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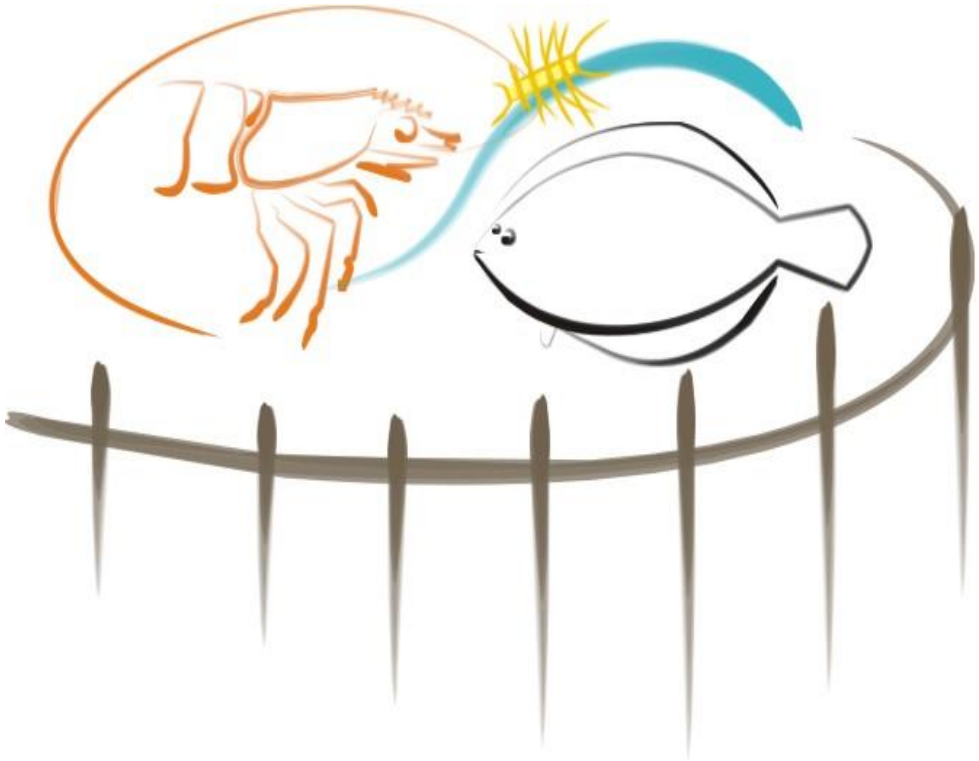
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA



BIANCA DE OLIVEIRA RAMIRO

ANÁLISE DO DESENVOLVIMENTO DAS BACTÉRIAS NITRIFICANTES PRESENTES EM SISTEMA DE BIOFLOCOS E ESTRATÉGIAS DE CULTIVOS DE *Penaeus vannamei* EM BFT E RAS

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Tese apresentada como parte dos requisitos para obtenção do grau de Doutora em Aquicultura no Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande – FURG.

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2024

ATA DE APROVAÇÃO

Pós Graduação em AQUICULTURA
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ATA DE DEFESA DA 89ª TESE DE DOUTORADO EM AQUICULTURA
No dia três de dezembro de dois mil e vinte e quatro, às uma hora e meia da tarde, reuniu-se a Banca Examinadora de Tese de Doutorado em Aquicultura, de **BIANCA DE OLIVEIRA RAMIRO**, orientada pelo Prof. Dr. Dariano Krummenauer, composta pelos seguintes membros: Prof. Dr. Dariano Krummenauer (Orientador – IO/FURG), Prof. Dr. Wilson Wasielesky Jr (Co Orientador – IO/FURG), Prof. Dr. Luke Roy (Universidade de Auburn, EUA), Dr. Fernando Gonçalves (Virgínia Tech, EUA) e Prof. Dr. Geraldo Foés (FURG). Título da Tese: **"ANÁLISE DO DESENVOLVIMENTO DAS BACTÉRIAS NITRIFICANTES PRESENTES EM SISTEMA DE BIOFLOCOS E ESTRATÉGIAS DE CULTIVOS DE *Penaeus vannamei* EM BFT E RAS".** Dando início à defesa, o Coordenador do PPGAq Prof. Dr. Ricardo Vieira Rodrigues, passou a presidência da sessão ao Prof. Dr. Dariano Krummenauer, que na qualidade de orientador, passou a palavra para a candidata apresentar a Tese. Após ampla discussão entre os membros da Banca e o candidato, a Banca se reuniu sob a presidência do Coordenador. Durante esse encontro ficou estabelecido que as sugestões dos membros da Banca Examinadora devem ser incorporadas na versão final da Tese, ficando a cargo do Orientador o cumprimento desta decisão. A candidata **BIANCA DE OLIVEIRA RAMIRO** foi considerada **APROVADA**, devendo a versão definitiva da Tese ser entregue a Secretaria do PPGAq, no prazo estabelecido nas Normas Complementares do Programa. Nada mais havendo a tratar, foi lavrada a presente ata, que após lida e aprovada, será assinada pela Banca Examinadora, pela candidata e pelo Coordenador do PPGAq.

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ÍNDICE

	Página
1. Introdução geral	14
2. Objetivos	19
2.1. Objetivo Geral	19
2.2. Objetivo Específico	19
Referências bibliográficas	**20**
Capítulo I	25
1. Introduction	27
2. Material and Methods	29
2.1. Design and Experimental Conditions	29
2.2. Water Quality Variables	31
2.3. Composition of the Microbial Community	31
2.4. Metagenomic Analysis	32
2.5. Proximate Composition	32
2.6. Feed Management and Shrimp Growth	33
2.7. Data Analysis	33
3. Results	34
3.1. Water Quality Variables	34
3.2. Composition of the Microbial Community	37
3.2.1 Water	37

3.2.2 Biofilm 40

3.3. Metagenomic Analysis 42

3.4. Proximate Composition of the Microbial Floc 45

3.5. Shrimp Growth 45

4. Discussion 46

5. Conclusion 50

References 51

Capitulo II 62

1. Introduction 63

2. Material and Methods 64

2.1. Design and Experimental Conditions 64

2.2. Phase 1 65

2.3. Phase 2 67

2.4. Water Quality Variables 67

2.5. Microbial Community Composition 67

2.6. Feed Management 68

2.7. Shrimp Growth 68

2.8. Data analysis 68

3. Results 70

3.1. Phase 1 70

3.1.1. Water quality 70

3.1.2. Phytoplankton and zooplankton community composition 71

3.1.3. Bacterial community composition 73

3.1.3.1. Water 73

3.1.3.2. Biofilm 73

3.1.4. Shrimp growth 75

3.2. Phase 2 75

3.2.1. Water quality 75

3.2.2. Phytoplankton and zooplankton community composition 78

3.2.3. Bacterial community composition 80

3.2.3.1. Water 80

3.2.3.2. Biofilm 82

3.2.4. Shrimp growth 83

4. Discussion 84

5. Conclusion 88

References 89

Capitulo III 98

1. Introduction 99

2. Material and Methods 100

2.1. Design and Experimental Conditions 100

2.2. Water Quality Variables 101

2.3. Vibrio Community Composition 102

2.4. Feed Management 102

2.5. Shrimp Growth, Survival and Water Use 102

2.6. Partial Budget analysis 103

2.7. Data Analysis 103

3. Results 104

3.1. Water Quality 104

3.2. Vibrio Community Composition 107

3.3. Shrimp Growth, Survival and Water Use 108

3.4. Partial Budget Analysis 108

4. Discussion 110

5. Conclusion 113

References 115

4. Discussão Geral 122

5. Conclusões 124

6. Perspectivas Futuras 125

DEDICATÓRIA

E eu desejo amar todos que eu cruzar pelo meu caminho
Como eu sou feliz, eu quero ver feliz
Quem andar comigo**.**

Maria Bethânia

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RESUMO

Em sistemas de produção de *Penaeus vannamei* que utilizam a tecnologia de bioflocos (Biofloc Technology System - BFT), a nitrificação desempenha um papel fundamental no controle das concentrações de compostos nitrogenados. No entanto, o estabelecimento e a eficiência das bactérias nitrificantes nesses sistemas podem ser influenciados por fatores físicos, químicos e biológicos, especialmente durante o cultivo. Dessa forma, esta tese teve como objetivo avaliar os efeitos dos diferentes tipos de sistema de aeração e de manejo de substratos artificiais no desenvolvimento das bactérias nitrificantes presentes no biofilme e na água e estabelecer práticas de manejo mais eficientes para a reutilização do biofilme durante cultivos superintensivos de *Penaeus vannamei* em sistema de bioflocos. Ainda foram avaliadas a qualidade da água e o crescimento de *Penaeus vannamei* em sistemas de bioflocos e sistemas de recirculação (RAS) usando alta densidade de estocagem. O primeiro estudo investigou o impacto do uso de nano e microbolhas como estratégias de aeração sobre o processo de nitrificação e crescimento do camarão *P. vannamei* em sistemas superintensivos com bioflocos. Os resultados demonstraram que o tratamento com a combinação de nano e microbolhas foi o mais efetivo, proporcionando melhor controle da amônia, maior abundância de bactérias benéficas no biofilme e promoveu o crescimento e a sobrevivência do camarão. O segundo estudo avaliou diferentes estratégias de manejo de substratos artificiais no processo de nitrificação e no crescimento do *P. vannamei*, conduzido em duas fases com substratos artificiais pré-colonizados. Observou-se que manter os substratos submersos em água foi uma abordagem eficaz que não comprometeu o processo de nitrificação entre os ciclos de cultivo. A exposição dos substratos ao ar também não comprometeu a nitrificação, promovendo a recuperação da comunidade bacteriana nitrificantes. Por fim, o terceiro estudo comparou os sistemas de BFT e RAS em termos de qualidade da água, crescimento dos camarões e viabilidade econômica. O BFT apresentou maior peso final, produtividade e controle de *Vibrio*, além de vantagem econômica sobre o RAS. Embora o RAS tenha demonstrado melhor controle de compostos nitrogenados, o BFT se destacou pela maior eficiência no uso de alimentos e água e apresentou maior lucratividade.

Palavras-Chave: Nitrificação, Bioflocos, Compostos Nitrogenados, Sistemas de Cultivo, Microrganismos.

ABSTRACT

In *Penaeus vannamei* production systems using biofloc technology (Biofloc Technology System - BFT), nitrification plays a key role in controlling nitrogen compound concentrations. However, the establishment and efficiency of nitrifying bacteria in these systems can be influenced by physical, chemical and biological factors, especially during cultivation. Therefore, this thesis aimed to evaluate the effects of different types of aeration systems and artificial substrate management on the development of nitrifying bacteria present in the biofilm and culture water and to establish more efficient management practices for the reuse of the biofilm during super-intensive cultivation of *Penaeus vannamei* in biofloc systems. The water quality and growth of *Penaeus vannamei* were also evaluated in biofloc systems and recirculating systems (RAS) using high stocking density. The first study investigated the impact of the use of nano and microbubbles as aeration strategies on the nitrification process and growth of *P. vannamei* shrimp in super-intensive systems with biofloc. The results demonstrated that the treatment with the combination of nano and microbubbles was the most effective, and provided better ammonia control, greater abundance of beneficial bacteria in the biofilm, and promoted shrimp growth and survival. The second study evaluated different strategies for managing artificial substrates in the nitrification process and growth of *P. vannamei*, conducted in two phases with pre-colonized artificial substrates. It was observed that keeping the substrates submerged in water was an effective approach that did not compromise the nitrification process between culture cycles. Exposing the substrates to air also did not compromise nitrification and promoted the recovery of the nitrifying bacterial community. Finally, the third study compared the BFT and RAS systems in terms of water quality, shrimp growth and economic viability. BFT showed greater final weight, productivity and *Vibrio* control, in addition to an economic advantage over RAS. Although RAS demonstrated better control of nitrogen compounds, BFT stood out for its greater efficiency in the use of food and water and greater profitability.

Keywords: Nitrification, Bioflocs, Nitrogen Compounds, Cultivation Systems, Microorganisms.

1. INTRODUÇÃO GERAL

Atualmente, a aquicultura vem se direcionando para sistemas de cultivo que visem o aumento da produtividade e a sustentabilidade ambiental da produção, reduzindo assim os impactos da atividade, como a redução dos lançamentos de efluentes no ambiente natural. Nesse sentido, diferentes estratégias de produção aplicadas para o cultivo de camarões marinhos vêm sendo estudadas. Dentre essas tecnologias destaca-se o sistema de bioflocos (Biofloc Technology System – BFT). O sistema BFT visa o aumento da produtividade com o cultivo em elevadas densidades de estocagem. Este sistema além de melhorar o controle ambiental através das baixas ou nenhuma renovação de água, o que promove ainda o aumento da biossegurança (Avnimelech, 2009; Krummenauer et al., 2011), e servem como fonte de alimento suplementar para os organismos produzidos (Wasielesky et al., 2006; Cardona et al., 2015). Outra vantagem é a possibilidade em reutilização da água do cultivo por diversos ciclos (Avnimelech et al., 2007; Krummenauer et al., 2014).

Os bioflocos são compostos por restos de alimentos, fezes, microalgas, protozoários, rotíferos, ecdises e bactérias. A sua formação favorece a manutenção da qualidade da água por meio da remoção dos compostos nitrogenados pelas bactérias heterotróficas e nitrificantes. O acúmulo de compostos nitrogenados pode ocorrer durante o ciclo de produção em altas densidades devido à excreção dos organismos e decomposição de matéria orgânica oriundas de ração não consumida e fezes (Timmons & Ebeling, 2010). Níveis inadequados destes compostos tóxicos, sobretudo de amônia e nitrito, podem induzir ao estresse e alterações fisiológicas nos organismos cultivados, afetando o crescimento e sobrevivência, prejudicando a produção (Vinatea et al., 2010; Ebeling, 2010). Desta forma, as bactérias nitrificantes presentes nos bioflocos apresentam papel importante no controle das concentrações dos compostos nitrogenados tóxicos em sistemas de BFT, uma vez que atuam na oxidação destes para compostos menos tóxicos para o camarão, como o nitrato (Del'Ducca et al., 2019).

As bactérias nitrificantes incluem dois grupos principais: as bactérias amônia-oxidantes (AOB), como *Nitrosomonas* e *Nitrosospira*, que convertem amônia em nitrito, e as bactérias nitrito-oxidantes (NOB), como *Nitrobacter* e *Nitrospira*, que transformam nitrito em nitrato (Ebeling et al., 2006; Madigan et al., 2016). Além das bactérias do domínio *Bacteria*, estudos recentes apontam a presença de microrganismos do domínio *Archaea*, como as Archaea amônia-oxidantes (AOA), que desempenham um papel

significativo no processo de nitrificação, especialmente em ambientes com condições extremas de temperatura, salinidade e baixos níveis de oxigênio (Ward, 2013; Hatzenpichler, 2012).

A eficiência das nitrificantes depende de fatores como temperatura, salinidade e concentração de oxigênio dissolvido. Em sistemas BFT, a manutenção de concentrações adequadas de oxigênio é crítica, pois baixos níveis podem limitar a nitrificação, levando ao acúmulo de compostos tóxicos (Souza et al., 2019; Zhu et al., 2008). Além disso, parâmetros como o uso de sistemas de aeração avançados, incluindo nanobolhas, têm demonstrado potencial para acelerar a formação dos bioflocos e otimizar a remoção de nitrogênio (Krummenauer et al., 2021; Lim et al., 2021).

As nitrificantes também colonizam biofilmes que se formam em superfícies submersas, oferecendo uma área adicional para seu desenvolvimento. Esses biofilmes, estruturados em camadas, proporcionam condições microambientais únicas, como a presença de zonas anóxicas que podem influenciar a atividade microbiana (Vlaeminck et al., 2010). A utilização de substratos artificiais para aumentar a área de adesão do biofilme é uma prática que melhora a qualidade da água e serve como fonte suplementar de alimento para os camarões (Ferreira et al., 2016; Morais et al., 2020).

O processo de formação do biofilme e dos bioflocos, em geral, é lento, podendo levar semanas para atingir a estabilidade, e assegurar assim a eficiência do processo de nitrificação (Krummenauer et al., 2014; Ruiz et al., 2020). Desta forma, a reutilização de inóculos de água maturada de sistemas de bioflocos de cultivos anteriores acelera o estabelecimento da comunidade microbiana nitrificante em um novo cultivo e contribui para um controle mais rápido das concentrações de amônia e nitrito durante o ciclo de cultivo de *Penaeus vannamei* (Krummenauer et al., 2014).

Somado aos inóculos, o uso de substratos artificiais em sistemas de BFT é uma estratégia de manejo que pode ser empregada visando aumentar a área disponível para a fixação do biofilme, contribuindo como uma fonte suplementar de alimento (Ferreira et al., 2016) e para a manutenção da qualidade de água no cultivo. Morais et al. (2020) demonstraram que a utilização de substratos colonizados com biofilme levou a valores mais baixos nas concentrações de amônia e nitrito quando comparados com os valores no sistema BFT sem o uso do substrato. A escolha do substrato ideal é crucial, pois diferentes materiais influenciam a formação, estabilidade e composição dos biofilmes, impactando diretamente a eficácia das bactérias nitrificantes no sistema BFT.

Assim, os substratos artificiais previamente colonizados com biofilme podem ser reutilizados em diferentes ciclos de cultivo, desempenhando um importante papel no sistema como um todo. Entretanto, estudos que avaliem esta possibilidade, e qual o manejo mais adequado para os substratos entre os ciclos de produção, ainda não foram realizados. Além das bactérias nitrificantes clássicas, pesquisas recentes com ferramentas moleculares têm identificado novos grupos de microrganismos com potencial nitrificante, como bactérias heterotróficas que possuem atividade amônia-oxidante em condições específicas. Essas descobertas ampliam a compreensão do papel das comunidades microbianas nos sistemas BFT e destacam a importância de estudos contínuos para otimizar esses processos (Holl et al., 2019; Lu et al., 2020).

O uso de ferramentas metagenômicas tem revolucionado a compreensão das comunidades nitrificantes em sistemas de aquicultura. Por meio dessas técnicas, é possível identificar a composição microbiana, avaliar a diversidade funcional e monitorar a dinâmica das bactérias sob diferentes condições de manejo. Isso oferece subsídios para a otimização dos sistemas de produção, contribuindo para a maior eficiência no controle de compostos nitrogenados.

A formação do biofilme e a eficiência da nitrificação depende de alguns fatores físicos, químicos e interações biológicas. Alguns parâmetros de qualidade de água como a temperatura, os níveis de oxigênio dissolvido, bem como os tipos de sistemas de aeração utilizados são alguns desses fatores que podem afetar a comunidade nitrificante dentro do ambiente de produção (Chen et al., 2006). Em sistemas BFT, um sistema de aeração eficiente é importante não só para manter o suprimento de oxigênio, tanto para os organismos produzidos quanto para a comunidade bacteriana, como também para a manutenção dos bioflocos em suspensão, garantindo a estabilidade do sistema. As bactérias nitrificantes são sensíveis a variações de oxigênio dissolvido (Souza et al, 2019), que quando em baixas concentrações limitam ou suprimem a nitrificação (Zhu et al., 2008; Avnimelech, 2009), uma vez que esses microrganismos demandam oxigênio para atividade celular, crescimento e reprodução. Além disso, Morais et al. (2020) observaram que o processo de nitrificação no biofilme, especialmente nas NOB, é menos eficiente na ausência de um sistema de aeração adequado, ainda que sejam mantidos níveis elevados de oxigênio dissolvido na água. Os autores justificaram esses resultados ao fato de que a ausência ou pouca movimentação da água pode limitar a transferência de oxigênio ao longo do biofilme, levando a presença de área anóxicas ou hipóxicas nas regiões mais

internas (Vlaeminck et al., 2010), onde as NOB em geral são encontradas (Gieseke et al., 2003).

Atualmente, há uma grande variedade de dispositivos de aeração utilizados na aquicultura, sendo que em sistemas BFT, o Nozzle a3® e as mangueiras micro perfuradas (Aerotube®) são os mais utilizados. Diferentes sistemas de aeração podem interferir na formação e tamanho do floco, além de afetar a abundância e diversidade de microrganismos presentes nos cultivos (Krummenauer et al., 2011; Lara et al., 2017). Lim et al. (2021) demonstraram que a utilização de sistema de aeração que produz nano bolhas acelerou a formação de bioflocos e levou a um melhor controle de compostos nitrogenados, a partir de uma conversão mais rápida de amônia a nitrito, e posteriormente, a nitrato. As interações mecânicas geradas pelos sistemas de aeração influenciam diretamente na velocidade de formação dos bioflocos, além disso, o tamanho das bolhas de ar determina a área de superfície interfacial, a velocidade de ascensão das bolhas e o coeficiente de transferência de massa. Portanto, as nanobolhas fornecem uma área de superfície maior para a adesão das bactérias (Abdelrahman & Veverica, 2016; Krummenauer et al., 2021).

Para traçar um paralelo entre as tecnologias que visam preservar os recursos hídricos e reduzir os impactos ambientais, a aquicultura tem avançado significativamente ao incorporar métodos sustentáveis. Entre essas abordagens, além do BFT, destaca-se os sistemas de recirculação (RAS), ambos operandos com uso mínimo de água (Verdegem et al., 2006; De Schryver et al., 2008). A prática de cultivo intensivo de *P. vannamei*, minimizando a troca de água, responde às crescentes preocupações ambientais e é impulsionada pelo conceito de desenvolvimento sustentável, que visa integrar princípios de prudência ecológica, eficiência econômica e equidade social em todas as atividades humanas (Macintosh e Phillips, 1992; Primavera, 1994; Rosenthal, 1994).

O RAS proporciona um alto nível de controle sobre o ambiente aquático, permitindo uma produção mais eficiente em termos de uso de espaço e mão de obra, além de reduzir substancialmente o consumo de água em relação à biomassa produzida. Segundo Timmons et al. (2009), esses sistemas facilitam economias de escala, possibilitando alta produção de camarão em comparação a outros métodos de aquicultura. A configuração dos sistemas RAS inclui dispositivos para tratamento e reuso de água, como decantadores, filtros mecânicos e filtros biológicos (Xiao et al., 2019). O uso de

filtros permite a remoção de resíduos sólidos, incluindo restos de ração e fezes, enquanto os filtros biológicos promovem a ação de bactérias nitrificantes para controlar os níveis de amônia e nitrito na água (Martins et al., 2011). Assim, esses sistemas podem atingir altos rendimentos com riscos ambientais mínimos, tornando-os uma das tecnologias mais promissoras para a aquicultura moderna.

O sistema BFT representa um ambiente complexo e dinâmico, caracterizado por uma diversidade microbiana abrangente (Hargreaves, 2013; Robles-Porchas et al., 2020). Este sistema adota uma abordagem ecologicamente responsável que favorece o reuso da água em vários ciclos, resultando em benefícios ambientais significativos, como a redução da poluição em áreas costeiras (Krummenauer et al., 2014). Além disso, a implementação do BFT demonstrou promover rendimentos otimizados em culturas de *P. vannamei* em altas densidades de estocagem (Krummenauer et al., 2011; Silveira et al., 2020). Portanto, essa estratégia não apenas aumenta a produtividade, mas também melhora o controle ambiental ao minimizar ou eliminar a necessidade de trocas de água, contribuindo assim para a sustentabilidade do setor de aquicultura (Krummenauer et al., 2011; Samocha, 2019; Schveitzer et al., 2023).

Tanto o sistema de recirculação de água (RAS) e tecnologia de bioflocos (BFT) na aquicultura são interconectados e impulsionados pela crescente necessidade de sustentabilidade e produtividade. O uso desses sistemas é essencial para mitigar os impactos ambientais da aquicultura intensiva e promover uma abordagem mais responsável ao uso dos recursos hídricos. No entanto, informações sobre os efeitos de altas densidades de estocagem nas condições de qualidade da água nesses sistemas ainda são escassas e precisam ser estudadas para que o manejo desses sistemas seja otimizado.

