

PRISCILLA CELES MACIEL DE LIMA

**CULTIVO INTEGRADO DO CAMARÃO *Litopenaeus vannamei* COM A OSTRÁ
Crassostrea sp. EM SISTEMA DE BIOFLOCOS**

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PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM RECURSOS PESQUEIROS E AQUICULTURA

**CULTIVO INTEGRADO DO CAMARÃO *Litopenaeus vannamei* COM A OSTRA
Crassostrea sp. EM SISTEMA DE BIOFLOCOS**

Priscilla Celes Maciel de Lima

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

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Tese julgada adequada para obtenção do título de doutora em Recursos Pesqueiros e Aquicultura. Defendida e aprovada em 20/01/2022 pela seguinte Banca Examinadora.

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Dedicatória

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Resumo

Atualmente, a aquicultura busca implantar técnicas de produção onde ocorra um melhor aproveitamento dos nutrientes e possibilitem aumento da biosseguridade e da produtividade. Por esse motivo, a aquicultura multitrófica integrada (IMTA) tem sido bastante estudada. Nesse sentido, o objetivo do estudo foi avaliar o uso de *Crassostrea* sp. em sistema multitrófico integrado com o camarão *Litopenaeus vannamei* na fase berçário. Para tal, foram realizados dois experimentos. No primeiro foi avaliada a influência da densidade de *Crassostrea* sp. no sistema multitrófico com camarões *L. vannamei* e no segundo avaliou-se a utilização da recirculação e sedimentação no cultivo multitrófico intensivo de *Crassostrea* sp. com *L. vannamei*. Ambos os experimentos contaram com quatro tratamentos, todos em triplicata. O primeiro experimento foi formado por um controle sem ostras e três tratamentos com ostras nas densidades de 100, 200 e 300 ostras m⁻² (BM-100, BM-200 e BM-300), com duração de 45 dias. No segundo, os tratamentos foram: Monocultura de camarão (controle sem ostras); IMTA (camarões e ostras juntos no mesmo tanque de cultivo); IMTA-R (camarões com ostras em tanque adjacentes com recirculação); e IMTA-RS (camarões com ostras em tanque adjacentes com recirculação e uso de sedimentador acoplado), com duração de 42 dias. Para qualidade da água, no primeiro experimento, os tratamentos com sistemas de bioflocos multitróficos apresentaram os menores valores de sólidos sedimentáveis, resultando em um menor tempo de uso do sedimentador e consumo de água nesses tratamentos, quando comparado ao controle. No entanto, a elevada densidade de ostras (300 ostras m⁻²) elevou os níveis de NAT e N-nitrito, prejudicando assim o desempenho zootécnico dos animais nesse tratamento. Por esse motivo, a sobrevivência das ostras (83,33%) e os valores de peso final (0,94 g), fator de conversão alimentar (1,17) e produtividade (2,26 Kg m⁻³) dos camarões foi significativamente menor no tratamento BM-300. Ainda no primeiro estudo, também observou-se que as ostras podem auxiliar na redução da concentração de *Vibrio* spp., onde os tratamentos com ostras apresentaram menores concentrações quando comparados ao tratamento controle. No segundo experimento, os menores valores de sólidos suspensos totais e sólidos sedimentáveis também foram encontrados nos tratamentos de cultivo multitrófico, mas a retirada excessiva de sólidos, causada pela combinação das ostras com o uso de sedimentador, resultou em maiores os níveis de NAT e N-nitrito no tratamento IMTA-RS. Nesse experimento, o desempenho zootécnico dos camarões (1,59g de peso final e 4,63 kg m⁻³ de produtividade) e das ostras (18,05 g de peso final, 1,25 g de peso de parte mole e 4,69 mm mês⁻¹ de

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Palavras-chave: *Crassostrea*, IMTA, sistema intensivo, *Vibrio*, sólidos.

Abstract

Currently, aquaculture seeks to implement production techniques where there is a better use of nutrients and enable an increase in biosecurity and productivity. For this reason, integrated multitrophic aquaculture (IMTA) has been extensively studied. In this sense, the aim of the study was to evaluate the use of *Crassostrea* sp. in a multitrophic system integrated with *Litopenaeus vannamei* shrimp in the nursery stage, and for this two experiments were carried out. In the first, the influence of the density of *Crassostrea* sp. in the multitrophic system with *L. vannamei* shrimp was evaluated and in the second, the use of recirculation and settling chamber in intensive multitrophic cultivation of *Crassostrea* sp. with *L. vannamei*. Both experiments had four treatments, all in triplicate. The first experiment consisted of a control without oysters and three treatments with oysters at densities of 100, 200 and 300 oysters m⁻² (BM-100, BM-200 and BM-300), with a duration of 45 days. In the second, the treatments were: Shrimp monoculture (control without oysters); IMTA (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tank with recirculation); and IMTA-RS (shrimp and oysters in adjacent tank with recirculation and use of attached settling chamber), lasting 42 days. For water quality, in the first experiment, the treatments with multitrophic biofloc systems showed the lowest value of settleable solids, resulting in a lower use of settling chamber and water consumption in these treatments, when compared to the control. However, the high density of oysters (300 oysters m⁻²) increased the levels of TAN and nitrite-N, thus harming the zootechnical performance of the animals in this treatment. For this reason, the survival of oysters (83.33%) and the values of final weight (0.94 g), feed conversion factor (1.17) and yield (2.26 kg m⁻³) of shrimp were significantly smaller. in the BM-300 treatment. Still in the first study, it was also observed that oysters can help to reduce the concentration of *Vibrio* spp., where treatments with oysters had lower concentrations when compared to the control treatment. In the second, the lower values of total suspended solids and settleable solids were also found in the multitrophic systems treatments, but the excessive removal of solids caused by the combination of oysters and settling chamber use in the IMTA-RS treatment increased the levels of TAN and nitrite-N. In this experiment, the performance of shrimp (1.59g final weight and 4.63 kg m⁻³ yield) and oysters (18.05 g final weight, 1.25 g oyster meat weight and 4.69 mm month⁻¹ of mean growth) was significantly

higher in the IMTA treatment. For the analysis of planktonic community, a significant difference was observed in relation to the cultivation time and between treatments, where the initial samples differed from the others and at 42 days the treatments with *Crassostrea* sp. showed lower abundance compared to the control treatment. Thus, it is recommended the density of 100 and 200 oysters m⁻² and multitrophic culture integrated with the two species in the same production unit, since in this system the oysters are able to help control solids and reduce *Vibrio* spp. in addition to promoting a supplementary food source for shrimp.

Key words: *Crassostrea*, IMTA, intensive system, *Vibrio*, solids.

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1. Introdução

A carcinicultura é o ramo da aquicultura responsável por uma produção de aproximadamente 9,4 milhões de toneladas, correspondendo a US\$ 69,3 bilhões, onde o camarão marinho *Litopenaeus vannamei* a principal espécie, sendo responsável por 52,9% desse total (FAO, 2020). No entanto, a carcinicultura tem apresentado oscilações na produção, associadas com o ciclo de expansão e queda, e uma redução na taxa de crescimento nos últimos anos, principalmente pelo impacto dos surtos de doença de etiologia viral, como a Síndrome da mancha-branca, Mionecrose infecciosa e Necrose infecciosa hipodermal e hematopoiética, e bacteriana, como a Hepatopancreatite necrosante bacteriana e Necrose hepatopancreática aguda (Morales-Covarrubias et al., 2018; Asche et al., 2020; Dey et al., 2020; FAO, 2020).

No Brasil, a carcinicultura é uma atividade econômica muito importante em vários estados. Em 2020, o cultivo dessa espécie atingiu uma produção de 63,2 mil toneladas, registrando crescimento pelo terceiro ano consecutivo, de 11,4%, 18,8% e 14,1% em 2018, 2019 e 2020, respectivamente (IBGE, 2019, 2021a, 2021b), demonstrando uma recuperação às perdas causadas pelas doenças. No país o destaque está na Região Nordeste, responsável por 99,6% da produção nacional, destacando-se os estados Rio Grande do Norte (22 mil toneladas – 34,8%) e Ceará (21 mil toneladas – 33,2%) (IBGE, 2021a).

Após as perdas significativas causadas por patógenos, os sistemas de cultivo intensivo com mínima troca de água somado ao controle das variáveis ambientais e a reutilização da água tornaram-se alternativas promissoras para minimizar as perdas econômicas e aumentar a biossegurança, em função do menor risco da entrada de patógenos (Samocha, 2019).

O sistema de cultivo intensivo com bioflocos baseia-se na manipulação

adequada de microrganismos (bactérias heterotróficas e nitrificantes), a partir de uma relação carbono:nitrogênio (C:N) na água, para uma melhor manutenção da qualidade da água (Emerenciano et al., 2013). A utilização desse sistema possibilita trocas mínimas de água, diminuindo a propagação de doenças e também a emissão de efluentes diretamente no meio ambiente (Krummenauer et al., 2012), e a utilização de altas densidades de estocagem, resultando em uma maior produtividade (Taw, 2010; Crab et al., 2012). Com a adição de carbono as bactérias heterotróficas transformam o nitrogênio amoniacal total, provenientes da ração não consumida e da excreta dos animais cultivados, em biomassa microbiana (Ebeling et al, 2006. Samocha et al., 2019), enquanto que as nitrificantes são responsáveis por converter NAT em nitrito e este em nitrato, mediante ao controle da alcalinidade total (Ebeling et al, 2006).

Recentemente, o uso de prebióticos e probióticos associados a processos de fertilização simbiótica também tem sido alvo de estudos nos cultivos em sistema intensivo, devido à busca por estratégias para promover melhores índices zootécnicos e diminuir a incidência de *Vibrio* spp. através do aumento de bactérias benéficas (*Bacillus* spp.) nos cultivos de peixes e camarões (Romano et al., 2018; Santos et al., 2022). Nesses cultivos, os carboidratos são pré-tratados a partir de processos aeróbicos e/ou anaeróbicos com bactérias probióticas e leveduras (Dawood e Koshio, 2019; Andrade et al., 2021; Pimentel et al., 2022). No pré-tratamento, os microrganismos decompõem os polissacarídeos aumentando assim sua solubilidade em água, o teor de açúcares solúveis totais e proteína bruta, além de reduzir a fibra bruta desses carboidratos (Dawood e Koshio, 2019; Romano et al., 2018).

Os flocos dos sistemas intensivos são macroagregados formados por vários materiais orgânicos, como bactérias, microalgas, zooplâncton, protozoários, nematoides, fungos, sobras de ração e fezes, que podem ser utilizados como fonte suplementar de

alimento para animais aquáticos capazes de coletar e consumir essas partículas em suspensão, como camarões e peixes (Long et al., 2015; Bossier e Ekasari, 2017; Krummenauer et al., 2020).

Embora os flocos microbianos forneçam benefícios à produção, no cultivo intensivo de camarão recomenda-se que os sólidos sedimentáveis e os sólidos suspensos totais sejam mantidos entre 10 - 14 mL L⁻¹ e 250 - 350 mg L⁻¹, respectivamente (Samocha, 2019). Devido às altas densidades de estocagem e redução da troca de água, ocorre o acúmulo de nutrientes, principalmente nitrogênio e fósforo, na água de cultivo em biofoco (Krummenauer et al., 2011), especialmente quando há reuso de água de ciclo anterior (Krummenauer et al., 2014). Na monocultura, os nutrientes fornecidos nos alimentos não são aproveitados de maneira eficiente, uma vez que o camarão *L. vannamei* aproveita apenas 39,1% do nitrogênio e 35% do fósforo que entram no sistema (Silva et al., 2013). Com isso, em sistema de cultivo intensivo com biofoco encontra-se altos níveis de sólidos suspensos totais e voláteis (Vinatea et al., 2010), lodo (Coyle et al., 2011) e demanda química e bioquímica de oxigênio (Mishra et al., 2008). Para que ocorra o controle das quantidades de sólidos suspensos durante o ciclo de produção, pode-se fazer uso de tanques de sedimentação (Ray et al., 2010; Gaona et al., 2016) ou utilizar outras espécies como biofiltros (cultivo multitrófico) (Jones et al., 2001; 2002).

A aquicultura multitrófica integrada é o cultivo de duas ou mais espécies pertencentes a diferentes níveis tróficos em uma unidade de cultivo ou em unidades adjacentes (Angel e Freeman, 2009). No sistema integrado pode-se utilizar peixes (Holanda et al., 2020), moluscos bivalves (Oliveira et al., 2014), macroalgas (Samocha et al., 2015; Brito et al., 2016), e outros organismos, e com este cultivo é possível não só tratar os resíduos dos efluentes gerados pela aquicultura, mitigando os impactos da

carcinicultura intensiva, mas também diversificar a produção, como também, aumentar o retorno econômico e proporcionar a sustentabilidade dos empreendimentos (Soto, 2009; Troell et al., 2009; Barrington et al., 2010; Ahmed e Glaser, 2016). No entanto, é necessária uma combinação apropriada das espécies para a transformação de resíduos em biomassa, já que devem ser levadas em consideração as funções que o organismo desempenhará no cultivo (Barrington et al., 2009). Rocha et al. (2008) encontraram um excelente crescimento do camarão *Litopenaeus vannamei* em função da melhoria da qualidade da água realizada pelas ostras e macroalgas.

Na escolha das espécies há uma preferência pelos moluscos bivalves quando comparados a outros organismos, devida à adaptabilidade destes organismos ao meio (Martínez-Cordova e Martínez-Porchas, 2006). As tentativas para melhorar a qualidade de água do cultivo utilizando bivalves vêm sendo estudadas como parte do desenvolvimento de uma aquicultura integrada, que além de melhorar a qualidade da água (Jones et al., 2001) podem ainda resultar em incremento econômico na produção (Lefebvre et al., 2000).

As ostras são grandes candidatas ao cultivo integrado, melhorando significativamente a qualidade de água dos efluentes dos viveiros de camarão, pois auxiliam na remoção de pequenas partículas em suspensão reduzindo assim sua carga orgânica, visto que as mesmas filtram até 200 litros dia^{-1} (Ward, 1996). Jones et al. (2001) observaram que as ostras reduziram os sólidos solúveis totais de 0,17 para 0,02 mg L^{-1} do efluente do cultivo comercial de *Penaeus japonicus* em sistema tradicional. Além disso, Jones et al. (2002) encontraram que a ostra *Saccostrea commercialis* foi capaz de reduzir a concentração de bactéria (de 12,3 para $4,8 \times 10^9 \text{ L}^{-1}$), clorofila (de 35,6 para $10,7 \mu\text{g L}^{-1}$), nitrogênio total (de 134 para $86 \mu\text{M}$) e fósforo total (de 5,8 para $3,2 \mu\text{M}$) do efluente do cultivo de *P. japonicus* em raceways. Azevedo et al. (2015)

afirmam que a ostra *C. rhizophorae* cultivada em efluente, proveniente do tanque de sedimentação do cultivo de *L. vannamei*, além de melhorar a qualidade da água, utilizaram os nutrientes do meio para o crescimento, apresentando bom crescimento e sobrevivência, similares aos obtidos nos cultivos tradicionais de ostras.

No Brasil o cultivo de ostras desenvolveu-se com base na pesquisa e produção de três espécies de ostras, sendo uma espécie exótica, a *Crassostrea gigas*, e duas espécies nativas, a *Crassostrea brasiliana* e a *Crassostrea rhizophorae* (Poli, 2004; Lazoski et al., 2011; Tureck et al., 2014). Estas espécies são atrativas para o cultivo devido a sua resistência às variáveis de qualidade de água (Dybas, 2014; Funo, 2016; Horodesky et al., 2019), rápido crescimento e rentabilidade (Hernandez, 1998). As ostras são candidatas ao cultivo em sistema intensivo, uma vez que seu crescimento também já foi comprovado em efluentes da aquicultura em sistema intensivo (Lefebvre et al., 2000), e devido ao hábito alimentar filtrador, estes organismos são capazes de aproveitar o alimento natural disponível no ambiente nesse sistema de cultivo (Grant et al., 1995).

Segundo Loosanoff e Tommers (1948) estes organismos podem se alimentar em águas turvas, no entanto há uma redução da filtração com o aumento da quantidade de sólidos do ambiente, e 1 g L^{-1} pode resultar numa redução de 80% na taxa de filtração desses organismos, e assim, reduzindo sua alimentação. As ostras filtram partículas em suspensão, incluindo partículas orgânicas e inorgânicas, fitoplâncton e bactérias, reduzindo a turbidez da água (Ward, 1996; Martínez-Cordova e Martínez-Porchas, 2006). Sendo capazes também de reduzir os níveis de nitrogênio (72%), fósforo (86%) e bactérias patogênicas presentes na água (Jones et al., 2001; Tendencia, 2007).

1.1 Objetivos

1.1.1 Objetivo geral

Avaliar a utilização de ostra *Crassostrea* sp. em sistema multitrófico intensivo com o camarão *Litopenaeus vannamei*.

1.1.2 Objetivos específicos

- Avaliar o efeito da densidade da ostra *Crassostrea* sp. no cultivo multitrófico integrado com *L. vannamei* em sistema intensivo;
- Avaliar diferentes técnicas para o cultivo multitrófico integrado de *L. vannamei* com a ostra *Crassostrea* sp. em sistema intensivo;
- Avaliar a performance produtiva do cultivo multitrófico integrado de *L. vannamei* com a ostra *Crassostrea* sp. em sistema intensivo;
- Avaliar a qualidade da água durante o cultivo multitrófico integrado de *L. vannamei* com a ostra *Crassostrea* sp. em sistema intensivo.

1.2 Hipótese

A ostra *Crassostrea* sp. melhora a qualidade da água do cultivo de *Litopenaeus vannamei* em sistema multitrófico intensivo, além de contribuir para o melhor desempenho zootécnico de juvenis de *L. vannamei*.

2. Artigo científico I

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1 **Effect of stocking density of *Crassostrea* sp. in a multitrophic biofloc system with**
2 ***Litopenaeus vannamei* in nursery**

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Abstract

In recent years, the coculture of organisms from different trophic levels has been practiced to improve water quality and nutrient consumption. In this context, this study evaluated the effect of *Crassostrea* stocking density on zootechnical performance and water quality of *Litopenaeus vannamei* reared in a multitrophic biofloc system during the nursery phase. The experimental design was completely randomized with four treatments: control (shrimp monoculture) and three treatments with oysters (3.21 ± 1.26 cm) at densities of 100, 200 and 300 oyster m^{-2} , for 45 days. Water quality, zootechnical performance parameters of shrimp and oysters, proximate composition of microbial flocs, *Vibrio* count from water, shrimp and oysters samples and total hemocyte counts from shrimp and oyster samples were evaluated. For water quality, multitrophic biofloc system treatments had the lowest amounts of settleable solids, resulting in lower settling chamber use than the control. However, a high density of oysters (300 oysters m^{-2}) increased total ammonia nitrogen and nitrite-N levels, which had maximum values of 4.01 and 4.63 $mg L^{-1}$, respectively. The final average weight and cumulative growth of oysters were $16.23 \pm 3.45g$ and $18.91 \pm 5.24\%$, respectively, corresponding to an average growth of $4.07 \pm 0.34 mm month^{-1}$. In shrimp performance, the higher density treatment (300 oysters m^{-2}) had the lowest values of final weight and yield and the lowest nutritional quality of the microbial floc (14.96% protein and 3.71% lipid). The *Vibrio* count was significantly lower in all the multitrophic biofloc system treatments compared to the control, with an average reduction of 37.32% in water and 75.69% in shrimp hepatopancreas. Stocking densities of up to 200 oysters m^{-2} did not affect shrimp growth in the multitrophic biofloc systems evaluated. The multitrophic biofloc system used in this research is a viable option for controlling nitrogen compounds and solids and reducing *Vibrio* in a shrimp biofloc system during the nursery phase.

42

43 **Keywords:** Shrimp, oyster, IMTA, water quality, *Vibrio* spp.

44

45 **1. Introduction**

46 Shrimp farming has grown at a reduced rate in recent years (FAO, 2018), mainly
47 due to viral outbreaks (White Spot Syndrome Virus and Infectious Myonecrosis Virus)
48 and bacterial disease (Acute Hepatopancreatic Necrosis Disease - AHPND), which have
49 caused significant losses totaling approximately US\$ 15 billion dollars in the production
50 of *Litopenaeus vannamei* (Costa et al, 2009; Shinn et al., 2018; Hostins et al., 2019;
51 Samocha, 2019). *L. vannamei* is the world's most reared crustacean (52.9%), with
52 approximately 4.97 million tons produced in 2018 (FAO, 2020). In Brazil, 45.8
53 thousand tons were farmed in 2018, production grew 11.4% over 2017, indicating a
54 recovery in losses caused by the White Spot Syndrome Virus (IBGE, 2019).

