**CLARISSA VILELA FIGUEIREDO DA SILVA CAMPOS**

# UTILIZAÇÃO DE *Daphnia* SP. ALIMENTADA COM *Haematococcus pluvialis* E *Chlorella vulgaris* NO CULTIVO BERÇÁRIO DO CAMARÃO LITOPENAEUS VANNAMEI EM SISTEMA DE BIOFLOCOS

**RECIFE,**

**2022**

**UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO**
**PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO**
**PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA**

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# USE OF *Daphnia* sp. FED WITH *Haematococcus pluvialis* AND *Chlorella vulgaris* IN NURSERY CULTURE OF SHRIMP *Litopenaeus vannamei* IN A BIOFLOC SYSTEM

**Clarissa Vilela Figueiredo da Silva Campos**

Tese apresentada ao Programa de Pós-Graduação em Recursos Pesqueiros e Aquicultura da Universidade Federal Rural de Pernambuco como exigência para obtenção do título de Doutora.

**Prof.(a) Dr.(a) Alfredo Olivera Gálvez**
Orientador

**Prof.(a) Dr.(a) Suzianny Maria Bezerra Cabral da Silva**
Coorientadora

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Tese julgada adequada para obtenção do título de Doutora em Recursos Pesqueiros e Aquicultura. Defendida e aprovada em 30/11/2022 pela seguinte Banca Examinadora.

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**Prof(a). Dr. ALFREDO OLIVERA GÁLVEZ - Orientador**
[Departamento de Pesca e Aquicultura]
[Universidade Federal Rural de Pernambuco - Sede]

---

**Prof(a). Dr PAULO GUILHERME DE OLIVEIRA (Membro interno) Titular**
[Departamento de Pesca e Aquicultura]
[Universidade Federal Rural de Pernambuco – Sede]

---

**Prof(a). Dra. GELCIRENE DE ALBUQUERQUE COSTA (Membro externo). -Titular**
[Departamento de Pesca e Aquicultura]
[Universidade Federal Rural de Pernambuco - Sede]

---

**Prof(a). Dra. JÉSSIKA LIMA DE ABREU (Membro externo) – Titular**
[Departamento de Pesca e Aquicultura]
[Universidade Federal Rural de Pernambuco – Campus Serra Talhada]

---

**Prof(a). Dr. PAULO ROBERTO CAMPAGNOLI DE OLIVEIRA FILHO (Membro interno) – Titular**
[Departamento de Pesca e Aquicultura]
[Universidade Federal Rural de Pernambuco - Sede]

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**Ficha catalográfica**

Dados Internacionais de Catalogação na Publicação
Universidade Federal Rural de Pernambuco
Sistema Integrado de Bibliotecas

Gerada automaticamente, mediante os dados fornecidos pelo(a) autor(a)

C198u Campos, Clarissa Vilela Figueiredo da Silva
UTILIZAÇÃO DE Daphnia sp. ALIMENTADA COM *Haematococcus pluvialis* E *Chlorella vulgaris* NO CULTIVO BERÇÁRIO DO CAMARÃO LITOPENAEUS VANNAMEI EM SISTEMA DE BIOFLOCOS / Clarissa Vilela
Figueiredo da Silva Campos. - 2022.
106 f. : il.
Orientador: Alfredo Olivera Galvez.
Coorientadora: Suzianny Maria Bezerra Cabral da Silva.
Inclui referências.
Tese (Doutorado) - Universidade Federal Rural de Pernambuco, Programa de Pós-Graduação em Recursos Pesqueiros
e Aquicultura, Recife, 2023.
1. Daphnia. 2. Chlorella. 3. Haematococcus. 4. Sustentabilidade. 5. bioflocos. I. Galvez, Alfredo Olivera, orient. II.
Silva, Suzianny Maria Bezerra Cabral da, coorient. III. Título
CDD 639.3

**Sumário**

**Dedicatória**

*Dedico este trabalho a minha família.*

**Agradecimentos**

Gostaria de agradecer primeiramente a Deus por ter me ajudado e me dado forças para continuar e finalizar mais uma etapa na minha vida profissional.

A minha família, em especial ao meu pai, minha madrasta, minha sogra e meu sogro por sempre me ajudarem e darem o apoio cuidando da minha filha Júlia quando em muitos momentos não pude cuidar dela e tive que ir à faculdade.

Ao meu esposo Lucilo por toda compreensão e apoio diante de várias situações difíceis.

À CAPES pela bolsa concedida.

À família LAPAVI e LAMARSU principalmente aos estagiários que sempre estiveram a postos para a resolução de problemas e oferta de ajuda.

Aos meus orientadores (Alfredo Olivera Gálvez e Suzianny Maria Bezerra Cabral da Silva) pela ajuda e conselhos oferecidos para obter sempre bons resultados.

Ao prof. Luís Otávio Brito pelas orientações sempre bem passadas e que esteve sempre disposto a ajudar.

Ao engenheiro de pesca Djaci pela concessão de tilápias da base de pesca.

A todos aqui mencionados e aqueles que por ventura esqueci, ficam aqui os meus agradecimentos.

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## Resumo

Medidas para o reúso de efluentes aquícolas têm sido priorizadas a fim de obter uma aquicultura sustentável. A utilização de efluentes aquícolas para produção de alimento vivo pode ser uma alternativa promissora. Dentro deste panorama, este estudo avaliou o uso do efluente do cultivo de tilápia do Nilo em sistema de bioflocos para a produção da pulga d'água *Daphnia similis* e *D. magna* a partir de dois momentos experimentais: 1) Influência do processamento do efluente sedimentado e não sedimentado combinado com diferentes salinidades (1, 2, 3 e 4) no cultivo da *D. similis;* e 2) Influência do sistema de cultivo autotrófico (sem efluente) e mixotrófico (com efluente) combinados com diferentes dietas microalgais: *Chlorella vulgaris* e *Haematococcus pluvialis* (fase cística e vegetativa) no crescimento e composição nutricional da *D. magna*. No primeiro experimento, foi reportado que o uso do efluente não sedimentado combinado com a salinidade 3 obteve melhor crescimento de biomassa, enquanto que o efluente sedimentado na salinade 2 houve melhor biorremediação a partir da redução dos compostos nitrogenados e ortofosfato. No segundo momento experimental, a utilização de sistema mixotrófico combinado com o uso de *Chlorella vulgaris* possibilitou melhores resultados de crescimento, concentrações de lipídeos (7,8%) e proteínas (61,2%) para *D. magna*, Os cultivos com a oferta de *H. pluvialis* na fase cística apresentou maiores reduções de compostos nitrogenados e ortofosfato, apesar de que não obteve sucesso de crescimento populacional da pulga d'água, pois houve morte de 100% dos indivíduos ao quarto dia de cultivo. Desta forma, os achados desta pesquisa contribuem para uma melhor avaliação da utilização de efluentes para a produção de alimento vivo para a aquicultura bem como no tratamento desses resíduos através da biorremediação, fomentando a aquisição de uma aquicultura mais sustentável para o setor produtivo e novas fontes alternativas de proteínas.

Palavras chave: *Daphnia*, *Chlorella*, *Haematococcus*, sustentabilidade, bioflocos, aquicultura.

## Abstract

Management for the reuse of aquaculture effluents have been prioritized in order to achieve sustainable aquaculture. The use of aquaculture effluents for the production of live food can be a promising alternative. Within this scenario, this study evaluated the use of effluent from the cultivation of Nile tilapia in a biofloc system for the production of the water flea *Daphnia similis* and *D. magna* from two experimental moments: 1) Use of effluent treatment (sedimentation and no-sedimentation) combined with different salinities (1, 2, 3 and 4) in the cultivation of *D. similis*; and 2) Influence of autotrophic (without effluent) and mixotrophic (with effluent) culture system combined with different microalgae diets: *Chlorella vulgaris* and *Haematococcus pluvialis* (cystic and vegetative phase) on the growth and nutritional composition of *D. magna*. In the first experiment, it was reported that no-sedimentation of effluent combined with salinity 3 had the best growth of water flea, while the sedimentation of effluent in salinity 2 had better bioremediation from the reduction of nitrogen compounds and orthophosphate. In the second experimental moment, the mixotrophic system using *C. vulgaris* as feed had better results of growth and increase of lipids and protein for *D. magna*. The cultures with *H pluvialis* in cystic phase as feed reported best reduction of nitrogen componds and orthophosphate, despite it had not achieved success on water flea growth, there was death of 100% of population of individuals on day 4 of cultivation. In this way, the findings of this research contribute to the management of production of live food for aquaculture and biorremediation of these wastewater promoting a better sustainable aquaculture for productive sector and new possibilities for alternative protein sources.

Key words: *Daphnia, Chlorella, Haematococcus*, sustainability, biofloc, aquaculture.

## INTRODUÇÃO

O sistema de bioflocos, a partir da formação dos flocos microbianos, tem como características o crescimento de microrganismos que auxiliam na manutenção da qualidade de água, redução do fator de conversão alimentar e competição com patógenos através da relação carbono:nitrogênio existente no sistema (EMERENCIANO et al., 2017). Os bioflocos são ricos em nutrientes, como: vitaminas e proteínas e apresentam atratividade para os camarões (SILVA et al., 2013), servindo como alimento complementar, e, em algumas espécies aquícolas, aumentando a taxa de crescimento (AVNIMELECH, 1999; BURFORD et al. 2004).

Esses benefícios deste sistema vêm sendo expressos em diferentes trabalhos com diversas espécies de camarões como *Litopenaues vannamei*, *Penaues monodon*, *Farfantepenaeus brasiliensis* e *Macrobrachium rosenbergii* (EMERENCIANO et al., 2012; ESPARZA-LEAL et al., 2016; KHATOON et al., 2016; XU et al., 2016; HUANG et al., 2017; MIAO et al., 2017). Shao et al. (2017) relataram que a substituição de 15% da farinha de peixe pala farinha do biofloco não proporcionou diferenças significativas no crescimento de *L. vannamei* (7,76 ± 0,61 g), podendo assim ser um ingrediente adequado para a formulação de rações.

Os flocos microbianos também têm demonstrado importância principalmente nas fases iniciais de desenvolvimento do camarão, onde o alimento natural possui grande relevância. Suita et al. (2016) comprovaram a partir de análise de isótopos estáveis de C e N que a contribuição do biofloco para o crescimento de músculo corporal das pós-larvas de L. vannamei variou de 47 a 54 % durante os estágios de desenvolvimento de PL1 a PL30 e atingindo um peso final de 36 ± 16 mg, mostrando assim que o camarão se alimenta do biofloco. Bons resultados produtivos também são observados nos cultivos de

L. vannamei em baixa salinidade em sistema de biofloco, como demonstrado por Esparza-Leal et al. (2017), onde pós-larvas de *L.vannamei* (0,09 g) atingiram em 28 dias de cultivo em salinidade de aproximadamente 9 $g.L^{-1}$ sobrevivência de 78%, peso final de 0,72 ± 0,08 g e produtividade de 0,17 kg.m-3. Este fato indica a possibilidade de cultivo desse crustáceo também em baixas salinidades, já que possui uma ampla faixa de tolerância 0,5 a 45 g.L-1 (TSANG e AGUILLÓN 2008).

Porém, um gargalo do biofloco é justamente as baixas concentrações de lipídeos, principalmente os ácidos graxos polinsaturados (PUFA). Mesmo utilizando diferentes fontes de carbono, as concentrações de lipídeos no sistema ainda são baixas, como relatado em Khanjani et al. (2017), onde o biofloco apresentou 0,86%, 1,14% e 2,18% de lipídeos (matéria seca) nos sistemas que utilizaram como fonte de carbono o melaço, amido e farelo de trigo, respectivamente.

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Devido a este fato, a manipulação de um alimento vivo no sistema proporciona uma elevação do conteúdo nutricional, refletindo em melhores taxas de crescimento e sobrevivência (BRITO et al., 2015). Dentro dos organismos ofertados destaca-se o zooplâncton. Este por sua vez apresenta-se como um importante elo entre o fitoplâncton e os outros níveis tróficos (LAVENS e SORGELOOS, 1996) servindo de alimento vivo e também como bioencapsulador. Brito et al. (2015) encontraram melhor desempenho zootécnico de *L.vannamei* cultivado em sistema de biofloco na fase berçário com a adição de microalga *Navicula* sp. e rotífero *Brachionus plicatilis* como fonte de alimento natural para o camarão.

Desta forma, um zooplâncton com grande potencial para a alimentação do camarão pode ser a *Daphnia* sp. Conhecido como “pulga d’água”, esse cladócero é muito utilizado como opção de alimento vivo na aquicultura, principalmente na piscicultura. Porém ainda

não avaliaram a sua utilização como alimento vivo para o camarão, já que náuplios de Artemia sp. é comumente ofertada. Além disso, morfologicamente, o neonato de *Daphnia* sp. possui aproximadamente o mesmo tamanho de um náuplio de *Artemia* recém eclodido, 500 μm (HOFF e SNELL 2004). Este tamanho encontra-se dentro do recomendado por Van Wyk (1999), onde para camarões com peso variando de 0,002 a 0,02 g é recomendada a oferta de alimento de tamanho de 400 a 600 μm.

Além dessas semelhanças, a *Daphnia* sp. também atua no aumento da resistência a agentes patógenos. Chiu et al. (2015) identificaram maior resistência em larvas de Lates calcarifer a Aeromonas hydrophila quando alimentadas com farinha de *Daphnia similis* (50 e 100 g.kg-1) o que pode ser explicada pelas elevadas quantidades de quitosana que elas possuem, como é o caso da *D. longispina* a qual apresenta uma variação de 75-76% de quitosana (KAYA et al., 2014), já que essa substância é considerada imune estimulante (CAHÚ et al., 2012).

Com relação aos parâmetros nutricionais, a Daphnia e o náuplio de Artemia são muito parecidos. Segundo Barrera et al. (2003), Daphnia sp., em matéria seca, possui um elevado valor proteico (50%) e valores de ácidos graxos da ordem de 20-27%. Em termos de perfil de aminoácidos, em peso seco, este microscrustáceo apresenta: arginina (10,26%), cistina (1,17%), histidina (2,69%), metionina (3,45%), triptofano (3,62%) e tirosina (4,27%) (TORRENTERA e TACON 1989). Já os náuplios de *Artemi*a apresentam aproximadamente 42,5% de proteína e de 12-32% de ácidos graxos (HOFF e SNELL 2004).

Porém, o valor nutricional de um zooplâncton está estritamente relacionado à sua dieta. E os melhores alimentos que se podem ofertar para um zooplâncton são as microalgas. Dentre as microalgas ofertadas na dieta destes cladóceros, destacam-se as clorofíceas *Haematococcus pluvialis* e *Chlorella vulgaris*. Alcántara-Azuara et al. (2014)

cultivando D. pulex sob diferentes dietas de microalgas obtiveram densidades de 1395 ± 24 ind.L-1 quando alimentadas com *C. vulgaris* e 1933 ± 60 ind.L-1 quando alimentadas com H. pluvialis. Porém, a utilização da oferta de H. pluvialis na fase de cistos como dieta para Daphnia sp. ainda não foi documentada.

A *H.pluvialis*, tanto na fase vegetativa (verde) quanto na fase cística (vermelha) produzem astaxantina, porém as maiores concentrações são documentadas na fase cística, variando um rendimento de 14 a 5,5 mg.L-1dia-1 dependendo do tipo de cultivo (KANG et al., 2009; HONG et al., 2016). Este carotenoide tem as propriedades de ser antioxidante (POGORZELSKA et al., 2018), imune estimulante e anticancerígeno (AMBATI et al., 2014) além de promover a pigmentação do tecido muscular do animal (YOUNG et al., 2016). Já a *C. vulgaris* apresenta aproximadamente, em peso seco, 30% de proteína e 10% de ácidos graxos (VILLARRUEL-LÓPEZ et al., 2017), onde, da quantidade total de ácidos graxos, 40,8% corresponde aos ácidos graxos poli-insaturados (PUFA) (TIBBETTS et al., 2017).

Além disso, a *Daphnia* sp. pode ser uma ótima opção para cultivos de *L.vannamei* em baixa salinidade, tolerando até 6 g.L-1 (EBERT 2005) e uma alternativa para o seu cultivo pode ser em utilizar o próprio bioflocos como meio de cultura. Campos (2017) reutilizou água de cultivo de biofloco de tilápia a 2 g.L-1 como meio de cultura para *Daphnia similis*, a qual foi alimentada com *C. vulgaris,* e obteve crescimento de até 800% maior do que em água clara, mostrando assim que esse cladócero pode ser facilmente cultivado reutilizando o próprio biofloco.

Nesse contexto, torna-se relevante avaliar a contribuição da inoculação do microcrustáceo *Daphnia* sp. alimentado com microalga *Haematococcus pluvialis* e/ou *Chlorella vulgaris* no cultivo berçário do camarão *Litopenaeus vannamei* em sistema de bioflocos.

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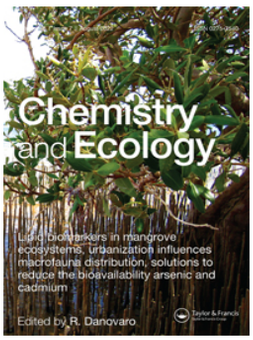
# CAPÍTULO I

Artigo Científico publicado na revista Chemistry and Ecology

**Consórcio *Chlorella-Daphnia* como uma ferramenta promissora para a biorremediação da criação em efluente de cultivo de tilápia do Nilo**

***Chlorella-Daphnia* consortium as a promising tool for bioremediation of Nile tilapia farming wastewater**

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ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/gche20

# *Chlorella-Daphnia* consortium as a promising tool for bioremediation of Nile tilapia farming wastewater

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**To cite this article:** Clarissa Vilela Figueiredo da Silva Campos, Carlos Yure B. Oliveira, Elizabeth Pereira dos Santos, Jéssika Lima de Abreu, William Severi, Suzianny Maria Bezerra Cabral da Silva, Luis Otavio Brito & Alfredo Olivera Gálvez (2022): *Chlorella-Daphnia* consortium as a promising tool for bioremediation of Nile tilapia farming wastewater, Chemistry and Ecology, DOI: 10.1080/02757540.2022.2120612

**To link to this article:** https://doi.org/10.1080/02757540.2022.2120612

Published online: 12 Sep 2022.

