for *C. vulgaris*. This result suggests that Flopam efficiency may have been improved by the compression of the double layer, reducing the required polymer dosage (Gregory, 2013). Restabilisation was mostly observed for *N. oculata*, indicating that Flopam (which is linear) undergoes coiling because of the high ionic strength of marine medium, as suggested by Bilanovic et al. (1988).

However, Garzon-Sanabria et al. (2013) compared flocculation of *N. salina* in medium with 5 and 35 g L^{-1} of NaCl and concluded that Flopam efficiency was not affected by salinity, relating it with the positive effect of the double layer compression caused by an increase in ionic strength. Possibly this difference can be related to the fact that Garzon-Sanabria et al. (2013) employed NaCl instead of synthetic sea salt, as in the present work. Recently, König et al. (2014) reported that Flopam efficiency was negatively impacted by salinity when harvesting the diatom *Conticribra weissflogii* in natural seawater.

Contrarily to Flopam, Tanfloc was not affected ($P > 0.05$) by salinity as efficiencies were described by the same dose-response curve. Besides, efficiencies were similar for both freshwater *C. vulgaris* and marine *N. oculata* (Fig. 3E-H). The fact that the

flocculation efficiency of Tanfloc does not differ between freshwater and marine medium may be due to different secondary structure of Tanfloc in comparison to Flopam, being Tanfloc a branched rather than a linear polymer. As a result, it may suffer less from coiling than Flopam at high ionic concentrations. Palomino et al. (2012) compared the efficiency of linear and branched cationic polymers on flocculating latex particles in solution containing monovalent salts. The authors reported that the branched polymer performed better than the linear polymer.

In a previous study, Roselet et al. (2015b) screened twenty-five cationic polymers for *C. vulgaris* and *N. oculata* flocculation and reported no differences in efficiency for Tanfloc in synthetic sea salt. However, in another work Roselet et al. (2015a) obtained a different result when comparing Tanfloc efficiency for harvesting *N. oculata* in natural seawater. In overall, reducing salinity from 30 to 10 PSU increased flocculation 41%. The results from the present work reveal that conducting flocculation experiments with synthetic seawater or NaCl may incur to conclusions not applicable to real case scenarios. According to Schlesinger et al. (2012), due to the shortage of freshwater much of the culturing of microalgae will utilize sea- or brackish water. Thus, the present results recommend that any flocculation procedure must be performed with natural seawater.

3.4. Effect of biomass concentration

In general, the different biomass concentrations resulted in significantly ($P < 0.05$) different dose-response curves (Fig. 4). For lower biomass concentrations, lower polymer dosages resulted in good flocculation whereas higher dosages were required for higher biomass. For example, Flopam FO 4990 SH (Fig. 4C) achieved 100% efficiency at 5 mg L^{-1} in low biomass (1x) whereas increasing biomass to 4x required a dosage increase to 20 mg L^{-1}. However, at 100 mg L^{-1} dosage flocculation at 1x biomass was reduced to ~75% whereas, at 4x biomass the efficiency was still more than 90%. This result was expected, as polymer absorption should not be too low otherwise charge neutralization or bridging will not be effective. Conversely, polymer absorption should not be too high, otherwise the particle surfaces will become so highly covered that charge reversal will occur, resulting in restabilisation (Bolto and Gregory, 2007).

Similar results were obtained by Garzon-Sanabria et al. (2012), who employed different initial cell concentrations to study *N. oculata* flocculation with $AlCl_3$. The authors concluded that cell concentration had an effect on the dosage requirement. However, the $AlCl_3$ concentration per cell required to achieve at least 90% removal was not proportional to cell density as different ratios were obtained. Tenney et al. (1969) tested several biomass concentrations (100, 200 and 350 mg L^{-1}) but reported a linear relationship between the cationic polyamine and the *Chlorophyta* studied. Contrarily to Flopam, at high Tanfloc dosages no effect of biomass concentration was observed for both *C. vulgaris* and *N. oculata* (Fig. 4E-H).

3.5. Effect of polymer dosage

As observed in the previous sections, increasing Flopam dosage resulted in restabilisation (Figs. 1-4, A-D). It is acknowledged that high molecular weight polymers like Flopam act better as bridging agents (Molina Grima et al., 2003) where a segment of the polymer binds to the microalgae cell and the remainder is free to interact with other cells. However, in excess of polymer the microalgae cell surface becomes so covered that a reversal in surface charge may occur, resulting in steric repulsion and restabilisation of the cell (Bolto and Gregory, 2007). Tenney et al. (1969) employed synthetic organic polymers to remove algae from water and wastewater, reporting that optimal flocculation occurred at approximately 50% coverage of microalgae cells, with restabilisation occurring at higher surface coverage ratios.

Contrarily, increasing the concentration of Tanfloc up to 100 mg L^{-1} did not resulted in restabilisation (Figs. 1-4, E-H). Considering the aggregation mechanism, low molecular weight polymers like Tanfloc act mostly by charge neutralization (Ebeling et al., 2005). However, at high cationic polymer dosages, the microalgae becomes positively charged and restabilisation occurs (Gregory, 2013). The same trend was observed by Beltrán-Heredia and Sánchez-Martín (2009), who employed up to 600 mg L^{-1} of Tanfloc to remove turbidity of wastewater and restabilisation was not observed. Gutiérrez et al. (2015) employed Tanfloc from 10-60 mg L^{-1} for harvesting freshwater microalgae, also reporting that increasing dosage improved flocculation from 51.6 to 93.3%. However, Wang et al. (2013) reported restabilisation when employed a modified tannin extract to

flocculate *M. aeruginosa.* Based on the results from this and previous works, it is likely that lack of restabilisation is an exclusive feature of Tanfloc.

According to Graham et al. (2008), due to the small charge density of Tanfloc at pH 9, therefore above its point of zero charge, the aggregation mechanism was principally by adsorption or enmeshment by precipitated Tanfloc (i.e. sweep flocculation). This result is noteworthy, as sweep flocculation mechanism has only been reported for hydrolyzing metal salts, indicating that charge neutralization may not be the major aggregation mechanism (Gregory, 2013).

3.6. Polymer toxicity

Figure 5 presents the effects of Flopam and Tanfloc on the maximum quantum yields of photosystem II of *C. vulgaris* and *N. oculata.* No significant (P > 0.05) effect of Flopam was observed for *N. oculata* and either Tanfloc had no effect for *C. vulgaris* or *N. oculata.* Gutiérrez et al. (2015) recently assessed Tanfloc potential toxicity for freshwater microalgae using biochemical methane potential tests, reporting that doses up to 50 mg L^{-1} did not affect anaerobic digestion. However, significant (P < 0.05) effect of Flopam was observed for *C. vulgaris* where concentrations above 20 mg L^{-1} resulted in a constant decrease of the maximum quantum yield (Fig. 5 A, C). Therefore, it appears that Flopam has short-term effects on the viability of *C. vulgaris.* However studies must elucidate why Flopam interfered only with the maximum quantum yields of photosystem II of *C. vulgaris* and not with *N. oculata*

4. Conclusions

The results indicated that the efficiency of Flopam and Tanfloc was regulated by several factors. Flopam was affected by salinity, AOM and biomass concentration. Over dosage induced restabilisation. Decrease in the maximum quantum yields of *C. vulgaris* was observed, indicating toxicity. Tanfloc was affected by pH, AOM and biomass concentration. The absence of restabilisation and efficient flocculation above the point of zero charge indicate that the aggregation mechanism of Tanfloc may be other than charge neutralization alone. The effective removal of AOM is essential for successful

microalgae flocculation. Tanfloc appears to be an efficient polymer for marine microalgae flocculation.