Nesse contexto, a sustentabilidade da aquicultura não se limita apenas a aspectos ambientais, mas também envolve a viabilidade econômica das práticas empregadas. A análise econômica desempenha um papel crucial na sustentabilidade da aquicultura, especialmente em sistemas inovadores como o BFT e o RAS. Avaliar os custos e benefícios associados às diferentes estratégias de manejo permite aos produtores identificar práticas mais eficientes e economicamente viáveis, promovendo a rentabilidade a longo prazo. A Partial Budget Analysis (PBA) emerge como uma ferramenta fundamental nesse contexto, pois facilita a comparação entre tecnologias e práticas de produção, destacando os impactos financeiros de mudanças específicas no

sistema. Além de fornecer subsídios para decisões estratégicas, a análise econômica contribui para o entendimento do custo-benefício de elementos como o uso de substratos, sistemas de aeração avançados e a incorporação de técnicas de manejo que melhoram a qualidade da água. Assim, integrar a PBA em estudos sobre aquicultura intensiva, como no cultivo de *Penaeus vannamei*, é essencial para otimizar a sustentabilidade econômica e ambiental do setor, garantindo maior competitividade no mercado global.

Diante do exposto, a aquicultura avança em direção a sistemas que garantem produtividade e sustentabilidade ambiental, destacando-se o sistema de bioflocos (BFT) e o sistema de aquicultura de recirculação (RAS) por seu potencial em reduzir o uso de água e os impactos ambientais. O papel das bactérias nitrificantes é crucial na manutenção da qualidade da água e no controle de compostos nitrogenados tóxicos. Assim, entender as interações entre os parâmetros ambientais e a atividade dessas bactérias é essencial para otimizar o manejo dos cultivos. Este trabalho investiga essas interações, visando aprimorar as práticas na aquicultura, especialmente no cultivo de *Penaeus vannamei*.

2. OBJETIVOS

2.1. Objetivo Geral

Avaliar os efeitos dos diferentes tipos de sistema de aeração no desenvolvimento das bactérias nitrificantes presentes no biofilme e na água e estabelecer práticas de manejo mais eficientes para a reutilização do biofilme durante cultivos superintensivos de *Penaeus vannamei* em sistema de bioflocos. Ainda buscamos avaliar a qualidade da água e o crescimento de *Penaeus vannamei* em sistemas de bioflocos e sistemas de recirculação (RAS) usando alta densidade de estocagem.

2.2 Objetivos Específicos

2.2.1. Analisar o desenvolvimento do biofilme e avaliar o crescimento e sobrevivência do *P. vannamei* criado com BFT e com o uso de diferentes sistemas de aeração.

2.2.2. Estabelecer o manejo mais adequado para a reutilização do biofilme em cultivos de *P. vannamei* com BFT.

2.2.3. Avaliar a qualidade de água, crescimento e sobrevivência do *P. vannamei* em sistemas de BFT e RAS, com ênfase na eficiência da nitrificação.

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CAPÍTULO I: The effect of using nano and microbubbles as aeration strategies on the nitrification process, microbial community composition, and growth of *Penaeus vannamei* in a super-intensive biofloc system.

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The effect of using nano and microbubbles as aeration strategies on the nitrification process, microbial community composition, and growth of *Penaeus vannamei* in a super-intensive biofloc system

Abstract

The aim of this study was to evaluate the effect of using nano and microbubbles as aeration strategies on the nitrification process, microbial community composition of water and biofilm, microbial flocs proximate composition, and growth of Penaeus vannamei in a pilot scale super-intensive system with biofloc technology. A grow-out (stocking density: 450 shrimp m^{-3}) was carried out for 74 days in a greenhouse with nine 35 m^3 tanks, using the following treatments: T1: nanobubbles, T2: microbubbles, T3: mixed (nano +microbubbles). The nanobubbles were generated by nozzle-type air injectors and the microbubbles were generated by microperforated hoses. Artificial substrates, with an area equivalent to 200% of the lateral surface, were used into the experimental units. Molasses was used as an organic carbon source at a carbon:nitrogen ratio of 15:1. The main morphotypes of bacteria in the water and in the biofilm were analyzed using direct counting and a profile of the bacterial composition of the water was performed using metagenomic analysis. Control of total ammonia nitrogen (TAN) was better in treatments T2 and T3 than in T1. At the end of the experiment, treatment T3 showed a higher total abundance of bacteria in the water than T1 and T2. Bacillus abundance in the biofilm was higher in the T3 treatment than in T1 and T2. The relative abundance of nitrifying bacteria was <1.0 % in all treatments. Treatment T1 had a higher relative abundance of nitrite-oxidizing bacteria than the others. However, the T3 treatment had a better distribution of nitrifying bacteria species. T3 treatment showed a higher diversity and evenness of operational taxonomic units. The microbial flocs in the T2 had a higher crude protein content than in the T1. These results indicate that the use of a mixed aeration system promotes a higher load of microorganisms, which has proven to improve water quality and can influence shrimp growth. The shrimp final weight was higher in T1 (12.57 g) and T3 (12.54 g) than in T2 (11.70 g), survival was higher in T3 (97.17%) than in T1 (80.33%), and yield was higher in T3 (4.61 Kg $m^{3)}$ than in T1 (4.06 Kg m^3) and T2 (4.21 Kg m^3). The treatment that used nano and microbubble as aeration strategy (T3) proved to be the best as it provided better TAN control, higher abundance of bacillus bacteria in the biofilm, higher diversity and evenness of bacteria, and even higher shrimp growth and survival.

1. Introduction

Shrimp farming with biofloc systems (BFT) is a strategy that allows increasing the productivity of marine shrimp *Penaeus vannamei* cultures with the use of high stocking densities, in addition to improving environmental control through the reduction or absence of water exchange, carried out by the manipulation of the carbon:nitrogen (C:N) ratio (Avnimelech, 1999; Ebeling et al., 2006; Krummenauer et al., 2011; Samocha, 2019; Schveitzer et al., 2023). In these systems, the accumulation of nitrogenous compounds is associated with feed intake, shrimp excretion, and decomposition of organic matter (e.g., unconsumed feed and feces) (Robles-Porchas et al., 2020). Inadequate levels of total ammonia nitrogen (TAN) and nitrite (NO_2^-) cause stress and physiological changes in cultured organisms, affecting growth and survival, impairing production (Vinatea et al., 2010; Ebeling et al., 2006). In this way, nitrifying bacteria present in the BFT play a key role in controlling concentrations of toxic nitrogenous compounds in the system. This chemoautotrophic bacteria act in the transformation of these compounds to less toxic forms of nitrogen for the shrimp, such as nitrate (NO_3^-) (Del'Duca et al., 2019). Two groups of nitrifying bacteria are responsible for this process in the BFT system. First, ammonia-oxidizing bacteria (AOB) grow with the function of oxidizing TAN to NO_2^- (Abakari et al., 2021). Next, nitrite-oxidizing bacteria (NOB) grow to oxidize NO_2^- to NO_3^- in a process that consumes alkalinity and reduces the pH of the water (Ebeling et al., 2006).

In intensive shrimp culture systems, nitrifying bacteria colonize biofilm that develops on submerged substrates (Thompson et al., 2002). On these artificial substrates, cell adhesion and interaction trigger the synthesis of extracellular polysaccharides that form the matrix for biofilm growth (Madigan et al., 2016). The use of artificial substrates in cultures with BFT systems is a management strategy to increase the area available for the establishment of a nitrifying bacteria community, acting in the maintenance of water quality in the culture, and contributing as a supplementary food source for the animals (Ballester et al., 2007; Lara et al., 2021). For example, Morais et al. (2020) tested the effect of different aeration intensities in Penaeus vannamei intensive culture systems with artificial substrates and reported that the nitrification process was more efficient in the treatment with the presence of biofilm and a higher aeration intensity. Biofilm formation and nitrification efficiency depend on some physical, chemical, and biological interactions such as the concentrations of toxic nitrogenous compounds, pH, alkalinity,

and dissolved oxygen (Abakari et al., 2021). Dissolved oxygen and its different forms of diffusion in water are one of the factors that can affect the nitrifying community within the production environment (Chen et al., 2006). In shrimp cultures with BFT, an efficient aeration system is important not only to maintain the oxygen supply for the animals and for the microorganisms in the system, but also to keep the bioflocs in suspension, guaranteeing the stability of the system (Krummenauer et al., 2011). Nitrifying bacteria are sensitive to variations in dissolved oxygen in water (Souza et al., 2019) since at low concentrations they limit or suppress the nitrification process (Zhu et al., 2016; Avnimelech, 2009). These microorganisms require oxygen for cellular activity, growth, and reproduction. Thus, the absence of an efficient aeration system or one that promotes little water movement can limit the transfer of oxygen throughout the biofilm, where NOB are generally found, leading to the presence of areas with low oxygen concentrations, affecting nitrification process (Gieseke et al., 2003; Vlaeminck et al., 2010; Morais et al., 2020).

In aquaculture there is a wide variety of aeration devices, in intensive shrimp farming systems with BFT, Nozzle a3® and Aerotube® micro- perforated hoses are the commonly used to generate nano and microbubbles (Krummenauer et al., 2021). The use of different types of bubbles can interfere with the formation and size of the biofloc, in addition to affecting the abundance and diversity of microorganisms present within medium (Krummenauer et al., 2011). Lim et al. (2021) demonstrated that the use of an aeration system that produces nano bubbles accelerated biofloc formation and led to a better control of nitrogenous compounds, from a faster conversion of TAN to NO NO_2^- and then, to NO_3^-. Mechanical interactions generated by aeration directly influence the speed of biofloc formation. Furthermore, the size of the air bubbles determines the interfacial surface area, the bubble rise velocity, and the mass transfer coefficient (Abdelrahman and Veverica, 2016). Therefore, nanobubbles can provide a larger surface area for bacteria adhesion (Krummenauer et al., 2021).

One of the advantages of using nanobubbles provided by air injectors is that there is no need for aeration devices at the bottom of the tank, since the injector only needs a centrifugal pump to recirculate the water through the tank. Upon returning, the water passes through the injectors which are enriched by atmospheric air through a snorkel attached to the injector. This process promotes the generation of nanobubbles, resulting in a highly efficient transfer of dissolved oxygen. Additionally, the directed flow originated by the nanobubbles causes horizontal and vertical movements in the water,

ensuring the suspension of bioflocs. This aspect is fundamental for the success of the BFT system. On the other hand, microperforated hoses operate through an air blower, generating substantial amounts of air under low pressure conditions, which materialize as microbubble (Lara et al., 2017; Krummenauer et al., 2021).

Although different studies show that aeration affects the nitrification process, information about the effect of the size of the bubble on the microbial composition of water and biofilm in intensive shrimp culture systems are still scarce. Thus, the aim of this study was to analyze the effect of using nano and microbubbles generated by nozzle-type air injectors and microperforated hoses, respectively, as aeration strategies on the nitrification process, microbial composition of water and biofilm, proximate composition of microbial flocs, and growth of *Penaeus vannamei* in a pilot scale super-intensive system with biofloc technology.

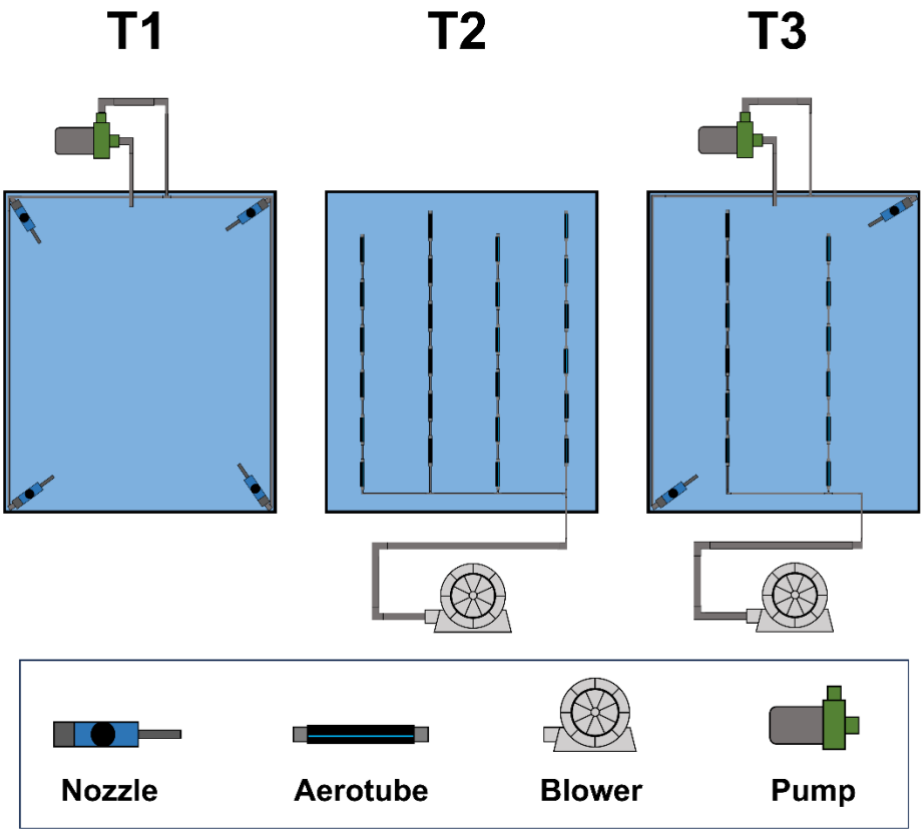
2. Materials and methods

2.1 Design and experimental conditions

A *Penaeus vannamei* shrimp grow-out was carried out during 74 days at Marine Aquaculture Station of the Federal University of Rio Grande. Post-larvae of *P. vannamei* were acquired from a commercial hatchery (Aquatec® LTDA, Brazil). Shrimp were initially kept in a 30-day nursery until reached a weight of 1.26 ±0.83 g and then were stocked in the experimental units at a density of 450 shrimp m^{-3}. The experiment was carried out in a greenhouse with nine rectangular tanks with 35 m^3 (7.0 m long x 5.0 m wide x 1.0 m deep), equipped with Needlona® artificial substrates with an area equivalent to 200% of the lateral surface of the tank (70 m^2), arranged vertically inside of the tank.

Three treatments were tested with three repetitions each and in a randomized experimental design: T1: nanobubbles, T2: microbubbles, T3: mixed (nano +microbubbles). In treatment T1, the nanobubbles were provided by an aeration system with four Nozzle® a3 air injectors (positioned at each corner of the tank). In the treatment T2, the microbubbles were provided by an aeration system composed of twenty- four pieces of microperforated hoses (Aerotube®) with 15 cm long arranged at the bottom of the tank. Treatment T3 used a mixed aeration with nano and microbubbles that were provided by two Nozzle® injectors (positioned at each end of the tank) and twelve pieces of microperforated hoses (Aerotube®) with 15 cm long inside the same tank. The

466 treatments that used microbubbles were supplied by a 7.5 HP blower (Ibram®) and 1.0 HP
467 centrifugal pump (Schneider®) per tank was used for the nanobubbles treatment (Fig. 1).
468 Seawater (salinity close to 35 g L^{-1}) used for the experiment was chlorinated with sodium
469 hypochlorite at a concentration of 1.0 g m^{-3} and dechlorinated after three h with ascorbic
470 acid at a concentration of 1 g m^{-3} (Moore et al., 2021). To stimulate biofloc development,
471 each experimental unit was inoculated with 3500 L (10% of total volume) with biofloc
472 from a previous culture according to the methodology described by Krummenauer et al.
473 (2014). The matured biofloc water used as inoculum had the following characteristics:
474 pH of 7.64, 0.20 mg L^{-1} of total ammonia nitrogen (TAN), 0.08 mg L− 1 (NO_{2-} -N),
475 20.00 mg L− 1 (NO_{3-} -N), 0.90 mg L^{-1} ($PO4^{3-}$), an alkalinity of 200.00 mg CaCO3 $L^{-1,}$ and total suspended solid of 335.00 mg L^{-1}. The nominal C:N ratio was maintained at
477 15:1 considering sugar cane molasses as supplementary carbon source (38% of carbon).
478 Furthermore, corrections were made when TAN exceeded 1 mg L^{-1}. To do this, for each
479 gram of ammonia nitrogen in the tank, 6g of carbon were added (Avnimelech, 1999;
480 Ebeling et al., 2006).



481

Fig.1. Aeration structure diagram of a *Penaeus vannamei* super-intensive grow- out using nano and microbubbles as aeration strategies in a biofloc system. T1 : nanobubbles; T2: microbubbles; T3: mixed (nano +microbubbles).

2.2 Water quality variables

Temperature (◦C), dissolved oxygen (DO, mg L^{-1}) (dissolved oxygen meter YSI® EcoSense DO200A), pH (Seven 2GO - Mettler Toledo®), total ammonia nitrogen (TAN, mg L^{-1}; UNESCO, 1983) and nitrite nitrogen (NO_2– -N, mg L^{-1}; Strickland and Parsons, 1972) were measured daily. Nitrate nitrogen (NO_3– -N, mg L^{-1}; García-Robledo et al., 2014), orthophosphate ($PO4^{3-}$, mg L^{-1}; Aminot and Chaussepied, 1983), total alkalinity (mg CaCO3 L^{-1}; APHA, 2012), turbidity (NTU; Hach® 2100P turbidimeter), and carbon dioxide (CO2, mg L^{-1}; Timmons and Ebeling, 2013) were measured weekly. Total suspended solids (TSS) were determinate weekly using the methodology adapted from Strickland and Parsons (1972) where 20 mL of water was filtered through a glass fiber filter with 1.6 μm of mean retention (Whatman GF/A) in a vacuum pump. When the TSS concentration exceeded 400 mg L^{-1}, clarifiers were used to remove excess solids following Ray et al. (2010) and Gaona et al. (2011). Settleable solids (SS, mL L^{-1}) quantification was carried out weekly using an Imhoff cone, with readings after 15 to 20 min, following the methodology described by Eaton et al. (1995) and adapted by Avnimelech (2007). Alkalinity was assessed weekly following APHA (2012). When the alkalinity concentration dropped below 150 mg CaCO3 L^{-1} and pH below 7.5, as recommended by Ebeling et al. (2006), corrections were made using hydrated lime, as described by Furtado et al. (2011) at a concentration of 0.15 g L^{-1}.

2.3 Composition of the microbial community

For the bacterial community characterization at the beginning and end of the experimental time, samples of 18 mL of water and fragments of 2.0 cm^2 of substrate (adapted from Silva et al., 2008) were collected from each experimental unit. Samples were stored and fixed in 4% formalin for later analysis. Artificial substrate samples were previously sonicated (Ultrasonic Homogeneizer 4710 Series, model CP50) to disaggregate biofilm, with three pulses of 30 s, using a frequency of 10 kHz. An interval of 30 s among pulses was adopted. For quantification and identification of the bacterial community, samples were filtered through polycarbonate membrane filters with 0.2 μm

of mean retention previously darkened with irgalan black and stained with 0.1% of acridine orange, following Hobbie et al. (1977). Bacteria were photographed with a camera attached to an epifluorescence microscope (Axioplan-Zeiss) at a final magnification of 1000×. Abundance was determined in organisms mL^{-1} by counting 30 random fields. Bacteria were identified in the following morphotypes: coccoid, vibrio, free and attached filamentous, bacillus, and prosthecate.

2.4 Metagenomic analysis

At the end of the experimental time, a water sample from each treatment (samples for each repetition were pooled in one sample) was collected for sequencing analysis. An aliquot of 1 mL was filtered through a 0.2 μm of mean retention membrane and stored in a chaotropic buffer solution for later analysis by Agrega Pesquisa e Desenvolvimento em Biotecnologia, Brazil. DNA extraction was performed with the DNeasy PowerWater extraction kit (Qiagen). DNA concentration and purity were determined on a NanoDrop® 2000 spectrophotometer (Thermo Scientific). DNA Integrity was checked on 1% agarose gel electrophoresis. Metabarcoding PCR was performed for the V3-V4 region of the bacterial 16S rRNA gene using primers 341F and 806R (Caporaso et al., 2011; Sundberg et al., 2013). Sequencing was performed on the Illumina NovaSeq platform with a 250 bp paired-end strategy. Biological sequences were inferred with the DADA2 package default configuration (Callahan et al., 2016) in R software (R Core Team, 2023). Operational Taxonomic Units (OTUs) were obtained with the DECIPHER package (Firth et al., 2009) and taxonomically classified up to genus level using the SILVA SSU r138 database (Yilmaz et al., 2014).

2.5 Proximate composition

At the end of the trial, samples of microbial flocs were collected with a 50 μm mesh. Samples were dried in an oven at a 105 °C until constant weight and macerated for subsequent analysis of proximate composition. Analysis was performed in triplicate at the Aquatic Organisms Nutrition Laboratory (LANOA) at the Federal University of Rio Grande (FURG), following standard protocol of the Association of Official Analytical Chemists (AOAC, 2007). Crude protein (CP) was measured from the determination of nitrogen (N × 6.25), using the Kjeldahl method (TE – 0363, Tecnal®, Sao Paulo, Brazil). Lipid content was determined using ether extraction with the aid of a Soxhlet extractor

548 (XT10, ANKON®, New York, USA). Moisture was determined by the gravimetric
549 method in an oven (AC -035, ACLabor®, Americana, SP, Brazil). Ash was determined
550 by incinerating samples in a muffle (1200DM/G, SPLabor, Sao Paulo, Brazil) at 600 ◦C
551 for three h.
552

553 *2.6 Feed management and Shrimp growth*

554 Shrimp were fed twice a day using Guabi® commercial feed with 35% crude
555 protein. To control consumption, 10% of the feed was offered in feeding trays and the
556 rest distributed in the tank. The feeding rate was adjusted weekly following Jory et al.
557 (2001). Samples were performed weekly and at the end of the experimental time to
558 determine shrimp weight (g), weekly growth rate (WGR; g $week^{-1}$), feed conversion ratio
559 (FCR), survival (%), and yield (kg m^{-3}).
560

561 *2.7 Data analysis*

562 For water quality variables, data were tested for normality with the Shapiro-Wilk
563 test and homoscedasticity with the Levene test. The repeated measures analysis of
564 variance (ANOVA) was applied to assess significant differences among treatments.
565 When repeated measures ANOVA was significant (p-value <0.05), Tukey's test was
566 applied. When necessary, data were transformed to fulfill parametric assumptions. For
567 non-parametric data (NO_{2-} -N, alkalinity, and DO), the Friedman test was used, followed
568 by the Conover multiple comparisons test with the Bonferroni correction.
569 In the metagenomics data, to avoid richness results biased by the size difference
570 among the treatment samples, abundance data of OTUs were rarefied to sample with the
571 lowest number of reads (14064). Rarefaction curves, richness, and diversity indices were
572 calculated using the vegan package (Oksanen et al., 2022).
573 Microbial composition of the water and biofilm, proximate composition of the
574 microbial flocs, and shrimp growth data were tested for normality with the Shapiro-Wilk
575 test, and homoscedasticity with the Levene test. One-way ANOVA was used to assess
576 whether there were significant differences among treatments. When one-way ANOVA
577 was significant (p-value <0.05), Tukey's test was applied. Survival percentage data were
578 arcsine transformed before analysis (Zar, 2010). When necessary, data were transformed
579 to fulfill parametric assumptions. For non-parametric data (total abundance of bacteria -

water - initial, total abundance of bacteria - biofilm - final, free, and attached filamentous - water - initial and final, vibrio - water - final, bacillus - water - initial and final, prosthecate - water - initial and final, filamentous - biofilm - initial and final; vibrio - biofilm - final; bacillus - biofilm - initial and final; prosthecate - biofilm - initial and final), the Kruskal- Wallis test was applied followed by the Dunn test with the Bonferroni correction.

One-way ANOVA, Tukey, Kruskal-Wallis, Dunn, Friedman, and Conover tests were performed in the R software (R Core Team, 2023) using car (Fox and Weisberg, 2019), dunn.test (Dinno, 2017), and PMCMRplus (Pohlert, 2022) packages. The repeated measures ANOVA and its post hoc were performed using Past 4.03 software (Hammer et al., 2001). Graphs were built using ggplot2 (Wickham, 2016) and Rmisc (Hope, 2022) packages of the R software (R Core Team, 2023).