55 Despite increased production, culture systems must employ better nutrient
56 recycling, and have minimal temperature fluctuations and water exchanges. Among the
57 techniques used in aquaculture one that can be highlighted intensive culture systems
58 with minimal water exchange and integrated aquaculture or integrated multitrophic
59 (Dauda, 2019; Hostins et al., 2019; Poli et al., 2019).

60 In the minimum water exchange system, nutrients are continuously recycled by
61 heterotrophic and nitrifying bacteria, and the water can be reused between culture cycles
62 (Emerenciano et al., 2013). This technology promotes better zootechnical performance
63 (Samocha et al., 2017) and an increase in the innate immune response (Kim et al.,
64 2014), while also permitting high shrimp densities, maintaining water quality and
65 greater biosecurity in culture, due to reduced water exchange (Samocha et al., 2017).

66 However, in non-multitrophic biofloc system, high stocking densities and

67 reduced water exchange lead to an accumulation of suspended and settleable solids,
68 nutrients (nitrogen and phosphorus) and heavy metals that may cause a proliferation of
69 *Vibrio* (Samocha et al., 2017). High levels of solids negatively affect shrimp health,
70 causing clogged gills and even mortality (Emerenciano et al., 2013; Hargreaves, 2013).

71 In this context, strategies such as encouraging beneficial bacteria, through the
72 combination of probiotics and prebiotics (Romano et al., 2018), multitrophic culture and
73 recirculating aquaculture system can improve water quality. The use of *Bacillus* sp. as a
74 probiotic can enhance zootechnical performance and resistance to stress (Hoseinifar et
75 al., 2016). The multitrophic culture system is a method for raising two or more species
76 belonging to different trophic levels in the culture unit or adjacent units with
77 recirculating system (Angel and Freeman, 2009; Chopin, 2010; Poli et al., 2019).
78 Culturing more than one species in the same water is often better than monocultures. By
79 the addition of a secondary species and recirculating system it is possible to improve the
80 efficiency of nutrients, maximize the reuse of water and may result in an economic
81 increase in production (Martins et al., 2010; Rosa et al., 2020). Several groups have
82 potential for multitrophic system, such as seaweeds, mollusks, fish and polychaetes.
83 However, a suitable combination of species is necessary to transform waste into
84 biomass, since the functions performed by each organism must be considered
85 (Barrington et al., 2009).

86 Multitrophic culture can consume excess nutrients and organic matter, since the
87 removal of organic and inorganic matter can be done using mollusks (Modesto et al.,
88 2010; Brito et al., 2018; Poli et al., 2019; Omont et al., 2020; Rosa et al., 2020),
89 especially oysters of the genus *Crassostrea* sp., which has potential for use in this type
90 of system (Barrington, et al., 2009). Attempts to improve water quality in culture using
91 bivalves have been studied as part of the development of integrated aquaculture (Jones

92 et al., 2001; Smaal et al., 2019). Oysters are good candidates for a multitrophic biofloc
93 system due to their resistance to poor water quality. Oysters are known as non-specific
94 filter feeders and can significantly improve water quality since they reduce suspended
95 particles, including organic and inorganic particles, phytoplankton and bacteria,
96 reducing water turbidity, as they filter around 200 liters day⁻¹ (Ward, 1996; Martínez-
97 Cordova and Martínez-Porchas, 2006).

98 Despite the wide variety of functions of oysters in traditional systems, their
99 performance in multitrophic biofloc systems is still unknown. For this reason, the
100 objective of this study was to evaluate the stocking density of oysters *Crassostrea* sp. in
101 a multitrophic biofloc system with the shrimp *Litopenaeus vannamei* on the
102 zootechnical performance (oysters and shrimp), water quality, *Vibrio* count and
103 microbial floc proximate composition.

104

105 **2. Material and methods**

106 The study was conducted at the Sustainable Mariculture Laboratory
107 (LAMARSU) of the Fisheries and Aquaculture Department (DEPAq) of the Rural
108 Federal University at Pernambuco (UFRPE), Recife, Brazil.

109

110 *2.1 Preparation of the matrix tank*

111 A matrix tank with water salinity of 30g L⁻¹ was chlorinated with 13 mg L⁻¹
112 (chlorine) and after 72 hours of aeration received inorganic fertilization (single
113 application) with urea (4.5 g m⁻³ N), triple superphosphate (0.3 g m⁻³ P) and sodium
114 silicate (0.23 g m⁻³ Si). After two days, organic fertilization was begun through 12
115 applications of product for 24h in an anaerobic phase followed by an aerobic phase
116 (24h). The organic fertilizer was composed of rice bran (20 g m⁻³), molasses (2 g m⁻³),

117 sodium bicarbonate (4 g m^{-3}) and a bacteria-based product (0.05 g m^{-3}), containing
118 *Bacillus subtilis*, *B. licheniformis*, *Saccharomyces* sp. and *Pseudomonas* sp. at a total of
119 5.5 to $6.5 \times 10^7 \text{ CFU g}^{-1}$ (Kayros Ambiental e Agrícola, Brazil). It was added with a
120 three-day interval between applications. The C-CHO was calculated from the
121 carbohydrate component of rice bran (17.15% wet matter) and molasses (32.23% wet
122 matter), and total nitrogen was determined from the protein content of the rice bran
123 (3.05% wet matter) and molasses (0.06% wet matter) and the nitrogen in the urea (46%
124 N). At the end of fertilization, the water C:N ratio was 4.13.

125

126 2.2 Seed and juvenile oyster survival test at different levels of settleable solids

127 To evaluate the survival of seeds ($0.81 \pm 0.6 \text{ cm}$) and juveniles ($3.55 \pm 1.20 \text{ cm}$)
128 of *Crassostrea* oysters at different concentrations of settleable solids, a previous
129 experiment was conducted over 15 days. The oysters were stocked at a density of 300
130 oysters m^{-2} in tanks with 12 liters of useful volume (30 cm in diameter). Five amounts
131 of settleable solids were tested (0, 2.5, 5, 10 and 20 mL L^{-1}), all in triplicate, in a
132 randomized factorial design. During the test, daily survival and water quality parameters
133 were checked and settleable solids were measured and adjusted.

134

135 2.3 Experimental conditions of multitrophic biofloc culture

136 To evaluate the effect of the density of the oyster *Crassostrea* sp., four
137 treatments were tested, a control (Shrimp monoculture) and three treatments of
138 multitrophic biofloc shrimp and juvenile oyster culture ($3.21 \pm 1.26 \text{ cm}$), at densities of
139 100 oysters m^{-2} (MB-100); 200 oysters m^{-2} (MB-200) and 300 oysters m^{-2} (MB-300), all
140 in triplicate and in a completely randomized design, for 45 days. The *L. vannamei*
141 postlarvae (pL₁₅– $9.64 \pm 0.23 \text{ mg}$) from commercial hatchery (Ocean Vitória, Paraíba,

142 Brazil) were stocked at a density of 2,500 pL's m⁻³ (100 shrimp per experimental unit)
143 in 60 L tank (40 liters useful volume).

144 The experimental units consisted of two compartments: (1) a 60 L tank for
145 shrimp (40 liters useful volume – 0.2 m²) and, (2) a 20 L adjacent tank for oysters (8 L
146 of useful volume – 0.1 m²), both rectangular. The water recirculation was performed by
147 air-lift, from the shrimp tank to the oyster tank and was returned by gravity (Figure 1).
148 Each day, 20% of the shrimp tank volume was renewed, corresponding to 100% of the
149 oyster tank volume with a 24-hour retention time in the tanks. The shrimp culture unit
150 contained a 100 W heater connected to a thermostat to maintain the temperature at 29 ±
151 1 °C and an aeration system with three air stones to keep the flocs in suspension and the
152 oxygen above 5 mg L⁻¹.

153

154 2.4 Management of production units

155 The postlarvae were fed four times a day (at 08:00 a.m., 11:00 a.m., 01:00 p.m. and
156 04:00 p.m.), with a commercial shrimp feed (0.4 to 0.8 mm in diameter) with 45%
157 crude protein and 9.5% lipid (In vivo Animal Nutrition and Health) and the amount of
158 feed was adjusted according to the estimate of consumption and mortality. The organic
159 fertilizer (10 g m⁻³ of rice bran, 1 g m⁻³ of molasses, 2 g m⁻³ of sodium bicarbonate and
160 0.05 g m⁻³ of a bacteria-based product, containing *Bacillus subtilis*, *B. licheniformis*,
161 *Saccharomyces* sp. and *Pseudomonas* sp. in a total of 5.5 to 6.5 x 10⁷ CFU g⁻¹ - Kayros
162 Ambiental e Agrícola, Brazil) was added every three days to the experimental units,
163 following the same preparation methodology described in item 2.1.

164 Sodium bicarbonate was added to maintain alkalinity >120 mg L⁻¹ and pH >7.5,
165 where 0.20 g L⁻¹ increases on average to 0.25 pH and 100 mg L⁻¹ alkalinity (Furtado et
166 al., 2011). No water changes were made during the experiment, only replacement of

167 evaporated water. The concentration stipulated as a limit for settleable solids was 5 mL
 168 L⁻¹, if this concentration was reached, the settling chamber was used. The settling
 169 chamber use (SC) was also evaluated, using the equation: $SC \text{ (h Kg}^{-1}\text{)} = \text{total time of use}$
 170 $\text{of settling chamber (h)} / \text{final shrimp biomass (Kg)}$ (Lima et al., 2019).

171

172 2.5 Water quality

173 Dissolved oxygen, temperature, salinity, pH and total dissolved solids (YSI
 174 model 100, Yellow Springs, Ohio, USA) were monitored twice a day (at 08:00 a.m. and
 175 04:00 p.m.). Total ammonia nitrogen (TAN) (APHA, 2012), nitrite-N (NO₂-N) (Fries,
 176 1971) and alkalinity (CaCO₃) (APHA, 2012), total suspended solids (TSS) (APHA,
 177 1995) and volatile suspended solids (VSS) (APHA, 1995) were measured weekly.
 178 Orthophosphate (APHA, 2012) and nitrate-N (NO₃-N) (APHA, 2012) were measured
 179 every two weeks. Settleable solids (SS) (Imhoff cone) were monitored three times a
 180 week (Avnimelech, 2015).

181

182 2.6 Zootechnical performance

183 Shrimp weight was monitored weekly, from the 15th day of culture, to determine
 184 shrimp growth and adjust the amount of feed offered. At the end of the experiment,
 185 biomass gain, mean final weight, specific growth rate (SGR), feed conversion ratio
 186 (FCR), survival and yield were determined based on the following equations: *Biomass*
 187 $\text{gain (g)} = \text{final biomass (g)} - \text{initial biomass (g)}$; *Final weight (g)} = \text{final biomass}
 188 $\text{(g)/number of individuals at the end of evaluation period}$; $\text{SGR (\% day}^{-1}\text{)} = 100 \times [\ln$
 189 $\text{final weight (g)} - \ln \text{initial weight (g)}] / \text{time (days)}$; $\text{FCR} = \text{feed supplied/biomass gain}$;
 190 $\text{Survival (\%)} = (\text{number of individuals at the end of the evaluation period/initial number}$
 191 $\text{of individuals}) \times 100$; $\text{Yield (kgm}^{-3}\text{)} = \text{final biomass (kg)/volume of experimental unit}$*

192 (m³).

193 During the experiments, oyster weight and length (Galtsoff, 1964) were
194 monitored daily (30% of the population) in each experimental unit. All oysters were
195 counted weekly in each experimental unit for available survival. Cumulative growth
196 (Cg) was calculated using the formula: $Cg (\%) = [(final\ length - initial\ length) / initial$
197 $length] \times 100$.

198

199 2.7 Proximate composition

200 For analysis of proximate composition (crude protein, lipid, ash and fiber) the
201 floc samples (30g) were collected with a cylindrical mesh net of 50 µm for retention of
202 solids, at the beginning and end of the experiment, in triplicate. The samples were
203 analyzed in the Animal Nutrition Laboratory of the Animal Science Department of the
204 Federal Rural University of Pernambuco. Protein was determined by measuring
205 nitrogen (N x 6.25) using the Kjeldahl method (AOAC, 2012), total lipid by hexane
206 extraction using Soxhlet (AOAC, 2012), fiber by acid and alkaline digestion (AOAC,
207 2012) and the moisture content by drying the material in an oven at 105 °C, until
208 reaching a stable weight (AOAC, 2012).

209

210 2.8 *Vibrio* count

211 The sampling to quantify *Vibrio* spp. in the water, shrimp and oysters was
212 performed at the beginning and end of the experiment. For water analysis, 50 mL were
213 collected from each experimental unit, of which 500 µL was diluted in 4.5 mL of
214 peptone water (2%), thus making the dilution 10⁻¹. After homogenization, serial
215 dilutions of 10⁻¹ to 10⁻⁵ were performed.

216 For quantification in animal samples, 10 pL₁₅ (beginning) (Vandenberghe et al.,

217 1999), 0.07g shrimp hepatopancreas (final) and 1 g of oyster meat (beginning and final)
218 were used. The shrimp and oysters were disinfected by immersion in 70% ethanol for
219 15 seconds, followed by immersion for 15 minutes in sodium hypochlorite solution
220 (1.5%) with 0.1% tween-80 and rinsed with sterile distilled water. Subsequently, the
221 biological samples were weighed, macerated and homogenized with a solution of
222 peptone water (2%) at a 1:10 ratio, for a 10^{-1} dilution, and serially diluted from 10^{-1} to
223 10^{-5} (1/10). Finally, a 0.1mL aliquot of the sample dilutions (water, shrimp and oysters)
224 was inoculated in petri dishes containing Thiosulphate Citrate Bile Sucrose (TCBS)
225 agar, in triplicate. The dishes were incubated at 35 °C for 24h for further quantification
226 (CFU mL^{-1} or CFU g^{-1}), classification according to the use of sucrose in the medium
227 (negative sucrose - green colony and positive sucrose - yellow colony) and counted
228 between 30 and 300 colonies using a colony counter.

229

230 *2.9 Total hemocyte count for shrimp and oysters*

231 The oysters' hemolymph (100 μ L) was withdrawn from the posterior adductor
232 muscle at the end of the experiment using a 1 ml syringe. After collecting the
233 hemolymph, the cell density of hemocytes was determined using a hemocytometer.

234 Shrimp hemolymph collection (100 μ L) was conducted at the end of the
235 experiment from the ventral sinus with a 1 ml sterile syringe, with 200 μ l of precooled
236 anticoagulant solution (modified Alsever solution (MAS) (336 mmol L^{-1} NaCl, 115
237 mmol L^{-1} glucose, 27 mmol L^{-1} sodium citrate, 9 mmol L^{-1} EDTA, pH 7.2) in the
238 proportion of 1:2 (v:v). To perform total hemocyte count, triplicates of 0.8 ml of diluted
239 hemolymph were counted for the number of hemocytes using a hemocytometer under a
240 light microscope (Guertler et al., 2013).

241

242 2.10 Statistical analysis

243 A one-way ANOVA was used to analyze production parameters, proximate
244 composition and total hemocyte count, after confirming homoscedasticity (Cochran $p <$
245 0.05) and normality (Shapiro–Wilk $p <$ 0.05). Tukey's test ($p <$ 0.05) was performed to
246 compare and rank mean results from the three treatments and the control. Water quality
247 parameters were analyzed by performing repeated measures ANOVA and Tukey's test
248 ($p <$ 0.05). For non-parametric statistical data (seed and juvenile oyster survival test and
249 *Vibrio* count), the Kruskal–Wallis ($\alpha <$ 0.05) and Dunn tests ($\alpha <$ 0.05) were used to
250 compare and rank median results from the three treatments and the control. The
251 Spearman correlation (ρ) was also applied for the survival test of oyster seeds and
252 juveniles at different levels of settleable solids. Data analyses were performed using
253 Statistica 10 software.

254

255 3. Results

256 3.1 Seed and juvenile oyster survival test at different levels of settleable solids

257 In the survival test, it was observed that the settleable solids have a negative ($\rho =$
258 -0.79) and significant ($p <$ 0.05) correlation with seed and juvenile oyster survival.
259 Higher mortality was associated to higher amounts of settleable solids. For both sizes of
260 oyster, the lowest mortalities were observed at 0, 2.5 and 5 mL L⁻¹ of settleable solids
261 (Figure 2). In addition, survival was also influenced by size, where juveniles were more
262 resistant than seeds to settleable solids.

263

264 3.2 Water quality

265 The water quality parameters are described in Table 1. During the experiment,
266 no significant differences were found between the treatments for temperature ($29.58 \pm$

267 0.21 °C), dissolved oxygen ($5.39 \pm 0.15 \text{ mg L}^{-1}$), pH (7.98 ± 0.06), alkalinity (122.84
268 mg L^{-1}) and orthophosphate ($27.53 \pm 2.07 \text{ mg L}^{-1}$). Differences were found for nitrogen
269 compounds in the treatments with oysters at densities of 100 and 200 oysters m^{-2} , which
270 had lower values than the control and MB-300. The MB-300 had the highest values of
271 these compounds, reaching up to 4.01 and 4.63 mg L^{-1} of TAN and nitrite-N
272 respectively, in the last two weeks of culture (Figure 3).

273 For solids, differences were observed between treatments with oysters compared
274 to the control. The control had higher total suspended solids (431.13 mg L^{-1}), volatile
275 suspended solids (217.49 mg L^{-1}) and settleable solids (13.19 mL L^{-1}). In addition, all
276 treatments had an increase in solids over time, while in the control treatment, the total
277 suspended solids exceeded 350 mg L^{-1} in the fourth week of culture (Figure 4).

278 The control treatment had the longest time of use of the settling chamber
279 ($p < 0.05$) as well as the highest amount of water use per kilogram of shrimp produced
280 (Table 1). Considering the total water replenishment, the control treatment also differs
281 significantly from the others ($1.37 \pm 0.02 \text{ \% day}^{-1}$).

282

283 *3.3 Shrimp and oyster zootechnical performance*

284 At 45 days, significant differences ($p < 0.05$) were observed for the shrimp
285 zootechnical performance, between MB-300 and the other treatments for final weight,
286 FCR and yield (Table 2). For the oysters, the survival rate was higher in the MB-100
287 and MB-200 than in the MB-300. The total shell length and total weight increased and
288 mollusk soft parts did not differ significantly ($p > 0.05$) among the treatments. The
289 oysters had continuous growth during the culture, from 3.21 cm to 3.82 cm, for an
290 average growth of $18.91 \pm 5.24\%$.

291

292 3.4 Proximate composition

293 The average values of the proximate composition of the microbial flocs are
294 summarized in Table 3. The moisture results ranged from 87.71 to 88.96%, protein from
295 13.32 to 20.15%, lipid from 3.43 to 6.33%, fiber from 4.09 to 4.42%, and ash from
296 22.87 to 26.53%. There was a significant difference ($p < 0.05$) between treatments and
297 the lowest levels of protein and lipid were observed in the treatment with the highest
298 density of oysters (MB-300), as well as the highest ash level.

299

300 3.5 *Vibrio* count

301 The initial mean concentration (CFU mL⁻¹) of the *Vibrio* spp. in the water was
302 0.79×10^3 CFU mL⁻¹, with 83.5% negative sucrose colonies (green), however, at the
303 end of the culture there was an increase in the total concentration ($p < 0.05$), to between
304 0.85 and 1.42×10^3 CFU mL⁻¹, but the number of negative sucrose colonies declined to
305 $0.00 - 27.77\%$ (Table 4). The same occurred for mean concentration of *Vibrio* spp.
306 (CFU g⁻¹) in shrimp hepatopancreas and oyster. In addition, at the end of the culture
307 there was a significant difference ($p < 0.05$) between shrimp monoculture and the
308 multitrophic biofloc treatments in the water and shrimp samples, where the treatments
309 with *Crassostrea* sp. had a lower concentration of *Vibrio*, but they did not differ from
310 each other ($p > 0.05$).

311

312 3.6 Total hemocyte count for shrimp and oysters

313 No significant differences were observed for the total hemocyte count, with an
314 average of 33.73 and 2.65×10^6 cells mL⁻¹, for shrimp and oysters, respectively (Figure
315 5).

316

317 **4. Discussion**

318 Even though oysters can feed in turbid waters, it was observed that the level of
319 solids influences the survival of these bivalves, regardless of size, which was confirmed
320 by the negative correlation found between the survival of oysters and the amount of
321 settleable solids. From the results obtained in the survival test at different levels of
322 solids, juvenile oysters (3.5 cm in length) were selected to conduct the experiment in a
323 multitrophic biofloc system.

324 The water quality variables remained within the ideal range for the shrimp
325 culture during the experimental period (Samocha et al., 2017). Due to the application of
326 sodium bicarbonate, fluctuations in pH and alkalinity were not observed during culture
327 and they remained in the ideal range for nitrification ($100 - 150 \text{ mg CaCO}_3 \text{ L}^{-1}$), while
328 these variables tend to decline in intensive systems (Ebeling et al. 2006; Samocha et al.,
329 2017) because of the microbial biomass consumption.

