UNIVERSIDADE FEDERAL DO RIO GRANDE - FURG
INSTITUTO DE OCEANOGRAFIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

OTIMIZAÇÃO DA PRODUÇÃO DE CONCENTRADOS DA MICROALGA *Nannochloropsis oceanica*

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RIO GRANDE - RS
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Tese apresentada como parte dos requisitos para obtenção do grau de doutor em Aquicultura no Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande.

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1 RESUMO GERAL

A presente tese teve por objetivo otimizar a produção de concentrados da microalga *Nannochloropsis oceanica.* O trabalho foi dividido em três capítulos. No primeiro capítulo foi determinada a influência da presença de bactérias e matéria orgânica no aparecimento de DMS ao longo da vida útil dos concentrados. Para isso, foi analisada a vida útil em duas temperaturas de armazenamento (24 e -4°C) de dois concentrados, um controle e um concentrado que passou pelo processo de lavagem. O processo de lavagem conseguiu estender consideravelmente a vida útil dos concentrados de 17 para 85 dias em -4°C. O segundo capítulo comparou o uso do *skimmer* e do processo de lavagem como formas de minimizar a densidade bacteriana nos concentrados ao longo do tempo. Foram testados quatro tratamentos mais um controle, além dos tratamentos com *skimmer* e lavagem, foi testada a combinação entre os dois tratamentos e uma variação do processo de lavagem. Os tratamentos que apresentaram os melhores resultados foram a combinação dos dois métodos (*skimmer* + lavagem) e o processo de lavagem repetido mais vezes. As duas variações do processo de lavagem foram estatisticamente diferentes. No terceiro capítulo, o processo de lavagem foi otimizado para produzir concentrados visando aumentar a eficiência de remoção de bactérias sem influenciar na viabilidade das células e melhor a relação benefício-custo do processo para torná-lo viável. Para viabilizar o processo foi necessário aumentar o preço de venda do concentrado em 10%. A combinação que gerou o melhor resultado foi repetir o processo 5 vezes em 3,405 de força g, em 24 °C por 20 minutos.

2 ABSTRACT

This thesis aimed to optimize the production of concentrates from the microalgae *Nannochloropsis oceanica*. The work was divided into three chapters. The first chapter determined the influence of the presence of bacteria and organic matter on the appearance of DMS throughout the shelf life of the concentrates. For this purpose, the shelf life at two storage temperatures (24 and -4°C) of two concentrates was analyzed, a control and a concentrate that underwent the washing process. The washing process managed to considerably extend the shelf life of the concentrates from 17 to 85 days at -4°C. The second chapter compared the use of the skimmer and the washing process as ways to minimize bacterial density in the concentrates over time. Four treatments plus a control were tested. In addition to the treatments with skimmer and washing, the combination of the two treatments and a variation of the washing process was tested. The treatments that presented the best results were the combination of the two methods (skimmer + washing) and the washing process repeated more often. The two variations of the washing process were statistically different. In the third chapter, the washing process was optimized to produce concentrates, aiming to increase the efficiency of bacteria removal without affecting cell viability and improve the benefit-cost ratio of the process to make it viable. To make the process viable, it was necessary to increase the sale price of the concentrate by 10%. The combination that generated the best result was to repeat the process 5 times at 3.405 g-force, at 24 °C for 20 minutes.

3 INTRODUÇÃO GERAL

3.1 Microalgas

Alga é um termo utilizado para designar organismos aquáticos autotróficos unicelulares, coloniais ou filamentosos. Esse termo não apresenta valortaxonômico, descrevendo organismos muito diferentes pertencentes aos reinos Bacteria, Plantae, Chromista e Protozoa com cerca de 72.500 espécies descritas, as quais podem ser classificadas como macroalgas ou microalgas (Guiry, 2012). As microalgas são organismos unicelulares fotossintetizantes que possuem clorofila *a* que podem ser procariontes ou eucariontes. São os principais responsáveis pela produção primária nos ambientes aquáticos e a base da cadeia trófica nesses ambientes (Riviers, 2006). Apesar de mais abundantes em ambientes marinhos, habitam praticamente todos os ambientes existentes incluindo ambientes extremos como fontes termais e calotas polares (Riviers, 2006).

As microalgas são uma fonte promissora de matéria-prima renovável para produtos de alto valor agregado em diferentes ramos da indústria, como alimentos, energia, cosméticos e fertilizantes (Patil et al., 2020). Devido a sua diversidade taxonômica, possuem uma grande variação no seu conteúdo de proteínas, cinzas, lipídios e ácidos graxos (Moura Junior et al., 2007). Enquanto as microalgas marinhas são as principais produtoras de ácidos graxos poliinsaturados de cadeia longa (LC-PUFA) de extrema importância como os ácidos araquidônico (AA - C20:4), eicosapentaenóico (EPA - C20:5) e docosahexanóico (DHA - C22:6), as microalgas de água doce produzem predominantemente ácidos graxos saturados ou monossaturados (Patil et al., 2007). Além disso, são importantes fontes de compostos bioativos, compostos antioxidantes e vitaminas (Borowitzka, 2013).

A composição das microalgas depende das condições de cultivo, como variação de luz, temperatura e a concentração de nutrientes (Niccolai et al., 2019; Thompson, 1999). Assim, a seleção da espécie e do método deve estar de acordo com o propósito da biomassa produzida, por exemplo, uso como alimento na aquicultura, extração de compostos de interesse para a indústria, tratamento de águas residuais e sequestro de CO_2 (Patil et al., 2020). Atualmente, um novo conceito tem sido amplamente abordado na produção das microalgas, a biorrefinaria. Conceito que propõe a remediação de resíduos como o uso de efluentes e sequestro de CO2 no cultivo e o total aproveitamento da

biomassa para que cada componente das microalgas seja extraído, processado e valorizado (Patil et al., 2020).

3.2 *Nannochloropsis oceanica*

As Eustigmatophyceaes são algas unicelulares com cerca de 30 espécies descritas. Produzem apenas clorofila *a* e são geralmente esféricas. Embora essa classe seja predominantemente composta por microalgas de água doce, possui representantes de ambientes extremos, de água salobra e salgada (Elias et al., 2016). Um dos gêneros mais importantes para a indústria é o *Nannochloropsis,* principalmente por produzir lipídios neutros (TAG) e LC-PUFAs, especialmente EPA (Ma et al., 2016).

A *Nannochloropsis oceanica* (Figura 1) é uma espécie de microalga que apresenta um diâmetro de 2 a 8 µm e possui uma rápida taxa de crescimento. Além disso, é tolerante às mais diversas variações de luz, temperatura, pH e nutrientes (Wang et al., 2014). Dependendo das condições de cultivo, essas microalgas podem acumular até 60% de lipídios em relação a sua biomassa seca com altos teores de ácidos graxos saturados, o que as torna ainda uma ótima opção para a produção de biocombustível (Bi & He, 2020; Islam et al., 2017; Ma et al., 2016). Na aquicultura, são usadas principalmente na alimentação direta e indireta de larvas de peixes e crustáceos, no cultivo de bivalves, como forma de suplementação de alimentos vivos como rotíferos e artêmia e como substitutos parciais da farinha de peixe nas rações (Knutsen et al., 2019; Ludwig et al., 2008; Sales et al., 2016; Serrano et al., 2021; Sirakov et al., 2015).

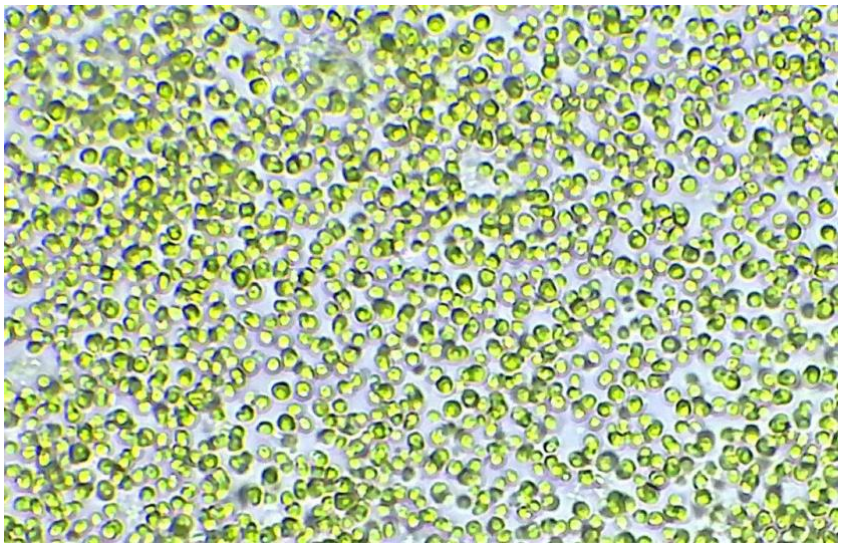

Figura 1: *Nannochloropsis oceanica*. Fonte: Arquivo AlgaSul

3.3 Cultivo de microalgas em grande escala

Em termos de utilização de área e velocidade de crescimento, as microalgas apresentam uma alta produtividade quando comparadas a outras matérias primas (Borowitzka, 2013). A única forma viável de obtenção de biomassa de microalgas é através do cultivo em larga escala, já que são necessários grandes volumes de cultivo para produzir uma pequena quantidade de biomassa (Camacho-Rodríguez et al., 2013; Roselet et al., 2017, 2013). De maneira geral, a produtividade vai depender do quão próximas as condições correspondem ao requerido pela espécie alvo. Diferente do que é observado em pequenos volumes de cultivo (10 mL – 1 L), a produtividade das microalgas em larga escala é influenciada por fatores que não são relevantes em pequena escala. Por exemplo, distribuição da luz, mistura, trocas gasosas, acúmulo de compostos produzidos pelas microalgas e contaminação por outras microalgas, zooplâncton e bactérias (Jerney and Spilling, 2020; Kumar et al., 2013).

Há duas estratégias que podem ser abordadas para cultivar microalgas em grande escala: cultivo aberto e fechado. A principal diferença entre esses dois tipos de sistema está relacionada com a forma que eles operam, vulnerabilidade à influência externa e custos para construir e operar o sistema (Patil et al., 2020). Independentemente do tipo, o sistema precisa prover luz o suficiente e mistura para evitar gradientes nos sistemas (Posten & Walter, 2012). Os *raceways* e tanques circulares (Figura 2a e b) são exemplos de sistemas abertos que são amplamente utilizados na produção de microalgas como a *Arthrospira platenses*, já os Fotobiorreatores (PBR – Figura 2c) são estruturas de cultivo fechado construídos de forma que sua geometria permita um melhor aproveitamento de luz e espaço podendo apresentar diferentes formatos tubulares ou em placas (Merchuk, 2020; Zeriouh et al., 2017). Para aplicações comerciais, os sistemas abertos são os mais utilizados, pois além de economicamente eficientes são mais fáceis de manter (Stephenson et al., 2010). Os sistemas fechados por sua vez necessitam de mais manutenção e são muito mais caros. No entanto, garantem um maior controle das condições ambientais e da entrada de organismos invasores (Roselet et al., 2013).

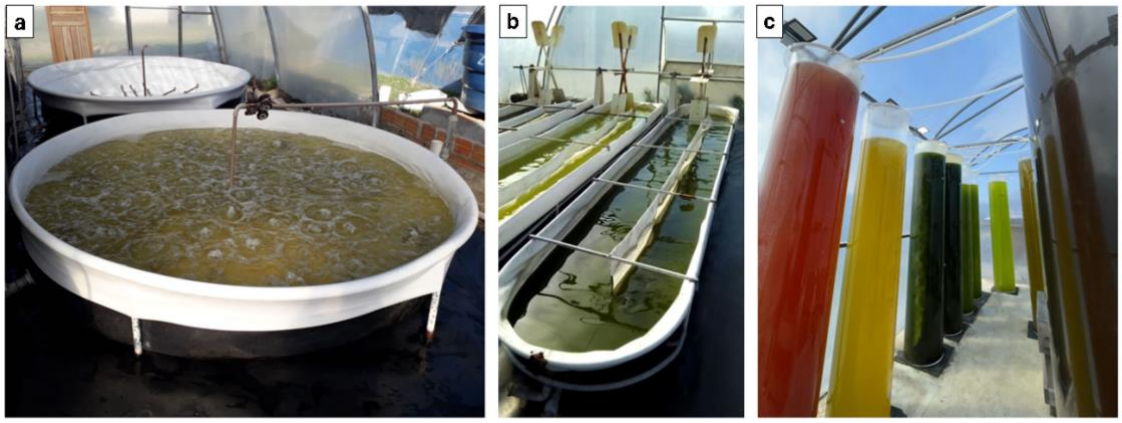

Figura 2: Exemplos de sistemas de cultivo de microalgas. Sistema aberto: (a) Tanque circular e (b) Raceways e sistema fechado: (c) Fotobioreatores. Fonte: Arquivo AlgaSul

Cultivar microalgas em larga escala é uma atividade desafiadora. A disponibilidade de luz e a temperatura são considerados os principais fatores que podem afetar a lucratividade do sistema(Jerney and Spilling, 2020). Em sistemas de cultivo *outdoor*, a temperatura e a incidência luminosa dependem da condição do próprio ambiente, gerando grandes variações nesses parâmetros, o que pode diminuir consideravelmente a produtividade de sistema (López-Elías et al., 2005; Roselet et al., 2013). Esses fatores são dependentes tanto de fatores ambientais quanto da própria geografia do local. Já em cultivos i*ndoor*, essas condições são controladas para aumentar a produtividade (Jerney and Spilling, 2020). Assim, embora esse sistema aumente consideravelmente a quantidade de biomassa produzida, os custos de produção muitas vezes inviabilizam financeiramente a produção da microalga.

3.4 Presença de bactérias nos cultivos em larga escala

Além dos problemas associados aos custos e as condições, as contaminações impactam diretamente na produtividade dos cultivos, mesmo em sistemas fechados. O que faz com que mesmo após a aplicação de protocolos de desinfecção das estruturas, materiais, água e até do ar, a produção industrial de microalgas seja conduzida em condições não axênicas (Giraldo et al., 2019). A comunidade microbiana é altamente dinâmica, dependendo da fase de crescimento e das condições externas (Giraldo et al., 2019). Além de outras espécies de microalgas e zooplâncton, as bactérias são as principais fontes de contaminação nos cultivos. Essas bactérias em associação com as microalgas podem induzir a formação de biofilme e bioincrustação nas paredes dos reatores,

atrapalhando a penetração de luz, e assim, diminuindo a produtividade e aumentando os custos de produção (Giraldo et al., 2019; Zeriouh et al., 2017). Além disso, as bactérias presentes nesse tipo de sistema usam compostos produzidos pelas microalgas para o seu próprio metabolismo, o que pode piorar a qualidade da biomassa produzida e aumentar a produção de matéria orgânica (Roselet et al., 2019). O ciclo do enxofre é um exemplo desse tipo de interação, que tem início na produção do dimetilsulfoniopropionato (DMSP), um composto organossulfurado produzido por microalgas marinhas (Charlson et al., 1987a). Na célula, desempenha um importante papel de osmólito, crioprotetor, antioxidante e dissuasor de pastoreio (Bates et al., 1987; Charlson et al., 1987a; Raina et al., 2010; Sunda et al., 2002; X.-H. Zhang et al., 2019). Pode ser liberado no meio ambiente por meio de ataques virais e bacterianos à célula, predação, exsudação ou autólise celular. Uma vez liberado, o DMSP pode ser assimilado por outras microalgas ou por bactérias que possuem a enzima DMSP liase, responsável por assimilar carbono e consequentemente liberar dimetilsulfeto (DMS) no meio ambiente (Charlson et al., 1987a, 1987b). Assim como o DMSP, o DMS é um composto organossulfurado, porém é volátil e possui baixo limiar de odor, mesmo em baixas concentrações, sua presença exala um odor forte semelhante ao cheiro de ovo podre (Charlson et al., 1987a). Na indústria, esse composto é extremamente indesejado. Além do forte cheiro de enxofre, em altas concentrações, o DMS pode causar dores de cabeça, congestão pulmonar, irritação cutânea e respiratória, vômitos, asfixia e, em casos extremos, pode levar à morte (Bouillon and Miller, 2005). Em culturas de microalgas, o DMS está presente em uma concentração tão baixa que não é detectado pelos métodos de quantificação (Liu et al., 2014; Yao et al., 2019).

Embora muitos trabalhos avaliem a interação entre as bactérias e microalgas nos cultivos (Berthold et al., 2019; Fulbright et al., 2018; Giroldo et al., 2007; Natrah et al., 2014), há poucos trabalhos que buscam formas de remediar esse problema como o controle do pH, limitação de nutrientes ou pela retirada da matéria orgânica do cultivo (Pleissner et al., 2020; Roselet et al., 2019). Na aquicultura, o fracionador de espuma (*Skimmer*) é amplamente utilizado em combinação com outros métodos para a remoção de matéria orgânica dissolvida e de partículas finas em sistemas de recirculação (Buckley et al., 2022; Gregersen et al., 2021; Kovács et al., 2023; Samocha, 2019), onde há pouca ou nenhuma troca de água (Ray et al., 2017). Seu funcionamento é dependente principalmente da presença de surfactantes, substâncias que podem ser produzidas pelas

microalgas (Garrett et al., 2008). Embora muitos estudos avaliem o uso do *skimmer* na produção de peixes e camarões, pouco se sabe sobre o uso do *skimmer* no cultivo das microalgas e o seu papel na remoção de matéria orgânica e de bactérias. M. Roselet et al. (2019) avaliou o efeito do *skimmer* (Figura 3) na remoção de matéria orgânica de um cultivo no fim da fase exponencial, e concluiu que o *skimmer* é uma forma eficiente de remoção de matéria orgânica. Não há trabalhos que avaliem o efeito do *skimmer* na remoção das bactérias que podem se aderir à matéria orgânica. Além de diminuir a produtividade do sistema, a presença de bactérias e matéria orgânica pode ainda influenciar na qualidade final da biomassa e atrapalhar na coleta (Roselet et al., 2019). O que para propósitos comerciais está distante do ideal, já que a coleta pode representar mais da metade do custo total de produção (Borowitzka, 2013).

Figura 3: *Skimmer* utilizado em cultivos de microalgas. Fonte: Arquivo AlgaSul.

3.5 Formas de colheita e comercialização

Existem diferentes métodos de colheita das microalgas, os mais utilizados são a centrifugação, sedimentação e floculação (Esteves et al., 2020). No entanto, não existe um método universal. A forma de colheita vai depender do tamanho da célula, densidade, propriedade da superfície celular e aplicação final da biomassa (Patil et al., 2020). O método de coleta de escolha precisa ter uma alta eficiência para que não tenham perdas de biomassa e econômicas (Esteves et al., 2020). Após coletada, a biomassa pode ser

comercializada de diferentes formas, seca, liofilizada ou em pastas dependendo da finalidade (Borowitzka, 2013).

A liofilização, processo que consiste na retirada de praticamente toda a água da célula por formação de vácuo em ambiente hermético, resulta em uma biomassa com pouca água, impossibilitando a ação bacteriana (Lee et al., 2013; Ryckebosch et al., 2012). Normalmente esse tipo de biomassa possui uma vida útil bem mais extensa, e dispensa o uso de armazenamento frio (Ryckebosch et al., 2012). No entanto, apesar de muito utilizada na produção de biomassa para análises laboratoriais (D'Ippolito et al., 2015; Vella et al., 2019), geralmente os custos dos equipamentos e consequentemente da produção da biomassa liofilizada (Figura 5) são tão altos que inviabilizam sua comercialização em grande escala e as microalgas acabam sendo comercializadas na forma de biomassa úmida (pastas e concentrados).

Figura 4: Biomassa liofilizada de diferentes espécies de microalgas. Fonte: Arquivo AlgaSul

No geral, as pastas e concentrados de microalgas (Figura 5) são utilizados como suplemento proteico na alimentação animal e na indústria aquícola para a alimentação de larvas de peixes, camarões e em todo o ciclo de produção de moluscos bivalves (Borowitzka, 2013, 1997). Diferente das biomassas liofilizadas, as pastas concentradas de microalgas necessitam ser conservadas sob condições especiais, pois quando mal armazenadas, ou guardadas por um longo tempo, podem sofrer perdas progressivas na sua qualidade nutricional, principalmente devido a lise e degradação das células que podem ocorrer pela ação microbiana ou pela formação de cristais de gelo no interior das células (Chellappan et al., 2020; Heasman et al., 2000).

Figura 5: Concentrados de Nannochloropsis oceanica. Fonte: Arquivo AlgaSul

3.6 Vida útil dos concentrados

No geral, as proteínas e os lipídios são os componentes mais afetados ao longo do tempo (Heasman et al., 2000; Ponis et al., 2008; Welladsen et al., 2014), entretanto, de formas diferentes. Enquanto a degradação proteica acontece de forma mais lenta, a degradação dos lipídios é bem mais rápida (Welladsen et al., 2014). Os pigmentos também são componentes extremamente afetados pela degradação das células (Lorenzen, 1967). Um dos principais efeitos é a mudança na coloração da biomassa que está diretamente associada à degradação das clorofilas em feopigmentos como feofitinas e feoforbideos (Louda et al., 2011). Embora os fatores que causam a perda da qualidade serem comuns para todas as biomassas de microalgas, a forma como as estruturas celulares são afetadas muda de acordo com a diferenciação celular de cada espécie e com a forma que a biomassa é armazenada (Heasman et al., 2000; Welladsen et al., 2014).

Na indústria alimentícia, dois métodos são principalmente utilizados para aumentar a vida útil de concentrados de microalgas: o armazenamento em temperaturas controladas e o uso de conservantes (Welladsen et al., 2014). São considerados conservantes aditivos alimentares não tóxicos, como antioxidantes, ácidos alimentares e vitaminas que previnem a oxidação, inibem a autólise e decomposição microbiana, como o ácido ascórbico que se enquadra nestas três categorias (Heasman et al., 2000; Sales and Souza-santos, 2020) e os crioprotetores que são aditivos que evitam a formação de gelo no interior da célula (Chellappan et al., 2020; Heasman et al., 2000).

Embora alguns trabalhos avaliem o efeito combinado da adição de conservante com o armazenamento frio (Heasman et al., 2000; Ponis et al., 2008), o armazenamento em baixas temperaturas ainda é preferível, pois além de mais simples, não adiciona elementos que podem modificar a biomassa, apresenta menor custo e não necessita de mão de obra especializada (Chellappan et al., 2020; Heasman et al., 2000; Sales and Souza-santos, 2020). Nesse tipo de armazenamento, dois fatores são essenciais para a conservação da biomassa concentrada: o tempo de estocagem e a temperatura de armazenamento que deve ser ideal para cada espécie (Heasman et al., 2000). Enquanto temperaturas muito mais baixas podem danificar as células, temperaturas muito altas podem estimular a ação bacteriana, provocando a degradação química das células (Welladsen et al., 2014).

Avaliar a vida útil de um concentrado permite determinar por quanto tempo esse concentrado pode permanecer na temperatura de armazenamento sem que haja mudanças na sua composição (Heasman et al., 2000). Trabalhos anteriores determinaram que a vida útil dos concentrados de *Nannochloropsis* sp. é de 3 a 4 meses em 4°C, temperatura de refrigeração (Chellappan et al., 2020; Sales and Souza-santos, 2020; Verspreet et al., 2020). Embora essa microalga possua um alto percentual de lipídios e esteja mais sujeita a processos de rápida degradação como a lipólise e oxidação (Beacham et al., 2014; Ma et al., 2016), sua estrutura extremamente rígida consiste de duas camadas poliméricas compostas de celulose e algaenan, tornando essa microalga extremamente resistente e estável (Scholz et al., 2014), permanecendo intacta mesmo após a aplicação de uma grande quantidade de força mecânica (Bernaerts et al., 2019).

Apesar de a estabilidade na composição da *Nannochloropsis* sp. a tornar uma espécie ideal para a produção de concentrados (Chellappan et al., 2020; Heasman et al., 2000; Welladsen et al., 2014), outros fatores precisam ser levados em consideração para avaliar a vida útil de concentrados. Embora as microalgas tenham seu metabolismo reduzido quando armazenadas em baixas temperaturas, este continua ativo gerando produtos como os compostos orgânicos voláteis (VOC) e substâncias poliméricas extracelulares (EPS)(Coleman et al., 2023). Além disso, quando concentramos as microalgas, todos os organismos presentes são concentrados. O que, dependendo das condições do cultivo, pode apresentar contaminação de outras microalgas e principalmente de bactérias (Giraldo et al., 2019). Essas bactérias podem competir por nutrientes com as microalgas ou podem utilizar de substâncias excretadas pelas microalgas (EPS) para se desenvolverem (Garrett et al., 2008; Giraldo et al., 2019;

Giroldo et al., 2007), como é o caso das bactérias que utilizam DMSP e produzem DMS (Vila-Costa et al., 2014; X.-H. Zhang et al., 2019; X. H. Zhang et al., 2019).

Apesar da vasta literatura apontar a presença de DMS em ambientes naturais (Asher et al., 2017; Bates et al., 1987; Charlson et al., 1992, 1987a), existem poucos estudos que avaliam sua presença em culturas e concentrados de microalgas e associam esse aparecimento a outros fatores como a presença de matéria orgânica e bactérias. Como o DMS é um composto volátil, é liberado durante os processos de colheita da microalga. No entanto, como o seu aparecimento é progressivo, a remoção de DMS do produto inicial não garante que a sua produção será cessada. Para isso é necessário avaliar quais são os fatores que influenciam no seu aparecimento em concentrados de *N. oceanica* e determinar a vida útil desses concentrados.