CHEMISTRY AND ECOLOGY
https://doi.org/10.1080/02757540.2022.2120612

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# *Chlorella-Daphnia* consortium as a promising tool for bioremediation of Nile tilapia farming wastewater

Clarissa Vilela Figueiredo da Silva Campos [a], Carlos Yure B. Oliveira [a], Elizabeth Pereira dos Santos [a], Jéssika Lima de Abreu [a], William Severi [b], Suzianny Maria Bezerra Cabral da Silva [c], Luis Otavio Brito [d] and Alfredo Olivera Gálvez [a]

[a]Laboratório de Produção de Alimento Vivo, Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco, Pernambuco, Brazil; [b]Laboratório de Limnologia, Universidade Federal Rural de Pernambuco, UFRPE, Rua Dom Manoel de Medeiros, Pernambuco, Brazil; [c]Laboratório de Sanidade de Animais Aquáticos, Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco, Pernambuco, Brazil; [d]Laboratório de Carcinicultura, Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco, Pernambuco, Brazil

**ABSTRACT**

The objective of this study was to investigate the effluent treatment from Nile tilapia farming in a biofloc system with a consortium of microalgae (*Chlorella vulgaris*) and zooplankton (*Daphnia similis)*. Thus, integrated cultures of *C. vulgaris* and *D. similis* were performed in two forms of wastewater treatment: sedimentation (S) and non-sedimentation (NS), in four different salinities (1, 2, 3 and 4 g $L^{-1}$). Water quality, growth of *D. similis*, behaviour of *C. vulgaris,* efficiency of removal of nitrogen compounds, orthophosphate, and total suspended solids (TSS) were measured. *D. similis* had higher density in 3NS ($p < 0.05$), while population die-off occurred in 4S and 4NS. The 2S and 1NS combinations stood out in bioremediation, achieving removal of up to 70.37% nitrate, 75.74% orthophosphate, and 90.74% TSS. 2S and 3S cultures became self-sufficient from day 21. Thus, the *Chlorella-Daphnia* consortium using 3NS allowed better production of *D. similis*, whereas salinities 2 g $L^{-1}$ (S) and 1 g $L^{-1}$ (NS) provided better bioremediation, and the use of S wastewater improved the sustainability of the system. These results contribute to a better evaluation of cultures in consortia of organisms for the treatment of aquaculture wastewater and the production of live feed for aquaculture.

**Highlights:**

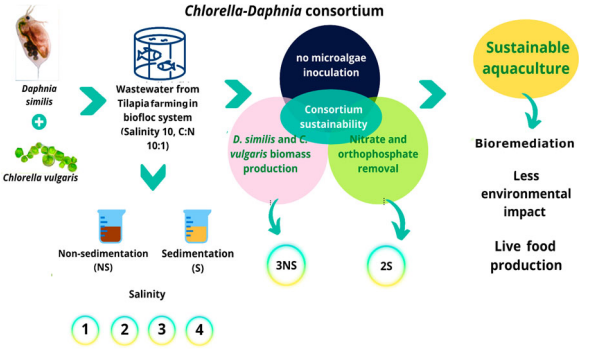
- Four salinities and two forms of biofloc wastewater processing were evaluated.
- Salinity 2 and sedimentation of biofloc wastewater showed better bioremediation.
- Salinity 3 and non-sedimentation of biofloc wastewater had better *D. similis* growth.
- *C. vulgaris* could grow in biofloc wastewater even with *Daphnia* predation.

**ARTICLE HISTORY**
Received 11 April 2022
Final Version Received 30 August 2022

**KEYWORDS**
Aquaculture; Nitrogen; Orthophosphate; BFT; Sustainability

**CONTACT** Clarissa Vilela Figueiredo da Silva Campos clarissavilela19@gmail.com Laboratório de Produção de Alimento Vivo, Departamento de Pesca e Aquicultura, Universidade Federal Rural de Pernambuco. Rua Dom Manoel de Medeiros, s/n, Dois irmãos, Recife, CEP: 52171-900, Pernambuco, Brazil

- *Chlorella-Daphnia* consortium is an option for bioremediation and live food production.




## 1. Introduction

In recent years, the farming of fish and shrimp in Biofloc systems (BFT) has emerged as an alternative to more sustainable aquaculture [1,2]. The use of this system can significantly reduce water consumption in aquaculture and the microbial flocs can provide an important food source for shrimp and fish. In addition, BFT allows better control of nitrogen compounds content, especially ammonia and nitrite, in addition to smaller area production, better animal health, reduction of pathological risks, and higher productivity [3–6]. Studies have reported the success of BFT in shrimp [2] and fish [7] farming. In particular, the Nile tilapia *Oreochromis niloticus* has shown satisfactory zootechnical performance when cultured in BFT, even in low salinity systems [8–11].

Despite these advantages, BFT has some bottlenecks that need more attention to ensure better efficiency of the system. One of them is the accumulation of nutrients such as nitrate [12] and phosphorus compounds [4,6] throughout the culture cycle. This problem makes it necessary to develop mechanisms to utilise these nutrients for valuable biomass production. Some studies have already been conducted using the effluent from BFT culture as a protein source for the production of feed [13], vegetables in hydroponics [14], microalgae [15,16], and zooplankton [17]. The cultivation of organisms using wastewater as a nutrient medium not only produces valuable biomass, but also improves the indices that indicate the quality of wastewater, using proven tools from ecological engineering. Therefore, the dissemination of practices that favour the development of the blue economy (i.e. optimised water use with low carbon dioxide emissions for sustainable, clean, and equitable food production) contributes to the sustainable development of green aquaculture [18,19].

The process of bioremediation is known for the use of beneficial microbiological agents to treat contaminated water or waste [20], where contaminated compounds are removed, reduced, or transformed by their own biological processes [21]. A consortium of microorganisms [22–24] may be an option for this process. In the consortium, the concepts of integrated multitrophic aquaculture (IMTA) are applied, which is based on the culture of species of different trophic levels sharing the same environment and

performing complementary functions that synergistically contribute to the maintenance of a balanced system [25,26].

The use of a consortium of microalgae and zooplankton in the process of bioremediation represents a promising option. On the one hand, microalgae are the basis of the food chain and convert inorganic nutrients (mainly nitrogen, phosphorus, and other trace elements) into valuable biomass [27–30]. On the other hand, zooplankton act as predators of bacteria, microalgae and detritus [31]. In this context, the microalgae *Chlorella vulgaris* and the microcrustaceans of the genus *Daphnia* are considered model organisms.

*C. vulgaris* is a green unicellular microalga that can grow under various conditions, either photoautotrophic, heterotrophic, or mixotrophic [32], and is one of the few microalgae capable of producing biomass and purifying aquaculture water [33–35]. Within the genus *Daphnia*, *D. magna* and *D. pulex* stand out for degrading organic matter, water solids, and heavy metals [36–38]. In addition, the diet of *Daphnia* spp. consists mainly of green microalgae, such as *Chlorella* spp. [39].

The use of *Chlorella* sp. in consortia with other algae, fungi, and bacteria has already been investigated for wastewater treatment [40,41]. For *Daphnia*, only one study has been carried out so far, dealing with the consortium of this microcrustacean with the macrophyte *Lemna minor* for the bioremediation of heavy metals [38]. Thus, the use of a consortium of *Chlorella* and *Daphnia* could be a promising option due to their trophic level. However, the efficiency of such a consortium has not yet been documented for the bioremediation of wastewater, especially low salinity aquaculture wastewater.

Therefore, the use of microalgae and zooplankton in consortia is a promising tool to obtain a new alternative for the use of tilapia farm effluent in low salinity BFT by combining the production of these organisms with the removal of inorganic compounds. As a case study on microbial consortia, our work aimed to evaluate the performance of intercropping *C. vulgaris* and *D. similis* when using wastewater from a low salinity BFT, in terms of (i) removal efficiency of nitrogen, phosphorus, and suspended solids compounds, (ii) production of these microorganisms, and (iii) equilibrium point of the consortium.

## 2. Material and methods

### 2.1. *Maintenance of strains of* Daphnia similis *and* Chlorella vulgaris

The *Daphnia similis* strain was maintained in a test tube (30 ml) and semi-continuous cultures were established in 2-L glass beakers with the microalga *Chlorella vulgaris* ad libitum every two days. Cultures were subjected to a natural photoperiod (12 h light, 12 h dark) with an irradiance of 30 µmols photons $m^{-2}$ $s^{-1}$ (10-W LED incandescent bulbs) adapted from Campos Clarissa Vilela et al. [17] and continuous aeration. A vitamin B solution (cyanocobalamin and biotin) was also added to the matrix cultures (0.2 mL $L^{-1}$). The water quality of the maintenance cultures was maintained at a pH of 7.2–7.8, a temperature of 25–27°C, an alkalinity of 35–50 mg $CaCO_3$ $L^{-1}$, and a salinity of 0.1–1.0 g $L^{-1}$.

The microalga *C. vulgaris* was cultured in Provasoli's culture medium (1 mL $L^{-1}$) [42] in an Erlenmeyer (2 L) with the addition of cyanocobalamin, thiamine, and biotin (0.2 mL $L^{-1}$) in volumes of 500 mL. Then, they were cultivated for production in larger volumes, namely 5 L tanks, in a semi-continuous system under constant light with an irradiance of 30 µmols photons $m^{-2}$ $s^{-1}$, adapted from Campos Clarissa Vilela et al. [17].

### 2.2. Experimental conditions

The entire experiment was conducted at the Laboratório de Produção de Alimento Vivo (LAPAVI [Laboratory of Live Food Production]) of the Departamento de Pesca e Aquicultura (DEPAq [Department of Fisheries and Aquaculture]) of the Universidade Federal Rural de Pernambuco (UFRPE [Federal Rural University of Pernambuco]). Culture of freshwater crustacean *D. similis* was conducted for 30 days in continuously aerated 1 L glass beakers using low salinity (10 g $L^{-1}$) effluent from the BFT culture of Nile tilapia. Adults (~1 mm in size) were used at a density of six organisms $L^{-1}$ [43]. Cultures were exposed to a natural photoperiod (12 h light, 12 h dark) with an irradiance of 30 µmol photons $m^{-2}$ $s^{-1}$ adapted from Campos Clarissa Vilela et al. [17]. Density was determined by counts every three days using the volumetric method, with five counts for each experimental unit [44]. On the same days of the counts, the microalga *Chlorella vulgaris* was inoculated in natura at a density of $1 \times 10^5$ cells $mL^{-1}$ *Daphnia*$^{-1}$ (adapted from Buratini, Aragão, [43] as needed, maintaining this minimum concentration of *C. vulgaris* cells *Daphnia*$^{-1}$.

### 2.3. Experimental design

Four salinities (1, 2, 3, and 4 g $L^{-1}$) and simple wastewater processing (sedimentation or non-sedimentation) were analyzed. Therefore, a $4 \times 2$ factorial design was used, with three replicates for each combination, resulting in 24 experimental units.

### 2.4. Wastewater treatment

The effluent from the BFT culture of Nile tilapia (*O. niloticus*) had the following characteristics: C:N of 10:1, 40 days of culture, stocking density of 40 fish $m^{-3}$, mean fish weight $30.57 \pm 10.4$ g, 25.66°C temperature, 7.55 pH, 8 mL $L^{-1}$ settleable solids, 110 mg $CaCO_3$ $L^{-1}$ alkalinity, 10 g $L^{-1}$ salinity, 6.45 mg $L^{-1}$ dissolved oxygen, 0.075 mg $L^{-1}$ nitrite, 12.51 mg $L^{-1}$ nitrate, 2.71 mg $L^{-1}$ total ammonia nitrogen, 0.227 mg $L^{-1}$ total suspended solids, and 6.09 mg $L^{-1}$ orthophosphate. The wastewater was treated by two methods: sedimentation (S) and non-sedimentation (NS). For the S effluent, the decantation time was 30 min, after which the supernatant was separated from the solids near the bottom and then used. The NS wastewater was used in raw form.

### 2.5. Adjustment of the salinity

After treatment (or non-treatment), the salinity of BFT wastewater (10 g $L^{-1}$) was adjusted using a handheld salinity refractometer (Kavasaki, model RHS – 10ATC) and administering freshwater and seawater (chlorinated 2 mL $L^{-1}$, dechlorinated with thiosulfate 0.3 mL $L^{-1}$, and filtered with filter paper of 80 g $m^{-2}$). Initially, the same volume of BFT wastewater (100 mL, salinity of 10 g $L^{-1}$) was used as a standard for the adjustment. To obtain the volume of 1 L (experimental units), the respective salinities (1, 2, 3 and 4 g $L^{-1}$) were adjusted in the following ratio: 1 g $L^{-1}$ (100 mL BFT wastewater and 900 mL freshwater), 2 g $L^{-1}$ (100 mL BFT wastewater, 100 mL seawater, and 800 mL freshwater), 3 g $L^{-1}$ (100 mL BFT wastewater, 200 mL seawater, and 700 mL freshwater), and 4 g $L^{-1}$ (100 mL BFT wastewater, 300 mL seawater, and 600 mL freshwater).

The salinity gradient up to 4 g $L^{-1}$ was taken as the basis because it is within the minimum tolerance range of the genus *Daphnia*, which ranges from 4 to 7 g $L^{-1}$ depending on the species [45].

### 2.6. Water quality

Dissolved oxygen (mg $L^{-1}$), salinity (g $L^{-1}$), pH, total suspended solids (TSS, mg $L^{-1}$), and temperature (°C) were selected to analyze water quality status. They were monitored with a multiparameter (YSI Model 100; Yellow Springs, OH, USA) daily at 10:00 am.

### 2.7. Growth of D. similis and residuals of C. vulgaris

Analysis of *D. similis* growth included determination of specific growth rate (SGR), doubling time (DT), yield (Y), maximum average density (MAD), and maximum density day (MDD), based on Otero et al. [46]. SGR, DT, and Y were calculated up to the MDD. SGR Equation (1), DT Equation (2), and Y Equation (3) were calculated using the following equations:

$$SGR = [(LnNt1\text{-}LnNt0)/t1] \times 100 \quad (1)$$

Where: Nt1: Final number of individuals. Nt0: Initial number of individuals. t1: Day of maximum density.

$$DT = Ln2/k \quad (2)$$

Where: Ln2: Natural logarithm of 2. k: Specific growth rate (SGR)

$$Y = Nt1\text{-}Nt0/t1 \quad (3)$$

Where: Nt1: Final number of individuals. Nt0: Initial number of individuals. t1: Day of maximum density.

The remains of *C. vulgaris* were examined every three days using a Neubauer chamber under a binocular microscope (magnification: 400x). The algal inoculum, Equation (4, 5), was added to the experimental units only when necessary to maintain the predetermined *Chlorella-Daphnia* concentration ($10^5$ cells $mL^{-1}$ *Daphnia*$^{-1}$).

$$AIn = Ct - (Nd \times 10^5) \quad (4)$$

Where: AIn: Algal inoculum (cells $mL^{-1}$) Ct: Algal concentration in the *Daphnia* tank culture (cells $mL^{-1}$) Nd: number of *Daphnia* individuals in the tank culture (Ind.) $10^5$: Predetermined algal concentration (cells $mL^{-1}$ *Daphnia*$^{-1}$) AIn $\geq$ 0 (zero) algal inoculum is not required. AIn < 0 = algal inoculum is necessary.

When the algal inoculum was necessary, the addition was made according to the following equation:

$$I = AIn \times Vt \ / \ Cm \quad (5)$$

Where: I: volume of the inoculum (L) AIn: algal inoculum (cells $mL^{-1}$) Vt: Volume of *Daphnia* tank culture (L) Cm: Algae concentration in the algae production tank (cells $mL^{-1}$)

Thus, the self-sustainability of the system could be inferred from the need or lack thereof for the addition of the microalgae. The fact that microalgae did not need to be

added indicates that the system was able to maintain itself even in the presence of predators of *D. similis* due to microalgae growth.

### 2.8. Efficiency of nitrogen and orthophosphate removal by the Chlorella-Daphnia consortium

Total ammonia nitrogen (TAN), nitrite ($NO_2^-$), nitrate ($NO_3^-$), total suspended solids (TSS), and orthophosphate ($PO_4^{-3}$) were determined at the beginning and at the end of each experiment, respectively, according to the methods described in [47–51]. The difference between the initial and final determination of nitrogen and orthophosphate compounds was used to calculate the efficiency of wastewater treatment (removal efficiency rate [%]).

### 2.9. Statistical evaluation

Data were subjected to Bartlett's and Shapiro–Wilk tests to determine homoscedasticity and normality, respectively, and then log-transformed (x + 1) for normalisation. Factorial (4 × 2) analyses of variance were performed: Tukey's test ($p < 0.05$) for *D. similis* growth data and Friedman's test followed by CANOVA ($p < 0.05$) for water quality variables. A polynomial regression curve was constructed to determine the effect of salinity on MAD. Spearman's correlation coefficient was proposed using previously log-transformed data (log (x + 1)). Statistical analyses were performed using R 3.4 software [52]. Residual *C. vulgaris* was investigated every three days using a Neubauer chamber under binocular microscope (magnification: 400x). The algal inoculum, Equation (4, 5), was added to the experimental units only when needed to maintain the pre-established *Chlorella-Daphnia* concentration ($10^5$ cells $mL^{-1}$ *Daphnia*$^{-1}$).

## 3. Results

### 3.1. Water quality

Water quality parameters of the *Chlorella-Daphnia* consortium in the effluent from Nile tilapia farming in BFT with low salinity at different processing forms S and NS and salinities from 1 to 4 g $L^{-1}$ are shown in Table 1. Significant differences ($p < 0.05$) between combinations were found only for salinity (an experimental variable). DO, pH and temperature ranged from 5.68–6.31 mg $L^{-1}$, 7.60–7.85, and 27.15–27.27°C, respectively.

### 3.2. Growth of the microorganisms

The growth curves of *D. similis* at different salinity levels in S and NS wastewater are shown in Figure 1. The higher salinity resulted in lower growth performance compared to salinities between 1 and 3 g $L^{-1}$. At a salinity of 4 g $L^{-1}$, individuals died after 2 and 6 days of culture in the S and N forms, respectively, so only one microalga inoculum was added to the 4S treatment and two inoculums to the 4NS treatment.