330 The nitrogen compounds remained within the ideal range for the shrimp culture
331 (Samocha et al., 2017), indicating their efficient transformation (Ebeling et al., 2006),
332 using organic fertilization (anaerobic and aerobic) and added bacteria. But in the
333 treatment at a density of $300 \text{ oysters m}^{-2}$, the TAN values in the last weeks were above
334 the ideal range recommended by Lin and Chen (2001) and Samocha (2019), and the
335 nitrite-N and nitrate-N values were also higher. This increase may be the result of the
336 intense filtration rate of oysters in the treatment with greater density, where the higher
337 removal of solids, and consequently of bacteria present in the flocs can harm the
338 nitrification processes, increasing ammonia and nitrite levels (Ebeling et al. 2006;
339 Samocha et al., 2017). The higher density also contributed to an increase in the amount
340 of oyster excreta in the system.

341 Thus, the treatments with lower densities more effectively maintained water

342 quality, and had lower levels of TAN and nitrite-N than the monoculture (Control).
343 Control of these variables is extremely important, especially TAN, as it is toxic and can
344 damage shrimp gills, reduce growth and lead to mortality (Samocha et al., 2017).
345 Ramos et al. (2010) also found that oysters (*Crassostrea rhizophorae*), were able to
346 reduce nitrogen compounds in the effluent from shrimp culture.

347 Intensive systems tend to accumulate solids in their environment due to reduced
348 water exchange, addition of organic carbon, uneaten feed, and fecal matter (Samocha,
349 2019). All treatments showed this increase, but in multitrophic biofloc treatments with
350 *Crassostrea* sp. the total suspended solids (TSS) and settleable solids (SS) were lower
351 than in the shrimp monoculture ($P < 0.05$). In multitrophic biofloc treatments, the solids
352 remained controlled by the filtering action of the mollusks, which can reduce the
353 suspended particles. Oysters have the ability to improve water quality by transforming
354 suspended particulate matter, coagulating small particles into larger, more complex
355 particles, through the aggregation of expelled material (Smaal et al., 2019; Kautsky and
356 Evans, 1987). Furthermore, as observed in this study, Omont et al. (2020), in a
357 multitrophic system, observed that the presence of oysters (*Crassostrea gigas*)
358 decreased settleable solids by half the level found in a traditional shrimp monoculture
359 system. In an intensive system, Ekasari et al. (2014) found that the mussel (*Perna*
360 *viridis*) can consume 36.0 to 57.2 g of TSS per kilogram of animal (wet weight),
361 regardless of the floc size.

362 Particles suspended in water are ingested as food, passing through the gills,
363 which act as a filter, concentrating organic particles, microalgae and planktonic
364 organisms (Ward, 1996; Smaal et al., 2019). For this reason, the lowest value of VSS
365 (organic portion of suspended solids) was found in the MB-300 treatment ($83.76 \pm$
366 10.09 mg L^{-1}), while the highest was found in the control treatment ($217.49 \pm 28.37 \text{ mg}$

367 L⁻¹).

368 Settling chambers were used to control solids, however, there was nonetheless
369 an increase in solids throughout the culture period in all of the treatments (Figure 4). In
370 the shrimp monoculture (Control), these solids accumulated faster than the others,
371 reaching up to 20 mL L⁻¹ in the last week of culture. Consequently, the control
372 treatments had a longer settling chamber use (190.21 ± 10.73 h Kg shrimp⁻¹) and water
373 consumption (235.42 ± 8.34 L Kg shrimp⁻¹). Strategies to reduce suspended particles
374 can efficiently control solids, providing a substantial improvement in the concentration
375 of the other water quality parameters, and consequent improvement in shrimp
376 production (Arantes et al., 2017).

377 The efficiency in conserving culture water can be measured by the amount of
378 water used to produce shrimp biomass (Browdy and Moss, 2005). During culture, there
379 was no water replacement, water was only added to compensate for evaporation losses,
380 resulting in low water use rates (approximately 1 % day⁻¹). In this study, due to the
381 lower level of solids found in the oyster treatments and the final weights, the water
382 consumption in the treatments with 100 and 200 oysters m⁻² was lower than control and
383 MB-300. Which were also lower than reported by Hargreaves (2013) and Samocha
384 (2019) for shrimp nursery in non-multitrophic biofloc system (200 to 400 L Kg shrimp⁻¹).
385 ¹).

386 In relation to shrimp zootechnical performance, all treatments were similar to the
387 results of other culture studies of *L. vannamei* in intensive systems, with final weights
388 between 0.69 -1.08 g, productivity between 1.21 – 2.42 Kg m⁻³, FCR up to 1.37 and
389 survival between 71.3 – 95.6% (Brito et al., 2016; Abreu et al., 2019), with density from
390 2,500 to 3,000 shrimp m⁻³. The multitrophic biofloc system with *Crassostrea* sp. at
391 stocking densities of 100 and 200 oysters m⁻² did not affect the performance of the

392 shrimp, however, the treatment with 300 oysters m^{-2} had the lowest values for final
393 weight (0.94 g) and productivity (2.26 kg m^{-3}), and the highest feed conversion factor
394 (1.17), probably, due to the high values of nitrogen compounds.

395 Oyster survival was greater than 80% in all treatments, even when the settleable
396 solids exceeded the limit established in the pre-test (5 mL L^{-1}). However, density
397 influenced survival and the MB-300 treatment had the highest mortality. Modesto et al.
398 (2010), when culturing oysters (*C. rhizophorae*) in shrimp pond effluents, also found
399 that an increase in stocking density reduced survival, there highest density (750 oysters
400 m^{-2}) had 83.4% mortality, compared to 55.4% at 250 oysters m^{-2} after three months. As
401 for the growth of oysters, proximate composition of particles is important during
402 filtration, thus, the microbial flocs, as the only food source for the oysters in the
403 multitrophic system, could be a limiting factor, as reported by Omont, et al. (2020). In
404 this study, there was no influence of stocking density and the average growth in length
405 was 6.1 mm over 45 days, similar to that reported by Modesto et al. (2010) for *C.*
406 *rhizophorae* culture ($4.3 - 4.4 \text{ mm month}^{-1}$).

407 In terms of the proximate composition of microbial flocs, it was observed that
408 the stocking density of oysters influenced the protein, lipid and ash contents. The lowest
409 levels of protein and lipid were found in the treatment with a density of 300 oysters m^{-2} ,
410 while for ash content the opposite was observed, where the highest value was found in
411 this treatment. The filtration of particles captured, ingested or rejected by mollusks, can
412 affect the quantity and quality of material that is recycled in the water column (Deslous-
413 Paoli et al., 1992; Smaal et al., 2019). This is because the nutritional value of particles
414 from solubilized pseudofeces is lower than that of the original particles, because the
415 organic content is lower than the mineral content (Deslous-Paoli et al., 1992).

416 The microbial flocs in an intensive system have greater fluctuations in their

417 proximate composition, varying according to the carbon source, proximal feed
418 composition, environmental conditions, culture time, and other factors (Emerenciano et
419 al., 2013; Ekasari et al., 2014). *L. vannamei* juveniles are able to satisfy up to 30% of
420 their needs with microbial flocs (Burford et al. 2004), however, these are insufficient for
421 meeting the entire nutritional requirements of shrimps (Marinho et al., 2014). In this
422 study, the values found in treatments, except in MB-300, were close to those found by
423 Abreu et al. (2019) in the *L. vannamei* culture under biofloc system with the addition of
424 *Navicula* sp. and molasses fertilization (17.2 to 22% crude protein and 6.3 to 9.8%
425 lipid).

426 In the intensive system, high densities and quantities of organic matter favor the
427 survival and spread of pathogens, such as *Vibrio* and *Aeromonas* (Yanong and Erlacher-
428 Reid, 2012). The genus *Vibrio* spp. occurs naturally in marine environments, and has
429 also been reported as one of the bacteria present in the intestinal and hepatopancreatic
430 bacterial community of shrimp (Huang et al., 2016; Aguilera-Rivera et al., 2019) and to
431 be predominant in microbial aggregates (floc) (Manan et al., 2017). However, these
432 microorganisms can reduce shrimp growth and survival (Tran et al., 2013). Some
433 species of this genus, microbiologically characterized as negative sucrose, are
434 associated with lethal bacterial infections in aquatic organisms, such as *Vibrio*
435 *vulnificus*, *V. harveyi* and *V. parahaemolyticus* (Thompson et al., 2006; Chatterjee and
436 Haldar, 2013). Colony coloration is due to the ability to consume sucrose from the
437 medium, where *Vibrio* that are able to use sucrose will form yellow colonies (positive
438 sucrose), while strains that are unable will form green colonies (negative sucrose)
439 (Thompson et al., 2006).

440 In this study, the amount of *Vibrio* spp. increased throughout the culture period,
441 due to the increase in solids, however, there was a change in the proportion of groups.

442 In the beginning, most were composed of negative sucrose colonies (59.93 to 83.54%),
443 while in the end, positive sucrose colonies were predominant (62.97 to 100%), this was
444 observed for water, shrimp and oysters. Organic fertilized (anaerobic and aerobic) and
445 the addition of beneficial microorganisms, such as *Bacillus* and *Saccharomyces*, may
446 have suppressed the development of negative sucrose bacteria (*Vibrio* spp.) by specific
447 competitions. The bacteria used in the formulation of probiotics and bioremediators can
448 help control outbreaks of *Vibrio* spp. (Aguilera-Rivera et al., 2014; Hostins et al., 2017),
449 because the addition of beneficial species increases competition for nutrients and
450 substrate (Zorriehzahra et al., 2016).

451 At 45 days of culture, the colony-forming unit in oysters had grown 188.92% to
452 357.00% above the level at the beginning of culture. The microbiota of oyster meat may
453 be directly associated with the environment in which it originates. Since mollusks, as
454 filtering organisms, have the ability to retain particles and concentrate bacteria present
455 in water in their intestine (Vieira et al., 2007), they function as a reservoir of
456 innumerable pathogens (Burge et al., 2016). For this reason, in multitrophic biofloc
457 culture with oysters the concentration of colony-forming units was lower than the
458 control, in both water and the shrimp hepatopancreas, with a reduction of 37.32 and
459 75.69%, respectively. The *Vibrio* genus includes more than 30 species, and at least 12
460 are pathogenic to humans (Thompson et al., 2006), however, there is no legislation that
461 regulates the tolerable levels of *Vibrio* spp. in raw bivalve mollusks, only for *V.*
462 *parahaemolyticus* (1×10^4 per gram), *V. cholerae* (presence of organism) and *V.*
463 *vulnificus* (30 Most Probable Number per gram) (FDA, 2019).

464 Crustaceans and mollusks have only innate immune systems and hemocytes are
465 the main immunocompetent cell in these organisms (Pipe and Coles, 1995;
466 Jiravanichpaisal et al., 2006). In an intensive culture, the microbial community and the

467 bioactive compounds in the flocs can have a positive effect on shrimp health (Xu and
468 Pan, 2013), stimulating the immune system (Kim et al., 2014) and increasing the total
469 hemocyte count.

470 In this study, the multitrophic biofloc system with oysters did not influence the
471 hemocyte count of shrimp, and the average values found in this study were close to
472 those found by Abreu et al. (2019), from 22.84 to 41.25 x 10⁶ cells mL⁻¹, and higher
473 than those found by other authors in the traditional system (12.4 x 10⁶ cells mL⁻¹)
474 (Javahery et al., 2019). Meanwhile, for oysters, there is a high inter-individual
475 variability in the number of circulating hemocytes. In this study the average found was
476 2.65 x 10⁶ cells mL⁻¹, which is close to that found by other authors for healthy oysters of
477 the genus *Crassostrea* sp., 2.50 and 2.52 x 10⁶ cells mL⁻¹, to *C. gigas* and *C.*
478 *hongkongensis*, respectively (Simões et al., 2015; Li et al., 2018).

479

480 **5. Conclusions**

481 This study shows that in the shrimp and oyster multitrophic biofloc system,
482 stocking densities of 100 and 200 oysters m⁻² can be used without compromising shrimp
483 growth, survival and yield. In addition, this is a viable alternative for maintaining
484 nitrogen compounds and controlling solids in a shrimp nursery with a biofloc system.
485 However, high oyster density (300 oysters m⁻²) can reduce water quality and flocs
486 nutritional value due to intense filtration rate. The technology developed in this study
487 creates possibilities for use of multitrophic biofloc shrimp and oyster cultures. Further
488 research is needed on multitrophic biofloc system with oysters at other phases of
489 culture, as well as the commercial application of this system and an economic
490 evaluation.

491

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Figure Captions

Figure 1. Experimental units for multitrophic biofloc treatments with *Crassostrea* sp. and *L. vannamei*. A: Shrimp tank (40L useful volume); B: Oyster juvenile tank (8L useful volume); C: Settling chamber (800 mL useful volume); D: Air supply hose; E: Water return pipe from the settler; F: Water inlet pipe of the settler and; G: Air-lift.

Figure 2. Survival of seeds (A) and juveniles (B) oysters of *Crassostrea* sp. at different concentrations of settleable solids, during 15 days. Error bars indicate the standard deviation.

Figure 3. Variations in the average concentrations of total ammonia nitrogen (A), nitrite-N (B) and nitrate-N (C) in multitrophic biofloc system during 45 days. Error bars indicate the standard deviation. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

Figure 4. Total suspended solids (A), volatile suspended solids (B), settleable solids (C) and settling chamber use (D) in a multitrophic biofloc system over the 45 days. ANOVA followed by Tukey's means comparison test, with a significance level of p of 0.05. Error bars indicate the standard deviation. * - Limit found in the juvenile survival test (5 mL L^{-1}). Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

Figure 5. Total hemocyte count of shrimp (A) and oysters (B), at 45 days in a multitrophic biofloc system. ANOVA followed by Tukey's means comparison test, with a significance level of p of 0.05. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

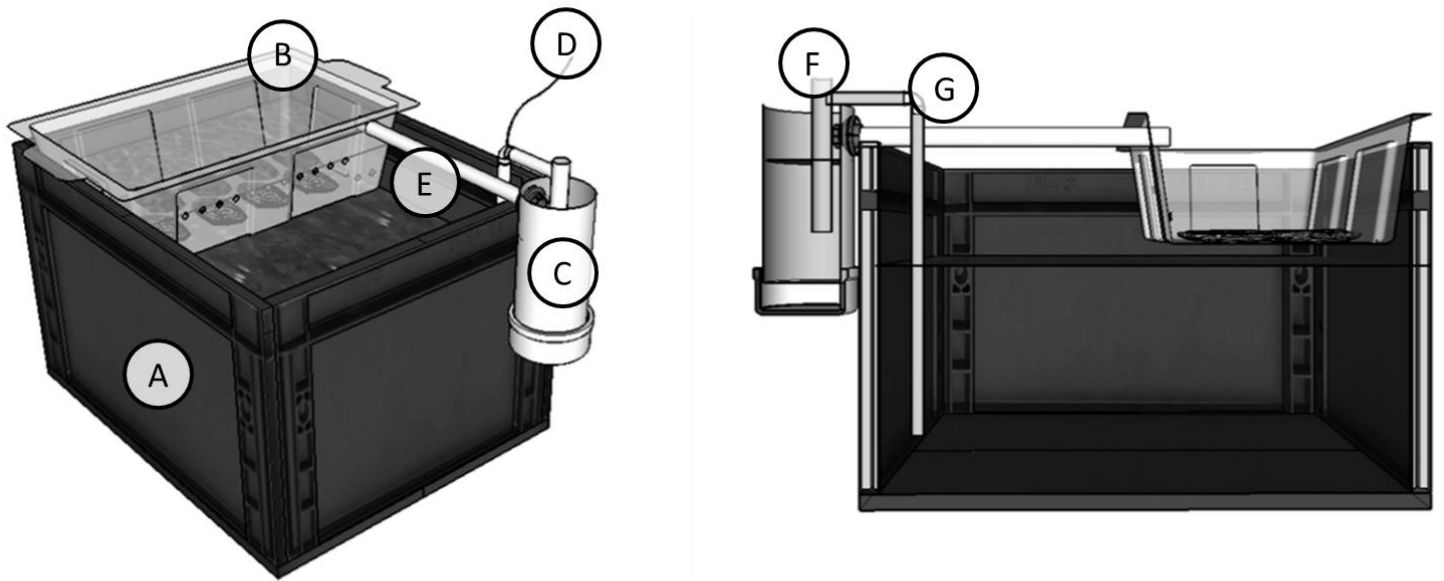


Figure 1.

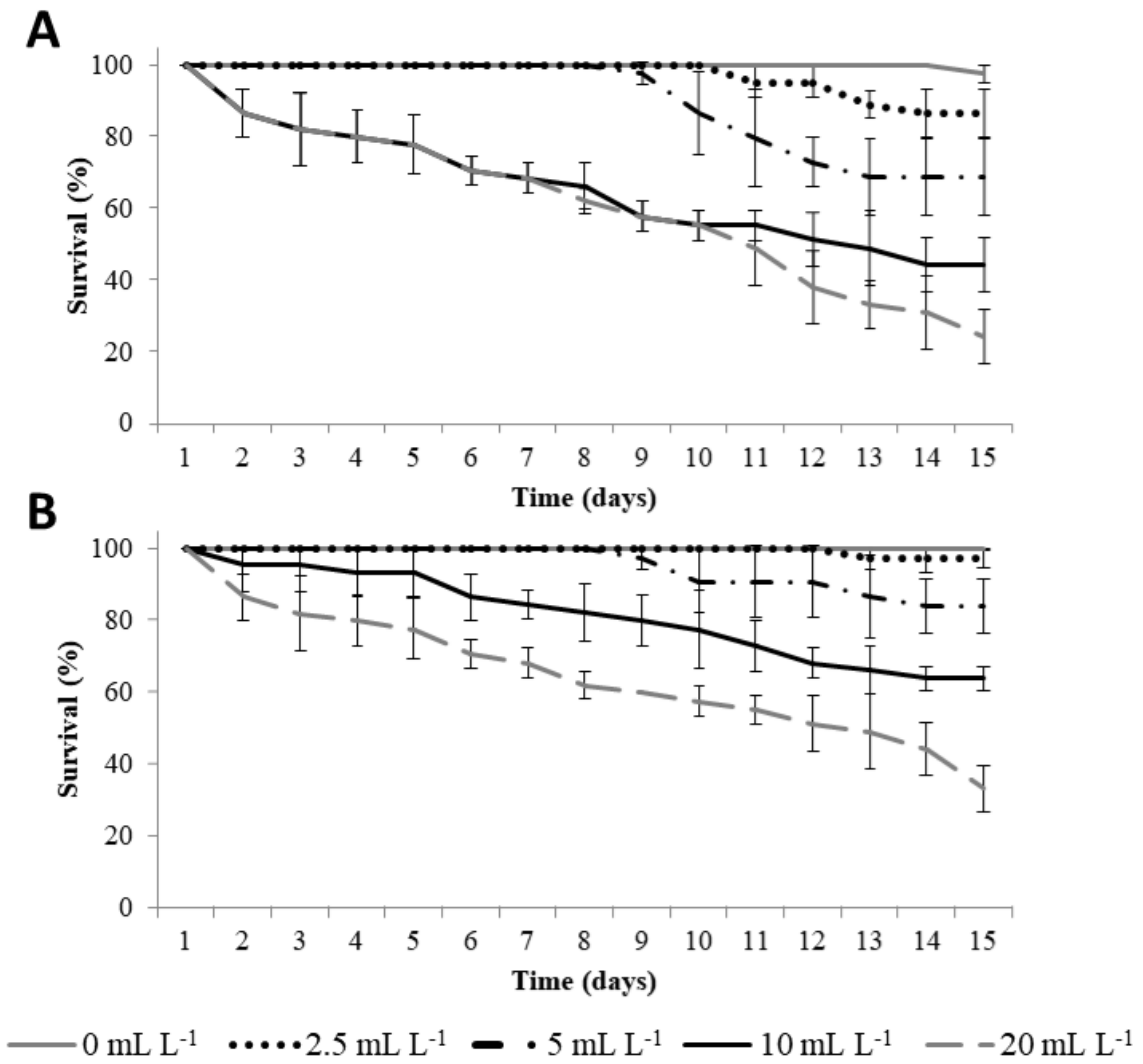


Figure 2.