3 Results

3.1. Water quality variables

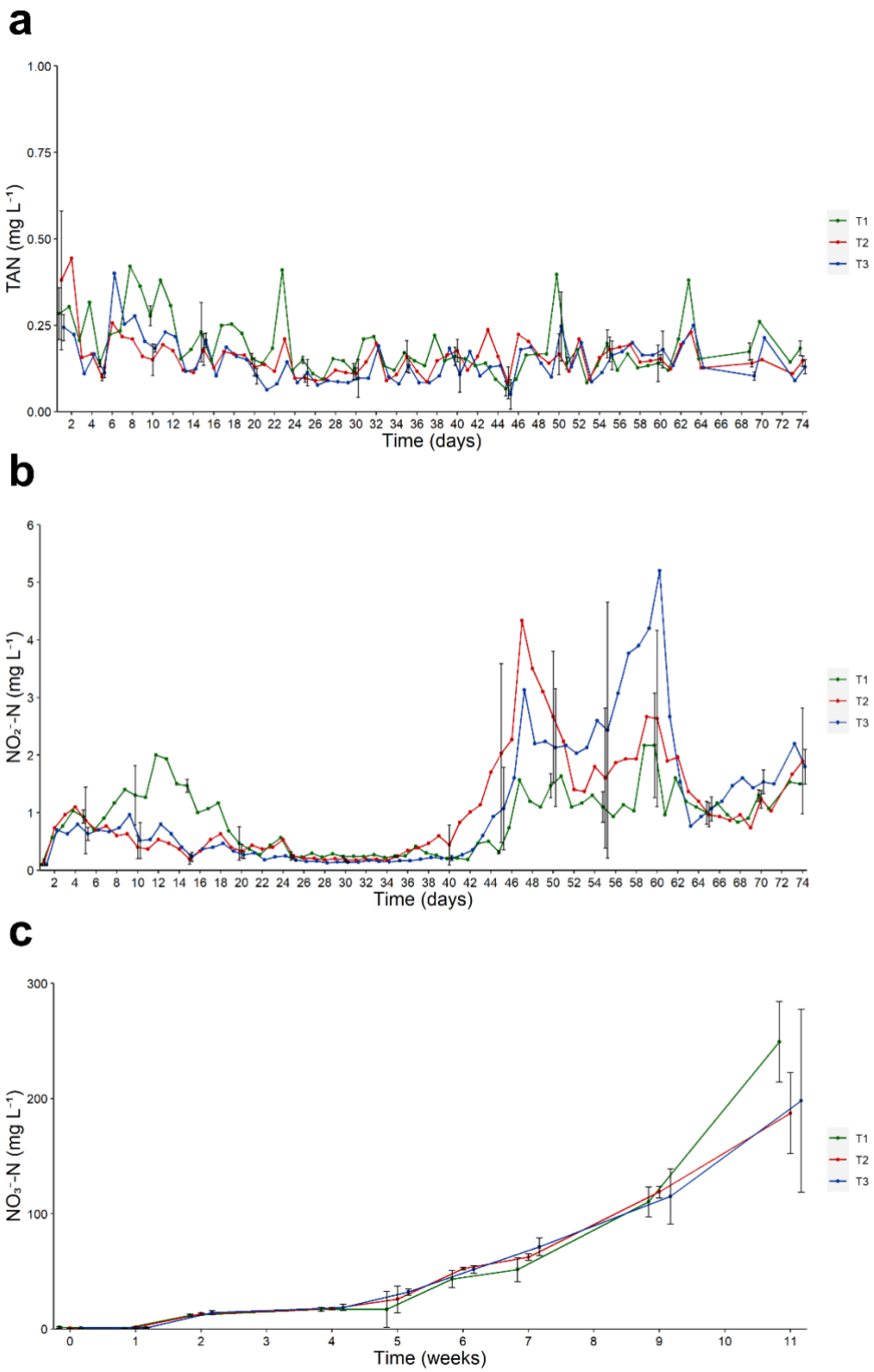
Temperature was between 26.62 and 27.38 °C and was higher in treatments T1 and T3 than in T2 (Table 1). DO was higher in treatment T3 than in T1 and T2 (Table 1). The mean pH varied between 7.39 and 7.47 during the trial (Table 1). TAN was lower in treatment T2 and T3 than in T1. In all treatments, mean concentrations were below 0.5 mg L^{-1} (Fig. 2a). Spikes in $NO2^{-}$ -N concentration were observed from day 40 of the trial, where treatment T2 recorded a mean concentration of 4.33 mg L^{-1} on day 47 and treatment T3 a mean concentration of 5.20 mg L^{-1} on day 60 (Fig. 2b). NO_{3}^{-} -N tended to increase in concentration over the experiment without significant differences among treatments (Fig. 2c). Alkalinity and CO2 were higher in treatment T1 than in T2 and T3 (Table 1). SS was higher in treatments T2 and T3 when compared to treatment T1 (Table 1).

Table 1. Water quality variables during a *Penaeus vannamei* super-intensive grow-out using nano and microbubbles as aeration strategies in a biofloc system.

Variables	Treatments		
	T1	T2	T3
Temperature (°C)	27.38 ± 1.00^{a}	26.62 ± 0.93^{b}	27.31 ± 0.84^{a}
DO (mg L^{-1})	4.72 ± 0.71^{b}	4.75 ± 0.73^{b}	5.29 ± 0.46^{a}

pH	7.39 ± 0.13[a]	7.45 ± 0.13[a]	7.47 ± 0.16[a]
TAN (mg L^{-1})	0.19 ± 0.10[a]	0.16 ± 0.07[b]	0.15 ± 0.07[b]
NO_2^--N (mg L^{-1})	0.87 ± 0.59[a]	1.04 ± 1.12[a]	1.10 ± 1.58[a]
NO_3^--N (mg L^{-1})	55.85 ± 78.01[a]	53.35 ± 61.13[a]	55.94 ± 66.49[a]
PO_4^{3-} (mg L^{-1})	2.50 ± 2.01[a]	2.73 ± 2.35[a]	2.36 ± 1.81[a]
Alkalinity (mg L^{-1})	304.00 ± 111.66[a]	173.00 ± 31.55 [b]	176.00 ± 32.68[b]
CO_2 (mg L^{-1})	26.46 ± 14.74[a]	12.93 ± 6.65[b]	12.60 ± 7.06[b]
TSS (mg L^{-1})	359.3 ± 175.35[a]	354.10 ± 131.66[a]	321.40 ± 114.06[a]
Turbidity (NTU)	225.10 ± 94.37[a]	173.20 ± 63.96[a]	201.90 ± 78.54[a]
SS (mL L^{-1})	4.72 ± 2.20[b]	10.38 ± 6.75[a]	10.95 ± 7.62[a]

Data are mean ± standard deviation. Superscript letters indicate the result of Tukey's test or Conover's multiple comparison test with Bonferroni correction. DO: dissolved oxygen; TAN: total ammonia nitrogen; NO_2^--N: nitrite nitrogen; NO_3^--N: nitrate nitrogen; PO_4^{3-}: orthophosphate; CO_2: carbon dioxide; TSS: total suspended solids; SS: settleable solids. T1: nanobubbles; T2: microbubbles; T3: mixed (nano + microbubbles).



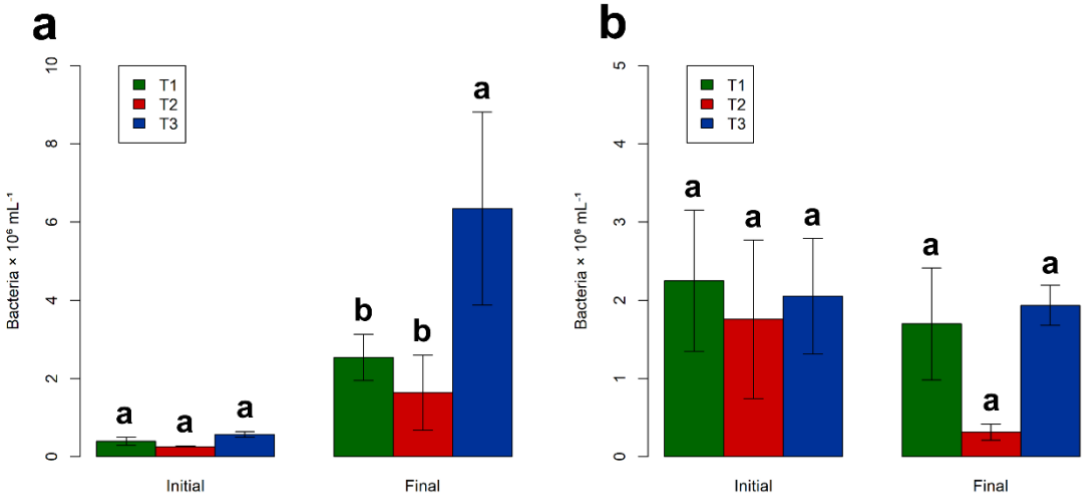
615

616 **Figure 2.** Total ammonia nitrogen (TAN, a), nitrite (NO_2^--N, b) and nitrate (NO_3^--N, c)
617 patterns during a *Penaeus vannamei* super-intensive grow-out using nano and
618 microbubbles as aeration strategies in a biofloc system. T1: nanobubbles; T2:
619 microbubbles; T3: mixed (nano + microbubbles).

620 *3.2. Composition of the microbial community*

621 *3.2.1. Water*

622 The mean total abundance of bacteria (final sample) was higher in treatment T3
623 ($6.35 \times 10^6 \pm 2.47 \times 10^6$ bacteria mL^{-1}) than T1 ($2.53 \times 10^6 \pm 5.88 \times 10^5$ bacteria mL^{-1})
624 and T2 ($1.64 \times 10^6 \pm 9.60 \times 10^5$ bacteria mL^{-1}) (Figure 3a). An increase in the total
625 abundance of bacteria was observed between the initial and final samples of the trial
626 (Figure 3a).



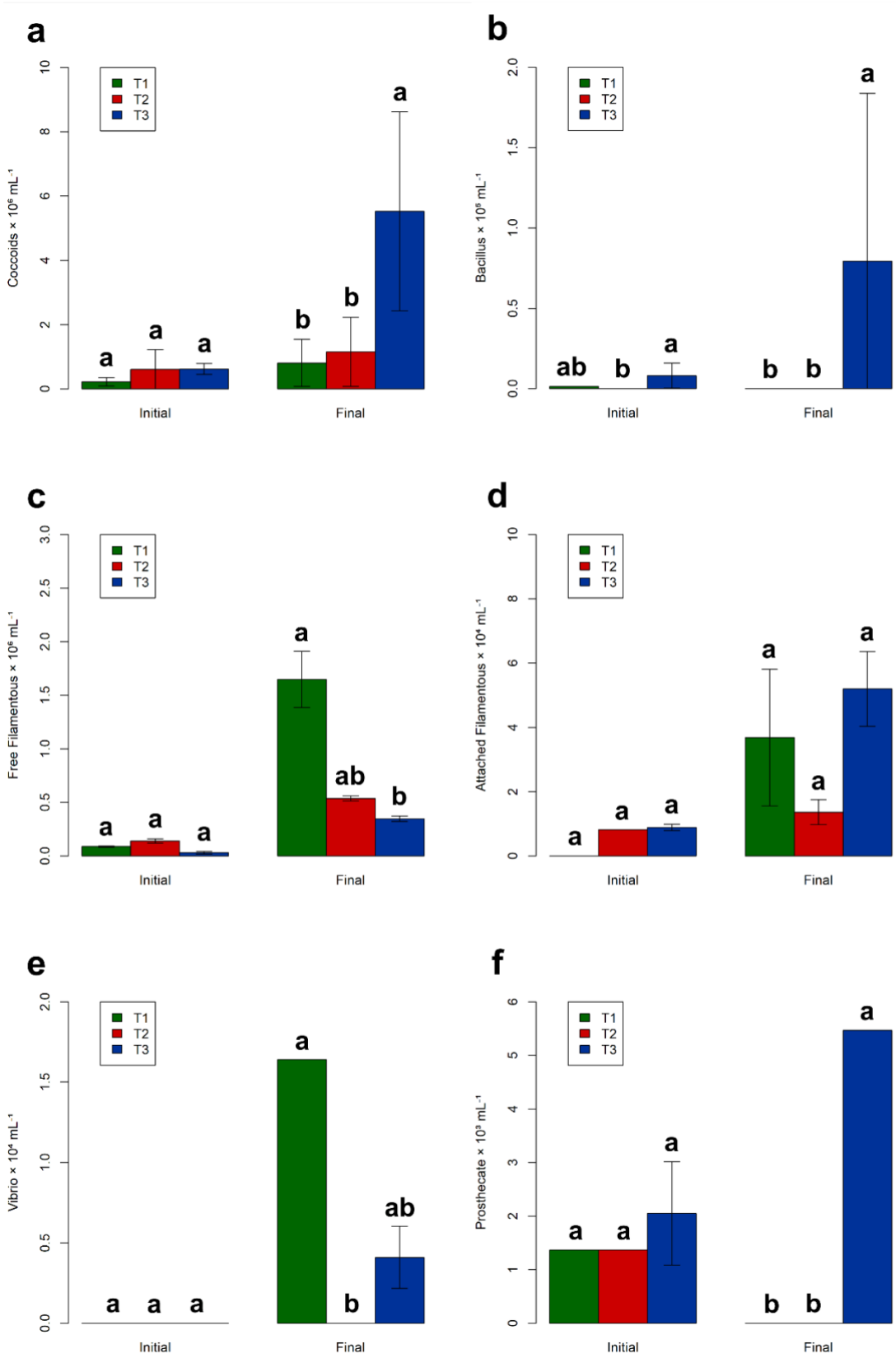
627

628 **Figure 3.** Total abundance (mean ± standard deviation) of bacteria found in water (a) and
629 biofilm (b) of a *Penaeus vannamei* super-intensive grow-out using nano and
630 microbubbles as aeration strategies in a biofloc system. T1: nanobubbles; T2:
631 microbubbles; T3: mixed (nano + microbubbles).

632 The abundance of coccoid bacteria was higher in treatment T3 than in treatments
633 T1 and T2 (Figure 4a). The abundance of coccoid bacteria increased throughout the
634 experiment in treatment T3 (Figure 4a). In the initial sampling, the abundance of bacillus
635 was higher in treatment T3 than in treatment T2 (Figure 4b). At the final sampling,
636 treatment T3 had higher abundance of bacillus than treatments T1 and T2 (Figure 4b),
637 treatment T1 had more free filamentous bacteria when compared to treatment T3 (Figure
638 4c). Also, treatment T1 showed a higher abundance of vibrio than treatment T2 (Figure
639 4e). An increase in the abundance of free filamentous bacteria and vibrio was observed
640 throughout the trial in the T1 treatment (Figure 4 c and e). Treatment T3 had a higher

641 abundance of prosthecate bacteria than treatments T1 and T2 at the end of the trial (Figure
642 4f).

643



644

645 **Figure 4.** Abundance (mean ± standard deviation) of coccoid (a), bacillus (b), free
646 filamentous (c), attached filamentous (d), vibrio (e) and prosthecate (f) bacteria found in

647 water of a *Penaeus vannamei* super-intensive grow-out using nano and microbubbles as
648 aeration strategies in a biofloc system. T1: nanobubbles; T2: microbubbles; T3: mixed
649 (nano + microbubbles).

650 *3.2.2. Biofilm*

651 During the trial, no significant differences were observed among treatments in the
652 total abundance of bacteria (Figure 3b). Treatment T3 had a higher abundance of coccoid
653 bacteria than treatment T2 (Figure 5a). Along the experimental time, treatment T3 showed
654 higher abundance of bacillus when compared to treatments T1 and T2 and its abundance
655 reduced at the end of the experiment (Figure 5b). In the initial sampling, treatment T3 had
656 a higher abundance of filamentous bacteria than treatments T2. At the end of the trial,
657 treatments T1 showed a higher abundance of filamentous bacteria than treatment T2 and
658 T3 (Figure 5c). At the initial sampling, treatment T3 had a higher concentration of vibrio
659 than treatments T1 and T2 (Figure 5d). At the final sampling, T3 treatment had higher
660 abundance of vibrio when compared to the T1 treatment (Figure 5d). However, vibrio
661 abundance reduced throughout the trial in the biofilm (Figure 5d). Prosthecate bacteria
662 were more abundant at the beginning of the trial in treatment T2 than in treatments T1
663 and T3 (Figure 5e). At the final sampling, prosthecate bacteria were more abundant in
664 treatment T3 than in treatments T1 and T2 (Figure 5e).

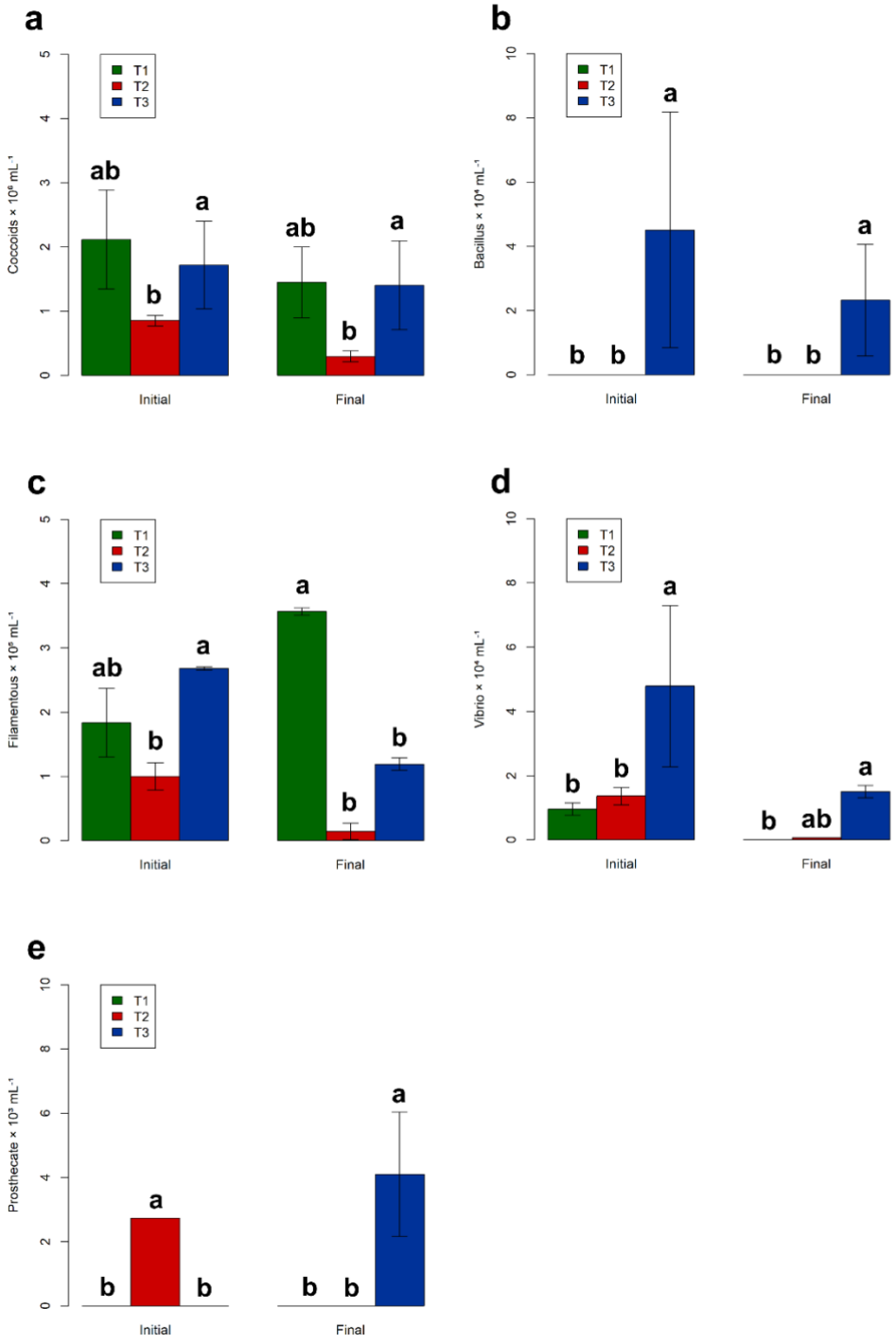
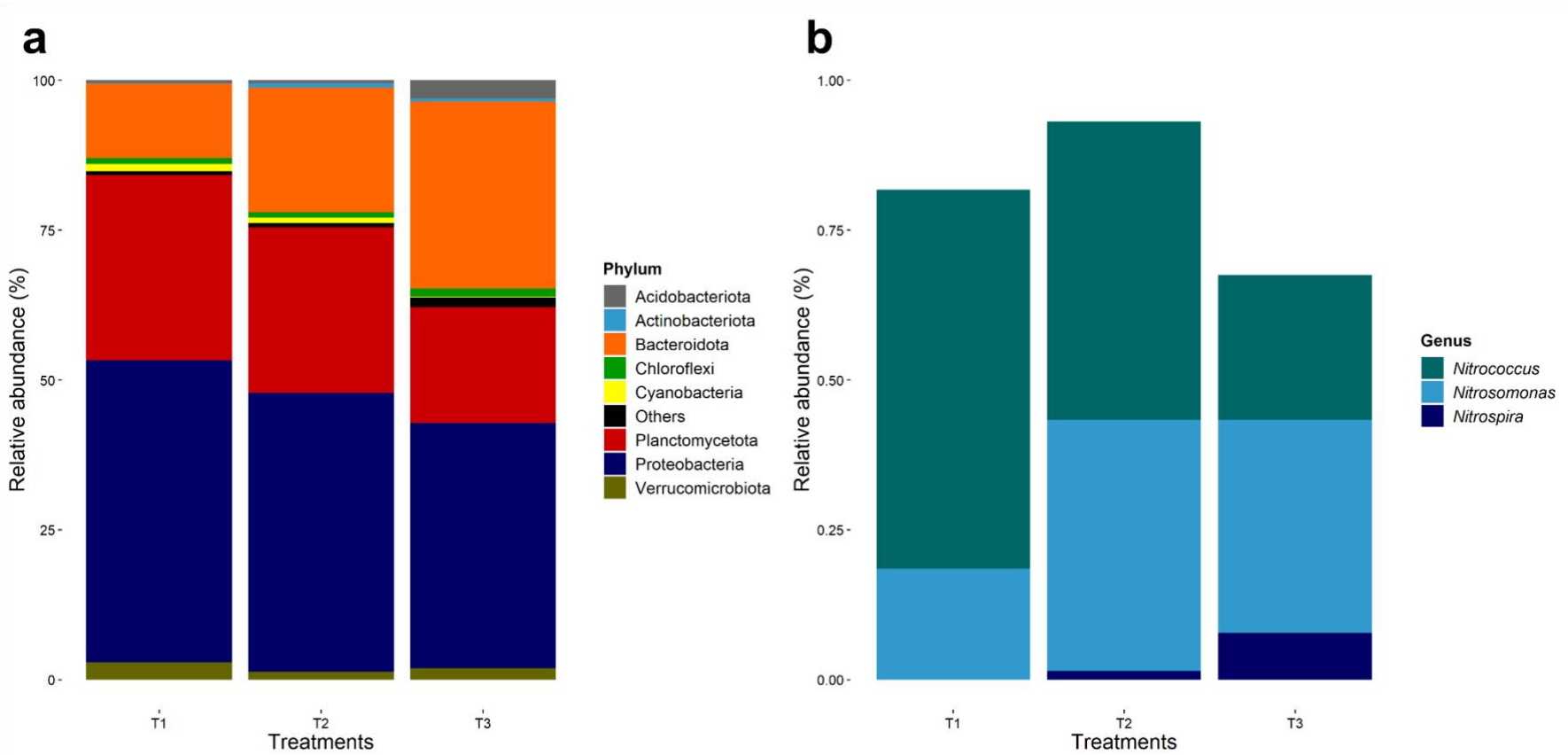


Figure 5. Abundance (mean ± standard deviation) of coccoid (a), bacillus (b), filamentous (c), vibrio (d), and prosthecate (e) bacteria found in the biofilm present in a *Penaeus vannamei* super-intensive grow-out using nano and microbubbles as aeration strategies in a biofloc system. T1: nanobubbles; T2: microbubbles; T3: mixed (nano + microbubbles).

3.3. Metagenomic analysis

The sequencing of the water bacterial community generated a total of 42,192 sequences, which were grouped into 189 OTUs. Metagenomic analysis showed that the bacterial community was mainly composed of 8 phyla (Figure 6a). In the treatments, the most abundant phyla were Proteobacteria followed by Planctomycetota, Bacteroidota, and Verrucomicrobiota (Figure 6a). The relative abundance of Bacteroidota phylum showed a decreasing pattern from treatment T3 (31.25%) to T1 (12.42%). The opposite was observed for the Planctomycetota which reduced its relative abundance from the T1 (30.88%) treatment to T3 (19.29%) (Figure 6a). Cyanobacteria showed a low relative abundance, being higher in treatment T1 (1.23%) than in treatments T2 (0.94%) and T3 (0.15%) (Figure 6a).