Salinity showed significant differences ($p < 0.05$) for MAD, SGR and Y with lower values for salinity 4 g $L^{-1}$. However, the factor wastewater treatment was significant ($p < 0.05$) for

**Table 1.** Mean ± standard deviation of the water quality variables in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g $L^{-1}$.

| | Sedimentation (S) | | | | Non-sedimentation (NS) | | | | Factors | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Salinity (Sa) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | Sa | W | Sa x W |
| **DO (mg $L^{-1}$)** | 6.12 ± 0.40 | 6.00 ± 0.33 | 5.68 ± 0.42 | 6.10 ± 0.28 | 6.00 ± 0.31 | 6.23 ± 0.26 | 6.06 ± 0.27 | 6.31 ± 0.31 | ns | ns | ns |
| **pH** | 7.76 ± 0.22 | 7.67 ± 0.17 | 7.70 ± 0.14 | 7.85 ± 0.17 | 7.66 ± 0.17 | 7.60 ± 0.19 | 7.64 ± 0.22 | 7.76 ± 0.34 | ns | ns | ns |
| **Sal. (g $L^{-1}$))** | 1.03 ± 0.02 | 2.09 ± 0.05 | 3.06 ± 0.05 | 4.10 ± 0.14 | 1.05 ± 0.03 | 2.08 ± 0.08 | 3.06 ± 0.06 | 4.15 ± 0.16 | * | ns | ns |
| **Temp. (°C)** | 27.15 ± 0.38 | 27.17 ± 0.34 | 27.24 ± 0.37 | 27.27 ± 0.36 | 27.19 ± 0.37 | 27.19 ± 0.37 | 27.26 ± 0.38 | 27.27 ± 0.38 | ns | ns | ns |

*Significant differences among the factors in two-way ANOVA followed by Tukey's test ($p < 0.05$). 'ns', not significant difference ($p > 0.05$); DO, dissolved oxygen; Sal., salinity; Temp., temperature.

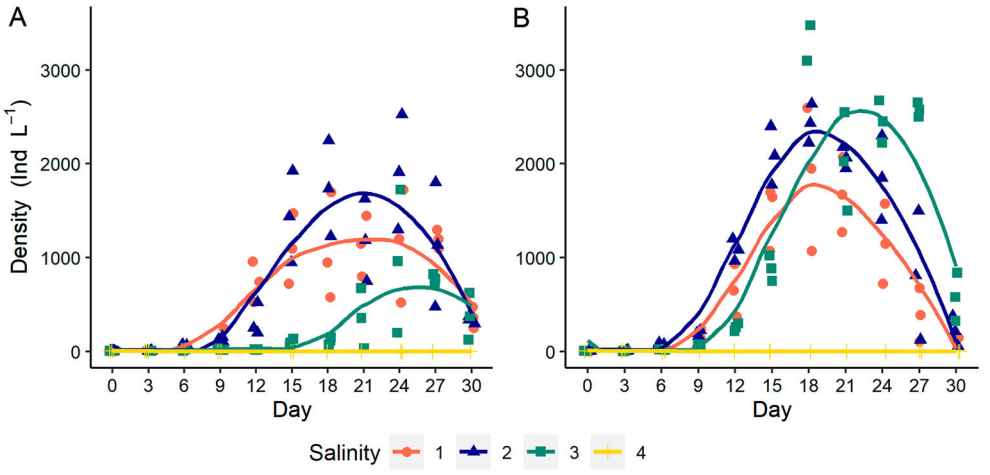



**Figure 1.** Growth curve of *Daphnia similis* in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g L$^{-1}$.

**Table 2.** Mean ± standard deviation of the growth variables obtained in each treatment of *D. similis* growth in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g L$^{-1}$.

| Wastewater (W) | Sedimentation (S) | | | | Non-sedimentation (NS) | | | | Factors | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Salinity (Sa) | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | Sa | W | SaxW |
| **MAD (ind L$^{-1}$)** | 1,200 ± 100a | 1,912 ± 612a | 962 ± 762b | 6 ± 0c | 1,875 ± 765a | 2,432 ± 208a | 3,475 ± 375b | 10 ± 5c | * | * | ns |
| **SGR (% day$^{-1}$)** | 24.8 ± 1.4ab | 26.8 ± 1.5a | 16.8 ± 7.3b | 0c | 27.1 ± 2.1a | 28.6 ± 0.4a | 30.3 ± 0.5b | 12.6 ± 22.8a | * | ns | ns |
| **Y (ind L$^{-1}$ d$^{-1}$)** | 54 ± 15a | 96 ± 28a | 17 ± 15b | 0c | 104 ± 43a | 135 ± 12a | 193 ± 21b | 1 ± 2c | * | * | * |
| **DT (day)** | 4.03 ± 0.23a | 3.73 ± 0.20a | 7.10 ± 4.01a | 0b | 3.71 ± 0.30a | 3.49 ± 0.05a | 3.30 ± 0.06a | 3.93 ± 0.56a | ns | ns | ns |
| **MDD (day)** | 27th | 24th | 24th | 0 | 18th | 18th | 18th | 3th | | | |

*Different letters between the columns show significant differences in two-way ANOVA followed by Tukey's test ($p$ <0.05). 'ns', not significant difference ($p$ > 0.05); MAD, maximum average density; SGR, specific growth rate; Y, yield; DT, doubling time; MDD, maximum density day.

MAD and Y with higher values for NS. In addition, Y was significantly different ($p$ < 0.05) when the interaction of factors was analyzed (Table 2).

In addition, NS wastewater had higher growth of *D. similis* at salinity levels of 1, 2, and 3 g L$^{-1}$, reaching mean values of 1,875 ± 765; 2,432 ± 207; and 3,475 ± 375 ind L$^{-1}$, respectively (Table 2). However, no significant differences ($p$ > 0.05) were observed in the 3NS treatment for MAD, SGR, Y, and DT compared with 1NS and 2NS for these two combinations (Table 2). MDD varied between salinity levels for S wastewater on days 27 (for 1S) and 24 (for 2S and 3S). In contrast, the use of NS wastewater on day 18 showed the same MDD for four salinity levels (Table 2). In contrast to the growth of *D. similis,* the microalga *C. vulgaris* showed higher growth at a salinity of 4 g L$^{-1}$ in both processing

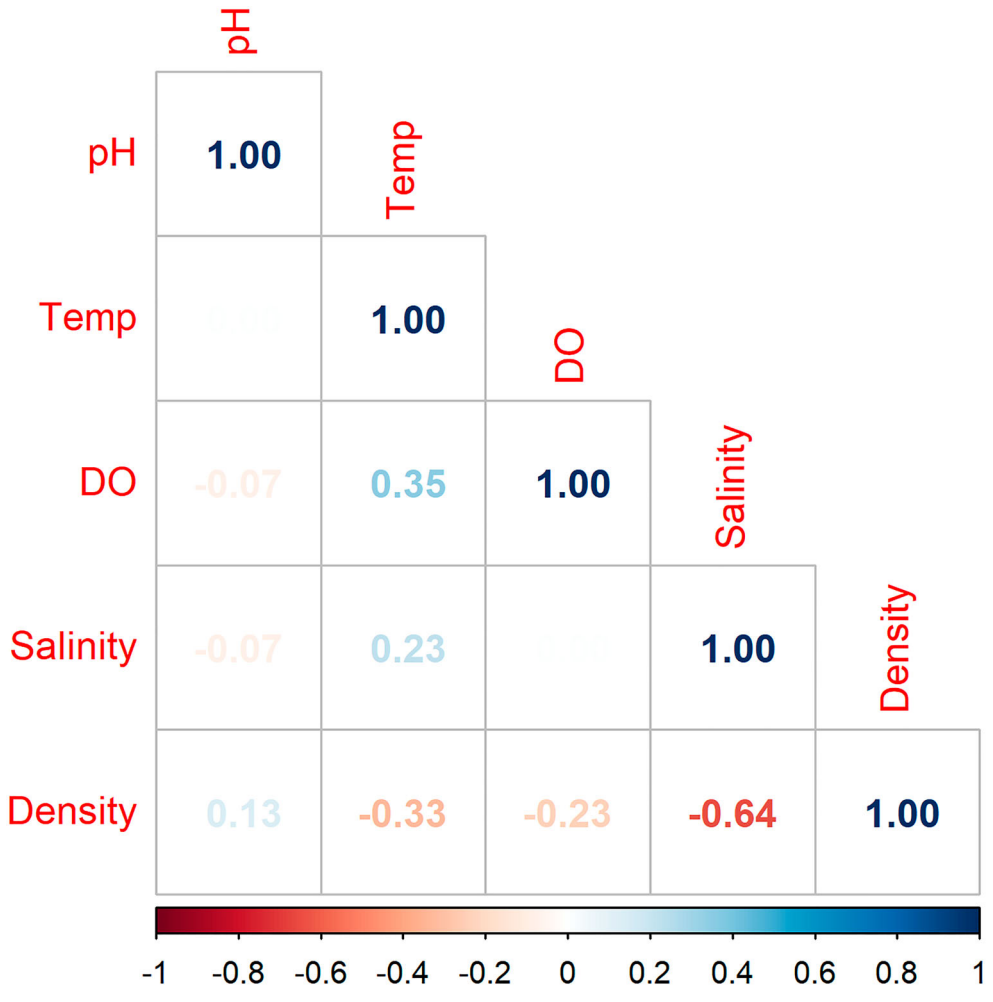



**Figure 2.** Spearman correlation (r, $p < 0.05$) of water quality variables: pH, temperature (Temp), dissolved oxygen (DO) and density of *D. similis* population in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (S) and non-sedimentation (NS) combined with four salinities: 1, 2, 3 and 4 g $L^{-1}$.

wastewater forms. Addition of *C. vulgaris* was more common in the higher density treatments of *D. similis* (Table 2).

Spearman correlation showed higher influences of temperature (r = −0.33) and salinity (r = −0.64) on maximum densities of *D. similis* (Figure 2). The regression curves allowed the conclusion of a positive relationship between the two wastewater treatment forms NS ($r^2 = 0.743$), S ($r^2 = 0.688$), and salinity. A maximum point on the trajectory of the parabolic model shows that higher *Daphnia* density can be achieved when both NS wastewater and salinity between 2 and 3 g $L^{-1}$ are used (Figure 3).

### *3.3. Efficiency of nutrient removal by the* Chlorella-Daphnia *consortium*

The initial and final levels of $NO_2^-$, $NO_3^-$, TAN, $PO_4^{-3}$, and TSS are shown in Table 3. Significant differences were found for initial and final nutrient levels. TAN, $PO_4^{-3}$

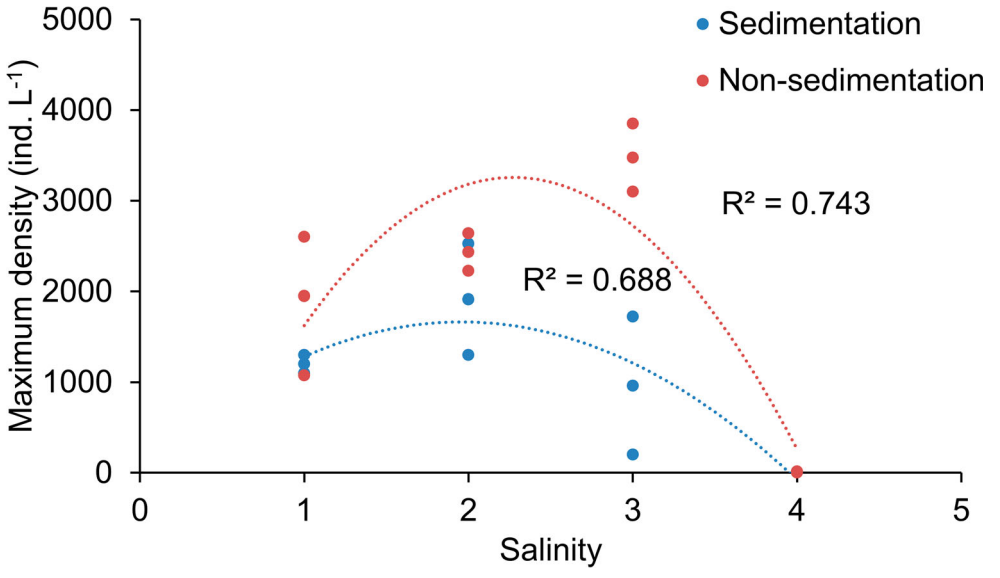



**Figure 3.** Salinity (1, 2, 3, and 4 g $L^{-1}$) and two forms of effluent treatment (sedimentation and non-sedimentation) effects on maximum average density (ind $L^{-1}$) of *D. similis* in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity.

and TSS obtained differences in processing effluent factor (S and NS) at initial conditions, unlike $NO_2^-$ and $NO_3^-$, which did not differ statistically. In the end, all nutrients showed significant differences ($p < 0.05$) among the treatments for all factors (Table 3).

Removal efficiencies of $NO_2^-$, $NO_3^-$, TAN, and orthophosphate varied between salinity levels and wastewater treatment form (Figure 4). Negative values indicate an increase and positive values indicate a decrease in nutrient levels. Higher rates of $NO_2^-$ and $NO_3^-$ removal were found at salinity 4 in both forms of wastewater: S (68.89% and 79.28%, respectively) and NS (81.30% and 97.86%, respectively). On the other hand, an increase in $NO_2^-$ was observed in both 3S (−276.28%), while in $NO_3^-$ a reasonable increase (~ 5%) was observed only in NS (at salinities 1, 2 and 3 g $L^{-1}$). As for TAN, removal was observed in NS wastewater at all salinities, highlighting 3 NS (91.79%); however, in S wastewater, a reduction in TAN values was observed only at salinities 2 and 3 g $L^{-1}$ (Figure 4). Finally, a higher removal of orthophosphate was observed in 1S (95.33%) and 4S (79.64%). In addition, all combinations had lower TSS values. At a salinity of 1 g $L^{-1}$, higher reduction rates were observed in both S (82.29%) and NS (90.74%).

### *3.4. Balance in the* Chlorella-Daphnia *consortium for self-sustaining of the system*

The actual requirement for microalgal inoculation in the different experimental combinations could be determined based on the residual density of *C. vulgaris* in the experimental units. Thus, the balance of the *Chlorella-Daphnia* consortium was determined by the self-sustaining of the system, as the last day of *C. vulgaris* inoculation was observed on day 18 for treatments 1NS, 2S, 2NS, and 3S, and on day 21 for treatments 1S and 3NS

**Table 3.** Mean ± standard deviation of the initial and final quantities of $NO_2^-$, $NO_3^-$, $PO_4^{-3}$, TAN, and TSS in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g $L^{-1}$.

| | | Sedimentation (S) | | | | Non-sedimentation (NS) | | | | Factors | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | Sa | W | Sa x W |
| **$NO_2^-$** | Initial | 33.85 ± 8.41 | 32.71 ± 12.28 | 32.03 ± 4.99 | 33.94 ± 15.26 | 35.47 ± 1.84 | 34.91 ± 2.65 | 35.99 ± 1.04 | 34.72 ± 1.96 | ns | ns | ns |
| **(µg $L^{-1}$)** | Final | 11.16 ± 0.20 | 12.41 ± 3.67 | 127.37 ± 63.85 | 10.53 ± 0.61 | 6.78 ± 3.88 | 6.64 ± 1.63 | 13.66 ± 3.87 | 6.49 ± 0.61 | * | * | * |
| **$NO_3^-$** | Initial | 1,265.18 ± 6.52 | 1,205.03 ± 17.21 | 1,248.13 ± 3.74 | 1,244.25 ± 8.75 | 1,214.86 ± 95.28 | 1,228.84 ± 105.98 | 1,216.69 ± 118.22 | 1,231.48 ± 101.02 | ns | ns | ns |
| **(µg $L^{-1}$)** | Final | 1097.45 ± 51.34 | 356.99 ± 153.47 | 700.33 ± 256.15 | 257.79 ± 136.43 | 1251.00 ± 4.41 | 1281.83 ± 15.45 | 1281.01 ± 7.25 | 26.35 ± 17.06 | * | * | * |
| **$PO_4^{-3}$** | Initial | 1,190.22 ± 227.58 | 1,187.21 ± 304.86 | 1,179.44 ± 245.24 | 1,184.08 ± 217.55 | 1,793.91 ± 477.70 | 1,785.85 ± 236.12 | 1,782.24 ± 344.53 | 1,791.56 ± 498.80 | ns | * | ns |
| **(µg $L^{-1}$)** | Final | 55.61 ± 22.30 | 288.01 ± 174.31 | 252.32 ± 183.11 | 241.03 ± 53.48 | 555.27 ± 177.24 | 996.00 ± 84.51 | 1171.96 ± 58.69 | 820.87 ± 3.52 | * | * | * |
| **TAN** | Initial | 83.59 ± 10.47 | 85.25 ± 16.89 | 80.98 ± 17.96 | 84.78 ± 7.25 | 539.74 ± 280.94 | 531.46 ± 480.95 | 533.98 ± 216.78 | 537.02 ± 195.23 | ns | * | * |
| **(µg $L^{-1}$)** | Final | 89.23 ± 5.84 | 78.95 ± 5.11 | 48.50 ± 18.97 | 445.82 ± 207.24 | 76.37 ± 4.38 | 92.36 ± 29.92 | 43.86 ± 15.32 | 282.77 ± 62.76 | * | * | * |
| **TSS (mg $L^{-1}$)** | Initial | 0.024 ± 0.005 | 0.024 ± 0.006 | 0.023 ± 0.004 | 0.023 ± 0.008 | 0.063 ± 0.020 | 0.062 ± 0.035 | 0.063 ± 0.022 | 0.063 ± 0.016 | ns | * | ns |
| | Final | 0.004 ± 0.001 | 0.006 ± 0.001 | 0.014 ± 0.004 | 0.019 ± 0.007 | 0.006 ± 0.003 | 0.014 ± 0.004 | 0.014 ± 0.000 | 0.016 ± 0.003 | * | * | * |

*Significant differences among the factors in two-way ANOVA followed by Tukey's test ($p < 0.05$). 'ns', not significant difference ($p > 0.05$); $NO_2^-$, nitrite; $NO_3^-$, nitrate; $PO_4^{-3}$, orthophosphate; TAN, total ammonia nitrogen; TSS, total suspended solids.