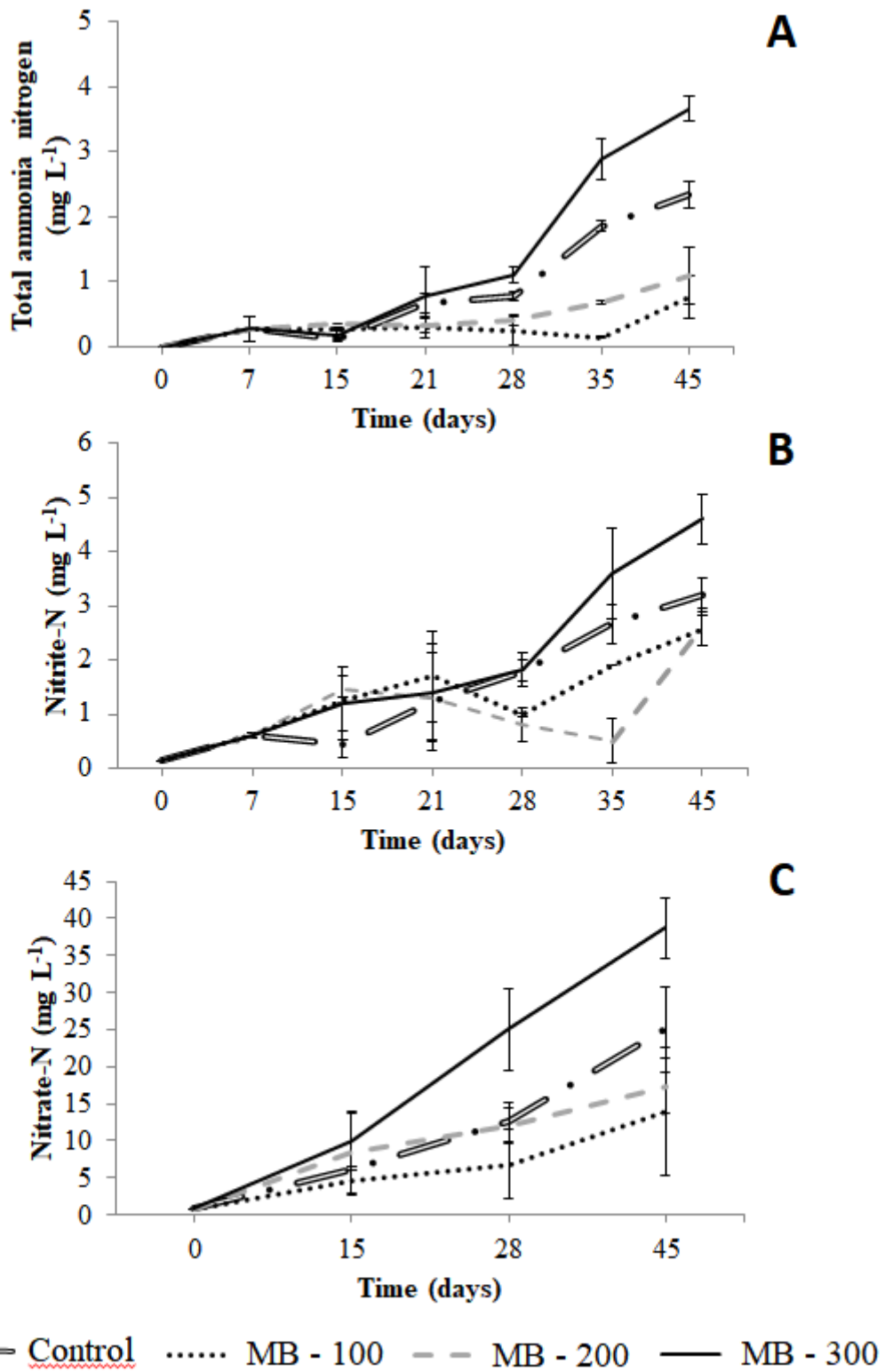


Figure 3.

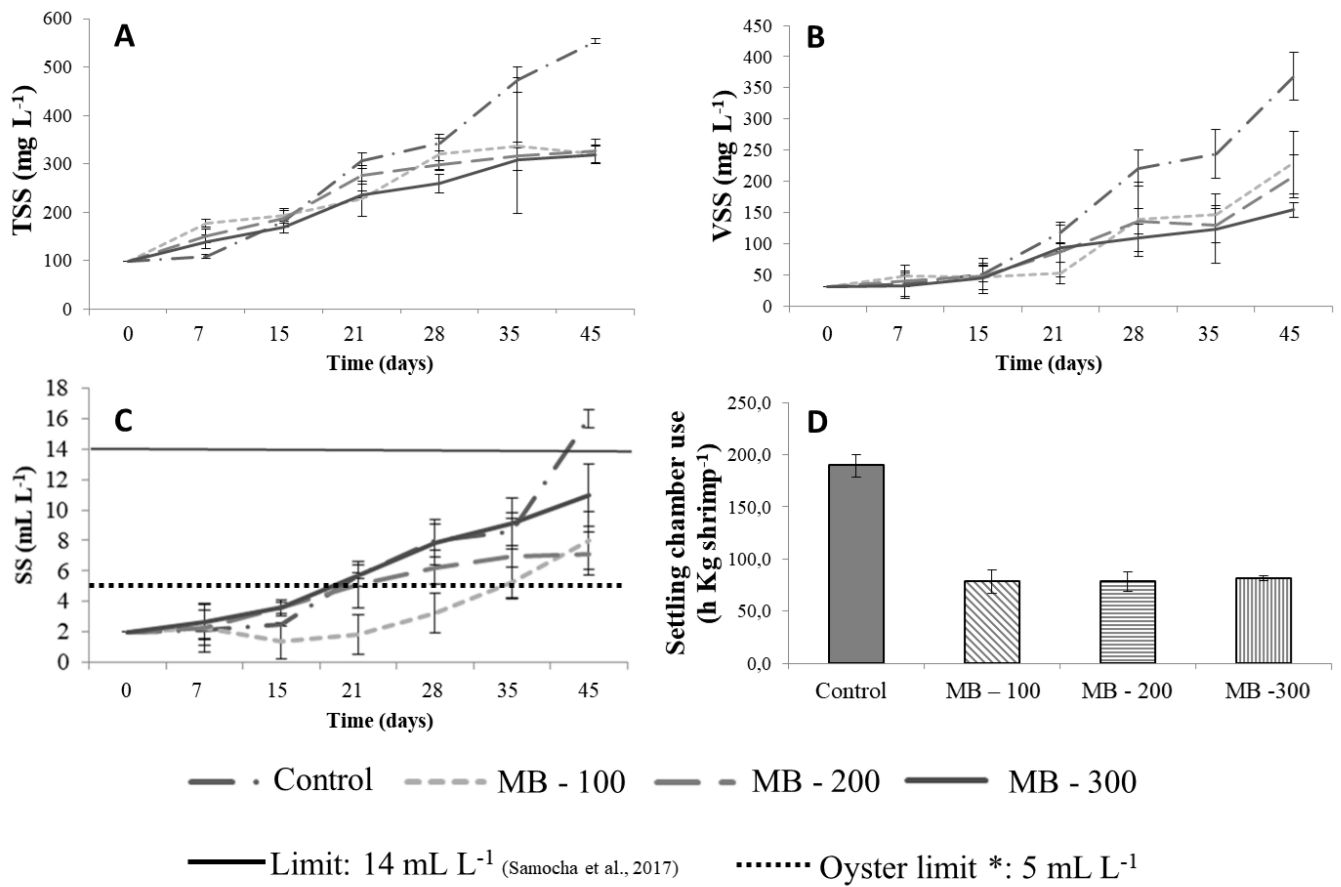


Figure 4.

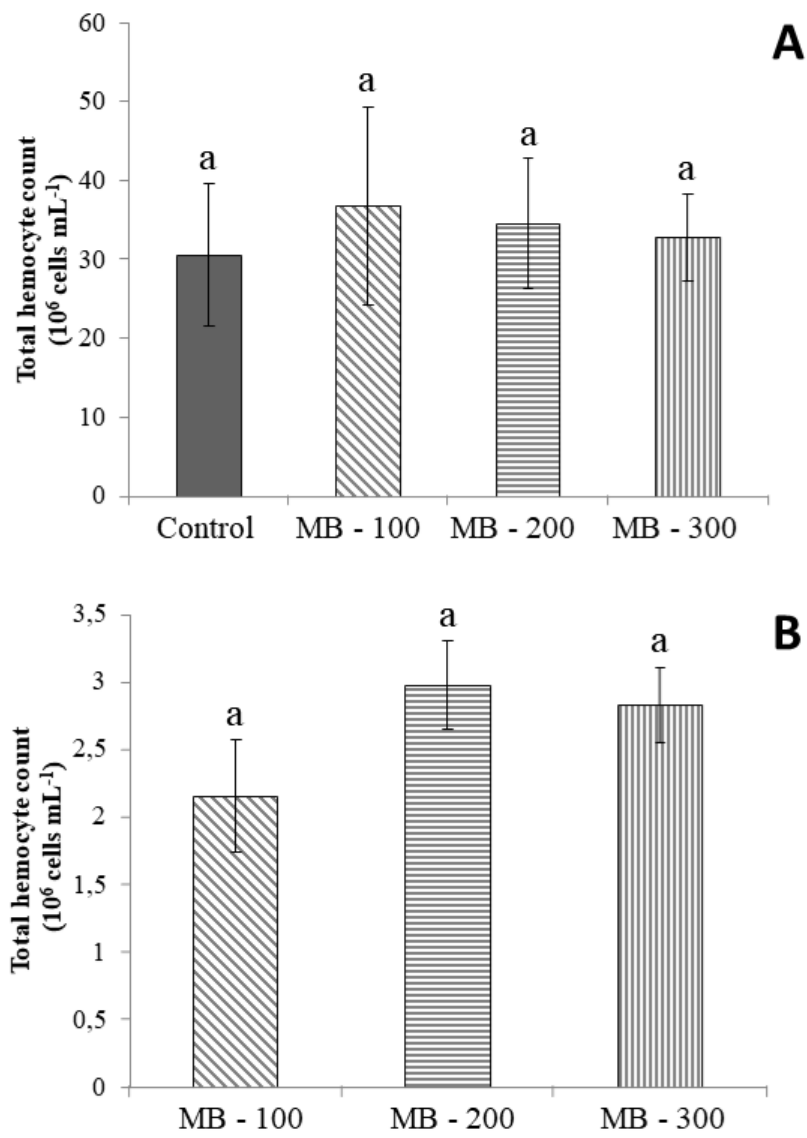


Figure 5.

Table 1. Water quality in multitrophic biofloc culture of *Litopenaeus vannamei* and *Crassostrea* sp., during 45 days.

Variables	Treatments			
	Control	MB -100	MB - 200	MB - 300
Temperature (°C)	29.84 ± 0.12 ^a	29.83±0.09 ^a	29.96±0.10 ^a	29.70±0.15 ^a
DO (mg L ⁻¹)	5.40±0.14 ^a	5.35±0.0.9 ^a	5.33±0.05 ^a	5.37±0.11 ^a
Salinity (g L ⁻¹)	28.86±0.51 ^a	28.19±0.19 ^a	28.59±0.44 ^a	28.55±0.21 ^a
pH	7.94±0.11 ^a	7.89±0.14 ^a	7.99±0.09 ^a	7.87±0.14 ^a
Alkalinity (mg L ⁻¹)	127.33±7.19 ^a	122.61±8.19 ^a	120.67±8.86 ^a	120.75±7.89 ^a
TAN (mg L ⁻¹)	0.66±0.07 ^b	0.35±0.06 ^c	0.44±0.06 ^c	1.50±0.02 ^a
Nitrite-N (mg L ⁻¹)	1.37±0.21 ^b	1.14±0.37 ^c	1.15±0.15 ^c	1.58±0.04 ^a
Nitrate-N (mg L ⁻¹)	11.11±2.24 ^{ab}	7.17±1.31 ^b	8.45±2.49 ^b	22.91±6.60 ^a
Orthophosphate (mg L ⁻¹)	24.00±2.57 ^a	29.02±1.97 ^a	25.35±2.14 ^a	28.12±2.04 ^a
SS (mL L ⁻¹)	13.19±0.55 ^a	6.36±0.44 ^b	6.90±0.53 ^b	6.95±0.52 ^b
TSS (mg L ⁻¹)	431.13±69.87 ^a	309.22±60.67 ^b	312.77±59.10 ^b	306.98±59.10 ^b
VSS (mg L ⁻¹)	217.49±28.37 ^a	107.71±24.22 ^b	108.92±25.67 ^b	83.76±10.09 ^c
Settling chamber use (h Kg shrimp ⁻¹)	190.21 ± 10.73 ^a	79.01 ± 11.27 ^b	78.86 ± 9.29 ^b	82.08 ± 2.30 ^b
Water consumption (L Kg shrimp ⁻¹)	235.42 ± 8.34 ^a	171.08 ± 8.26 ^c	167.77 ± 11.33 ^c	203.38 ± 7.66 ^b
Water replenishment (% day ⁻¹) ¹	1.37 ± 0.02 ^a	1.07 ± 0.05 ^b	1.00 ± 0.10 ^b	1.02 ± 0.11 ^b

The data correspond to the mean of thirty replicates ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly ($p < 0.05$). Results from one-way ANOVA and Tukey's test. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m⁻²); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m⁻²) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m⁻²).

Abbreviations: DO = Dissolved oxygen; TAN = Total ammonia nitrogen; SS = Settleable solids; TSS = Total suspended solids; VSS = Volatile suspended solids. ¹Water replenishment (% day⁻¹) is the percentage of freshwater used to compensate the evaporation losses.

Table 2. Shrimp and oyster performance cultured in nursery multitrophic biofloc system after a 45-day trial.

Parameters	Control	MB -100	MB - 200	MB - 300
Shrimp				
Initial weight (mg)	9.64 ± 0.23 ^a	9.64 ± 0.23 ^a	9.64 ± 0.23 ^a	9.64 ± 0.23 ^a
Final weight (g)	1.13 ± 0.05 ^a	1.18 ± 0.04 ^a	1.17 ± 0.04 ^a	0.94 ± 0.03 ^b
Survival (%)	92.67 ± 2.85 ^a	89.00 ± 1.53 ^a	90.67 ± 1.86 ^a	93.33 ± 1.76 ^a
FCR	1.00 ± 0.05 ^a	1.05 ± 0.03 ^a	1.01 ± 0.04 ^a	1.17 ± 0.03 ^b
SGR (% dia ⁻¹)	10.59 ± 0.11 ^a	10.69 ± 0.08 ^a	10.56 ± 0.07 ^a	10.18 ± 0.17 ^a
Yield(Kg m ⁻³)	2.59 ± 0.13 ^a	2.60 ± 0.08 ^a	2.65 ± 0.09 ^a	2.26 ± 0.05 ^b
Oyster				
Survival (%)	-	93.94 ± 3.03 ^a	96.43 ± 3.57 ^a	83.33 ± 5.52 ^b
Final weight(g)	-	18.69 ± 0.59 ^a	12.66 ± 2.03 ^a	17.35 ± 1.20 ^a
Oyster meat weight (g)	-	1.30 ± 0.09 ^a	0.99 ± 0.41 ^a	1.11 ± 0.03 ^a
Final length (cm)	-	3.81 ± 0.17 ^a	3.77 ± 0.29 ^a	3.87 ± 0.07 ^a
Cumulative growth (%)	-	18.58 ± 5.41 ^a	17.65 ± 1.44 ^a	20.50 ± 2.17 ^a

The data correspond to the mean of thirty replicates ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly ($p < 0.05$). Results from one-way ANOVA and Tukey's test. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m⁻²); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m⁻²) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m⁻²).

Abbreviations: FCR= feed conversion ratio; SGR= specific growth rate.

Table 3. Proximate composition (% dry weight) of microbial floc from multitrophic biofloc system culture of *Litopenaeus vannamei* and *Crassostrea* sp. oyster.

Proximate composition ¹ (%)	Treatments			
	Control	MB -100	MB - 200	MB - 300
Moisture	88.40±0.34 ^a	88.88±0.02 ^a	88.46±0.12 ^a	88.46±0.23 ^a
Crude protein	19.67±0.29 ^a	18.88±0.55 ^a	17.99±0.51 ^b	14.96±0.94 ^c
Lipid	6.14±0.51 ^a	6.06±0.37 ^a	5.68±0.26 ^a	3.71±0.22 ^b
Fiber	4.12±0.94 ^a	4.22±0.55 ^a	4.35±0.47 ^a	4.39±0.95 ^a
Ash	22.03±0.32 ^b	22.01±0.11 ^b	22.97±0.31 ^b	26.15±0.22 ^a

The data correspond to the mean of thirty replicates ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly ($p < 0.05$). Results from one-way ANOVA and Tukey's test. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

¹Except for moisture (%), the other values are expressed in % of dry matter.

Table 4. *Vibrio* count of water, shrimp and oyster samples from multitrophic biofloc system.

	Initial	Final			
		Control	MB - 100	MB - 200	MB - 300
Water (10³ CFU mL⁻¹)					
Positive sucrose	0.13 (16.46%)	1.42 (100%)	0.85 (100%)	0.92 (100%)	0.65 (72.22%)
Negative sucrose	0.66 (83.54%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.25 (27.77%)
<i>Total</i>	<i>0.79</i>	<i>1.42^a</i>	<i>0.85^b</i>	<i>0.92^b</i>	<i>0.90^b</i>
Shrimp (10³ CFU g⁻¹)					
Positive sucrose	0.13 (35.14%)	3.95 (78.06%)	1.21 (93.08%)	1.47 (96.71%)	0.84 (95.45%)
Negative sucrose	0.24 (64.86%)	1.11 (21.94%)	0.09 (6.92%)	0.05 (3.29%)	0.04 (4.55%)
<i>Total</i>	<i>0.37</i>	<i>5.06^a</i>	<i>1.30^b</i>	<i>1.52^b</i>	<i>0.88^b</i>
Oyster (10³ CFU g⁻¹)					
Positive sucrose	1.23 (40.07%)	-	6.87 (62.97%)	6.30 (71.03%)	9.54 (68.00%)
Negative sucrose	1.84 (59.93%)	-	4.04 (37.03%)	2.57 (28.97%)	4.49 (32.00%)
<i>Total</i>	<i>3.07</i>	-	<i>10.91^a</i>	<i>8.87^a</i>	<i>14.03^a</i>

The data correspond to the mean \pm standard deviation of triplicate determinations by treatments. Results from Kruskal-Wallis and Dunn's test. Mean values in the same row with different superscript letters differ significantly ($\alpha < 0.05$).

- : There are no oysters in this treatment.

Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m⁻²); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m⁻²) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m⁻²).

3. Artigo científico II

Artigo científico a ser encaminhado à Aquaculture (ISSN: 0044-8486)

Todas as normas de redação e citação, deste artigo, atendem aquelas estabelecidas pela referida revista.

1 **Effect of recirculating aquaculture system and settling chamber on the integrated**
2 **culture of *Litopenaeus vannamei* and *Crassostrea* sp. in a nursery synbiotic system**

3

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15

16 **Abstract**

17 The objective of this work was evaluating the use of recirculation and settling chamber
18 in synbiotic multitrophic culture of *Crassostrea* sp. with *Litopenaeus vannamei*. Four
19 treatments were tested: shrimp monoculture (control without oysters); IMTA (shrimp
20 and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks
21 with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with
22 recirculation and use of attached settling chamber), in triplicate. Shrimp post-larvae
23 (pL₁₀ - 2.22 ± 0.09 mg) and oyster juveniles (3.12 ± 1.02 cm) of *Crassostrea* sp. were
24 stocked at a density of 3,000 pL m⁻³ and 200 oysters m⁻², respectively, and reared for 42
25 days. For water quality, treatments with multitrophic systems showed lower values of
26 settleable solids, when compared to the control one. However, the excessive removal
27 caused by the combined use of oysters and settling chamber in the IMTA-RS treatment
28 increased the levels of TAN (1.15 mg L⁻¹) and nitrite-N (3.47 mg L⁻¹), differing from
29 the other treatments. At the end of culture, the performance of shrimp (1.59 g final
30 weight and 4.63 kg m⁻³ yield) and oysters (18.05 g final weight) was significantly
31 higher in the IMTA treatment. Regarding the planktonic community analysis,
32 significant difference was observed in relation to the culture time and among treatments
33 (ANOSIM), where the initial samples differed from the others (21st and 42nd days of
34 culture) and at 42 days the treatments with *Crassostrea* sp. showed lower abundance
35 compared to the control treatment, mainly for the Cyanophyta, Euglenophyta and
36 Dinophyta divisions. Thus, the integrated multitrophic culture with the two species in
37 the same productive unit is recommended, since the system promotes a supplementary
38 food source for the shrimp, and oysters are able to help in the control of solids.

39

40 **Keywords:** Oyster, Integrated Multi-Trophic Aquaculture, Solids, Shrimp.

41 **1. Introduction**

42 Currently, aquaculture seeks to implement modern systems that meet the global
43 demand for more efficient production and greater biosecurity, making it necessary to
44 incorporate culture strategies capable of reducing the incidence of diseases and
45 maintaining water quality, by using culture systems with minimal water exchange and
46 better use of nutrients, such as in intensive culture (Samocho, 2019).