De um ponto de vista industrial, a otimização de processos é fundamental para garantir a eficácia e viabilidade econômica na formulação de um novo produto. Um fator crucial a considerar é a vida útil do produto, que influencia diretamente na satisfação do consumidor e na gestão do estoque. Ao desenvolver um produto com uma vida útil prolongada é possível reduzir custos associados ao desperdício e armazenamento. Além disso, o conceito de TRL (Technology Readiness Level) ajuda a avaliar em que estágio de desenvolvimento a tecnologias se encontra (Figura 6), permitindo uma alocação mais eficiente de recursos e esforços nas fases de concepção e produção. O objetivo primordial é atingir um produto finalizado que não apenas atenda às necessidades do mercado, mas também seja otimizado em termos de qualidade e custo, garantindo assim um retorno sobre o investimento mais elevado e a sustentabilidade do produto a longo prazo. No caso dos concentrados, para aumentar o TRL foi primeiro determinada a relação entre a vida útil e as concentrações de bactérias, matéria orgânica e DMS. Além disso, foram comparadas formas de diminuir essas concentrações e por fim o método que apresentou o melhor resultado foi otimizado e foi feita uma análise de custo.

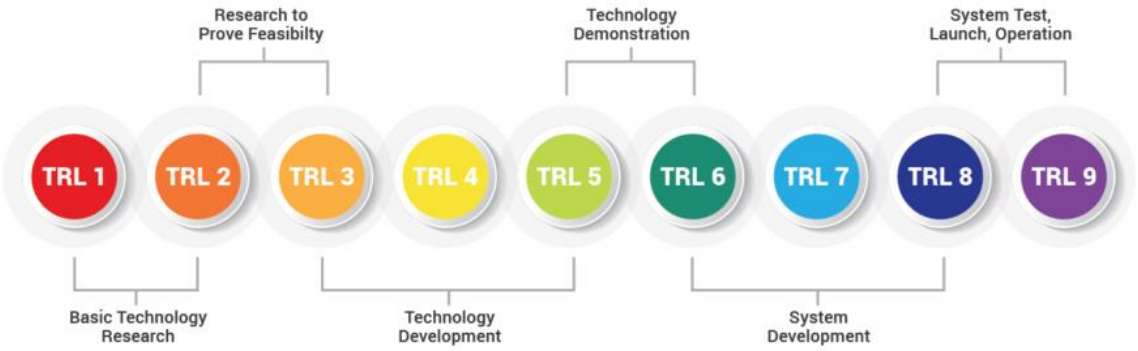

Figura 6: Níveis de maturidade tecnológica na inovação. Fonte: https://biominas.org.br/blog/technology-readiness-level-trl/

574 **REFERÊNCIAS**

575 Asher, E.C., Dacey, J.W.H., Stukel, M., Long, M.C., Tortell, P.D., 2017. Processes driving
576 seasonal variability in DMS, DMSP, and DMSO concentrations and turnover in
577 coastal Antarctic waters. Limnol Oceanogr 62, 104–124.
578 https://doi.org/10.1002/lno.10379

579 Bates, T.S., Charlson, R.J., Gammon, R.H., 1987. Evidence for the climatic role of marine
580 biogenic sulphur. Nature 329, 319–321. https://doi.org/10.1038/329319a0

581 Beacham, T.A., Bradley, C., White, D.A., Bond, P., Ali, S.T., 2014. Lipid productivity and
582 cell wall ultrastructure of six strains of *Nannochloropsis*: Implications for biofuel
583 production and downstream processing. Algal Res 6, 64–69.
584 https://doi.org/10.1016/j.algal.2014.09.003

585 Bernaerts, T.M.M., Gheysen, L., Foubert, I., Hendrickx, M.E., Van Loey, A.M., 2019.
586 Evaluating microalgal cell disruption upon ultra high pressure homogenization.
587 Algal Res 42, 101616. https://doi.org/10.1016/j.algal.2019.101616

588 Berthold, D.E., Shetty, K.G., Jayachandran, K., Laughinghouse, H.D., Gantar, M., 2019.
589 Enhancing algal biomass and lipid production through bacterial co-culture.
590 Biomass Bioenergy 122, 280–289.
591 https://doi.org/10.1016/j.biombioe.2019.01.033

592 Bi, Z., He, B., 2020. Biodiesel from microalgae, Handbook of Microalgae-Based
593 Processes and Products: Fundamentals and Advances in Energy, Food, Feed,
594 Fertilizer, and Bioactive Compounds. Elsevier Inc.
595 https://doi.org/10.1016/B978-0-12-818536-0.00013-0

596 Borowitzka, M.A., 2013. High-value products from microalgae — their development and
597 commercialisation 743–756. https://doi.org/10.1007/s10811-013-9983-9

598 Borowitzka, M.A., 1997. Microalgae for aquaculture: Opportunities and constraints. J
599 Appl Phycol 9, 393–401. https://doi.org/10.1023/A:1007921728300

600 Bouillon, R.-C., Miller, W.L., 2005. Photodegradation of Dimethyl Sulfide (DMS) in
601 Natural Waters: Laboratory Assessment of the Nitrate-Photolysis-Induced DMS
602 Oxidation. Environ Sci Technol 39, 9471–9477. https://doi.org/10.1021/es048022z

603 Buckley, T., Xu, X., Rudolph, V., Firouzi, M., Shukla, P., 2022. Review of foam
604 fractionation as a water treatment technology. Separation Science and Technology
605 (Philadelphia) 57, 929–958. https://doi.org/10.1080/01496395.2021.1946698

606 Camacho-Rodríguez, J., Cerón-García, M.C., González-López, C.V., Fernández-Sevilla,
607 J.M., Contreras-Gómez, A., Molina-Grima, E., 2013. A low-cost culture medium for
608 the production of *Nannochloropsis gaditana* biomass optimized for aquaculture.
609 Bioresour Technol 144, 57–66. https://doi.org/10.1016/j.biortech.2013.06.083

610 Charlson, R.J., Anderson, T.L., McDuff, R.E., 1992. 13 The Sulfur Cycle, in: International
611 Geophysics. Academic Press Inc., pp. 285–300. https://doi.org/10.1016/S0074-
612 6142(08)62696-0

613 Charlson, R.J., Lovelock, J.E., Andreae, M.O., Warren, S.G., 1987b. Oceanic
614 phytoplankton, atmospheric sulphur, cloud albedo and climate. Nature 326, 655–
615 661. https://doi.org/10.1038/326655a0

616 Chellappan, A., Thangamani, P., Markose, S., Thavasimuthu, C., Thangaswamy, S.,
617 Mariavincent, M., 2020. Long-term Preservation of Micro-algal Stock for Fish
618 Hatcheries. Aquac Rep 17, 100329. https://doi.org/10.1016/j.aqrep.2020.100329

619 Coleman, B., Van Poucke, C., De Witte, B., Casciaro, V., Moerdijk-Poortvliet, T.,
620 Muylaert, K., Robbens, J., 2023. The effect of drying, cell disruption and storage on
621 the sensory properties of *Nannochloropsis* sp. Algal Res 71, 103092.
622 https://doi.org/10.1016/j.algal.2023.103092

623 D'Ippolito, G., Sardo, A., Paris, D., Vella, F.M., Adelfi, M.G., Botte, P., Gallo, C.,
624 Fontana, A., 2015. Potential of lipid metabolism in marine diatoms for biofuel
625 production. Biotechnol Biofuels 8, 1–10. https://doi.org/10.1186/s13068-015-
626 0212-4

627 Elias, M., Amaral, R., Fawley, K.P., Nemcova, Y., 2016. Handbook of the Protists,
628 Handbook of the Protists. https://doi.org/10.1007/978-3-319-32669-6

629 Esteves, A.F., Almeida, C.J., Ana Luísa, G., Pires, J.C., 2020. Microalgae harvesting
630 techniques, Handbook of Microalgae-Based Processes and Products: Fundamentals
631 and Advances in Energy, Food, Feed, Fertilizer, and Bioactive Compounds.
632 https://doi.org/10.1016/B978-0-12-818536-0.00010-5

633 Fulbright, S.P., Robbins-Pianka, A., Berg-Lyons, D., Knight, R., Reardon, K.F.,
634 Chisholm, S.T., 2018. Bacterial community changes in an industrial algae production
635 system. Algal Res 31, 147–156. https://doi.org/10.1016/j.algal.2017.09.010

636 Garrett, T.R., Bhakoo, M., Zhang, Z., 2008. Bacterial adhesion and biofilms on surfaces.
637 Progress in Natural Science 18, 1049–1056.
638 https://doi.org/10.1016/j.pnsc.2008.04.001

639 Giraldo, J.B., Stock, W., Dow, L., Roef, L., Willems, A., Mangelinckx, S., Kroth, P.G.,
640 Vyverman, W., Michiels, M., 2019. Influence of the algal microbiome on biofouling
641 during industrial cultivation of *Nannochloropsis* sp. in closed photobioreactors.
642 Algal Res 42, 101591. https://doi.org/10.1016/j.algal.2019.101591

643 Giroldo, D., Ortolano, P.I.C., Vieira, A.A.H., 2007. Bacteria-algae association in batch
644 cultures of phytoplankton from a tropical reservoir: The significance of algal
645 carbohydrates. Freshw Biol 52, 1281–1289. https://doi.org/10.1111/j.1365-
646 2427.2007.01764.x

647 Gregersen, K.J. de J., Pedersen, L.-F., Pedersen, P.B., Syropoulou, E., Dalsgaard, J., 2021.
648 Foam fractionation and ozonation in freshwater recirculation aquaculture systems.
649 Aquac Eng 95, 102195. https://doi.org/10.1016/j.aquaeng.2021.102195

650 Guiry, M.D., 2012. How many species of algae are there? J Phycol 48, 1057–1063.
651 https://doi.org/10.1111/j.1529-8817.2012.01222.x

652 Heasman, M., Diemar, J., Connor, W.O., Sushames, T., Foulkes, L., 2000. Development
653 of extended shelf-life microalgae concentrate diets harvested by centrifugation for
654 bivalve molluscs ± a summary 637–659.

655 Islam, M.A., Heimann, K., Brown, R.J., 2017. Microalgae biodiesel: Current status and
656 future needs for engine performance and emissions. Renewable and Sustainable
657 Energy Reviews 79, 1160–1170. https://doi.org/10.1016/j.rser.2017.05.041

658 Jerney, J., Spilling, K., 2020. Large Scale Cultivation of Microalgae: Open and Closed
659 Systems. Methods in Molecular Biology 1980, 1–8.
660 https://doi.org/10.1007/7651_2018_130

661 Knutsen, H.R., Johnsen, I.H., Keizer, S., Sørensen, M., Roques, J.A.C., Hedén, I.,
662 Sundell, K., Hagen, 2019. Fish welfare, fast muscle cellularity, fatty acid and body-

663 composition of juvenile spotted wolffish (Anarhichas minor) fed a combination of
664 plant proteins and microalgae (Nannochloropsis oceanica). Aquaculture 506, 212–
665 223. https://doi.org/10.1016/j.aquaculture.2019.03.043

666 Kovács, B.D., de Jesus Gregersen, K.J., Rüppel, F., von Danwitz, A., Pedersen, L.F.,
667 2023. Evaluating protein skimmer performance in a commercial seawater
668 recirculating aquaculture system (RAS). Aquac Eng 103.
669 https://doi.org/10.1016/j.aquaeng.2023.102369

670 Kumar, K., Sirasale, A., Das, D., 2013. Use of image analysis tool for the development
671 of light distribution pattern inside the photobioreactor for the algal cultivation.
672 Bioresour Technol 143, 88–95. https://doi.org/10.1016/j.biortech.2013.05.117

673 Lee, Y.L., Chuang, Y.C., Su, H.M., Wu, F.S., 2013. Freeze-dried microalgae of
674 *Nannochloropsis oculata* improve soybean oil's oxidative stability. Appl Microbiol
675 Biotechnol 97, 9675–9683. https://doi.org/10.1007/s00253-013-5183-4

676 Liu, C., Gao, C., Zhang, H., Chen, S., Deng, P., Yue, X., Guo, X., 2014. Production of
677 dimethylsulfide and acrylic acid from dimethylsulfoniopropionate during growth of
678 three marine microalgae. Chinese Journal of Oceanology and Limnology 32, 1270–
679 1279. https://doi.org/10.1007/s00343-015-4029-6

680 López-Elías, J.A., Voltolina, D., Enríquez-Ocaña, F., Gallegos-Simental, G., 2005. Indoor
681 and outdoor mass production of the diatom *Chaetoceros muelleri* in a mexican
682 commercial hatchery. Aquac Eng 33, 181–191.
683 https://doi.org/10.1016/j.aquaeng.2005.01.001

684 Lorenzen, C.J., 1967. Determination of Chlorophyll and Pheo-Pigments:
685 Spectrophotometric Equations. Limnol Oceanogr 12, 343–346.
686 https://doi.org/10.4319/lo.1967.12.2.0343

687 Louda, J.W., Mongkhonsri, P., Baker, E.W., 2011. Chlorophyll degradation during
688 senescence and death-III: 3-10yr experiments, implications for ETIO series
689 generation. Org Geochem 42, 688–699.
690 https://doi.org/10.1016/j.orggeochem.2011.03.018

691 Ludwig, G.M., Rawles, S.D., Lochmann, S.E., 2008. Effect of rotifer enrichment on
692 sunshine bass morone chrysops × M. saxatilis larvae growth and survival and fatty

693 acid composition. J World Aquac Soc 39, 158–173. https://doi.org/10.1111/j.1749-
694 7345.2008.00148.x

695 Ma, X.N., Chen, T.P., Yang, B., Liu, J., Chen, F., 2016. Lipid production from
696 *Nannochloropsis*. Mar Drugs 14. https://doi.org/10.3390/md14040061

697 Merchuk, J.C., 2020. Photobioreactor design, Handbook of Microalgae-Based Processes
698 and Products: Fundamentals and Advances in Energy, Food, Feed, Fertilizer, and
699 Bioactive Compounds. Elsevier Inc. https://doi.org/10.1016/B978-0-12-818536-
700 0.00005-1

701 Miller, T.R., Belas, R., 2004. Dimethylsulfoniopropionate Metabolism by Pfiesteria -
702 Associated *Roseobacter* spp. Appl Environ Microbiol 70, 3383–3391.
703 https://doi.org/10.1128/AEM.70.6.3383-3391.2004

704 Moura Junior, A.M., Bezerra Neto, E., Koening, M.L., Eskinazi Leça, E., 2007. Chemical
705 compositon of three microalgae species for possible use in mariculture. Brazilian
706 Archives of Biology and Technology 50, 461–467. https://doi.org/10.1590/S1516-
707 89132007000300012

708 Natrah, F.M.I., Bossier, P., Sorgeloos, P., Yusoff, F.M., Defoirdt, T., 2014. Significance of
709 microalgal-bacterial interactions for aquaculture. Rev Aquac 6, 48–61.
710 https://doi.org/10.1111/raq.12024

711 Niccolai, A., Chini Zittelli, G., Rodolfi, L., Biondi, N., Tredici, M.R., 2019. Microalgae
712 of interest as food source: Biochemical composition and digestibility. Algal Res 42.
713 https://doi.org/10.1016/j.algal.2019.101617

714 Patil, R.A., Kausley, S.B., Joshi, S.M., Pandit, A.B., 2020. Process intensification applied
715 to microalgae-based processes and products, in: Handbook of Microalgae-Based
716 Processes and Products. Elsevier, pp. 737–769. https://doi.org/10.1016/B978-0-12-
717 818536-0.00027-0

718 Patil, V., Källqvist, T., Olsen, E., Vogt, G., Gislerød, H.R., 2007. Fatty acid composition
719 of 12 microalgae for possible use in aquaculture feed. Aquaculture International 15,
720 1–9. https://doi.org/10.1007/s10499-006-9060-3

721 Pleissner, D., Lindner, A.V., Ambati, R.R., 2020. Techniques to Control Microbial
722 Contaminants in Nonsterile Microalgae Cultivation. Appl Biochem Biotechnol 192,
723 1376–1385. https://doi.org/10.1007/s12010-020-03414-7

724 Ponis, E., Parisi, G., Chini Zittelli, G., Lavista, F., Robert, R., Tredici, M.R., 2008.
725 *Pavlova lutheri*: Production, preservation and use as food for Crassostrea gigas
726 larvae. Aquaculture 282, 97–103. https://doi.org/10.1016/j.aquaculture.2008.06.010

727 Posten, C., Walter, C., 2012. Microalgal biotechnology: Potential and production,
728 Microalgal Biotechnology: Potential and Production.
729 https://doi.org/10.1515/9783110225020

730 Raina, J.B., Dinsdale, E.A., Willis, B.L., Bourne, D.G., 2010. Do the organic sulfur
731 compounds DMSP and DMS drive coral microbial associations? Trends Microbiol
732 18, 101–108. https://doi.org/10.1016/j.tim.2009.12.002

733 Ray, A.J., Drury, T.H., Cecil, A., 2017. Comparing clear-water RAS and biofloc systems:
734 Shrimp (*Litopenaeus vannamei*) production, water quality, and biofloc nutritional
735 contributions estimated using stable isotopes. Aquac Eng 77, 9–14.
736 https://doi.org/10.1016/j.aquaeng.2017.02.002

737 Riviers, B. de, 2006. Biologia e Filogenia das algas. Artmed, Porto Alegre.

738 Roselet, F., Maicá, P., Martins, T., Abreu, P.C., 2013. Comparison of open-air and semi-
739 enclosed cultivation system for massive microalgae production in sub-tropical and
740 temperate latitudes. Biomass Bioenergy 59, 418–424.
741 https://doi.org/10.1016/j.biombioe.2013.09.014

742 Roselet, F., Vandamme, D., Roselet, M., Muylaert, K., Abreu, P.C., 2017. Effects of pH,
743 Salinity, Biomass Concentration, and Algal Organic Matter on Flocculant Efficiency
744 of Synthetic Versus Natural Polymers for Harvesting Microalgae Biomass.
745 Bioenergy Res 10, 427–437. https://doi.org/10.1007/s12155-016-9806-3

746 Roselet, M., Roselet, F., Abreu, P.C., 2019. Foam fractionator as a tool to remove
747 dissolved organic matter and improve the flocculation of the marine microalga
748 *Nannochloropsis oceanica*. J Appl Phycol 31, 2911–2919.
749 https://doi.org/10.1007/s10811-019-01801-0

Ryckebosch, E., Muylaert, K., Foubert, I., 2012. Optimization of an analytical procedure for extraction of lipids from microalgae. JAOCS, Journal of the American Oil Chemists' Society 89, 189–198. https://doi.org/10.1007/s11746-011-1903-z

Sales, R., Mélo, R.C.S., de Moraes, R.M., da Silva, R.C.S., Cavalli, R.O., do Amaral Ferraz Navarro, D.M., de Souza Santos, L.P., 2016. Production and use of a flocculated paste of *Nannochloropsis oculata* for rearing newborn seahorse *Hippocampus reidi*. Algal Res 17, 142–149. https://doi.org/10.1016/j.algal.2016.04.024

Sales, R., Souza-santos, L.P. De, 2020. Production of concentrated inocula from the microalgae *Nannochloropsis oculata*. 1609–1620.

Samocha, T.M., 2019. Sustainable Biofloc Systems for Marine Shrimp. Elsevier. https://doi.org/10.1016/C2018-0-02628-6

Scholz, M.J., Weiss, T.L., Jinkerson, R.E., Jing, J., Roth, R., Goodenough, U., Posewitz, M.C., Gerken, H.G., 2014. Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall. Eukaryot Cell 13, 1450–1464. https://doi.org/10.1128/EC.00183-14

Serrano, E., Simpfendorfer, R., Medina, A., Sandoval, C., Martínez, A., Morales, R., Davies, S.J., 2021. Partially replacing fish oil with microalgae (*Schizochytrium limacinum* and *Nannochloropsis oceanica*) in diets for rainbow trout (*Oncorhynchus mykiss*) reared in saltwater with reference to growth performance, muscle fatty acid composition and liver ultrast. Aquac Res 52, 4401–4413. https://doi.org/10.1111/are.15279

Sirakov, I., Velichkova, K., Stoyanova, S., Staykov, Y., 2015. The importance of microalgae for aquaculture industry . Review . Int J Fish Aquat Stud 2, 81–84.

Stephenson, A.L., Kazamia, E., Dennis, J.S., Howe, C.J., Scott, S.A., Smith, A.G., 2010. Life-cycle assessment of potential algal biodiesel production in the united kingdom: A comparison of raceways and air-lift tubular bioreactors. Energy and Fuels 24, 4062–4077. https://doi.org/10.1021/ef1003123

Sunda, W.G., Kieber, D., Kiene, R.P., 2002. An antioxidant function of DMSP and DMS in marine algae oceanic dimethylsulfide (DMS) photolysis. Nature 418, 317–320.

780 Thompson, P., 1999. The response of growth and biochemical composition to variations
781 in daylength, temperature, and irradiance in the marine diatom *Thalassiosira*
782 *pseudonana* (Bacillariophyceae). J Phycol 35, 1215–1223.
783 https://doi.org/10.1046/j.1529-8817.1999.3561215.x

784 Vella, F.M., Sardo, A., Gallo, C., Landi, S., Fontana, A., D'Ippolito, G., 2019. Annual
785 outdoor cultivation of the diatom *Thalassiosira weissflogii*: productivity, limits and
786 perspectives. Algal Res 42, 101553. https://doi.org/10.1016/j.algal.2019.101553

787 Verspreet, J., Soetemans, L., Bastiaens, L., 2020. Searching for Appropriate Storage
788 Conditions for Short-Term Wet Preservation of *Porphyridium purpureum*. Applied
789 Sciences 10, 8315. https://doi.org/10.3390/app10238315

790 Vila-Costa, M., Rinta-Kanto, J.M., Poretsky, R.S., Sun, S., Kiene, R.P., Moran, M.A.,
791 2014. Microbial controls on DMSP degradation and DMS formation in the Sargasso
792 Sea. Biogeochemistry 120, 295–305. https://doi.org/10.1007/s10533-014-9996-8

793 Wang, D., Ning, K., Li, J., Hu, J., Han, D., Wang, H., Zeng, X., Jing, X., Zhou, Q., Su,
794 X., Chang, X., Wang, A., Wang, W., Jia, J., Wei, L., Xin, Y., Qiao, Y., Huang, R.,
795 Chen, J., Han, B., Yoon, K., Hill, R.T., Zohar, Y., Chen, F., Hu, Q., Xu, J., 2014.
796 *Nannochloropsis* Genomes Reveal Evolution of Microalgal Oleaginous Traits. PLoS
797 Genet 10. https://doi.org/10.1371/journal.pgen.1004094

798 Welladsen, H., Kent, M., Mangott, A., Li, Y., 2014. Shelf-life assessment of microalgae
799 concentrates: Effect of cold preservation on microalgal nutrition profiles.
800 Aquaculture 430, 241–247. https://doi.org/10.1016/j.aquaculture.2014.04.016

801 Yao, S., Lyu, S., An, Y., Lu, J., Gjermansen, C., Schramm, A., 2019. Microalgae-bacteria
802 symbiosis in microalgal growth and biofuel production: a review. J Appl Microbiol
803 126, 359–368. https://doi.org/10.1111/jam.14095

804 Zeriouh, O., Reinoso-Moreno, J.V., López-Rosales, L., Cerón-García, M. del C.,
805 Sánchez-Mirón, A., García-Camacho, F., Molina-Grima, E., 2017. Biofouling in
806 photobioreactors for marine microalgae. Crit Rev Biotechnol 37, 1006–1023.
807 https://doi.org/10.1080/07388551.2017.1299681

808 Zhang, X.-H., Liu, J., Liu, J., Yang, G., Xue, C.-X., Curson, A.R.J., Todd, J.D., 2019.
809 Biogenic production of DMSP and its degradation to DMS—their roles in the global

sulfur cycle. Sci China Life Sci 62, 1296–1319. https://doi.org/10.1007/s11427-018-9524-y

Zhang, X.H., Liu, Ji, Liu, Jingli, Yang, G., Xue, C.X., Curson, A.R.J., Todd, J.D., 2019. Biogenic production of DMSP and its degradation to DMS—their roles in the global sulfur cycle. Sci China Life Sci 62, 1296–1319. https://doi.org/10.1007/s11427-018-9524-y

4 OBJETIVO GERAL

Otimizar a produção de concentrados da microalga *Nannochloropsis oceanica*.

5 OBJETIVOS ESPECÍFICOS

- Determinar a influência da presença de bactérias e matéria orgânica no aparecimento de DMS ao longo da vida útil de concentrados de *N. oceanica;*
- Testar o uso do *skimmer* e do método de lavagem para minimizar bactérias em concentrados de *N. oceanica;*
- Otimizar o processo de lavagem na produção de concentrados de *N. oceanica* visando diminuir a concentração de bactérias e melhorar a relação benefício-custo do processo, preservando a integridade celular.

6 CAPÍTULO I

Effect of removing bacteria and dissolved organic matter on DMS production in *Nannochloropsis oceanica* concentrates

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Abstract

The aim of this study was to determine the influence of bacteria and organic matter on the occurrence of dimethyl sulfide (DMS) over the shelf life of Nannochloropsis oceanica concentrates. Two concentrates were compared: control treatment, composed of biomass collected by centrifugation and diluted to a cell density of 5×109 cells·mL-1, and a washed concentrate treatment, where the biomass underwent successive centrifugations to remove dissolved organic matter (DOM) and bacteria before being diluted to a cell density of 5×109 cells·mL-1. Both concentrates were stored at two temperatures: 23°C for 10 days and 4°C for 85 days. Cellular viability, bacterial density, DOM, and DMS concentration were assessed. The washing process was responsible for removing approximately 84% of the bacterial load from the concentrates. This significant reduction played a crucial role in decreasing the concentrations of DOM and DMS over the storage period. Furthermore, the process did not impair the cellular viability of microalgae. The findings underscore the importance of carefully managing bacterial and organic components in microalgal concentrates, especially when considering the preservation of freshness and cellular viability, which are key factors in applications such as larviculture.