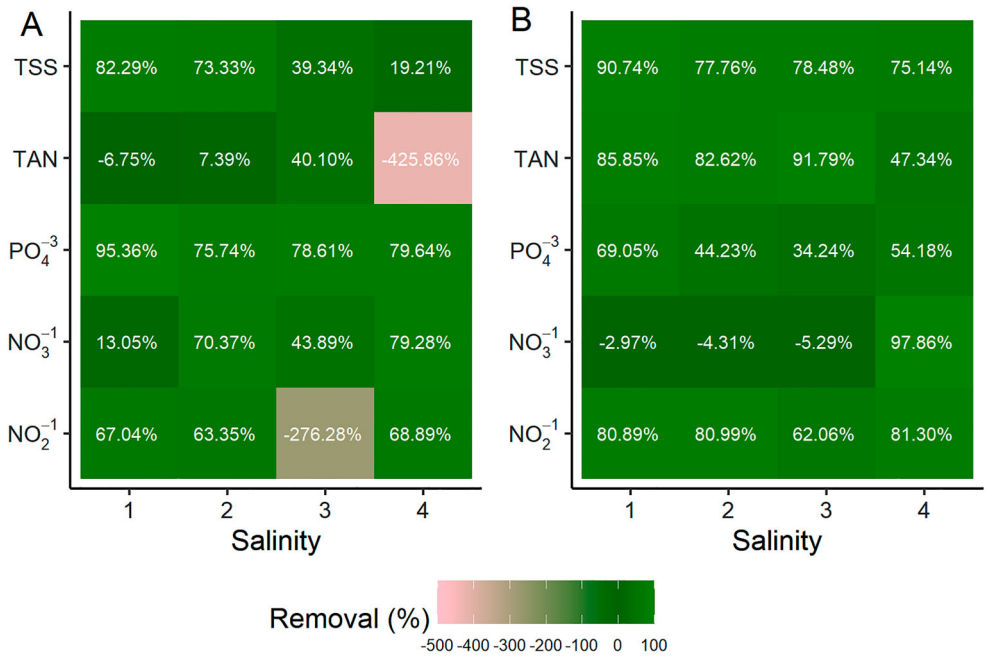



**Figure 4.** Removal efficiency (%) of nitrite ($NO_2^-$), nitrate ($NO_3^-$), total ammonia nitrogen (TAN), orthophosphate ($PO_4^{-3}$) and total suspended solids (TSS) in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g $L^{-1}$. Negative values indicate increase of nutrient quantities and positive ones indicate reduction.

(Figure 5). In contrast, for the wastewater processing form, a new microalgal inoculation was required to maintain the previously established cell concentration in the effluent observed on day 24 for 1NS and 2NS, and on day 27 for treatments 1NS, 2NS, and 3NS.

When evaluating only the factorial combinations in which both *Chlorella* and *Daphnia* grew, i.e. excluding the salinity of 4 g $L^{-1}$ at which the microcrustaceans died, the combinations 3NS, 2NS, and 2S stood out for the meaningful values of biomass production of *D. similis* and/or bioremediation of the effluent and/or sustainability of the system (Figure 6).

## 4. Discussion

### *4.1. Water quality*

The dissolved oxygen and pH provided ideal conditions for the growth of *Daphnia similis*, in contrast to the temperature, which was above 26°C. According to [47], the ideal temperature range for culturing *D. similis* is 24–26°C.

### *4.2. Growth of* Daphnia similis

First, simultaneous biomass production (Table 2) and bioremediation were observed in the *Chlorella-Daphnia* consortium examined in this study due to the reduction of TAN,

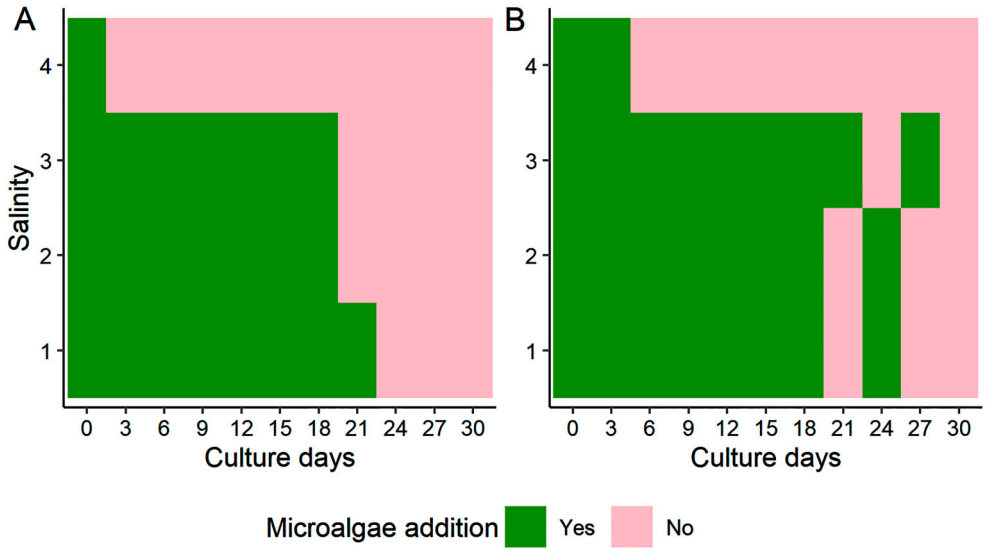



**Figure 5.** Status of self-sustainability of the system by need (green) or non-need (pink) of microalgae addition over days in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g $L^{-1}$. The day of non-need of microalgae addition suggests a status of self-sustainability of the system.

nitrite, nitrate, and orthophosphate levels (Figure 4). In addition, microcrustaceans of the genus *Daphnia* are common in environments with high concentrations of organic matter (debris), where bacteria, yeasts, and microalgae proliferate and use them as food [53,54]. Therefore, effluents from BFT-based systems provide favourable conditions for *Daphnia* species [17].

The maximum average density of *D. similis* reached in 3NS was higher than that reported by Campos Clarissa Vilela et al. [17] (1,234 ± 286 ind $L^{-1}$), which also used effluent from the BFT culture of Nile tilapia (*O. niloticus*) with a C:N ratio of 12:1 at zero salinity. In contrast, the highest density reached in the present study was similar to that reported by Mota et al. [55] (3,433 ± 267 ind $L^{-1}$), culturing *D. magna* in wastewater from the BFT culture of Nile tilapia (*O. niloticus*) with a C:N ratio of 10:1, similar to this study, but at zero salinity. These findings show the influence of salinity and C:N ratios on the physiological system of *Daphnia* spp., providing higher or lesser growth rates.

Salinities 1, 2, and 3 g $L^{-1}$ combined with non-sedimentation wastewater had higher growth of *D. similis* (Table 2). This may be linked to the orthophosphate levels, as well as the use of raw form of effluent, preserving characteristics of the BFT and its microbial community. According to Barsanti, Gualtieri [56], phosphorus levels up to 1 mg $L^{-1}$ stimulate the reproduction of *D. pulex*, and between 5–7 mg $L^{-1}$ stimulate the reproduction of *D. magna*. In addition, *Daphnia* spp. feed on various groups of bacteria, yeasts, microalgae, as well as debris and dissolved organic matter [57], all of these present in the BFT culture water.

The reason *D. similis* did not survive at 4-salinity is associated with its tolerance to variation in salinity, from 4 to 7 g $L^{-1}$ [58–60], as salinity is inversely proportional to the

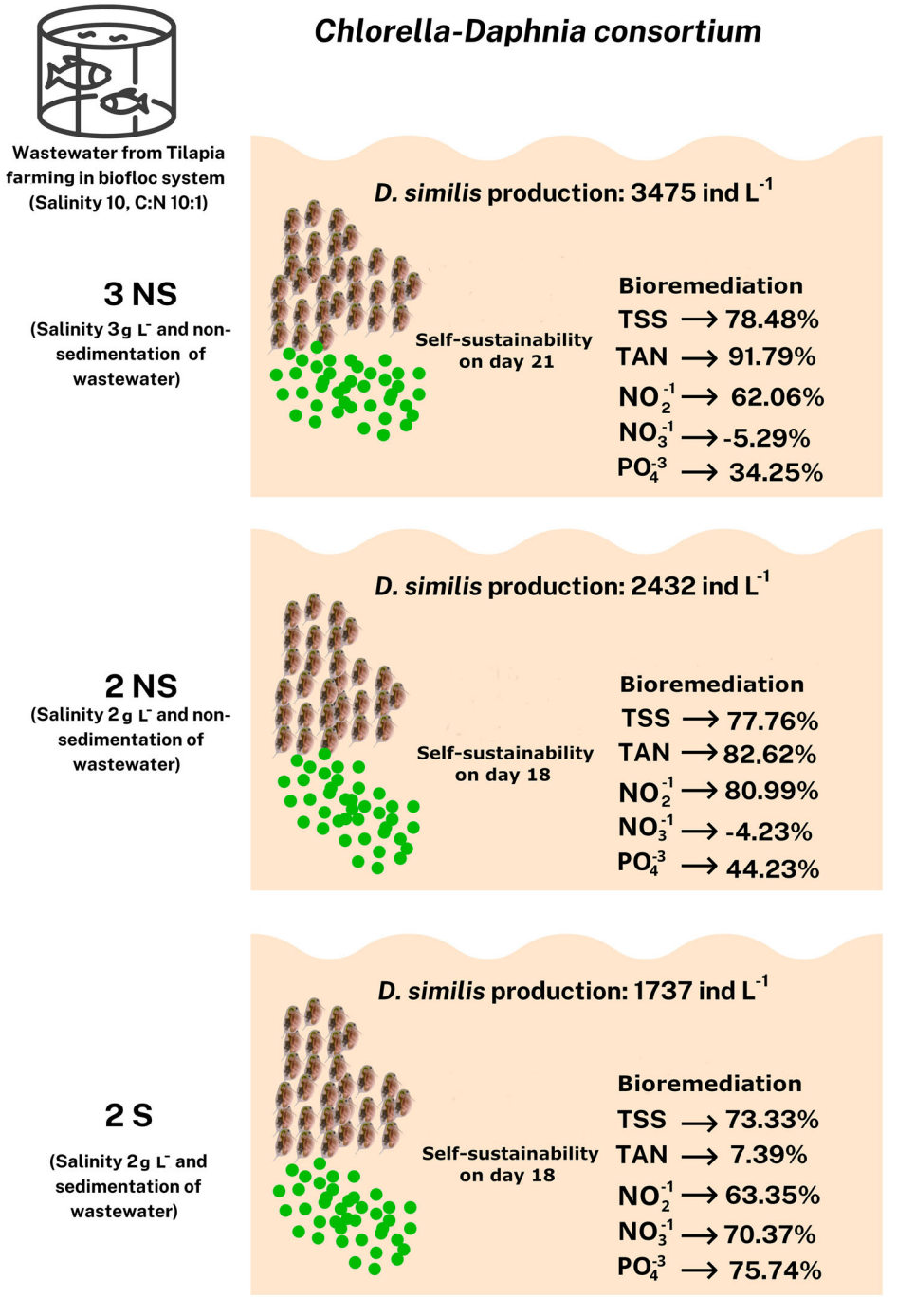



**Figure 6.** Main treatments (3NS, 2S, and 2NS) in *Chlorella-Daphnia* consortium in BFT wastewater from Nile tilapia farming in low salinity using two forms of wastewater treatment: sedimentation (A) and non-sedimentation (B) combined with four salinities: 1, 2, 3 and 4 g $L^{-1}$. The day of non-need of microalgae addition suggests a status of self-sustainability of the consortium. There is emphasis in the *D. similis* production, bioremediation and the day that was identified a self-sustaining system. Negative values indicate increase of nutrient quantities and positive one indicates reduction.

species growth (Figure 2). The influence of salinity is related to the cultivation conditions evaluated. The physiological adaptations to increasing salinity is strictly linked to the capacity for osmoregulatory control of the hemolymph. However, species like *D. magna* and *D. pulex* can tolerate higher salinities, up to 8 g $L^{-1}$, but above this, its hemolymph becomes isosmotic to the external environment, leaving the microcrustacean unable to survive any further increase in salinity [58].

Temperature was also inverse to the growth of *D. similis* (Figure 2). Values between 24 and 26°C are the most suitable for this species [45]. However, this study reported mean temperatures above 27°C (Table 1). Other studies also report the influence of temperature increase on the growth of *Daphnia*. Starke et al. [61] reported 100% death of *D. pulicaria* population in temperatures higher than 28°C.

### *4.3. Nutrient removal efficiency in the* Chlorella-Daphnia *consortium*

At beginning and end of experiment, the processing of wastewater was the most evident factor for significant differences found among treatments (Table 3). First, the microbial flocs and organic matter in the wastewater in NS treatments can be the reason for the differences of orthophosphate, TAN and TSS variables in initial conditions. According to Timmons and Ebeling [62], about 1.3% of the dry matter of aquaculture waste corresponds to total phosphorus. In addition, Boyd [27] explain that organic matter decomposition is the main source of ammonia production in aquaculture systems. Regarding TSS, the processing of the aquacultural wastewater by sedimentation could have aided the reduction of solids, leading S to have lower values of TSS than NS.

At the end, the wastewater processing form and salinity influenced on decrease/increase and removal efficiency of nitrogen, phosphate compounds and solids (TSS) (Table 3, Figure 4). Two combinations that used the S wastewater (1S and 4S) had an increase in TAN concentrations. One reason for this may be a lower number of nitrifying bacteria that reduce ammonia to nitrite, which causes the loss of much of the particulate organic matter in the sedimentation process and shows an increase in ammonia level. Microbial flocs are important sources of organic carbon and serve as a substrate for bacterial growth [4], mainly for heterotrophic bacteria. 4S had a highlight: it presented a TAN increase of more than 400%, which may also be linked to not only the processing of wastewater but to the deaths of both *Daphnia* and algal cells.

On the other hand, dissolved inorganic carbon (mainly in the form of carbon dioxide) is the primary energy source for nitrifying bacteria [62]. Therefore, these bacteria can compete with algae, which also assimilate carbon dioxide to perform the oxygen photosynthesis. Heterotrophic bacteria were not well established, as they assimilate ammonia, transforming it into bacterial biomass [63], which may have influenced the increase in ammonia levels as well.

The reverse also occurred regarding nitrite. The combination that showed a higher nitrite level had a reduction in ammonia level, as 3S combination (Figure 4 (A)), confirming the action of nitrifying bacteria, such as the genus *Nitrosomonas*, oxidising ionised ammonia to nitrite [61]. Additionally, reductions in nitrite concentrations may be more closely related to bacterial activity, since in the nitrification process other groups of bacteria, such as *Nitrobacter*, are responsible for the oxidation of nitrite to nitrate [63,27].

Thus, greater ammonia and nitrite removal efficiencies are linked to a better-established bacterial community in the medium. This was also evidenced by Pous et al. [36], who cultivated *D. magna* in reactors for domestic effluent treatment for one year and attributed the reductions in ammonia and total nitrogen in the system to bacteria through the nitrification process as well.

Regarding the nitrate removal, the best results were in salinity 4 g $L^{-1}$, both in S (79.28%) and NS (97.86%) forms, where there was no consortium, since *D. similis* could not survive in this salinity. The second-best results were found in salinity 2 g $L^{-1}$ in the S form of wastewater process (70.37%, *Chlorella-Daphnia* consortium). Thus, the presence of green algae *C. vulgaris* might be the main responsible for this, since nitrate is one of the most assimilable forms of oxidised nitrogen by microalgae [64]. By this reason, the absence of predation (4 S/NS) combined with bioavailable nutrients (2S) in the water led to better microalgal growth, providing a more effective bioremediation process for this nitrogenous compound.

Gil-lzquierdo et al. [41] found that the green microalgae consortium *Monoraphidium* sp., *Desmodesmus subspicatus* and *Nannochloris* sp. achieved a nitrate removal of 89.9% in wastewater from the dry riverbed El Albujón. Pous et al. [36] attributed the nitrate reductions to the macrophyte *Lemna* sp. which was occasionally present in *Daphnia* magna reactors for effluent treatment. This demonstrates that algae and macrophytes are in fact the most biologically suitable for removing nitrates in effluent treatments when compared to genus *Daphnia*, since this microcrustacean has not been attributed the ability to remove nitrate in the literature. The performance of *Daphnia* in a consortium for effluent treatment was documented only by Fikirdesici-Ergen et al. [38], who combined *D. pullex* with the macrophyte *Lemna minor* to remove heavy metals Fe (27.7%), Al (76 .5%) and Ba (91.8%) on a laboratory scale.

In addition to the evident nitrate removals, there was also high efficiency of orthophosphate removal in salinity 1 g $L^{-1}$ for both forms of processing, which might be due to the *C. vulgaris* and *D. similis* consortium. The former has the capacity to remove more than 98% of total phosphorus from wastewaters [65] and the latter can contribute to phosphorus removal by up to 12% in domestic sewage effluent [31]. In fact, in all salinities and processing forms, with the presence of *Daphnia*, there was a reduction in orthophosphate levels, especially in the 1S and 1NS, which had better results when compared with 4S and 4NS, where there was no *Daphnia* (Figure 4). This fact strengthens the importance that the consortium of microorganisms has for the removal of nutrients in aquaculture wastewaters, since phosphorus is one of the compounds that tend to accumulate in crop water, especially in BFT [12].

As with orthophosphate, all combinations showed a reduction in TSS concentrations, highlighting again the 1S (82.29%) and 1NS (90.74%) with better results (Figure 4). Removal efficiency in NS for TSS can be related in part to sedimentation of the organic matter that occurs naturally over time, even with constant aeration in the tanks of *D. similis* culture. However, the addition of *C. vulgaris* to the wastewater was a successful strategy to optimise the consumption of solids by *D. similis,* according to Campos Clarissa Vilela et al. [17]. Other studies also reported the use of *Daphnia* sp. as a bioremediation agent regarding the concentration of solids present in water [29,30]. Pau et al. [31] tested the role of *Daphnia* in the tertiary treatment of wastewater and reported a solids reduction from 10.1% to 29.4%, which was related to *Daphnia* population in

densities of 10 and 50 ind $L^{-1}$, respectively. In addition, they reported that the size range of the ingested particles suspended ranged from 2.5–30 µm, similar to what was found by Burns [66], who detected that 35 µm was the maximum diameter an ingestible particle could have to be consumed by *Daphnia*. Although the floc size in this study was not measured, Meenakshisundaram et al. [67] found a range of 5–30 µm in the floc size in tilapia culture (freshwater) using a C:N ratio of 10:1.

Combining the *Daphnia* production with the water bioremediation process, there is another panorama, where 1S and 1NS had great reduction of orthophosphate and the TSS and 2S combination showed high removal efficiency of nitrogen compounds (Figure 4), mainly nitrate (70.37%), the main nitrogen compound accumulated in the BFT wastewater [4,68], which may cause the death of animals at high concentrations [69]. In fact, when it is evaluated using the *Chlorella-Daphnia* consortium, the 2S had better balance for nutrients efficiency removal for all variables analyzed (Figure 4 (A)). In addition, the 2S combination was also favourable to the growth of *D. similis*, but with lower values when compared to the 3NS and 2NS ones (Figures 4, 5; Table 2). Thus, depending on the focus (biomass production or bioremediation) the cultivation conditions can be managed to provide better results that are more advantageous for the interest of the aquaculture industry.