47 In intensive culture systems, bacterial processes tend to dominate over algae
48 processes (Avnimelech, 2015), because through the addition of organic carbon,
49 microbial biomass is encouraged to recycle nitrogen compounds, controlling ammonia
50 and nitrite concentrations without the need for large volumes of water exchange, while
51 providing cultured animal with microbial biomass (flocs) (Krummenauer et al., 2020).
52 However, the choice of carbon source can substantially affect the formation and
53 nutritional value of the flocs, depending on their composition and water solubility. For
54 this reason, in recent years, new strategies for the pre-treatment of carbohydrates
55 (synbiotic) have emerged, such as bran, from aerobic (respiration) and/or anaerobic
56 (fermentation) processes with microorganisms (bacteria, probiotics and yeast) (Dawood
57 and Koshio, 2019; Andrade et al., 2021; Da Silva et al., 2021; Lima et al, 2021; Silva et
58 al., 2021; Pimentel et al., 2022).

59 During the preparation of synbiotic fertilizers, microorganisms break down
60 complex carbohydrates (polysaccharides) into simpler molecules, decreasing fiber
61 content and increasing crude protein content and water solubility, thus improving
62 carbon utilization by floc-associated bacteria (Al-Mashhadani, 2019; Dawood and
63 Koshio, 2019; Romano et al., 2018). In addition, this strategy can also reduce the
64 abundance of pathogenic bacteria, such as *Vibrio* spp., through the increase of beneficial
65 bacteria in the environment, e.g. *Bacillus* spp. (Aguilera-Rivera et al., 2014; Andrade et

66 al., 2021).

67 In intensive systems, due to high feed rates, reduced exchange and reuse of
68 culture water, fecal matter and flocs maturation, there is an increase in total solids,
69 dissolved nutrients and heavy metals throughout the culture (Samocha, 2019). The
70 accumulation of solids may have negative effects on shrimp health and performance,
71 cause the proliferation of pathogenic organisms and increase biochemical oxygen
72 demand (Emerenciano et al., 2013; Hargreaves 2013), for this reason, excess solids
73 must be controlled during culture.

74 An alternative for both the control of suspended particles and the optimization of
75 nutrients is the integrated multitrophic culture (IMTA), where there is an integration of
76 two or more species of different trophic levels in a single system (Angel and Freeman,
77 2009; Chopin, 2010). With the addition of a secondary species capable of harnessing
78 these dissolved nutrients, a much larger portion of these resources can be converted to
79 biomass. Furthermore, this concept can improve economic diversity, producing other
80 species with added value (Chopin et al., 2010).

81 Mollusks are the most tested organisms as secondary species in multitrophic
82 cultures, since these organisms are capable of taking advantage of nutrients and filtering
83 suspended particulate organic material (Chopin et al., 2012; Granada et al., 2016; Rosa
84 et al., 2020). Moreover, they are capable of improving water quality and providing an
85 increase in economic value (Ramos et al., 2010; Biswas et al., 2020). Oysters, in
86 particular, are known as non-specific biofilters capable of filtering organic particles and
87 converting them into biomass (Jones et al., 2001; Dué et al., 2010; Omont et al., 2020).
88 In addition, their growth has been proven in intensive culture (Lima et al., 2021) and in
89 effluents (Costa et al., 2021).

90 However, few studies are available on synbiotic multitrophic culture with

91 *Crassostrea* sp. For this reason, the aim of the present study was to evaluate the use of
92 this species in intensive multitrophic culture with *Litopenaeus vannamei* shrimp, and
93 the zootechnical performance of oysters and shrimp and water quality, in a nursery
94 synbiotic system and using settling chamber to assist in the management of solids.

95

96 **2. Material and methods**

97 The study was conducted at the Sustainable Mariculture Laboratory -
98 LAMARSU of the Fisheries and Aquaculture Department - DEPAq of the Federal Rural
99 University of Pernambuco, Brazil.

100

101 *2.1 Preparation of the matrix tank*

102 In a matrix tank (1.2 m³), the water (30g L⁻¹) was chlorinated with 13 mg L⁻¹ of
103 active chlorine. Then, a single inorganic fertilization was carried out using urea (4.5 g
104 m⁻³ N), triple superphosphate (0.30 g m⁻³ P) and sodium silicate (0.23 g m⁻³ Si). Organic
105 fertilization was carried through ten applications of a synbiotic fertilizer composed of
106 rice bran (30 g m⁻³), molasses (3 g m⁻³), sodium bicarbonate (6 g m⁻³), a bacteria-based
107 product (0.5 g m⁻³), containing *Bacillus subtilis*, *B. licheniformis*, *Saccharomyces* sp.
108 and *Pseudomonas* sp. at a total of 5.5 to 6.5 x 10⁷ CFU g⁻¹ (Kayros Ambiental and
109 Agrícola, Brazil), and marine water (30 g L⁻¹) in the proportion of 1:10 (m:v) of the
110 amount of the rice bran, following a protocol adapted from Lima et al. (2021). The
111 synbiotic fertilizer was prepared with 24h in an anaerobic phase, followed by an aerobic
112 phase (24h) and added every three days. During initial fertilization, the diatom
113 *Chaetoceros calcitrans* was inoculated weekly, at a concentration of 10 × 10⁴ cells mL⁻¹
114 ¹, corresponding to an addition of approximately 250 mL of microalgae culture per
115 experimental unit.

116 At the end of synbiotic fertilization, the total organic carbon:nitrogen ratio in
117 water ratio was 6.66. To calculate the ratio, carbon to carbohydrate (C-CHO) was
118 calculated from the carbohydrate of rice bran (46.24% of dry matter) and molasses
119 (98.74% of dry matter), and total nitrogen was determined from the protein content of
120 rice bran (21.62% dry matter) and molasses (0.44% dry matter). Three days before
121 stocking the animals, water from the matrix tank (TAN 0.57 mg L⁻¹, N-NO₂ 0.88 mg L⁻¹,
122 N-NO₃ 0.65 mg L⁻¹, alkalinity 159.31 mg L⁻¹, pH 7.88, orthophosphate 1.29 mg L⁻¹,
123 total suspended solids 112.80 mg L⁻¹, settleable solids 3.1 mL L⁻¹) was homogenized
124 and distributed equally to fill the experimental units (40 L).

125

126 2.2 Experimental conditions

127 In order to evaluate the culture of shrimp with *Crassostrea* sp., four treatments
128 were tested, a shrimp monoculture (control without oysters) and three IMTA treatments
129 of shrimp and oyster juveniles in different culture systems: IMTA (shrimp and oysters
130 together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with
131 recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation
132 and use of attached settling chamber), in triplicate and completely randomized design,
133 for 42 days.

134 Juveniles of *Crassostrea* sp. were collected in a natural environment
135 (Mamanguape, Paraíba, Brazil) and transported to LAMARSU, where they were
136 acclimated to laboratory conditions for 10 days. During this period, the oysters were
137 kept in marine water tanks (350 L - 30g L⁻¹), at a density of 200 oysters m⁻², with
138 constant aeration and fed daily with effluent from matrix tank, equivalent to 5% of the
139 tank volume. After acclimatization, they were stocked in the experimental units at a
140 density of 200 oysters m⁻² (3.12 ± 1.02 cm). Post-larvae of *L. vannamei* (pL₁₀ - 2.22 ±

141 0.09 mg) from a commercial larviculture (Aquasul, Rio Grande do Norte, Brazil), were
142 stocked at a density of 3,000 pL's m^{-3} in the experimental units with useful volume of 40
143 liters ($0.04 m^3$), totaling 120 shrimp per experimental unit.

144 The experimental units consisted of a 40 L tank of useful volume (50cm x 35cm
145 x 30cm) where the shrimp were stocked, and in the IMTA-RS and IMTA-R treatments
146 another tank of 8 L of useful volume (30cm x 34cm x 15cm) was used for oysters (Fig.
147 1). In these treatments, water recirculation was carried out through an air-lift. In the
148 IMTA-R treatment, water from the shrimp tank went to the oyster tank and returned by
149 gravity (Fig. 1C, 1D), while in the IMTA-RS treatment water from the shrimp tank was
150 first sent to the settling chamber, coupled to the experimental unit with a capacity of
151 800 mL, before going to the oyster tank (Fig. 1E, 1F). The recirculation ratio was 10%
152 of the shrimp tank volume per day, at a flow rate of $1 L h^{-1}$, corresponding to 50% of the
153 oyster tank, and a retention time in the oyster tanks of 24h. The shrimp culture units
154 contained a 100 W heater connected to a thermostat to maintain the temperature at $30 \pm$
155 $1^\circ C$, an aeration system consisting of three air stones (diameter 24 mm) and covered by
156 a plastic mesh with 2 mm mesh to prevent shrimp escape.

157

158 *2.3 Management of experimental units*

159 The shrimps were fed four times a day (8 a.m., 11 a.m., 2 p.m. and 5 p.m.) with
160 commercial feed (45% crude protein, 9.5% lipids, 13% moisture, 9.5% crude fiber and
161 4, 0% ash – In vivo Animal Nutrition and Health Ltda.) and the amount of feed was
162 adjusted according to the estimate of consumption and mortality. For the maintenance
163 of fertilization, the synbiotic fertilizer ($15 g m^{-3}$ of rice bran, $1.5 g m^{-3}$ of molasses, 3 g
164 m^{-3} of sodium bicarbonate and $0.5 g m^{-3}$ of the bacteria-based product) was added every
165 three days, following the preparation methodology described in the fertilization of the

166 matrix tank (item 2.2).

167 No water changes took place during the experiment, only the addition to replace
168 water lost through evaporation. Water consumption and daily replacement rate were
169 calculated using the equations: Water consumption (L Kg shrimp^{-1}) = *Total water used*
170 (L) / *final shrimp biomass* (kg) (Lima et al., 2018; Lima et al., 2019); Water
171 replenishment ($\% \text{ day}^{-1}$) = [$(\text{Total water (L)} / \text{Useful volume of the experimental unit}$
172 (L)) / *Culture time* (days)] * 100. Settling chambers were used to control settleable
173 solids, except for IMTA-RS treatment, where these were used daily (4 h day^{-1}). For the
174 other treatments, the use of the settling chamber was evaluated through the equation:
175 Settling chamber use (h Kg shrimp^{-1}) = *total time of use of settling chamber* (h) / *final*
176 *shrimp biomass* (kg) (Lima et al., 2019). In addition, sodium bicarbonate was added to
177 maintain alkalinity $> 150 \text{ mg L}^{-1}$ and $\text{pH} > 7.5$, according to Furtado et al. (2011), where
178 0.20 g L^{-1} increases on average 0.25 pH and 100 mg L^{-1} alkalinity.

179

180 2.4 Water quality

181 Temperature, salinity, dissolved oxygen and pH were monitored twice a day (at
182 8:00 a.m. and 4:00 p.m.) with a YSI 556 multiparameter probe (Yellow Springs
183 Incorporated, Ohio, USA). Oxidation-reduction potential (ORP) and settleable solids
184 (SS) were monitored three times a week using a probe (model ORP-619, YIERYI,
185 Guangdong, China) and an Imhoff cone (Avnimelech, 2015), respectively. Total
186 ammonia nitrogen (TAN) (APHA, 2012), nitrite-N (FRIES, 1971) and alkalinity
187 (APHA, 2012) were measured weekly, while nitrate-N (APHA, 2012) and
188 orthophosphate (APHA, 2012) were measured every two weeks. Total suspended solids
189 (TSS) and volatile suspended solids (VSS) were also analyzed weekly according to
190 APHA (2012), using a $0.47 \mu\text{m}$ filter.

191

192 *2.5 Zootechnical performance of shrimp and oysters*

193 The zootechnical performance of shrimps was evaluated through biometrics,
194 from the 15th day of culture, to determine shrimp growth and adjust the amount of feed
195 offered. On 42nd culture day, mean final weight, survival, feed conversion ratio (FCR),
196 specific growth rate (SGR) and yield were determined based on the following
197 equations: Final weight (g) = *final biomass* (g) / *number of individuals at the end of*
198 *evaluation period*; Survival (%) = (*number of individuals at the end of evaluation*
199 *period* / *initial number of stored individuals*) x 100; Feed conversion factor = *feed*
200 *supplied* / *biomass gain*; Specific growth rate (% day⁻¹) = 100 x [(*ln final weight* (g) - *ln*
201 *initial weight* (g) / *time* (days)]; Yield (Kg m⁻³) = *final biomass* (kg) / *volume of*
202 *experimental unit* (m³).

203 During the experiment, all oysters from the experimental units were counted
204 daily to assess mortality. The growth was evaluated from weekly biometrics of 50% of
205 the population from each experimental unit (randomly sampled), when the length
206 (Galtsoff, 1964) and the weight of the oysters were measured. At the end of culture,
207 after recording the length and weight of the oysters, the soft tissue was removed and its
208 wet weight recorded. Later, cumulative growth (Cg) and monthly growth (Mg) were
209 calculated using the formulae: Cg (%) = [(*Final length* - *Initial length*) / *Initial length*]
210 × 100 (El-Wazzan and Scarpa, 2009); and Mg (mm month⁻¹) = (*Final length* - *Initial*
211 *length*) / *time* (months).

212

213 *2.6 Presumptive diagnosis of oysters*

214 At the end of the experiment, ten oysters were dissected. The valves were
215 opened using a sterile spatula, sterile scalpel and scissors, and the gills and the mantle

216 were removed to observe the degree of stomach fullness of the oysters. These were
217 classified, according to their fullness degree, as “empty”, “almost empty”, “almost full”
218 and “full” according to the methodology from Dué (2010). Then, the gills removed from
219 the ten oysters were observed under a microscope to verify changes and accumulation
220 of solids.

221

222 *2.7 Total hemocyte count for shrimp and oysters*

223 Shrimp hemolymph (100 μL) was collected on 42 days of the experiment,
224 through the hemocele of the abdominal region with a 1-mL syringe containing 200 μL
225 of precooled anticoagulant modified Alsever solution (MAS - 336 mmol L^{-1} NaCl, 115
226 mmol L^{-1} glucose, 27 mmol L^{-1} sodium citrate, 9 mmol L^{-1} EDTA, pH 7.2), in a 1:2
227 ratio (hemolymph:anticoagulant). For the total count of shrimp hemocytes, the number
228 of cells was estimated using a hemocytometer under a light microscope, in triplicate
229 (Guertler et al., 2013).

230 The oysters' hemolymph (100 μL) was withdrawn at the end of the experiment,
231 close to the adductor muscle using a cooled sterile syringe (1 mL), to prevent hemocytic
232 aggregation. After collecting the hemolymph, the cell density of hemocytes was
233 determined using a hemocytometer in triplicate.

234

235 *2.8 Monitoring of phytoplankton and zooplankton communities*

236 Water samples (500 mL) were collected from each experimental unit, close to
237 the aeration, on the 1st, 21st and 42nd days of culture. The collected volume was filtered
238 through a cylindrical net with 250, 125 and 70 μm cylindrical mesh to reduce suspended
239 solids in the sample, then they were filtered through a 50 μm mesh to retain the
240 zooplankton, and a 15 μm mesh for phytoplankton retention, and concentrate to 25 mL.

241 Then, a 2.5 mL aliquot was fixed in 4% formalin and stored for further analysis. For
242 identification and quantification, a Sedgewick-Rafter chamber and binocular optical
243 microscope, with a 1,000x magnification were used. The organisms were identified
244 based on cell morphology at the genus level with the aid of identification keys for
245 phytoplankton (Bicudo and Menezes, 2006; Hoek et al., 1995) and zooplankton
246 (Bradford-Grieve et al., 1999; Foissner et al., 1999). Phytoplankton abundance was
247 expressed in cells per milliliter (cells mL^{-1}) following the methodology described by
248 Hötzel and Croome (1999), and zooplankton was expressed in organisms per milliliter
249 (org mL^{-1}) following the methodology described in APHA (2012).

250

251 2.9 Statistical analyses

252 Statistical analyses were performed using the Statistica 10 software (StatSoft).
253 Data were checked for homogeneity of variances using the Cochran test and normality
254 using the Shapiro-Wilk test. For normal and homogeneous data, parametric one-way
255 ANOVA (zootechnical performance and total hemocyte count) and ANOVA of
256 repeated measures (water quality) were used, followed by Tukey's mean comparison
257 test ($p \leq 0.05$), when differences were observed. For non-normal data (planktonic
258 community), the Kruskal-Wallis test ($\alpha < 0.05$) was applied, followed by the Dunn test
259 ($\alpha < 0.05$).

260 The phytoplankton and zooplankton communities were analyzed using the
261 PRIMER 6.0 program, with previously logarithm transformed ($\log(x + 1)$) data for
262 analysis of similarity (two-factor ANOSIM) ($p < 0.05$) with 999 permutations to
263 identify differences within and between groups in relation to species abundance (Clarke,
264 1993). Cluster analysis (Bray-Curtis similarity) was used to determine similarities in the
265 temporal and spatial scales and to identify the possible formation of groups, and

266 similarity percentage analysis (SIMPER) was performed to identify the main typifying
267 species of the groups.

268

269 **3. Results**

270 *3.1 Water quality*

271 Water quality parameters are summarized in Table 1. Throughout the
272 experiment, no significant differences were found between treatments for temperature
273 (29.65 ± 0.09 °C), dissolved oxygen (5.36 ± 0.10 mg L⁻¹), pH (7.88 ± 0.07), and
274 orthophosphate (9.11 ± 1.59 mg L⁻¹). On the other hand, for nitrogen compounds
275 significant differences were found.

276 Treatments with shrimp and oysters together in the same tank (IMTA) presented
277 lower values of TAN and nitrite-N differing from the other treatments, while the
278 treatment with shrimp and oysters in adjacent tanks with recirculation and use of
279 attached settling chamber (IMTA-RS) presented higher values of ORP and a higher
280 time of the use of settling chamber ($p < 0.05$), resulting in a higher amount of water per
281 kg of shrimp yield (Table 1).

282 For solids (SS, TSS, and VSS), differences were observed between treatments
283 multitrophic when compared with the control – shrimp monoculture presented higher
284 values of SS, TSS, and VSS. In all treatments, there was an increase in solids over time,
285 but in the control treatment (shrimp monoculture) this increase was faster when
286 compared to the other treatments (IMTA, IMTA-R and IMTA-RS), reaching 560.55 mg
287 L⁻¹ (TSS) and 18.30 mL L⁻¹ (SS) in the last week of culture (Fig. 2). This negatively
288 influenced the results of settling chamber use time, water consumption and water
289 replenishment.

290 For alkalinity, from the fourth week in the control treatment (shrimp

291 monoculture) there was a greater decrease when compared to the other treatments. For
292 this reason, it was necessary to add a smaller amount of sodium bicarbonate to control
293 alkalinity in IMTA treatments (Fig. 3).

294

295 *3.2 Zootechnical performance*

296 At 42 days, significant differences ($p < 0.05$) between treatments were observed
297 for final weight, SGR, FCR and yield (Table 2), when the best results were found in the
298 IMTA treatment. Regarding oysters, there was a significant difference ($p < 0.05$) for
299 survival and final weight, where the IMTA treatment differed from the others (Table 2).

300

301 *3.3 Presumptive diagnosis of oysters*

302 For the degree of stomach fullness there was a higher incidence of full stage
303 individuals in the IMTA treatment (100.0%), while the lowest was found in the IMTA-
304 RS, where no oysters had a full stomach – 60% of them had an intermediate stomach
305 (30 % almost empty and 30% almost full) (Table 3).

306 Fresh microscopic examination of the oyster gills from all treatments indicated
307 the presence of ciliates and flagellates. Lesions on the lamellae and accumulation of
308 solids were observed only in oysters from the IMTA treatment.

309

310 *3.4 Total hemocyte count for shrimp and oysters*

311 No significant differences were observed for the total hemocyte count, with an
312 average of 15.50 and 2.83 10^6 cells mL^{-1} , for shrimp and oysters, respectively (Fig. 4).

313

314 *3.5 Plankton community*

315 *3.5.1 Phytoplankton structure*

316 A total of 26 and 35 genera were respectively identified at the beginning and the
317 end of culture, divided into: Heterokontophyta (9 and 13), Chlorophyta (6 and 11),
318 Cyanophyta (7 and 7), Euglenophyta (2 and 2), and Dinophyta (2 and 2), respectively.
319 At the end of the experiment, IMTA-treatments showed lower density of Cyanophyta,
320 Euglenophyta and Dinophyta, differing from control (shrimp monoculture). Moreover,
321 these treatments also presented a lower phytoplankton cell density when compared to
322 the control, which ranged between 6,673.10 (IMTA) and 14,774.16 cells mL⁻¹ (control).
323 Regardless of treatment, the most frequent genera were *Chaetoceros*, *Skeletonema*,
324 *Mychonastes*, *Aphanocapsa* and *Oscillatoria* (Fig. 5).