Nitrifying bacteria identified in the water showed a relative abundance below 1% in all treatments (Figure 6b). The presence of bacteria of the genus *Nitrospira* was not observed in the T1 treatment (nanobubbles). However, in this treatment a higher abundance of NOB (*Nitrococcus* + *Nitrospira*) (0.63%) was observed in relation to treatments T2 (0.51%) and T3 (0.32%) (Figure 6b). Treatment T2 showed the highest relative abundance of nitrifying bacteria (0.93%). The T3 treatment showed a better balance among nitrifying bacteria species (Figure 6b).



689

690 **Figure 6.** Relative abundance of the main phyla of bacteria (a) and genus of nitrifying bacteria (b) found in the water of a *Penaeus vannamei* super-
691 intensive grow-out using nano and microbubbles as aeration strategies in a biofloc system. T1: nanobubbles; T2: microbubbles; T3: mixed (nano
692 + microbubbles).

Bacterial community richness was higher in treatment T2 (136 OTU's), followed by treatment T3 (122 OTU's) and T1 (95 OTU's) (Figure 7a). According to the Shannon and the Simpson diversity indices, treatment T3 (3.51 and 0.942, respectively) had higher diversity of OTU's than treatments T2 (3.43 and 0.938, respectively) and T1 (3.02 and 0.915, respectively) (Figure 7b and c). The Pielou index shows that, at the end of the experimental time, treatment T3 had a higher evenness (0.731) in the distribution of OTUs observed in the sample than treatments T2 (0.699) and T1 (0.664) (Figure 7d).

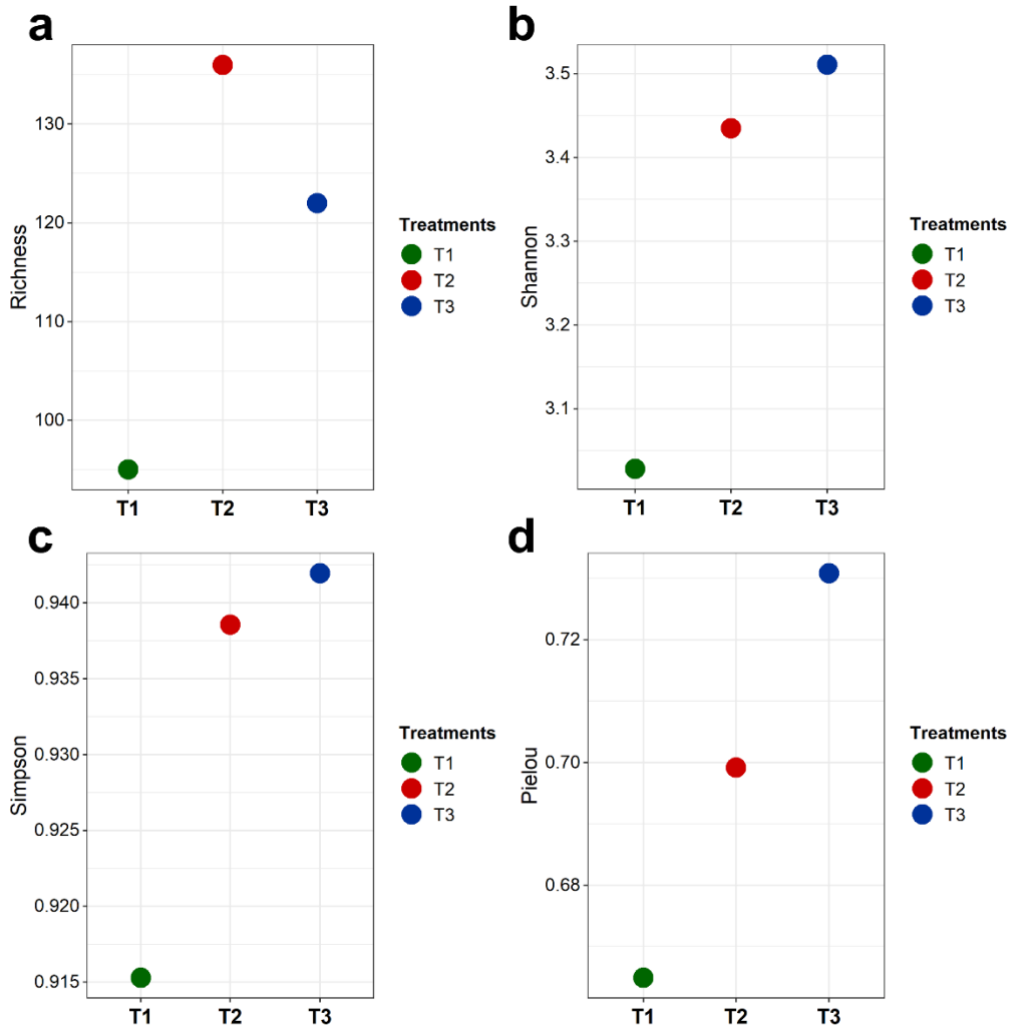


Figure 7. Richness (a), Shannon (b), Simpson (1-D; c) and Pielou (d) indices of the bacterial community at the end of a *Penaeus vannamei* super-intensive grow-out using nano and microbubbles as aeration strategies in a biofloc system. T1: nanobubbles; T2: microbubbles; T3: mixed (nano + microbubbles).

706 *3.4. Proximate composition of the microbial floc*

707 The percentage of crude protein in the microbial floc was between 25.23 and
708 28.86% (Table 2). Microbial floc from the T2 treatment had a higher crude protein content
709 when compared to the T1 treatment (Table 2). The percentage of lipids in the biofloc was
710 between 0.97 and 1.28% (Table 2).

711 **Table 2.** Proximate composition of the microbial flocs grown at the end of a *Penaeus*
712 *vannamei* super-intensive grow-out using nano and microbubbles as aeration strategies in
713 a biofloc system.

	Treatments		
	T1	T2	T3
Crude protein (%)	25.23 ± 2.98^{b}	28.86 ± 3.54^{a}	26.28 ± 2.64^{ab}
Lipids (%)	1.28 ± 0.82^{a}	0.97 ± 0.32^{a}	1.22 ± 0.78^{a}
Fibers (%)	5.47 ± 2.33^{a}	6.22 ± 2.55^{a}	5.46 ± 1.81^{a}
Ashes (%)	51.04 ± 2.99^{a}	53.83 ± 3.24^{a}	48.88 ± 1.93^{a}
Moisture (%)	1.16 ± 0.27^{a}	1.06 ± 0.45^{a}	0.86 ± 0.39^{a}

714 Data are mean ± standard deviation. Superscript letters indicate the Tukey test result. T1:
715 nanobubbles; T2: microbubbles; T3: mixed (nano + microbubbles).

716 *3.5. Shrimp growth*

717 The shrimp mean final weight varied between 11.70 and 12.57 g (Table 3). Shrimp
718 from treatments T1 and T3 had higher final weight and weekly growth rate (WGR) than
719 treatment T2 (Table 3). FCR was between 1.76 and 1.97 and was lower in treatment T3
720 than in T1 (Table 3). T3 treatment showed a higher survival when compared to the T1
721 treatment (Table 3). Yield was higher in treatment T3 than in T1 and T2 (Table 3).

722

723 **Table 3.** Growth of *Penaeus vannamei* at the end of a super-intensive grow-out using
724 nano and microbubbles as aeration strategies in a biofloc system.

	Treatments		
	T1	T2	T3
Initial weight (g)	1.26 ± 0.83	1.26 ± 0.83	1.26 ± 0.83
Final weight (g)	12.57 ± 0.17^{a}	11.70 ± 0.26^{b}	12.54 ± 0.40^{a}
WGR (g week^{-1})	1.08 ± 0.02^{a}	1.00 ± 0.02^{b}	1.08 ± 0.04^{a}

FCR	1.97 ± 0.01^{a}	1.84 ± 0.04^{ab}	1.76 ± 0.09^{b}
Survival (%)	80.33 ± 1.67^{b}	90.04 ± 5.61^{ab}	97.17 ± 4.29^{a}
Yield (Kg m^{-3})	4.06 ± 0.03^{b}	4.21 ± 0.20^{b}	4.61 ± 0.003^{a}

Data are mean ± standard deviation. Superscript letters indicate Tukey test result. WGR: weekly growth rate; FCR: feed conversion ratio. T1: nanobubbles; T2: microbubbles; T3: mixed (nano + microbubbles).

4. Discussion

In this study, water quality variables were maintained within the recommended for the *P. vannamei* shrimp culture (Ponce-Palafox et al., 1997; Van Wyk et al., 1999). Temperature is a crucial factor for shrimp growth and survival. The species *P. vannamei* can tolerate temperatures between 15°C and 35°C (Van Wyk et al., 1999). In treatments T1 and T3, the temperature was maintained above 27.3°C, which was within the ideal range for shrimp growth (Ponce-Palafox et al., 1997). It is important highlight that the nozzle-type air injector captures the heated air inside the greenhouse from the snorkel and generates nanobubbles, while the micro-perforated hoses capture the external air to be supplied through them (During the trial, the mean external temperature was 22.12 ± 2.09°C. Data retrieved from the Brazilian Coast Monitoring System - SiMCosta). This difference may have contributed to the maintenance of higher temperatures in the treatments where nanobubbles was used (T1 and T3). On the other hand, capturing air from inside the greenhouse may have some disadvantages, since the CO_2 produced by the system tends to accumulate in these environments (Browdy et al., 2012) and can be reintroduced into the water through the nozzle injectors. This was observed because the concentration of CO_2 in the water was higher in the T1 treatment when compared to the others. These high concentrations in this treatment may have negatively influenced the shrimp survival. According to Kurihara (2008), chronic exposure of *Palaemon pacificus* shrimp to high concentrations of CO_2 can result in a reduction in the survival, growth, and possibility of reproduction of these organisms. Furtado et al. (2017) found that safe level of CO_2 for *Penaeus vannamei* is 5.9 mg L^{-1}, considering 23.8 mg L^{-1} as non-lethal concentration. Levels between 20 and 60 mg L^{-1} are not lethal but can impact CO_2 exchange in the gills (Van Wyk et al., 1999).

Another important factor for the success of intensive marine shrimp farming is the dissolved oxygen. The treatment with a mixed aeration strategy (T3 – nanobubbles +

754 microbubbles) was more efficient in maintaining dissolved oxygen concentrations in the
755 system (> 5 mg L^{-1}). This concentration is considered ideal both for the biomass of
756 cultured shrimp and for the development of bacteria involved in the process of nitrogen
757 assimilation and nitrification (Timmons and Ebeling, 2010; Van Wyk et al., 1999).

758 In fact, the aeration system in the T2 and T3 treatment proved to have influenced
759 the TAN oxidation to nitrite, providing lower TAN concentrations than T1 treatment. The
760 presence of mature bioflocs in the system, containing an active bacterial community, may
761 have contributed to the maintenance TAN and nitrite levels stable and within the
762 recommended limits for *P. vannamei* culture throughout most of the experiment (Van
763 Wyk et al., 1999; Lin and Chen, 2001; Lin and Chen, 2003; Timmons and Ebeling, 2010).
764 Variations in nitrite concentration observed in treatments T2 and T3 over the
765 experimental time may be related to the efficiency of the NOB community. The activity
766 of these bacteria is limited by several factors such as, variations in the concentration of
767 organic matter, alkalinity, pH, temperature, and dissolved oxygen (Robles-Porchas et al.,
768 2020). Despite variations over the trial, the nitrification process occurred in all treatments
769 since there was an accumulation of nitrate over the experimental time.

770 The temporal pattern found in the concentration of nitrogenous compounds is
771 reinforced by the results found in the relative abundance of nitrifying bacteria. Although
772 the highest relative abundance of AOB was found in the T2 treatment (only
773 microbubbles), the T3 treatment (nanobubbles + microbubbles) better controlled the TAN
774 concentration. The results also indicate a possible better control of nitrite in the T1
775 treatment (nanobubbles), as in this treatment there was a higher relative abundance of
776 bacteria of the genus *Nitrococcus*, which are NOB (Prosser, 2005). Also, in this treatment,
777 the highest mean nitrate concentration was observed at the end of the trial.

778 In a BFT system, solids control is one of the key factors for the correct
779 management of the system. Solids overproduction can have negative consequences for
780 water quality and animal growth (Schveitzer et al., 2013; El-Sayed, 2021). In this study,
781 lower concentrations of settleable solids in treatment T1 may have occurred due to the
782 higher presence of free filamentous bacteria in the water than in the others. Filamentous
783 bacteria hinder the sedimentation of bioflocs (Hargreaves, 2013) making the reading of
784 settleable solids biased.

In BFT shrimp production, microorganisms play a key role in the functioning of the system (Ebeling et al., 2006). Aeration systems have a direct influence on the microbial composition in the system since the size of the bubbles provided by each device plays a different role in the microbial aggregates formation (Silveira, 2017). The reduced size of the aeration bubbles is associated with an accelerated maturation of the bacterial community, as a result of the increased residence time of the bubbles in the water column. This promotes more efficient gas exchange and contributes to the formation of bioflocs (Abdelrahman and Veverica, 2016; Krummenauer et al., 2021). Microorganisms, in addition to leading the control route of nitrogenous compounds, also increase the availability of food for the animals being cultured (Emerenciano et al., 2013).

The use of a mixed aeration, with nanobubbles and microbubble, provided higher growth of coccoid bacteria and bacillus both in the water and in the biofilm. Coccoid bacteria, in addition to participate in the biofloc formation, are a valuable nutrient source, abundant in proteins and lipids for shrimp (Rocha et al., 2012). *Bacillus* contribute to the microbiological balance of the culture medium, working to maintaining water quality and preventing problems arising from the accumulation of organic matter, as they can act on its decomposition and nutrient cycling (Decamp et al., 2002).

Regarding *Vibrios*, different patterns were found in the water and in the biofilm. It is important to highlight that the presence of these bacteria in the system can be a disadvantage since some species can be pathogenic and cause diseases for shrimp, such as vibriosis (Tan et al., 2014). However, *Vibrio* was not dominant in the system, since the abundance of these microorganisms was lower than the other morphotypes of bacteria found in the water. It is crucial to properly monitor and control *Vibrio* communities in the water, in addition to the use of good management practices, when seeking to ensure the health of farmed shrimp (Defoirdt et al., 2011; Chen et al., 2019).

Metagenomic analysis carried out in our study showed a microbial community formed by phyla that play important ecological roles in aquatic systems. Proteobacteria is a broad phylum composed of gram-negative bacteria. They perform a variety of functions in the environment, including nitrogen fixation, which is an important process in regulating the availability of this element in aquatic environments (Falkiewicz-Dulik et al., 2005). On the other hand, Bacteroidetes are gram-positive bacteria with great metabolic diversity which could degrade complex biopolymers, such as carbohydrates,

proteins, and lipids, being important in the nutrient cycling and decomposition of organic materials (Madigan et al., 2016). Thus, the amount of organic matter in the system can limit the development of this phylum of bacteria and this can be one of the possible explanations for the pattern found among treatments, evidencing the negative role of the accumulation of organic matter in the system. The availability of inorganic phosphorus can also affect the abundance of Bacteroidetes, as they contribute to the cycling of this nutrient (Grossart et al., 2007). The phylum Planctomycetota includes bacteria that can carry out nitrification and denitrification processes (Ward, 2013). However, it is important to consider other factors, such as microbial interactions and specific conditions of the culture system, for a more complete understanding of changes in the bacterial community (Rofner et al., 2016).

When comparing the two types of bubbles separately, it is observed that the use of microbubbles (T2) moves the water vertically, while the nanobubbles (T1) promotes both horizontal and vertical circulation. This generates higher water circulation in the tanks, while avoiding the formation of areas with low oxygen concentration (Krummenauer et al., 2015). The strategy of associated use of nano and microbubbles (T3) can promote good water mixing, which is reflected in the microbial composition of the system. This could be observed in this study, where treatment T3 showed higher diversity and evenness of OTU's than treatments T1 and T2. Thus, a system that, in addition to supplying the system's high demand for oxygen, ends up being more diverse and ecologically efficient for shrimp production.

In BFT systems, microbial growth sustained by the addition of organic carbon and cycling of nitrogenous compounds reverts to the production of biomass with high nutritional value (Emerenciano et al., 2016). Tacon et al. (2002), stated that the bacterial flocs contain high levels of protein and other important compounds that complement the shrimp nutrition. The presence of a high level of protein in bacterial flocs is relevant for shrimp nutrition, as protein is an essential nutrient for the healthy growth and development of these animals (Crab et al., 2012). The availability of a supplementary food source for shrimp is essential to meet their nutritional needs and promote good growth performance by reducing feed use. Our results showed that, despite T2 treatment having shown a higher percentage of crude protein, this did not influence shrimp growth.

Finally, treatments T1 and T3 (that have used nanobubbles) provided higher final weight and weekly growth to shrimp. Still, the T3 treatment enabled higher survival and yield. These results reinforce the importance of using a system that provides higher water mixing and higher transfer of oxygen to the water. This is possible with the addition of nanobubbles in the system. This aeration system provides a higher surface area for the adhesion of microorganisms, which allows the rapid bacterial flocs formation (Krummenauer et al., 2021). The effect of microorganisms on shrimp growth can be observed in the T3 treatment, since it had higher abundance and diversity of bacteria and better results for animal growth. Our results strongly indicate that the use of an aeration system that provides nano and microbubbles, promotes better system stability, microbial loop development, and shrimp growth.

5. Conclusion

The use of nano and microbubbles (T3) proved to be the best aeration strategy for super-intensive marine shrimp culture. Using this system provided better nitrogen compounds control, higher abundance of coccoid bacteria and bacillus in the water and in the biofilm, and higher diversity, and species evenness than in treatments where these aeration systems were used separately. In addition to these benefits, the use of nanobubbles also stimulates a more intense colonization of nitrite oxidizing bacteria. This better control of water quality variables and better development of the microbial community resulted in better shrimp growth, since the T3 treatment allowed higher final weight, survival, and yield.

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CrediT authorship contribution statement

Bianca de Oliveira Ramiro: Conceptualization, Investigation, Methodology, Data Curation, Visualization, Writing – original draft. **Wilson Wasielesky Jr.:** Supervision,

Funding acquisition, Project Administration, Resources, Writing – review & editing. **Otávio Augusto Lacerda Ferreira Pimentel:** Investigation, Data Curation, Formal Analysis, Visualization, Writing – review & editing. **Luis Henrique da Silva Poersch:** Supervision, Funding acquisition, Project Administration, Resources, Writing – review & editing. **Bob Advent:** Writing – review & editing. **Genes Fernando Gonçalves Junior:** Investigation. **Dariano Krummenauer:** Conceptualization, Methodology, Supervision, Funding acquisition, Project Administration, Resources, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

Data will be made available on request.

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CAPÍTULO II: Different management strategies for artificial substrates on nitrifying bacteria development in *Penaeus vannamei* super-intensive culture with biofloc technology

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Different management strategies for artificial substrates on nitrification, microbial composition, and growth of *Penaeus vannamei* in a super-intensive biofloc system

Abstract

The aim of this study was to test the effect of different management strategies for artificial substrates on the nitrification process, microbial composition of water and biofilm, and growth of *Penaeus vannamei* in a super-intensive biofloc system. The research was conducted over 60 days using experimental units of 200L and had the following treatments: T1 – artificial substrate + biofloc + shrimp + aeration (control), T2 - artificial substrate + water + aeration, T3 - artificial substrate + water, and T4 - only artificial substrate. The experiment was divided into two phases. Phase 1 involved maintaining the artificial substrates under the management strategy for 30 days. Phase 2 involved the use of substrates from phase 1 in a shrimp nursery for 30 days. Pre colonized artificial substrates (Needlona®) were employed at a ratio of 200% of the tank's lateral area to promote biofilm growth. In the phase 1, T1 used a stocking density of 500 shrimp m^{-3} (9.72 ± 0.50g). In phase 2, shrimp weighing 0.10 ± 0.05g were stocked at a density of 1750 shrimp m^{-3} and all treatments included artificial substrates from phase 1, aeration, water, and shrimp. Molasses was the carbon source organic used. In phase 2, ammonia control was observed in treatments T2, T3, and T4 from the 10^{th} day onwards. T4 had a nitrite spike, controlled from day 14 onwards, suggesting recovery of the nitrifying bacteria community. At the end of the phase 2, T2 and T4 showed higher abundance of coccoid bacteria in the biofilm compared to T1 and T3. T4 also had more bacillus. The shrimp final weight was higher in T2 compared to the other treatments. These findings suggest that maintaining the substrate submerged in water (T3) can be considered a practical management for artificial substrates and that it does not limit the nitrification process between culture cycles. Furthermore, exposure of artificial substrates to air (T4) also did not affect the nitrification process, leading to the recovery of the bacterial community, and the proliferation of various bacterial groups.

Keywords: Biofilm; Nitrifying bacteria; Heterotrophic bacteria; Zooplankton.

1. Introduction

The biofloc system (BFT) is made up of a vast microbial community, including not only bacteria, but also algae, protozoa, and organic matter (Hargreaves, 2013, Robles-Porchas et al., 2020). Furthermore, the BFT adopts an ecologically responsible approach that allows the reuse of water for various cycles, which contributes to reducing pollution in coastal areas (Krummenauer et al., 2014) and by stimulating high yields in the *Penaeus vannamei* culture using high stocking densities (Krummenauer et al., 2011, Silveira et al., 2020).

The presence of the microbial community in the BFT system, as heterotrophic and chemoautotrophic bacteria, plays a key role in the control of nitrogenous waste (i.e., ammonia and nitrite) in the system, since these microorganisms act in the constant removal of these compounds from the water (Del'Duca et al., 2019). Heterotrophic bacteria assimilate the ammonia produced by shrimp excretion and feed waste into microbial biomass, generating an accumulation of solids in the system (Khanjani et al., 2022). One of the most important groups of bacteria for the BFT are nitrifying bacteria, which oxidize the ammonia present in the system into nitrite (by the ammonia-oxidizing bacteria – AOB) and oxidize nitrite into nitrate (result of the activity of nitrite-oxidizing bacteria – NOB) (Robles-Porchas et al., 2020). These bacteria grow in the system and prefer colonizing substrates and forming biofilms, as occurs when a submerged artificial substrate is introduced.

Biofilm is an organic matrix that is inhabited by bacteria, protozoa, fungi, and algae attached to emersed surfaces in aquatic environments (Ferreira et al., 2016), and its development occurs in distinct phases. The first stage involves creating an organic film that forms on any immersed surface. Then, the bacterial cells adhere to the substrate through their interaction with the organic film. Soon after, the process of colonization and biofilm growth begins, which includes the addition of a layer of mucus (polysaccharides) and the presence of other microorganisms, such as protozoa and microalgae (Madigan et al. 2016).

The use of artificial substrates is a strategy that can be adopted with the aim of increase the surface available for biofilm attachment. This contributes not only as an additional source of feed, but also to improve the maintenance of water quality in the culture (Ferreira et al., 2016). The use of artificial substrates with biofilm resulted in

reduced levels of ammonia and nitrite compared to the BFT system without the use of substrate (Morais et al., 2020). The artificial substrate contributes to improving shrimp growth parameters by providing higher natural productivity. Furthermore, the use of vertical substrates helps to relatively reduce stocking density (Lara et al., 2021).

The development of biofilm in artificial substrates, in general terms, is a gradual process that may require weeks to reach stability, thus ensuring the effectiveness of the nitrification process (Krummenauer et al., 2014; Ruiz et al., 2020). In this context, the reuse of artificial substrates from previous cultures in biofloc systems stands out as an effective strategy to accelerate the establishment of the nitrifying community. This, in turn, contributes to a prompt regulation of ammonia and nitrite concentrations during a *P. vannamei* culture. Keeping the substrates submerged in water without aeration, submerged in aerated water or exposed to air are strategies that can be adopted when managing this tool that is important in super-intensive shrimp farming. However, between culture cycles, it is not yet known which strategy is most appropriate to maintain artificial substrates without compromising the microbial community, especially the activity of nitrifying bacteria. Therefore, the aim of this study was to test the effect of different management strategies for artificial substrates on the nitrification process, microbial composition of water and biofilm, and growth of *P. vannamei* in a super-intensive biofloc system.

2. Material and methods

This study was carried out for 60 days at the "Estação Marinha de Aquacultura" of the "Universidade Federal do Rio Grande – FURG", Brazil.