Thus, the removal of nitrogen, phosphate and solid compounds performed by the synergistic mechanisms of *Chlorella* and *Daphnia* in the consortium significantly improved the levels of these nutrients in BFT effluent at low salinity. *Chlorella* genus is also active in the heavy metals removal (Cu, Pb, Zn, Cd, Cr) [70–72], pharmaceuticals and personal care products such as antibiotics [40], hormones [73], antimicrobials [74], and nonsteroidal anti-inflammatory drugs [75], which have better results when there is a consortium with other organisms [76]. *Daphnia* also acts in the reduction of heavy metals, such as Cu (26%) [36] and Pb (75.3% to 97.2%) [37]. This demonstrates that it is still possible to obtain even more benefits from this consortium (*Chlorella-Daphnia*) in a promising way for the bioremediation of effluents from the aquaculture industry, since this study did not carry out analyzes of heavy metals.

### *4.4. Balance in the* Chlorella-Daphnia *consortium for self-sustaining of the system*

The importance of the *Chlorella-Daphnia* consortium is focused on solids, nitrogen and phosphorous removal, and increasing biomass production (*Chlorella* and *Daphnia*) simultaneously. Similar to integrated multitrophic aquaculture systems, consortium cultures of microorganisms have been gaining relevance in recent years as an alternative as well for the effluents treatments [24]. Several studies reported the success of several consortia of microorganisms, such as bacteria-bacteria [23], bacteria-algae [22], bacteria-algae-fungi [77], and algae-fish [78,79].

The balance of the consortium in the present study took place from the moment it was no longer necessary to inoculate *C. vulgaris* in the system. In other words, there was a production of algal biomass from the bioremediation of the wastewater even with its predation by *D. similis*. Thus, there was a balance in the consortium promoted by the self-sustainability status of the system. The maximum density of *D. similis* for the use of S wastewater was achieved after the identification of the

consortium equilibrium (Table 2; Figure 5). The use of S wastewater also showed the highest removal rates of nutrients, especially nitrate and phosphorus, important for algae growth.

Although the 1S combination removed about 95% of the orthophosphate (the highest among all the consortium combinations), the nitrate removal was only about 13% (the lowest among the consortium combinations). This may have contributed to reaching the later self-sustainability day (24th) when compared to 2S and 3S combinations.

On the other hand, NS wastewater combinations did not have a consistent balance and a new inoculation of *C. vulgaris* was necessary on average every three days (Figure 5). That is, algae production did not become self-sustained in the system. The reason for this is the low nitrate assimilation, despite having presented considerable orthophosphate removal rates, possibly posing an imbalance between phosphorus and nitrogen in the cell, hindering an ideal growth of *C. vulgaris*. In addition, NS wastewater has shown higher amounts of TSS, which may have influenced the light penetration in the water, consequently reducing photosynthesis, since light is a fundamental requirement for energy conversion [56, 64].

In this way, the production of *D. similis* in consortium with *C. vulgaris* using aquaculture wastewater from BFT systems has proved to be an interesting strategy to make aquaculture more sustainable. Apart from promoting the production of high commercial value biomass, it also contributes to a reduction of environmental impacts, since wastewaters can be bioremediated by these organisms and discarded with lower nitrogen and phosphorous concentrations. Furthermore, biomasses of both species are suitable for feeding fish larvae or for feed manufacturing, for example.

## 5. Conclusions

In summary, the *Chlorella-Daphnia* consortium in a wastewater from Nile tilapia cultivation in crude form (i.e. non-sedimentation) at 3-salinity allowed a better production of *D. similis*, while using processed wastewater (i.e. sedimented) at 2-salinity allowed a higher removal of inorganic compounds, therefore promoting a self-sustaining system by a solid balance through algal growth. These results contribute towards a better evaluation of cultures in consortia of organisms for the treatment of aquaculture wastewater and production of live food for aquaculture. Future research that can analyze the efficiency of removal of heavy metals in aquaculture effluents using the *Chlorella-Daphnia* consortium may contribute to a better understanding of the effectiveness of this consortium.

## Acknowledgements

The authors are grateful to all the laboratory members for the preparation of the experimental material and technical assistance.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

## Funding

This work was supported by CAPES: [Grant Number 88882.436231/2019-01,88882.436234/2019-01,88887.497047/2020-00]; CNPQ: [Grant Number PQ 308063/2019-8, PQ PQ309669/2021-9]; Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco (FACEPE): [Grant Number BFP-0186-5.06/20].

## Compliance with ethical standards

### *Ethical approval*

The experiment was in accordance with Brazilian Law no. 11.794/2008.

## Data availability statement

Research data are not shared.

## Notes on contributors

***Clarissa Vilela Figueiredo da Silva Campos*** is a Doctorate candidate in the Fishing Resources and Aquaculture Postgraduate Program at Universidade Federal Rural de Pernambuco (UFRPE), Brazil. She earned a Bachelor's degree in Fishing Engineering in 2014 and a Master's degree in Fishing Resources and Aquaculture in 2017, both at UFRPE.

***Carlos Yure B. Oliveira*** is a Doctorate candidate in the Fishing Resources and Aquaculture Postgraduate Program at Universidade Federal Rural de Pernambuco (UFRPE), Brazil. He earned a Bachelor's degree in Fishing Engineering in 2018 (also at UFRPE), and a Master's degree in Aquaculture in 2020 at Universidade Federal de Santa Catarina, Brazil.

***Elizabeth Pereira dos Santos*** is a Doctorate student of the Fishing Resources and Aquaculture Postgraduate Program at Universidade Federal Rural de Pernambuco (UFRPE), Brazil. She earned a Bachelor's degree in Fishing Engineering in 2014 and has experience in Fishery Resources and Fisheries Engineering, focusing on sea culture.

***Suzianny Maria Bezerra Cabral da Silva*** is a professor of Fishing Engineering in the Department of Fisheries and Aquaculture at the Universidade Federal Rural de Pernambuco, Brazil. She earned a Bachelor's in Fishing Engineering (2007), a Master's in Aquaculture (2010), and a Doctorate degree in Fishery Resources and Aquiculture (2014), all at Universidade Federal Rural de Pernambuco (UFRPE).

***Jéssika Lima de Abreu*** is a professor of Fishing Engineering in the Department of Fisheries and Aquaculture at the Universidade Federal Rural de Pernambuco, Brazil. She earned a Bachelor's in Fishing Engineering (2014), a Master's in Aquaculture (2016), and a Doctorate degree in Fishery Resources and Aquiculture (2020), all at Universidade Federal Rural de Pernambuco (UFRPE).

***William Severi*** is a professor of Fishing Engineering in the Department of Fisheries and Aquaculture at the Universidade Federal Rural de Pernambuco, Brazil. He earned a Bachelor's degree in Fishing Engineering at the Universidade Federal Rural de Pernambuco (1980), and a Master's degree (1991) plus a Doctorate (1997) in Ecology and Natural Resources at the Universidade Federal de São Carlos, Brazil.

***Luis Otavio Brito*** is a professor of Fishing Engineering in the Department of Fisheries and Aquaculture at the Universidade Federal Rural de Pernambuco, Brazil. He earned a Bachelor's degree in Fishing Engineering and then a Doctorate in Fishing Resources and Aquaculture in 2013, both at the Universidade Federal Rural de Pernambuco.

***Alfredo Olivera Gálvez*** is a professor of Fishing Engineering in the Department of Fisheries and Aquaculture at the Universidade Federal Rural de Pernambuco, Brazil. He earned a Bachelor's degree in Biology at Universidad Ricardo Palma (Peru) and then a doctorate in Biology of Aquatic Organisms and Aquaculture in 1998, at the Universidade Estadual Paulista Júlio de Mesquita Filho (Brazil).

## ORCID

*Clarissa Vilela Figueiredo da Silva Campos* http://orcid.org/0000-0002-7697-7964
*Carlos Yure B. Oliveira* http://orcid.org/0000-0001-9237-1869
*Elizabeth Pereira dos Santos* http://orcid.org/0000-0002-5268-6994
*Jéssika Lima de Abreu* http://orcid.org/0000-0002-5614-0454
*William Severi* http://orcid.org/0000-0001-8873-5965
*Suzianny Maria Bezerra Cabral da Silva* http://orcid.org/0000-0002-7571-3465
*Luis Otavio Brito* http://orcid.org/0000-0002-6971-3020
*Alfredo Olivera Gálvez* http://orcid.org/0000-0001-5149-2605

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# CAPÍTULO II

Artigo Científico submetido à revista Science of the Total Environment

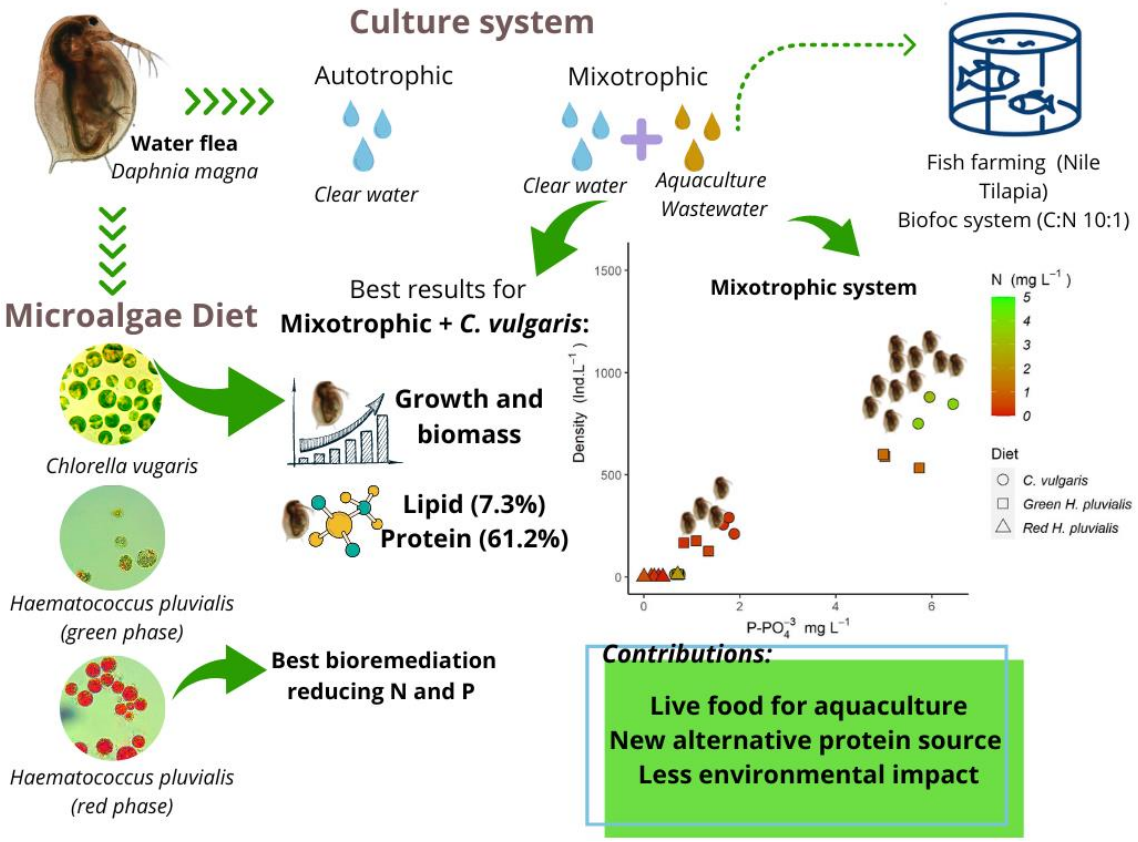
**Cultivo da pulga d'água *Daphnia magna* alimentada com diferentes dietas microalgais utilizando efluente da psicultura**

**The Water flea *Daphnia magna* culture fed with different microalgae using wastewater from fish farming**

# THE WATER FLEA *Daphnia magna* CULTURE FED WITH DIFFERENT MICROALGAE USING WASTEWATER FROM FISH FARMING

Clarissa Vilela Figueiredo da Silva Campos[1*]

## Graphical abstract



## Highlights

- Reusing aquaculture wastewater for production of water flea *D. magna* was studied.
- Microalgae *C. vulgaris* and *H. pluvialis* (green and red phases) were used as diet.
- *H. pluvialis* on red phase showed relevant biorremediation reducing N and P to zero.
- Best *D. magna* growth was found in aquaculture wastewater medium added *C. vulgaris*.
- *D. magna* fed with *C. vulgaris* had highest lipid (7.3%) and protein (61.2%) content.

## Abstract

This study aimed to evaluate the cultivation of the water flea *Daphnia magna* using different microalgal diets: *Chlorella vulgaris* (C) and *Haematococcus pluvialis* in the vegetative (HV) and cystic (HC) phases; and two cultivation systems: autotrophic (A), using clearwater, and mixotrophic (M), using wastewater from Nile tilapia farming in biofloc. During 18 days of cultivation, *D. magna* was fed every two days, and the water quality parameters were analyzed. Better growth results of *D. magna* were evidenced for MC, followed by MHV. Both cultures (A and M) fed with HC were not successful, and the entire population died on the fourth day of cultivation. Lower levels of TAN, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$. and orthophosphate were observed for the AHC and MHC combinations. Higher concentrations of lipids and proteins in the water flea were found in MC. Maximum average density, biomass, lipids and proteins were significantly correlated (R-values ranging from 0.57 to 1) with N and P concentrations in the water. Thus, it was possible to conclude that the cultivation system and the type of diet directly influenced the growth and nutritional composition of *D. magna*, in which the MC is more suitable for *D. magna* biomass growth, rich in proteins and lipids. These results contribute to a better evaluation of possible microalgae diets for water flea cultures in different cultivation systems that provide better biomass yields and nutritional composition through the reuse of fish farming effluent, aiming at its use as live food for aquaculture and new possibilities for alternative protein sources.



Keywords: *Daphnia*, *Chlorella*, *Haematococcus*, sustainability, bioflocs, aquaculture.

## Introduction

One of the key factors for the successful cultivation of aquatic organisms is nutrition, and within this universe, there is plankton, which is made up of phytoplankton (microscopic algae) and zooplankton (small animals) that are carried by currents of water (Brierley 2017). Within the planktonic community, microalgae and microcrustaceans stand out, with the microalgae *Haematococcus pluvialis* and *Chlorella vulgaris* as well as the microcrustacean *Daphnia magna* (water flea) being evident both in terms of nutritional content and immunological benefits.

The search for new alternative sources for animal and human feed has increased in recent years, and in this scenario, live food (i.e., microalgae, fungi, and zooplankton) that has been of great importance in aquaculture industry, has been pointed out as promising organisms towards the replacement of certain traditional grains, such as soy, corn, and others. Microalgae are a very diverse group of photosynthetic organisms (including eukaryotes and procaryotes cells) rich in high-value compounds (Oliveira et al., 2022). On the other hand, the zooplankton feed on microalgae, and other organic particles, in the natural environment, and are used in aquaculture, as for example the water flea *Daphnia magna* that is widely used to feed fish larvae (Chiu et al., 2015; Abo-Taleb et al., 2021).. Recently, a study used the cladoceran *Eurycercus beringi* as flour replacing fish meal in the feed in the feeding of post-larvae shrimp (Aravind et al., 2021) obtaining good productive results.

Given this scenario, the use of the microalgae *Haematococcus pluvialis* and *Chlorella vulgaris* in the diet of the microcrustacean *D. magna* (water flea) is presented as a good alternative. The microalgae *H. pluvialis* has a peculiarity during its growth, going through two growth phases with distinct morphological characteristics: the first is the vegetative phase, in which the microalgae has a green hue due to the chlorophyll

pigment and mobility through flagella; the second is the cystic or aplanospore phase, which is when the microalgae lose their flagella, present reddish colors, radial morphology having a cyst shape, and start to produce large amounts of carotenoids, including astaxanthin, approximately 4% of the cellular content (Chekanov et al., 2014; Hagen et al. 2022) The use of *H. pluvialis* in the vegetative phase in feeding *D. pulex* was documented by Alcántara-Azuara et al. (2014) however, neither the nutritional content nor the supply of *H. pluvialis* in the cyst stage as a diet for *Daphnia* sp.

Astaxanthin is red carotenoid widely used in the pharmaceutical, nutraceutical, cosmetic and food industries, as it has antioxidant, anti-inflammatory, antitumor, antidiabetic and immunomodulatory properties, in addition to being used in aquaculture, both for pigmentation and to improve the immune response and the zootechnical performance of shrimp and fish (Ding et al., 2018; Kim et al., 2015; Xie et al., 2018). The microalga *C. vulgaris* presents proteins and lipids, in dry matter, of approximately 30% - 61.6% (Villarruel-López et al., 2017; Ahmed et al., 2020; Turcihan et al. 2022) and of 11,.3 to 12.5% of lipids depending on the culture medium (Turcihan et al. 2022; Ahmed et al., 2020). On the other hand, *H. pluvialis* in the vegetative phase can reach up to 45% and 25% of the dry weight of proteins and lipids, respectively, while in the cystic stage it reaches approximately 23% of protein and 37% of lipid (Kim et al., 2015; Shah et al., 2018).

The cladoceran *D. magna*, has a high amount of crude protein (approximately 60 to 68%) and a lower content of lipids (about 6 to 8%) (Herawati et al., 2017), in addition to an essential amino acid profile (Torrentera and Tacon 1989). In addition, they have concentrations of chitin and chitosan, approximately 75% (Kaya et al., 2014), which are sources of glucans and have immunostimulant properties. The benefits of chitin and chitosan present in *D. similis* were investigated by Tseng et al. (2021) for the zootechnical

103 performance of *Penaeus vannamei*, that reported adding these substances to the feed
104 during cultivation enabled greater weight gain and specific growth rate, in addition to
105 stimulating the production of digestive enzymes such as trypsin, lysine and pepsin.