Acknowledgements

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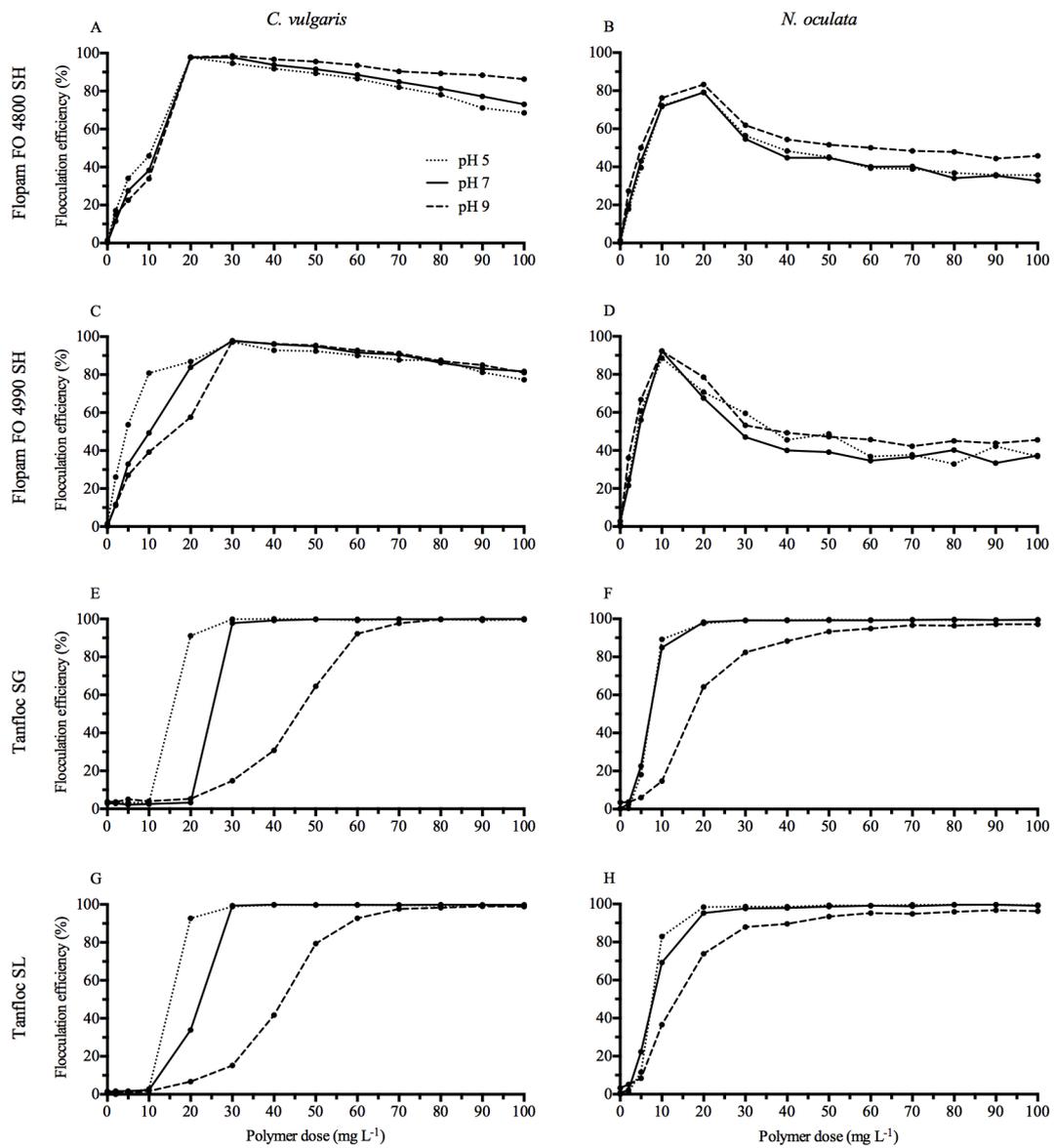
Fig. 1: Effect of pH (5, 7 and 9) and polymer dosage on flocculation efficiency of *Chlorella vulgaris* and *Nannochloropsis oculata*.
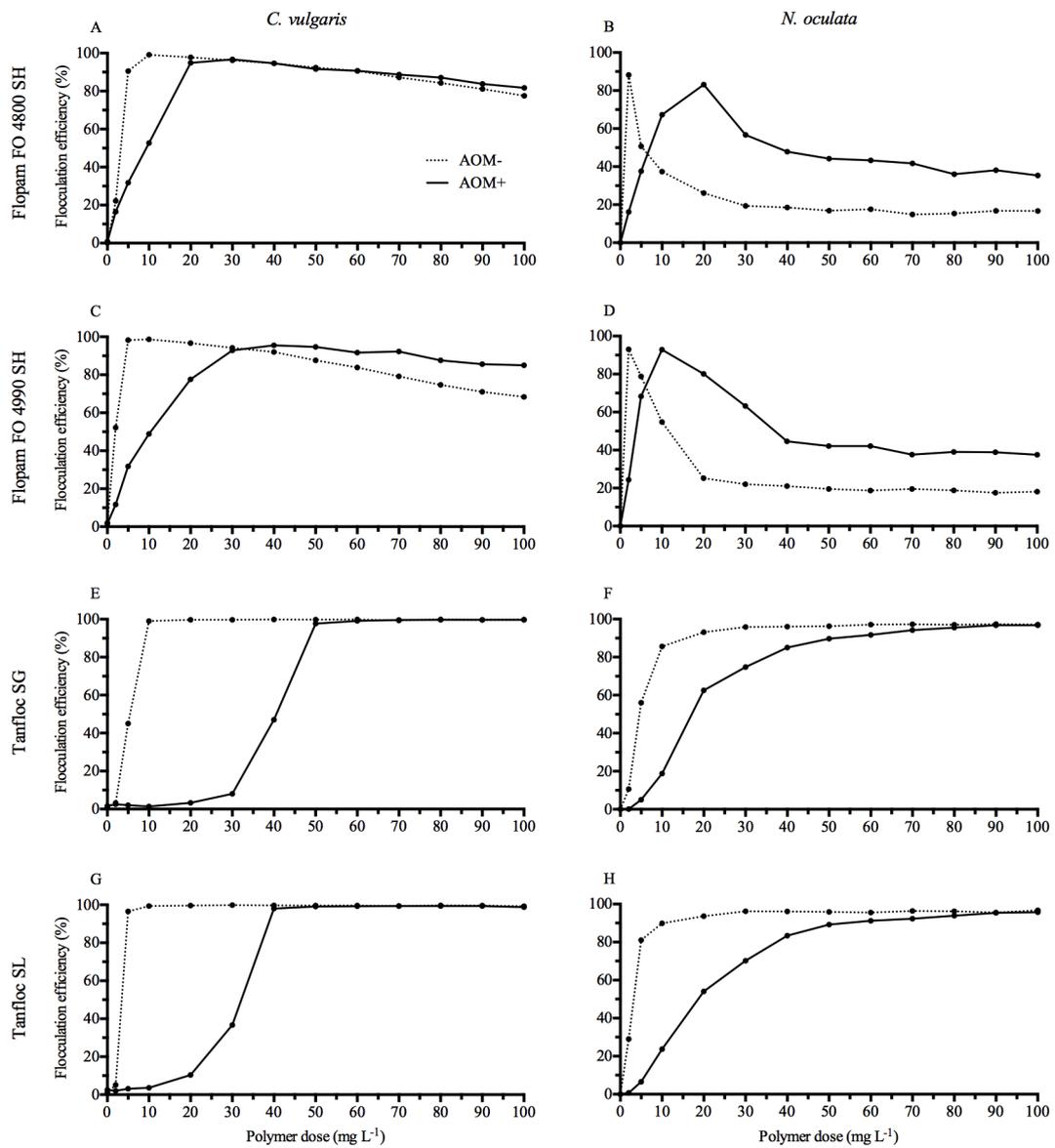
Fig. 2: Effect of AOM and polymer dosage on flocculation efficiency of *Chlorella vulgaris* and *Nannochloropsis oculata*.
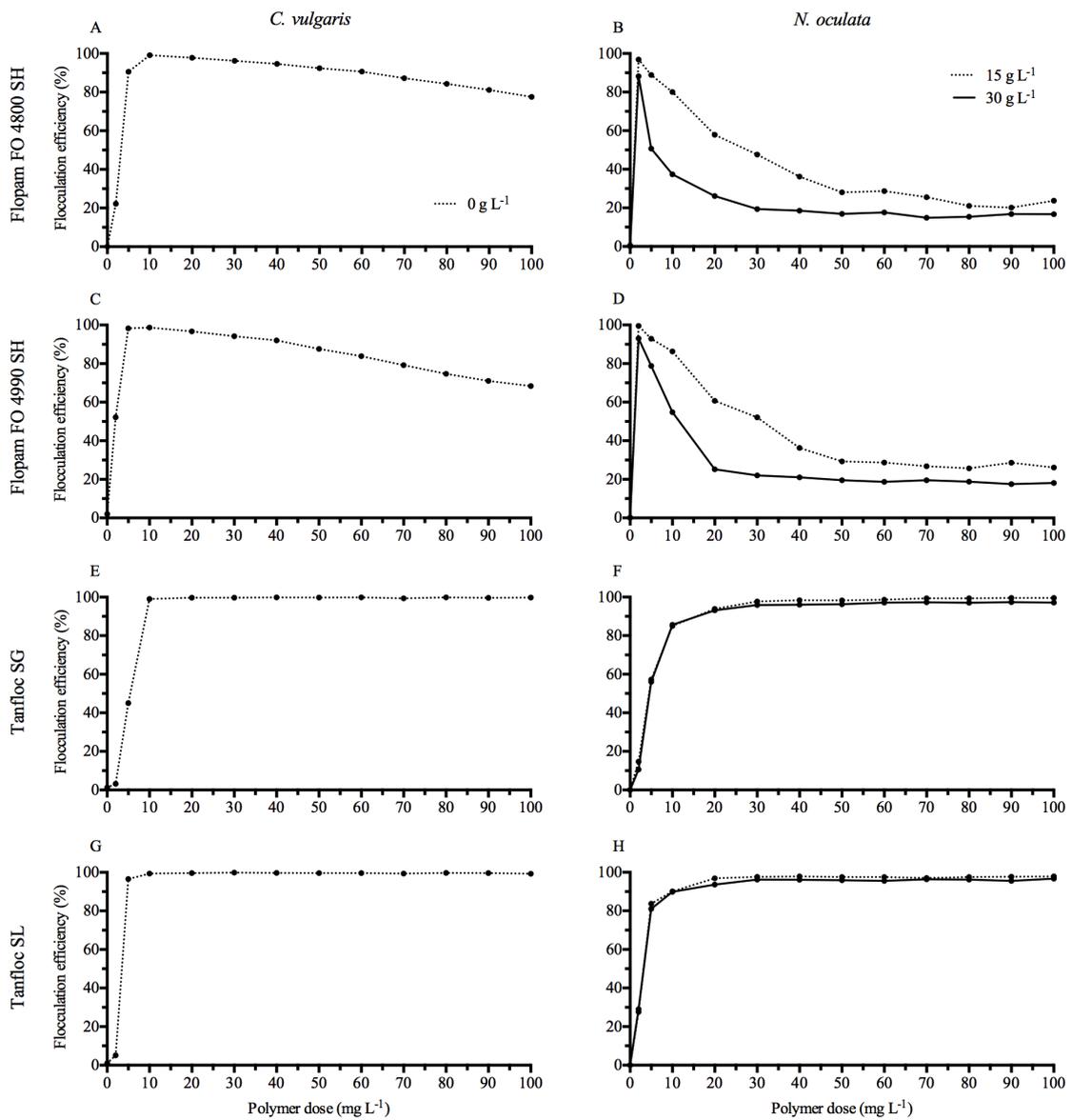