325 Analysis of similarity (ANOSIM) showed significant differences in the
326 phytoplankton community between treatments (R global= 0.838; p=0.01) and time of
327 culture (R global = 0.944; p = 0.01) (Table 4). The days 21 and 42 showed a complete
328 difference when compared to the beginning of culture (R global = 1; p<0.05).
329 Moreover, it was observed that in the treatments of integrated multitrophic culture with
330 oysters the abundance was lower, completely differing from the shrimp monoculture
331 one (R global = 1). This difference was also proven by Cluster analysis, where the
332 formation of two groups was observed, both for time and for treatments. The percentage
333 similarity analysis (SIMPER) showed that several species contributed to the
334 differentiation between groups, with a low contribution of each genus (between 2.61
335 and 8.69%).

336

337 3.5.2. Zooplankton structure

338 The zooplankton community was composed of 11 and 17 (beginning and end)
339 identified genera, distributed in the following groups: Protozoa (4 and 3), Cladocera (0
340 and 2), Copepoda (2 and 4), Rotifera (3 and 6), Cirripedia (1 and 1), and Nematoda (1

341 and 1). The zooplankton total density varied from 5.17 to 7.33 organisms mL⁻¹ at the
342 end of cultures, *Brachionus* sp. being the most abundant taxa (Fig. 6).

343 ANOSIM for zooplankton also showed significant differences between
344 treatments (R global = 0.68; p = 0.01) and time of culture (R global = 0.483; p = 0.01)
345 (Table 5). Samples from days 21 and 42 were different when compared to the beginning
346 of culture (R global = 1; p < 0.05). When analyzing the difference between treatments,
347 it was also found that treatments with *Crassostrea* sp. differed from the control (shrimp
348 monoculture). SIMPER showed that *Brachionus* sp. (15.86 to 48.59%) and *Arcella* sp.
349 (11.46 to 13.95%) were the typified genera for all treatments.

350

351 **4. Discussion**

352 For nitrogen compounds, the nitrification process was efficient during the
353 experimental period, since nitrate-N levels were higher when compared to nitrite-N and
354 TAN, and these two nitrogen compounds remained within the levels recommended by
355 Samocha (2019). The use of inorganic and synbiotic fertilization in the preparation of
356 biofloc matrix and the synbiotic ones during culture contributed to the good
357 development of heterotrophic and nitrifying bacteria, and similar results have been
358 reported by Da Silva et al. (2021) and Santos et al. (2022). However, the treatment with
359 shrimp and oysters at the same culture unit (IMTA) showed lower values of TAN and
360 nitrite-N over the 42 days, as oysters can take advantage of some dissolved nutrients, as
361 was also reported by Lima et al. (2021).

362 In multitrophic treatments, oysters probably played the role of a natural substrate
363 for the development of a consortium of microorganisms (biofilm). Where this biofilm
364 may have accelerated the nitrification processes (Arnold et al., 2009; Zhang et al.,
365 2016). Bacteria and microalgae present in the biofilm are able to use inorganic nitrogen,

366 which can help to improve water quality levels (Ballester et al., 2006). In the IMTA-RS
367 treatment, the use of settling chamber in multitrophic culture removed excessive solids,
368 which may have contributed to an imbalance in the bacterial community, since bacteria
369 are attached to solids, resulting in an increase in the concentration of nitrogen
370 compounds.

371 During the experiment alkalinity was reduced due to the consumption of
372 inorganic carbon by shrimp, phytoplankton, and bacteria in all treatments, but this
373 reduction did not affect shrimp development or bacterial activity, as it was controlled
374 through weekly additions of sodium bicarbonate. However, all treatments with oysters
375 presented lower reduction in alkalinity levels when compared to the shrimp
376 monoculture. Possibly this fact is associated with the calcium carbonate present in shells
377 that provided alkalinity to the system, as observed by Magri et al. (2013) when
378 evaluating the potential for plugging oyster shells in aerobic reactors.

379 There are many sources of solids in aquaculture systems, making the
380 implementation of strategies to reduce suspended particles one of the main steps to
381 maintain water quality, and consequently the success in shrimp production (Arantes et
382 al., 2017; Mendoza-López et al., 2017; Bao et al., 2019). According to Chopin et al.
383 (2001), bivalves in IMTA are a viable option for solids reduction. Besides, another
384 efficient and practical method for solids removal is the use of settling chamber (Zemor
385 et al., 2019). In aquaculture intensive system there is an increase in the amount of solids
386 due to the reduction in water exchange, the accumulation of organic matter, and the
387 development of the bacterial community (Samocha, 2019), as observed in all treatments
388 in the present study. However, in shrimp monoculture this increase was greater than in
389 treatments with *Crassostrea* sp. For all treatments, TSS and SS levels remained in the
390 range recommended by Samocha (2019), which varied from 250 to 350 mg L⁻¹ and up

391 to 14 mL L⁻¹, for TSS and SS, respectively. While in monoculture, the concentration of
392 TSS exceeded this limit at the fourth week of culture, reaching up to 560.55 mg L⁻¹ at
393 the last week. The same was observed for SS, where this treatment presented
394 concentrations above the recommended limit since the fifth week of culture. In
395 treatments with oysters, solids remained controlled by natural filtering action of bivalve
396 mollusks, since these animals are able to coagulate small inorganic particles into larger,
397 more complex ones (Kautsky and Evans, 1987).

398 Similar results of solids dynamics in integrated systems with shrimp and oysters
399 have been previously reported (Lima et al., 2021), and also when used for the treatment
400 of wastewater from *L. vannamei* culture (Ramos et al., 2009; Brito et al., 2018). Thus,
401 using the multitrophic system with oysters can be a solution to the problem of excess
402 solids in intensive systems, since *Crassostrea* sp. can reduce this excess of solids,
403 transforming it into biomass with economic value.

404 For the shrimp performance, the best results were found in the treatment in
405 which oysters were cultured together with shrimps in the same tank. In this treatment,
406 the oysters having acted as natural substrates, they may have provided food supplement
407 for the shrimp, through the biofilm formed on the shells surface. Biofilm is an organic
408 community adhered to any submerged substrate, colonized by algae, bacteria, and fungi
409 (Costerton and Irvin, 1981). The use of substrates for biofilm formation in aquaculture
410 systems has been demonstrated as an efficient option for providing supplemental food
411 for *L. vannamei* (Moss and Moss, 2004; Santos et al., 2020). Thus, biofilm can
412 contribute to a better zootechnical performance of animals, since these microorganisms
413 are part of the natural diet of shrimp (Ballester et al., 2007).

414 Nevertheless, the zootechnical performance of shrimp showed similar results to
415 those found in other studies in *L. vannamei* culture in intensive systems using synbiotic

416 fertilization strategy at a density of 3,000 shrimp m^{-3} over 42-45 days of culture, such as
417 final weight between 0.61 – 1.26 g; yield between 1.60 – 3.42 Kg m^{-3} ; FCR up to 1.50,
418 and survival between 77.08 – 95.00% (Andrade et al., 2021; Da Silva et al., 2021; Silva
419 et al., 2021). Pre-treatment by using bran with beneficial microorganisms, such as
420 *Bacillus* spp., with anaerobic and aerobic processes for fertilization increases the
421 availability of nutrients, which contributes to improving the development of cultured
422 animals (Dawood and Koshio, 2019; Romano et al., 2018).

423 Bivalve mollusks (such as oysters) ingest the particles suspended in water as
424 food, using the gills as a filter. These organisms can concentrate organic particles,
425 microalgae and other planktonic organisms (Pearse et al. 1987; Ward 1996). In the
426 present study, examining the degree of stomach fullness of the oysters, it was observed
427 that they consumed the bioflocs, since most of the individuals analyzed had full or
428 intermediate (almost empty + almost full) stomachs – 50 to 100%. In the IMTA
429 treatment, 100% of the oysters had a full stomach. This fact reflected in the
430 development of oysters in this treatment, which had a higher final weight. Protozoa
431 (ciliates and flagellates) were abundantly found in the oyster, and this fact corroborates
432 findings reported by Costa et al. (2021) when evaluating the action of the oyster
433 (*Crassostrea gasar*) on the microbial community present in the bioflocs. On the other
434 hand, even with solids not exceeding the maximum limits during the experiment, and
435 sufficient aeration for keeping solids in suspension, in the IMTA treatment, high solids
436 concentrations in the oyster gills were observed. This fact is probably associated with
437 oysters position at the bottom of the experimental unit, and possibly in contact with an
438 excess of decanted solids. Thus, it is likely that this fact has negatively impacted the
439 survival of oysters in the IMTA treatment.

440 The zootechnical performance of the animals directly depends on the system

441 management and health status of the animals in the culture environment. The cellular
442 immune response of crustaceans and mollusks is mediated by hemocytes. These
443 organisms only have an innate immune system where hemocytes constitute the first line
444 of defense (Pipe and Coles, 1995; Barraco et al., 2008). Thus, the total hemocyte count
445 is one of the best indicators of the animals' immune status. In the present study, the
446 multitrophic culture with oysters did not influence the hemocyte count of shrimp and
447 oysters, indicating that the culture system used did not compromise shrimp health. The
448 mean values found in the present study were close to those found by other authors,
449 between $11.2 - 41.25 \times 10^6$ cells mL⁻¹ for shrimp (Abreu et al., 2019; Da Silva et al.,
450 2021), and 2.50×10^6 cells mL⁻¹ for *Crassostrea gigas* (Simões et al., 2015).

451 In relation to the plankton community, there was an increase in the plankton
452 density over the 42 days of culture in all treatments evaluated, similar to that reported
453 by Silva et al. (2021). This increase justifies the significant differences in both
454 phytoplankton and zooplankton community structure, as proved by temporal ANOSIM.
455 Furthermore, ANOSIM has also shown differences in community structures between
456 treatments, where integrated treatments (i.e., shrimp and oysters) differed from shrimp
457 monoculture, this difference is associated with differences in density of some species.
458 The treatments with oysters presented lower density of phytoplankton, especially
459 Cyanophyta, Euglenophyta and Dinophyta, when compared to the control. In the
460 analysis of stomach contents of *Crassostrea rhizophorae* cultured in an estuary,
461 phytoplankton (>50%) was predominant and among several organisms the diatom group
462 was dominant with 63% of total phytoplankton ingested (Dué et al., 2010).

463 In the IMTA treatments, microalgae were not only food for oysters, but may also
464 have been a supplementary source of food for shrimp. Considering that oysters acted as
465 a natural substrate for biofilm development, some of the main phytoplankton taxa (e.g.,

466 *Nitzschia* sp., *Anabaena* sp. and *Aphanocapsa* sp.) found in this study may be associated
467 with this biofilm, since they are frequently reported in the composition of biofilms
468 (Felisberto and Rodrigues, 2010).

469 At the beginning, the Heterokontophyta division was predominant, with the
470 taxon *Chaetoceros* sp. responsible for this dominance, due to inoculations of
471 *Chaetoceros calcitrans* cultures during fertilization process. However, during culture
472 other taxa also became abundant, such as *Mychonastes* sp. (Chlorophyta) and
473 *Oscillatoria* sp. (Cyanophyta). At the end, Heterokontophyta (26.77 – 29.22%),
474 Chlorophyta (26.59 – 42.94%) and Cyanophyta (27.79 – 44.81%) were the predominant
475 divisions, similar to results reported by Silva et al. (2021). These changes in the
476 community structure between the beginning and end of the experiment, probably
477 occurred due to the accumulation of nutrients and organic matter resulting from
478 fertilization, feed residues, and excretion of cultured organisms, which can favor the
479 more resistant algae groups, such as Cyanophyta. These algae present diverse
480 biochemical, physiological, genetic, and reproductive adaptations for development
481 under adverse conditions, such as low light, and tolerance to high levels of nitrogen and
482 phosphorus compounds (Reichwaldt and Ghadouani, 2012; Yusoff et al., 2010).

483 In relation to zooplankton, the treatments with oysters showed lower organisms
484 density, especially of the Protozoa division (*Paramecium* sp. and *Leptotintinnus* sp.),
485 than in the control group, with a reduction of approximately 50% in the density of
486 Protozoa. The divisions with higher relative abundance were Rotifera and Protozoa,
487 even in multitrophic culture treatments, which may be related to the adaptation of these
488 organisms to nutrient-rich environments. Similar results were reported by Silva et al.
489 (2021) and Andrade et al. (2021). High concentrations of rotifers and protozoa, as
490 observed in this study, may have benefited shrimp performance (Thompson et al.,

491 2002), as these organisms have already been reported in studies of the intestinal content
492 of *L. vannamei* (Loureiro, et al. 2012). Furthermore, when evaluating the stomach
493 content of the oyster *Crassostrea gasar* cultured using wastewater from *L. vannamei*
494 culture, Costa et al. (2021) observed the predominance of flagellates in the stomach
495 contents of oysters. In the present culture, the reduction of these microorganisms in the
496 water column of treatments with integrated multitrophic culture (IMTA, IMTA-RS and
497 IMTA-R) may be attributed to oyster consumption.

498

499 **5. Conclusions**

500 The study shows that the multitrophic system with *L. vannamei* and *Crassostrea*
501 sp. using synbiotic fertilization strategy can be developed with both species at the same
502 production unit without compromising the zootechnical performance of either species.
503 In addition, the use of oysters in multitrophic culture with synbiotic, cultured together
504 with shrimps in the same tank or in an adjacent recirculation pond, is an alternative for
505 the maintenance of nitrogen compounds, control of solids and, when cultured to the
506 same environment, they can provide a supplementary food source as a natural substrate
507 for biofilm formation. However, further research on the multitrophic system with
508 oysters to assess their resistance to other stages of culture, as well as the composition of
509 the biofilm formed in oysters cultured in an intensive system are needed.

510

511

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758 **Figure Captions**

759

760 Figure 1. Schematic detailing of the experimental units of integrated multitrophic
761 culture of shrimp (*Litopenaeus vannamei*) with oysters (*Crassostrea* sp.) under
762 recirculation and/or settling chamber. A-B: Treatment without recirculation and settling
763 chamber (IMTA); C-D: Treatment with only recirculation (IMTA-R); E-F: Treatment
764 without recirculation and settling chamber (IMTA-RS).

765 I: Shrimp tank (40L useful volume); II: Oyster tank (8L useful volume); III: Air-lift; IV:
766 Settling chamber (800 mL useful volume); V: Water return pipe from the settling
767 chamber.

768 Figure 2. Variations in the average concentrations of ORP (A), total suspended solids
769 (B), and settleable solids (C) in a multitrophic synbiotic system over 42 days, in the
770 different treatments: Monoculture (control without oysters); IMTA (shrimp and oysters
771 together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with
772 recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation
773 and use of attached settling chamber). Different letters represent significant differences
774 ($p < 0.05$) between treatments by ANOVA followed by Tukey's *post-hoc* test. Error bars
775 indicate standard deviation.

776 Figure 3. Alkalinity (lines) and amount of sodium bicarbonate added (bars) in a
777 multitrophic synbiotic system over 42 days of culture in the different treatments:
778 Monoculture (control without oysters); IMTA (shrimp and oysters together in the same
779 tank); IMTA-R (shrimp and oysters in adjacent tanks with recirculation); and IMTA-RS
780 (shrimp and oysters in adjacent tanks with recirculation and use of attached settling
781 chamber). Error bars indicate standard deviation.

782 Figure 4. Total hemocyte count of shrimp and oysters, after 42 days in monoculture and
783 multitrophic synbiotic system. Monoculture (control without oysters); IMTA (shrimp
784 and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks
785 with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with
786 recirculation and use of attached settling chamber). Error bars indicate the standard
787 deviation.

788

789 Figure 5. Phytoplankton density in the culture of *Litopenaeus vannamei* integrated with
790 *Crassostrea* sp. in a synbiotic system. Monoculture (control without oysters); IMTA
791 (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent
792 tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with
793 recirculation and use of attached settling chamber).

794 Figure 6. Zooplankton density in the culture of *Litopenaeus vannamei* integrated with
795 *Crassostrea* sp. in a synbiotic system. Monoculture (control without oysters); IMTA
796 (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent
797 tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with
798 recirculation and use of attached settling chamber).

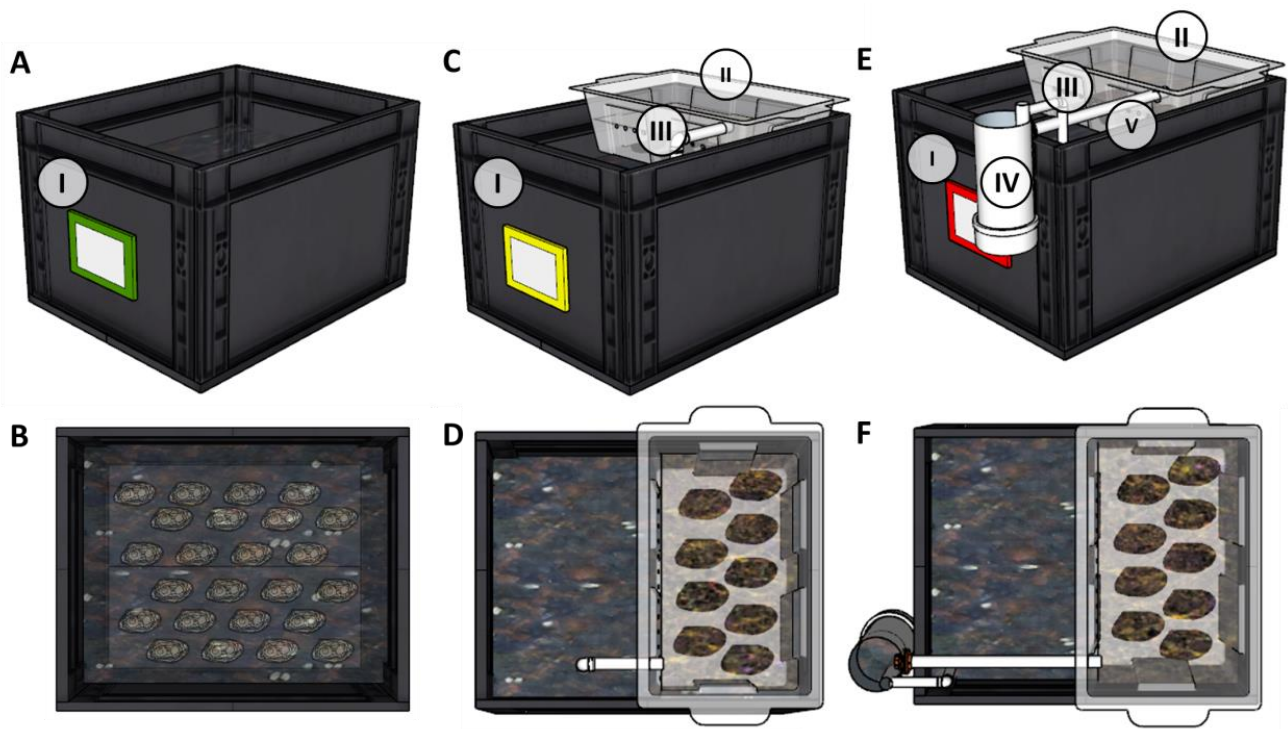


Figure 1.