106 In parallel to this recurrent quest for better production and nutrition of aquatic
107 animals, there is the problem of aquaculture sustainability in wastewater reusability. Fish
108 and shrimp farming in Biofloc systems (BFT) has emerged as an alternative to more
109 sustainable aquaculture (Khanjani et al., 2016; El-Sayed 2020), but at the end of culture
110 there is a lot of quantity of nitrate and phosphorus. BFT allows better control of nitrogen
111 compounds content, especially ammonia and nitrite, in addition to smaller area
112 production, better animal health, reduction of pathological risks, and higher productivity
113 (Avnimelech 2012; Hargreaves 2013; Emerenciano et al., 2013). Studies have reported
114 the success of BFT in shrimp (El-Sayed 2020) and fish farming (Azim and Little 2008).
115 The possibilities of reuse of aquaculture effluent in a BFT system have been documented,
116 such as its use as a source of protein in feed formulation (Lobato et al., 2019), cultivation
117 of vegetables in hydroponics (Fimbres-Acedo et al., 2020), cultivation of microalgae
118 (Abreu et al., 2016) and zooplankton (Mota et al., 2019; Campos et al., 2020; 2022).
119 Campos et al. (2020) and Mota et al. (2019) proved that *D. similis* and *D. magna*,
120 respectively, grow well in a culture medium reusing the wastewater from Nile tilapia
121 cultivation in a biofloc system with *C. vulgaris* in the diet. However, they did not
122 investigate *D. magna* grown in this system with different microalgae diets or the
123 nutritional content of the water flea when grown in this medium and fed with *C. vulgaris*.

124 Thus, evaluating the effect of microalgae, *Chlorella vulgaris* and *Haematococcus*
125 *pluvialis* (vegetative and cystic phase) on the feeding of the microcrustacean *Daphnia*
126 *magna* (water flea) is extremely important to obtain a better evaluation of possible
127 microalgal diets for water flea cultures in different cultivation systems that provide better

biomass yields and nutritional composition through the reuse of Nile tilapia effluent in a biofloc system.

## Material and Methods

*Experimental design*

Two types of *Daphnia magna* production system were analyzed (factor 1): autotrophic (A) and mixotrophic (M); and three diets with microalgae (factor 2): *Haematococcus pluvialis* in the vegetative phase (HV), *Haematococcus pluvialis* in the cystic phase (HC) and *Chlorella vulgaris* (C). The combination of factors (2x3), with three repetitions each, totaled 18 experimental units, distributed in a completely randomized design. Thus, the resulting combinations were: AHV (autotrophic system with vegetative *H. pluvialis* as diet), AHC (autotrophic system with cystic *H. pluvialis* as diet), AC (autotrophic system with C. vulgaris as diet), MHV (mixotrophic system with vegetative *H. pluvialis* as diet), MHC (mixotrophic system with cystic *H. pluvialis* as diet) and MC (mixotrophic system with *C. vulgaris* as diet).

*Experimental conditions*

The entire experiment was conducted at the Living Food Production Laboratory – LAPAVI, located at the Department of Fisheries and Aquaculture – DEPAq at the Federal Rural University of Pernambuco – UFRPE. Cultures of the freshwater crustacean *D. magna* for 18 days were carried out in polyethylene containers of 5 L, with a useful volume of 2 L, continuously aerated, natural photoperiod (12 h of light) under 30 µmols photos $m^{-2}$ $s^{-1}$ irradiance. Adult individuals (~1 mm in size) were stocked at a density of 12 organisms $L^{-1}$, adapted from Campos et al. 2020. The density of the organism was determined by counting every two days by the volumetric method, performing five counts for each experimental unit (Manso, 2006). On the same days of counting the population

of *D. magna*, the microalgae *C. vulgaris* and *Haematococcus pluvialis* were inoculated in natura at a density of 1 x $10^5$ cells $mL^{-1}$ $ind^{-1}$ for both species (Campos, et al. 2020).

*Maintenance of stock cultures of D. magna, C. vulgaris, H. pluvialis and algal inoculation regime*

The stock culture of *D. magna* was maintained in a mixotrophic system through the fermentation of chicken manure (0.3 g $L^{-1}$) with dry bread yeast (*Saccharomyces cerevisiae*) (0.3 g $L^{-1}$) adapted from Herawati et al (2017), 15 L tanks with a useful volume of 12 L maintaining the variables alkalinity (100-120 mg $CaCO_3$ $L^{-1}$), pH 7-8, temperature (26-28 ºC), constant aeration, at the same light regime, fed with the microalgae *C. vulgaris* every two days *ad libitum* (Campos et al., 2020)

The stock production of microalgae *C. vulgaris* and *H. pluvialis* was carried out in a semi-continuous system in polyethylene containers of 5 and 2 L, respectively, using NPK culture medium (agricultural fertilizer in the proportion 20:10:20) at a concentration of 2 mL $L^{-1}$, vitamin B complex solution (cyanocobalamin and biotin) (0.2 mL $L^{-1}$) and trace metal solution (1 mL $L^{-1}$). The amounts of N, P, K, and vitamins present in the medium were calculated according to the Bold's Basal medium (Kanz and Bold 1969). The metal solution used followed the amounts described by Renstrom et al. (1981) with some adaptations. The description of the NPK culture medium and metal solution is listed below (Table 1).

Table 1 Amounts of macronutrients and micronutrients present in the NPK culture medium and metal solution.

| NPK Medium | Stock solution (g $L^{-1}$) | Use (mL $L^{-1}$) |
|---|---|---|
| NPK (20:10:20) | 50 | 2 |
| Trace metals | | |
| $Co(NO_3)2\ 6H_2O$<br>$CuSO_4\ 5H_2O$<br>$NaMoO_4\ 5H_2O$<br>$ZnSO_4\ 7H_2O$<br>$MnCl_2\ 4H_2O$<br>$NH_4VO_3$<br>$H_3BO_3$ | 0.145<br>0.125<br>0.12<br>0.29<br>1.98<br>0.01<br>0.62 | 0.1 |

The microalgae were subjected to integral photoperiod (i.e., 24 h of light) under 30 µmol photons $m^{-2}\ s^{-1}$ irradiance (10W LED lamps), continuously aerated at pH 7.2-7.8 and temperature 25-27 ºC. To induce the transition from the vegetative phase to the cystic phase of the microalgae *H. pluvialis*, sodium acetate (1,96 mg $L^{-1}$ was added on the ninth day of cultivation and on the tenth day of cultivation, the irradiance was increased to 70 µmols photons $m^{-2}\ s^{-1}$. The algae inoculation methodology in the system was in accordance with Campos et al. (2022), based on the following equations:

$$InA = Ct - (Nind \times 10^5)$$

Where:

InA: microalgae inoculum (cells $mL^{-1}$); Ct: microalgae concentration in the *D. magna* culture tank (cells $mL^{-1}$); Nind: number of individuals of *D. magna* (Ind) $10^5$: predetermined algal concentration (cells $mL^{-1}$ $Daphnia^{-1}$) $InA \geq 0$ (zero) algal inoculum will not be necessary. $InA \leq 0$ algae inoculum will be required. When necessary, the inoculum was calculated following the equation below:

$$I = InA \times Vt / Cm$$

Where I: inoculum volume (L); InA: Seaweed inoculum (cell $mL^{-1}$); Vt: volume of the *D. magna* culture tank; Cm: algae concentration in the production tanks (cell $mL^{-1}$).

On the first day of cultivation, 4 h after the diet was offered, the individuals were photographed to observe the intestinal tract and confirm the ingestion of microalgae.

The wastewater from the cultivation of Nile tilapia (*Oreochromis niloticus*) in was conducted on a C:N of 10, stocking density of 40 fish $m^{-3}$ (Azim and Little 2008), (25.66 ºC, pH 7.55 , sedimentable solids of 15 mL $L^{-1}$, alkalinity of 105 mg $CaCO_3$ $L^{-1}$, 5.45 mg $L^{-1}$ of dissolved oxygen, NAT 1.55 mg $L^{-}$, N-$NO_2$ 0.5 mg $L^{-1}$, N-$NO_3$ 4 mg $L^{-}$, orthophosphate of 20.55 mg $L^{-1}$, 105 was used as a constituent of the water flea culture medium. 200 mL of wastewater was used (10% of the useful volume of the experimental units, 2 L) and 1800 mL of previously treated, chlorinated, dechlorinated and aerated water, pH 7.3, in the mixotrophic treatments. The wastewater was used *in natura*, excluding any previous processing. Regarding the autotrophic system, 2 L of clear water, treated with chlorine, dechlorinated and aerated. All experimental units were adjusted to an alkalinity of 100 mg $CaCO_3$ $L^{-}$.

*Water quality*

Temperature and pH were monitored (YSI model 100; Yellow Springs, OH, USA) every other day (at 9:00 am). Total ammonia nitrogen (NAT), N - nitrite (N-$NO_2^{-}$), N - nitrate (N-$NO_3^{-}$), and orthophosphate ($PO_4^{-3}$) were monitored at the beginning (day 0), middle (day 8) and at the end (day 18) of the experiment following the methods described

by Koroleff (1976), Golterman et al. (1978), Mackereth et al. (1978), Felföldy et al. (1987), respectively.

*Protein and lipid analysis*

The biochemical composition of algal biomasses (i.e., *C. vulgaris*, and *H. pluvialis* in both growth phase), and *D. magna* fed with different algal diets were evaluated in terms of total protein and crude lipids according to the micro-Kjeldahl (Association of Official Agricultural Chemists [AOAC], 2012) and Bligh and Dyer (1959) methods, respectively.

*D. magna growth*

The analysis of the growth of *D. similis* was verified through the variables specific growth rate (TCE); doubling time (TD); Yield (Y) and maximum average density (MAD) and maximum density day (DMD) which were determined according to Campos et al., (2020). In addition to these variables, the wet biomass generated at the end of cultivation was also quantified.

*Statistical analysis*

Homoscedasticity (Bartlett's test) and normality (Shapiro-Wilk) were used to check the data, followed by log transformation (x + 1) for data normalization. Factorial analyzes of variance (2 x 3) were performed; Tukey's test ($p<0.05$) for the variables of growth of *D. magna*, percentage of lipids, proteins, and water quality data. In addition, the coefficient Pearson's correlation test for the variables that stood out in the study. Statistical analysis was performed using the R 3.4 software (R Core Team 2021).

## Results

*Water quality*

The water quality variables in the cultivation of *D. magna* with different microalgae diets using wastewater from the cultivation of Nile tilapia in a biofloc system are shown in Table 2. Significant differences ($p < 0.05$) were found for the both factors (i.e., system and diet) for most variables except for NAT, pH, and temperature. However, regarding the interaction between these factors, only significant differences ($p < 0.05$) were obtained for the variables $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, N, $P\text{-}PO_4^{-3}$ and $PO_4^{-3}$ (Table 2). The amount of N and $P\text{-}PO_4^{-3}$ present in the water at the beginning, middle and end of cultivation can be seen in Figures 1 and 2. In most of the results, the highest N values were documented at the beginning of cultivation (day 0) for both systems, except for the autotrophic that had mixotrophic *C. vulgaris* as a diet. However, for $P\text{-}PO_4^{-3}$ in most combinations there were higher amounts on the last day of cultivation (18), except for the combinations that had *H. pluvialis* in the cystic phase (Red H) as a diet. In the end, the latter practically had their N and P concentrations zeroed (Figure 1, 2).

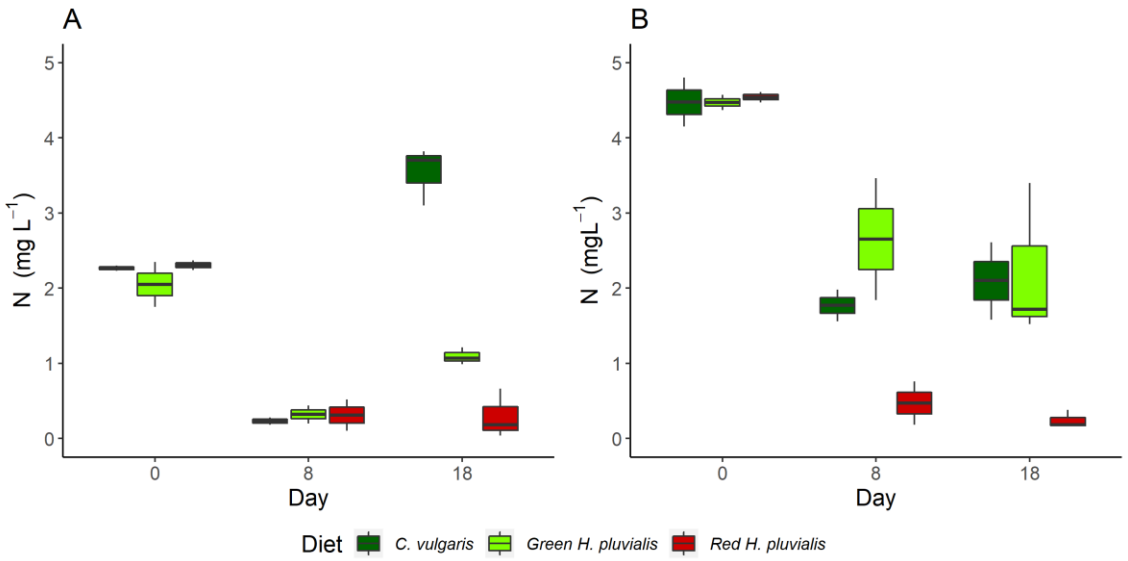



*Figure 1 Amounts of nitrogen present at the beginning (day 0), middle (day 8), and end (day 18) in the water of the cultivation for the autotrophic (A) and mixotrophic (B) system with the respective diets. Mixotrophic system was made using wastewater from Nile tilapia culture in biofloc system and autotrophic system with clear water.*

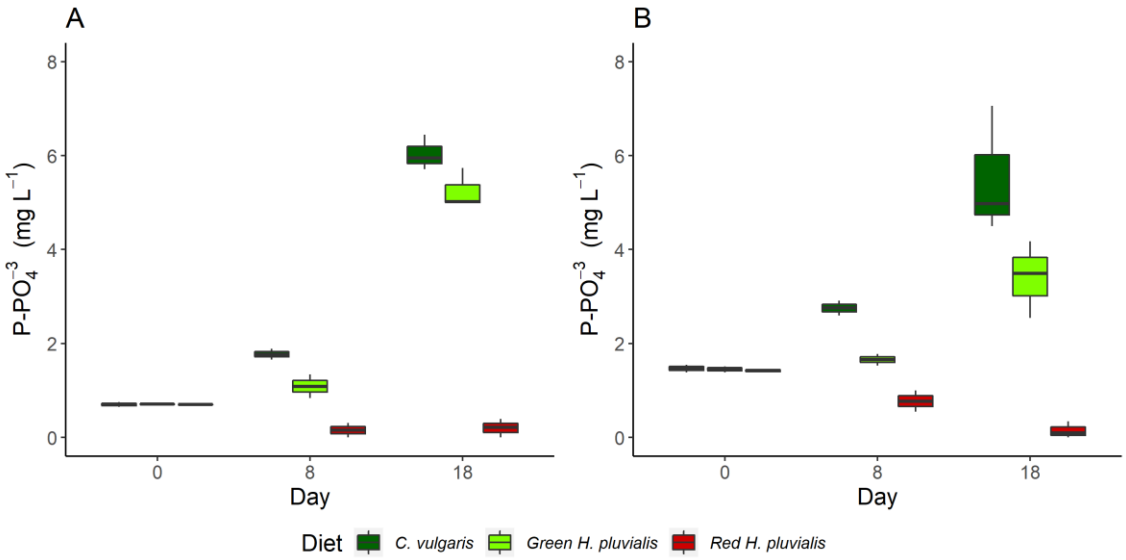



*Figure 2 Amounts of P-$PO_4^{-3}$ present at the beginning (day 0), middle (day 8) and end (day 18) of the culture for the autotrophic (A) and mixotrophic (B) system with the respective diets. Mixotrophic system was made using wastewater from Nile tilapia culture in biofloc system and autotrophic system with clear water.*

*Growth and protein and lipid content of Daphnia magna*

The values found for the variables MAD, MDD, SGR, DT, Y and biomass are described in Table 3. All variables showed significant differences ($p < 0.05$) for the factors and interactions between them. The combinations that had *H. pluvialis* as a diet in the cystic phase (Red H.) in both systems (Autotrophic and Mixotrophic) did not obtain growth of *D. magna*, presenting the total death of the individuals on the fourth day of cultivation. At the beginning of cultivation, it was detected that the individual's intestinal tracts were filled with algal biomass (Figure 3). For this reason, all growth variables for the red AH and red MH combinations are described with zero amounts (0.00). After identifying the death of individuals in these combinations, no more was added to the diet (*H. pluvialis* in the cystic phase – red). The highest MAD values were documented in combinations where *C. vulgaris* was in the diet, with day 14 having the highest density for both culture systems, reaching average values of 825 ± 67 (AC) and 1333 ± 88 ind $L^{-1}$ (MC) (Table 3). The *D. magna* growth curves are plotted in Figure 4.