Fig. 3: Effect of salinity (0, 15 and 30 g L^{-1}) and polymer dosage on flocculation efficiency of *Chlorella vulgaris* and *Nannochloropsis oculata.*
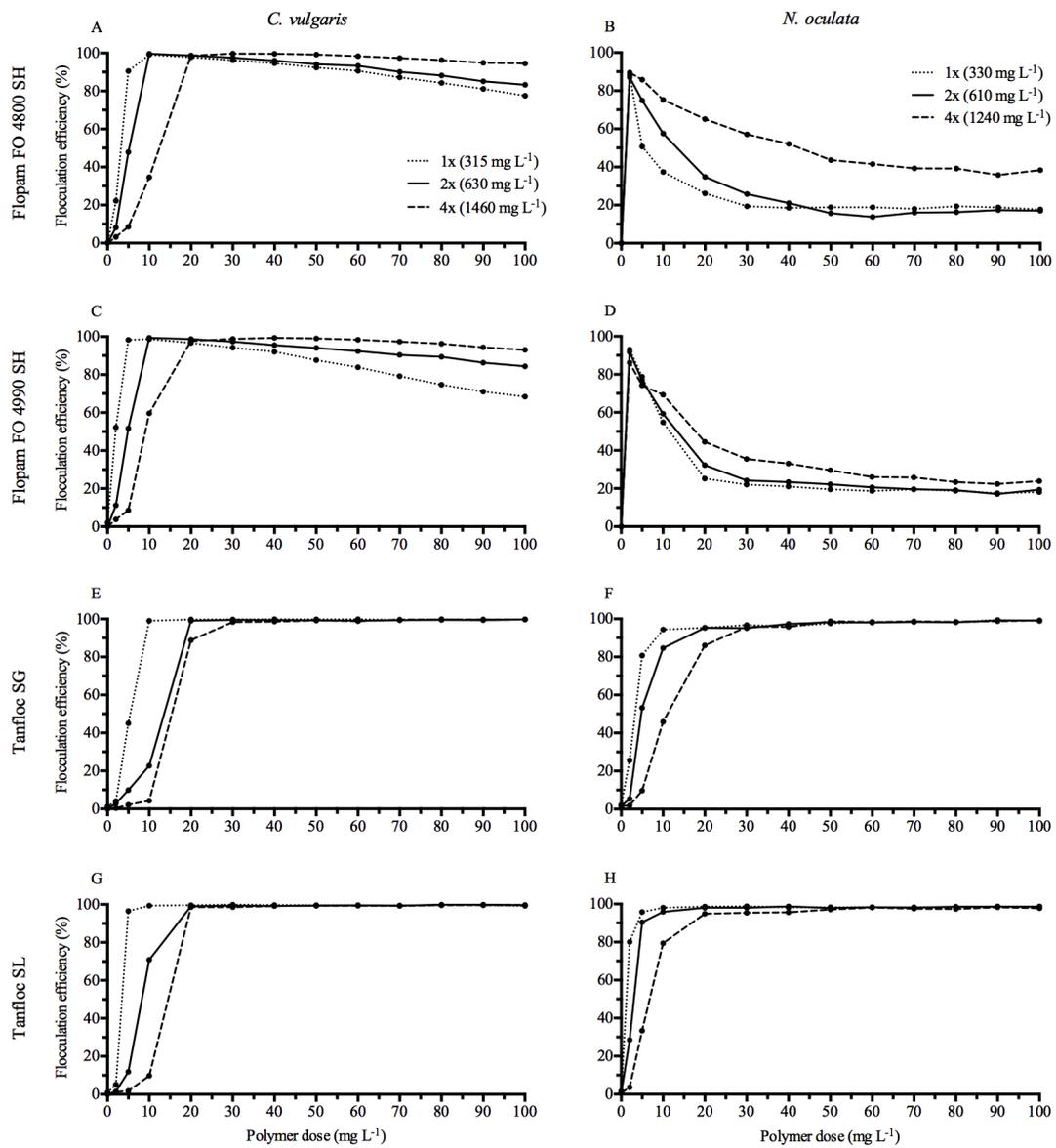
Fig. 4: Effect of biomass concentration (1, 2 and 4x) and polymer dosage on flocculation efficiency of *Chlorella vulgaris* and *Nannochloropsis oculata*.
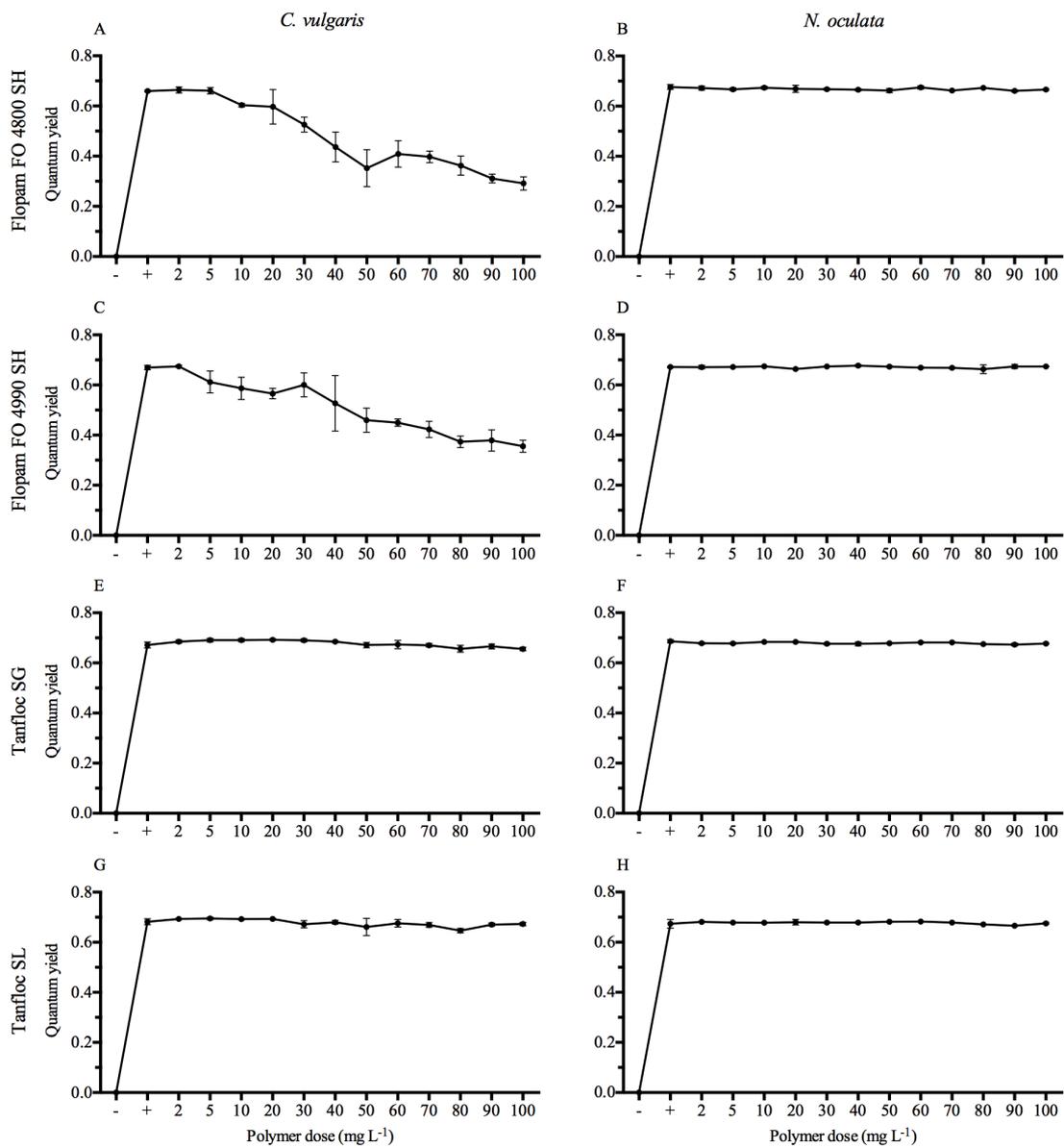
Fig. 5: Effect of polymer dose on the maximum quantum yields of photosystem II of *Chlorella vulgaris* and *Nannochloropsis oculata*.