2.1. Design and experimental conditions

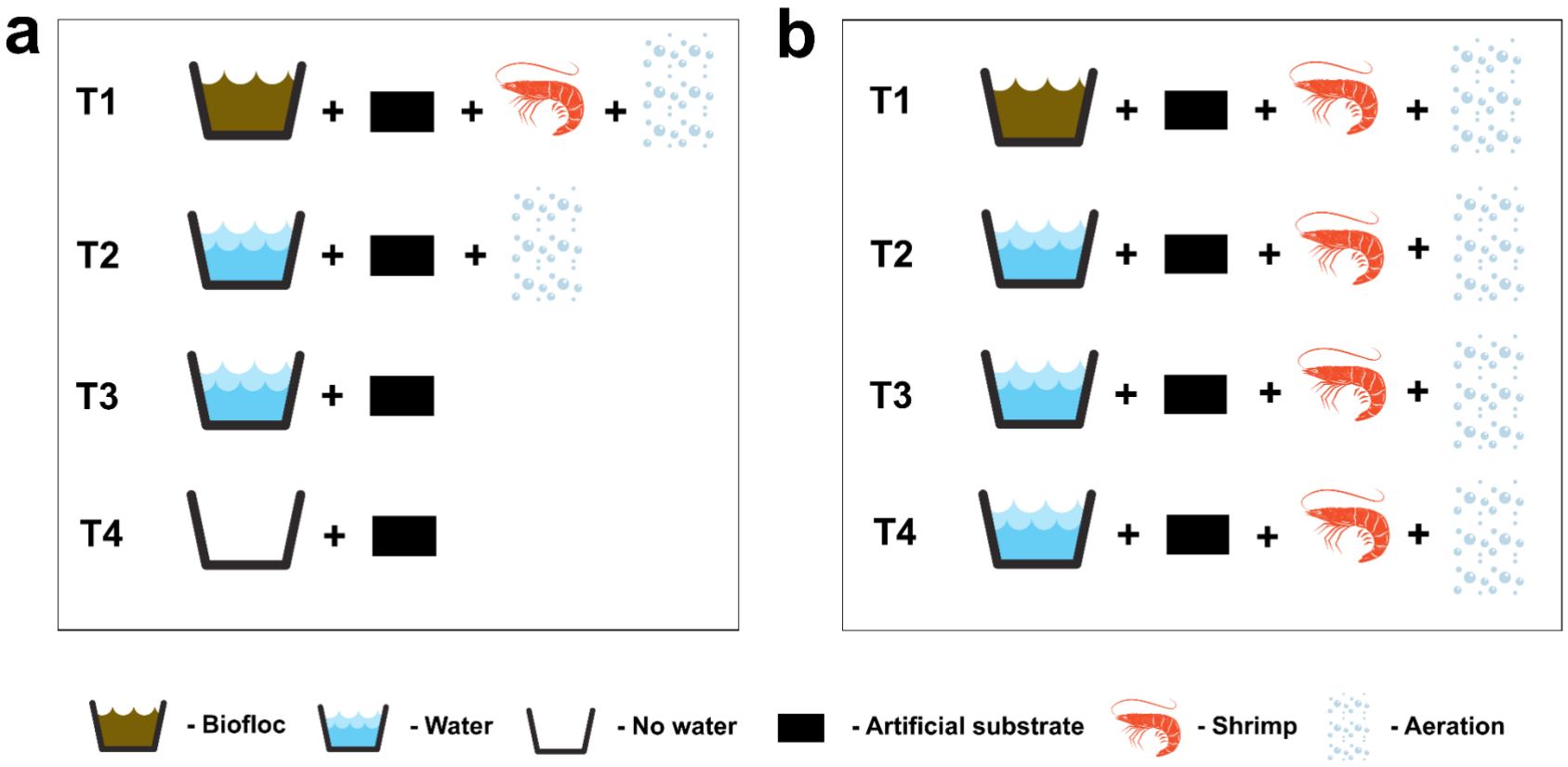
The experiment was carried out in experimental units with 200L. Seawater (salinity 29 g L^{-1}) was initially treated with 10 g m^{-3} of sodium hypochlorite and subsequently dechlorinated using 1 g m^{-3} of ascorbic acid.

A non-floating artificial vertical substrate (Needlona®, 1.4 mm thick) was used in a proportion of 200% of the lateral area of the tank, to allow biofilm growth (Ferreira et al., 2016; Lara et al., 2021). The substrates were previously colonized within a *Penaeus vannamei* biofloc system and then randomly distributed in the experimental units.

The experiment was divided into two phases. Phase 1 consisting of maintaining the artificial substrates in different managements strategies for a period of 30 days. Phase 2 consisted of the use of artificial substrates maintained in phase 1 in a *P. vannamei* nursery for 30 days.

2.2. Phase 1

During phase 1, the following treatments were established, with four replicates, in a completely randomized experimental design: T1 – artificial substrate + biofloc + shrimp + aeration (control), T2 - artificial substrate + water + aeration, T3 - artificial substrate + water, and T4 - only artificial substrate (Figure 1a). Throughout phase 1, in T4, the substrate was kept completely out of the water, without receiving sunlight, vertically positioned inside the tank, and at no time was it in contact with water. In the Phase 1, only treatment T1 (control) was stocked with *P. vannamei* at a density of 500 shrimp m^{-3}, with a mean weight of 9.72 ± 0.50g. Furthermore, T1 received 10% of water inoculum from previous culture cycles. The matured biofloc water used as inoculum, had the following characteristics: total ammonia nitrogen (TAN): 0.20 mg L^{-1}; nitrite nitrogen (NO_2^--N): 0.08 mg L^{-1}; nitrate nitrogen (NO_3^--N): 20.00 mg L^{-1}; orthophosphate (PO_4^{3-}): 0.90 mg L^{-1}; alkalinity: 200.00 mg L^{-1}; total suspended solids (TSS): 335.00 mg L^{-1}; pH: 7.64.



1292

1293 **Figure 1.** Diagram of the experimental design for phases 1 (a) and 2 (b) of a *Penaeus vannamei* super-intensive culture using different artificial
1294 substrates management strategies to promote the nitrifying bacteria development.

2.3. Phase 2

In this phase, a 30-day nursery trial was carried out using the artificial substrates managed in the phase 1. At this phase, all treatments had aeration, shrimp, water, and artificial substrates from phase 1 (Figure 1b). In treatment T1 (control), the same water with bioflocs and artificial substrates were maintained from phase 1. The experimental units of the treatments T2, T3, and T4 were filled with filtered, chlorinated, and dechlorinated seawater.

In all treatments, biofloc growth was stimulated by maintaining a carbon:nitrogen (C:N) ratio of 15:1 (Ebeling et al., 2006; Avnimelech, 2012), through the addition of molasses once the concentration of total ammonia nitrogen (TAN) reached 1 mg L^{-1}. At this phase, the shrimp, with a mean weight of 0.10 ± 0.05g, were counted individually and stocked at a density of 1750 shrimp m^{-3}.

2.4. Water quality variables

Temperature (°C), dissolved oxygen (DO, mg L^{-1}) (dissolved oxygen meter YSI® EcoSense DO200A), pH (Seven 2GO – Mettler Toledo®), TAN (mg L^{-1}) (UNESCO, 1983), and NO_2^--N (mg L^{-1}) (Strickland and Parsons, 1972) were measured daily in both phases.

The NO_3^--N (mg L^{-1}) (García-Robledo et al., 2014), PO_4^{3-} (mg L^{-1}) (Aminot and Chaussepied, 1983), and TSS (mg L^{-1}) (Strickland and Parsons, 1972) were measured weekly in both phases.

Alkalinity (mg L^{-1}) (APHA, 2012) was analyzed weekly. When pH and alkalinity were below 7.5 and 150 mg L^{-1} respectively, adjustment were made with the application of sodium bicarbonate ($NaHCO_3$), following Furtado et al. (2011).

2.5. Microbial community composition

For the phytoplankton and zooplankton characterization, samples of 18 mL of water were collected from the experimental units and preserved in formalin in a final concentration of 4%. In phase 1, samples were collected at the end of the trial. In phase 2, samples were collected at the beginning and end of the experiment. The counting and identification of the main groups of microorganisms were carried out through direct counting in a sedimentation chamber, using an inverted microscope (Nikon, Eclipse

TS100), at a final magnification of 200× (Utermöhl, 1958). Phytoplankton and zooplankton abundance were expressed in cells mL^{-1} and organisms mL^{-1}, respectively.

The bacterial community characterization was carried out by sampling 18 mL of water and fragments of 2.0 cm^2 of substrate (adapted from Silva et al., 2008) from each experimental unit. The fragments of the substrate were randomly selected, covering both the deepest and most superficial layers of the substrate. In phase 1, samples were collected at the end of the trial. In phase 2, samples were collected at the beginning and end of the experiment. Samples were preserved in 4% formalin for later analysis. Substrate samples were previously sonicated (Ultrasonic Homogenizer 4710 Series, model CP50) to disaggregate biofilm. Three pulses of 30 seconds, using a frequency of 10 kHz were used. An interval of 30 seconds among pulses was adopted. Samples were filtered through polycarbonate membrane filters with 0.2 µm of mean retention, previously darkened with irgalan black and stained with 0.1% acridine orange (Hobbie et al., 1977). Bacteria were photographed with a camera attached to an epifluorescence microscope (Axioplan-Zeiss) at a final magnification of 1000×. Abundance was determined in organisms mL^{-1} from counting 30 random fields.

2.6.Feed management

In phase 1, shrimp were fed twice a day using Guabi® commercial feed with 35% crude protein. To control consumption, 10% of the feed was offered in feeding trays and the rest distributed in the tank. In phase 2, shrimp were fed three times a day using Guabi® commercial feed with 40% crude protein. In both phases, the feeding rate was adjusted weekly following Jory et al. (2001).

2.7. Shrimp growth

In both phases, at the initial and end of the experimental time, shrimp growth was evaluated to determine final weight (g), survival (%), specific growth rate (SGR, % day^{-1}), weekly growth rate (g $week^{-1}$), and yield (kg m^{-3}). A net and a digital scale were used to capture and weigh the animals, respectively.

2.8. Data analysis

Water quality data from phases 1 and 2 were tested for normality and homoscedasticity with the Shapiro-Wilk and the Levene tests, respectively. For these

data, a repeated measures analysis of variance (ANOVA) was used followed by the Tukey test to assess significant differences among treatments. When necessary, data were log (TAN, NO_2^--N, NO_3^--N, and TSS - phase 1, and PO_4^{3-} - phase 2), sine (DO - phase 1, pH – phase 2), tangent (alkalinity - phase 1), sine of tangent (pH - phase 1), cosine (DO - phase 2), cosine of log (NO_3^--N - phase 2) transformed to fulfill parametric assumptions. For non-parametric data (TAN and NO_2^--N - phase 2), the Friedman test was used followed by the Conover multiple comparison test with Bonferroni correction.

Phytoplankton, zooplankton, bacterial composition (each sampled time analyzed separately), and shrimp growth data in phases 1 and 2 were tested for normality and homoscedasticity with the Shapiro-Wilk and the Levene tests, respectively. A one-way ANOVA followed by Tukey's test was used to test for significant differences among treatments. Survival and SGR percentage data of the phase 2 were arcsine transformed before analysis (Zar, 2010). When necessary, data were log (flagellates and ciliates - phase 1, coccoid - water - phase 1, free filamentous - water and biofilm - phase 1, ciliates - initial - phase 2, coccoid - water - initial - phase 1, free filamentous - water - initial - phase 2, vibrio - biofilm - final - phase 2, filamentous - biofilm - final - phase 2), square root (rotifers - phase 1, bacillus – water – phase 2, attached filamentous – water – initial – phase 2, vibrio – water – initial – phase 2, amoeba – water - final – phase 2), cosine (Chlorophyta – initial – phase 2), tangent of square root (attached filamentous – water – phase 1, flagellates – initial – phase 2), cosine of cube (vibrio – water – phase 1, final weight – phase 2), sine of log (coccoid – biofilm – phase 1, coccoid – biofilm - initial – phase 2), cosine of sine (bacillus – water – initial – phase 2), and sine of square root (vibrio – biofilm – initial – phase 2) transformed to fulfill parametric assumptions. For non-parametric data (Chlorophyta – phase 1, Nematodes – phase 1, Bacillus – artificial substrate – phase 1, Rotifers – phase 2 – initial, Nematodes – phase 2 – initial, Amoeba – water – phase 2 – initial, Vibrio – water – phase 2 – final, and Bacillus – artificial substrate – phase 2 – initial and final), the Kruskal-Wallis test was used followed by the Dunn test with Bonferroni correction.

The graphs, ANOVA one way, Kruskal-Wallis, Friedman test, and post hoc tests were run in the software R version 4.2.3 (R core team, 2023) using ggplot2 (Wickham, 2016), Rmisc (Hope, 2022), car (Fox and Weisberg, 2019), stats (R core team, 2023), PMCMRplus (Pohlert, 2022), and dunn.test (Dinno, 2017) packages. Repeated measures

ANOVA and its post hoc was performed using Past 4.03 2020 software (Hammer et al., 2001).

3. Results

3.1. Phase 1

3.1.1. Water quality

During phase 1, the temperature was maintained between 27.83 and 28.65 °C (Table 1). DO was lower in the treatment T3 (Table 1). The pH varied between 7.57 and 8.06. TAN was higher in T1 than in T2 (Table 1). NO_2^--N was below 1 mg L^{-1}. NO_3^--N, PO_4^{3-}, and TSS were higher in T1 than in T2 and T3 (Table 1). Alkalinity was maintained above 100 mg L^{-1}

Table 1. Water quality variables in the phase 1 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies.

Variables	**Treatments**				***p*-value**
	T1	**T2**	**T3**	**T4**	
Temperature (°C)	28.65 ± 1.17[a]	28.26 ± 2.20[a]	27.83 ± 2.16[a]	-	0.129
DO (mg L^{-1})	5.34 ± 0.28[a]	5.70 ± 0.39[a]	3.91 ± 1.34[b]	-	< 0.01
pH	7.57 ± 0.27[a]	8.06 ± 0.08[a]	7.88 ± 0.07[a]	-	0.211
TAN (mg L^{-1})	0.19 ± 0.08[a]	0.12 ± 0.18[b]	0.20 ± 0.17[ab]	-	0.036
NO_2^--N (mg L^{-1})	0.10 ± 0.07[a]	0.09 ± 0.16[a]	0.08 ± 0.09[a]	-	0.595
NO_3^--N (mg L^{-1})	126.33 ± 52.54[a]	9.03 ± 3.78[b]	5.25 ± 2.85[b]	-	<0.01
PO_4^{3-} (mg L^{-1})	4.17 ± 1.49[a]	2.00 ± 1.51[b]	1.22 ± 0.58[b]	-	<0.01
Alkalinity (mg L^{-1})	104.40 ± 73.84[a]	105.60 ± 15.04[a]	113.10 ± 8.73[a]	-	0.489
TSS (mg L^{-1})	718.40 ± 167.19[a]	76.25 ± 79.86[b]	36.25 ± 45.00[b]	-	<0.01

Data are mean ± standard deviation. DO: dissolved oxygen; TAN: total ammonia nitrogen; NO_2^--N: nitrite nitrogen; NO_3^--N: nitrate nitrogen; PO_4^{3-}: orthophosphate; TSS: total suspended solids. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.1.2. Phytoplankton and zooplankton community composition

At the end of phase 1, only Chlorophyta was found in the Phytoplankton composition, where treatment T1 had a higher abundance than the others (Table 2). The Zooplankton community included flagellates, ciliates, rotifers, and nematodes, where treatment T1 had a higher abundance than the others (Table 2).

Table 2. Phytoplankton and zooplankton community composition at the end of the phase 1 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies.

Phytoplankton					
	Treatments				***p*-value**
	T1	**T2**	**T3**	**T4**	
Chlorophyta (cells mL^{-1})	$6.77 \times 10^{2} \pm 2.87 \times 10^{2}$ [a]	$1.24 \times 10^{1} \pm 1.58 \times 10^{1}$ [b]	$3.31 \times 10^{1} \pm 1.91 \times 10^{1}$ [b]	-	0.014
Zooplankton					
	Treatments				***p*-value**
	T1	**T2**	**T3**	**T4**	
Flagellates (orgs mL^{-1})	$6.54 \times 10^{3} \pm 2.56 \times 10^{3}$ [a]	$2.81 \times 10^{2} \pm 1.02 \times 10^{2}$ [b]	$2.85 \times 10^{2} \pm 1.10 \times 10^{2}$ [b]	-	<0.01
Ciliates (orgs mL^{-1})	$4.31 \times 10^{3} \pm 7.04 \times 10^{2}$ [a]	$1.57 \times 10^{2} \pm 7.20 \times 10^{1}$ [b]	$1.73 \times 10^{2} \pm 9.59 \times 10^{1}$ [b]	-	<0.01
Rotifers (orgs mL^{-1})	$1.65 \times 10^{2} \pm 6.61 \times 10^{1}$ [a]	$0.41 \times 10^{1} \pm 0.82 \times 10^{1}$ [b]	0.00 ± 0.00 [b]	-	<0.01
Nematodes (orgs mL^{-1})	$6.61 \times 10^{1} \pm 0.00$ [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	-	0.018

Data are mean ± standard deviation. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.1.3. Bacterial community composition

3.1.3.1. Water

At the end of the phase 1, the abundance of coccoid, bacillus, free filamentous bacteria, and vibrio in the water was higher in treatment T1 than in the others (Table 3). Attached filamentous bacteria were more abundant in the water of the treatment T1 than in treatment T3 (Table 3).

3.1.3.2. Biofilm

At the end of phase 1, treatment T3 had a lower abundance of coccoid bacteria than the other treatments (Table 3). Bacillus were not found in treatments T1 and T2 and were found in treatments T3 and T4. (Table 3). Furthermore, treatments T2 and T4 showed a higher abundance of filamentous bacteria and vibrio than in treatments T1 and T3 at the end of the trial (Table 3).

Table 3. Bacterial community composition in the water and biofilm at the end of the phase 1 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies.

Water

	Treatments				*p*-value
	T1	**T2**	**T3**	**T4**	
Coccoid (orgs mL^{-1})	$1.02 \times 10^{7} \pm 2.17 \times 10^{6}$ [a]	$1.87 \times 10^{5} \pm 1.75 \times 10^{4}$ [b]	$1.06 \times 10^{5} \pm 2.43 \times 10^{4}$ [c]	-	<0.01
Bacillus (orgs mL^{-1})	$1.15 \times 10^{5} \pm 3.47 \times 10^{4}$ [a]	$7.38 \times 10^{3} \pm 1.73 \times 10^{3}$ [b]	$1.39 \times 10^{4} \pm 1.96 \times 10^{4}$ [b]	-	0.013
Free filamentous (orgs mL^{-1})	$5.74 \times 10^{4} \pm 1.89 \times 10^{4}$ [a]	$5.28 \times 10^{3} \pm 3.77 \times 10^{3}$ [b]	$2.39 \times 10^{3} \pm 8.82 \times 10^{2}$ [b]	-	<0.01
Attached filamentous (orgs mL^{-1})	$8.20 \times 10^{3} \pm 1.30 \times 10^{4}$ [a]	$1.13 \times 10^{3} \pm 1.30 \times 10^{3}$ [ab]	$3.41 \times 10^{2} \pm 2.61 \times 10^{2}$ [b]	-	0.048
Vibrio (orgs mL^{-1})	$5.47 \times 10^{3} \pm 3.86 \times 10^{3}$ [a]	$4.10 \times 10^{2} \pm 1.36 \times 10^{2}$ [b]	$4.44 \times 10^{2} \pm 6.44 \times 10^{2}$ [b]	-	0.040

Biofilm

	Treatments				*p*-value
	T1	**T2**	**T3**	**T4**	
Coccoid (orgs mL^{-1})	$1.84 \times 10^{6} \pm 1.12 \times 10^{5}$ [a]	$6.37 \times 10^{5} \pm 9.24 \times 10^{4}$ [a]	$2.73 \times 10^{5} \pm 4.83 \times 10^{4}$ [b]	$7.71 \times 10^{5} \pm 2.43 \times 10^{5}$ [a]	<0.01
Bacillus (orgs mL^{-1})	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	$1.64 \times 10^{3} \pm 3.86 \times 10^{2}$ [a]	$1.59 \times 10^{3} \pm 3.94 \times 10^{2}$ [a]	0.017
Filamentous (orgs mL^{-1})	$2.73 \times 10^{3} \pm 1.93 \times 10^{3}$ [b]	$1.68 \times 10^{4} \pm 7.18 \times 10^{3}$ [a]	$1.37 \times 10^{3} \pm 4.73 \times 10^{2}$ [b]	$1.30 \times 10^{4} \pm 1.37 \times 10^{3}$ [a]	<0.01
Vibrio (orgs mL^{-1})	0.00 ± 0.00 [b]	$3.64 \times 10^{3} \pm 1.04 \times 10^{3}$ [a]	$7.28 \times 10^{2} \pm 1.57 \times 10^{2}$ [b]	$2.77 \times 10^{3} \pm 1.22 \times 10^{3}$ [a]	<0.01

Data are mean ± standard deviation. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.1.4. Shrimp growth

Shrimp in treatment T1 reached a final weight of 15.09 ± 1.81 g (mean ± standard deviation), a weekly growth rate of 1.34 ± 0.34 g $week^{-1}$, and a survival of 77.33 ± 5.51%. The final yield was 5.71 ± 0.86 kg m^{-3}.

3.2. Phase 2

3.2.1. Water quality

During phase 2, the temperature was maintained between 26.80 and 27.98 °C. DO was maintained above 5 mg L^{-1} and the pH was between 7.77 and 7.91. TAN was higher in treatments T2 and T3 than in treatment T1 (Table 4). Treatment T4 had a higher variation in TAN throughout the experimental time (Figure 2a). TAN stabilization in treatments T2, T3, and T4 occurred from day 10, remaining below 0.5 mg L^{-1} until the end of the trial (Figure 2a). NO_2^--N was lower in treatment T1 than in the others. A NO_2^--N spike was observed in the T4 treatment and was only controlled from day 14 of the trial (Figure 2b). NO_3^--N was higher in T1 treatment (Table 4). A pattern of increasing NO_3^--N concentration was observed in all treatments throughout the experimental period (Figure 2c). PO_4^{3-} and TSS were higher in treatment T1 than in the others (Table 4). Alkalinity was higher in treatments T2 and T4 than in treatment T1 (Table 4).

Table 4. Water quality variables in the phase 2 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies.