Keywords: Storage time; Storage temperature; DMSP; Cell Viability; DOM

6.1 Introduction

Microalgae are extremely important in aquaculture as a source of high nutritional quality food. They are ideal for feeding bivalves, larvae of fish, crustaceans, and as a way of enriching artemia and rotifers (Sales et al., 2019; Sorgeloos and Lavens, 1996). Microalgae production requires high cost, a suitable location, specialized labor, and strict control against contamination (Fernández et al., 2020). For this reason, the production of microalgae in aquaculture facilities has been left aside and increasingly efforts have been made to acquire commercialized biomass. *Nannochloropsis* sp. is one of the most used species for this purpose, because it has high percentages of fatty acids, mainly eicosapentaenoic acid (EPA), and has the technology required for cultivation, harvesting and storage (Faé Neto et al., 2018; Kubelka et al., 2018; Roselet et al., 2017, 2019; Safafar et al., 2017).

As microalgae culture have extremely low densities that can reach up to 0.05% of dry weight, large volume of culture would be required to produce a small amount of biomass (Camacho-Rodríguez et al., 2013; Mayers et al., 2014; Roselet et al., 2013, 2017). So, for commercial purposes, it is necessary to harvest this biomass. Regardless of the method used, the biomass obtained is concentrated and can therefore be sold in a dry or wet form. Freeze-drying and spray-drying the biomass keeps its composition stable for longer (Coleman et al., 2023), however, this process requires high energy expenditure due to the use of freeze-dry or spray-dry, making its cost unfeasible. Although wet biomass has a lower cost when compared to dry biomass, degradation is faster due to the high-water activity (Coleman et al., 2023; Welladsen et al., 2014). The speed at which these processes will deteriorate the biomass depends mainly on the storage temperature (Balduyck et al., 2016; Dalheim et al., 2021). These degradation processes are mainly metabolic (protein denaturation, lipolysis and lipid peroxidation) (Balduyck et al., 2016; Bernaerts et al., 2019; Coleman et al., 2023), and processes is caused by bacterial contamination (Dan et al., 2018; Verspreet et al., 2020).

Although in large-scale cultivation several protocols are used to reduce contamination, bacteria are ubiquitous (Giraldo et al., 2019; Lian et al., 2022). When this biomass is harvested, all the organic load present in the cultivation can be concentrated (Roselet et al., 2019). These bacteria compete with microalgae for nutrients or can use the compounds produced by microalgae for their own metabolism (Giroldo et al., 2007; Lian et al., 2021), an example, are bacteria that use an important osmolyte, antioxidant and cryoprotectant for microalgae, dimethylsulfoniopropianate (DMSP), and produce dimethylsulfide (DMS) (Zhang et al., 2019; Zhuang et al., 2011), a volatile compound highly unwanted in industry due to its strong sulfur smell and the problems it can cause to human health (Charlson et al., 1987). In microalgae cultures, DMS is present in such a low concentration that it is not detected by quantification methods (Liu et al., 2014; Malin, 2006; Yao et al., 2019). As it is a volatile compound, when microalgae are harvested and concentrated, the existing portion of DMS is released. However, this does not prevent its subsequent appearance during storage periods, mainly in the presence of bacteria and dissolved organic matter.

DMS in marine environments has been extensively studied over the past 40 years due to its importance in the sulfur cycle (Asher et al., 2017; Bates et al., 1987; Bouillon and Miller, 2005; Charlson et al., 1992; Raina et al., 2010; Vila-Costa et al., 2014). To date, there are few studies that evaluate its appearance and relevance in microalgae cultures and concentrates and associating this appearance with other factors such as the presence of organic matter and bacteria (Levasseur et al., 1996; Zhuang et al., 2011). Thus, the objective of this study is to determine the influence of bacteria and organic matter on the appearance of DMS throughout the shelf life of *Nannochloropsis oceanica* concentrate.

6.2 Material and methods

6.2.1 Microalgae cultivation and harvesting

The microalgae *Nannochloropsis oceanica* (strain NANN OCEA_1) was obtained from the collection of the Laboratory of Phytoplankton and Marine Microorganisms, of the Federal University of Rio Grande (FURG). The microalgae were cultivated in 330 L closed bubble columns photobioreactors with a diameter of 0.55 m and water column of 1.5 m. Natural seawater (30 $g \cdot L^{-1}$) was treated following a protocol adapted from Kubelka et al. (Kubelka et al., 2017). Briefly, seawater was sequentially filtered through 1.0 µm and 0.5 µm polypropylene filter cartridges, and then sterilized by ultraviolet light (UVC). Salinity was adjusted to 28 $g \cdot L^{-1}$ using freshwater, followed by addition of hypochlorite 12 % (NaClO, 0.50 $mL \cdot L^{-1}$) for 48 h, and then neutralized with ascorbic acid ($C_6H_8O_6$, 30 $mg \cdot L^{-1}$) (Harrison and Berges, 2005). A fertilizer-based medium (YCB) was employed (Couto et al., 2021). A 10 % inoculum was added, reaching a final cell density of 4.25×10^7 cells$\cdot mL^{-1}$, and the culture was homogenized through continuous atmospheric air injection (4.8 $L \cdot min^{-1}$) (Kubelka et al., 2017).

The culture was kept in a greenhouse under natural environmental conditions (temperature, irradiance and photoperiod). The fed-batch cultivation was conducted in August 2022 (late winter in the southern hemisphere), lasting 18 days with medium additions at every 5 days. Growth was monitored twice a week by optical density at 750 nm, and pH was monitored and corrected to 8.00 by CO_2 injection, at the same frequency. The microalgae were collected by centrifugation in a continuous disk centrifuge (FJ 130 EPR, Janschitz Gmbh, Althofen, Austria) with a flow rate of 20 $L \cdot h^{-1}$ and rotation of 9,500

RPM, resulting in a 25% dry matter paste. The paste was stored at 4ºC for less than 48 h until the beginning of the experiment.

6.2.2 Experimental design

To understand the evolution of DMS in microalgae concentrates, the presence of bacteria and organic matter were evaluated at different temperatures and storage times (at 23°C for 0, 2, 4, 6, 8, and 10 days, and at 4ºC for 0, 17, 34, 51, 68, and 85 days). A control group containing bacteria and organic matter (concentrate C) was set to observe the natural evolution of DMS, where the paste was simply diluted in autoclaved seawater until a concentration of 5×10^9 cells·mL-1. A treatment with reduced quantities of bacteria and organic matter (concentrate W) was set by sequentially washing the paste (Roselet et al., 2019). Briefly, about 20 g of paste were resuspended in 450 mL of autoclaved seawater and centrifuged for 18 minutes at 2,169 g, then the supernatant was discarded. This process was repeated 4 times, then the paste was diluted in autoclaved seawater until a concentration of 5×10^9 cells·mL^{-1}. Finally, the concentrates C and W were packed in 50 mL transparent LDPE stand up airtight pouch bags and stored at the experimental temperatures, being manually homogenized every 2 days to avoid sedimentation (Figure 1a and c). In both concentrates, no chemical additives were employed to extend the shelf life. For each storage time, cell viability, bacterial density, DOM and DMS concentrations were determined.

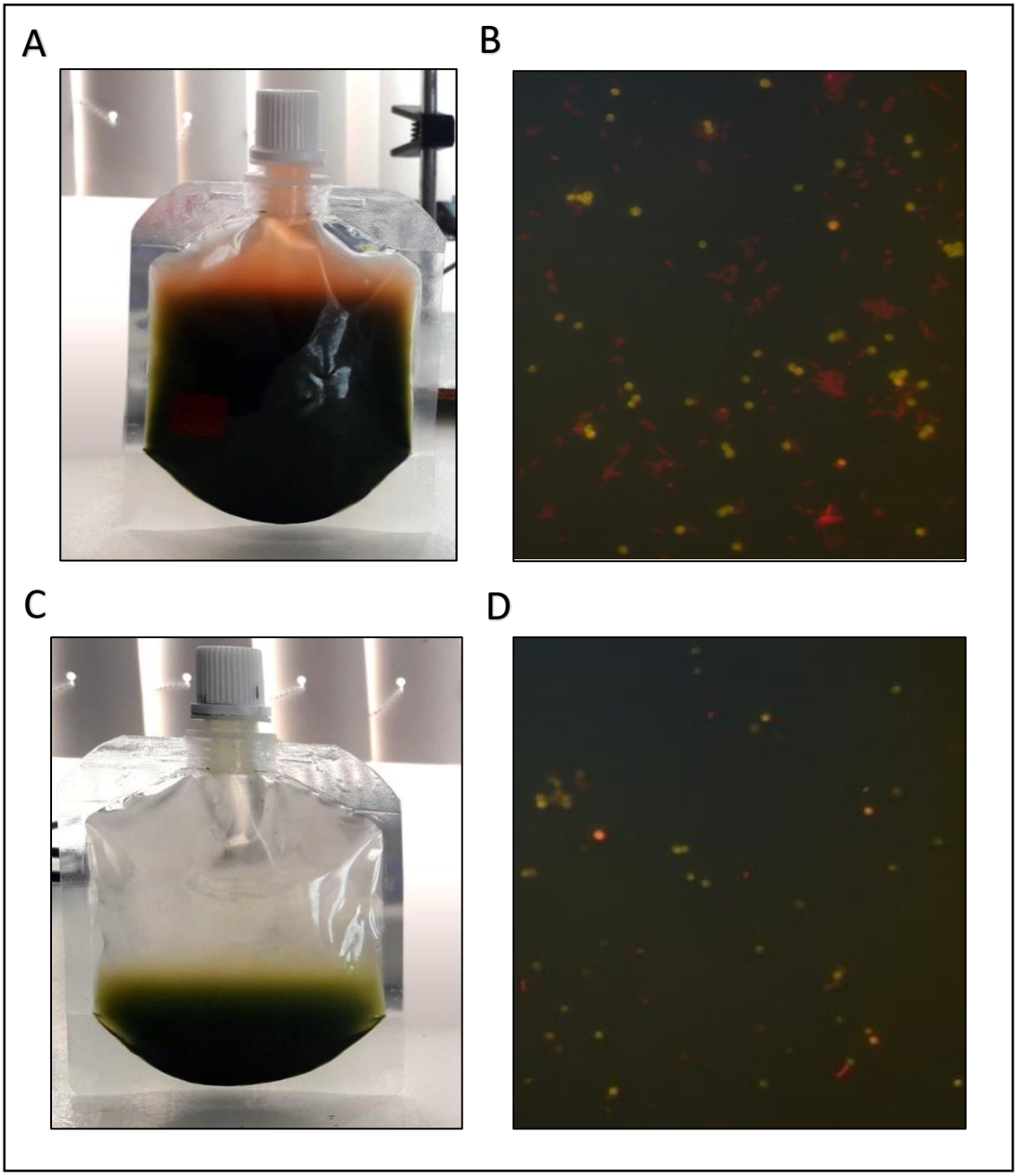

Figure 1: Macro (A and C) and micro (B and D) images of the 85 days concentrates. Control (A and B) and Washing process (C and D). In green the microalgae cells and in red/orange bacteria.

6.2.3 Dissolved organic matter (DOM)

For the DOM quantification, 20 mL samples of the concentrates C and W were first centrifuged for 18 min at 2,169 g to remove the bulk microalgae, then the supernatants were filtered through 0.7 µm to remove any remaining microalgae. The DOM fraction was finally obtained by passing the filtrate through a 0.45 µm filter. DOM samples were then stored in a freezer (−21°C) until analysis. Dissolved organic carbon (DOC) was

analyzed as a proxy for DOM. A Shimadzu TOC–CVSP analyzer (Tokyo, Japan) was used for DOM determinations. The instrument was calibrated with potassium hydrogen phthalate (total carbon standard, TC) and sodium hydrogen carbonate and anhydrous sodium carbonate (inorganic carbon standards, IC). TC and IC standards ranged from 0 to 100 $mg \cdot L^{-1}$. DOC concentration was calculated as the difference between TC and IC measurements. All measurements were performed in triplicate. TC and IC standards with concentrations of 10 and 50 $mg \cdot L^{-1}$ were used as controls.

6.2.4 Bacterial density

Bacterial density of concentrates C and W was determined by direct cell counting under an epifluorescence microscope. Hence, 10 mL samples were stored in 4% formaldehyde solution at room temperature until analysis. As the samples were highly concentrated (5×10^9 $cells \cdot mL^{-1}$), a dilution rate of 10,000× using sterilized seawater was necessary. For the preparation of counting slides, 1 mL of samples were filtered through 0.2 µm pore-size Nuclepore polycarbonate filters previously darkened with Irgalan Black, with a vacuum pressure <5 cmHg (Hobbie et al., 1977). The samples were incubated for 10 minutes with a 0.1 % Acridine Orange solution (Nishino, 1986), then the filters were removed from the filtration apparatus and let dry. After drying, they were placed on a slide with immersion oil and observed in Zeiss Axioplan epifluorescence microscope (1,000× final magnification, λEx = 460 nm and λEm = 490 nm) (Hobbie et al., 1977). Bacteria were counted in 20 random fields until reaching a total of at least 100 cells, and then separated into morphotypes (cocci, bacilli and filament). The bacterial density was calculated using Equation 1.

$$Total\ Bacterial\ Cell\ Count/\mu L = \frac{A \times (\frac{(\frac{B}{C})}{D})}{1000} \qquad \text{Equation 1}$$

where A = average count per field, B = filtration area (mm2), C = count field area (mm2), and D = sample volume (mL).

6.2.5 DMS

A headspace-gas chromatography/mass spectrometry (HS-GC/MS) method was developed for quantifying DMS. Briefly, 10 mL samples were pipetted to 20 mL headspace vials sealed with magnetic screw-capped lids lined with PTFE-silicone septa.

The vials were then closed and placed in the AOC 6000 auto-sampler, where the headspace extraction was automatically carried out. The samples were incubated at 70°C with agitation at 250 rpm for 20 minutes for transferring of DMS from sample to the headspace. Afterwards, 1000 µL of headspace gas was drawn with a gas-tight syringe and injected in the splitless mode into the GC for analysis. DMS determination was carried out by a Shimadzu gas chromatograph tandem mass spectrometry, equipped with a Combipal AOC 6000 autosampler, GC-2010 Plus column oven and a TQ8050 mass spectrometer detector with a triple quadrupole type mass filter. Carrier gas was helium (99.999 % purity) at constant flow rate of 0.87 $mL \cdot min^{-1}$. The injector temperature was 60°C. A capillary column model RTX®-Wax (25 m × 0.25 mm × 0.25 µm) (Restek, Bellefonte, PA, USA) was used for separating DMS. The oven temperature program started at 30°C, which was kept for 2 min. Afterward, the temperature increased up to 180°C at $15°C \cdot min^{-1}$, totaling 12 min of analysis. In this condition, retention time for DMS was 1.760 min. Mass spectrometry was performed in the electron impact mode with collision energy of 70 eV. Interface and ion source temperatures were 220°C and 200°C, respectively. The selective ion monitoring using the third quadrupole filter mode was used for identifying DMS, whose most intense ion was used for quantification (62 m/z) while the other two most intense ions were used for confirmation (47 and 61 m/z, respectively). Equipment manipulation and data collection and treatment were performed by the GCMSsolution software, version 4.45 SP1 (Shimadzu, Japan). The limit of quantification was assessed experimentally by the lowest concentration with a signal to noise ratio of 10, while the limit of detection was that concentration divided by 3.3. The analytical curves were daily prepared by proper dilution of the DMS standard at seven concentration levels, from the limit of quantification, which was 1 $\mu g \cdot L^{-1}$ up to 100 $\mu g \cdot L^{-1}$. The linear regression was used for construction of the analytical curves, and the equation Y = 269,033.3192x + 98.57896258 was used for the determinations, whose determination coefficient (R2) was > 0.999. Accuracy was assessed by a recovery assay, by addition of the DMS standard at different concentrations. Recoveries between 85 to 107% with relative standard deviation lower than 8.8% were achieved.

6.2.6 Cell viability

To determine effects of washing, temperature and storage times on cell viability, the concentrates C and W were inoculated (in triplicate) at 6.25×10^6 $cells \cdot mL^{-1}$ in 10 mL test

tubes containing f/2 medium (Guillard, 1975) and incubated for six days at 21°C, with a light intensity of 100 µmol-photons·m^{-2}·s^{-1} and a 12 h light period. A fresh N. oceanica culture in the exponential growth phase was employed as an inoculum control (I). The test tubes were manually homogenized daily. Microalgae density was measured by cell counting initially (D) and on day six (D0) using an improved Neubauer chamber at 400× magnification, and growth rate (K) was calculated as a proxy for cell viability (equation 2).

$$K(division\ d^{-1}) = \frac{3.322}{(t-t0)\times Log(\frac{D}{D0})} \quad \text{Equation 2}$$

6.2.7 Statistical analysis

The presence of bacteria and organic matter in the formation of DMS in N. oceanica concentrates was studied at two different temperatures and six storage times (23ºC × 0, 2, 4, 6, 8, 10 days, and 4ºC × 0, 17, 34, 51, 68, 85 days). Data on cell viability, bacterial density, dissolved organic matter and DMS concentrations were first analyzed for normality and homogeneity using the Kolmogorov-Smirnov and Lavene tests, respectively. Then, Student's t-test or one-way analysis of variance followed by a Tukey post hoc test was performed, both with a significance level of 5%. Analyzes were performed separately by temperatures, comparing responses between treatments within the same storage time and comparing responses within treatments over storage time.

6.3 Results

6.3.1 Dissolved organic matter

Microalgae naturally release DOM containing DMSP, which is absorbed by bacteria and broken down into DMS (Charlson et al., 1992). Thus, controlling the initial DOM concentration in microalgae concentrates could be an approach to reduce bacterial growth and, consequently, DMS production. Figure 2 presents the effect of washing, temperature and storage times on DOM concentrations (expressed as DOC mg·L^{-1}). At room temperature, a significant difference ($P<0.0001$) was observed for the initial concentration of DOM between treatment washed W (12.76 ± 1.57 mg·L^{-1}) and control C (38.73 ± 1.32 mg·L^{-1}), indicating that the washing process was efficient (Figure 2a). DOM concentrations increased over time in treatment W and control C, remaining

significantly different (P<0.0001). DOM concentrations in treatment W stabilized from day 4 onwards (67.85 ± 13.16 mg·L^{-1}), whereas control C stabilized on day 6 (175.02 ± 29.03 mg·L^{-1}). The maximum DOM concentrations achieved in 10 days for treatment W and control C were 80.81 ± 6.87 mg·L^{-1} and 196.43 ± 29.71 mg·L^{-1}, respectively. In overall, a similar pattern was observed with concentrates kept under refrigeration for 85 days (Figure 1b). Both treatment W and control C presented a steady DOM increase over time, attaining maximum concentrations of 114.90 ± 6.80 mg·L^{-1} and 227.03 ± 32.67 mg·L^{-1}, respectively. Comparing the effect of storage temperatures, DOM concentrations for treatment W at 23ºC (51.78 ± 26.09 mg·L^{-1}) and at 4ºC (63.97 ± 36.48 mg·L^{-1}) were not significantly different (P=0.536). For control C at 23ºC (126.52 ± 59.50 mg·L^{-1}) and at 4ºC (125.90 ± 67.61 mg·L^{-1}), DOM concentrations were also not significantly different (P=0.987). These results indicate that the washing process was efficient in reducing the initial DOM concentrations. Furthermore, refrigeration also played a significant role in slowing DOM production, as no significant difference (P=0.125) was observed for control C after 85 days at 4ºC (227.03 ± 32.67 mg·L^{-1}) and after 10 days at 23ºC (176.37 ± 31.65 mg·L^{-1}). Regarding treatment W, no significant difference (P=0.052) was observed up to 68 days at 4ºC (95.17 ± 0.63 mg·L^{-1}) and 10 days at 23ºC (60.82 ± 21.79 mg·L^{-1}). After 68 days, DOM concentration was significantly different (P=0.014) at 4ºC (114.90 ± 6.80 mg·L^{-1}).

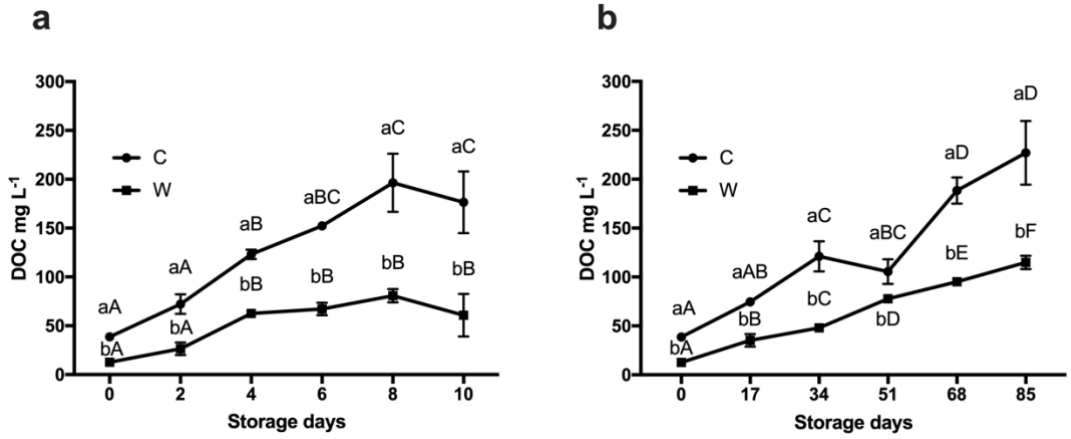

Figure 2: Mean ± standard deviation of concentrations of DOM (as DOC mg·L^{-1}) at 23°C (a) and at 4°C (b) for treatment W and control C. Different lowercase letters indicate statistically significant differences (p<0.05) among treatment W and control C within the same storage day (n = 3). Different uppercase letters indicate statistically significant differences (p<0.05) within treatment W and control C throughout the storage period (n = 3).

6.3.2 Bacterial density

Figure 3 presents the effect of washing, temperature, and storage times on bacterial densities (in cells·µL^{-1}). A significant increase is noticeable in the total bacterial densities in control C at 23ºC (P=0.001, Figure 3a) and at 4ºC (P=0.001, Figure 3b) during the storage time. The maximum bacterial densities for control C were attained at the end of the experiment, being $8.98\times10^4 \pm 1.10\times10^4$ cells·µL^{-1} at 23ºC (10th day), and $7.28\times10^4 \pm 7.33\times10^3$ cells·µL^{-1} at 4ºC (85th day). No significant difference (P=0.093) was observed for control C at 23ºC and 4ºC. Although a significant increase is observable in the total bacterial densities in treatment W at 23ºC (P=0.016, Figure 3a) and at 4ºC (P=0.039, Figure 3b), those densities ($3.09\times10^3 \pm 6.91\times10^2$ cells·µL^{-1}) were significantly lower (P<0.0001) than in the control C ($8.05\times10^4 \pm 7.73\times10^3$ cells·µL^{-1}). These results indicate that the washing process was efficient in reducing the total bacterial density. Moreover, refrigerating the concentrates delayed bacterial growth for up to 85 days.

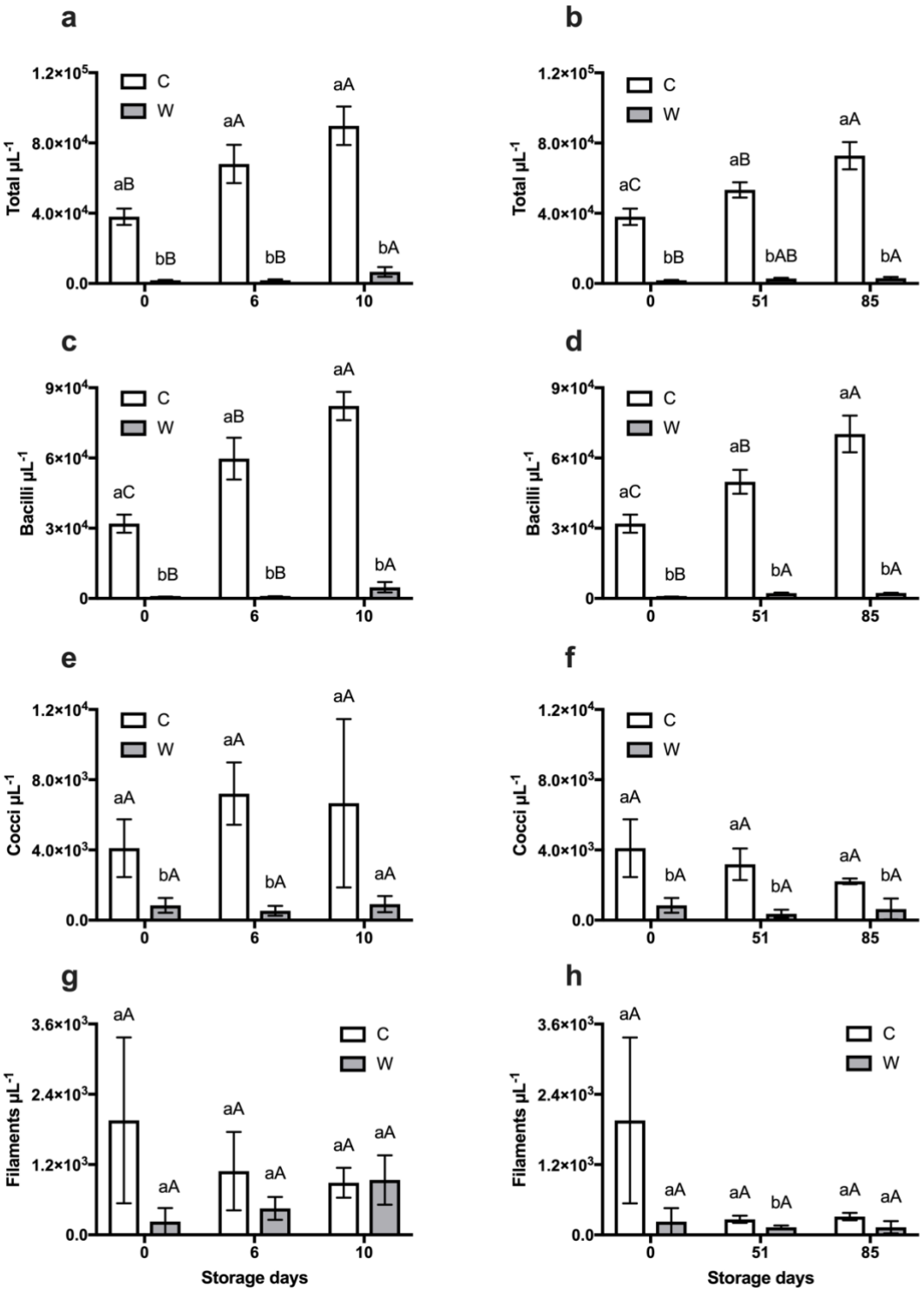

Figure 3: Mean ± standard deviation of concentrations (μL^{-1}) of total, bacilli, cocci and filamentous bacteria at 23°C (a, c, e, g) and at 4°C (b, d, f, h) for treatment W and control C. Different lowercase letters indicate statistically significant differences ($p<0.05$) among treatment W and control C within the same storage day (n = 3).