Bench and pilot scale flocculation of *Nannochloropsis oculata* using a natural tannin-based cationic polymer

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Abstract

Harvesting is one of the major bottlenecks to the large-scale expansion of microalgae massive cultures for production of food, feed, bulk chemicals, or biofuels. The objective of this study was to evaluate the efficiency of Tanfloc, a low molecular weight tannin-based cationic polymer, for harvesting the marine microalgae *Nannochloropsis oculata*. A 2^4 full factorial bench scale experiment determined the effects of salinity (10 and 30), pH (6 and 8), polymer dosage (1 and 10 mg L^{-1}), and biomass concentration (200 and 400 mg L^{-1}) on flocculation efficiency. The results from the full factorial experiment were replicated at pilot scale (250 L) to verify the up-scaling reproducibility. Tanfloc achieved 98% efficiency with 10 mg L^{-1} of polymer in brackish and acidic conditions. Salinity and pH were the two factors that most influenced the Tanfloc efficiency. The flocculation efficiencies obtained in the bench scale were highly reproducible at pilot scale. Moreover, it was observed that Tanfloc and synthetic polymers performed similarly for microalgae harvesting.

Keywords: Microalgae, Coagulation, Biopolymer, Harvesting, Dewatering

1. Introduction

It is commonly acknowledged that microalgae are a promising feedstock for food, feed, bulk chemicals, or biofuels [1]. Marine microalgae have high productivity rates, synthesize bioproducts such as carotenoids or poly-unsaturated fatty acids, do not occupy fertile lands and can be grown using seawater supplemented with commercial fertilizers or using domestic and/or industrial effluents as a nutrient source [2-4].

The marine microalga *Nannochloropsis oculata* is a widely documented feedstock for biofuel and other bioproducts [5-7]. It is a robust species with high growth rates that tolerates a broad range of environmental conditions. Roselet et al. [8] successfully cultured *N. oculata* in an open-air production system in a sub-tropical region (32º S; 52º W) under uncontrolled environmental conditions from autumn to spring. Also, *N. oculata* can accumulate from 4% under normal conditions to up to 53% lipid under nutrient stress with lipid productivity up to 90 g m^{-3} day^{-1} [6, 9]. However, harvesting has been one of the major bottlenecks to the commercial expansion of this and other

microalgae production for low value bioproducts [4], accounting for up to 30% of the total cost in open ponds [10].

Microalgae are unicellular and microscopic organisms, ranging from 3 to 30 μm in diameter, achieving low densities even in high production systems as photobioreactors [2]. Additionally, they have negative surface charges and low sedimentation rates, consequently forming stable suspensions [4,11]. These characteristics make it difficult to concentrate microalgae. The current costs for harvesting must drastically decrease to make microalgae production of low value bioproducts commercially feasible [1]. Currently, harvesting is achieved by centrifugation [12], although up scaling this technology is energy intensive [4], being only justified for high value products [11, 12].

On the contrary, flocculation is a promising low-cost technology that is widely employed in water treatment to separate small amounts of impurities from a large volume of liquid [11]. However, flocculation of marine microalgae faces several limitations due to chemical properties that hinder the action of flocculating agents [13]. For instance, the salinity of the culture medium reduces the chemical activity of polymers, masking its functional sites and changing its molecular structure, resulting in low effectiveness and raising the required dosage [11, 14]. Culture pH also affects flocculation due to changes in the surface charges of the microalgae cells, the extent of coiling and the degree of ionization of the polymers [11, 15].

In practice, flocculation is achieved by the action of chemical additives that destabilize and aggregate the suspended particles. Two broad classes of additives are widely used, hydrolyzing metal salts and organic polymers [16]. Hydrolyzing metal salts, regardless of being widely used in water treatment, are not appropriate for microalgae due to the high concentrations of metals in the harvested biomass [11]. Organic polymers can be further separated into synthetic and natural polymers. Most of the polymers commercially employed are synthetic polyacrylamide-based as these can easily be synthesized in a tailor-made fashion [17]. However, these may contain traces of toxic un-polymerized monomers, apart from their relatively high cost and low degree of

biodegradability [18]. On the other hand, natural polymers are regarded as being toxin free [11].

Recently, a new natural tannin-based polymer entered the market for water treatment [18]. Tanfloc is a natural low molecular weight cationic polymer manufactured by Tanac (Brazil) and extracted from the black wattle tree (*Acacia mearnsii*). Tanfloc was successfully used for dye [19] and sodium dodecyl benzene sulfonate (SDBS) [20] removal and in the treatment of surface water [21, 22] and municipal wastewater [22, 23]. However, Tanfloc has never been used to flocculate freshwater or marine microalgae.

This study investigated the performance of Tanfloc for harvesting the marine microalgae *N. oculata*. A 2^4 full factorial bench scale experiment accessed the effects of salinity, pH, polymer dose, and biomass concentration on flocculation efficiency. The results from the full factorial bench experiment were replicated at pilot scale (250 L) to evaluate the up scaling reproducibility.

2. Materials and methods

2.1. Microalgae cultivation

The microalga used in this study, *Nannochloropsis oculata* (Eustigmatophyceae), is a marine species and was obtained from the microalgae collection of the Laboratory of Phytoplankton and Marine Microorganisms, from Federal University of Rio Grande (Brazil), registered as NANN OCUL-1. This microalga was cultured in filtered and sterilized seawater (salinity 30) enriched with a fertilizer medium composed of ammonium sulphate, urea, calcium superphosphate, and ferric chloride and supplemented with vitamins B_1, B_6 and B_{12} adapted from Yamashita and Magalhães [24]. The culture was stirred by continuous atmospheric air injection (20 L min^{-1}) and maintained under natural light and photoperiod, in a 1,200 L open-air cultivation system as described in a previous study [8]. Experiments were carried out when the culture achieved late exponential phase. Microalgae biomass was monitored daily by measuring absorbance at 750 nm. Optical density was calibrated against dry weight measured

gravimetrically on pre-weighed GF/F glass fiber filters, according to the following formula ($R^2 = 0.987$, Eq. 1):

$$y = 941.03x - 8.41 \qquad (1)$$

Where x is the optical density at 750 nm and y is the dry biomass (mg L^{-1}). The microalgae were washed with 0.5 M ammonium formate prior to filtration to remove salts absorbed on the cell surface. The biomass concentration, measured by optical density, after 10 days of culturing was 400 mg L^{-1}.