Variables	Treatments				*p*-value
	T1	T2	T3	T4	
Temperature (°C)	27.88 ± 3.22[a]	27.74 ± 3.70[a]	27.98 ± 3.47[a]	26.80 ± 4.05[a]	0.844
DO (mg L^{-1})	5.75 ± 0.57[a]	5.79 ± 0.65[a]	5.74 ± 0.60[a]	5.81 ± 0.72[a]	0.485
pH	7.77 ± 0.16[a]	7.89 ± 0.20[a]	7.91 ± 0.18[a]	7.88 ± 0.17[a]	0.665
TAN (mg L^{-1})	0.15 ± 0.04[b]	0.31 ± 0.27[a]	0.28 ± 0.21[a]	0.36 ± 0.46[ab]	<0.01
NO_2^--N (mg L^{-1})	0.41 ± 0.64[b]	1.28 ± 1.01[a]	1.03 ± 0.64[a]	1.99 ± 2.64[a]	<0.01
NO_3^--N (mg L^{-1})	99.98 ± 53.82[a]	26.67 ± 17.09[b]	23.64 ± 16.18[b]	22.45 ± 16.87[b]	<0.01
PO_4^{3-} (mg L^{-1})	7.83 ± 5.71[a]	1.71 ± 1.06[b]	1.63 ± 0.95[b]	2.75 ± 1.14[b]	<0.01
Alkalinity (mg L^{-1})	107.50 ± 20.87[b]	125.00 ± 27.81[a]	117.00 ± 22.03[ab]	125.50 ± 21.64[a]	0.025
TSS (mg L^{-1})	748.10 ± 280.81[a]	290.90 ± 232.33[b]	265.60 ± 205.30[b]	230.70 ± 180.85[b]	<0.01

Data are mean ± standard deviation. DO: dissolved oxygen; TAN: total ammonia nitrogen; NO_2^--N: nitrite nitrogen; NO_3^--N: nitrate nitrogen; PO_4^{3-}: orthophosphate; TSS: total suspended solids. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

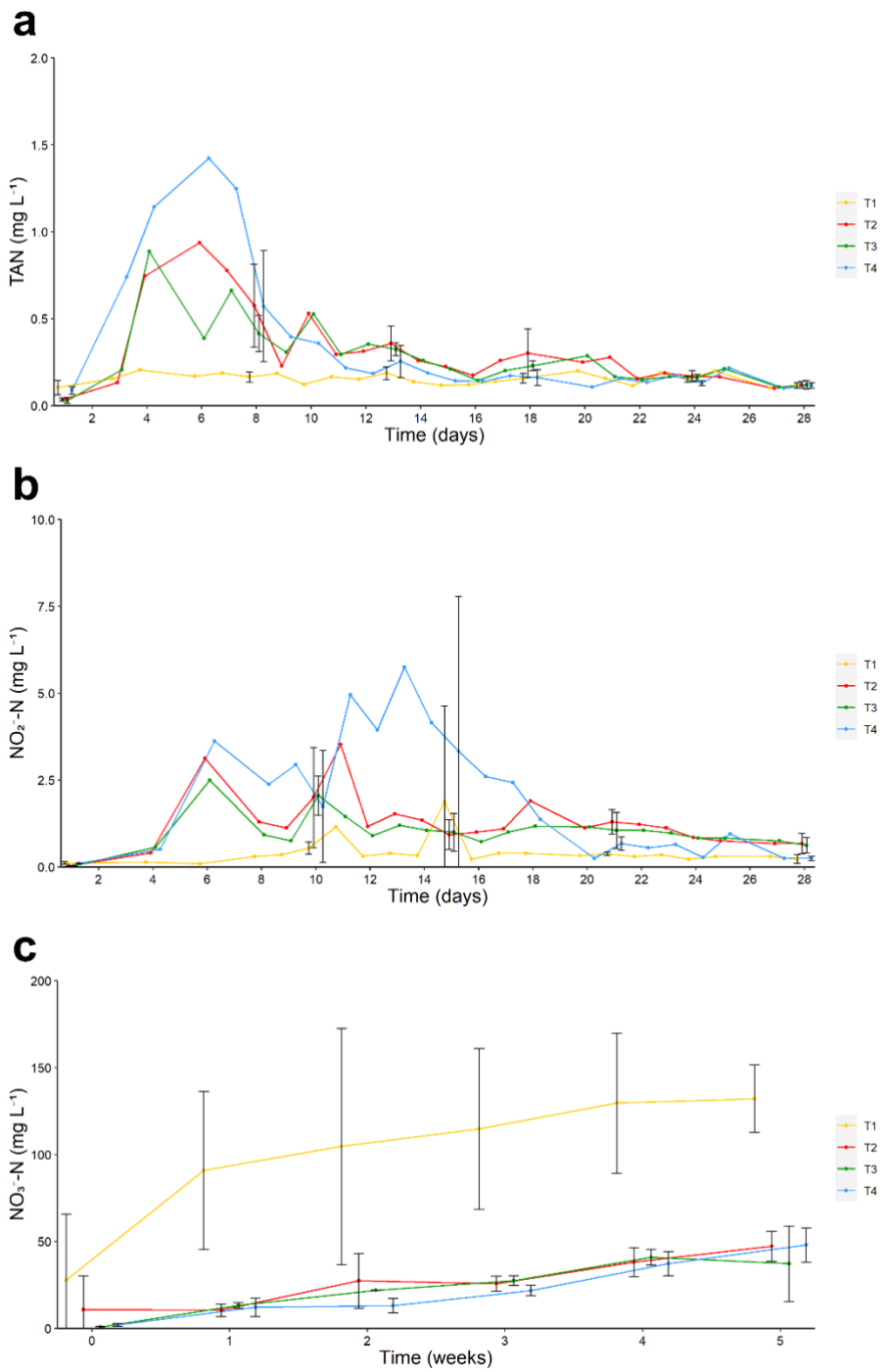


Figure 2. Concentration (mean ± standard deviation) of total ammonia nitrogen (TAN, a), nitrite nitrogen (NO_2^--N, b), and nitrate nitrogen (NO_3^--N, c) during phase 2 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies. T1: artificial substrate + biofloc + shrimp + aeration (control);

T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.2.2. Phytoplankton and zooplankton community composition

At the beginning of phase 2, there were more Chlorophyta in treatments T1, T2, and T4 than in treatment T3 (*p*-value = 0.004. Figure 3a).

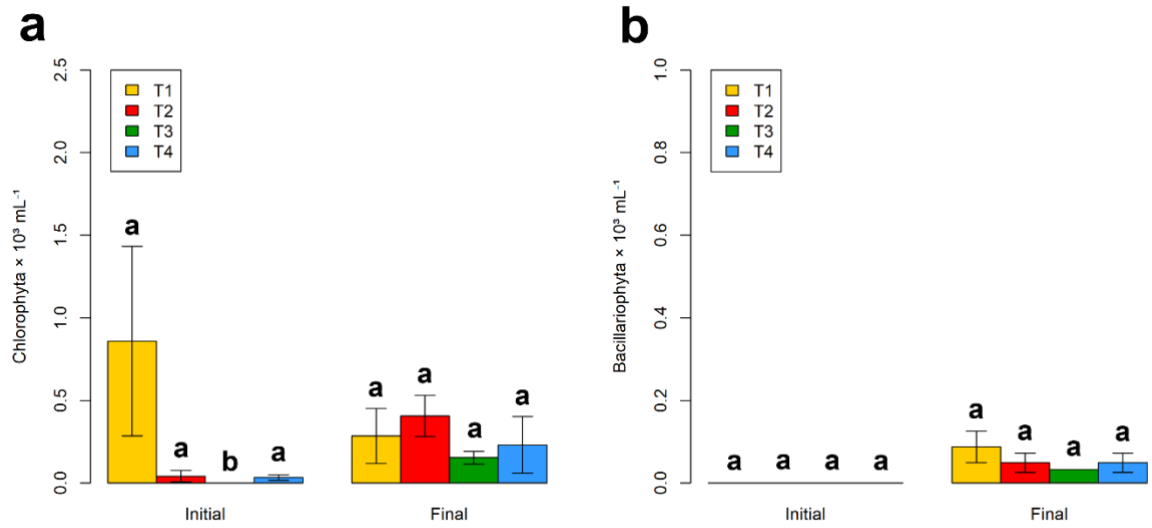


Figure 3. Abundance of Chlorophyta (a) and Bacillariophyta (b) during the phase 2 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

At the beginning of phase 2, treatment T1 had a higher abundance of flagellates, ciliates, and rotifers than the other treatments (*p*-value < 0.01. Figure 4). At the end of the trial, T1 and T2 treatments had more ciliates than the T3 and T4 treatments (*p*-value = 0.003. Figure 4b). Furthermore, treatment T1 had more rotifers and nematodes than the other treatments at the final sample (*p*-value = 0.01 and 0.005, respectively. Figure 4c and d). At the end of the experiment, treatment T2 had more amoeba than treatments T1 and T3 (*p*-value = 0.004. Figure 4e).

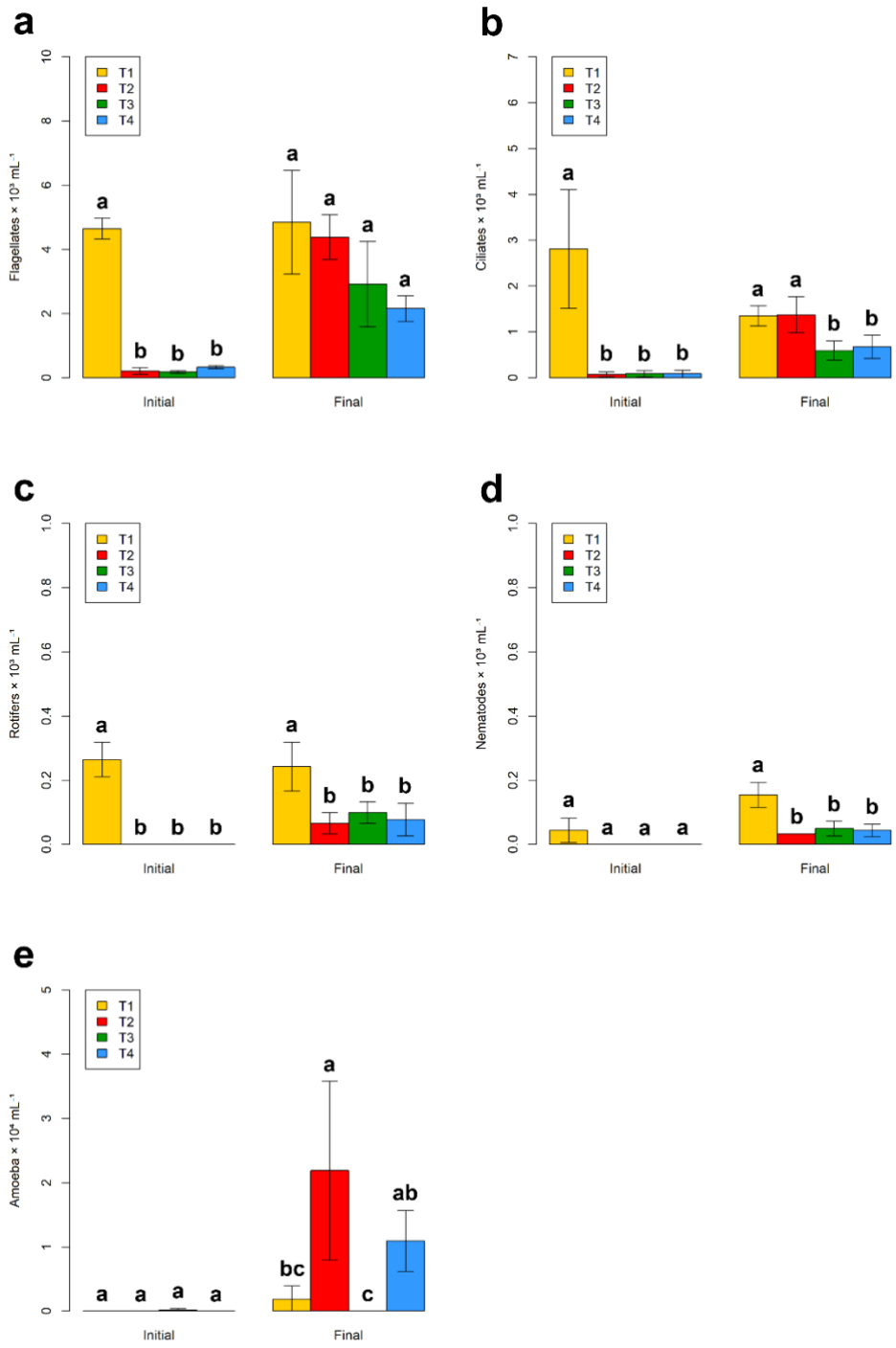


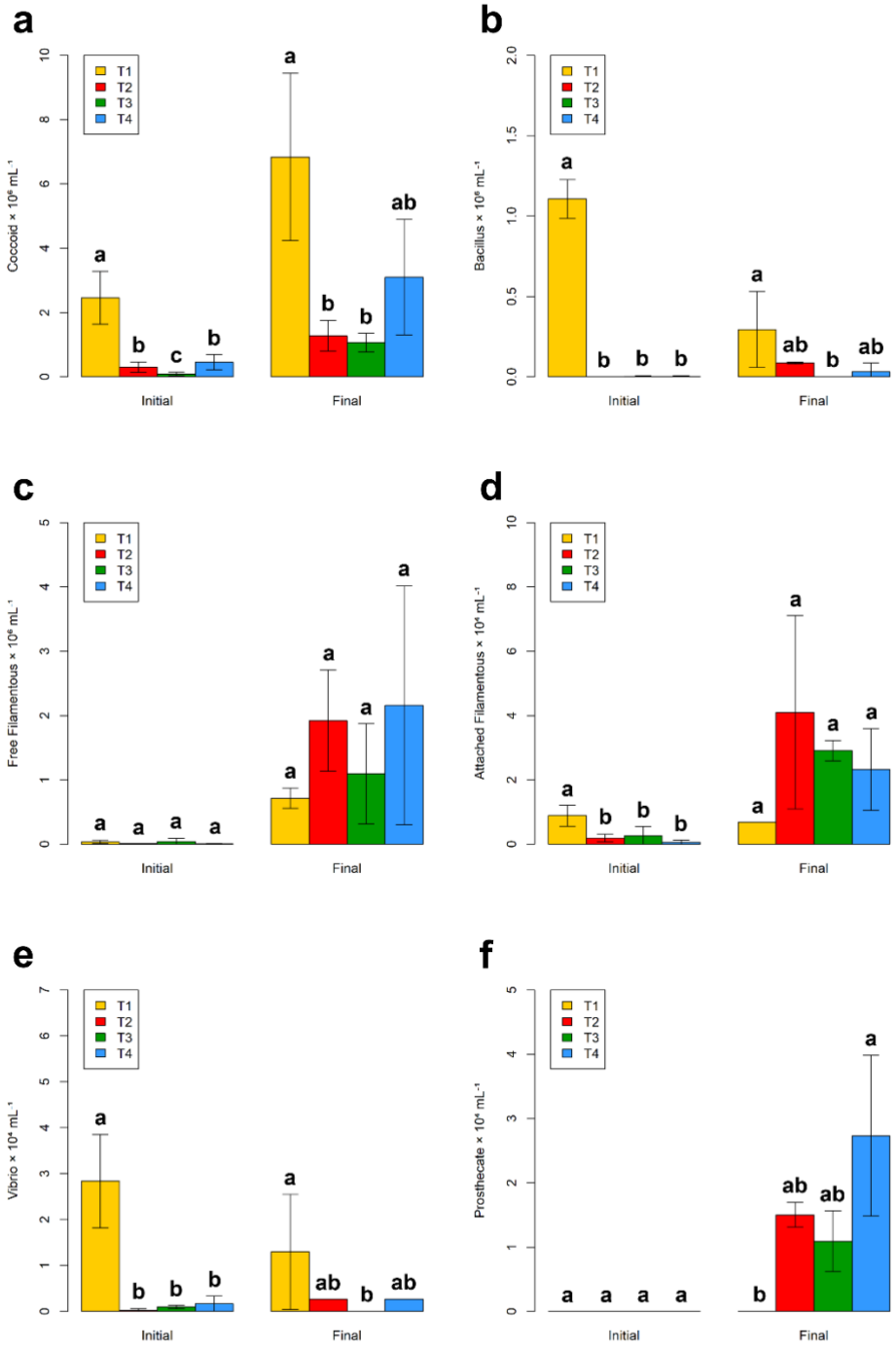
Figure 4. Abundance of flagellates (a), ciliates (b), rotifers (c), nematodes (d), and amoeba (e) during the phase 2 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies. T1: artificial substrate +

biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.2.3. Bacterial community composition

3.2.3.1. Water

At the beginning of phase 2, treatment T1 had a higher abundance of coccoid bacteria, bacillus, attached filamentous bacteria, and vibrio in the water than the other treatments (p-value < 0.01. Figure 5). At the end of the trial, the abundance of coccoid bacteria in the water was higher in treatment T1 than in treatments T2 and T3 (p-value = 0.008. Figure 5a). At the end of the experiment, treatment T1 had a higher abundance of bacillus and vibrio in the water than treatment T3 (p-value = 0.024 and 0.047, respectively. Figure 5b and e). Furthermore, T4 treatment had more prosthecate bacteria than the T1 (p-value = 0.041. Figure 5f).



1502

1503 **Figure 5.** Abundance of coccoid (a), bacillus (b), free filamentous (c), attached (d), vibrio
1504 (e), and prosthecates (f) bacteria in the water of the phase 2 of a *Penaeus vannamei* super-
1505 intensive biofloc system using different artificial substrates management strategies. T1:

artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.2.3.2. Biofilm

At the initial sample, treatments T1, T2, and T4 had more coccoid bacteria than T3 (*p*-value <0.01. Figure 6a). At the end of the trial, coccoid bacteria were more abundant in the treatments T2 and T4 than in the T1 and T3 (*p*-value = 0.002. Figure 6a). At the beginning, treatment T4 had a higher abundance of bacillus than treatments T1 and T2 (*p*-value = 0.017. Figure 6b). Treatment T4 had more bacillus than the other treatments at the final sample (*p*-value = 0.013. Figure 6b). At the initial sample, T2 and T4 had more filamentous bacteria than T1 and T3 (*p*-value <0.01. Figure 6c). Filamentous bacteria were more abundant in the T2 than the other treatments at the end of the trial (*p*-value <0.01. Figure 6c). At the initial sample, vibrio was more abundant in the T2 treatment than in the T3 treatment (*p*-value = 0.048. Figure 6d).

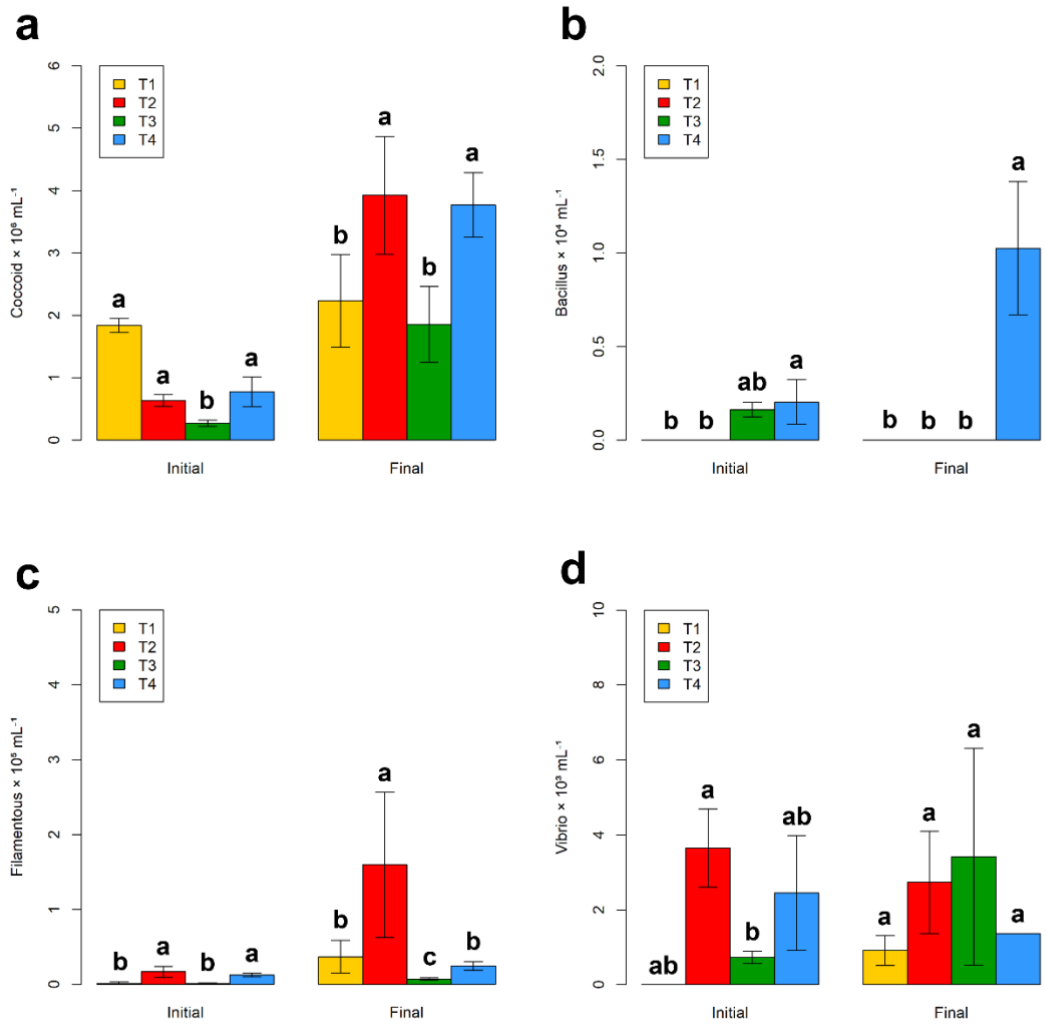


Figure 6. Abundance of coccoid bacteria (a), bacillus (b), filamentous (c), and vibrio (d) present in the biofilm of phase 2 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

3.2.4. Shrimp growth

At the end of phase 2, the shrimp final weight was higher in T2 (1.74g) than the other treatments (Table 5). Survival was higher than 80% in all treatments and was higher in T3 (99.62%) than in the others (Table 5). SGR was higher in the T2 treatment than in

the T4 treatment (Table 5). Yield varied between 2.17 Kg m^{-3} in T4 and 2.38 Kg m^{-3} in T3 (Table 5).

Table 5. Performance of *Penaeus vannamei* at the end of the phase 2 of a *Penaeus vannamei* super-intensive biofloc system using different artificial substrates management strategies.

	Treatments				*p*-value
	T1	**T2**	**T3**	**T4**	
Initial weight (g)	0.10 ± 0.05	0.10 ± 0.05	0.10 ± 0.05	0.10 ± 0.05	-
Final weight (g)	1.53 ± 0.14^{b}	1.74 ± 0.11^{a}	1.52 ± 0.14^{b}	1.41 ± 0.17^{b}	0.037
Survival (%)	87.21 ± 2.38^{b}	86.00 ± 6.00^{b}	99.62 ± 1.57^{a}	91.62 ± 1.15^{b}	<0.01
SGR (% day^{-1})	9.25 ± 0.31^{ab}	9.69 ± 0.21^{a}	9.34 ± 0.27^{ab}	8.97 ± 0.41^{b}	0.048
Yield (kg m^{-3})	2.22 ± 0.16^{a}	2.35 ± 0.06^{a}	2.38 ± 0.18^{a}	2.17 ± 0.25^{a}	0.467

Data are mean ± standard deviation. SGR: specific growth rate. T1: artificial substrate + biofloc + shrimp + aeration (control); T2: artificial substrate + water + aeration; T3: artificial substrate + water, and T4: only artificial substrate.

4. Discussion

In Phase 1, managing the artificial substrate without oxygenation (T3) resulted in limited water circulation and low oxygen concentrations compared to the other treatments. This condition could establish a gradient in the concentration of oxygen throughout the biofilm (Gieseke et al., 2003; Vlaeminck et al., 2010). Consequently, hypoxic, or anoxic areas may form in the deeper sections of the biofilm, as discussed by Vlaeminck et al. (2010). Under these circumstances, the activity of nitrite-oxidizing bacteria can significantly decrease, leading to nitrogen accumulation in the water upon reusing the artificial substrate. Furthermore, the predominance of anaerobic decomposition pathways promotes an increase in the production and release of reduced toxic compounds in the water column, including ammonia, sulfides, manganese, methane, and nitrite (Alongi et al., 1999; Jiménez-Montealegre et al., 2002; Avnimelech and Ritvo, 2003). These factors can compromise the functioning of the system and the growth of animals under culture.

The water quality conditions found in the control treatment (T1) help explain our findings on plankton composition during phase 1 of this study. The high concentrations of NO_3^--N and PO_4^{3-} found in T1 show that a nutrient-rich culture medium can sustain a high load of microorganisms that perform essential functions in a biofloc system. NO_3^--N is fundamental as a source of inorganic nitrogen, directly related to the amount of carbon required by the system (Lovera et al., 2017). This nutrient directly impacts the phytoplankton composition in bioflocs, as it is quickly absorbed by algae (Esteves et al., 2011). The higher abundance of Chlorophyta in T1 served as the basis for the growth of all zooplankton, which can directly prey on this Phytoplankton group (González, 2000). The participation of Chlorophytes in biofloc systems constitutes a relevant aspect in the dynamics of these aquatic environments, as they contribute to the production of oxygen (Avnimelech, 2015) and can influence the structure and formation of the biofilm, providing a surface conducive to the growth of a diverse microbial community (Crab et al., 2007). However, the presence of chlorophytes can result in competition for nutrients with other microorganisms, such as bacteria, which influences the dynamics of the microbial community in the biofloc system (Kuhn et al., 2009).

The presence of nutrients in the T1, associated with the effect of aeration, facilitates the assimilation of nutrients and microbial growth (Avnimelech, 2007). These are some of the factors that can explain the abundance patterns found in the bacterioplankton community both in the water and in the biofilm. In biofloc systems, coccoid bacteria present specific adaptations to the environment, using a gelatinous matrix (biofilm) as a favorable substrate for adhesion and growth (Crab et al., 2012). This preference of coccoid bacteria for being attached to substrates may explain their higher abundance in the biofilm in treatments T1, T2, and T4 during phase 1. The presence and activity of these bacteria can influence water quality, affecting nitrogen concentration (Crab et al., 2012; Emerenciano et al., 2013). The interaction between coccoid bacteria and other microbial communities presents in the biofilm and in the water is important for the microbial dynamics and diversity of the BFT system (Kuhn et al., 2010).