Regarding bacterial morphotypes, bacillus was the most predominant, showing the same behavior regardless of storage temperature (Figure 3c and d). The average densities in control C and treatment W were $5.88\times10^4 \pm 1.92\times10^4$ cells·µL^{-1} and $2.19\times10^3 \pm 1.64\times10^3$ cells·µL^{-1}, respectively, being significantly lower ($P<0.0001$) for treatment W. In general, densities significantly increased ($P<0.01$) over time, though no differences were observed comparing storage temperatures for control C ($P=0.187$) and for treatment W ($P=0.659$). Coccus was the second most predominant morphotype (Figure 3e and f). Briefly, no significant difference was observed in densities in relation to temperature and storage time for treatment W ($P=0.368$). For control C, a significant difference ($P=0.011$) was observed for temperature, being lower at 4ºC. The average densities in control C and treatment W were $4.67\times10^3 \pm 2.18\times10^3$ cells·µL^{-1} and $6.59\times10^2 \pm 2.27\times10^2$ cells·µL^{-1}, respectively, being significantly lower ($P<0.0001$) for treatment W. Filamentous bacteria were the least predominant morphotype and no significant difference was observed between control C ($9.02\times10^2 \pm 6.88\times10^2$ cells·µL^{-1}) and treatment W ($3.74\times10^2 \pm 3.40\times10^2$ cells·µL^{-1}) for temperature and storage time (Figure 3g and h).

6.3.3 DMS

The washing process demonstrated to be efficient in removing DOM and bacteria, therefore the hypothesis is that the production of DMS in the concentrates would be lower than in the control not subjected to this process. As shown in Figure 3a, DMS was not detected (< 1 µg·L^{-1}) until day 2 in treatment W and control C. From day 2 onwards, DMS concentrations significantly increased ($P<0.0001$) in both samples, reaching a maximum of 21.37 ± 6.11 µg·L^{-1} and 56.00 ± 8.75 µg·L^{-1} in treatment W and control C, respectively. DMS concentrations at 23ºC were significantly ($P<0.01$) lower in treatment W than in control C. For samples stored at 4ºC (Figure 4b), DMS concentrations increased significantly ($P<0.001$) on day 17, then stabilizing and reaching a maximum of 8.50 ± 0.95 µg·L^{-1} and 39.90 ± 19.77 µg·L^{-1} in treatment W and control C, respectively. As observed at 23ºC, DMS concentrations were significantly ($P<0.01$) lower in treatment W than in control C. It is interesting to note that DMS concentrations after 10 days at 23ºC were almost double than after 85 days at 4ºC, being significantly different ($P<0.01$). These results demonstrate that the washing process efficiently reduce DMS production, remaining at low levels for up to 85 days when refrigerated.

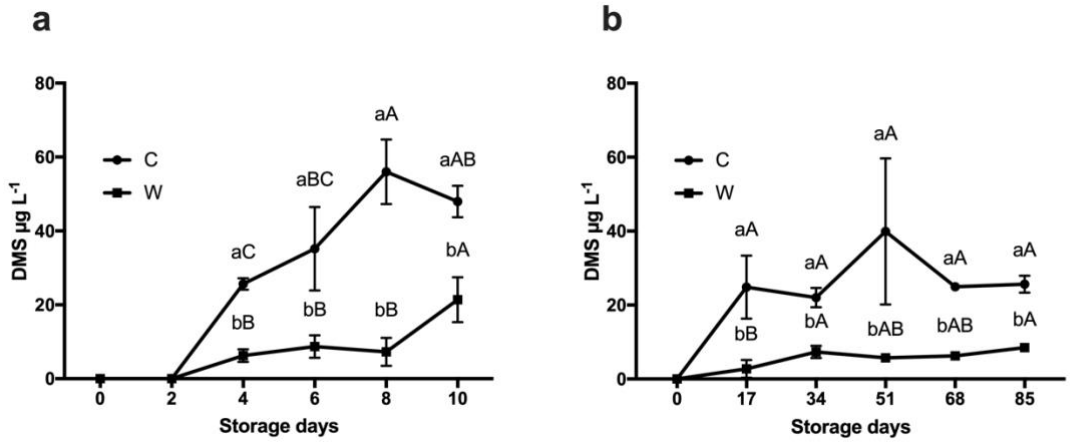

Figure 4: Mean ± standard deviation of concentrations of DMS ($\mu g \cdot L^{-1}$) at 23°C (a) and at 4°C (b) for treatment W and control C. Different lowercase letters indicate statistically significant differences ($p<0.05$) among treatment W and control C within the same storage day (n = 3). Different uppercase letters indicate statistically significant differences ($p<0.05$) within treatment W and control C throughout the storage period (n = 3).

As DMS is an extremely volatile compound (Charlson et al., 1992). In general, the presence of odor was perceived at every sampling time. In the control, except for day 0, all samples had a strong sulfur smell. Already in treatment W, a smell resembling fresh products from the sea, such as seaweed, was observed. Thus, can be concluded that under these conditions, the shelf life of concentrates can be extended by up to 4 days in 23°C, from 4 to 8 days, and 68 days in 4°C, from 17 to 85 days. As shown in figure 1 (a and c), there is a visual difference in color between the two concentrates. Whereas the control showed a reddish/orange color (Figure 1a), W treatment was clear (Figure 1c), which through a microscopic analysis (Figure 1b and d), was shown to be directly related to the amount of bacteria. Samples were collected and the bacterial composition was analyzed by metagenomics (unpublished data). Among the groups found, two possible groups responsible for the red-orange color were identified, *Erythrobacter* (15.3%) and *Roseobacter* (2.3%).

6.3.4 Cell viability

Although the washing process has been shown to be efficient in reducing DMS production, it should not negatively affect the quality of the microalgae. Figure 5 presents the effects of washing, temperature and storage times on cell viability, expressed as

growth rate (k, duplications·day^{-1}). As observed, the average growth rate was 0.290 ± 0.040·day^{-1} and no statistically significant differences (P>0.05) were observed among or between treatment W and controls C and I (inoculum) at both temperature and storage times (Figure 5a and b). The only difference observed was on day 6 at 23ºC, where a significant difference (P=0.003) was observed for treatment W (0.339 ± 0.013·day-1) versus control C and I (0.267 ± 0.035·day^{-1}, Figure 5a). These results indicate that the concentrates remained viable for 10 and 85 days at 23ºC and 4ºC, respectively.

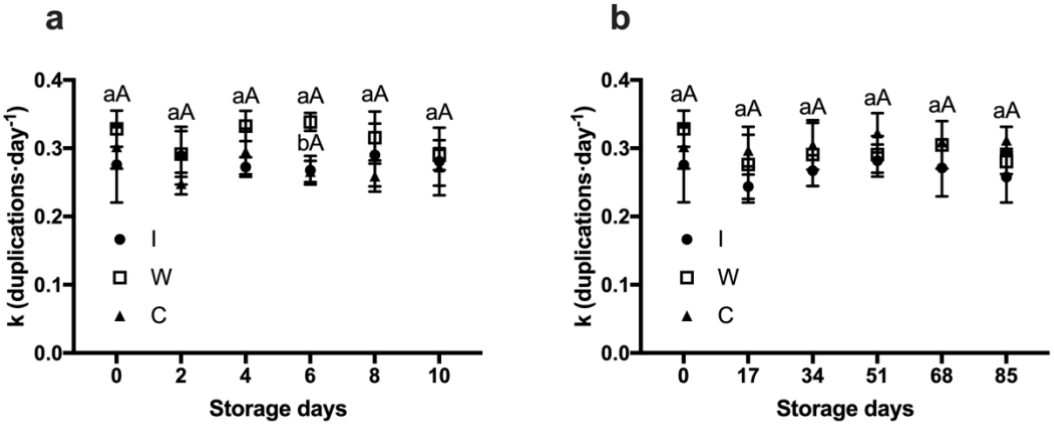

Figure 5: Mean ± standard deviation of growth rate (duplications·day^{-1}) at 23°C (a) and at 4°C (b) for treatment W and control C. Different lowercase letters indicate statistically significant differences (p<0.05) among treatment W and control C within the same storage day (n = 3). Different uppercase letters indicate statistically significant differences (p<0.05) within treatment W and control C throughout the storage period (n = 3).

6.4 Discussion

The washing process allowed the organic load to be considerably reduced, generating commercially acceptable product, and contributed to enhance understanding about shipping time and shelf life of *Nannochloropsis oceanica* concentrates developed and commercialized by AlgaSul Biotecnologia de Microalgas. In parallel, this developed protocol allows a decrease in DMS concentrations. This is not a direct product of microalgae metabolism, but a result of the interaction between microalgae and bacteria, since both concentrates had the same concentration of microalgae cells (5×10^{9}·mL^{-1}) and there is a relationship between the amount of bacteria, organic matter and DMS concentration.

Despite the implementation of several protocols aimed at disinfection and decrease of contamination in cultivation, achieving this in large-scale is nearly impossible, particularly in an open production system (Fulbright et al., 2018; Giraldo et al., 2019; Lian et al., 2022, 2021). This is primarily due to the favorable conditions for bacterial groups to thrive in the cultivation environment, where they can compete with microalgae for nutrients or utilize byproducts from the algae's metabolism for their own benefit (Fulbright et al., 2018; Giraldo et al., 2019; Lian et al., 2022). *Erithrobacter* was found as the most abundant group, an ovoid to rod-shaped bacteria with red or orange pigments (Shiba and Simidu, 1982). In luminous environments, this group makes photosynthesis and plays an essential role in the carbon cycle (Shiba and Imhoff, 2015), which explains the increase in organic matter in the control over time, (item 3.4) but not the increase in DMS concentration. *Roseobacter* in turn, is a gram-negative bacteria group shaped like bacilli. They can produce colored pigments, generally pink or orange, like *Erithrobacter*, but among other groups of bacteria, this is an important group in the sulfur cycle for producing DMS from DMSP (Geng and Belas, 2010; Miller and Belas, 2004).

As the quantification limit of the method used was 1 $\mu g \cdot L^{-1}$, it is impossible to state that in the initial sampling and on day 2 at 23°C, the DMS concentration was even equal to zero, since on the day 4 the means of W and C were 6.23 ± 1.70 $\mu g \cdot L^{-1}$ and 25.67 ± 1.56 $\mu g \cdot L^{-1}$, respectively. There is no consensus in the literature regarding the levels of detection of DMS. According to Demchuk et al. (2018), comparing several studies, the detection level can vary from 0.16 to 98 $\mu g \cdot L^{-1}$. DMS odor was detected in all control samples, except for the initial sample. In the washing treatment, the odor was much less pronounced and unpleasant. But even in the worst-case scenario, the DMS concentration was no more than 70 $\mu g \cdot L^{-1}$, on day 8 in 23°C.

As DMS in the concentrates is not from the microalgae, it is possible to decrease its production by reducing the DOM concentration or stop the bacteria's metabolism. Despite the washing method allowing the reduction of organic load, with the use of successive centrifugation, cells may be susceptible to rupture due to shear stress, compromising viability (Balduyck et al., 2017; Bernaerts et al., 2019). However, *Nannochloropsis* is known for its robust cell wall, composed by two polymeric layers of algaenan and

cellulose (Beacham et al., 2014; Scholz et al., 2014), which renders the cell extremely resistant to shear stress (Balduyck et al., 2017; Bernaerts et al., 2019). Another potential manner to reduce the organic load is by using a skimmer, a foam fractionator used in aquaculture plants in RAS systems (Kovács et al., 2023), during microalgae cultivation (Roselet et al., 2019). But, unlike the present work, these authors used this method to remove organic matter directly from microalgae cultivation, prior to harvesting and did not evaluate its implications on the shelf life of the microalgae.

Both washing and skimming are proven methods for reducing organic load, but do not guarantee its complete removal. In industry, usually, the treatment of bacteria is made by thermal treatment, either cold or hot (Dan et al., 2018; Khalili et al., 2019; Savvidou et al., 2016; Welladsen et al., 2014). Although studies demonstrate effectiveness in decreasing the bacteria load, this type of treatment considerably increases the costs. It can also cause biomass to lose its freshness and cell viability, becoming inert. While fresh microalgae concentrate maintain water quality in aquaculture, freeze-dried or pasteurized inert microalgae increase the levels of nitrogen compounds in the water, deteriorating cultivation conditions (Sales et al., 2019). Thus, the use of fresh microalgae is more suitable. Another way is to use an antibacterial additive, such as ascorbic acid (Khalili et al., 2019). The use of antibacterial additives, besides extending the storage time of concentrates, can also prevent lipid peroxidation. Hence, this additive can also be combined with the washing process to decrease bacterial density throughout the shelf life of microalgae concentrates.

6.5 Conclusions

The presence of DMSP in the organic matter of microalgae deserves attention due to its impact on the shelf life of concentrates, as its bacterial metabolism generates DMS, a compound with a strong sulfurous odor. The washing process is a great alternative to reduce the development of bacteria, generation DOM and DMS in concentrates. Also is responsible for improving the sensorial characteristics of the concentrate, such as color and odor, without any damage to cell viability. The washing process provides concentrates with a longer shelf life, remaining for up to 8 days at 23°C and for 85 days at 4°C. In

instance, current research provided information to collaborating company define maximum shipping time and warranty time for its clients.

References

Asher, E.C., Dacey, J.W.H., Stukel, M., Long, M.C., Tortell, P.D., 2017. Processes driving seasonal variability in DMS, DMSP, and DMSO concentrations and turnover in coastal Antarctic waters. Limnol Oceanogr 62, 104–124. https://doi.org/10.1002/lno.10379

Balduyck, L., Bijttebier, S., Bruneel, C., Jacobs, G., Voorspoels, S., Van Durme, J., Muylaert, K., Foubert, I., 2016. Lipolysis in *T-Isochrysis lutea* during wet storage at different temperatures. Algal Res 18, 281–287. https://doi.org/10.1016/j.algal.2016.07.003

Balduyck, L., Stock, T., Bijttebier, S., Bruneel, C., Jacobs, G., Voorspoels, S., Muylaert, K., Foubert, I., 2017. Integrity of the microalgal cell plays a major role in the lipolytic stability during wet storage. Algal Res 25, 516–524. https://doi.org/10.1016/j.algal.2017.06.013

Bates, T.S., Charlson, R.J., Gammon, R.H., 1987. Evidence for the climatic role of marine biogenic sulphur. Nature 329, 319–321. https://doi.org/10.1038/329319a0

Beacham, T.A., Bradley, C., White, D.A., Bond, P., Ali, S.T., 2014. Lipid productivity and cell wall ultrastructure of six strains of *Nannochloropsis*: Implications for biofuel production and downstream processing. Algal Res 6, 64–69. https://doi.org/10.1016/j.algal.2014.09.003

Bernaerts, T.M.M., Gheysen, L., Foubert, I., Hendrickx, M.E., Van Loey, A.M., 2019. Evaluating microalgal cell disruption upon ultra high pressure homogenization. Algal Res 42, 101616. https://doi.org/10.1016/j.algal.2019.101616

Bouillon, R.-C., Miller, W.L., 2005. Photodegradation of Dimethyl Sulfide (DMS) in Natural Waters: Laboratory Assessment of the Nitrate-Photolysis-Induced DMS Oxidation. Environ Sci Technol 39, 9471–9477. https://doi.org/10.1021/es048022z

Camacho-Rodríguez, J., Cerón-García, M.C., González-López, C.V., Fernández-Sevilla, J.M., Contreras-Gómez, A., Molina-Grima, E., 2013. A low-cost culture medium for

1297 the production of *Nannochloropsis gaditana* biomass optimized for aquaculture.
1298 Bioresour Technol 144, 57–66. https://doi.org/10.1016/j.biortech.2013.06.083

1299 Charlson, R.J., Anderson, T.L., McDuff, R.E., 1992. 13 The Sulfur Cycle, in:
1300 International Geophysics. Academic Press Inc., pp. 285–300.
1301 https://doi.org/10.1016/S0074-6142(08)62696-0

1302 Charlson, R.J., Lovecook, J.E., Andreae, M.O., Warren, S.G., 1987. Ocean
1303 phytoplankton, atmospheric sulfur, cloud albdeo and climate. Nature 326, 655–661.

1304 Coleman, B., Van Poucke, C., De Witte, B., Casciaro, V., Moerdijk-Poortvliet, T.,
1305 Muylaert, K., Robbens, J., 2023. The effect of drying, cell disruption and storage on
1306 the sensory properties of *Nannochloropsis* sp. Algal Res 71, 103092.
1307 https://doi.org/10.1016/j.algal.2023.103092

1308 Couto, C., Hernández, C.P., Alves Sobrinho, R.C.M., Mendes, C.R.B., Roselet, F., Abreu,
1309 P.C., 2021. Optimization of a low-cost fertilizer-based medium for large-scale
1310 cultivation of the coastal diatom *Conticribra weissflogii* using response surface
1311 methodology and its effects on biomass composition. J Appl Phycol 33, 2767–2781.
1312 https://doi.org/10.1007/s10811-021-02519-8

1313 Dalheim, L., Svenning, J.B., Eilertsen, H.C., Vasskog, T., Olsen, R.L., 2021. Stability of
1314 lipids during wet storage of the marine diatom *Porosira glacialis* under semi-
1315 preserved conditions at 4 and 20 °C. J Appl Phycol 33, 385–395.
1316 https://doi.org/10.1007/s10811-020-02292-0

1317 Dan, S., Ashidate, M., Yamashita, T., Hamasaki, K., 2018. Effects of thermal disinfection
1318 and autoclave sterilisation on the quality of microalgae concentrates. Aquac Res 49,
1319 3559–3568. https://doi.org/10.1111/are.13822

1320 Demchuk, E., Ball, S.L., Le, S.L., Prussia, A.J., 2018. Concentration-time extrapolation
1321 of short-term inhalation exposure levels: dimethyl sulfide, a case study using a
1322 chemical-specific toxic load exponent. Inhal Toxicol 30, 448–462.
1323 https://doi.org/10.1080/08958378.2018.1551444

1324 Faé Neto, W.A., Borges Mendes, C.R., Abreu, P.C., 2018. Carotenoid production by the
1325 marine microalgae *Nannochloropsis oculata* in different low-cost culture media.
1326 Aquac Res 49, 2527–2535. https://doi.org/10.1111/are.13715

Fernández, F.G.A., Fernández-Sevilla, J.M., Moya, B.L., Grima, E.M., 2020. Microalgae production systems, in: Handbook of Microalgae-Based Processes and Products. Elsevier, pp. 127–163. https://doi.org/10.1016/B978-0-12-818536-0.00006-3

Fulbright, S.P., Robbins-Pianka, A., Berg-Lyons, D., Knight, R., Reardon, K.F., Chisholm, S.T., 2018. Bacterial community changes in an industrial algae production system. Algal Res 31, 147–156. https://doi.org/10.1016/j.algal.2017.09.010

Geng, H., Belas, R., 2010. Molecular mechanisms underlying *roseobacter*–phytoplankton symbioses. Curr Opin Biotechnol 21, 332–338. https://doi.org/10.1016/j.copbio.2010.03.013

Giraldo, J.B., Stock, W., Dow, L., Roef, L., Willems, A., Mangelinckx, S., Kroth, P.G., Vyverman, W., Michiels, M., 2019. Influence of the algal microbiome on biofouling during industrial cultivation of *Nannochloropsis* sp. in closed photobioreactors. Algal Res 42, 101591. https://doi.org/10.1016/j.algal.2019.101591

Giroldo, D., Ortolano, P.I.C., Vieira, A.A.H., 2007. Bacteria-algae association in batch cultures of phytoplankton from a tropical reservoir: The significance of algal carbohydrates. Freshw Biol 52, 1281–1289. https://doi.org/10.1111/j.1365-2427.2007.01764.x

Guillard, R.R.L., 1975. Culture of Phytoplankton for Feeding Marine Invertebrates, in: Culture of Marine Invertebrate Animals. https://doi.org/10.1007/978-1-4615-8714-9_3

Harrison, P.J., Berges, J.A., 2005. Marine Culture Media, in: Algal Culturing Techniques. Elsevier, pp. 21–33. https://doi.org/10.1016/B978-012088426-1/50004-4

Hobbie, J., Daley, R.J., Jasper, S., 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33, 1225–1228.

Khalili, M.A., Abedian Kenari, A., Rezaei, M., Mirzakhani, M.K., 2019. Antioxidant and Antibacterial Effects of Vitamins C and E Alone or a Combination on Microalgae (*Nannochloropsis oculata*) Paste Quality during Cold Storage. Journal of Aquatic Food Product Technology 28, 1051–1062. https://doi.org/10.1080/10498850.2019.1683108

Kovács, B.D., de Jesus Gregersen, K.J., Rüppel, F., von Danwitz, A., Pedersen, L.F., 2023. Evaluating protein skimmer performance in a commercial seawater recirculating aquaculture system (RAS). Aquac Eng 103. https://doi.org/10.1016/j.aquaeng.2023.102369

Kubelka, B.G., Pinto, W.T., Abreu, P.C., 2017. Hydrodynamic performance of two air nozzles diameters on the massive microalgae culture: Computational and experimental approaches. Algal Res 27, 318–324. https://doi.org/10.1016/j.algal.2017.09.024

Kubelka, B.G., Roselet, F., Pinto, W.T., Abreu, P.C., 2018. The action of small bubbles in increasing light exposure and production of the marine microalga *Nannochloropsis oceanica* in massive culture systems. Algal Res 35, 569–576. https://doi.org/10.1016/j.algal.2018.09.030

Levasseur, M., Michaud, S., Egge, J., Cantin, G., Nejstgaard, J.C., Sanders, R., Fernandez, E., Solberg, P.T., Heimdal, B., Gosselin, M., 1996. Production of DMSP and DMS during a mesocosm study of an *Emiliania huxleyi* bloom: influence of bacteria and Calanus finmarchicus grazing. Mar Biol 126, 609–618. https://doi.org/10.1007/BF00351328

Lian, J., Schimmel, P., Sanchez-Garcia, S., Wijffels, R.H., Smidt, H., Sipkema, D., 2021. Different co-occurring bacteria enhance or decrease the growth of the microalga *Nannochloropsis* sp. CCAP211/78. Microb Biotechnol 14, 1159–1170. https://doi.org/10.1111/1751-7915.13784

Lian, J., Steinert, G., de Vree, J., Meijer, S., Heryanto, C., Bosma, R., Wijffels, R.H., Barbosa, M.J., Smidt, H., Sipkema, D., 2022. Bacterial diversity in different outdoor pilot plant photobioreactor types during production of the microalga *Nannochloropsis* sp. CCAP211/78. Appl Microbiol Biotechnol 106, 2235–2248. https://doi.org/10.1007/s00253-022-11815-3

Liu, C., Gao, C., Zhang, H., Chen, S., Deng, P., Yue, X., Guo, X., 2014. Production of dimethylsulfide and acrylic acid from dimethylsulfoniopropionate during growth of three marine microalgae. Chinese Journal of Oceanology and Limnology 32, 1270–1279. https://doi.org/10.1007/s00343-015-4029-6

1387 Malin, G., 2006. New Pieces for the Marine Sulfur Cycle Jigsaw. Science (1979) 314,
1388 607–608. https://doi.org/10.1126/science.1133279

1389 Mayers, J.J., Flynn, K.J., Shields, R.J., 2014. Influence of the N:P supply ratio on biomass
1390 productivity and time-resolved changes in elemental and bulk biochemical
1391 composition of *Nannochloropsis* sp. Bioresour Technol 169, 588–595.
1392 https://doi.org/10.1016/j.biortech.2014.07.048

1393 Miller, T.R., Belas, R., 2004. Dimethylsulfoniopropionate Metabolism by Pfiesteria -
1394 Associated *Roseobacter* spp. Appl Environ Microbiol 70, 3383–3391.
1395 https://doi.org/10.1128/AEM.70.6.3383-3391.2004

1396 Nishino, S.F., 1986. Direct Acridine Orange counting of bacteria preserved with acidified
1397 Lugol Iodine. Appl Environ Microbiol 52, 602–604.