Table 2 Mean ± standard deviation, minimum and maximum of the variables TAN, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $PO_4^{-3}$, pH, temperature and alkalinity in the production of *D. magna* present in the combinations of the analyzed factors: factor 1- System of cultivation (autotrophic and mixotrophic) (S) and factor 2 – Diet (D): microalgae *C. vulgaris*, *H. pluvialis* in the vegetative (green) and cyst (red) phases. The culture medium in an autotrophic system consisted of clear water + microalgae and the mixotrophic medium of the wastewater from Nile tilapia cultivation in a biofloc system + algae.

| **Variables** | **System of cultivation (S)** | | | | | | **Fators** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Autotrophic** | | | **Mixotrophic** | | | | | |
| | **Diet (D)** | | | **Diet (D)** | | | **D** | **S** | **D * S** |
| | ***C. vulgaris*** | ***Green H*** | ***Red H*** | ***C. vulgaris*** | ***Green H*** | ***Red H*** | | | |
| **TAN (mg $L^{-1}$)** | 1.453 ± 1.081 a<br>0.04 – 2.92 | 0.617 ± 0.772 a<br>0 – 1.89 | 0.702 ± 0.622 a<br>0 – 1.48 | 0.933 ± 0.426 a<br>0.4 – 1.4 | 1.319 ± 0.668 a<br>0.08 – 2.46 | 0.628 ± 0.593 a<br>0 – 1.38 | ns | ns | ns |
| **$N\text{-}NO_2^-$ (mg $L^{-1}$)** | 0.453 ± 0.284 a<br>0.12 – 0.94 | 0.536 ± 0.365 a<br>0.20 – 1.15 | 0.197 ± 0.202 a<br>0.02 – 0.50 | 0.696 ± 0.353 a<br>0.25 – 1.35 | 0.473 ± 0.292 a<br>0.08 – 0.94 | 0.252 ± 0.318 a<br>0 – 0.78 | * | ns | * |
| **$N\text{-}NO_3^-$ (mg $L^{-1}$)** | 0.106 ± 0.235 b<br>0 – 0.69 | 0.0 ± 0.00 b<br>0 – 0 | 0.071 ± 0.213 ab<br>0 – 0.64 | 1.151± 1.122 ab<br>0 – 2.60 | 1.319 ± 0.980 a<br>0 – 2.68 | 0.870 ± 1.224 ab<br>0 – 2.54 | * | * | * |
| **$PO_4^{-3}$ (mg $L^{-1}$)** | 8.697 ± 7.516 ab<br>2.00 – 19.75 | 7.207 ± 6.732 ab<br>2.13 – 17.60 | 1.081 ± 0.894 b<br>0 – 2.16 | 9.950 ± 5.876 a<br>4.25 – 21.65 | 6.654 ± 3.119 ab<br>4.25 – 12.80 | 2.397 ± 1.754 ab<br>0 – 4.49 | * | ns | * |
| **N (mg $L^{-1}$)** | 2.012 ± 1.459 ab<br>0.18 – 3.82 | 1.153 ± 0.770 ab<br>0.20 – 2.35 | 0.969 ± 0.970 a<br>0.04 – 2.37 | 2.781 ± 1.319 ab<br>1.56 – 4.80 | 3.111 ± 1.106 b<br>1.52 – 4.57 | 1.750 ± 2.101 ab<br>0.16 – 4.61 | ns | * | * |
| **P- $PO_4^{-3}$ (mg $L^{-1}$)** | 2.836 ± 2.451 ab<br>0.652 – 6.440 | 2.350 ± 2.195 ab<br>0.695 – 5.739 | 0.352 ± 0.291a<br>0.00 – 0.704 | 3.245 ± 1.756 b<br>1.386 – 7.060 | 2.170 ± 1.017 ab<br>1.386 – 4.174 | 0.782 ± 0.572 a<br>0.00 – 1.464 | * | ns | * |
| **pH** | 7.61 ± 0.269 a<br>7.40 – 8.00 | 7.574 ± 0.264 a<br>7.40 – 8.00 | 7.52 ± 0.269 a<br>7.30 – 8.00 | 7.38 ± 0.0 a<br>7.30 – 7.50 | 7.397 ± 0.067 a<br>7.30 – 7.50 | 7.382 ± 0.066 a<br>7.30 – 7.50 | ns | ns | ns |
| **Temperature (ºC)** | 29.689 ± 0.285 a<br>28.2 – 29.00 | 28.622 ± 0.186 a<br>28.3 – 8.00 | 28.522 ± 0.172 a<br>28.3 – 28.8 | 28.656 ± 0.283 a<br>28.3 – 29.0 | 28.600 ± 0.269 a<br>28.4 – 29.0 | 28.633 ± 0.292 a<br>28.3 – 29.2 | ns | ns | ns |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Alkalinity (mg $CaCO_3$ $L^{-1}$)** | 117.333 ± 17.861 a<br>100 – 134 | 103.778 ± 7.563 a<br>100 - 119 | 101.111 ± 3.333 a<br>100 - 110 | 118.889 ± 17.266 a<br>100 – 139 | 108.000 ± 12.971 a<br>100 - 135 | 111.111 ± 11.591 a<br>100 – 130 | ns | ns | ns |

$N\text{-}NO_2^-$ , nitrite nitrogen; $N\text{-}NO_3^-$ , nitrate nitrogen; $PO_4^{-3}$, orthophosphate; TAN, total ammoniacal nitrogen; * Significant differences between factors according to two-way ANOVA analysis of variance followed by Tukey's test ($p < 0.05$). “ns” indicate absence of significance between the factors. Different letters between the columns indicate statistical differences ($p < 0.05$) between the combinations for the analyzed variable.

Table 3 Mean ± standard deviation of the variables maximum average density (MAD), day of maximum density (DMD), specific growth rate (SGR), doubling time (DT), yield (Y) and biomass in the production of *D. magna* present in the combinations of analyzed factors: factor 1- Cultivation system (autotrophic and mixotrophic) (S) and factor 2 – Diet (D): microalgae *C. vulgaris*, *H. pluvialis* in the vegetative (green) and cyst (red) phases. The culture medium in an autotrophic system consisted of clear water + microalgae and the mixotrophic medium of the wastewater from Nile tilapia cultivation in a biofloc system + algae.

| **Variables** | **System of cultivation (S)** | | | | | | **Factors** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Autotrophic** | | | **Mixotrophic** | | | | | |
| | **Diet (D)** | | | **Diet (D)** | | | **D** | **S** | **D * S** |
| | ***C. vulgaris*** | ***Green H*** | ***Red H*** | ***C. vulgaris*** | ***Green H*** | ***Red H*** | | | |
| **MAD (Ind $L^{-}$)** | 825 ± 67 a | 574 ± 36 b | 12 ± 0.0 c | 1333 ± 88 d | 688 ± 28 ab | 12 ± 0.0 c | * | * | * |
| **DMD (day)** | 14 | 16 | 0 | 14 | 14 | 0 | - | - | - |
| **SGR (% $day^{-}$)** | 30.20 ± 0.59 a | 24.17 ± 0.40 b | 0.00 ± 0.00 c | 33.63 ± 0.48 d | 28.92 ± 0.288 e | 0.00 ± 0.00 c | * | * | * |
| **DT (day)** | 0.023 ± 0.000 a | 0.029 ± 0.000 b | 0.00 ± 0.00 c | 0.021 ± 0.000 d | 0.024 ± 0.000 e | 0.00 ± 0.00 c | * | * | * |
| **Y (Ind $L^{-}$ $day^{-}$)** | 58 ± 5 ae | 35 ± 3 b | 0.00 ± 0.00 c | 94 ± 6 d | 48 ± 8 e | 0.00 ± 0.00 c | * | * | * |
| **Biomass (g)** | 6.37 ± 0.520 a | 4.43 ± 0.479 b | 0.00 ± 0.00 c | 10.3 ± 0.682 d | 5.32 ± 0.617 ab | 0.00 ± 0.00 c | * | * | * |

* Significant differences between factors according to two-way ANOVA analysis of variance followed by Tukey's test ($p < 0.05$). “ns” indicate absence of significance between the factors. Different letters between the columns indicate statistical differences ($p < 0.05$) between the combinations for the analyzed variable.


*Figure 3 Visualization of the intestinal tract filled with algal biomass in water fleas D. magna cultivated in autotrophic and mixotrophic systems with the microalgae diet C. vulgaris (a), H. pluvialis in the vegetative phase (b) and H. pluvialis in the cystic phase (c). Image recorded 4 h after offering the diet on the first day of cultivation.*

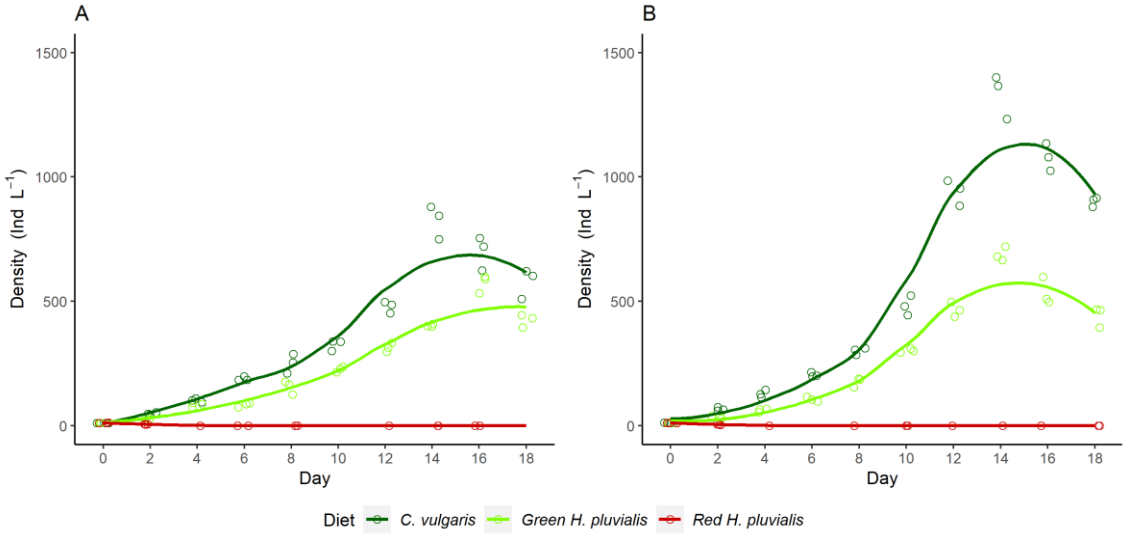



*Figure 4 D. magna growth curve in the autotrophic (A) and mixotrophic (B) systems with the respective diets. Mixotrophic system was made using wastewater from Nile tilapia culture in biofloc system and autotrophic system with clear water.*

*D. magna* showed significant differences ($p < 0.05$) for the percentage amounts of proteins and lipids for the combinations. Higher values of proteins and lipids were reported in the MC combination, reaching 61.20 ± 5.74 and 7.28 ± 1.34%, respectively, followed by MHV with 55.75 ± 1.31% of proteins and AHV with 4 .79% ± 0.48 (Figure 5). The amounts of proteins and lipids present in the diets are described in the table 4.

Table 4 Proteins and lipids contents in the microalgae *Chlorella vulgaris*, *Haematococcus pluvialis* – vegetative (green) and cyst (red) phases cultured in NPK culture medium.

| Diet | Lipids (%) | Protein (%) |
|---|---|---|
| *Chlorella vulgaris* | 14.31 ± 1.79 a | 26.87 ± 0.85 a |
| *Haematococcus pluvialis* (Green H) | 5.07 ± 1.55 b | 40.11 ± 0.75 b |
| *Haematococcus pluvialis* (Red H) | 22.03 ± 4.16 c | 21.49 ± 1.61 c |

Different letters between the lines indicate statistical differences ($p < 0.05$) between the combinations for the variable analyzed after one-way ANOVA followed by Tukey's test ($p < 0.05$).

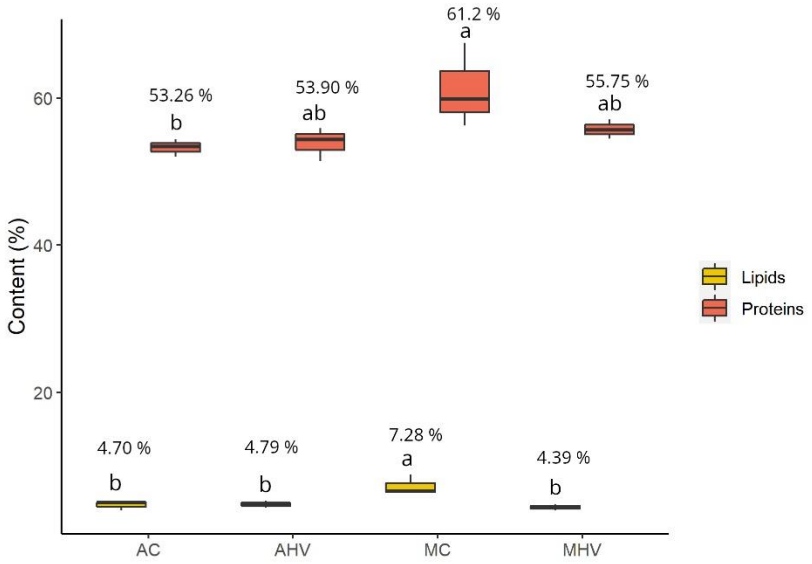



*Figure 5 Total protein and crude lipid contents (%) contained in dry biomass of D. magna cultivated in effluent from Nile tilapia culture in biofloc system (mixotrophic culture) and in clear water (autotrophic) and different microalgae diets. AHV (autotrophic system with vegetative H. pluvialis as diet), AC (autotrophic system with C. vulgaris as diet), MHV (mixotrophic system with vegetative H. pluvialis as diet MC (mixotrophic system with C. vulgaris as diet). Analysis of proteins and lipids was not performed for experimental combinations AHC and MHC because there was population death on the fourth day of cultivation. Different letters between the combinations indicate statistical differences ($p < 0.05$).*

*Correlation between growth variables, water quality and nutritional composition*

From the Pearson correlation test ($p<0.05$) it was possible to identify a high direct correlation between the variables Biomass, MAD, DT, SGR, Y, N, P-$PO_4^{-3}$, Lipids and proteins, presenting the lowest significant value of r=0.53 (N and P-$PO_4^{-3}$). The correlation between the Y and biomass variables obtained an r = 1.00, also for the biomass and MAD variables; and MAD and Y (Figure 6). This result is complemented by the interaction between the variables N, P-$PO_4^{-3}$ and MAD present in autotrophic and mixotrophic systems (Figure 7). MAD was higher in the presence of higher amounts of N and P-$PO_4^{-3}$. However, it is important to point out that there was a certain limit regarding N, where above 5 mg $L^{-}$ the MAD of *D. magna* in mixotrophic culture did not obtain high MAD.

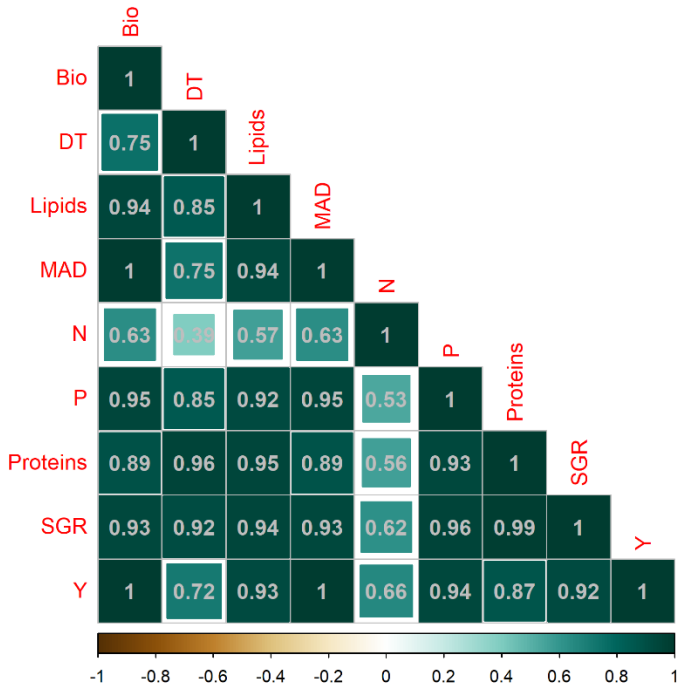
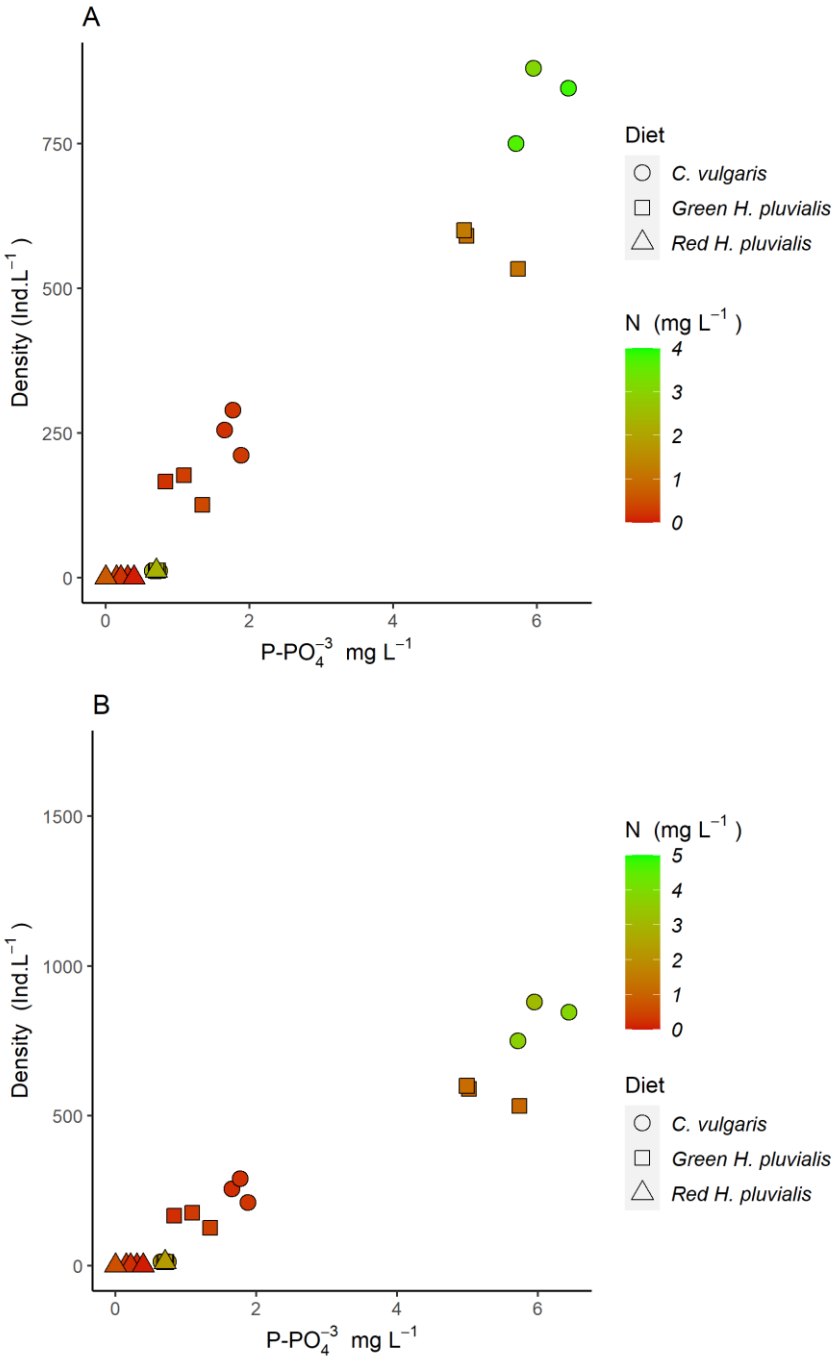



*Figure 6 Pearson correlation (p < 0.05) between the variables biomass (Bio), maximum average density (MAD), doubling time (DT), specific growth rate (SGR), yield (Y), nitrogen (N), phosphorus present in the orthophosphate (P-PO4-3), Lipids and proteins present in the cultivation of the water flea D. magna cultivated in wastewater from the cultivation of Nile tilapia in a biofloc system (mixotrophic cultivation) and in clear water (autotrophic ) and different microalgae diets: C. vulgaris, H. pluvialis (vegetative phase and cystic phase).*

349




350

351 *Figure 7 Interactive behavior between the variables N, P-PO4-3 and MAD present in the autotrophic (A) and*
352 *mixotrophic (B) culture systems of the water flea D. magna with different microalgae diets: C. vulgaris, H. pluvialis in*
353 *vegetative phase (green H.) and H. pluvialis in the cystic phase (red H.).*

354

## Discussion

*Water quality*

Analyzing the results obtained in terms of water quality, it was possible to verify that the diet factor was the main factor for obtaining significant differences between the experimental combinations. Despite some differences between factors in some variables, mainly nitrogenous compounds, the amounts of these compounds were still within the acceptable range for the species (Hoff and Snell 2006). The highest mean values of N present in the mixotrophic system, regardless of the microalgae in the diet, are primarily responsible for $N\text{-}NO_3^-$, practically absent in the autotrophic system and quite present in the mixotrophic system. This may be due to the fact that in these combinations, the wastewater from fish farming was conducted in a biofloc system, which has high amounts of nitrate (Robles-Porchas et al. 2020) as this compound tends to accumulate in the system (Emerenciano et al., 2017, Poli et al. 2019) transferring these amounts to the mixotrophic cultivation of *D. magna*.