2.2. Flocculation experiments

Tanfloc is a natural low molecular weight quaternary ammonium polymer based on tannins extracted from the black wattle tree (*Acacia mearnsii*) and manufactured by Tanac (Brazil). Tanfloc was supplied as powder and a 1 g L^{-1} stock solution was prepared by adding deionized water and mixing for 1 hour.

Jar test experiments were used to quantify the efficiency of *N. oculata* flocculation. During addition of Tanfloc, the microalgae suspensions were intensively mixed (500 rpm) for 5 min, to allow uniform polymer dispersal, followed by gentler mixing (100 rpm) for 15 min to allow floc formation. Subsequently, the suspensions were allowed to settle and then samples were collected in the middle of the clarified zone. Optical density at 750 nm was measured prior to polymer addition (OD_i) and after 30 min settling (OD_f) and the flocculation efficiency (η_a) was calculated as Equation 2:

$$\eta_a = \frac{OD_i - OD_f}{OD_i} \times 100 \qquad (2)$$

2.3. Bench scale experiments

A 2^4 full factorial design was performed to determine the effect of salinity, pH, polymer dosage, and biomass concentration on flocculation efficiency, the response variable (Table 1). Each factor was tested at low (-1) and high (+1) levels at bench scale (300 mL) in duplicate (Table 1). In a previous study, Roselet et al. [8] reported the minimum and maximum values for pH, in an open-air cultivation system, as being around 5.6 and 8.0. Therefore, in the present study, pH was set at 6 and 8 by addition of 1 M NaOH or HCl solutions. Salinity was tested in brackish (10) and marine (30) conditions as microalgae cultured outdoors are subjected to great oscillations due to precipitation,

mostly during rainy seasons [8]. Both salinity (based on electrical conductivity) and pH were measured with an YSI 556 Handheld Multiparameter (Yellow Springs Instrument, OH, USA). Biomass concentration was evaluated at 200 mg L^{-1} and 400 mg L^{-1} as this range was reported in previous work for the same cultivation system [8]. Regarding Tanfloc dose, preliminary results achieved good flocculation with doses between 1-10 mg L^{-1} (unpublished data).

2.4. Pilot scale experiments

A second experiment was performed, after the bench scale full factorial experiment, to verify the reproducibility of flocculating *N. oculata* at a pilot scale (250 L). Therefore, the microalgae were cultivated in 250 L circular tanks as previously described (Section 2.1), except that salinity was adjusted at the time of inoculation according to the results obtained from the bench scale experiment (salinity 10). pH was adjusted to 6 prior to flocculation. The microalgae biomass was monitored daily by measuring the absorbance at 750 nm and the flocculation experiments were performed in the same tank when the biomass achieved the intermediate concentration of 300 mg L^{-1}. The Tanfloc dose was employed based on the full factorial results. Polymer was added and mechanically homogenized as previously described and the flocculation efficiency was measured by optical density after sedimentation. In total, three pilot scale experiments were independently repeated (in duplicates) with fifteen days intervals.

2.5. Statistical analysis

The comparisons among treatments were performed using a four-way ANOVA ($P < 0.05$) followed by a Tukey's multiple comparisons test (Table 1). The significance of each factor studied and its interactions were confirmed by the statistical parameters *t*-test and *P*-value (Table 2). A four-way ANOVA was performed and an empirical model describing the flocculation efficiency was established. The resulting model was used to generate a contour diagram for the analysis of the variable effects on flocculation efficiency. Data normality and homoscedasticity were verified for each data set using Shapiro-Wilk and Bartlett's test.

3. Results and discussion

3.1. Effect of pH

In any flocculation process, consideration must be given to the hydrogen ion concentration as it influences not only the action of the polymer but the microalgae cell surface as well [11, 15]. In this study, the flocculation efficiency of Tanfloc was compared in acidic (pH 6) and alkaline (pH 8) microalgae cultures. Increasing pH presented a detrimental affected on flocculation ($P < 0.05$, Table 2). For example, a decrease from 95.2% to 33.0% can be observed in Table 1 for runs 5 and 6, respectively. Overall, increasing the pH accounted for 20% of the interactions (Table 2). This can be explained by the decrease in Tanfloc zeta potential due to pH increase. According to the manufacturer, at pH 6 the zeta potential of Tanfloc is +22 mV whereas at pH 8 it decreases to +5 mV, and the point of zero charge is around pH 8.17.

Beltrán-Heredia et al. [20] also worked with Tanfloc on the removal of SDBS, a dangerous and pollutant anionic surfactant, and tested the effect of Tanfloc in a pH gradient between 4 and 10. According to their results, Tanfloc was less effective as the pH became higher. The authors considered that lowering the pH enhanced the cationic character of Tanfloc. Similarly, Sánchez-Martín et al. [21] used Tanfloc for surface water treatment in conditions with variable pH (4 - 9) and found that flocculation was most effective at acidic pH (near 4), although in a neutral pH, the effectiveness was still high enough. According to these authors, this loss of effectiveness was likely due to the structural nature of Tanfloc, which was denatured at an alkaline pH [21]. Graham et al. [18] were the first to study Tanfloc's characteristics and properties, using kaolin suspensions in a series of flocculation tests. The charge density of Tanfloc was found to be pH dependent. At pH 4, the charge density was 3.1 mequiv g^{-1}, whereas at pH 9, it decreased to 0.2 mequiv g^{-1}, due to amine de-protonation. That decrease in charge density resulted in a dosage increase, consistent with a charge neutralization mechanism between the polymer and the negatively charged kaolin suspension. According to the authors, at pH 9, the polymer had very little cationic charge and the mechanism of action would most likely be enmeshment in Tanfloc precipitates (i.e., sweep flocculation). Wang et al. [25] used modified tannin to harvest *Microcystis aeruginosa.* At pH 6, the modified tannin presented a removal efficiency of more than 97%, whereas

a further increase to pH 9 resulted in less than 10% removal. After analyzing the effects of pH on zeta potential, the modified tannin had a positive zeta potential (>15 mV) at pH 6, although it attained the point of zero charge at pH 9. Consequently, this decrease in zeta potential resulted in the loss of positively charged groups.

It is important to highlight that all of the previous cited studies tested Tanfloc or tannin-based polymers in freshwater conditions, whereas the present study was performed in natural seawater. However, in spite of the salinity conditions, the results obtained here were consistent with those studies conducted in freshwater.

3.2. Effect of salinity

Due to the chemical properties of marine waters (salinities up to 36, ionic strength of 0.7 M and high magnesium, calcium and phosphate ions concentrations), the flocculation of microalgae faces several limitations. The reduction of the chemical activity of polymers, the masking of functional sites, the changes in the molecule structure as a random coiled configuration all result in a lower effectiveness and a higher dosage demand of polymers [11, 14]. The present study evaluated the effect of brackish (salinity 10) and marine (salinity 30) waters on *N. oculata* flocculation using Tanfloc (Table 1). As shown in Table 2, a highly negative effect (-41.7%, $P < 0.05$) of increased salinity upon flocculation was observed. These results clearly demonstrated that flocculation in brackish water attained higher efficiencies (98.3% ± 0.4) than in saltwater (50.6% ± 1.5) (runs 9 and 11, Table 1).

As stated before, to date, Tanfloc was used only in freshwater studies to remove dye [19], SDBS [20], color and humic material [18] and to clean surface water [21, 22], municipal wastewater [22, 23] and petrol wastewater [26]. Apart from Tanfloc, few authors have used tannin-based polymers for drinking water treatment [27] or to harvest the freshwater cyanobacteria *Microcystis aeruginosa* [25].

Sukenik et al. [13] compared the flocculation of microalgae in fresh and seawater using chitosan, a natural cationic polymer that is efficient in freshwater flocculation. The authors found that chitosan was effective only when the ionic strength of the medium

was lower than 0.1 M, when the polymer was highly hydrated and linearly extended. In marine conditions, chitosan was ineffective due to shifts in its molecular configuration and dimension, masking its active sites. Similarly, Bilanovic et al. [28] compared the flocculation of the marine *Chlorella stigmatophora* with that of the freshwater *C. vulgaris* using chitosan. For the freshwater species*,* removal efficiencies higher than 90% were obtained with 5 mg L^{-1} of chitosan, whereas poor removal was obtained for the marine species, even at concentrations above 20 mg L^{-1}. Thus, changing the culturing conditions from marine to brackish resulted in *N. oculata* flocculation improvement with Tanfloc.