Regarding biofilm, treatments T2 and T4 showed a significant presence of filamentous bacteria and vibrio. It is possible that the absence of aeration, combined with the lower density of predators (Zooplankton) in these treatments, favored the increase in

abundance and adherence of these groups of bacteria to the biofilm. It is interesting to highlight that bacterial survival was observed in the substrate of the T4 treatment, where it was kept exposed to air, without water, and aeration. This suggests that its reuse can be carried out without the need for specific management between culture cycles.

During phase 2, all water quality parameters were within limits considered safe for *Penaeus vannamei* (Van Wyk and Scarpa, 1999; Gaona et al., 2011; Furtado et al., 2011; Zhang et al., 2020). At this phase, treatment T1 clearly had the best water quality condition, demonstrating a good nitrification process, as it had lower TAN compared to T2 and T3, lower NO_2^--N, and higher NO_3^--N (with accumulation throughout the trial). This possibly happened due to the maintenance of the biofloc and artificial substrate in a common culture during phase 1, without any significant variation in water quality conditions that could compromise the nitrifying bacteria community.

In treatment T4, TAN showed the highest variation throughout the experimental time. However, it was possible to observe a recovery of the ammonia-oxidizing bacteria community in this treatment since TAN was controlled from day 10 of the trial and in the same period there was a spike in the NO_2^--N concentration. During this spike, the mean concentration of NO_2^--N did not exceed 6 mg L^{-1}, remaining within the safe limit considering the salinity (Lin and Chen, 2003). The control of NO_2^--N in this treatment throughout the phase 2 demonstrates the activity of the community of nitrite-oxidizing bacteria. These findings are reinforced by the accumulation of nitrate throughout the experimental time in the treatments where artificial substrates received different management.

Regarding microbial community found in phase 2, the highest abundances of nematodes and rotifers found in T1 indicate a better microbial loop development. This probably happened because this treatment presents a nutrient-rich medium during phase 1, enabling adequate conditions for the growth of microorganisms during phase 2. On the other hand, T2 was the one that best provided the growth of phytoplankton and zooplankton, as it had higher abundance of ciliates and amoebas. In aquaculture environments, ciliated protozoa act as indicators of water quality (Decamp et al., 1999), nematodes and flagellates stand out as lipid sources, while coccoid bacteria are source of protein for shrimp (Silva et al., 2008; Rocha et al., 2012). The influence of the predation

rate exerted by cultivated organisms can have a significant impact on the abundance of these organisms (Ray et al., 2010). These interactions between microorganisms, for example, may explain the reduction in ciliates in T1 between the initial and final samples.

Treatments T2 and T4 demonstrated a recovery of the community in the biofilm over the phase 2. This was confirmed by the higher abundance of free coccoid, bacillus, and filamentous bacteria in these treatments compared to the others at the end of the trial. These findings are very relevant, since coccoid bacteria act in the formation of biofilm (Rocha et al., 2012), while the presence of bacillus can positively influence water quality, due to their ability to degrade organic matter in the system. (Verschuere et al., 2000). The higher abundance of vibrio in the T2 compared to the T3 treatment shows that maintaining the artificial substrate in water without oxygen can be effective in controlling this group of bacteria that can be pathogenic. However, the abundance of vibrio in phase 2, both in the water and in the biofilm, was lower than that found for other groups of bacteria, such as coccoid and bacillus. This shows an antagonistic effect between these bacterial groups. Antagonism is recognized as a robust mechanism of action of probiotics, being characterized by competitive exclusion, where probiotic bacteria compete for nutrients (Gatesoupe, 1999), for adhesion sites in the intestinal tract (Mohapatra et al., 2013; Hostins et al., 2017) and for production of various toxins (Gatesoupe, 1999; Gillor et al., 2008). Furthermore, it is important to highlight that not all species of the genus *Vibrio* are pathogenic (Gomez-Gil et al., 1998). Previous studies have characterized *Vibrio alginolyticus* as beneficial for the *P. vannamei* culture (Gomez-Gil et al., 2000).

Finally, the use of artificial substrates in aquaculture offers several advantages, including increasing the availability of natural food, contributing to shrimp nutrition (Thompson et al., 2002; Abreu et al., 2007; Ballester et al., 2007), and promoting the development of microbial communities with probiotic effects on cultivated organisms (Azim et al., 2001). Furthermore, different management strategies for artificial substrates have significant impacts on water quality variables and the microbial composition of the system, positively reflecting on shrimp growth and survival of the shrimp. Treatments T2 and T3 demonstrated the best performance, presenting higher final weight and survival, respectively. The insights brought in this study on the reuse of pre-colonized artificial

substrates have practical implications for the management of these tools that play a key role in the super-intensive culture of *P. vannamei* with biofloc systems.

5. Conclusion

The different management strategies on the artificial substrate in *Penaeus vannamei* intensive culture with a biofloc system is a viable alternative to improve water quality and the microbial community composition. Maintaining the substrate submerged only in water (T3) does not limit the nitrification process between culture cycles. Also, maintaining artificial substrates exposed to air (T4) proved not to compromise the nitrification process. The microbial community recovers, with the growth of a high load of coccoid, bacilli, and filamentous bacteria.

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CrediT authorship contribution statement

Bianca de Oliveira Ramiro: Conceptualization, Investigation, Methodology, Data Curation, Visualization, Writing – original draft. **Wilson Wasielesky Jr.:** Supervision, Funding acquisition, Project Administration, Resources, Writing – review & editing. **Otávio Augusto Lacerda Ferreira Pimentel:** Investigation, Data Curation, Formal Analysis, Visualization, Writing – review & editing. **Natália Pereira da Silva:** Investigation, Writing – review & editing. **Lucélia do Valle Borges:** Investigation Writing – review & editing**. Dariano Krummenauer:** Conceptualization, Methodology, Supervision, Funding acquisition, Project Administration, Resources, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data availability

Data will be made available on request.

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CAPITULO III: Assessment of Water Quality, Growth of *Penaeus vannamei,* and Partial Budget in Super-Intensive BFT and RAS: A Comparison Between Sustainable Aquaculture Systems

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Assessment of Water Quality, Growth of *Penaeus vannamei*, and Partial Budget in Super-Intensive BFT and RAS: A Comparison Between Sustainable Aquaculture Systems

Abstract

This study evaluated water quality, growth, and partial budget analysis (PBA) for *Penaeus vannamei*, comparing super-intensive Biofloc Technology (BFT) and Recirculating Aquaculture Sys- tems (RAS). The 69-day trial used 100 L units with two treatments (RAS and BFT), each with three replicates. Shrimp were initially reared in a 30-day nursery to a weight of 0.10 ± 0.04 g and then stocked at 500 shrimp m−3. Biofloc growth in BFT was promoted by maintaining a C:N ratio of 15:1, adding dextrose when total ammonia nitrogen (TAN) reached 1 mg L−1. Probiotics (3 g m−3) were administered daily to both groups. TAN levels in BFT initially spiked but stabilized after 36 days. *Vibrio* abundance was initially higher in RAS, but by the end of the trial, it was higher in BFT. Final weight, weekly growth ratio, and yield were greater in BFT, whereas feed conversion ratio (FCR) and water use were higher in RAS. Survival rates were 83.33% in BFT and 88% in RAS. BFT achieved a superior net benefit/cost compared to RAS. Although RAS more effectively controlled nitrogenous compounds, BFT exhibited better growth performance, with higher final weights, lower FCR, and better *Vibrio* management. The partial budget analysis indicated an economic advantage for BFT, with a net positive benefit of $2270.09 when shifting from RAS to BFT due to lower operating costs and higher shrimp yield. Among these two sustainable production systems, BFT was more productive while utilizing less natural resources.

Keywords: recirculating aquaculture system; biofloc; nitrogenous compounds; *Vibrio*; sustainable shrimp production

1. Introduction

Aquaculture has advanced significantly by incorporating innovative technologies that aim to preserve water resources and reduce environmental impacts. Among these approaches, Recirculating Aquaculture Systems (RAS) and Biofloc Technology System (BFT) operate with minimal water use [1,2]. The practice of *Penaeus vannamei* intensive and super-intensive culture minimizing water exchange meets growing environmental concerns driven by the concept of sustainable development, which seeks to integrate principles of ecological prudence, economic efficiency, and social equity into all human activities [3–5].

RAS provides a high level of control over the aquatic environment, allowing for more efficient production in terms of space and labor use, in addition to substantially reducing water consumption in relation to the biomass produced. According to Timmons et al. [6], these systems facilitate economies of scale, enabling high shrimp production compared to other aquaculture methods. The configuration of RAS includes devices for water treatment and reuse, such as decanters, mechanical filters, and biological filters [7]. The use of mechanical filters allows the removal of solid waste, including feed remains and feces, whereas biological filters promote the action of nitrifying bacteria to control the levels of ammonia and nitrite in the water [8]. Thus, these systems can achieve high yields with minimal environmental risks, making them one of the most promising technologies for modern aquaculture.

The BFT system represents a complex and dynamic environment characterized by comprehensive microbial diversity, comprising not only bacteria but also algae, protozoa, and organic matter [9,10]. This system adopts an ecologically responsible approach that favors the reuse of water in several cycles, resulting in significant environmental benefits such as reducing pollution in coastal areas [11]. Furthermore, the implementation of BFT has been shown to promote optimized yields in *P. vannamei* cultures at high stocking densities [12,13]. Therefore, this strategy not only increases productivity but also improves environmental control by minimizing or eliminating the need for water changes, thus contributing to the sustainability of the aquaculture sector [12,14,15].

In the context of these systems, the accumulation of nitrogenous compounds results mainly from the ingestion of food by shrimp, their excretion, and the decomposition of organic matter present in the culture environment, including unconsumed feed and feces [10]. Maintaining inadequate levels of total ammonia nitrogen (TAN) and nitrite (NO_2^-) can induce stress and physiological changes in cultured organisms, harming their growth and survival, with a consequent negative impact on production [16]. Therefore, nitrifying bacteria present in bioflocs play a key role in controlling these toxic nitrogenous compounds, facilitating their oxidation to less harmful forms such as nitrate (NO_3^-) [17].

When using RAS and BFT systems in aquaculture, an interconnected approach has emerged, driven by the growing need for sustainability and productivity. Using these systems is essential to mitigate the environmental impacts of intensive aquaculture and promote a more responsible approach to the use of water resources. Although there are studies on BFT and RAS, this research explores, in an unprecedented way, the comparison of specific production costs under super-intensive aquaculture conditions, highlighting the economic and microbiological advantages of bioflocs. This study aimed to evaluate the differences in water quality parameters, *Penaeus vannamei* growth, and partial budget analysis (PBA) between BFT and RAS systems, emphasizing economic factors and *Vibrio* control throughout the cultivation cycle.

2. Materials and Methods

2.1. Design and experimental conditions

A *P. vannamei* shrimp grow-out was carried out for 69 days at the Virginia Seafood Agricultural Research and Extension Center, Virginia Polytechnic Institute and State Uni- versity, Hampton, VA, USA. *P. vannamei* post-larvae were acquired from Homegrown Shrimp, LLC, Indiantown, FL, USA. Shrimp were initially kept in a 30-day nursery until they reached a weight of 0.10 ± 0.04 g (mean ± standard deviation) and then were stocked in the 6 experimental units at a density of 500 shrimp m−3.

The experiment was carried out in 100 L experimental units and divided into two treat- ments, all with three repetitions: RAS (Recirculating Aquaculture System) and BFT

(Biofloc Technology System). Seawater (salinity between 28 and 30 g L−1) obtained from mixing tap fresh water with artificial salt (Instant Ocean Sea Salt, Blacksburg, VA, USA) was ini- tially treated with 10 g m−3 of sodium hypochlorite and subsequently dechlorinated using ClorAm-x (Reed Mariculture, Campbell, CA, USA).

In the RAS treatment, the water was driven by a 0.75 hp centrifugal pump (Doheny's, model 2601, flow rate of 180 L h−1) to a mechanical Bubble bead filter and then to a biological filter as steps of water treatment before being recirculated among experimental units. The biological filter was composed of K1 Kaldnes Biological Media (Evolution Aqua, Green Brook, NJ, USA) and was constantly aerated with an air injector (Nozzle®, model a3, Detroit, MI, USA). Additionally, two air stones were installed inside each RAS tank to maintain optimal oxygen levels. The total volume of the RAS system was approximately 600 L.

In the BFT treatment, biofloc growth was stimulated by maintaining a carbon:nitrogen (C:N) ratio of 15:1 [16,18], by adding dextrose (based on Serra et al. [19]) once the total ammonia nitrogen (TAN) concentration reached 1 mg L−1. In the BFT tanks, a structure containing four porous stones (7.6 cm long × 2.5 cm wide × 2.5 cm deep) coupled to a central hose was arranged in each tank to provide oxygenation by an air pump (Intertek, model AP-60, Chickasaw, AL, USA).

In both treatments, the commercial probiotic Sanolife MIC (Inve® Aquaculture, Den- dermonde, Belgium), composed of *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus pumilus* (concentration: 5 × 1010 colony forming units (CFU) g−1) was administered daily at a dosage of 3 g m−3. The probiotics were diluted in water and incorporated into the feed before each feeding.

2.2. Water quality variables

Temperature (◦C), dissolved oxygen (DO, mg L−1) (YSI Pro 2030), pH (Hanna, model HI98107), TAN (mg L−1) [20], and nitrite nitrogen (NO_2^--N, mg L−1) (Hach method 8507) were measured daily. The concentration of nitrate nitrogen (NO_3^--N, mg L−1) (Hach method 8039), total suspended solids (TSS, mg L−1) (Hach portable multiparameter colorimeter, model DR900), settleable solids (SS, mL L−1) [21], CO_2 (mg L−1) [22], and alkalinity (mg L−1) [23] were measured weekly. When pH and

alkalinity were below 7.5 and 150 mg L−1, respectively, adjustments were made with sodium bicarbonate (NaHCO3) application, following Furtado et al. [24]. On day 51, a sampling error occurred for NO2−-N in both treatments. Therefore, we decided to present the data only up to day 50.

2.3. Vibrio community composition

Water samples were collected from experimental units on days 0, 14, 28, 42, and 63 of the trial to assess the abundance of *Vibrio* spp. in the systems. From each tank, at least two 10 mL water samples were drawn as biological replicates. Samples were collected using sterile techniques to prevent contamination.

The samples were subjected to a standard plate count method to enumerate *Vibrio* spp. Initially, each sample was diluted using sterile phosphate-buffered saline (PBS) to prepare serial dilutions appropriate for counting. The diluted samples were then plated on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar, a selective medium for the isolation of *Vibrio* species. Plates were incubated at 35 ◦C for 24 h. After the incubation, colonies characteristic of *Vibrio* spp. were counted on the TCBS agar plates. The counts were expressed as colony-forming units (CFU) mL −1, according to the methodology proposed by FDA BAM [25].

2.4 Feed management

The shrimp were fed Ziegler® commercial feed (Gardners, PA, USA), with 35% crude protein, distributed manually three times a day. The amount of feed offered to the animals was calculated according to Jory et al. [26], which is based on the animal's weight and the temperature of the culture water.

2.5. Shrimp growth, survival, and water use

At the end of the experimental time, shrimp sampling was carried out to determine final weight (g), feed conversion ratio (FCR), survival (%), weekly growth ratio (WGR, g week−1), yield (Kg m−3), and water use (m3 Kg−1).

2.6. Partial Budget analysis

A partial budget analysis (PBA) was performed considering the costs and revenue associated with shrimp production in RAS compared to BFT systems. Partial budget analysis measures the net benefit from the difference between the benefits and costs for a small change in the operation [2

7]. In this case, the RAS and BFT systems have different equipment requirements and procedures that impact on the costs of water use, salt, sodium bicarbonate, dextrose, feed, electricity, labor, operating interest, and depreciation.

The PBA considered two different scenarios to estimate the net benefit cost of a RAS system turning into a BFT system (RAS to BFT) and a BFT system turning into a RAS system (BFT to RAS), based on the performance obtained in the grow-out experiment. The indicators utilized in the PBA are similar to those used by Krummenauer et al. [28] and are described as follows. Additional revenue was estimated based on the difference in gross receipts between RAS and BFT systems for each scenario. Reduced costs were estimated based on the difference in the input supply items, operating interest, and equip- ment depreciation between RAS and BFT systems. Total additional benefits = Additional revenue + Reduced costs. In each scenario, additional costs were estimated based on the difference in input supply items, operating interest, and equipment depreciation between RAS and BFT systems. Reduced revenue was estimated based on the difference in gross receipts between RAS and BFT systems for each scenario. Total additional cost = Re- duced revenue + Additional costs. Net benefit/cost = Total additional benefits-Total additional costs.

2.7. Data analysis

Water quality data were tested for normality and homoscedasticity with the Shapiro- Wilk and the Levene test, respectively. Differences between treatments were tested with a repeated measures analysis of variance (ANOVA). When necessary, data were transformed to fulfill parametric assumptions. For non-parametric data (NO_2^--N), the Friedman test was used.

Vibrio abundance (analyzed separately for each day sampled), shrimp growth, and water use data were tested for normality and homoscedasticity with the Shapiro-Wilk and the Levene test, respectively. Differences between treatments were tested with the T-test.

When necessary, data were transformed to fulfill parametric assumptions. Non-parametric data (*Vibrio*—day 28 and survival) were analyzed with the Wilcoxon test.

The graphs, T-test, Wilcoxon, and Friedman tests were performed in the software R 4.3.1 [29] using the packages car [30], stats [29], rstatix [31], and ggplot2 [32]. Repeated measures ANOVA was performed using Past 4.03 2020 software [33].

3. Results

3.1. Water quality

Temperature was 28.21 ◦C in RAS and 29.82 ◦C in the BFT System, dissolved oxygen was above 5 mg L−1, and pH was close to 8 in both treatments (Table 1).

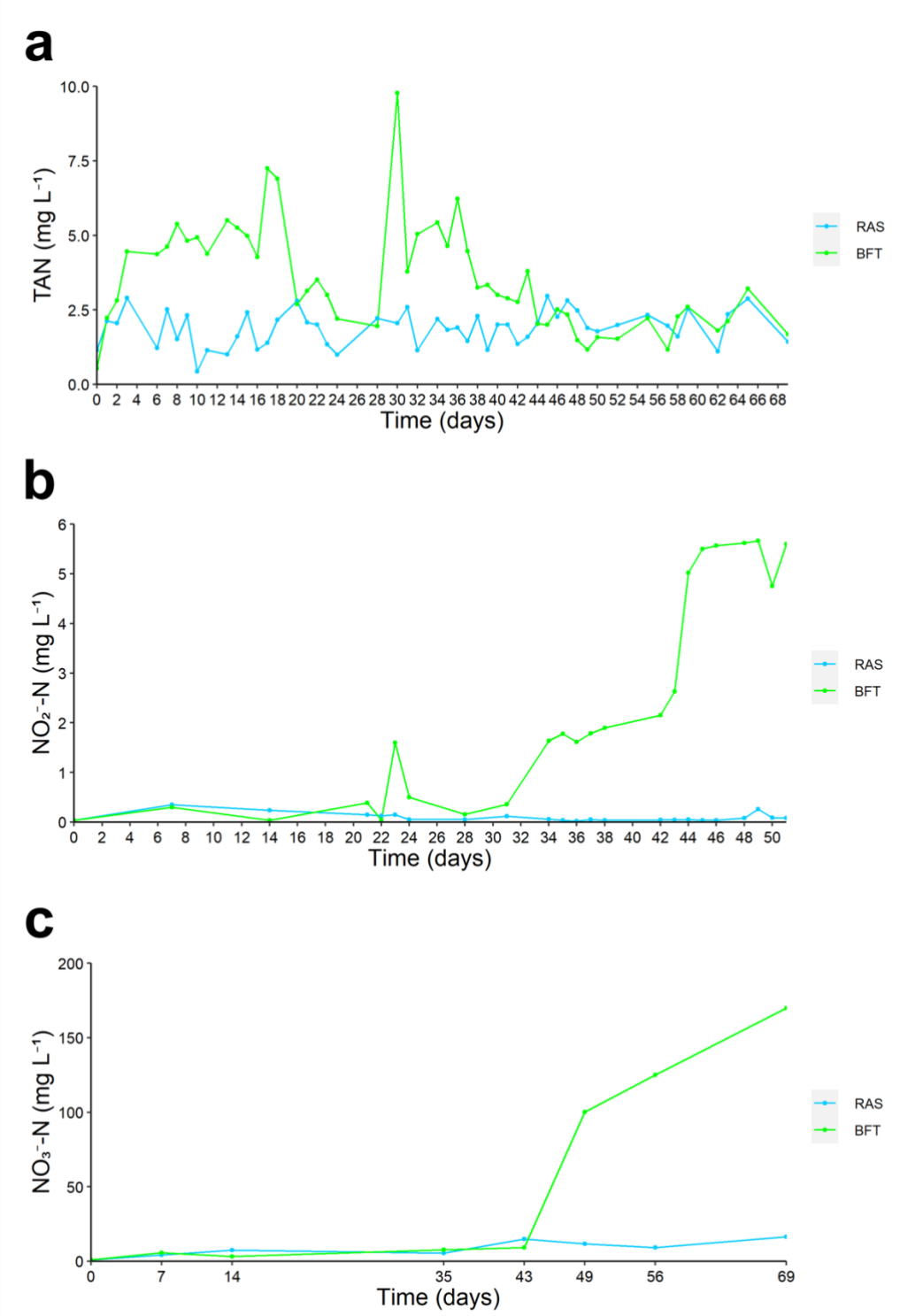
TAN, NO2−-N, and NO3−-N, and turbidity were higher in the BFT treatment than in the RAS treatment (Table 2). The BFT treatment had spikes in TAN concentration on days 17 (7.25 mg L−1) and 30 (9.78 mg L−1), being controlled from day 36 of the trial (Figure 1a). NO2−-N and NO3−-N showed an increasing pattern throughout the experimental time in the BFT, while RAS remained stable (Figure 1b,c).

Table 1. Water quality variables during a Penaeus vannamei super-intensive grow-out with biofloc technology (BFT) and recirculating aquaculture systems (RAS). Different letters in the same line indicate significant differences between treatments ($p < 0.05$).

Variables	Treatments	
	RAS	BFT
Temperature (°C)	28.21 ± 1.12	29.82 ± 0.79
DO (mg L^{-1})	5.75 ± 0.07	5.51 ± 0.16
pH	8.10 ± 0.17	8.11 ± 0.21
TAN (mg L^{-1})	1.89 ± 0.60^{b}	3.52 ± 2.00^{a}
NO_2^--N (mg L^{-1})	0.09 ± 0.11^{b}	2.38 ± 2.23^{a}
NO_3^--N (mg L^{-1})	8.85 ± 5.18^{b}	52.73 ± 65.80^{a}
Alkalinity (mg L^{-1})	166.20 ± 31.17	199.00 ± 34.62
CO_2 (mg L^{-1})	2.17 ± 0.35	2.69 ± 1.28

TSS (mg L^{-1})	31.39 ± 28.75^{b}	217.10 ± 114.95^{a}
SS (mL L^{-1})	0.00 ± 0.00^{b}	14.56 ± 15.28^{a}
Turbidity (NTU)	20.38 ± 17.19^{b}	179.90 ± 104.25^{a}

Data are mean ± standard deviation of values. DO: dissolved oxygen; TAN: total ammonia nitrogen; NO_2^--N: nitrite nitrogen; NO_3^--N: nitrate nitrogen; CO_2: carbon dioxide: TSS: total suspended solids; SS: settleable solids.



2092

2093 **Figure 1.** Concentration of total ammonia nitrogen (TAN, (**a**)), nitrite nitrogen (NO2 −N,
2094 (**b**)), and nitrate nitrogen (NO3−−N, (**c**)) during a *Penaeus vannamei* super-intensive
2095 grow-out with biofloc technology (BFT) and recirculating aquaculture systems (RAS).

The TSS was higher in the BFT treatment, with a tendency to increase throughout the trial (Table 1, Figure 2). The SS was higher in the BFT treatment compared to the RAS (Table 1).

Table 2. *Penaeus vannamei* growth, survival, and water use at the end of a super-intensive grow-out with biofloc technology and recirculating aquaculture systems.