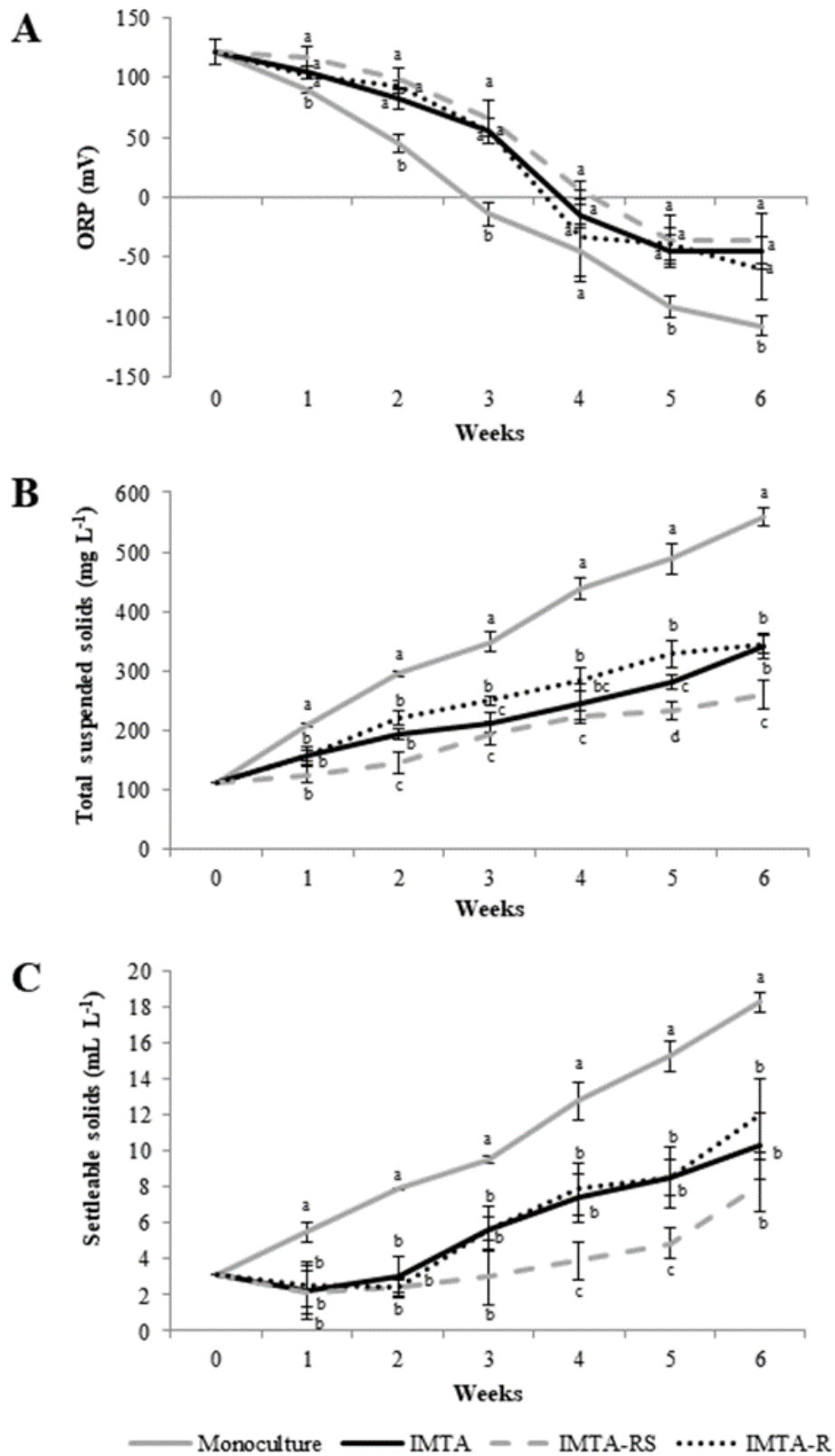


Figure 2.

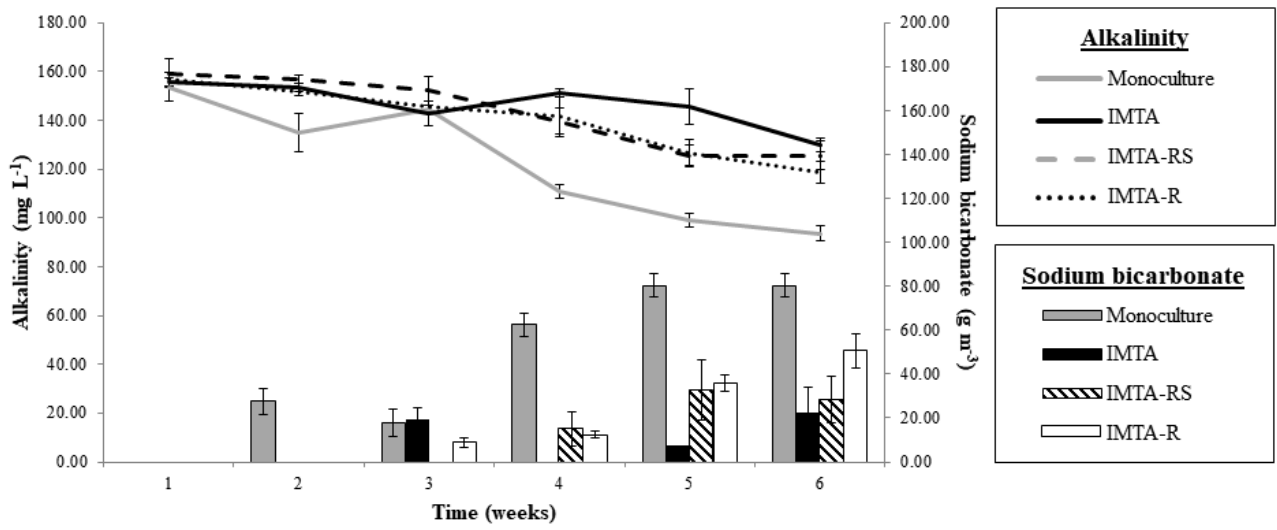


Figure 3.

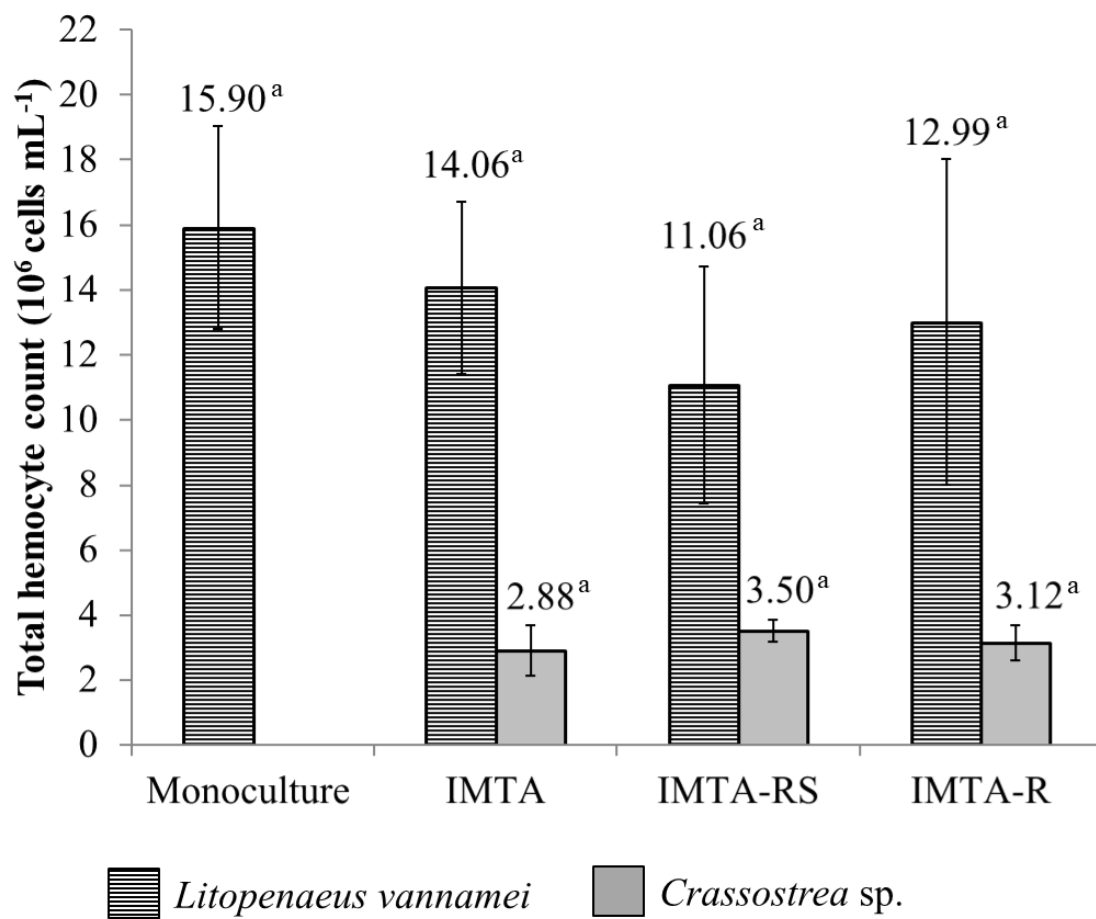


Figure 4.

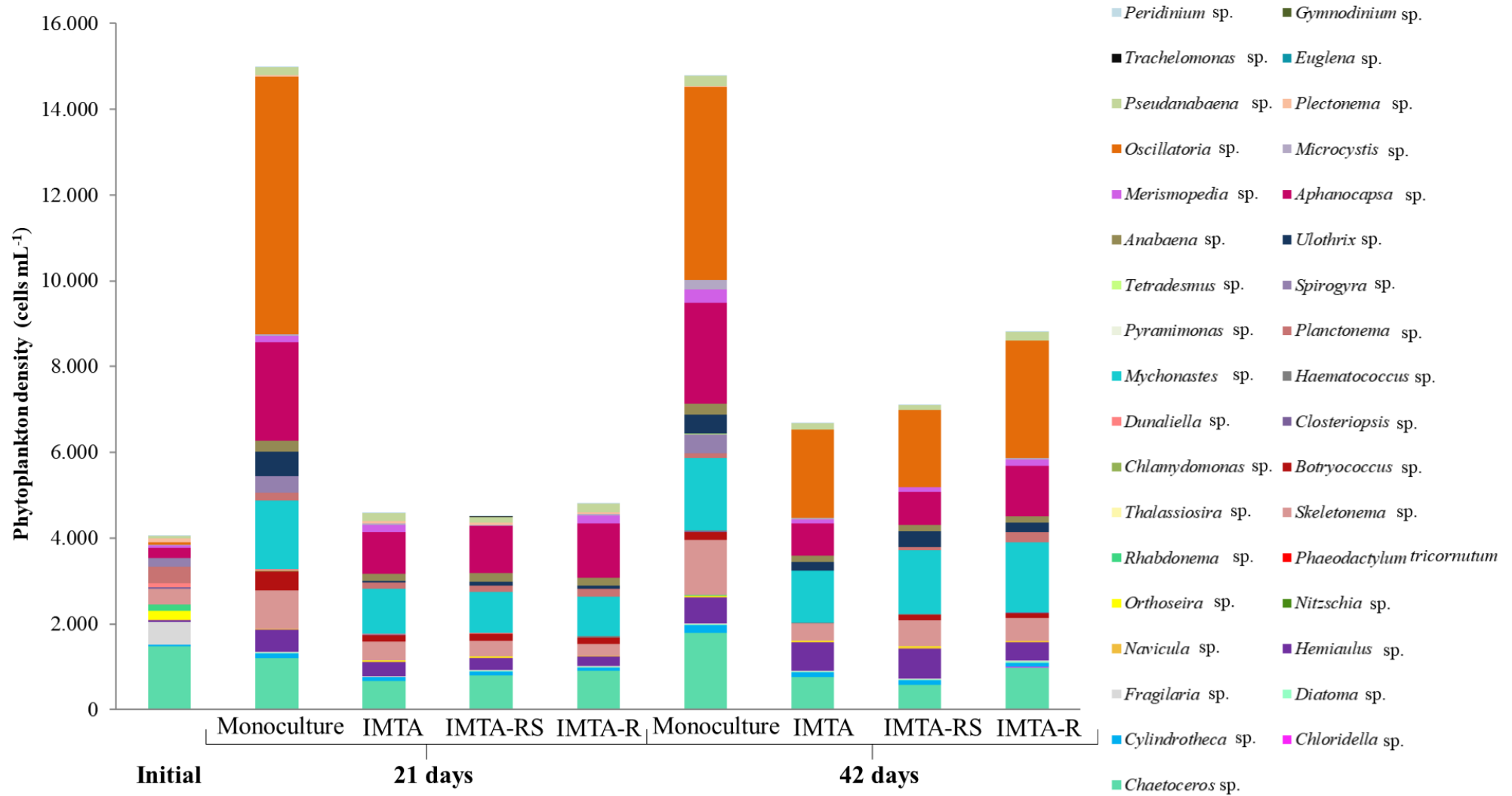


Figure 5.

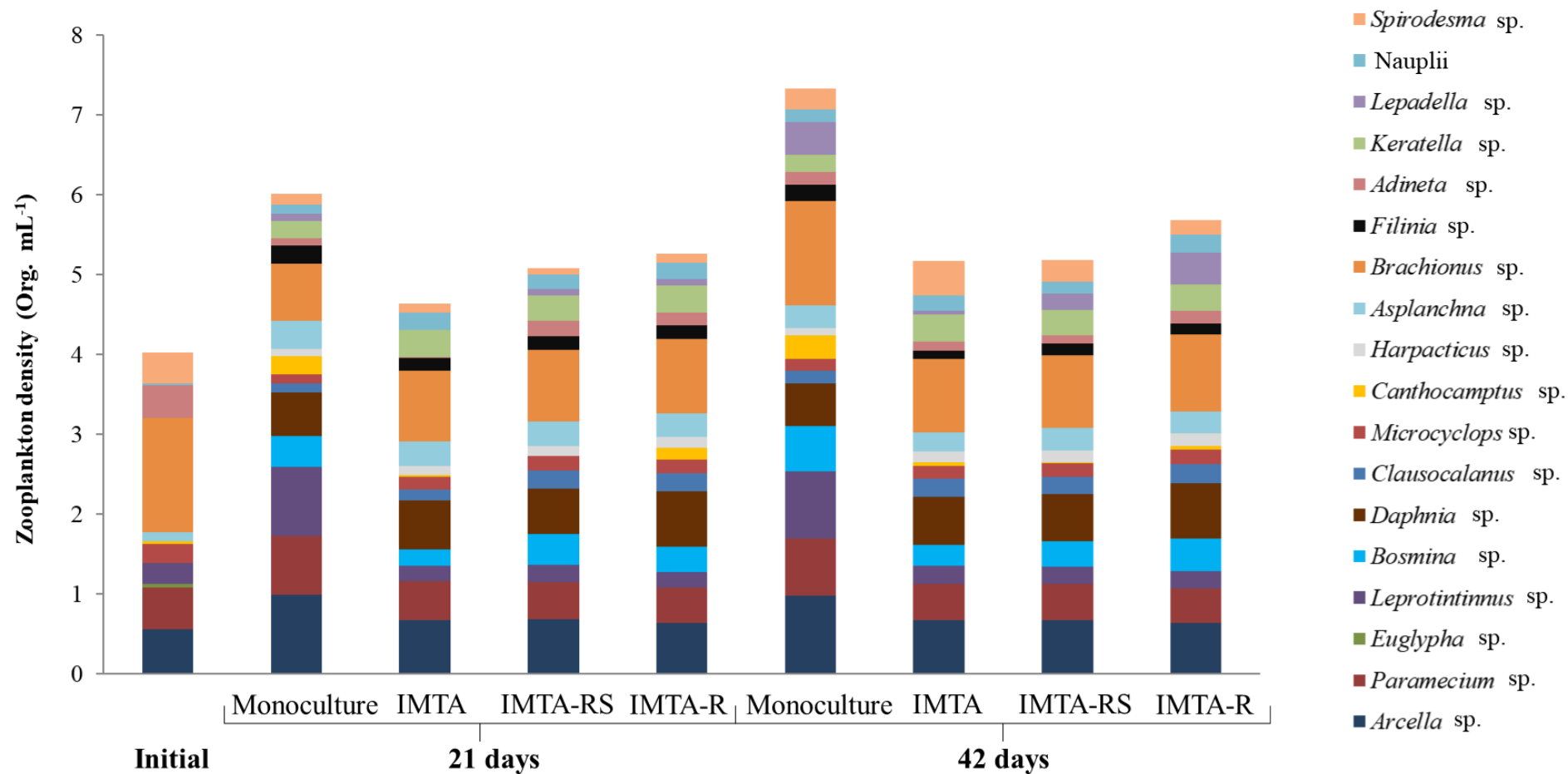


Figure 6.

Table 1. Water quality parameters of the multitrophic culture of *Litopenaeus vannamei* and *Crassostrea* sp. in a symbiotic system for 42 days.

Variables	Treatments				p-value
	Monoculture	IMTA	IMTA-RS	IMTA-R	
Temperature (°C)	29.51 ± 0.35 ^a	29.70 ± 0.11 ^a	29.61 ± 0.21 ^a	29.65 ± 0.09 ^a	0.688
Dissolved oxygen (mg L ⁻¹)	5.35 ± 0.20 ^a	5.45 ± 0.19 ^a	5.64 ± 0.35 ^a	5.39 ± 0.08 ^a	0.336
Salinity (g L ⁻¹)	28.99 ± 0.33 ^a	28.90 ± 0.25 ^a	28.97 ± 0.77 ^a	28.85 ± 0.62 ^a	0.919
pH	7.92 ± 0.09 ^a	7.91 ± 0.12 ^a	7.90 ± 0.10 ^a	7.95 ± 0.14 ^a	0.406
TAN (mg L ⁻¹)	0.92 ± 0.03 ^b	0.60 ± 0.05 ^c	1.15 ± 0.04 ^a	0.90 ± 0.04 ^b	0.009
Nitrite-N (mg L ⁻¹)	2.56 ± 0.44 ^b	1.35 ± 0.45 ^c	3.47 ± 0.62 ^a	2.49 ± 0.53 ^b	0.001
Nitrate-N (mg L ⁻¹)	15.91 ± 1.15 ^a	9.04 ± 1.1 ^c	13.86 ± 1.08 ^b	12.15 ± 5.78 ^b	0.026
Orthophosphate (mg L ⁻¹)	13.23 ± 1.27 ^a	14.04 ± 1.25 ^a	12.18 ± 1.29 ^a	11.44 ± 1.18 ^a	0.264
Water use (L) ¹	67.56 ± 1.24 ^a	56.80 ± 1.58 ^b	58.77 ± 0.38 ^b	56.93 ± 0.55 ^b	0.002
Water replenishment (% day ⁻¹) ²	1.64 ± 0.07 ^a	1.00 ± 0.09 ^b	1.12 ± 0.02 ^b	1.01 ± 0.03 ^b	0.003
Water consumption (L Kg ⁻¹)	507.61 ± 7.88 ^a	306.46 ± 6.40 ^c	444.81 ± 27.94 ^{ab}	418.05 ± 20.61 ^b	0.003
Settling chamber use (h Kg shrimp ⁻¹)	189.28 ± 6.05 ^b	0.00 ± 0.00 ^d	1,232.70 ± 49.31 ^a ¥	44.19 ± 2.49 ^c	0.001
Settleable solids (mL L ⁻¹)	10.54 ± 1.23 ^a	5.77 ± 0.62 ^b	3.95 ± 0.58 ^b	6.02 ± 0.67 ^b	0.010
Total suspended solids (mg L ⁻¹)	350.39 ± 54.31 ^a	220.78 ± 42.66 ^b	185.34 ± 38.12 ^c	243.07 ± 45.22 ^b	0.002
Volatile suspended solids (mg L ⁻¹)	220.78 ± 26.03 ^a	72.15 ± 20.05 ^c	73.96 ± 25.11 ^c	100.35 ± 13.20 ^b	0.001

The data correspond to the mean ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly (p<0.05) by repeated ANOVA measures followed by Tukey's *post-hoc* test. Monoculture (control without oysters); IMTA (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation and use of attached settling chamber).

Abbreviations: TAN = Total ammonia nitrogen.

¥: The settling chamber was used daily in this treatment at 4 h day⁻¹.

¹ Total water used during culture = experimental unit volume + volume used in replacement.

² Water replenishment (% day⁻¹) is the percentage of freshwater used to compensate the evaporation losses.

Table 2. Zootechnical performance of *Litopenaeus vannamei* and *Crassostrea* sp. cultured in a symbiotic multitrophic system with sedimentation and recirculation, after 42 days.

Parameters	Treatments				p-value
	Monoculture	IMTA	IMTA-RS	IMTA-R	
Shrimp performance					
Final weight (g)	1.15 ± 0.03 ^b	1.59 ± 0.05 ^a	1.20 ± 0.05 ^b	1.17 ± 0.08 ^b	0.011
Survival (%)	96.94 ± 0.28 ^{ab}	97.50 ± 0.83 ^a	94.72 ± 0.77 ^b	95.28 ± 0.32 ^{ab}	0.030
SGR (% day ⁻¹)	14.87 ± 0.07 ^b	15.64 ± 0.08 ^a	14.98 ± 0.09 ^b	14.90 ± 0.16 ^b	0.002
FCR	1.10 ± 0.03 ^a	0.97 ± 0.03 ^a	1.11 ± 0.04 ^a	1.10 ± 0.07 ^a	0.181
Yield (Kg m ⁻³)	3.32 ± 0.10 ^b	4.63 ± 0.12 ^a	3.41 ± 0.13 ^b	3.33 ± 0.23 ^b	0.001
Oyster performance					
Final weight (g)	-	18.05 ± 0.77 ^a	16.66 ± 1.01 ^b	17.01 ± 0.52 ^b	0.030
Survival (%)	-	82.984 ± 2.55 ^b	95.43 ± 3.02 ^a	90.71 ± 2.11 ^a	0.014
Oyster meat weight (g)	-	1.25 ± 0.05 ^a	0.89 ± 0.28 ^b	1.10 ± 0.19 ^{ab}	0.022
Final length (cm)	-	3.93 ± 0.17 ^a	3.83 ± 0.10 ^a	3.87 ± 0.14 ^a	0.401
Monthly growth (mm month ⁻¹)	-	4.69 ± 1.12 ^a	4.02 ± 0.66 ^a	4.25 ± 0.53 ^a	0.408
Cumulative growth (%)	-	21.78 ± 5.23 ^a	18.68 ± 3.07 ^a	19.76 ± 2.45 ^a	0.251
Yield (Kg of oyster meat m ⁻²)	-	0.21 ± 0.10 ^a	0.17 ± 0.07 ^a	0.19 ± 0.09 ^a	0.101

The data correspond to the mean ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly (p<0.05) by one-way ANOVA followed by Tukey's *post-hoc* test. Monoculture (control without oysters); IMTA (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation and use of attached settling chamber).