3.3. Effect of polymer dosage and microalgae biomass

It is generally agreed that the flocculation efficiency is determined by the extent to which the microalgae surface is covered with the polymer. Similarly, a variation in the microalgae concentration would appreciably influence the concentration of the polymer required for a given degree of flocculation [15]. Therefore, different polymer and microalgae biomass concentrations were tested to establish the optimal conditions for producing flocculation. The increase of the polymer dose from 1 mg L^{-1} to 10 mg L^{-1} improved flocculation in 21% in general ($P < 0.05$, Table 2). For example, in Table 1 efficiency increased from 19.9% to 50.6% only due to dosage increase (runs 3 and 11). However, the increase in microalgae biomass concentration in the range studied (200 mg L^{-1} to 400 mg L^{-1}) did not significantly affect the flocculation efficiency ($P > 0.05$, Table 2).

Sánchez-Martín et al. [21] employed Tanfloc to remove suspended matter from surface water achieving a turbidity reduction of 99% at relatively low dosages (10 mg/L). Their results were similar to those obtained in the present study, although experiments were conducted with freshwater. Garzon-Sanabria et al. [29] used a full factorial analysis to determine the relationship between the microalgae concentration and the required polymer dose on flocculating *N. oculata*. They reported that increasing the biomass concentration resulted in lower flocculation efficiency.

3.4. Factors interaction

The effect estimates for the interactions between the factors were determined and reported (Table 2). Only the interaction between pH and salinity at low levels was statistically significant ($P < 0.05$), increasing flocculation in 22%. From Table 1 we can observe that reducing both salinity and pH increased efficiency from 19% (salinity at +1) and 40% (pH at +1) to almost 90% (runs 3, 2 and 1, respectively). On the basis of the four-way ANOVA ($R^2 = 0.969$ and *F-ratio* = 135.85) an empirical model (Eq. 3) described the flocculation efficiency (η_a) as a function of pH (*A*), salinity (*B*) and polymer dosage (*C*).

$$\eta_a = 52.96 - 10.18{\times}A - 20.86{\times}B + 10.73{\times}C + 11.32{\times}A{\times}B \qquad (3)$$

Figure 1 presents the effects of the interaction between pH and salinity on flocculation efficiency. From the effects analysis, it was verified that the best efficiencies occurred at higher levels of polymer dosage (+1), lower pH (-1) and salinity (-1), being independent of the biomass concentration. Therefore, the optimum conditions were 10 mg L^{-1} of Tanfloc, pH 6 and salinity 10 (runs 9 and13). A deviation of less than 1% in relation to the empirical model (Eq. 3) was observed (run 5). Results were confirmed by the Tukey's multiple comparisons test (Table 1). Both pH and salinity at the low levels (-1) resulted flocculation efficiencies higher than 95% (runs 5, 9 and 13). The worst conditions to induce flocculation were pH 6, salinity 30 and 1 mg L^{-1} of Tanfloc for 400 mg L^{-1} of biomass.

3.5. Up-scaling reproducibility

Large scale microalgae harvesting is one of the major bottlenecks in upstream processing, potentially contributing to 20–30% of the total biomass production costs due to energy input [10]. Therefore, current commercial large-scale production is used solely for high value products [11, 12]. Research is underway to alleviate these costs for low value bioproducts [4]. However, most research on microalgae flocculation is performed at bench scale, and that performed at pilot scale are generally related to water treatment.

In the present study, the comparison between bench (300 mL) and pilot scale (250 L) experiments resulted in no significant difference ($P < 0.05$, Figure 2). The pilot scale conditions were previously determined in a 2^4 full factorial bench scale experiment (Table 1). The conditions yielding the highest efficiencies were pH 6, salinity 10 and 10 mg L^{-1} of Tanfloc with results ranging from 97% to 99%. Similarly, Sánchez-Martín et al. [22], who tested Tanfloc for water treatment at pilot plant scale, also reported that the efficacy was similar to or even better than that obtained in batch scale. The results obtained in the present experiment clearly demonstrated that up scaling is achievable with Tanfloc.

3.6. Comparison with synthetic polymers

Although natural polymers have the advantage of being toxic free, they are generally considered less effective than synthetic polymers [17]. Usually, doses lower than 10 mg L^{-1} are required only when synthetic polymers are employed [30]. However Tanfloc resulted in 98% flocculation applying only 10 mg L^{-1}, comparable with Flopam and Zetag, synthetic polymers recently reported for harvesting marine microalgae (Table 3). Comparing the prices of Tanfloc (US$ 1.50 kg^{-1}) with Flopam and Zetag (both at US$ 8.00 kg^{-1}) and considering that they have similar performances, one can suggest that Tanfloc is an economical alternative for microalgae flocculation.

4. Conclusions

Few authors have investigated tannins for flocculation and none have evaluated them on marine microalgae. The present study demonstrated that Tanfloc was highly efficient for *N. oculata*, achieving efficiency up to 98% at both bench and pilot scales. Based on full factorial results, increasing salinity and pH affected flocculation efficiency up to 41.7%. Therefore, for efficient flocculation *N. oculata* should be cultured in lower conditions of salinity and pH.

Acknowledgements

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Table 1: Coded levels and real values (in parentheses) for the 2^4 factorial experiment, flocculation efficiency (mean % ± SD) and Tukey's test. The optimal flocculation conditions are highlighted in bold.

Run	Factors				Efficiency (%)
	pH	Salinity	Biomass (mg L^{-1})	Dose (mg L^{-1})	
1	-1 (6)	-1 (10)	-1 (200)	-1 (1)	89.9 (±1.3) [b]
2	+1 (8)	-1 (10)	-1 (200)	-1 (1)	40.8 (±0.0) [e]
3	-1 (6)	+1 (30)	-1 (200)	-1 (1)	19.9 (±0.9) [i]
4	+1 (8)	+1 (30)	-1 (200)	-1 (1)	21.8 (±0.6) [hi]
5	**-1 (6)**	**-1 (10)**	**+1 (400)**	**-1 (1)**	**95.2 (±1.6) [a]**
6	+1 (8)	-1 (10)	+1 (400)	-1 (1)	33.0 (±0.4) [g]
7	-1 (6)	+1 (30)	+1 (400)	-1 (1)	12.3 (±0.6) [j]
8	+1 (8)	+1 (30)	+1 (400)	-1 (1)	24.9 (±0.2) [h]
9	**-1 (6)**	**-1 (10)**	**-1 (200)**	**+1 (10)**	**98.3 (±0.4) [a]**
10	+1 (8)	-1 (10)	-1 (200)	+1 (10)	69.3 (±0.8) [c]
11	-1 (6)	+1 (30)	-1 (200)	+1 (10)	50.6 (±1.5) [d]
12	+1 (8)	+1 (30)	-1 (200)	+1 (10)	49.4 (±0.3) [d]
13	**-1 (6)**	**-1 (10)**	**+1 (400)**	**+1 (10)**	**98.0 (±1.0) [a]**
14	+1 (8)	-1 (10)	+1 (400)	+1 (10)	66.3 (±0.1) [c]
15	-1 (6)	+1 (30)	+1 (400)	+1 (10)	41.0 (±0.3) [e]
16	+1 (8)	+1 (30)	+1 (400)	+1 (10)	36.9 (±1.3) [f]

[a] Different letters indicate significant differences between efficiencies ($p < 0.05$).

Table 2: Main effects and interaction analysis for *N. oculata* flocculation efficiency.

Factor	Effects	Std. Err.	*t*-value	*p*-value
pH (1)	-20.362	2.193	-9.284	<0.001[a]
Salinity (2)	-41.737	2.193	-19.284	<0.001[a]
Biomass (3)	-4.050	2.193	-1.846	0.079
Dose (4)	21.475	2.193	9.791	<0.001[a]
(1) x (2)	22.650	2.193	10.327	<0.001[a]
(1) x (3)	-0.987	2.193	-0.450	0.657
(1) x (4)	3.837	2.193	1.749	0.094
(2) x (3)	-2.587	2.193	-1.197	0.251
(2) x (4)	3.237	2.193	1.476	0.154
(3) x (4)	-2.300	2.193	-1.048	0.306

[a] Significant factors ($p < 0.05$).

Table 3: Comparison of Tanfloc with synthetic polymers recently reported for harvesting marine microalgae.