	Treatments	
	RAS	BFT
Initial weight (g)	0.102 ± 0.04	0.102 ± 0.04
Final weight (g)	8.14 ± 1.47^{b}	13.56 ± 1.22^{a}
WGR (g week^{-1})	0.80 ± 0.15^{b}	1.35 ± 0.12^{a}
FCR	2.81 ± 0.49^{a}	1.91 ± 0.12^{b}
Survival (%)	88.00 ± 0.00	83.33 ± 9.24
Yield (Kg m^{-3})	3.58 ± 0.65^{b}	5.62 ± 0.33^{a}
Water use (m^{3} Kg^{-1})	2.13 ± 0.36^{a}	1.82 ± 0.12^{b}

Data are mean ± standard deviation of values. WGR: weekly growth rate; FCR: feed conversion ratio.

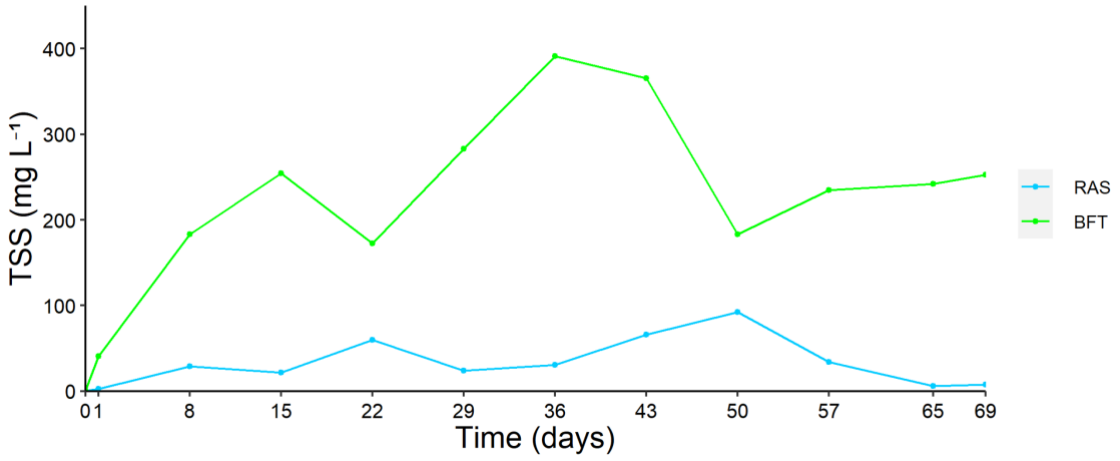


Figure 2. Concentration of total suspended solids (mg L−1) during a *Penaeus vannamei* super-intensive grow-out with biofloc technology (BFT) and recirculating aquaculture systems (RAS).

3.2. Vibrio community composition

At the beginning of the trial and on days 28 and 42, the RAS treatment had more *Vibrio* than BFT (Figure 3). On day 63, BFT had more *Vibrio* than RAS (Figure 3).

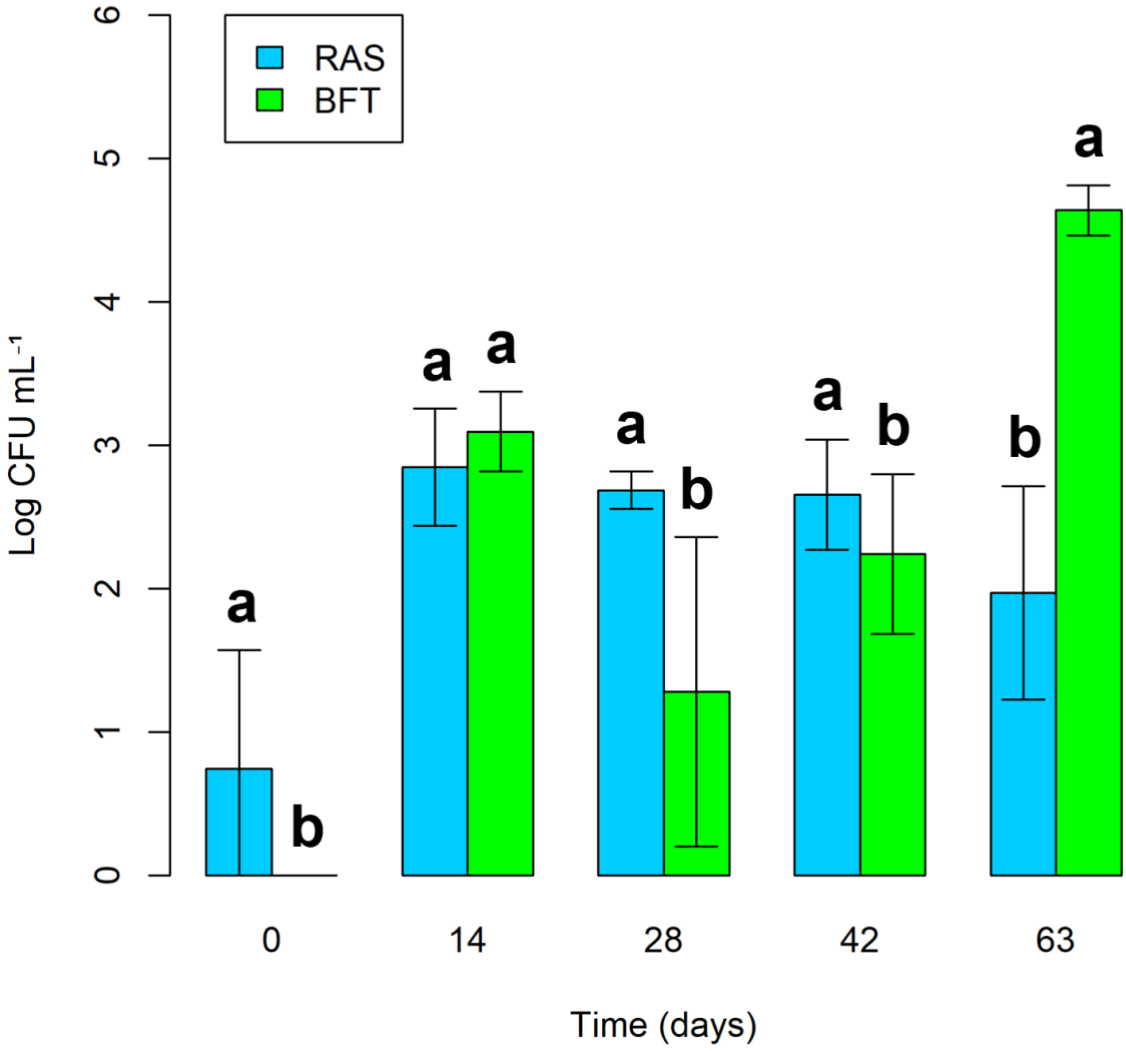


Figure 2. Abundance (Log CFU mL^{-1}) of *Vibrio* spp. during a *Penaeus vannamei* super-intensive grow-out with biofloc technology and recirculating aquaculture systems. Different letters letters indicate significant differences between treatments ($p<0.05$). indicate significant differences between treatments ($p < 0.05$).

3.3. Shrimp growth, survival, and water use

At the end of the trial, final weight, WGR, and yield were higher in the BFT treatment than in the RAS treatment (Table 3). FCR and water use were higher in the RAS treatment than in the BFT treatment (Table 3). Survival was 83.33% in the BFT treatment and 88% in the RAS treatment (Table 2).

Table 3. Price of items considered in the Partial Budget Analysis (PBA) for *Penaeus vannamei* production comparing RAS and BFT systems.

Input	Description	Unit Price ($)	Total cost ($)	
			RAS	BFT
Water	Rate per gallon	3.69	2.92	3.98
Salt	Box 27.2 Kg	50.00	143.77	196.14
Sodium Bicarbonate	Bag 22.68 Kg	34.46	0.07	0.03
Probiotic	500g	104.00	16.97	16.97
Dextrose	997g	18.00	-	6.45
Feed	25Kg	47.79	5.81	6.33
Labor	Wage/hour	12.00	346.00	407.00
Electricity	Rate per kWh	2.49	2,380.20	137.42
Operating interest	5% interest rate	-	144.79	38.72
Equipment	Description	Unit price ($)	RAS	BFT
Aeration pump	50 Watts	413.00	413.00	413.00
Water pump	0.75 HP	443.00	443.00	-
Sump	180 gallons	470.00	470.00	-
KMT media	per cu.ft.	45.00	45.00	-
Mechanical sand filter	Bubble beed filter	352.00	352.00	-
Equipment depreciation	$/year	172.30	32.57	6.16
Production	Description	Unit	RAS	BFT
Final biomass	Experiment yield	G	1,074.07	1,684.97
Sales price ($/kg)	Farmer's market	$/Kg	26.43	26.43
Revenue	From yield	$	28.39	44.58

3.4. Partial Budget Analysis

The PBA considered economic variables such as costs of water, electricity, labor, and other inputs. These data were collected for both systems and allowed the assessment of the economic cost-benefit comparison. The costs of supplies and equipment were quantified for the scale of the experiment based on the local market prices of goods and services. The items utilized to perform the PBA are listed in Table 3.

The Partial Budget Analysis (PBA) findings (Table 4) indicate that the BFT system is more advantageous compared to shrimp production in RAS. The scenario considering a change from RAS to BFT treatment had a positive net benefit cost of $2270.09. The scenario considering a change from BFT to RAS requires a series of equipment and extra costs, especially with electricity, which caused the negative net benefit cost of −$2270.09.

Table 4. Partial budget analysis which compared changing scenarios between RAS to BFT System to produce *Penaeus vannamei.*

Scenarios	Benefits			Costs			Net benefit/ cost
	Additional revenue	Reduced costs	Total additional benefits	Additional costs	Reduced revenue	Total additional costs	
RAS to BFT	16.19	2,375.31	2391.50	121.41	0	121.41	2270.09
BFT to RAS	0	121.41	121.41	2375.31	16.19	2391.50	-2270.09

Values expressed in dollars on a per-cycle basis and experimental scale.

4. Discussion

In this study, the water quality variables were maintained within the recommended ranges for *Penaeus vannamei* culture, as indicated by Ponce-Palafox et al. [34], Gaona et al. [35], Furtado et al. [24], Maicá et al. [36], and Van Wyk et al. [37]. Temperature, which is a crucial factor for shrimp growth and survival, was monitored carefully. *P. vannamei* can tolerate temperatures ranging from 15 to 35 ◦C [37]. In both treatments,

RAS and BFT, the temperature was maintained above 28.21 ◦C, which falls within the optimal range for shrimp growth [34].

RAS treatment provided the best conditions for controlling nitrogenous compounds. This was because of the constant mechanical and biological filtration processes used in this treatment. The presence of artificial substrates in one of the water treatment stages provides an increase in the surface area for the growth of chemoautotrophic bacteria, which are responsible for the transformation of toxic nitrogen compounds [38,39]. Furthermore, backwashing was responsible for discarding most of the nitrate produced in the system and maintaining a low concentration throughout the trial [40]. These results are in line with those found by Ray & Lotz [41], who compared the *P. vannamei* culture in RAS and BFT, using a density of 250 shrimp m−3, and found the highest control of ammonia and nitrite in RAS, and attributed this to the external filtration process.

In the BFT treatment, the nitrification process was observed because TAN concentra- tions were controlled from day 31 of the trial, and nitrate began to increase from day 43. The reduction in nitrite concentrations may not have occurred because nitrite-oxidizing bacteria were not fully established in the system. The observed TAN and nitrite spikes are typical of a newly started system [42]. This behavior was also reported by Ren et al. [43], who observed TAN spikes within the first two weeks of culture and elevated nitrite levels until the end of the seventh week of the trial. Biofloc development in aquaculture tanks requires a certain period until a stable maturity state is achieved [44]. A reliable BFT system is often established 30 days after the initial application of organic carbon to water [45]. Consequently, dangerous spikes in TAN and nitrite commonly occur during the initial weeks of BFT culture [43,46,47].

Under these conditions, management strategies are adopted to maintain the nitroge- nous compound concentration within the appropriate limits for the species being cul- tivated. In contrast, the nitrogen cycling process does not begin to be carried out by chemoautotrophic bacteria. High concentrations of TAN are controlled by manipulating the Carbon:Nitrogen (C:N) ratio of water with the addition of an organic carbon source, which stimulates TAN immobilization through the growth of heterotrophic bacteria [48]. Nitrite concentration can be managed by water changes, which is the most effective strat-

egy for eliminating part of this compound in the absence of the oxidation process of this compound by nitrite-oxidizing bacteria.

The composition of the *Vibrio* sp. community in the water exhibited several distinct pat- terns. It is important to highlight that the presence of these bacteria can be disadvantageous since some species have pathogenic potential and can cause diseases, such as vibriosis in shrimp [49]. Our findings revealed that the abundance of *Vibrio* was higher in the RAS treatment during most of the experiment. The difference in *Vibrio* abundance between the RAS and BFT systems throughout the experiment suggests that the characteristics of the bioflocs may play a significant role in bacterial control, especially in the initial days, with a lower abundance of *Vibrio* in the BFT. According to Decamp & Moriarty [50], the inclusion of *Bacillus* sp. as a probiotic in diets increases the survival of cultured shrimp and reduces the presence of *Vibrio* sp. in the water and sediment of the tank. Ferreira et al. [51] indicated that microbial bioflocs can serve as a source of Gram-positive probiotic bacteria of the genus *Bacillus* and are effective in controlling opportunistic *Vibrio* bacteria. It is essential to adequately monitor and control *Vibrio* communities in water, in addition to implementing good management practices, to ensure the health of cultured shrimp [11,52,53].

Probiotic inoculation contributed to microbial stability in both systems; however, the biofloc environment in the BFT may have enhanced the action of probiotics, providing more effective control of *Vibrio*. In addition to helping control water quality conditions, probiotic inoculation also improves the growth of aquatic organisms [54]. A study by Zokaeifar et al. [55] demonstrated that juvenile *Penaeus vannamei* that received probiotics containing *Bacillus subtilis* strains presented significantly higher values of final weight, weight gain, specific growth rate, and survival compared to systems that did not use probiotics. According to Balcázar et al. [56], probiotic bacteria can reduce or eliminate the incidence of pathogenic microorganisms in the intestine, which is extremely important for the animal's immune system, increasing nutrient absorption and, consequently, improving their performance. The use of probiotics in aquatic organisms has shown positive effects in several experiments and cultivation practices, including the control of bacterial diseases [55,57–59].

In terms of final weight and growth rate (WGR), the RAS system showed inferior performance. This result may be associated with the greater presence of *Vibrio* on days 0, 28, and 42 of the experimental time, despite the daily applications of probiotics. In the BFT system, the presence of microorganisms, combined with the inoculation of probiotics, contributed significantly to the growth of *Penaeus vannamei* [60,61]. In addition, the mi- crobiota in the BFT system, composed of protozoa, rotifers, and other microorganisms, promoted better growth performance for shrimp [62]. These findings contrast with the studies by Ray et al. [63] and Ray & Lotz [41], who compared the intensive cultivation of *P. vannamei* in the RAS and BFT systems, observing superior performance and survival in the RAS system. In our results, we observed that, on certain points, the RAS system presents a higher abundance of *Vibrio*, while on others, the BFT system has higher values. This suggests that the two systems influence the presence of *Vibrio* in different ways over time.

In the BFT system, in addition to the feed provided, the shrimp were able to benefit from the microbial flocs as an additional food source, increasing the culture yield. According to Jory et al. [26] and Tacon et al. [64], bioflocs have high levels of proteins and other essential nutrients that complement the shrimp diet [65]. When analyzing the nutritional influence using the stable isotope technique in the BFT system, it was observed that the microbial community present in the bioflocs was reflected in the tissues of *P. vannamei*, representing a nutritional contribution. These studies corroborate with our results, where the yield was higher in the BFT treatment than in the RAS.

The results obtained in this study revealed a significant difference in water use be- tween RAS and BFT in the super-intensive culture of *Penaeus vannamei*. The BFT system demonstrated superior efficiency, using only 1.82 m3 of water per kilogram of shrimp produced, while RAS required 2.13 m3 of water per kg of shrimp. By using less water per shrimp produced, BFT is more aligned with sustainability principles, as it reduces dependence on this natural resource. This aspect is especially important in a global sce- nario where the demand for aquatic products continuously increases while water resources become scarce due to climate change, urbanization, and population growth.

The overall growth performance was superior in the BFT system compared to RAS. The BFT advantage over RAS can be significantly improved by the addition of micro and nanobubble technology [66] and artificial substrates [67]. These management strategies for aeration and bacteria colonization improve the nitrification process, the microbial com- munity composition, and the growth of *Penaeus vannamei* [66,67]. However, the economic implications of the adoption of micro and nanobubble technology and artificial substrate in super-intensive biofloc systems have never been quantified.

The Partial Budget Analysis (PBA) findings reinforce the advantages of the BFT system over RAS from an economic standpoint. Even though the BFT system presented higher costs with water, salt, dextrose, feed, and labor, the increased productivity of the BFT system yielded larger shrimp, which can potentially access a different market price compared to RAS-produced shrimp. The RAS system's dependence on equipment such as water pumps, sump, KMT media, and sand filters adds challenges to investment capital allocation and increases energy consumption and electricity costs, causing a negative net benefit cost for this system. However, this analysis considered the scale of the experiment, and most of the equipment utilized is over-dimensioned for the experiment's needs. Even though this PBA is limited by the experimental scale and is not representative of commercial farms, this method serves as a tool for short-term financial evaluation and decision-making to improve efficiency and profitability [27]. However, aspects of the long-term viability and sustainability of the production systems should be assessed via a feasibility analysis [28], considering real-world farming conditions.

5. Conclusion

The results of this study indicate that the BFT system is technically and economically more advantageous than shrimp production in RAS, particularly under the specific conditions of the United States of America. The natural productivity from aggregates in BFT provides better zootechnical parameters, offering valuable insights for improving the management of intensive *Penaeus vannamei* culture. The use of probiotics and bioflocs is essential for controlling *Vibrio* sp. and enhancing productive indices.

However, these results are closely tied to the characteristics of local water, climate, and regulations in the U.S., which favor the effectiveness of BFT over RAS. Due

to the delayed maturation of nitri- fiers in BFT, it would be beneficial to initiate the development of stable biofloc before the introduction of shrimp, considering the specific temperature and water quality conditions in the region.

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4. DISCUSSAO GERAL

Esta tese investigou a produção intensiva de *Penaeus vannamei*, com foco na nitrificação, sistemas de aeração, manejo de substratos artificiais, composição microbiana do sistema através de análises de metagenômica e Partial Budget Analysis (PBA), comparando duas abordagens principais: o sistema de bioflocos (BFT) e o de recirculação de aquicultura (RAS). Os resultados obtidos destacam que, além de fatores ambientais, a viabilidade econômica é essencial no manejo desses sistemas. A PBA demonstrou ser uma ferramenta eficaz para avaliar e comparar tecnologias, revelando os benefícios econômicos do uso de substratos, sistemas de aeração avançados e técnicas que otimizam a qualidade da água, contribuindo para maior sustentabilidade e produtividade (Krummenauer et al., 2011; Morais et al., 2020).

No primeiro capítulo, a pesquisa discutiu a relevância do processo de nitrificação no BFT, além de analisar os avanços promovidos pelas tecnologias de aeração que utilizam nano e microbolhas no cultivo superintensivo de camarões. O uso combinado dessas tecnologias resulta em uma melhoria substancial no controle dos compostos nitrogenados, uma das principais dificuldades enfrentadas na aquicultura intensiva. As nano e microbolhas proporcionaram maior oxigenação, promoveram uma comunidade microbiana mais diversa e estável no biofilme e incentivam o crescimento de bactérias oxidantes de nitrito (Lim et al., 2021; Krummenauer et al., 2021). Esse ambiente de cultivo aprimorado se reflete em um aumento significativo no peso final dos camarões e em uma maior taxa de sobrevivência. Ademais, a adoção simultânea dessas tecnologias se mostrou mais eficiente economicamente do que o uso isolado de um único tipo de bolha, indicando que essa inovação não apenas melhora a qualidade ambiental, mas também otimiza os rendimentos econômicos dos produtores.

O segundo capítulo abordou o efeito do manejo de substratos artificiais na qualidade da água e na composição da comunidade microbiana em sistemas BFT aplicados ao cultivo intensivo de *P. vannamei*. Durante a Fase 1 do experimento, a insuficiência de oxigenação levou à formação de áreas hipóxicas no biofilme, prejudicando a atividade das bactérias oxidantes de nitrito e resultando no acúmulo de compostos tóxicos (Souza et al., 2019). Já na Fase 2, as variáveis de qualidade da água

foram adequadas para o cultivo dos camarões, observando-se uma melhora nos processos de nitrificação e na recuperação das populações de bactérias oxidantes de amônia e nitrito (Vlaemick et al., 2010; Ferreira et al., 2016). Além disso, o aumento na abundância de nematoides e rotíferos indicou um desenvolvimento eficiente da alça microbiana, abrangendo fitoplâncton e zooplâncton. Isso sugere que o manejo dos substratos, seja submerso ou exposto ao ar, podem contribuir para a melhoria da qualidade da água e da composição microbiana, resultando em benefícios diretos para o crescimento e a sobrevivência dos camarões.

No terceiro capítulo, os resultados evidenciaram que o sistema BFT apresenta vantagens consideráveis em relação ao RAS, tanto em termos de crescimento dos camarões quanto de uso eficiente da água. O sistema BFT fornece bioflocos ricos em nutrientes e facilita o controle de bactérias patogênicas, como o *Vibrio* sp., por meio da aplicação de probióticos, o que aprimora os indicadores zootécnicos dos camarões (Martins et al., 2011). Embora o RAS seja eficaz no controle de compostos nitrogenados, seu desempenho em termos de crescimento é inferior, possivelmente devido à maior prevalência de *Vibrio* sp. A maior eficiência do BFT no uso de água por quilograma de camarão produzido ressalta sua sustentabilidade, um aspecto crucial diante da crescente escassez de recursos hídricos. Apesar do RAS demandar menos insumos, seus custos elevados de manutenção e energia o tornam economicamente menos competitivo em relação ao BFT. Assim, a superioridade do BFT em termos de produtividade e sustentabilidade destaca esse sistema como a alternativa mais adequada para o cultivo intensivo de *P. vannamei* a longo prazo, proporcionando melhores resultados econômicos e ambientais.

Esses resultados reforçam o papel central das bactérias nitrificantes, das estratégias de manejo e das tecnologias avançadas, como a metagenômica e a PBA, no aprimoramento da aquicultura intensiva. Estudos futuros devem explorar a interação entre diferentes tecnologias e as condições ambientais para maximizar a sustentabilidade e a produtividade no cultivo de P. vannamei.

5. CONCLUSOES

O uso conjunto de nanobolhas e microbolhas demonstraram ser a estratégia de aeração mais eficiente para o cultivo superintensivo de camarão marinho. Este sistema favoreceu o controle dos compostos nitrogenados e estimulou uma maior abundância e diversidade de bactérias benéficas, tanto na água quanto no biofilme, além de otimizar o desenvolvimento das bactérias oxidantes de nitrito. Como resultado, observou-se um crescimento superior dos camarões, com maior peso final, taxa de sobrevivência e produtividade.

As estratégias de manejo de substratos artificiais mostraram-se alternativas viáveis para melhorar a qualidade da água e a composição da comunidade microbiana. A manutenção dos substratos submersos ou expostos ao ar não prejudicou o processo de nitrificação, indicando uma eficiente recuperação microbiana entre os ciclos de cultivo.

Ainda o sistema BFT oferece vantagens significativas em termos de crescimento e eficiência econômica quando comparado ao RAS. Além do controle eficaz de patógenos como *Vibrio* sp., a produtividade natural gerada pelos agregados do BFT proporciona melhores parâmetros zootécnicos e econômicos.

6. PERSPECTIVAS FUTURAS

Futuras pesquisas devem explorar como a salinidade pode afetar o desenvolvimento do sistema de bioflocos (BFT) e a atividade das bactérias nitrificantes. Além disso, é fundamental investigar o impacto da temperatura no desempenho e nas funções dessas bactérias, visando otimizar o manejo dos sistemas BFT em diferentes condições ambientais.

A inclusão da análise metagenômica em estudos futuros seria uma abordagem valiosa para identificar detalhadamente as bactérias presentes nos sistemas de cultivo e suas funções metabólicas. Essa técnica permitiria uma compreensão mais profunda das interações microbianas, especialmente em sistemas de bioflocos, onde a comunidade microbiana desempenha um papel crucial na manutenção da qualidade da água e na reciclagem de nutrientes. A metagenômica também ajudaria a identificar bactérias benéficas e patogênicas, favorecendo a otimização do sistema e o desenvolvimento de estratégias mais sustentáveis e eficientes para a carcinicultura.