1398 Raina, J.B., Dinsdale, E.A., Willis, B.L., Bourne, D.G., 2010. Do the organic sulfur
1399 compounds DMSP and DMS drive coral microbial associations? Trends Microbiol
1400 18, 101–108. https://doi.org/10.1016/j.tim.2009.12.002

1401 Roselet, F., Maicá, P., Martins, T., Abreu, P.C., 2013. Comparison of open-air and semi-
1402 enclosed cultivation system for massive microalgae production in sub-tropical and
1403 temperate latitudes. Biomass Bioenergy 59, 418–424.
1404 https://doi.org/10.1016/j.biombioe.2013.09.014

1405 Roselet, F., Vandamme, D., Roselet, M., Muylaert, K., Abreu, P.C., 2017. Effects of pH,
1406 Salinity, Biomass Concentration, and Algal Organic Matter on Flocculant Efficiency
1407 of Synthetic Versus Natural Polymers for Harvesting Microalgae Biomass.
1408 Bioenergy Res 10, 427–437. https://doi.org/10.1007/s12155-016-9806-3

1409 Roselet, M., Roselet, F., Abreu, P.C., 2019. Foam fractionator as a tool to remove
1410 dissolved organic matter and improve the flocculation of the marine microalga
1411 *Nannochloropsis oceanica*. J Appl Phycol 31, 2911–2919.
1412 https://doi.org/10.1007/s10811-019-01801-0

1413 Safafar, H., Langvad, S., Møller, P., Jacobsen, C., 2017. Storage Conditions Affect
1414 Oxidative Stability and Nutritional Composition of Freeze-Dried *Nannochloropsis*
1415 *salina*. European Journal of Lipid Science and Technology 119, 1–10.
1416 https://doi.org/10.1002/ejlt.201600477

Sales, R., Derner, R.B., Tsuzuki, M.Y., 2019. Effects of different harvesting and processing methods on *Nannochloropsis oculata* concentrates and their application on rotifer Brachionus sp. cultures. J Appl Phycol 31, 3607–3615. https://doi.org/10.1007/s10811-019-01877-8

Savvidou, M.G., Sotiroudis, T.G., Kolisis, F.N., 2016. Cell surface and cellular debris-associated heat-stable lipolytic enzyme activities of the marine alga *Nannochloropsis oceanica*. Biocatal Biotransformation 34, 24–32. https://doi.org/10.1080/10242422.2016.1212843

Scholz, M.J., Weiss, T.L., Jinkerson, R.E., Jing, J., Roth, R., Goodenough, U., Posewitz, M.C., Gerken, H.G., 2014. Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall. Eukaryot Cell 13, 1450–1464. https://doi.org/10.1128/EC.00183-14

Shiba, T., Imhoff, J.F., 2015. Erythrobacter, in: Bergey's Manual of Systematics of Archaea and Bacteria. Wiley, pp. 1–5. https://doi.org/10.1002/9781118960608.gbm00919

Shiba, T., Simidu, U., 1982. Bacterium Which Contains Bacteriochlorophyll a. Int J Syst Bacteriol 32, 211–217.

Sorgeloos, P., Lavens, P., 1996. Manual on the production and use of live food for aquaculture. Rome.

Verspreet, J., Kreps, S., Bastiaens, L., 2020. Evaluation of microbial load, formation of odorous metabolites and lipid stability during wet preservation of *Nannochloropsis gaditana* concentrates. Applied Sciences (Switzerland) 10. https://doi.org/10.3390/app10103419

Vila-Costa, M., Rinta-Kanto, J.M., Poretsky, R.S., Sun, S., Kiene, R.P., Moran, M.A., 2014. Microbial controls on DMSP degradation and DMS formation in the Sargasso Sea. Biogeochemistry 120, 295–305. https://doi.org/10.1007/s10533-014-9996-8

Welladsen, H., Kent, M., Mangott, A., Li, Y., 2014. Shelf-life assessment of microalgae concentrates: EFFECT of cold preservation on microalgal nutrition profiles. Aquaculture 430, 241–247. https://doi.org/10.1016/j.aquaculture.2014.04.016

Yao, S., Lyu, S., An, Y., Lu, J., Gjermansen, C., Schramm, A., 2019. Microalgae–bacteria symbiosis in microalgal growth and biofuel production: a review. J Appl Microbiol 126, 359–368. https://doi.org/10.1111/jam.14095

Zhang, X.H., Liu, Ji, Liu, Jingli, Yang, G., Xue, C.X., Curson, A.R.J., Todd, J.D., 2019. Biogenic production of DMSP and its degradation to DMS—their roles in the global sulfur cycle. Sci China Life Sci 62, 1296–1319. https://doi.org/10.1007/s11427-018-9524-y

Zhuang, G., Yang, G., Yu, J., Gao, Y., 2011. Production of DMS and DMSP in different physiological stages and salinity conditions in two marine algae. Chinese Journal of Oceanology and Limnology 29, 369–377. https://doi.org/10.1007/s00343-011-0046-2

7 CAPÍTULO II

SKIMMER AND WASHING METHOD IN PREVENTING THE APPEARANCE OF DMS IN *Nannochloropsis oceanica* CONCENTRATES

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Abstract

The aim of this study was to test the use of the skimmer and the washing method as ways to minimize the concentration of bacteria in *Nannochloropsis oceanica* concentrates and to evaluate their effect on the concentration of organic matter and dimethylsulfide (DMS) in the long term. For this purpose, a concentrate of *N. oceanica* with a density of 5 billion cells per mL (C) was compared to four treatments: skimmer (S), washing (W), skimmer + washing (SW) and removal (R) for 68 days. Initial collections were made on days 17, 34, 51 and 68 to evaluate cell viability, bacterial density, bacteria removal efficiency, dissolved organic matter (DOM) concentration and DMS. Cell viability was not influenced by the treatments over time. However, the bacterial removal rate was influenced from the initial sampling. The highest removal rates were observed in the SW and R treatments. The concentration of DOM increased in all treatments over time but was higher in the SW treatment. The DMS concentration showed the same pattern as the organic matter, however, the highest concentrations were observed in the S treatment. To reduce bacterial growth in the concentrates and consequently reduce the concentration of DOM and DMS, it is necessary to use the washing process either combined with the skimmer or repeating the process. These results point to the need to optimize the washing process to ensure a higher rate of bacteria removal and the viability of the process.

Keywords: Foam fractionator; Bacteria; Organic matter; Cell Viability; Bacteria removal efficiency; DMSP; Dimethyl sulfide

7.1 Introduction

Dimethylsulfide (DMS) is a volatile organosulfur compound with a low odor threshold (0.6 to 40 ppb) and is the most important form of marine sulfur (Keller 1988; Kiene and Bates 1990). Since the 1980s, it has gained great prominence as an important climate regulator (Charlson et al. 1987, 1992; Bates et al. 1987; Keller 1988; Zhang et al. 2019). It can be produced by some groups of microalgae and marine bacteria, besides their association (Levasseur et al. 1996; Cui et al. 2015; Zhang et al. 2019b). This association occurs through the precursor of DMS, dimethylsulfoniopropionate (DMSP), produced by microalgae of great economic importance, such as *Nannochloropsis oceanica* (Curson et al. 2017)**.**

DMSP acts on cellular osmotic balance, as an antioxidant, inhibitor of cysteine and methionine production, furthermore as a defense mechanism against predation (Kiene et al. 2000; Zhang et al. 2019). When released into the environment in the form of dissolved organic matter (DOM), it can be assimilated by bacteria, such as the genus *Roseobacter*, generating DMS into the environment (Zhang et al. 2019). Just as in the natural environment, this process can occur in microalgae cultivation if those bacteria are present (Miller and Belas 2004). However, even in high-density cultures of microalgae, DMS is often undetectable. This is a consequence of its volatile nature, which causes it to be constantly being released into the atmosphere. Thus, DMS is not a problem in microalgae cultivation.

For commercial applications of microalgae, it is necessary to concentrate them. As a result, the bacteria present in the cultures are also concentrated. Because DMS has a low odor threshold and presents an unpleasant odor like rotten eggs**,** giving the product a spoiled characteristic, its presence in microalgae concentrates is highly undesirable in industry (Giri et al. 2010). In this type of processing, although the initial DMS concentration is low, DMS formation is constant and accumulates over the storage time (Couto et al. 2024). Therefore, to avoid DMS formation, it is necessary to find ways to remove bacteria from the concentrates, either before or after collecting the microalgae.

In aquaculture, the skimmer (foam fractionator) is widely used in combination with other methods to decrease dissolved organic matter, turbidity, bacteria and fine particles in recirculating systems (RAS) like ozone, and most recently, in Biofloc Technologic System (BFT) where there is little or no water exchange. In these systems, it is essential to maintain control of nitrogen by controlling the biological demand for oxygen (Samocha 2019; Gregersen et al. 2021; Kovács et al. 2023). Its operation is mainly dependent on the presence of surfactants, chemical agent that reduces surface tension at the air-water interface. Some of these agents can be produced by microalgae (Chen et al. 1994; Buckley et al. 2022). Although many studies evaluate the use of the skimmer in fish and shrimp production, little is known about the use of the skimmer in microalgae cultivation (Csordas and Wang 2004; Figueiras Guilherme et al. 2020).

Unlike the skimmer, washing is a process that proceeds cultivation and harvesting of microalgae. The process consists of adding water to the concentrated microalgae and centrifuging. After that, the supernatant is discarded and the process is repeated (Roselet et al. 2019; Couto et al. 2024). The microalgae with a higher density settle in the centrifuge tube and the bacteria and organic matter remain in the supernatant. In previous studies, the skimmer and washing have proven their efficiency in the removal of organic matter and bacteria (Roselet et al. 2019; Couto et al. 2024). However, there is a lack of studies comparing and evaluating the effect of combining the two methods and determining their impact on the storage time of microalgae concentrates. Therefore, the objective of this study was to assess the use of the skimmer and the washing method to minimize bacteria from *Nannochloropsis oceanica* concentrates, and to evaluate their effect on the concentration of organic matter and DMS over time.

7.2 Material and methods

7.2.1 Microalgae cultivation and harvesting

Nannochloropsis oceanica strain (NANN OCEA_1) was obtained from the Phytoplankton and Marine Microorganisms Laboratory collection from Federal University of Rio Grande (FURG), cultivated in f/2 medium (Guillard 1975) in natural marine water (28 $g \cdot L^{-1}$), filtered (1 µm), and autoclaved (121 ºC for 15 minutes). The cultivation was carried out in a photobioreactor with a diameter of 0.55 m and a water column of 1.5 m with atmospheric air injection (4.8 $L \cdot min^{-1}$) (Kubelka et al. 2017) under natural environmental conditions of temperature, lighting, and photoperiod. The natural seawater (28 $g \cdot L^{-1}$) used, about 300 L, was filtered through 1.0 and 0.5 µm filters, sterilized with ultraviolet light and 12% hypochlorite (NaClO, 0.50 $mL \cdot L^{-1}$) for 48 hours, then it was neutralized with 20% sodium thiosulfate ($Na_2S_2O_3$, 0.50 $mL \cdot L^{-1}$) and enriched with YCB medium (Couto et al. 2021). An inoculum of 10% (30 L) of the total volume with an initial cell density of $8 \cdot 10^6$ $cells \cdot mL^{-1}$ was used. The cultivation was monitored by optical density, pH monitoring and correction for 15 days; when the pH reached 9.5 it was corrected to 8.0 by CO_2 injection. Additionally, every five days an extra YCB medium charge was added. When the cell density of the cultivation reached $5 \cdot 10^7$ $cells \cdot mL^{-1}$ on day 15, the microalgae was collected by centrifugation in a continuous disc

centrifuge (FJ 130 EPR, Janschitz Gmbh, Althofen, Austria) with a flow rate of 20 $L \cdot h^{-1}$ and rotation of 9,500 RPM, resulting in a 25% dry matter paste. The paste was stored at 4 °C for 24 h until the start of the experiment.

7.2.2 Experimental design

To determine the best way to remove bacteria and DOM in microalgae concentrates, we evaluated five conditions; a control (C) and four treatments, skimmer (S), washing (W), skimmer + washing (SW) and removal (R). In C, the paste did not undergo any treatment; after collection, it was diluted to a cell density of 5 billion cells per mL. To evaluate the use of the skimmer in the S and SW treatments, approximately 150 L of culture were treated with a skimmer for approximately 4 hours with a flow rate of 1000 $L \cdot h^{-1}$ (Roselet et al. 2019). In treatments W, SW and R, the washing method was used, which consisted of successive centrifugation of the pastes (60 g) in autoclaved seawater (28 $g \cdot L^{-1}$, 500 mL) and discarding the supernatant (Couto et al. 2024). In treatments W and SW the process was repeated 3 times. In R, the process was repeated 5 times, until the supernatant was completely clean with an optical density (OD) close to 0. The paste from each treatment was diluted to a concentration of 5 billion cells per mL and stored in Pouch-type bags with a useful volume of 50 mL at a temperature of 4 °C for 68 days with initial collection and on days 17, 34, 51 and 68, totaling 75 independent units (5 treatments x 5 sampling times x 3 replications) analyzed for cell viability, bacterial density, bacteria removal efficiency, concentration of DOM and DMS.

7.2.3 Cell viability

The determination of the viability of cells was carried out in treatments C, S, W, SW and R. Samples of the concentrates were inoculated in f/2 medium (Guillard 1975) in triplicate and their growth was compared to the growth of an inoculum (I) of the same microalgae in exponential growth phase. The volume of concentrates and inoculum used was adjusted to the initial cell density of 6.0×10^6 $cells \cdot mL^{-1}$ in 10 mL test tubes. Tubes were then incubated for six days at 21°C, with a light intensity of 100 μmol $photons \cdot m^{-2} \cdot s^{-1}$ and a 12 h light period. Daily, the test tubes were manually homogenized, and microalgae density was measured by cell counting initially (D_0) and on day six (D) using an improved Neubauer chamber at 400× magnification. Cell viability was then calculated by growth rate (K) (equation 1).

$$K(division\ d^{-1}) = \frac{3.322}{(t-t0)\times Log(\frac{D}{D0})} \quad \text{Equation 1}$$

7.2.4 Bacteria community

Bacterial density in concentrates was determined by direct cell counting under an epifluorescence microscope. For that, 10 mL samples were stored in 4% formaldehyde solution at room temperature until analysis. Before analysis, the samples were diluted (10,000x) using sterilized and filtered (0.2 µm) seawater. For the preparation of counting slides, 1 mL of samples were filtered through 0.2 µm pore-size darked Nuclepore polycarbonate filters, with a vacuum pressure <5 cmHg (Hobbie et al. 1977). The samples were then incubated for 10 minutes with a 0.1 % Acridine Orange solution (Nishino 1986). The filters were removed from the filtration apparatus and let dry at environmental temperature. Afterwards, they were placed on a slide with immersion oil and observed in a Zeiss Axioplan epifluorescence microscope (1,000× final magnification, λ_{Ex} = 460 nm and λ_{Em} = 490 nm) (Hobbie et al. 1977). Bacteria were counted in 20 random fields until reaching a total of at least 100 cells, and then separated into morphotypes (cocci, bacilli and filament). The bacterial density was calculated using Equation 2.

$$Total\ Bacterial\ Cell\ Count/\mu L = \frac{A \times (\frac{(\frac{B}{C})}{D})}{1000} \quad \text{Equation 2}$$

where A = average count per field; B = filtration area (mm^2); C = count field area (mm^2); and D = sample volume (mL).

7.2.5 Bacteria removal efficiency

To determine how effective each method was in removing bacteria, the bacteria removal efficiency (Kovács et al. 2023) was calculated according to Equation 3.

$$RE\ (\%) = \frac{(Bc-Bt)}{Bc}\times 100 \quad \text{Equation 3}$$

Where Bc is the total average number of bacteria in the control and Bt is the total number of bacteria in each treatment. All calculation was made with the values of each sampling time.

7.2.6 Dissolved Organic Matter

To analyze only the dissolved part of the organic matter, the concentrates needed to go through centrifugation and filtration processes. Firstly, 20 mL samples were centrifuged for 18 min at 2,169 g to remove the bulk microalgae, then the supernatants were filtered through 0.7 µm to remove any remaining microalgae. The DOM fraction was finally obtained by passing the filtrate through a 0.45 µm filter. DOM samples were then stored in a freezer (−21°C) until analysis. Dissolved organic carbon (DOC) was analyzed as a proxy for DOM. A Shimadzu TOC–CVSP analyzer (Tokyo, Japan) was calibrated with potassium hydrogen phthalate (total carbon standard, TC) and sodium hydrogen carbonate and anhydrous sodium carbonate (inorganic carbon standards, IC). TC and IC standards ranged from 0 to 100 $mg \cdot L^{-1}$. DOC concentration was calculated as the difference between TC and IC measurements. All measurements were performed in triplicate. TC and IC standards with concentrations of 10 and 50 $mg \cdot L^{-1}$ were used as controls.

7.2.7 DMS determination by HS-GC-MS/MS

The determination of DMS was carried out employing a properly Headspace Extraction and Gas Chromatography tandem Mass Spectrometry (HS-GC-MS) method, which was previously validated (Couto et al. 2024). The volatile compound DMS was handled carefully throughout the analysis process to avoid gas exchange. For analysis, samples were stored at -21 °C in sealed falcon tubes. Then, 10 mL of each sample was transferred to a 20 mL headspace vial, sealed with a magnetic screw-capped lid lined with PTFE-silicone septa. The vials were incubated at 70 °C with agitation for 20 minutes to extract DMS into the headspace. Headspace gas (1000 µL) was then injected into a Shimadzu GC-MS model TQ8050 for analysis. The GC-MS system utilized helium as the carrier gas with a constant flow rate of 0.87 $mL \cdot min^{-1}$ and a capillary column (RTX®-Wax) for separation. DMS was detected using selective ion monitoring (SIM) mode with the most intense ion (62 m/z) for quantification and two additional ions (47 and 61 m/z) for

confirmation. Calibration curves were constructed daily using DMS standards at concentrations ranging from 1 $\mu g \cdot L^{-1}$ to 100 $\mu g \cdot L^{-1}$.

7.2.8 Statistical analysis

The efficiency of the skimmer and washing methods were evaluated together and separately as ways to improve *Nannochloropsis oceanica* concentrates for 68 days. For this, data on cell viability, bacterial density, bacteria removal efficiency, DOM and DMS concentration were analyzed for normality and homogeneity using the Shapiro-Wilk and Brown-Forsythe tests, respectively. Then, the one-way ANOVA was performed followed by Tukey's post hoc test ($p < 0.05$) for time and for treatment.

7.3 Results

7.3.1 Cell viability

The cell viability of the microalgae *Nannochloropsis oceanica* was not influenced by the use of the skimmer or the washing process, presenting an average value of 0.39 ± 0.03 duplications·day^{-1} (Figure 1a). Thus, there was no significant difference between the inoculum (I) and the other treatments. On day 68, C (0.43 ± 0.02 duplications·day^{-1}) was significantly higher ($p = 0.0304$) than the SW treatment (0.36 ± 0.01 duplications·day^{-1}). For the treatments over time, the only one that showed a significant difference ($p = 0.0096$) was R, which on day 17 (0.35 ± 0.02 duplications·day^{-1}) was lower than on day 34 (0.40 ± 0.12 duplications·day^{-1}).

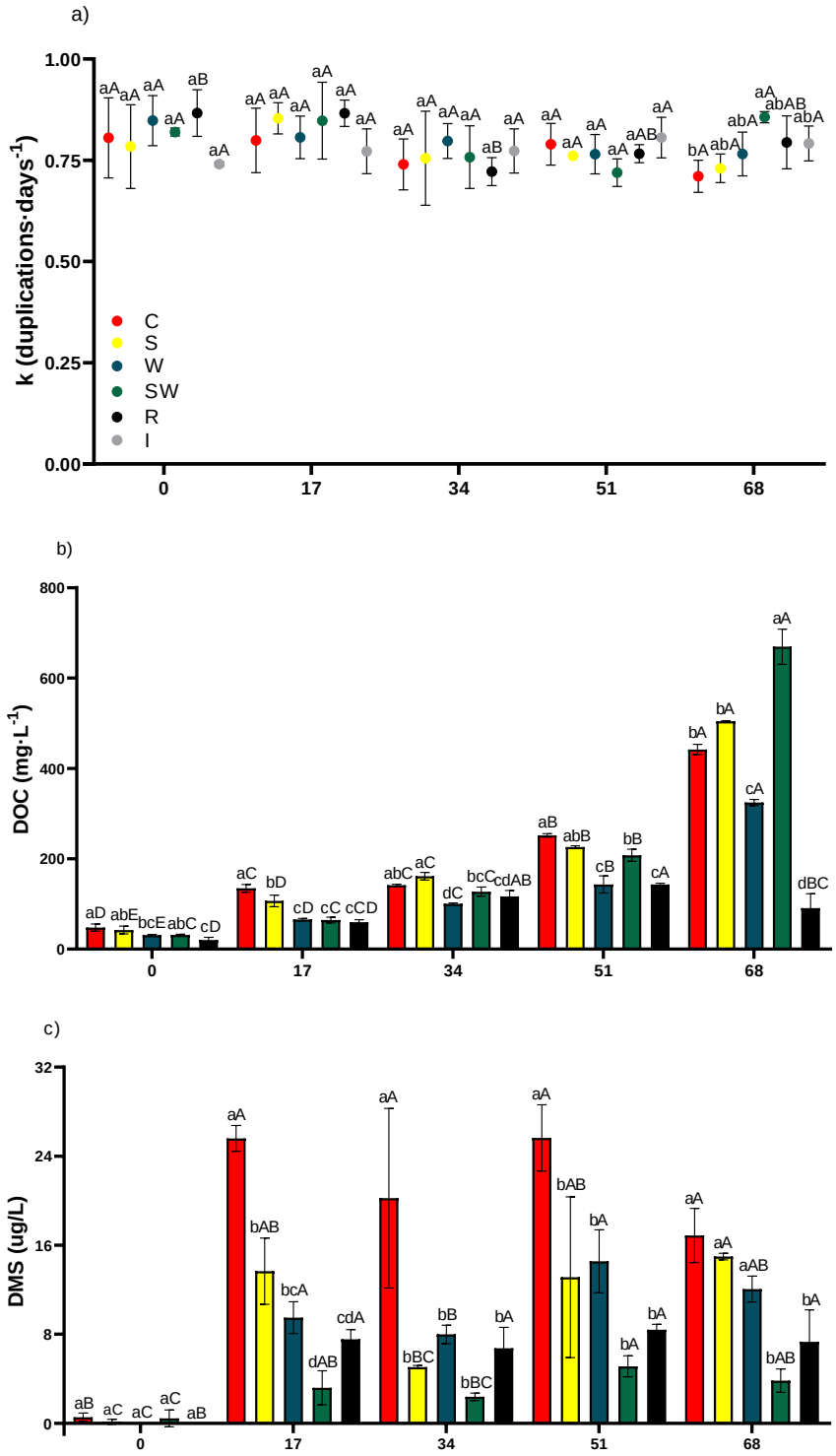
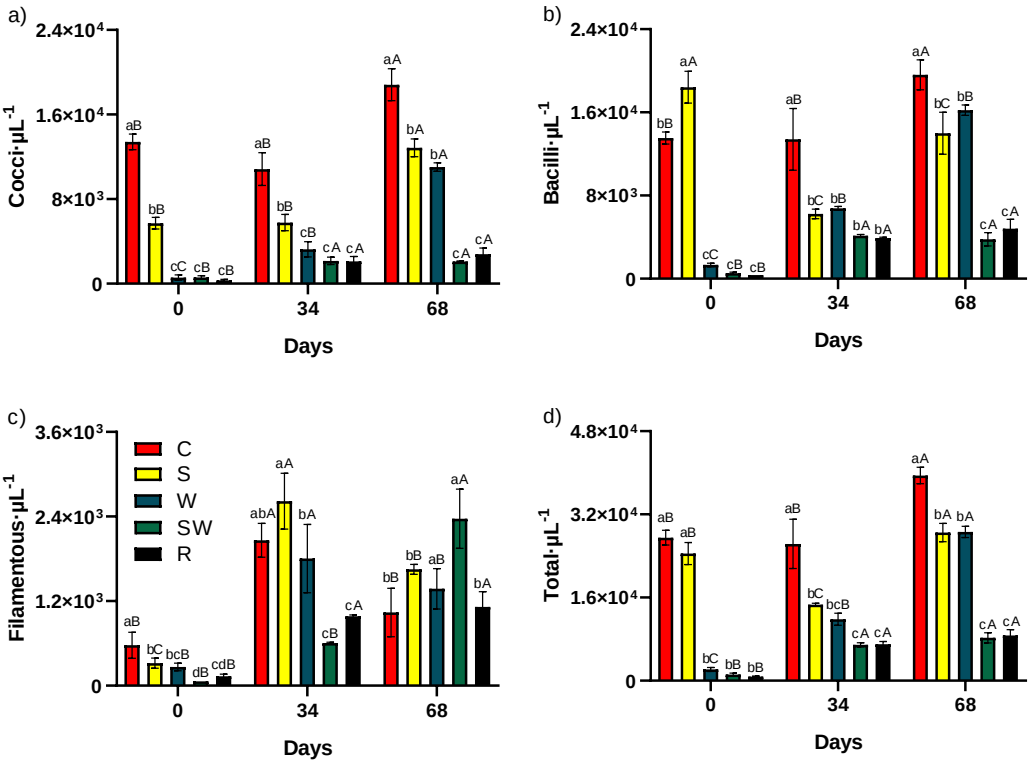


Figure 1: Cell viability (a), organic matter (b) and DMS (c) over time. C – control; S – skimmer; W – Washing; SW – skimmer + washing; R – Removal; I – culture inoculum. Letters indicate significant differences ($p<0.05$) in treatments (lowercase) and over time (uppercase).