Microalgae species	Polymer	Dose (mg L^{-1})	Recovery (%)	Reference
Conticribra weissflogii	Flopam FO4240SH	4	92	[31]
Nannochloropsis salina	Flopam FO4490SH	3	94	[33]
	Zetag 7570	10	20	[32]
Phaeodactylum tricornutum	Zetag 7557	10	98	[30]
Nannochloropsis oculata	Tanfloc	10	98	This study
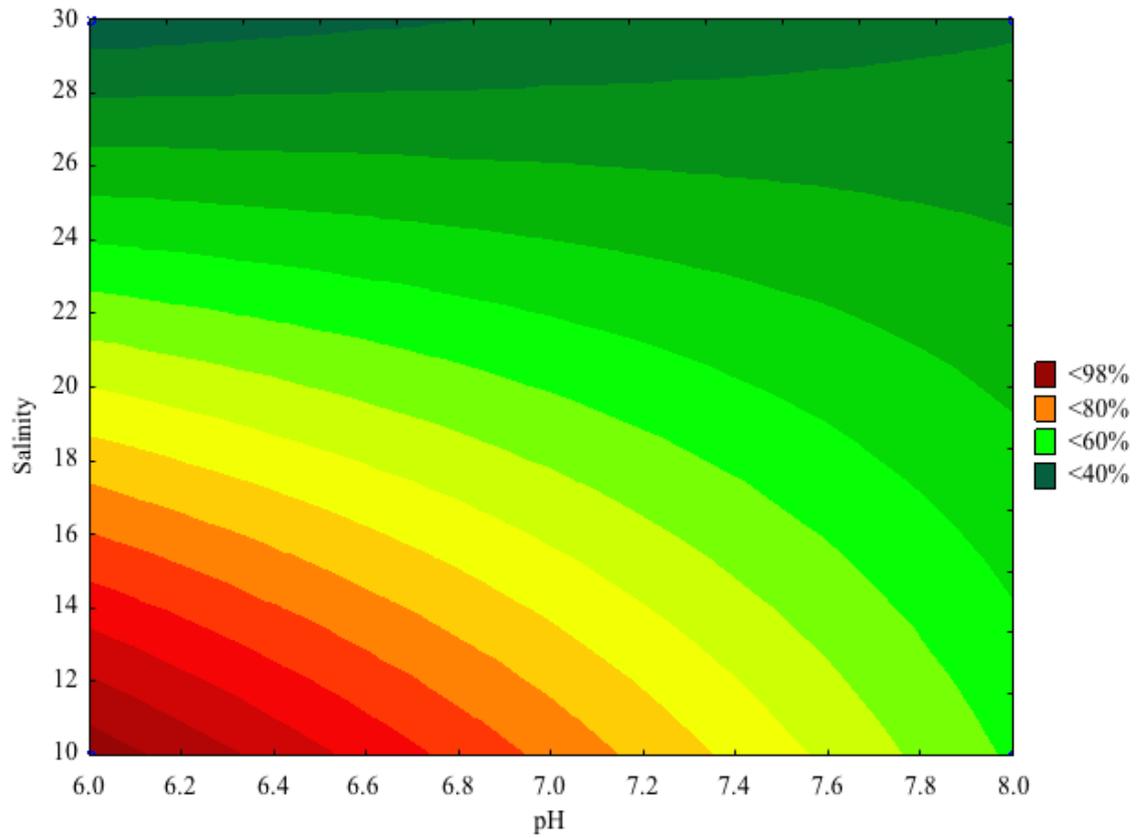
**Fig. 1:** Contour diagram of *Nannochloropsis oculata* flocculation efficiency as a function of pH (6-8) and salinity (10-30) with Tanfloc (10 mg L^{-1}), calculated after equation 3.
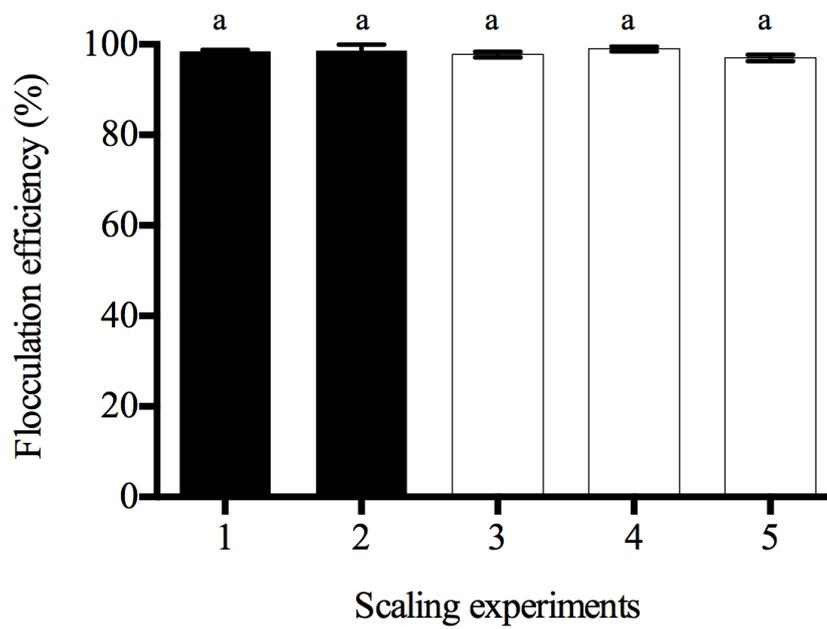
Fig. 2: *Nannochloropsis oculata* flocculation efficiency (mean % ± SD) at bench (columns 1 and 2) and pilot scale experiments (columns 3-5) with Tanfloc (10 mg L^{-1}). Columns with same the letter are statistically identical.

10. DISCUSSÃO GERAL

Apesar das microalgas serem reconhecidas como uma nova fonte para a produção de diversos bioprodutos (Borowitzka, 2013), a sua produção comercial ainda se restringe a poucas espécies, sendo a maioria de água doce (Milledge, 2010). No entanto, devido à escassez de água doce no planeta não é recomendável que estas espécies sejam cultivadas em larga escala (Schlesinger et al., 2012). Portanto, a produção de bioprodutos deveria ser direcionada para espécies capazes de crescer em água salina (Borowitzka e Moheinami, 2010). *Nannochloropsis oculata* é uma espécie marinha, que apresenta alta taxa de crescimento e tolera amplas variações ambientais (Moazami et al., 2012), além de produzir metabólitos de elevada importância comercial (Lubián et al., 2000; Gouveia e Oliveira, 2008; Borowitzka, 2013).

O presente estudo teve por objetivo realizar o escalonamento da produção de *N. oculata* para a extração de metabólitos de interesse comercial. Atualmente, sistemas abertos são empregados para o cultivo em larga escala, no entanto, estes sistemas possuem baixa produtividade devido a falta de controle dos parâmetros ambientais (Borowitzka e Moheinami, 2013), sendo recomendados apenas para regiões com condições ideais de irradiância e temperatura (López-Elías et al., 2005). Por outro lado, os sistemas fechados (fotobiorreatores) possuem alto controle dos parâmetros ambientais mas o seus custos de implantação e operação são elevados, dificultando o seu escalonamento (Waltz, 2009).

Desta forma, como primeiro trabalho desta Tese foi proposto, no Capítulo 1, um sistema intermediário entre o sistema aberto e o fechado, que pode ser empregado em regiões climáticas menos favoráveis, além de aliar baixo custo e alta produtividade. A instalação de tanques de 1.200 L dentro de uma estufa agrícola permitiu que *N. oculata* fosse cultivada durante o inverno na região Sul do Brasil. Neste sistema semifechado, a água se manteve 4ºC acima da temperatura registrada em tanques externos (sistema aberto). Esta diferença permitiu uma maior produtividade do sistema semifechado (360 mg L^{-1}) em relação ao sistema aberto (260 mg L^{-1}). Além do mais, durante o outono e o inverno, as altas taxas de pluviosidade acarretaram uma queda da produtividade do sistema aberto devido à diluição do cultivo e queda da salinidade. Na primavera, o

sistema semifechado atingiu a sua produção máxima de biomassa (830 mg L^{-1}), similar a produção de sistemas fechados como fotobioreatores (1.000 mg L^{-1}) (Olofsson et al., 2012). No entanto, os custos de produção com o sistema semifechado são mais baixos. Os resultados deste trabalho apresentado no Capítulo I permitem afirmar que o sistema semifechado proporcionou melhores condições para a produção em larga escala de *N. oculata* em regiões onde as condições ambientais são menos favoráveis devido às baixas temperaturas e maior pluviosidade.