The combinations that obtained the lowest values of N and P nutrients in the water were precisely those that had the microalgae *H. pluvialis* in its cystic phase, that is, the red H. as a diet (Figure 3, 4). Although these combinations did not show *D. magna* growth, they stood out in terms of the bioremediation process possibly carried out by the microalgae in the cyst stage, reaching zero concentrations of P and N at the end of the cultivation. This may have happened due to the absence of predation by the water flea, since there was population death on the fourth day of cultivation, allowing bioremediation by the microalgae. As a good part of the N in mixotrophic cultivation consists of $N\text{-}NO_3$, green microalgae have the ability to uptake nitrogen compounds when compared to $N\text{-}NO_2^-$ and TAN (Boyd 2015). In contrast, these last two compounds are better fixed by

nitrifying bacteria (Ebeling et al. 2006), which are possibly also present in the mixotrophic culture due to the wastewater inoculum.

On the other hand, the combinations with *C. vulgaris* and *H. pluvialis* in the vegetative phase (Green H.), when comparing the beginning and end of cultivation, the amounts of $P\text{-}PO_4^{-3}$ increased, for both systems. This could have occurred due to the predatory action of *D. magna* on these microalgae, since there was population growth in these experimental units. Thus, the microalgae were not present in sufficient quantities to significantly reduce the concentrations of P in the water, with the accumulation of this macronutrient.

However, for the amounts of N, only the autotrophic system showed higher values at the end of the cultivation with *C. vulgaris* as diet. This event demonstrates the importance of the mixotrophic system in the control of N present in the water, since with the inoculation of the wastewater from the cultivation of Nile tilapia in a biofloc system, there was possibly a plus of nitrifying bacteria previously established in the wastewater of the tilapia cultivation (Hargreaves 2013, Emerenciano 2013). These bacteria are essential for maintaining the N cycle in water, from the reduction of ammonia to nitrite, which is responsible for bacteria of the genus *Nitrosomonas*; and the reduction of nitrite to nitrate, a process carried out by bacteria of the genus *Nitrobacter*. (Ebeling et al. 2006; Boyd 2015).

*Growth of Daphnia magna, protein and lipid content*

In the cultivation of the water flea *D. magna*, both factors system (autotrophic and mixotrophic) and diet (the microalgae *C. vulgaris*, *H. pluvialis* in the vegetative and cystic phases) were predominant for the statistical differences between the combinations experimental data (as showed in Table 3 and Figure 4).

405 The benefits of using mixotrophic systems for the production of *D. magna* has
406 also been documented by other studies, using different sources of organic matter such as
407 fish farming wastewater in a biofloc system (e.g., Mota et al., 2019; Campos et al., 2020;
408 Campos et al., 2022), chicken manure (e.g., Paray and Al-Sadoon, 2016; Herawati et al.,
409 2017), quail and goat manure (e.g., Herawati et al., 2017).
410 One reason for this prominence of mixotrophic cultures in the production of the
411 water flea of the genus *Daphnia* using the wastewater from Nile tilapia cultivation in a
412 biofloc system is precisely its inorganic, organic and biological constitution. Population
413 growth *Daphnia* sp. it is stimulated in the presence of P in the water (Barsanti and
414 Gualtieri 2006); they are abundant in environments with a high concentration of organic
415 matter (debris) and where there is proliferation of bacteria, yeasts, and microalgae, as they
416 use these components as food (Monakov, 1972; Torrentera and Tacon 1989; Barrera et
417 al. 2003). Thus, the wastewater from aquaculture in the BFT system presents favorable
418 conditions for the production of this microcrustacean.
419 Campos et al. (2020) and Mota et al. (2019) reported high growth of *D. similis*
420 (1,234 ± 286 ind $L^{-1}$) and *D. magna* (3,433 ± 267 ind $L^{-1}$), respectively, in wastewater
421 from Nile tilapia cultivation in a biofloc system with C: N of 12 and 10, respectively,
422 being this work closer to the results obtained by Campos et al. (2020). A higher densities
423 of *D. magna* were reported by Herawati et al. (2017) (211,788.9 ind $L^{-}$) using chicken
424 manure fermented with tofu and bread waste and by Paray and Al-Sadoon (2016) in the
425 cultivation of *D. carinata* in medium with chicken manure (4,660 ± 523 ind $L^{-1}$).
426 Alcántara-Azuara et al. (2014) cultivated *D. pulex* using the microalgae *Sphaerocystis*
427 sp., *Chlorella vulgaris* and *Haematococcus pluvialis* as diet and achieved similar growth
428 results to this study for *C. vulgaris* (1,395 ± 24 ind $L^{-1}$), however, different for to *H.*
429 *pluvialis* in the vegetative phase (1,933 ± 60 ind $L^{-1}$). The results found in this study

confirm that the use of wastewater from Nile tilapia cultivation in a biofloc system is an alternative option for the production of the water flea *D. magna*, on a par with other mixotrophic cultures that use other sources of organic matter.

However, the present work was not successful in the production of *D. magna*, in both systems, with the presence of *H. pluvialis* in the cystic phase (Red H.) (Table 3, Figure 4). In this case, the diet factor was the cause of the statistical differences, since there was a population death of the water flea in the combinations (AHC and MHC) (Table 3, Figure 4). This result can be linked to the fact of the morphological characteristic of the microalgae, as there was decantation of algal biomass even in the presence of aeration. It is important to remember that the aeration in *Daphnia* cultures cannot be too intense, but must be mild or even without aeration with water renewal, which could affect the filtration efficiency (Serra et al., 2018; Serra et al. 2019).

The microalga *H. pluvialis* in this cyst phase presents high contents of carotenoids, mainly astaxanthin, loses mobility due to the absence of flagella and can form colonies, becoming "heavier" when compared to its vegetative phase, considerably reducing their permanence time in the water column. According to Hagen et al. (2002), *H. pluvialis* in the cyst stage (aplanospore) has a cell wall 2 to 3 times thicker than in the vegetative stage(Green H.). However, this does not mean that *D. magna* does not feed on *H. pluvialis* in the cyst stage, as can be seen in Figure 4. However, its use alone does not promote growth due to this fact detected during the cultivation in cyst stage. This was more worrying due to feeding frequency, which was every two days.

Then, it is assumed that the death of water fleas would be linked more to the absence of food and not to the characteristics of the adopted cultivation systems. Due to this fact, it can be suggested that its use is more for enrichment at the end of the cultivation and not for biomass production, since this microalgae presents several nutritional and

455 immunological benefits due to the production of the carotenoid astaxanthin (Pogorzelska
456 et al., 2018; Mota et al., 2022) and can be used as an immunostimulant in the production
457 of aquatic organisms. In this case, *D. magna* would act as a bioencapsulator agent to be
458 used as live food in aquaculture.

459 In contrast, *C. vulgaris* stood out as a better diet for *D. magna* biomass production,
460 both in autotrophic and mixotrophic systems (Table 3, Figure 4). The use of this
461 microalgae as a diet also provided good growth of the water flea *D. pulex* (Alcántara-
462 Azuara et al. 2014), *D. magna* (Mota et al. 2019) and *D similis* (Campos et al., 2020),
463 proving its food efficiency. This was reflected in the nutritional composition of *D. magna*
464 cultivated in a mixotrophic system and with *C. vulgaris* in the diet.

465 The amount of crude protein found in *D. magna* in a mixotrophic system with *C.*
466 *vulgaris*(Figure 5) is similar to that reported by Turcihan et al. (2022), that reported
467 52.40% of protein in *D. magna* fed with *C. vulgaris*. Similarly, Herawati et al. (2017)
468 reported contents of 68.85% of crude protein in *D. magna* using chicken manure
469 fermented with tofu and bread waste.

470 Regarding to lipids, the results found in this study in the MC combination (7.28%)
471 are similar to other works where the amount of lipids present in *D. magna* fed with *C.*
472 *vulgaris* reached 7.84% (Turcihan et al. 2022) and the one fed with chicken manure
473 fermented by tofu and bread waste, 7.16% (Herawati et al. 2017). Turcihan et al. (2022)
474 analyzed the fatty acids present in the water flea *D. magna* when fed on *C. vulgaris* and
475 identified the presence of approximately 31.89% of saturated fatty acids, 21.76% of
476 monounsaturated fatty acids, 6.50% of omega-3, 17.99% of omega-6 and 18.34% of
477 omega-9. On the other hand, Fung and Leung (2009) reported 24.2% of saturated fatty
478 acids, 46.8% of fatty acids monounsaturated and 34.2% polyunsaturated fatty acids.

Nevertheless, no studies were found that analyzed the nutritional content of *D. magna* fed with *H. pluvialis* in the vegetative or cystic phase.

The results found for proteins and lipids in algae produced with NPK were close to those found by other authors (Table 4), which demonstrates that the medium used can replace the means of traditional production cultures, reducing the cost. The microalga *C. vulgaris* presents concentrations of proteins and lipids, in dry matter, of approximately 30% - 61.6% (Villarruel-López et al., 2017; Ahmed et al., 2020; Turcihan et al. 2022) and of 11.3 to 12.5% of lipids depending on the culture conditions (Turcihan et al. 2022; Ahmed et al., 2020). On the other hand, *H. pluvialis* in the vegetative phase (H. verde), can reach up to 45% and 25% of the dry weight, of proteins and lipids, respectively, while in the cystic stage it reaches approximately 23% of protein and 37% and lipid (Kim et al., 2015; Shah et al., 2018).

Water fleas cultivated in the combinations with MC and MHV had a protein increase of 7.94 and 1.85%, respectively, when compared to the autotrophic combinations. In this case, the system was essential to increase the protein concentrations, since, being mixotrophic, it also has other protein sources present, such as bacteria and fungi that can also be filtered by *D. magna*.

According to Hargreaves (2013) biofloc system presents microbial aggregates (flocs) consisting of microalgae, bacteria, protozoa and other types of organic matter, which compiled can contain, in dry matter, about 30 - 45% of protein. Another important fact is that the microalga *Chlorella* spp. because it is smaller, 2 to 10 µm in diameter, and without mobility (Jin et al. 2015) it has a greater probability of adhering to the flake and perhaps that is why this percentage increase was greater in the combination with *C. vulgaris* than with *H. pluvialis* in the vegetative phase (H. verde) in this system. Campos et al. (2020) reported that *D. similis* was able to grow 40 ± 6 ind $L^{-1}$ during six days using

504 only Nile tilapia effluent in a biofloc system as a culture medium, indicating that the water
505 flea also feeds on these microbial aggregates.
506
507 *Correlation of growth variables, water quality and nutritional content in the cultivation*
508 *of D. magna*
509 The high correlations found in this study demonstrate how the analyzed variables
510 interact with each other (Figure 6). The high correlation between *D. magna* growth
511 variables (MAD, SGR, DT and Y) was also found in the work carried out by Mota et al.
512 (2019), which also produced this same species of water flea in wastewater from fish
513 farming in a biofloc system with C:N of 10:1. As the growth variables depend on the
514 number of individuals present, a high correlation between MAD, biomass, Y, SGR, DT
515 was expected (Figure 6).
516 In addition, as the highest growth results (MAD), lipid and protein content of the
517 water flea were in the MC combination, this high correlation was also expected. However,
518 it was interesting to find significant correlations between the amounts of N and P in the
519 water with the MAD, percentages of lipids and proteins contained in *D. magna* (Figure 6
520 and 7). Herawati et al. (2017) did not perform a correlational analysis between these
521 variables, but identified that precisely in the experimental units where the highest
522 percentages of N and P were found in the culture medium, it was exactly where there
523 were the highest densities of *D. magna* and also the highest biomass. Our study indicated
524 that the range of $P\text{-}PO_4^{-3}$ concentration between 4 and 6 mg $L^{-1}$ in the culture water was
525 where it presented the highest MAD (Figure 7). These concentrations were higher at the
526 beginning of the mixotrophic cultures (Figure 1), which could have been a stimulus for
527 the growth of the individuals, explaining the high MAD of *D. magna*, when compared to
528 the autotrophic culture. According to Barsanti and Gualtieri (2006), phosphorus

529 concentrations equal to or less than 1 mg $L^{-1}$ stimulate the reproduction of *D. pulex* and
530 between 5-7 mg $L^{-1}$ stimulate the reproduction of *D. magna*.

531 Serra et al. (2019) found no interference in *D. magna* filtration rate when exposed
532 to high concentration of P-$PO_4^{-3}$ in the range of 0 to 100 mg $L^{-1}$. Inorganic phosphate is
533 important for energy generation, constituting the molecules of adenosine diphosphate
534 (ADP) and adenosine triphosphate (ATP), important for metabolic processes in the cell
535 in addition to constituting the phospholipid layer of the cell membrane, DNA and RNA
536 (Boyd 2015).

537 The highest N concentrations in the mixotrophic system were mainly due to the
538 N-$NO_3^-$ concentrations found (Table 2). N-$NO_3^-$ on a scale of 0 to 100 mg $L^{-1}$ does not
539 interfere with *D. magna* filtration rate when compared to TAN and N-$NO_2^-$, which already
540 become lethal at concentrations of 35 and 20 mg $L^{-1}$, respectively (Serra et al. 2019).
541 Nitrogen, in organic form, is important for *Daphnia* growth, being required in large
542 amounts as a major component in the formation of peptides, proteins, enzymes,
543 chlorophyll, energy transfer molecules (ATP, ADP), DNA, RNA, and other cellular
544 constituents (Barsanti and Gualtieri 2006).

545

546 *Perspectives and contributions*

547 Based on what was found in this study regarding the nutritional aspects of *D.*
548 *magna*, in particular the high concentration of proteins, approximately 60% (Figure 5), it
549 can be suggested its applicability as a live food in aquaculture, mainly in larval stages of
550 fish, shrimp, etc. as well as in the production of feed for aquatic organisms as a substitute
551 for fish meal, since the demand for this product is high and raises several problems related
552 to the maintenance of fish stocks and natural resources. This theme needs to be better
553 investigated since there are few works that evaluated the use of microcrustaceans as an

554 alternative source of proteins, either in vivo or dry in the form of flour for the formulation
555 of feed for aquatic organisms (Chiu et al. 2015; Che et al. 2017; Abo-Taleb 2021;
556 Aravind et al. 2021).

557 The search for alternative protein sources is a worldwide concern, and needs to be
558 better explored. This research contributes to both aquaculture and fishing by stimulating
559 the blue transformation, a goal advocated by the Food and Agriculture Organization of
560 the United Nations (FAO) which aims to establish practices that ensure and improve the
561 contribution of aquatic foods (marine or inland) to food security, nutrition and healthy
562 diets for all (FAO 2022). In addition, the sustainable theme addressed in this work enables
563 the water reuse from Nile tilapia cultivation in a biofloc system for the production of live
564 food, which can be used in the aquaculture production chain itself, contributing to lower
565 impacts from the release of effluents in adjacent water resources. It is also important to
566 emphasize that all algae were cultivated using NPK agricultural fertilizer, lowering
567 production costs, making it more accessible to the producer. This research also
568 contributes to encouraging and achieving goals established by the important Sustainable
569 Development Goals (SDGs) in Agenda 2030, including: Ensuring sustainable production
570 and consumption standards; ensure the availability and sustainable management of water;
571 and conserve oceans, seas and marine resources for sustainable development.

572

573 ## Conclusions

574 Based on the results, the cultivation of *Daphnia magna* in a mixotrophic system
575 using fish farming wastewater (Nile tilapia) in bioflocs with the supply of *Chlorella*
576 *vulgaris* in the diet provided better growth and nutritional content from higher amounts
577 of biomass, yield, proteins and lipids, which were influenced by the highest
578 concentrations of N and P in the system.

Future researches evaluating the offer of the microalgae *H. pluvialis* in the transition from the vegetative to the cystic phase as diet for *D. magna* would be interesting to observe its benefits in relation to growth, biomass, and nutritional content in *Daphnia magna*. In addition, due to the high amount of protein present in *D. magna*, its evaluation as a substitute for fish meal in the formulation of feeds for aquatic organisms becomes relevant.

These results contribute to a better evaluation of possible microalgal diets for water flea cultures in different cultivation systems that provide better biomass yields and nutritional composition through the reuse of fish farming wastewater in a biofloc system, aiming at its use as live food for aquaculture and new possibilities for alternative protein sources.

## Acknowledgements

The authors are grateful to all the laboratory members for the preparation of the experimental material and technical assistance.

## Funding

This work was supported by CAPES: [Grant Number 88882.436231/2019-01,88882.436234/2019-01,88887.497047/2020-00]; CNPQ: [Grant Number PQ 308063/2019-8, PQ PQ309669/2021-9].

## Compliance with ethical standards

*Conflict of interest*: The authors declare that there is no conflict of interest about the publication of this article.

*Ethical approval:* The experiment was in accordance with Brazilian Law no. 11.794/2008.

## Data availability statement

Research data are not shared.

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## Considerações Finais

Futuros trabalhos avaliando a oferta da microalga *H. pluvialis* na fase de transição da vegetativa para a cística como dieta da *D. magna* seria interessante para observar seus benefícios em relação ao crescimento, biomassa e conteúdo nutricional em *Daphnia magna*.

Além disso, é importante a realização de futuros trabalhos que analisem o enriquecimento de *D. magna* com a *H. pluvialis* na fase cística e seu potencial como alimento vivo para organismos aquáticos e os benefícios que ela traz para a imunidade desses animais frente a doenças virais e bacterianas, uma vez que a *H. pluvialis* nesta fase é rica em astaxantina.

A possibilidade do uso da farinha de *D. magna* como substituta parcial ou integral da farinha de peixe na formulação de ração para organismos aquáticos carece de atenção haja vista o seu conteúdo elevado de proteínas, aproximadamente 61%.