7.3.2 Bacteria community

The density of cocci, bacilli, filamentous and total bacteria per µL in the different ways of removing bacteria were analyzed in three sampling times: initial (0), intermediary (day 34) and final (day 68) as showed in Figure 2. In the initial sampling of cocci (Figure 2a), the control C showed the highest ($p < 0.0001$) cocci density of $1.34 \pm 0.07 \times 10^4$ cells·µL^{-1}, followed by S with $5.72 \pm 0.55 \times 10^3$ cells·µL^{-1} which was higher than the W with $5.74 \pm 2.46 \times 10^2$ cells·µL^{-1}, SW $5.95 \pm 1.64 \times 10^2$ cells·µL^{-1} and R $3.18 \pm 1.64 \times 10^2$ cells·µL^{-1}. At day 34, the pattern was the same, C presented the higher ($p < 0.0001$) density of $1.08 \pm 0.15 \times 10^4$ cells·µL^{-1} than S with $5.77 \pm 0.77 \times 10^3$ cells·µL^{-1} which was higher than W $3.25 \pm 0.72 \times 10^3$ cells·µL^{-1}, SW ($2.16 \pm 0.35 \times 10^3$ cells·µL^{-1}) and R ($2.11 \pm 0.46 \times 10^3$ cells·µL^{-1}). Although in the day 68, C treatment ($1.88 \pm 0.15 \times 10^4$ cells·µL^{-1}) presented more cocci ($p < 0.0001$) than S ($1.29 \pm 0.08 \times 10^4$ cells·µL^{-1}), W ($1.10 \pm 0.04 \times 10^4$ cells·µL^{-1}) showed no difference from S but it was higher than SW ($2.09 \pm 0.06 \times 10^3$ cells·µL^{-1}) and R ($2.79 \pm 0.57 \times 10^3$ cells·µL^{-1}). Regarding treatments over time, in control (C), there was no significant difference ($p = 0.0009$) between days 0 and 34 that were lower than day 68. In S, days 0 and 34 were also similar ($p < 0.0001$) and lower than day 68. In W, there was a progressive increase ($p < 0.0001$) in the density of cocci, day 0 was lower than day 34 which was lower than day 68. In SW, day 0 was lower ($p = 0.0002$) than 34 and 68. And in R, day 0 was lower ($p = 0.0010$) than day 34 and day 68.



1725

1726 Figure 2: Bacterial density over time of cocci (a), bacilli (b), filamentous (c) and total bacteria (d). C –
1727 control; S – skimmer; W – Washing; SW – skimmer + washing; R – Removal. Letters indicate significant
1728 differences ($p<0.05$) in treatments (lowercase) and over time (uppercase).

1729

1730 For bacilli, in the initial sampling, S presented $1.84 \pm 0.15 \times 10^4$ cells·µL^{-1} which was the
1731 highest ($p < 0.0001$) density, followed by C with $1.35 \pm 0.06 \times 10^4$ cells·µL^{-1}, which was
1732 higher than W ($1.32 \pm 0.17 \times 10^3$ cells·µL^{-1}), SW ($5.33 \pm 1.03 \times 10^2$ cells·µL^{-1}) and R
1733 ($3.49 \pm 0 \times 10^2$ cells·µL^{-1}). On day 34, C presented $1.34 \pm 0.23 \times 10^4$ cells·µL^{-1}, that was
1734 the highest ($p < 0.0001$) density, followed by S with $6.22 \pm 0.46 \times 10^3$ cells·µL^{-1} and W
1735 with $6.77 \pm 0.18 \times 10^3$ cells·µL^{-1} which were higher than SW with $3.79 \pm 0.63 \times 10^3$
1736 cells·µL^{-1} and R with $4.81 \pm 0.91 \times 10^3$ cells·µL^{-1}. On day 68, C presented $1.96 \pm 0.14 \times$
1737 10^4 cells·µL^{-1} with more ($p < 0.0001$) bacilli than S ($1.40 \pm 0.20 \times 10^4$ cells·µL^{-1}) and W
1738 ($1.62 \pm 0.05 \times 10^4$ cells·µL^{-1}) which were more than what was found in SW (3.79 ± 0.63
1739 $\times 10^3$ cells·µL^{-1}) and R ($4.81 \pm 0.91 \times 10^3$ cells·µL^{-1}). Overtime, there was no significant
1740 difference in C between days 0 and 34 which were lower ($p = 0.0119$) than 68. In S ($p =$
1741 0.0002), the highest density was observed at the initial time, followed by time 68 with the
1742 lowest values at 34. W ($p < 0.0001$) presented the lowest density in 0, followed by 34 and

68, respectively. In SW ($p < 0.0001$), 0 was lower than the other times 34 and 68. Just like R ($p = 0.0001$), 0 is lower than 34 and 68.

In filamentous, in 0, C ($8.20 \pm 0.62 \times 10^2$ cells·μL^{-1}) presented the highest density ($p < 0.0001$), followed by S ($3.18 \pm 0.72 \times 10^2$ cells·μL^{-1}) and W ($2.67 \pm 0.54 \times 10^2$ cells·μL^{-1}), however, W was similar to R ($1.33 \pm 0.31 \times 10^2$ cells·μL^{-1}) which was similar to SW ($6.20 \pm 0 \times 10^1$ cells·μL^{-1}). In 34 ($p < 0.0001$), S ($2.62 \pm 0.40 \times 10^3$ cells·μL^{-1}) presented the highest density, similar to C ($2.06 \pm 0.24 \times 10^3$ cells·μL^{-1}) which was similar to W ($1.80 \pm 0.48 \times 10^3$ cells·μL^{-1}) and higher than SW ($6.05 \pm 0.10 \times 10^2$ cells·μL^{-1}) and R ($9.84 \pm 0.21 \times 10^2$) cells·μL^{-1} which were similar. In 68 ($p = 0.0016$), the highest density was observed in SW ($2.37 \pm 0.42 \times 10^3$ cells·μL^{-1}), followed by the other treatments C (1.04 ± 0.34 cells·μL^{-1}), S ($1.65 \pm 0.072 \times 10^3$ cells·μL^{-1}), W ($1.37 \pm 0.29 \times 10^3$ cells·μL^{-1}) and R ($1.12 \pm 0.21 \times 10^3$ cells·μL^{-1}). In C ($p = 0.0017$), there was no significant difference between the initial and the final time, with the highest density at 34. In S ($p < 0.0001$), the highest density was observed on day 34, followed by 68 and 0. In W ($p = 0.0030$), T0 was lower than the other times 34 and 68. SW, T0 and T34 were similar and lower than T68. In R ($p = 0.0001$) T0 was lower than the other times 34 and 68.

In the initial sampling ($p < 0.0001$), there was no significant difference between the total bacteria in C ($2.78 \pm 0.13 \times 10^4$ cells·μL^{-1}) and S ($2.45 \pm 0.21 \times 10^4$ cells·μL^{-1}), higher than W ($2.16 \pm 0.39 \times 10^3$ cells·μL^{-1}), SW ($1.19 \pm 0.27 \times 10^3$ cells·μL^{-1}) and R ($8.00 \pm 1.11 \times 10^2$ cells·μL^{-1}). In 34 ($p < 0.0001$), C presented $2.63 \pm 0.47 \times 10^4$ cells·μL^{-1}, which was the highest concentration, followed by S with $1.46 \pm 0.03 \times 10^4$ cells·μL^{-1} and W with $1.18 \pm 0.11 \times 10^4$ cells·μL^{-1}. W was similar to SW ($6.89 \pm 0.40 \times 10^3$ cells·μL^{-1}) and R ($7.01 \pm 0.52 \times 10^3$ cells·μL^{-1}). In 68, C ($3.95 \pm 0.16 \times 10^4$ cells·μL^{-1}) was superior to S ($2.85 \pm 0.17 \times 10^4$ cells·μL^{-1}) and W ($2.86 \pm 0.11 \times 10^4$ cells·μL^{-1}) followed by SW ($8.25 \pm 0.98 \times 10^3$ cells·μL^{-1}) and R ($8.72 \pm 1.11 \times 10^3$ cells·μL^{-1}). In C ($p = 0.0031$), there was no significant difference between time 0 and 34, and they were lower than 68. In S ($p = 0.0001$), the lowest density was found at time 34, then at the initial time, followed by 68. In W ($p < 0.0001$), the bacteria community has increased over time. 0 was lower than 34 which was lower than 68. In SW ($p < 0.0001$), the lowest value was found at the

initial time, which was lower than the other days 34 and 68. The same occurred in R, the lowest value was found at the initial time followed by days 34 and 68.

7.3.3 Bacteria removal efficiency

The bacteria removal efficiency rate was influenced by the treatment since the initial sampling (Figure 3). The concentrate that underwent treatment with the skimmer obtained a removal rate of 26.83 ± 32.53%, less efficient (p = 0.0024) than the other treatments, W with 91.05 ± 2.90 %, SW with 92.69 ± 5.17 % and R with 95.70 ± 2.70 %. On day 34, S presented 37.43 ± 13.23%, lower (p=0.0038) than SW with a rate of 74.60 ± 4.85% and R with 73.90 ± 6.67%, however it was similar to W with 58.29 ± 9.92% which was similar to the others. On day 68, there was no significant difference between S (27.01 ± 17.74 %) and W (24.07 ± 9.60 %), which were inferior to SW (74.34 ± 9.73%) and R (72.49 ± 11.04%). Overtime, S showed no significant difference (p=0.8195). W had a progressive decrease in removal efficiency (p = 0.0002), 0 was higher than 34, which was higher than 68. SW showed a decrease in efficiency on day 34 and it was maintained until day 68. The same happened with R, which showed a high removal efficiency on day 0, which was higher than on the days 34 and 68.

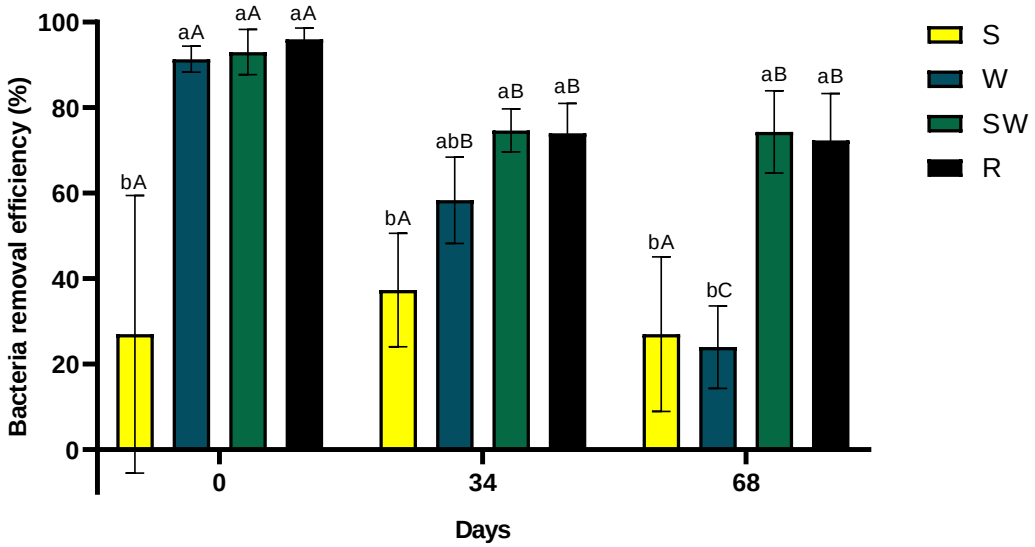


Figure 3: Bacteria removal efficiency rate over time. S – skimmer; W – Washing; SW – skimmer + washing; R – Removal. Letters indicate significant differences (p<0.05) in treatments (lowercase) and over time (uppercase).

7.3.4 Dissolved Organic Matter

In the initial sampling, there was no difference ($p = 0.0020$) between control C (47.58 ± 8.01 mg·L^{-1}) and treatments S (42.23 ± 8.66 mg·L^{-1}) and SW (31.44 ± 1.45 mg·L^{-1}), followed by W (30.76 ± 1.91 mg·L^{-1}) which was similar to S and SW and R (20.23 ± 6.02 mg·L^{-1}) (Figure 1b). On day 17 ($p < 0.0001$), C (134.40 ± 8.59 mg·L^{-1}) presented the highest concentration followed by S (106.75 ± 12.80 mg·L^{-1}) which was higher than W (65.49 ± 2.65 mg·L^{-1}), SW (64.41 ± 6.22 mg·L^{-1}) and R (59.46 ± 5.66 mg·L^{-1}). On day 34 ($p < 0.0001$), there was no difference between controls C (141.20 ± 2.36 mg·L^{-1}) and R (116.60 ± 13.36 mg·L^{-1}) and treatments S (161.33 ± 8.26 mg·L^{-1}), W (100.34 ± 1.88 mg·L^{-1}) and SW (87.31 ± 69.66 mg·L^{-1}). In 51 ($p < 0.0001$), C (251.97 ± 3.54 mg·L^{-1}) and S (225.93 ± 2.75 mg·L^{-1}) presented the highest concentrations, S was similar to SW (207.77 ± 13.20 mg·L^{-1}) which was higher than W (143.13 ± 18.85 mg·L^{-1}) and C (142.90 ± 3.03 mg·L^{-1}). On day 68, the highest ($p < 0.0001$) concentration was observed in SW (669.20 ± 38.90 mg·L^{-1}), followed by C (441.80 ± 11.35 mg·L^{-1}) and S (504.35 ± 1.25 mg·L^{-1}) which was higher than W (324.25 ± 6.95 mg·L^{-1}), and higher than R (90.54 ± 32.17 mg·L^{-1}).

Overall, organic matter increased over time. In C ($p < 0.0001$), the lowest concentration was found in the initial sample (47.58 ± 8.01 mg·L^{-1}), followed by days 17 (134.40 ± 8.59 mg·L^{-1}) and 34 (141.20 ± 2.36 mg·L^{-1}), which were lower than day 51 (251.97 ± 3.54 mg·L^{-1}), and day 68 (441.80 ± 11.35 mg·L^{-1}). In S ($p < 0.0001$), all days were different. The initial sampling presented the lowest concentration (42.23 ± 8.66 mg·L^{-1}), followed by day 17 (106.75 ± 12.80 mg·L^{-1}), which was lower than day 34 (161.33 ± 8.26 mg·L^{-1}), lower than day 51 (225.93 ± 2.75 mg·L^{-1}) which was lower than day 68 (504.35 ± 1.25 mg·L^{-1}). The same pattern was repeated in W ($p < 0.0001$); 0 (30.76 ± 1.91 mg·L^{-1}) was lower than 17 (65.49 ± 2.65 mg·L^{-1}), lower than 34 (100.34 ± 1.88 mg·L^{-1}), lower than 51 (143.13 ± 18.85 mg·L^{-1}), and 68 (324.25 ± 6.95 mg·L^{-1}). In SW ($p < 0.0001$) there was no significant difference between the initial sample (31.44 ± 1.45 mg·L^{-1}), day 17 (64.41 ± 6.22 mg·L^{-1}) and day 34 (87.31 ± 69.66 mg·L^{-1}) which were lower than 51 (207.77 ± 13.20 mg·L^{-1}), and lower than 68 (669.20 ± 38.90 mg·L^{-1}). In R, the highest ($p < 0.0001$) concentration was found on day 51 (142.90 ± 3.03 mg·L^{-1}) similar to day 34 (116.60 ±

13.36 mg·L^{-1}) which was similar to day 68 (90.54 ± 32.17 mg·L^{-1}), similar to 17 (59.46 ± 5.66 mg·L^{-1}), similar to 0 (20.23 ± 6.02 mg·L^{-1}).

7.3.5 DMS

In the initial sampling, in S and in R, no DMS was detected, while in the control (C = 0.54 ± 0.37 µg·L^{-1}), in the washing treatment (W = 0.13 ± 0.22 µg·L^{-1}) and in the combination of skimmer and washing (SW = 0.44 ± 0.76 µg·L^{-1}) the concentrations found did not differ significantly from S and R ($p = 0.3473$) (Figure 1c). On day 17, the highest concentration ($p<0.0001$) was found in C (25.60 ± 1.16 µg·L^{-1}) followed by S (13.67 ± 2.97 µg·L^{-1}) and W (9.49 ± 1.43 µg·L^{-1}) which were statistically similar, and R (7.54 ± 0.87 µg·L^{-1}) was similar to W, and to SW (3.19 ± 1.53 µg·L^{-1}) which presented the lowest average. On day 34, C (20.23 ± 8.07 µg·L^{-1}) presented the highest ($p = 0.0014$) concentration, followed by S (7.99 ± 0.83 µg·L^{-1}), W (5.04 ± 0.14 µg·L^{-1}), SW (2.38 ± 0.34 µg·L^{-1}) and R (8.40 ± 0.49 µg·L^{-1}), with no significant difference between treatments. The same happened on day 51 ($p = 0.0005$), C (25.65 ± 2.98 µg·L^{-1}) was superior to the other treatments S (14.55 ± 2.83 µg·L^{-1}), W (13.12 ± 7.22 µg·L^{-1}), SW (5.11 ± 0.95 µg·L^{-1}) and R (8.40 ± 0.49 µg·L^{-1}). On day 68 ($p < 0.0001$), the values of C (16.87 ± 2.44 µg·L^{-1}), S (12.06 ± 1.16 µg·L^{-1}) and W (14.99 ± 0.30 µg·L^{-1}) were similar, S was similar to R (7.31 ± 2.89 µg·L^{-1}) which was similar to SW (3.83 ± 1.05 µg·L^{-1}).

In general, all treatments increased their DMS concentrations over time. In the control (C), only the initial sampling (0.54 ± 0.37 µg·L^{-1}) was different from the other days, 17 (25.60 ± 1.16 µg·L^{-1}), 34 (20.23 ± 8.07 µg·L^{-1}), 51 (25.65 ± 2.98 µg·L^{-1}) and 68 (16.87 ± 2.44 µg·L^{-1}). In S, the day that presented the lowest concentration was the initial time (0.13 ± 0.22 µg·L^{-1}), which was similar to day 34 (7.99 ± 0.83 µg·L^{-1}), not differing from day 17 (13.67 ± 2.97 µg·L^{-1}) and 51 (14.55 ± 2.83 µg·L^{-1}), both similar to 68 (12.06 ± 1.16µg·L^{-1}). In W, although DMS was not detected initially, there was an increase in concentration. Day 34 (5.04 ± 0.14 µg·L^{-1}) was similar to day 68 (14.99 ± 0.30 µg·L^{-1}), which was similar to days 17 (9.49 ± 1.43 µg·L^{-1}) and 51 (13.12 ± 7.22 µg·L^{-1}). In SW, the initial sample (0.44 ± 0.76 µg·L^{-1}) was similar to day 34 (2.38 ± 0.34 µg·L^{-1}), which was similar to days 17 (3.19 ± 1.53 µg·L^{-1}) and 68 (3.83 ± 1.05 µg·L^{-1}) and had the highest value on day 51 (5.11 ± 0.95 µg·L^{-1}). No DMS was detected in R on day 0, and the other

days 17 (7.54 ± 0.87 $\mu g \cdot L^{-1}$), 34 (6.73 ± 1.89 $\mu g \cdot L^{-1}$), 51 (8.40 ± 0.49 $\mu g \cdot L^{-1}$) and 68 (7.31 ± 2.89 $\mu g \cdot L^{-1}$) did not differ statistically.

7.4 Discussion

The most efficient methods in removing bacteria and reducing the production of DOM and DMS throughout the storage of concentrates were washing repeated 5 times (R) and combining the use of the skimmer with washing (SW). Initially, the use of skimmer (S) was the only method that was not efficient, removing about 26% of the total bacteria, a concentration much lower than the average of other treatments, which were greater than 90%. However, although the washing process repeated only 3 times (W) showed a high initial bacterial removal rate, after 68 days of storage this rate dropped to around 24%.

An efficient method used to remove bacteria, in addition to allowing a high removal rate, it cannot influence the cellular viability of microalgae. It was observed in this and previous experiments (Couto et al. 2024) that the viability of *Nannochloropsis oceanica* is not influenced by the collection and cleaning method. *Nannochloropsis* ssp. is known to be a microalga with an extremely resistant cell wall (Beacham et al. 2014) remaining viable even after the use of high mechanical force (Bernaerts et al. 2019). For this reason, despite being an ideal species to be used in concentrates, it may not be the best biological model to test the effect of these methods on the viability of microalgae. The genus *Isochrysis*, on the other hand, in addition to being widely used in the production of concentrates, is susceptible to cell breakdown after the use of mechanical force (Balduyck et al. 2016, 2019).

Although at first glance it appears that the skimmer did not influence the bacterial community, it was efficient in reducing more than 50% of cocci (Figure 2a) and presenting more bacilli than even the control itself (Figure 2b), showing that the reduction of specific groups of bacteria favors the increase of other groups. In work not yet published, a metagenomic analysis was carried out to identify these bacteria. 13 genera were identified, among the most abundant are *Erythrobacter*, *Oceanicaulis* and *Roseobacter*, that was also identified and is mainly responsible to produce DMS.

Although the cultivation is in constant mixing because of aeration, dead zones can still be formed in the reactors where the cultivation remains static (Kubelka et al. 2017, 2018). In these zones, groups of bacteria may be favored by a lack of light and the presence of organic matter. In the reactors used in this experiment, the skimmer was installed in the upper region of the water column, where photosynthetic groups such as *Erithrobacter* bacteria would concentrate. Therefore, the removal of these bacteria can favor the growth of other groups such as *Roseobacter*, which is undesirable, since it is responsible for the production of DMS.

Although no external sources of nutrients were added to the concentrates, in all treatments there was an increase in bacterial density due to the increase in organic matter, which may have been caused by cell death and EPS production by microalgae (Koçer et al. 2021). In the final sampling, the SW treatment presented a higher concentration of organic matter than that found in the control, as the bacterial load in this treatment was low, the accumulation of organic matter may have occurred due to not having enough bacteria to use the organic matter for their metabolism as happened with control and treatment S.

7.5 Conclusion

The results presented here show that to reduce bacterial growth in concentrates and consequently reduce the concentration of DOM and DMS, it is necessary to use a washing process combined with the use of the skimmer or repeating this process more times. As seen, the use of the skimmer alone is not enough, despite buying a skimmer is expensive and as it is sensitive equipment, which requires specialized labor to operate. Washing has been shown to be a fundamental process and depending on the number of times it is repeated, it can significantly improve the quality of the concentrates. Therefore, it is necessary to optimize this method, seeking to increase bacterial removal and reduce costs.