Independente do sistema de cultivo empregado, a coleta da biomassa é um dos principais entraves para a produção comercial de microalgas (Vandamme et al., 2013), contribuindo com até 30% do custo total de produção (Gudin e Thepenier, 1986). A floculação é uma tecnologia de baixo custo, amplamente empregada no tratamento de grande volume de água para a remoção de partículas em suspensão. Até o momento, diversos estudos obtiveram sucesso na floculação de microalgas (Ebeling et al., 2005; Knuckey et al., 2006; Danquah et al., 2009; Granados et al., 2012; Lam et al., 2014). No entanto, todos estes estudos foram realizados apenas em escala de bancada, e muitos empregaram apenas espécies de microalgas de água doce. Desta forma, além de realizar o escalonamento do cultivo de *N. oculata*, a presente Tese também teve por objetivo avaliar a floculação de uma espécie marinha e escalonar o processo de floculação que, normalmente, é testado em escala de bancada.

Objetivou-se primeiramente selecionar os melhores polímeros para estudos subsequentes. Para isto, foram avaliados vinte e cinco polímeros de três fabricantes distintos de floculantes (Capítulo 2). Flopam e Zetag são polímeros sintéticos de alto peso molecular e com diferentes densidades de carga, enquanto que Tanfloc é um polímero natural de baixo peso molecular. A eficiência de cada polímero foi comparada entre *N. oculata* e *C. vulgaris*, uma espécie de água doce.

Das três séries analisadas, apenas Tanfloc apresentou alta eficiência para as duas espécies estudadas, demonstrando que este polímero natural não sofre o efeito da salinidade do meio de cultura. Contrariamente, os polímeros sintéticos apresentaram alta eficiência apenas para a microalga de água doce. Estes resultados podem ser

explicados pelas diferenças estruturais dos polímeros sintéticos e natural, pois Tanfloc possui estrutura ramificada, enquanto que os sintético são lineares, sofrendo um maior enovelamento devido a maior força iônica da água marinha (Palomino et al., 2012). Apesar disto, foi observado que os polímeros sintéticos com maior densidade de carga apresentaram melhores resultados que aqueles com densidade menor. Desta forma, a densidade de carga é um fator importante a ser considerado na floculação de microalgas marinhas. Garzon-Sanabria et al. (2013) e Udom et al. (2013) compararam vários polímeros de diferentes valores de densidade de carga e chegaram as mesmas conclusões. Além da análise de eficiência, o custo de cada séries de polímeros foi calculado. De acordo com os resultados, Tanfloc apresentou o menor preço, custando apenas US$ 36, enquanto que, Flopam e Zetag custaram US$ 186 e US$ 216, respectivamente. Portanto, além de ser eficiente para *N. oculata*, Tanfloc também se mostrou econômico.

No entanto, além do peso molecular e da densidade de carga, a eficiência dos polímeros é regulada por outros fatores como pH, salinidade, presença de matéria orgânica, concentração de biomassa. A própria dose de polímero empregada pode influenciar a sua eficiência pois, em baixa concentração o polímero não surte efeito enquanto que, em altas concentrações, as partículas podem se re-estabilizar e permanecer em suspensão. Desta forma, é importante que os efeitos de cada um destes fatores seja conhecido para que a eficiência dos polímeros seja otimizada. Além do mais, é necessário avaliar se os polímeros empregados podem apresentar toxicidade pois, eventualmente, a água da floculação pode ser descartada e os polímeros presentes podem provocar danos ambientais. Desta forma, no Capítulo 3 foram avaliados os efeitos de pH, salinidade, matéria orgânica, concentração de biomassa e dose na eficiência dos dois melhores polímeros das séries Flopam e Tanfloc, determinados no capítulo anterior. Também foi avaliada a potencial toxicidade destes polímeros.

De acordo com os resultados deste trabalho, verificou-se que o pH afetou apenas a eficiência do Tanfloc, provavelmente devido a perda da sua carga elétrica. Resultados similares foram observados por outros autores para este polímero natural, porém em água doce (Graham et al., 2008; Beltrán-Heredia et al., 2009; Wang et al., 2013). A

salinidade apresentou efeito negativo somente para Flopam, provavelmente em consequência da menor rigidez da sua estrutura linear em comparação ao polímero natural (Palomino et al., 2012). A presença de matéria orgânica afetou tanto Tanfloc quanto Flopam pois, de acordo com Henderson et al. (2008), a matéria orgânica também possui carga negativa, competindo com as microalgas pelos polímeros positivamente carregados. O aumento da concentração de biomassa acarretou no aumento da dosagem dos polímeros naturais e sintéticos. No entanto, para a mesma concentração de biomassa, o aumento da dosagem resultou na perda de eficiência apenas para os polímeros sintéticos, como também observado por Bolto e Gregory (2007). Beltrán-Heredia e Sánchez-Martín (2009) e Gutiérrez et al. (2015) também verificaram que Tanfloc não perde a eficiência quando aplicado em dosagens altas. Tanto Flopam quanto Tanfloc não apresentaram toxicidade para *N. oculata*. Apesar disto, apenas o polímero natural é indicado para a floculação de *N. oculata* pois não é afetado pela salinidade.

Finalmente, após definir qual o melhor polímero e quais fatores que influenciam a sua eficiência, foi realizado o escalonamento da floculação de *N. oculata* para tanques de 250 L (Capítulo 4). Como os testes em escala de bancada do capítulo anterior empregaram água marinha sintética, optou-se por repeti-los com água marinha natural para corroborar os resultados obtidos. Foi feito um experimento fatorial, que permitiu avaliar a ação isolada e as interações entre salinidade, pH, concentração de biomassa e dosagem do polímero natural. A eficiência de floculação obtida em escala de bancada foi plenamente reproduzida em escala piloto, provando que o escalonamento é viável. No entanto, diferentemente dos resultados anteriores com água marinha sintética, a eficiência de Tanfloc foi menor em água marinha natural. As altas concentrações de íons da água marinha natural podem acarretar a redução da atividade química dos polímeros, pelo mascaramento dos sítios ativos ou pela alteração da sua estrutura química (Sukenik et al., 1988; Vandamme et al., 2013). Além disso, a água marinha natural apresenta elevadas concentrações de matéria orgânica dissolvida (Ogawa e Tanoue, 2003), que podem reduzir a eficiência dos polímeros (Henderson et al., 2008). Desta forma, baseado nos resultados do Capítulo 4, é necessário avaliar o efeito da água do mar natural no processo de floculação de microalgas marinhas.

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11. CONCLUSÕES GERAIS

- O sistema de cultivo semifechado proporcionou melhores condições que o sistema aberto para o cultivo da microalga *N. oculata*, principalmente nos períodos de baixa temperatura e alta pluviosidade (Capítulo 1).

- Dos vinte e cinco polímeros comerciais sintéticos e naturais avaliados em escala de bancada, apenas os da série Tanfloc apresentaram alta eficiência de floculação para *N. oculata* em água marinha sintética. Flopam e Zetag foram eficientes apenas para a microalga de água doce. A densidade de carga dos polímeros sintéticos influenciou a sua eficiência, sendo que os de maior densidade foram mais eficientes (Capítulo 2).

- A eficiência de floculação dos polímeros sintéticos e naturais foi regulada de forma diferente pelo fatores avaliados. Em geral, a concentração de matéria orgânica e de biomassa afetaram todos os polímeros, entanto que a salinidade afetou apenas Flopam e o pH teve maior influência sobre o Tanfloc. Como Tanfloc não sofreu influência da salinidade (em água marinha sintética), este polímero foi recomendado para floculação de *N. oculata* (Capítulo 3).

- Nos experimentos em escala piloto com água marinha natural, 10 mg L^{-1} de Tanfloc resultaram em 98% de eficiência de floculação de *N. oculata* em cultivos com 300 mg L^{-1} de biomassa, em condições de salinidade 10. No entanto, em salinidade 30 a eficiência foi reduzida para 41%. Esta diferença indica que o uso de água marinha natural resultou em queda na performance de Tanfloc, quando comparado com água marinha sintética (Capítulo 4).

12. ESTUDOS FUTUROS

Baseado nos resultados obtidos na presente Tese, propõem-se que estudos futuros sejam realizados a fim de esclarecer a influência da água marinha natural na eficiência de floculação de *N. oculata*. Supõem-se que as propriedades químicas da água marinha, como alta salinidade, alta força iônica e altas concentrações de íons como ferro, magnésio, cálcio e fosfato, limitem a eficiência dos polímeros, reduzindo a sua atividade química, mascarando os seus sítios ativos e alterando a sua estrutura química. Também é recomendado que sejam estudados meios de remoção da matéria orgânica dissolvida na água marinha natural, pois esta é um importante fator que limita a floculação de microalgas. Acredita-se que, através do estudo destes fatores, seja possível realizar a floculação da microalga *N. oculata* em escala piloto sem que seja necessário realizar a redução da salinidade do cultivo.