References

Balduyck L, Bijttebier S, Bruneel C, et al (2016) Lipolysis in *T-Isochrysis lutea* during wet storage at different temperatures. Algal Res 18:281–287. https://doi.org/10.1016/j.algal.2016.07.003

Balduyck L, Dejonghe C, Goos P, et al (2019) Inhibition of lipolytic reactions during wet storage of *T-Isochrysis lutea* biomass by heat treatment. Algal Res 38:101388. https://doi.org/10.1016/j.algal.2018.101388

Bates TS, Charlson RJ, Gammon RH (1987) Evidence for the climatic role of marine biogenic sulphur. Nature 329:319–321. https://doi.org/10.1038/329319a0

Beacham TA, Bradley C, White DA, et al (2014) Lipid productivity and cell wall ultrastructure of six strains of *Nannochloropsis*: Implications for biofuel production and downstream processing. Algal Res 6:64–69. https://doi.org/10.1016/j.algal.2014.09.003

Bernaerts TMM, Gheysen L, Foubert I, et al (2019) Evaluating microalgal cell disruption upon ultra high pressure homogenization. Algal Res 42:101616. https://doi.org/10.1016/j.algal.2019.101616

Buckley T, Xu X, Rudolph V, et al (2022) Review of foam fractionation as a water treatment technology. Separation Science and Technology (Philadelphia) 57:929–958. https://doi.org/10.1080/01496395.2021.1946698

Charlson RJ, Anderson TL, McDuff RE (1992) 13 The Sulfur Cycle. In: International Geophysics. Academic Press Inc., pp 285–300

Charlson RJ, Lovecook JE, Andreae MO, Warren SG (1987) Ocean phytoplankton, atmospheric sulfur, cloud albdeo and climate. Nature 326:655–661

Chen S, Timmons MB, Bisogni JJ, Aneshansley DJ (1994) Modeling surfactant removal in foam fractionation: I - Theoretical development. Aquac Eng 13:163–181. https://doi.org/10.1016/0144-8609(94)90001-9

Couto C, Hernández CP, Alves Sobrinho RCM, et al (2021) Optimization of a low-cost fertilizer-based medium for large-scale cultivation of the coastal diatom *Conticribra weissflogii* using response surface methodology and its effects on biomass composition. J Appl Phycol 33:2767–2781. https://doi.org/10.1007/s10811-021-02519-8

Couto C, Roselet F, Kubelka B, et al (2024) Effect of removing bacteria and dissolved organic matter on DMS production in *Nannochloropsis oceanica* concentrates. Algal Res 80:103534. https://doi.org/10.1016/j.algal.2024.103534

1953 Csordas A, Wang JK (2004) An integrated photobioreactor and foam fractionation unit
1954 for the growth and harvest of *Chaetoceros* spp. in open systems. Aquac Eng 30:15–
1955 30. https://doi.org/10.1016/j.aquaeng.2003.07.001

1956 Cui Y, Suzuki S, Omori Y, et al (2015) Abundance and distribution of
1957 dimethylsulfoniopropionate degradation genes and the corresponding bacterial
1958 community structure at dimethyl sulfide hot spots in the tropical and subtropical
1959 Pacific Ocean. Appl Environ Microbiol 81:4184–4194.
1960 https://doi.org/10.1128/AEM.03873-14

1961 Curson ARJ, Liu J, Bermejo Martínez A, et al (2017) Dimethylsulfoniopropionate
1962 biosynthesis in marine bacteria and identification of the key gene in this process. Nat
1963 Microbiol 2:. https://doi.org/10.1038/nmicrobiol.2017.9

1964 Figueiras Guilherme M, Jesus Gregersen KJ, Pedersen L (2020) Effects of foam
1965 fractionation and chemical disinfection on the removal of different microalgae
1966 cultures. Aquac Res 51:3274–3283. https://doi.org/10.1111/are.14663

1967 Giri BS, Mudliar SN, Deshmukh SC, et al (2010) Treatment of waste gas containing low
1968 concentration of dimethyl sulphide (DMS) in a bench-scale biofilter. Bioresour
1969 Technol 101:2185–2190. https://doi.org/10.1016/j.biortech.2009.11.033

1970 Gregersen KJ de J, Pedersen L-F, Pedersen PB, et al (2021) Foam fractionation and
1971 ozonation in freshwater recirculation aquaculture systems. Aquac Eng 95:102195.
1972 https://doi.org/10.1016/j.aquaeng.2021.102195

1973 Guillard RRL (1975) Culture of Phytoplankton for Feeding Marine Invertebrates. In:
1974 Culture of Marine Invertebrate Animals

1975 Hobbie J, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by
1976 fluorescence microscopy. Appl Environ Microbiol 33:1225–1228

1977 Keller MD (1988) Dimethyl sulfide production and marine phytoplankton: the
1978 importance of species composition and cell size. Biological Oceanography 6:375–
1979 382. https://doi.org/10.1080/01965581.1988.10749540

1980 Kiene RP, Bates TS (1990) Biological removal of dimethyl sulphide from sea water.
1981 Nature 345:702–705. https://doi.org/10.1038/345702a0

1982 Kiene RP, Linn LJ, Bruton JA (2000) New and important roles for DMSP in marine
1983 microbial communities. J Sea Res 43:209–224. https://doi.org/10.1016/S1385-
1984 1101(00)00023-X

1985 Koçer AT, İnan B, Kaptan Usul S, et al (2021) Exopolysaccharides from microalgae:
1986 production, characterization, optimization and techno-economic assessment.
1987 Brazilian Journal of Microbiology 52:1779–1790. https://doi.org/10.1007/s42770-
1988 021-00575-3

1989 Kovács BD, de Jesus Gregersen KJ, Rüppel F, et al (2023) Evaluating protein skimmer
1990 performance in a commercial seawater recirculating aquaculture system (RAS).
1991 Aquac Eng 103:. https://doi.org/10.1016/j.aquaeng.2023.102369

1992 Kubelka BG, Pinto WT, Abreu PC (2017) Hydrodynamic performance of two air nozzles
1993 diameters on the massive microalgae culture: Computational and experimental
1994 approaches. Algal Res 27:318–324. https://doi.org/10.1016/j.algal.2017.09.024

1995 Kubelka BG, Roselet F, Pinto WT, Abreu PC (2018) The action of small bubbles in
1996 increasing light exposure and production of the marine microalga *Nannochloropsis*
1997 *oceanica* in massive culture systems. Algal Res 35:569–576.
1998 https://doi.org/10.1016/j.algal.2018.09.030

1999 Levasseur M, Michaud S, Egge J, et al (1996) Production of DMSP and DMS during a
2000 mesocosm study of an *Emiliania huxleyi* bloom: influence of bacteria and Calanus
2001 finmarchicus grazing. Mar Biol 126:609–618. https://doi.org/10.1007/BF00351328

2002 Miller TR, Belas R (2004) Dimethylsulfoniopropionate Metabolism by Pfiesteria -
2003 Associated *Roseobacter* spp. Appl Environ Microbiol 70:3383–3391.
2004 https://doi.org/10.1128/AEM.70.6.3383-3391.2004

2005 Nishino SF (1986) Direct Acridine Orange counting of bacteria preserved with acidified
2006 Lugol Iodine. Appl Environ Microbiol 52:602–604

2007 Roselet M, Roselet F, Abreu PC (2019) Foam fractionator as a tool to remove dissolved
2008 organic matter and improve the flocculation of the marine microalga
2009 *Nannochloropsis oceanica*. J Appl Phycol 31:2911–2919.
2010 https://doi.org/10.1007/s10811-019-01801-0

2011 Samocha TM (2019) Sustainable Biofloc Systems for Marine Shrimp. Elsevier

2012 Zhang XH, Liu J, Liu J, et al (2019) Biogenic production of DMSP and its degradation to
2013 DMS—their roles in the global sulfur cycle. Sci China Life Sci 62:1296–1319.
2014 https://doi.org/10.1007/s11427-018-9524-y

2015

2016

8 CAPÍTULO III

COST EFFECTIVE OPTIMIZATION OF BACTERIA REMOVAL IN *Nannochloropsis oceanica* CONCENTRATES

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Abstract

The objective of this study was to decrease bacterial contamination and improve the net benefit-cost of the process while preserving the integrity of microalgae cells. The washing process conditions were optimized to increase the rate of bacterial removal, making the process economically viable without impacting cell viability. To this, a Central Composite Rotational Design with four variables and two levels (CCRD 2^4) was performed, generating 27 experimental units (runs). Cell viability was determined by subculture in culture medium and growth rate calculation. The rate of bacterial removal was estimated by directly counting bacteria in the concentrate before and after undergoing the washing process, and a Partial Budget Analysis estimated the net benefit-cost for each run. The experimental models of each response were combined using the desirability index to find the combination that generated the best result in all responses. The best relation between cell viability, bacterial removal efficiency rate, and PBA was found by repeating the process 5 times at 3.405 g-force at 24°C for 20 minutes. To make the washing process economically viable, it was necessary to increase the sales price by 10%, which is justified by the increase in shelf life that the process generates.

Keywords: Microalgae; net benefit-cost; washing process; microalgae; bacteria; contamination; cost decrease

8.1 Introduction

Nannochloropsis oceanica is one of the microalgae with the greatest potential for large-scale production (Borowitzka, 2013). It is widely known for its potential in the production of biofuels and nutraceuticals. It presents aquaculture applications as a live feed due to its composition, mainly due to its high content of protein and fatty acids such as eicosapentaenoic acid – EPA and docasahexaenoic acid - DHA (Dewi et al., 2018; Saxena et al., 2023). In addition, it is a microalgae that withstands large environmental variations and can be cultivated in alternative media that allow for the reduction of its production costs (Couto et al., 2024).

However, in large-scale cultivation of this microalgae, it is nearly impossible to maintain an axenic culture, that is, without bacterial contamination. Some works showed that combining microalgae and bacteria is often better than using either alone. Some groups

of bacteria can produce compounds such as B vitamins that are not synthesized by microalgae, enhancing their growth. Like bacteria, microalgae can produce compounds that are essential for the growth of bacteria (Natrah et al., 2014). As microalgae culture becomes denser, cultivation conditions may become less favorable and nutrient depletion and shadowing effect may occur. In addition, the life cycle of microalgae reaches the end (Kubelka et al., 2018). Therefore, cells begin to die, causing organic matter to build up over time (Zuo, 2019), which in turn serves as substrates for bacterial growth (Giroldo et al., 2007). Other secondary metabolites from microalgae can also benefit and stimulate bacterial proliferation (Natrah et al., 2014).

While it is not possible to completely halt bacterial growth in crops, it is critical to minimize the bacterial load in the produced biomass (Couto et al., 2024). Beyond ensuring higher purity for microalgae products, it also minimizes the risks associated with excessive bacterial growth, which can shorten product shelf life and lead to the production of potentially toxic compounds (Dan et al., 2018).

Washing is a process that consists of resuspending the collected biomass in aseptic salt water and centrifuging until there is a separation between the microalgae that settle and other smaller particles, such as bacteria (Couto et al., 2024; Roselet et al., 2019). After centrifugation, this supernatant water containing bacteria is discarded, and the microalgae-settled pellet can be used. The efficiency of the washing process may depend on the frequency, centrifugation velocity, temperature, and process time. These conditions need to be optimum so that the microalgae settle without affecting the viability of the cells and maximum bacterial load is removed.

Given the numerous variables and responses, a highly effective approach is to use experimental design to optimize these conditions. This allows for the simultaneous evaluation of multiple variables in a single experiment while also identifying their interactions (Couto et al., 2021; Kirrolia et al., 2014). Using the desirability index, multiple responses can be combined to obtain the ideal condition that meets all needs.

Thus, this study aimed to reduce bacterial contamination and improve the process's net benefit-cost while preserving the integrity of microalgae cells.

8.2 Materials and methods

8.2.1 Obtaining the microalgae paste

In this experiment, a *Nannochloropsis oceanica* paste with approximately 25% dry matter produced by the company AlgaSul Biotecnologia de Microalgas was used. This microalgae was grown in a YCB medium (Couto et al., 2021) in natural seawater with a 28 $g \cdot L^{-1}$ salinity for 15 days under natural environmental conditions of light, photoperiod, and temperature. During cultivation, the pH was adjusted daily to 8, and a new load of YCB was added every 5 days. The microalgae were then collected in a disc centrifuge with a flow rate of 20 $L \cdot h^{-1}$, and the paste was stored at 4°C until the time of the experiment.

8.2.2 Experimental Design

A Central Composite Rotational Design with 4 factors and 2 levels (DCCR 2^4) were applied to optimize the washing process conditions. In this design, the number of process repetitions (X_1), centrifugation velocity in g-force (X_2), temperature in °C (X_3), and time in minutes (X_4) were analyzed, totaling 27 experimental units. These units were represented by falcon tubes (50 mL) with 40 mL of useful volume.

Approximately 8 g of paste and 40 mL of artificial seawater (VeroSal Corais) with a final density of 200 $g \cdot L^{-1}$ were placed in each experimental unit, and then each falcon was subjected to the conditions of its test defined in Table 1. After each centrifugation round, the efficiency was estimated as described in item 2.5.1. Then, the new paste obtained was diluted and adjusted to a concentration of 5 billion cells per mL, and a sample was collected to determine cell viability and bacterial density (items 2.3 and 2.4). From these results, the bacteria removal efficiency rate (item 2.3.4) and the process cost analysis (item 2.3.5) were calculated.

2132 Table 1: Central Composite Rotational Design (CCRD 24) with real and coded values of the number of process repetitions, centrifugation velocity (g-force), temperature (°C),
2133 time (min) and experimental responses for cell viability (duplications·day-1) and bacteria removal rate (%).

Run	Repetitions		g-Force		Temperature (°C)		Time (min)		k (division·d^{-1})	Bacteria removal rate (%)
1	2	(-1)	2.000	(-1)	6	(-1)	10	(-1)	0.102	78.15
2	2	(-1)	2.000	(-1)	6	(-1)	20	(+1)	0.098	69.63
3	2	(-1)	2.000	(-1)	18	(+1)	10	(-1)	0.147	70.67
4	2	(-1)	2.000	(-1)	18	(+1)	20	(+1)	0.073	69.09
5	2	(-1)	2.835	(+1)	6	(-1)	10	(-1)	0.000	72.64
6	2	(-1)	2.835	(+1)	6	(-1)	20	(+1)	0.015	73.29
7	2	(-1)	2.835	(+1)	18	(+1)	10	(-1)	0.114	68.92
8	2	(-1)	2.835	(+1)	18	(+1)	20	(+1)	0.104	70.07
9	4	(+1)	2.000	(-1)	6	(-1)	10	(-1)	0.000	83.40
10	4	(+1)	2.000	(-1)	6	(-1)	20	(+1)	0.124	56.80
11	4	(+1)	2.000	(-1)	18	(+1)	10	(-1)	0.135	80.39
12	4	(+1)	2.000	(-1)	18	(+1)	20	(+1)	0.112	60.95
13	4	(+1)	2.835	(+1)	6	(-1)	10	(-1)	0.089	76.02
14	4	(+1)	2.835	(+1)	6	(-1)	20	(+1)	0.093	63.30
15	4	(+1)	2.835	(+1)	18	(+1)	10	(-1)	0.086	74.55
16	4	(+1)	2.835	(+1)	18	(+1)	20	(+1)	0.146	72.04
17	1	(-2)	2.417	0	12	0	15	0	0.075	59.26

18	5	(+2)	2.417	0	12	0	15	0	0.092	68.87
19	3	0	1.583	(-2)	12	0	15	0	0.099	69.96
20	3	0	3.405	(+2)	12	0	15	0	0.074	67.12
21	3	0	2.417	0	0	(-2)	15	0	0.081	82.20
22	3	0	2.417	0	24	(+2)	15	0	0.124	74.99
23	3	0	2.417	0	12	0	5	(-2)	0.085	63.95
24	3	0	2.417	0	12	0	25	(+2)	0.022	46.31
25	3	0	2.417	0	12	0	15	0	0.103	50.74
26	3	0	2.417	0	12	0	15	0	0.087	57.78
27	3	0	2.417	0	12	0	15	0	0.055	53.85

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8.2.3 Cell viability

Cell viability was determined by calculating the specific growth rate (k) described in Equation 1. Samples from each experimental unit were inoculated in f/2 medium (Guillard, 1975) at 21°C with a light intensity of 100 μmol photons·m^{-2}·s^{-1} and a photoperiod of 12:12 light:dark with an initial cell density of 6.0×10^6 cells·mL^{-1}. The tubes were manually homogenized, and the cell density was determined initially (DOi) and after 6 days of culture (DOf) by cell counting in a Neubauer chamber at 400x magnification. Cell viability was then calculated using Equation 1 of the specific growth rate (k).

$$k(division\ d^{-1}) = \frac{3.322}{(t-t0)\times Log(\frac{DOf}{DOi})} \quad \text{Equation 1}$$

8.2.4 Bacteria removal efficiency

Bacterial density was determined by direct counting under an epifluorescence microscope. Samples from each experimental unit were diluted (10,000x) and stored in a 4% formaldehyde solution until analysis. Samples were then filtered through 0.2 μm pore-size darkened polycarbonate filters at a vacuum pressure of less than 5 cmHg (Giraldo et al., 2019). Samples were incubated for 10 min in a 0.1% acridine orange solution (Lian et al., 2022). The filters were removed, dried, and placed on a counting slide with immersion oil. Counts were performed in 20 random fields under a Zeiss Axiopla epifluorescence microscope. The final bacterial density was calculated using Equation 2.

$$Total\ Bacterial\ Cell\ Count/\mu L = \frac{A\times(\frac{(\frac{B}{C})}{D})}{1000} \quad \text{Equation 2}$$

Where A = average count per field; B = filtration area (mm^2); C = area of the counting field (mm^2); D = sample volume (mL).

The bacterial removal efficiency rate was determined from the relationship between the bacterial density in the concentrate before the washing process (Bc) and the density of each experimental unit (Bu), as described in Equation 3.

$$Removal\ efficiency\ (\%) = \frac{(Bc-Bu)}{Bc} x100 \qquad \text{Equation 3}$$

8.2.5 Partial Budget Analysis

A partial budget analysis (PBA) considering the costs and revenues associated with the addition of the washing process in the production of *Nannochloropsis oceanica* concentrates was performed to estimate the net benefit-cost of this process change (Engle, 2010). For this, the different revenue and associated costs were calculated for each of the 27 experimental trials. All the values utilized in this analysis were converted from Brazilian Reais (BRL) to dollars (USD) based on the exchange rate in May 2024, which was $1.00 BRL = $0.20 USD. Cost data used in the PBA are expressed in Table 2.

Table 2: Price of items considered in the Partial Budget Analysis (PBA) for the addition of the washing process in the production of *Nannochloropsis oceanica* concentrates

Item	**Description**	**Unit**	**$**
Centrifuge	Capacity of 3 L	$	6,183.00
Depreciation	Useful life of 10 years	$·hour^{-1}	0.07
Energy	Value for the month of May 2024	kW·hour^{-1}	0.17
Labor	Value for the year 2024	$·hour^{-1}	1.83

8.2.5.1 Total additional revenue

Calculating the total additional revenue required an initial assessment of biomass loss during centrifugation, which we determined by calculating the centrifugation efficiency. For this, two optical density measurements at 750 nm were made in each experimental unit, an initial (DOi) taken before centrifugation and a final (DOf), after each process established in its experimental test according to Equation 4.

$$Centrifugation\ efficiency\ (\%) = 100 - \frac{(DOf * 100)}{DOi}$$ Equation 4

To calculate the revenue (Equation 5), the selling price of 400g of *N. oceanica* paste in the form of concentrate ($406.37), and the centrifugation efficiency were considered.

$$Gross\ receipt\ (\$) = \frac{\$406.37 * Centrifugation\ efficiency}{100}$$ Equation 5

The revenue for each run was calculated by the difference between the reference sales price of 400g of the concentrate and gross receipt. When values are positive or negative, they are considered additional or reduced revenue, respectively (Equation 6).

$$Revenue\ (\$) = \$406.37 - Gross\ receipt$$ Equation 6

8.2.5.2 Total additional costs

Total additional costs (Equation 7) were calculated by adding the interest on investment (IOI), the cost of energy (EC) and labor (LC), 5% interest on operating costs (IOC), and the depreciation of equipment used in the process (DBP).

$$Total\ additional\ costs\ (\$) = IOI + EC + LC + IOC + DBP$$ Equation 7

To calculate the IOI (Equation 8), the depreciation of the equipment per hour of use was first calculated. Considering that the centrifuge costs $6,183.00 and has a useful life of 10 years, the average depreciation per hour of use was $0.07.

$$IOI\ (\$) = \$0.07 * time\ (h) * repetition$$ Equation 8

Energy cost (EC - Equation 9) was calculated taking into account the process repetitions, the cost of kWh in Brazil in USD in May 2024 ($0.17), the time in hours, and the average

maximum power in each experimental test. To calculate the power (kW) for each run, the average power was taken at each temperature and rotation setting. The power correlation is established based on the equipment's maximum capacity of 1.5 kW, where: The maximum rotation (4000 RPM) and the minimum temperature (-4°C) corresponded to 1.5 kW; The minimum rotation (2600 RPM) corresponded to 0.975 kW; The maximum temperature (24°C) corresponded to 0.051 kW.

$$EC\ (\$) = Repetition * kWh\ (\$) * time\ (h) * kW \quad \text{Equation 9}$$

To calculate the labor cost (LC – Equation 10), it was considered that the average wage for rural workers in Brazil in 2024, which was $1.83 per hour (Talent.com, 2024), multiplied by the time of each process, and the number of times the process was repeated.

$$LC\ (\$) = 1.83\ (\$) * repetition * time\ (h) \quad \text{Equation 10}$$

The IOC was calculated taking into account 5% interest on energy and labor costs (Equation 11).

$$IOC\ (\$) = (EC + LC) * 5\% \quad \text{Equation 11}$$

Finally, the depreciation per process (DBP – Equation 12) was calculated considering that the average depreciation of equipment per hour is $0.07 multiplied by the time in hours of each process and the number of times the process is repeated.

$$DBP\ (\$) = 0.0706\ (\$) * time\ (h) * repetition \quad \text{Equation 12}$$

The net benefit-cost of the washing process was determined by subtracting the total additional costs (reduced revenue and/or increased expenses) from the total additional benefits (increased revenue and/or cost savings).

$$Net\ Benefit - Cost = Total\ Additional\ Benefits - Total\ Additional\ Costs$$

Equation 13

8.2.6 Statistical analysis

First, the data were analyzed for normality and homogeneity using the Shapiro-Wilk and Levene tests. The effects and interactions of the factors number of process repetitions, centrifugation speed, temperature, and time, were evaluated by an ANOVA with a significance level of 5% in a Central Composite Rotational Design (CCRD). Based on the experimental results obtained, an empirical polynomial quadratic model was adjusted to correlate the independent variables and the response (Equation 14). The resulting models of the responses, cell viability, bacteria removal efficiency rate, and PBA, were then combined through the desirability index function to find the combination that meets all these responses. All analyses were performed using the design of experiments package for the STATISTICA software version 13.3 (TIBCO Software Inc, USA).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

Equation 14

Where Y represents the response variable (cell viability, bacteria removal efficiency rate, and PBA), β is the coefficient of the equation, and X_1, X_2, X_3, and X_4 are the coded levels of the independent variables.

8.3 Results

8.3.1 Cell viability

The data demonstrate that the only parameter that influenced cell viability was temperature ($p = 0.0190$; $R^2 = 0.62$), with a significant positive effect. The higher the temperature, the higher the viability, with an average of 0.089 ± 0.040 duplications·days^{-1}, ranging from 0 to 0.177. According to the generation generated by the model (Figure 1, Equation 15), the ideal temperature to maintain cell viability is 24°C.

$$k\ (duplications \cdot day^{-1}) = 0.0864 + 0.0069 * X_3 \qquad \text{Equation 15}$$

Where, X_3 = temperature in °C.

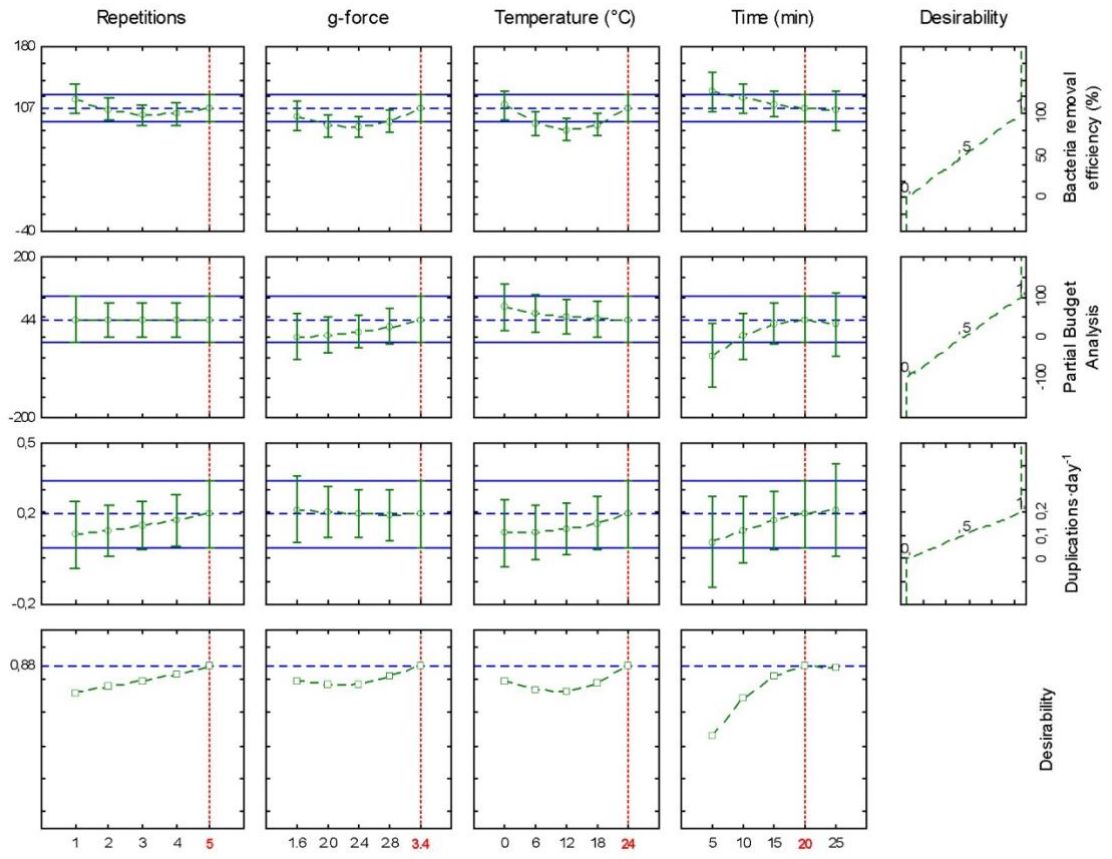

Figure 1: Profiles for predicted values and overall desirability as a function of washing process variables for Bacteria removal efficiency (%), Partial Budget Analysis, and Cell viability (duplications per day).

8.3.2 Bacteria removal efficiency

The data showed that time was the only variable that had a significant negative linear effect (p=0.0004). The number of repetitions (p=0.0049), centrifugation speed (p=0.0006), and temperature (p<0.0001) showed a positive quadratic effect. Two interactions were observed: a negative one between the number of repetitions and time (p=0.0106) and a positive one between centrifugation speed and time (p=0.0314). According to the model (R^2 = 0.86; Figure 1; Equation 16), the highest bacterial removal rate was obtained by repeating the process 4 times at 3.405 g-force, 24°C for 25 minutes.

$$Bacteria\ removal\ (\%) = 55.95 + 2.92 * X_1^2 + 4.03 * X_2^2 + 6.55 * X_3^2 - 4.37 * X_4 - 3.31 * X_1 * X_4 + 2.67 * X_2 * X_4 \quad \text{Equation 16}$$

Where, X_1 = number of repetitions of the process; X_2 = g-force; X_3 = temperature in °C and X_4 = time in minutes.

8.3.3 Partial Budget Analysis

The efficiency of centrifugation was influenced by the four variables analyzed in the process: number of repetitions, centrifugation speed, temperature, and time. These factors were tested to optimize the washing process of *Nannochloropsis oceanica* concentrates. The centrifugation efficiency was calculated in each experimental unit and ranged from 69.59% to 98.86 with an average of 92.38 ± 6.45% (Table 3). The results demonstrated that all variables influenced the centrifugation efficiency ($R^2 = 0.86$), either independently or through interactions. Centrifugation speed and time had a significant positive effect ($p<0.0001$), meaning that when increasing it, higher efficiency is observed. In contrast, temperature showed a significant negative effect ($p<0.0001$), indicating that higher temperatures reduced centrifugation efficiency. The number of repetitions, centrifugation speed, and time exhibited positive interactions with one another, while temperature demonstrated negative interactions with both the number of repetitions and time. According to the statistical model generated, the best result was obtained by repeating the process 5 times, with 3.405 g, 0°C for 25 min. Additionally, gross revenue was primarily affected by the loss of biomass centrifugation efficiency, resulting from biomass loss during the centrifugation process. This led to a revenue reduction ranging from $123.59 to $4.65 (Table 3). Meanwhile, the total process costs varied between $0.55 and $2.96 (Table 3).

In the current scenario (Table 4), with a selling price of $406.37, all net benefit/cost values were negative, indicating that this selling value does not cover the additional costs associated with the changes in the process. Therefore, a scenario of a 10% sales price increase was evaluated (Table 5) since the washing process extends the biomass shelf life (Couto et al., 2024). In this scenario, the optimal net benefit-cost was observed when

repeating the process 5 times at 3.405 g-force at 18°C for 25 minutes (Equation 17). Using the desirability index function (Figure 1), the combination for maximizing cell viability, bacteria removal efficiency, and PBA is to repeat the process 5 times at a 3.405 g-force at 24°C for 20 minutes.

$$PBA\ (\$) = 14.55 + 8.28 * X_2 - 7.93 * X_3 + 21.04 * X_4 - 10.88 * X_4^2 \quad \text{Equation 17}$$

Where, X_2 = g-force; X_3 = temperature in °C and X_4 = time in minutes.

Table 3: Benefits and costs considered in the PBA for each run. Centrifugation efficiency (CE), Gross receipt (GR), Reduced revenue (RR), Interest over investment (IOI), Cost with energy (EC), Labor cost (LC), Interest on operating costs (IOC), Depreciation by process (DP) and Additional costs (AC).

Run	Benefits			Costs					
	CE (%)	GR ($)	RR ($)	IOI ($)	EC ($)	LC ($)	IOC ($)	DP ($)	AC ($)
1	91.44	371.61	34.77	0.01	0.06	0.62	0.03	0.02	0.74
2	94.16	382.65	23.72	0.02	0.12	1.23	0.07	0.05	1.49
3	81.19	329.95	76.42	0.01	0.04	0.62	0.03	0.02	0.73
4	91.49	371.78	34.59	0.02	0.08	1.23	0.07	0.05	1.45
5	92.16	374.52	31.85	0.01	0.07	0.62	0.03	0.02	0.75
6	98.29	399.44	6.93	0.02	0.13	1.23	0.07	0.05	1.51
7	83.68	340.06	66.31	0.01	0.05	0.62	0.03	0.02	0.73
8	98.03	398.37	8.00	0.02	0.10	1.23	0.07	0.05	1.47
9	92.47	375.77	30.61	0.02	0.12	1.23	0.07	0.05	1.49
10	96.38	391.66	14.71	0.05	0.24	2.47	0.14	0.09	2.98
11	84.60	343.77	62.60	0.02	0.08	1.23	0.07	0.05	1.45
12	92.59	376.27	30.10	0.05	0.17	2.47	0.13	0.09	2.91
13	96.26	391.17	15.21	0.02	0.13	1.23	0.07	0.05	1.51
14	98.86	401.72	4.65	0.05	0.27	2.47	0.14	0.09	3.01
15	88.81	360.89	45.48	0.02	0.10	1.23	0.07	0.05	1.47
16	98.42	399.96	6.41	0.05	0.20	2.47	0.13	0.09	2.94
17	93.71	380.82	25.55	0.01	0.04	0.46	0.03	0.02	0.55

18	93.30	379.16	27.21	0.04	0.20	2.31	0.13	0.09	2.77
19	91.27	370.90	35.48	0.03	0.11	1.39	0.07	0.05	1.65
20	98.43	400.01	6.36	0.03	0.14	1.39	0.08	0.05	1.68
21	95.37	387.54	18.83	0.03	0.16	1.39	0.08	0.05	1.71
22	94.64	384.58	21.79	0.03	0.08	1.39	0.07	0.05	1.62
23	69.59	282.78	123.59	0.01	0.04	0.46	0.03	0.02	0.55
24	98.76	401.35	5.02	0.04	0.20	2.31	0.13	0.09	2.77
25	92.47	375.78	30.59	0.03	0.12	1.39	0.08	0.05	1.66
26	95.53	388.21	18.16	0.03	0.12	1.39	0.08	0.05	1.66
27	92.39	375.43	30.94	0.03	0.12	1.39	0.08	0.05	1.66

Table 4: PBA for the addition of the washing process in the production of *Nannochloropsis oceanica* concentrates. Additional revenue (AR), Reduced costs (RC), Total additional benefits (TAB), Additional costs (AC), Reduced revenue (RR) and Total additional costs (TAC).

Run	**Benefits**			**Costs**			**Net Benefit-cost ($)**
	AR ($)	**RC ($)**	**TAB ($)**	**AC ($)**	**RR ($)**	**TAC ($)**	
1	0	0	0	0.74	34.77	35.51	-35.51
2	0	0	0	1.49	23.72	25.21	-25.21
3	0	0	0	0.73	76.42	77.15	-77.15
4	0	0	0	1.45	34.59	36.04	-36.04
5	0	0	0	0.75	31.85	32.60	-32.60

6	0	0	0	1.51	6.93	8.44	-8.44
7	0	0	0	0.73	66.31	67.04	-67.04
8	0	0	0	1.47	8.00	9.47	-9.47
9	0	0	0	1.49	30.61	32.10	-32.10
10	0	0	0	2.98	14.71	17.69	-17.69
11	0	0	0	1.45	62.60	64.05	-64.05
12	0	0	0	2.91	30.10	33.00	-33.00
13	0	0	0	1.51	15.21	16.71	-16.71
14	0	0	0	3.01	4.65	7.66	-7.66
15	0	0	0	1.47	45.48	46.95	-46.95
16	0	0	0	2.94	6.41	9.35	-9.35
17	0	0	0	0.55	25.55	26.10	-26.10
18	0	0	0	2.77	27.21	29.99	-29.99
19	0	0	0	1.65	35.48	37.12	-37.12
20	0	0	0	1.68	6.36	8.05	-8.05
21	0	0	0	1.71	18.83	20.53	-20.53
22	0	0	0	1.62	21.79	23.42	-23.42
23	0	0	0	0.55	123.59	124.15	-124.15
24	0	0	0	2.77	5.02	7.79	-7.79
25	0	0	0	1.66	30.59	32.26	-32.26
26	0	0	0	1.66	18.16	19.83	-19.83
27	0	0	0	1.66	30.94	32.61	-32.61

Table 5: PBA for the addition of the washing process to the production of *Nannochloropsis oceanica* concentrates, considering a 10% increase in the selling price. Additional revenue (AR), Reduced costs (RC), Total additional benefits (TAB), Additional costs (AC), Reduced revenue (RR) and Total additional costs (TAC).

Run	Benefits			Costs			Net Benefit-cost ($)
	AR ($)	RC ($)	TAB ($)	AC ($)	RR ($)	TAC ($)	
1	2.37	0	2.37	0.74	0	0.74	1.63
2	14.53	0	14.53	1.49	0	1.49	13.04
3	0	0	0	0.73	43.45	44.17	-44.17
4	2.60	0	2.60	1.45	0	1.45	1.14
5	5.59	0	5.59	0.75	0	0.75	4.84
6	32.99	0	32.99	1.51	0	1.51	31.49
7	0.00	0	0	0.73	32.31	33.05	-33.05
8	31.83	0	31.83	1.47	0	1.47	30.36
9	6.98	0	6.98	1.49	0	1.49	5.49
10	24.46	0	24.46	2.98	0	2.98	21.48
11	0	0	0	1.45	28.20	29.66	-29.66
12	7.51	0	7.51	2.91	0	2.91	4.61
13	23.92	0	23.92	1.51	0	1.51	22.41
14	35.54	0	35.54	3.01	0	3.01	32.53
15	0	0	0	1.47	0	10.85	-10.85
16	33.57	0	33.57	2.94	0	2.94	30.64

17	12.52	0	12.52	0.55	0	0.55	11.97
18	10.69	0	10.69	2.77	0	2.77	7.91
19	1.61	0	1.61	1.65	0	1.65	-0.03
20	33.62	0	33.62	1.68	0	1.68	31.94
21	19.94	0	19.94	1.71	0	1.71	18.23
22	16.68	0	16.68	1.62	0	1.62	15.05
23	0	0	0	0.55	95.30	95.85	-95.85
24	35.09	0	35.09	2.77	0	2.77	32.32
25	6.98	0	6.98	1.66	0	1.66	5.31
26	20.66	0	20.66	1.66	0	1.66	18.99
27	6.62	0	6.62	1.66	0	1.66	4.96

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8.4 Discussion

The washing process was optimized to reduce bacterial contamination of *Nannochloropsis oceanica* concentrates and increase the net benefit-cost. To this end, four centrifugation variables were tested: number of times the process was repeated, centrifugation speed, temperature, and time. To be considered efficient, the washing process had to meet some requirements: not harm the cell viability of the microalgae, present a high rate of bacterial removal, and be economically viable. To meet all these requirements, it was necessary to evaluate different responses from the same design and find the combination that covers all these responses. In general, the number of repetitions, centrifugation speed, and time positively influenced the responses analyzed. The higher these values, the better the response obtained. The temperature, in turn, presented divergent effects. Although the decrease in temperature increased centrifugation efficiency, it decreased cell viability and the net benefit-cost. This difference made it necessary to combine the responses and thus obtain the ideal combination. The combination that provided the best result combining cell viability, bacteria removal efficiency and the net benefit-cost was repeating the process 5 times, with 3.405 g-force at 24°C for 20 minutes.

Washing is essential to maintain the quality of concentrates for longer (Couto et al., 2024). When centrifugation is not 100% efficient, it causes biomass losses that reduce expected revenue since the quantity sold is also lower. Thus, centrifugation efficiency directly impacts revenue and, consequently, the net benefit-cost of the process. Revenue is essential to making the process viable (Engle, 2010). In the case of concentrates, cell viability cannot be affected since they are preferred in aquaculture due to the viability of their cells (Dineshbabu et al., 2019) that help maintain water quality, unlike dry diets (Borowitzka, 2013; Dineshbabu et al., 2019). In addition, there was also a gain in the shelf life of the concentrate because it reduces the concentration of bacteria, which can allow an increase in the concentrate's sale price (Couto et al., 2024).

Bacteria can be removed in two ways. Since bacterial cells are lighter than microalgae cells, the microalgae settle at the correct centrifugation speed, and the bacteria remain in the water column. The other way is through cell disruption. Initially, increasing the

centrifugation speed was believed to increase the concentration of bacteria that sediment together with the microalgae. *N. oceanica* is a microalgae known for having an extremely resistant cell wall, which allows it to withstand the application of high mechanical force (Bernaerts et al., 2019; Lemahieu et al., 2016). Bacteria, in turn, have a simplified prokaryotic structure, making them more susceptible to cell disruption (Jaiganesh and Kumar, 2012). Although it is not clear which speed causes the bacteria to break, this pattern can be easily observed in Figure 1 in the second graph of the first row, where the bacteria removal efficiency was greater at the extreme centrifugation speeds. A g-force of 2.000 to 2.417 causes a bigger accumulation of bacteria in the concentrates. Although the lower centrifugation speed generates a biomass with less bacteria, this speed is not recommended because there is a lot of biomass loss.

Process optimization is an important tool in the construction of protocols and products that are highly relevant in research and industry (Wijffels and Barbosa, 2010). Especially for the industry, it is necessary to ensure the viability of the process. Several studies have used the optimization methodology in the process of the microalgae production chain (Kumaran et al., 2017; Ryckebosch et al., 2012; Seyed Hosseini et al., 2018) enabling increased productivity of systems and extraction of compounds of interest. Despite the fact that the information obtained in these studies is essential to make this production chain viable, an extremely important step in optimization is missing: cost analysis. Although microalgae have great potential for application in the food, pharmaceutical, and nutraceutical industries, the cost of production and collection is still much higher than the cost of other raw materials (Borowitzka, 2013; Ruiz et al., 2016).

The PBA showed negative net benefit-cost when the current selling price was considered. The revenue estimated for this pricing condition did not cover the costs associated with the washing process. Thus, a new scenario was investigated by considering a 10% increase in the selling price (Table 5), which yielded positive net benefit-cost results. This increase in the selling price is easily justified by the increased shelf life of the new concentrate from 17 to 85 days, as demonstrated in a previous work (Couto et al., 2024) . The PBA is a tool for making financial decisions regarding the adoption of a process change (Engle, 2010). However, this analysis considered only experimental conditions,

and a complete economic feasibility analysis considering commercial conditions is recommended to provide applicable insights to the industry.

8.5 Conclusions

The washing process was optimized to increase the efficiency rate of bacterial removal without affecting cell viability in *Nannochloropsis oceanica* concentrates. To achieve this, the concentrate needs to be centrifuged using a g-force of 3.405 at 24°C for 20 minutes five times. Although an optimization was made to make the process economically viable, the selling price of the concentrate should increase by 10%, which can be justified by the increase in shelf life.

References

Borowitzka, M.A., 2013. High-value products from microalgae-their development and commercialisation. J Appl Phycol 25, 743–756. https://doi.org/10.1007/s10811-013-9983-9

Couto, C., Hernández, C.P., Alves Sobrinho, R.C.M., Mendes, C.R.B., Roselet, F., Abreu, P.C., 2021. Optimization of a low-cost fertilizer-based medium for large-scale cultivation of the coastal diatom Conticribra weissflogii using response surface methodology and its effects on biomass composition. J Appl Phycol 33, 2767–2781. https://doi.org/10.1007/s10811-021-02519-8

Couto, C., Roselet, F., Kubelka, B., de Oliveira Arias, J.L., Primel, E.G., Bianchini, A., Abreu, P.C., Krummenauer, D., 2024. Effect of removing bacteria and dissolved organic matter on DMS production in Nannochloropsis oceanica concentrates. Algal Res 80, 103534. https://doi.org/10.1016/j.algal.2024.103534

Dan, S., Ashidate, M., Yamashita, T., Hamasaki, K., 2018. Effects of thermal disinfection and autoclave sterilisation on the quality of microalgae concentrates. Aquac Res 49, 3559–3568. https://doi.org/10.1111/are.13822

Dewi, I.C., Falaise, C., Hellio, C., Bourgougnon, N., Mouget, J.L., 2018. Anticancer, antiviral, antibacterial, and antifungal properties in microalgae, Microalgae in Health and Disease Prevention. Elsevier Inc. https://doi.org/10.1016/B978-0-12-811405-6.00012-8

Dineshbabu, G., Goswami, G., Kumar, R., Sinha, A., Das, D., 2019. Microalgae–nutritious, sustainable aqua- and animal feed source. J Funct Foods 62, 103545. https://doi.org/10.1016/j.jff.2019.103545

Engle, C.R., 2010. Aquaculture Economics and Financing, Universitas Nusantara PGRI Kediri. Wiley. https://doi.org/10.1002/9780813814346

Giraldo, J.B., Stock, W., Dow, L., Roef, L., Willems, A., Mangelinckx, S., Kroth, P.G., Vyverman, W., Michiels, M., 2019. Influence of the algal microbiome on biofouling during industrial cultivation of Nannochloropsis sp. in closed photobioreactors. Algal Res 42, 101591. https://doi.org/10.1016/j.algal.2019.101591

Giroldo, D., Ortolano, P.I.C., Vieira, A.A.H., 2007. Bacteria-algae association in batch cultures of phytoplankton from a tropical reservoir: The significance of algal carbohydrates. Freshw Biol 52, 1281–1289. https://doi.org/10.1111/j.1365-2427.2007.01764.x

Guillard, R.R.L., 1975. Culture of Phytoplankton for Feeding Marine Invertebrates, in: Culture of Marine Invertebrate Animals. https://doi.org/10.1007/978-1-4615-8714-9_3

Jaiganesh, R., Kumar, N.S.S., 2012. Marine Bacterial Sources of Bioactive Compounds, 1st ed, Marine Medicinal Foods. Elsevier Inc. https://doi.org/10.1016/B978-0-12-416003-3.00025-1

Kirrolia, A., Bishnoi, N.R., Singh, R., 2014. Response surface methodology as a decision-making tool for optimization of culture conditions of green microalgae Chlorella spp. for biodiesel production. Ann Microbiol 64, 1133–1147. https://doi.org/10.1007/s13213-013-0752-4

Kubelka, B.G., Roselet, F., Pinto, W.T., Abreu, P.C., 2018. The action of small bubbles in increasing light exposure and production of the marine microalga Nannochloropsis oceanica in massive culture systems. Algal Res 35, 569–576. https://doi.org/10.1016/j.algal.2018.09.030

Kumaran, J., Jose, B., Joseph, V., Bright Singh, I.S., 2017. Optimization of growth requirements of marine diatom Chaetoceros muelleri using Response Surface Methodology. Aquac Res 48, 1513–1524. https://doi.org/10.1111/are.12987

Lian, J., Steinert, G., de Vree, J., Meijer, S., Heryanto, C., Bosma, R., Wijffels, R.H., Barbosa, M.J., Smidt, H., Sipkema, D., 2022. Bacterial diversity in different outdoor pilot plant photobioreactor types during production of the microalga Nannochloropsis sp. CCAP211/78. Appl Microbiol Biotechnol 106, 2235–2248. https://doi.org/10.1007/s00253-022-11815-3

Natrah, F.M.I., Bossier, P., Sorgeloos, P., Yusoff, F.M., Defoirdt, T., 2014. Significance of microalgal-bacterial interactions for aquaculture. Rev Aquac 6, 48–61. https://doi.org/10.1111/raq.12024

Roselet, M., Roselet, F., Abreu, P.C., 2019. Foam fractionator as a tool to remove dissolved organic matter and improve the flocculation of the marine microalga

Nannochloropsis oceanica. J Appl Phycol 31, 2911–2919. https://doi.org/10.1007/s10811-019-01801-0

Ruiz, J., Olivieri, G., De Vree, J., Bosma, R., Willems, P., Reith, J.H., Eppink, M.H.M., Kleinegris, D.M.M., Wijffels, R.H., Barbosa, M.J., 2016. Towards industrial products from microalgae. Energy Environ Sci 9, 3036–3043. https://doi.org/10.1039/c6ee01493c

Ryckebosch, E., Muylaert, K., Foubert, I., 2012. Optimization of an analytical procedure for extraction of lipids from microalgae. JAOCS, Journal of the American Oil Chemists' Society 89, 189–198. https://doi.org/10.1007/s11746-011-1903-z

Saxena, R., Rodrı, R.M., Gonza, C., 2023. A plausible scenario for the third generation of biofuels from microalgae 107–121. https://doi.org/10.1016/B978-0-12-824318-3.00013-8

Seyed Hosseini, N., Shang, H., Scott, J.A., 2018. Optimization of microalgae-sourced lipids production for biodiesel in a top-lit gas-lift bioreactor using response surface methodology. Energy 146, 47–56. https://doi.org/10.1016/j.energy.2017.08.085

Talent.com, 2024. Salário Médio de Trabalhador Rural em Brasil 2024 [WWW Document]. URL https://br.talent.com/salary?job=trabalhador+rural#:~:text=Descobre qual é a média salarial de um(a) Trabalhador Rural&text=O salário médio de trabalhador,a ganhar R%2424.000 anuais (accessed 9.16.24).

Wijffels, R.H., Barbosa, M.J., 2010. An outlook on microalgal biofuels. Science (1979) 329, 796–799. https://doi.org/10.1126/science.1189003

Zuo, Z., 2019. Why algae release volatile organic compounds - The emission and roles. Front Microbiol 10, 1–7. https://doi.org/10.3389/fmicb.2019.00491

9 DISCUSSÃO GERAL

O presente estudo teve por objetivo otimizar a produção de concentrados da microalga *Nannochloropsis oceanica*, para isso, foram feitos três experimentos. No primeiro experimento foi estabelecida a relação entre a presença de bactérias e as concentrações de matéria orgânica (MO) e dimetilsulfeto (DMS) nos concentrados ao longo do tempo. Nos dois tratamentos avaliados, com e sem bactérias, houve um aumento da concentração de MO no decorrer do tempo. Porém, na presença de bactérias esses valores foram muito maiores, pois houve uma maior quantidade de MO produzida pelas próprias bactérias, além da MO que já era produzida pelas microalgas na forma de exopolissacarideos (EPS) e por compostos liberados devido à morte celular. Um desses compostos é o dimetilsulfoniopropriánato (DMSP) que na presença de grupos de bactérias como as *Roseobacter* é utilizado como substrato para o seu metabolismo liberando para o ambiente DMS, um composto orgânico volátil (VOC) que apresenta um cheiro desagradável de enxofre com baixo limiar, ou seja, é detectável mesmo em baixas concentrações (Miller & Belas, 2004; X.-H. Zhang et al., 2019). A presença do DMS diminuiu consideravelmente a vida útil dos concentrados pois conferiu um cheiro e aparência desagradável.

Para diminuir a carga orgânica (bactérias e matéria orgânica dissolvida) nos concentrados foi utilizado o método de lavagem. Esse método consiste em diluir os concentrados em água do mar e fazer sucessivas centrifugações, onde entre cada centrifugação o sobrenadante é descartado. Embora o método tenha sido eficiente para esse propósito, o uso da centrífuga acrescenta um custo elevado ao processo de produção de concentrados. Por esse motivo, foi analisada uma forma alternativa de remoção, o *skimmer,* que apresenta um valor de instalação bem menor que o valor de uma centrífuga. No segundo experimento, além de comparar o uso do *skimmer* com a lavagem, foi testada a junção dos métodos e uma segunda forma de lavagem com menos repetições do processo visando diminuir o custo de operação do equipamento. No entanto, o uso do *skimmer* não foi eficiente nesse trabalho, diferente do que foi observado em trabalhos anteriores em cultivo de *Nannochloropsis oceanica* (Roselet et al., 2019) onde o *skimmer* diminuiu consideravelmente a concentração de matéria orgânica no cultivo. Por outro lado, foram encontrados bons resultados na combinação do uso do *skimmer* com a lavagem que apesar de ter apresentado as maiores concentrações de matéria orgânica, diminuiu consideravelmente a concentração de bactérias e DMS. Além disso, os

resultados mostraram uma diferença entre os dois tratamentos com os métodos de lavagem à longo prazo. O tratamento com mais repetições foi significativamente mais eficiente que o tratamento com menos repetições. Assim, como a utilização do *skimmer* depende da utilização da lavagem e a lavagem feita nas condições ideais dispensa o uso do *skimmer*, no terceiro experimento foi feita uma otimização do método de lavagem visando aumentar a taxa de remoção das bactérias sem afetar a viabilidade das células e fazer uma análise de custo para viabilizar o processo. A análise de custo parcial (PBA) mostrou que o processo não era viável com o preço de venda estipulado. No entanto, como o processo aumenta a vida útil dos concentrados, um aumento no preço de venda é justificado. A partir desse aumento, uma otimização foi feita combinando as respostas de taxa de remoção de bactérias, viabilidade celular e relação benefício-custo. Repetir o processo 5 vezes em uma força g de 3,405 em 24°C por 20 minutos garante uma maior taxa de remoção de bactérias sem influenciar na viabilidade das células com uma boa relação de benefício-custo. Por fim, a combinação das respostas obtidas nos três experimentos garantiu um produto com uma vida útil mais longa a partir da identificação do problema, busca por soluções e de viabilizar um processo.

10 CONCLUSÕES GERAIS

- Há uma relação direta entre a densidade bacteriana e a produção de matéria orgânica dissolvida e DMS nos concentrados de *Nannochloropsis oceanica* que afetam diretamente as características sensoriais (cor e cheiro) e consequentemente a vida útil dos concentrados*;*
- Para garantir uma vida útil mais extensa dos concentrados é necessário diminuir a concentração de bactérias garantindo que os concentrados possam permanecer por até 8 dias em temperatura ambiente (23°C) e 85 dias em temperatura de refrigeração (4°C);
- O *skimmer* não foi considerado eficiente como forma de diminuir a densidade bacteriana e prolongar a vida útil dos concentrados;
- O processo de lavagem é uma ótima alternativa para diminuir o desenvolvimento de bactérias que geram matéria orgânica e DMS nos concentrados;
- Para viabilizar o processo de lavagem foi necessário aumentar o valor de venda dos concentrados em 10% com a justificativa do aumento da vida útil dos concentrados;

- A lavagem foi otimizada para manter as células das microalgas viáveis, ter a melhor taxa de remoção de bactérias e a melhor relação benefício-custo. Para isso, o processo precisa ser repetido 5 vezes em 3,405 de força g na temperatura de 24°C por 20 minutos.

11 TRABALHOS FUTUROS

Os resultados obtidos na presente tese apontaram para o efeito direto da presença das bactérias nas concentrações de DOM e DMS e o efeito da lavagem nessas bactérias, propõem-se estudos utilizando técnicas como a análise metagenômica e o *fish* para identificar os grupos de bactérias mais afetados pelo processo de lavagem e a sua relação direta com a presença de DOM e DMS ao longo do tempo de armazenamento dos concentrados.

Baseado nos resultados obtidos e na literatura que aponta para o efeito de DMS na predação das microalgas, novos estudos podem ser feitos para avaliar a utilização desses concentrados na aquicultura. Por exemplo, um estudo comparativo entre um concentrado sem lavagem e um concentrado que passou pelo processo de lavagem no cultivo de zooplânctons como *Brachionus plicatilis*.

Além disso, assim como a lavagem, o uso do *skimmer* também pode ser otimizado durante o cultivo das microalgas. Fatores como a frequência de uso e em que momento do cultivo o *skimmer* deve ser utilizado podem ser otimizados para obter um cultivo com menor concentração de DOM e DMS.

REFERÊNCIAS

Miller, T.R., Belas, R., 2004. Dimethylsulfoniopropionate Metabolism by Pfiesteria - Associated *Roseobacter* spp. Appl Environ Microbiol 70, 3383–3391. https://doi.org/10.1128/AEM.70.6.3383-3391.2004

Roselet, M., Roselet, F., Abreu, P.C., 2019. Foam fractionator as a tool to remove dissolved organic matter and improve the flocculation of the marine microalga *Nannochloropsis oceanica*. J Appl Phycol 31, 2911–2919. https://doi.org/10.1007/s10811-019-01801-0

Zhang, X.-H., Liu, J., Liu, J., Yang, G., Xue, C.-X., Curson, A.R.J., Todd, J.D., 2019. Biogenic production of DMSP and its degradation to DMS—their roles in the global sulfur cycle. Sci China Life Sci 62, 1296–1319. https://doi.org/10.1007/s11427-018-9524-y