Abbreviations: FCR = feed conversion ratio; SGR= specific growth rate.

Table 3. Degree of fullness of the stomach contents of the *Crassostrea* sp. oysters in a synbiotic multitrophic system with sedimentation and recirculation, after 42 days.

Treatment	Oysters number	Degree of fullness			
		Empty	Almost empty	Almost full	Full
Monoculture	-	-	-	-	-
IMTA	10	0.0%	0.0%	0.0%	100.0%
IMTA-RS	10	20.0%	30.0%	30.0%	20.0%
IMTA-R	10	10.0%	20.0%	30.0%	40.0%

Monoculture (control without oysters); IMTA (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation and use of attached settling chamber).

Table 4. Values concerning to statistic R (p-value) found in the analysis of similarity (ANOSIM) for phytoplankton communities from different treatments and days of culture.

Global R = 0.838	ANOSIM – Treatments		
	Monoculture	IMTA	IMTA-RS
Monoculture	-		
IMTA	1 (0.01)	-	
IMTA-RS	1 (0.01)	0.704 (0.05)	-
IMTA-R	1 (0.01)	0.833 (0.01)	0.815 (0.01)
Global R = 0.944	ANOSIM – Time (days)		
	0		22
0	-		
22	1 (0.004)		-
45	1 (0.004)		0.907 (0.001)

Monoculture (control without oysters); IMTA (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation and use of attached settling chamber).

Table 5. Values for statistic R (p-value) found in the analysis of similarity (ANOSIM) for zooplankton communities from different treatments and days of culture.

Global R = 0.68	ANOSIM – Treatments		
	Monoculture	IMTA	IMTA-RS
Monoculture	-		
IMTA	1 (0.012)	-	
IMTA-RS	0.91 (0.002)	0.27 (0.019)	-
IMTA-R	0.88 (0.002)	0.48 (0.004)	0.18 (0.008)
Global R = 0.483	ANOSIM – Time (days)		
	0		22
0	-		
22	1 (0.002)		-
45	1 (0.002)		0.13 (0.03)

Monoculture (control without oysters); IMTA (shrimp and oysters together in the same tank); IMTA-R (shrimp and oysters in adjacent tanks with recirculation); and IMTA-RS (shrimp and oysters in adjacent tanks with recirculation and use of attached settling chamber).

4. Considerações finais

Com os resultados obtidos durante as pesquisas realizadas para o desenvolvimento da tese, é possível concluir que a utilização de ostras *Crassostrea* sp. no sistema multitrófico intensivo com mínima troca de água, quando estocadas com densidade de 100 e 200 ostras m⁻², é uma alternativa para a manutenção de compostos nitrogenados, controle de sólidos e redução do *Vibrio* spp. sem comprometimento do crescimento, a sobrevivência e produtividade do camarão. Além disso, quando cultivados no mesmo ambiente, as ostras podem atuar como substrato natural para biofilme, fornecendo assim uma fonte suplementar de alimento como. No entanto, são necessárias mais pesquisas sobre o sistema multitrófico integrado com ostras. Dessa forma, recomenda-se a avaliação da resistência das ostras a outras fases de cultivo, como na engorda, do desenvolvimento e composição do biofilme formado sobre as ostras cultivadas em sistema intensivo, assim como uma análise da viabilidade econômica e a aplicação comercial deste sistema.

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ANEXO

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Effect of stocking density of *Crassostrea* sp. in a multitrophic biofloc system with
Litopenaeus vannamei in nursery

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Effect of stocking density of *Crassostrea* sp. in a multitrophic biofloc system with *Litopenaeus vannamei* in nursery

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ABSTRACT

In recent years, the coculture of organisms from different trophic levels has been practiced to improve water quality and nutrient consumption. In this context, this study evaluated the effect of *Crassostrea* stocking density on zootechnical performance and water quality of *Litopenaeus vannamei* reared in a multitrophic biofloc system during the nursery phase. The experimental design was completely randomized with four treatments: control (shrimp monoculture) and three treatments with oysters (3.21 ± 1.26 cm) at densities of 100, 200 and 300 oyster m^{-2} , for 45 days. Water quality, zootechnical performance parameters of shrimp and oysters, proximate composition of microbial flocs, *Vibrio* count from water, shrimp and oysters samples and total hemocyte counts from shrimp and oyster samples were evaluated. For water quality, multitrophic biofloc system treatments had the lowest amounts of settleable solids, resulting in lower settling chamber use than the control. However, a high density of oysters (300 oysters m^{-2}) increased total ammonia nitrogen and nitrite-N levels, which had maximum values of 4.01 and 4.63 $mg L^{-1}$, respectively. The final average weight and cumulative growth of oysters were 16.23 ± 3.45 g and $18.91 \pm 5.24\%$, respectively, corresponding to an average growth of 4.07 ± 0.34 mm month⁻¹. In shrimp performance, the higher density treatment (300 oysters m^{-2}) had the lowest values of final weight and yield (0.94 ± 0.03 g and 2.26 ± 0.05 Kg m^{-3}) and the lowest nutritional quality of the microbial floc (14.96% protein and 3.71% lipid). The *Vibrio* count was significantly lower in all the multitrophic biofloc system treatments compared to the control, with an average reduction of 37.32% in water and 75.69% in shrimp hepatopancreas. Stocking densities of up to 200 oysters m^{-2} did not affect shrimp growth in the multitrophic biofloc systems evaluated. The multitrophic biofloc system used in this research is a viable option for controlling nitrogen compounds and solids and reducing *Vibrio* in a shrimp biofloc system during the nursery phase.

1. Introduction

Shrimp farming has grown at a reduced rate in recent years (FAO, 2018), mainly due to viral outbreaks (White Spot Syndrome Virus and Infectious Myonecrosis Virus) and bacterial disease (Acute Hepatopancreatic Necrosis Disease - AHPND), which have caused significant losses totaling approximately US\$ 15 billion dollars in the production of *Litopenaeus vannamei* (Costa et al., 2009; Shinn et al., 2018; Hostins et al., 2019; Samocha, 2019). *L. vannamei* is the world's most reared crustacean (52.9%), with approximately 4.97 million tons produced in 2018 (FAO, 2020). In Brazil, 45.8 thousand tons were farmed in 2018, production grew 11.4% over 2017, indicating a recovery in losses caused by the White Spot Syndrome Virus (IBGE, 2018).

Despite increased production, culture systems must employ better

nutrient recycling, and have minimal temperature fluctuations and water exchanges. Among the techniques used in aquaculture one that can be highlighted intensive culture systems with minimal water exchange and integrated aquaculture or integrated multitrophic (Dauda, 2019; Hostins et al., 2019; Poli et al., 2019).

In the minimum water exchange system, nutrients are continuously recycled by heterotrophic and nitrifying bacteria, and the water can be reused between culture cycles (Emerenciano et al., 2013). This technology promotes better zootechnical performance (Samocha et al., 2017) and an increase in the innate immune response (Kim et al., 2014), while also permitting high shrimp densities, maintaining water quality and greater biosecurity in culture, due to reduced water exchange (Samocha et al., 2017).

However, in non-multitrophic biofloc system, high stocking

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densities and reduced water exchange lead to an accumulation of suspended and settleable solids, nutrients (nitrogen and phosphorus) and heavy metals that may cause a proliferation of *Vibrio* (Samocho et al., 2017). High levels of solids negatively affect shrimp health, causing clogged gills and even mortality (Emerenciano et al., 2013; Hargreaves, 2013).

In this context, strategies such as encouraging beneficial bacteria, through the combination of probiotics and prebiotics (Romano et al., 2018), multitrophic culture and recirculating aquaculture system can improve water quality. The use of *Bacillus* sp. as a probiotic can enhance zootechnical performance and resistance to stress (Hoseinifar et al., 2016). The multitrophic culture system is a method for raising two or more species belonging to different trophic levels in the culture unit or adjacent units with recirculating system (Angel and Freeman, 2009; Chopin et al., 2010; Poli et al., 2019). Culturing more than one species in the same water is often better than monocultures. By the addition of a secondary species and recirculating system it is possible to improve the efficiency of nutrients, maximize the reuse of water and may result in an economic increase in production (Martins et al., 2010; Rosa et al., 2020). Several groups have potential for multitrophic system, such as seaweeds, mollusks, fish and polychaetes. However, a suitable combination of species is necessary to transform waste into biomass, since the functions performed by each organism must be considered (Barrington et al., 2009).

Multitrophic culture can consume excess nutrients and organic matter, since the removal of organic and inorganic matter can be done using mollusks (Modesto et al., 2010; Brito et al., 2018; Omont et al., 2020; Rosa et al., 2020), especially oysters of the genus *Crassostrea* sp., which has potential for use in this type of system (Barrington et al., 2009). Attempts to improve water quality in culture using bivalves have been studied as part of the development of integrated aquaculture (Jones et al., 2001; Smaal et al., 2019). Oysters are good candidates for a multitrophic biofloc system due to their resistance to poor water quality. Oysters are known as non-specific filter feeders and can significantly improve water quality since they reduce suspended particles, including organic and inorganic particles, phytoplankton and bacteria, reducing water turbidity, as they filter around 200 L day⁻¹ (Ward, 1996; Martínez-Cordova and Martínez-Porchas, 2006).

Despite the wide variety of functions of oysters in traditional systems, their performance in multitrophic biofloc systems is still unknown. For this reason, the objective of this study was to evaluate the stocking density of oysters *Crassostrea* sp. in a multitrophic biofloc system with the shrimp *Litopenaeus vannamei* on the zootechnical performance (oysters and shrimp), water quality, *Vibrio* count and microbial floc proximate composition.

2. Material and methods

The study was conducted at the Sustainable Mariculture Laboratory (LAMARSU) of the Fisheries and Aquaculture Department (DEPAq) of the Rural Federal University at Pernambuco (UFRPE), Recife, Brazil.

2.1. Preparation of the matrix tank

A matrix tank with water salinity of 30 g L⁻¹ was chlorinated with 13 mg L⁻¹ (chlorine) and after 72 h of aeration received inorganic fertilization (single application) with urea (4.5 g m⁻³ N), triple superphosphate (0.3 g m⁻³ P) and sodium silicate (0.23 g m⁻³ Si). After two days, organic fertilization was begun through 12 applications of product for 24 h in an anaerobic phase followed by an aerobic phase (24 h). The organic fertilizer was composed of rice bran (20 g m⁻³), molasses (2 g m⁻³), sodium bicarbonate (4 g m⁻³) and a bacteria-based product (0.05 g m⁻³), containing *Bacillus subtilis*, *B. licheniformis*, *Saccharomyces* sp. and *Pseudomonas* sp. at a total of 5.5 to 6.5 × 10⁷ CFU g⁻¹ (Kayros Ambiental e Agrícola, Brazil). It was added with a three-day interval between applications. The C-CHO was

calculated from the carbohydrate component of rice bran (17.15% wet matter) and molasses (32.23% wet matter), and total nitrogen was determined from the protein content of the rice bran (3.05% wet matter) and molasses (0.06% wet matter) and the nitrogen in the urea (46% N). At the end of fertilization, the water C:N ratio was 4.13.

2.2. Seed and juvenile oyster survival test at different levels of settleable solids

To evaluate the survival of seeds (0.81 ± 0.6 cm) and juveniles (3.55 ± 1.20 cm) of *Crassostrea* oysters at different concentrations of settleable solids, a previous experiment was conducted over 15 days. The oysters were stocked at a density of 300 oysters m⁻² in tanks with 12 L of useful volume (30 cm in diameter). Five amounts of settleable solids were tested (0, 2.5, 5, 10 and 20 mL L⁻¹), all in triplicate, in a randomized factorial design. During the test, daily survival and water quality parameters were checked and settleable solids were measured and adjusted.

2.3. Experimental conditions of multitrophic biofloc culture

To evaluate the effect of the density of the oyster *Crassostrea* sp., four treatments were tested, a control (Shrimp monoculture) and three treatments of multitrophic biofloc shrimp and juvenile oyster culture (3.21 ± 1.26 cm), at densities of 100 oysters m⁻² (MB-100); 200 oysters m⁻² (MB-200) and 300 oysters m⁻² (MB-300), all in triplicate and in a completely randomized design, for 45 days. The *L. vannamei* postlarvae (pL₁₅-9.64 ± 0.23 mg) from commercial hatchery (Ocean Vitória, Paraíba, Brazil) were stocked at a density of 2500 pL's m⁻³ (100 shrimp per experimental unit) in 60 L tank (40 L useful volume).

The experimental units consisted of two compartments: (1) a 60 L tank for shrimp (40 L useful volume - 0.2 m²) and, (2) a 20 L adjacent tank for oysters (8 L of useful volume - 0.1 m²), both rectangular. The water recirculation was performed by air-lift, from the shrimp tank to the oyster tank and was returned by gravity (Fig. 1). Each day, 20% of the shrimp tank volume was renewed, corresponding to 100% of the oyster tank volume with a 24-h retention time in the tanks. The shrimp culture unit contained a 100 W heater connected to a thermostat to maintain the temperature at 29 ± 1 °C and an aeration system with three air stones to keep the flocs in suspension and the oxygen above 5 mg L⁻¹.

2.4. Management of production units

The postlarvae were fed four times a day (at 08:00 a.m., 11:00 a.m., 01:00 p.m. and 04:00 p.m.), with a commercial shrimp feed (0.4 to 0.8 mm in diameter) with 45% crude protein and 9.5% lipid (In vivo Animal Nutrition and Health) and the amount of feed was adjusted according to the estimate of consumption and mortality. The organic fertilizer (10 g m⁻³ of rice bran, 1 g m⁻³ of molasses, 2 g m⁻³ of sodium bicarbonate and 0.05 g m⁻³ of a bacteria-based product, containing *Bacillus subtilis*, *B. licheniformis*, *Saccharomyces* sp. and *Pseudomonas* sp. in a total of 5.5 to 6.5 × 10⁷ CFU g⁻¹ - Kayros Ambiental e Agrícola, Brazil) was added every three days to the experimental units, following the same preparation methodology described in item 2.1.

Sodium bicarbonate was added to maintain alkalinity > 120 mg L⁻¹ and pH > 7.5, where 0.20 g L⁻¹ increases on average to 0.25 pH and 100 mg L⁻¹ alkalinity (Furtado et al., 2011). No water changes were made during the experiment, only replacement of evaporated water. The concentration stipulated as a limit for settleable solids was 5 mL L⁻¹, if this concentration was reached, the settling chamber was used. The settling chamber use (SC) was also evaluated, using the equation: SC (h Kg⁻¹) = total time of use of settling chamber (h) / final shrimp biomass (Kg) (Lima et al., 2019).

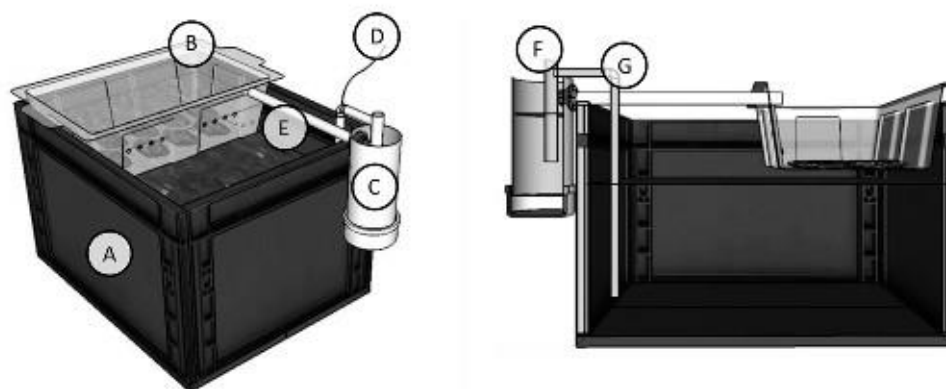


Fig. 1. Experimental units for multitrophic biofloc treatments with *Crassostrea* sp. and *L. vannamei*. A: Shrimp tank (40 L useful volume); B: Oyster juvenile tank (8 L useful volume); C: Settling chamber (800 mL useful volume); D: Air supply hose; E: Water return pipe from the settler; F: Water inlet pipe of the settler and; G: Air-lift.

2.5. Water quality

Dissolved oxygen, temperature, salinity, pH and total dissolved solids (YSI model 100, Yellow Springs, Ohio, USA) were monitored twice a day (at 08:00 a.m. and 04:00 p.m.). Total ammonia nitrogen (TAN) (APHA, 2012), nitrite-N ($\text{NO}_2\text{-N}$) (Fries, 1971) and alkalinity (CaCO_3) (APHA, 2012), total suspended solids (TSS) (APHA, 1995) and volatile suspended solids (VSS) (APHA, 1995) were measured weekly. Orthophosphate (APHA, 2012) and nitrate-N ($\text{NO}_3\text{-N}$) (APHA, 2012) were measured every two weeks. Settleable solids (SS) (Imhoff cone) were monitored three times a week (Avnimelech, 2015).

2.6. Zootechnical performance

Shrimp weight was monitored weekly, from the 15th day of culture, to determine shrimp growth and adjust the amount of feed offered. At the end of the experiment, biomass gain, mean final weight, specific growth rate (SGR), feed conversion ratio (FCR), survival and yield were determined based on the following equations: $\text{Biomass gain (g)} = \text{final biomass (g)} - \text{initial biomass (g)}$; $\text{Final weight (g)} = \text{final biomass (g)} / \text{number of individuals at the end of evaluation period}$; $\text{SGR (\% day}^{-1}\text{)} = 100 \times [\ln \text{final weight (g)} - \ln \text{initial weight (g)}] / \text{time (days)}$; $\text{FCR} = \text{feed supplied} / \text{biomass gain}$; $\text{Survival (\%)} = (\text{number of individuals at the end of the evaluation period} / \text{initial number of individuals}) \times 100$; $\text{Yield (kg m}^{-3}\text{)} = \text{final biomass (kg)} / \text{volume of experimental unit (m}^3\text{)}$.

During the experiments, oyster weight and length (Galtsoff, 1964) were monitored daily (30% of the population) in each experimental unit. All oysters were counted weekly in each experimental unit for available survival. Cumulative growth (Cg) was calculated using the formula: $\text{Cg (\%)} = [(\text{final length} - \text{initial length}) / \text{initial length}] \times 100$.

2.7. Proximate composition

For analysis of proximate composition (crude protein, lipid, ash and fiber) the floc samples (30 g) were collected with a cylindrical mesh net of 50 μm for retention of solids, at the beginning and end of the experiment, in triplicate. The samples were analyzed in the Animal Nutrition Laboratory of the Animal Science Department of the Federal Rural University of Pernambuco. Protein was determined by measuring nitrogen ($\text{N} \times 6.25$) using the Kjeldahl method (AOAC, 2012), total lipid by hexane extraction using Soxhlet (AOAC, 2012), fiber by acid and alkaline digestion (AOAC, 2012) and the moisture content by drying the material in an oven at 105 $^\circ\text{C}$, until reaching a stable weight (AOAC, 2012).

2.8. *Vibrio* count

The sampling to quantify *Vibrio* spp. in the water, shrimp and oysters was performed at the beginning and end of the experiment. For water analysis, 50 mL were collected from each experimental unit, of which 500 μL was diluted in 4.5 mL of peptone water (2%), thus making the dilution 10^{-1} . After homogenization, serial dilutions of 10^{-1} to 10^{-5} were performed.

For quantification in animal samples, 10 μL_{15} (beginning) (Vandenbergh et al., 1999), 0.07 g shrimp hepatopancreas (final) and 1 g of oyster meat (beginning and final) were used. The shrimp and oysters were disinfected by immersion in 70% ethanol for 15 s, followed by immersion for 15 min in sodium hypochlorite solution (1.5%) with 0.1% tween-80 and rinsed with sterile distilled water. Subsequently, the biological samples were weighed, macerated and homogenized with a solution of peptone water (2%) at a 1:10 ratio, for a 10^{-1} dilution, and serially diluted from 10^{-1} to 10^{-5} (1/10). Finally, a 0.1 mL aliquot of the sample dilutions (water, shrimp and oysters) was inoculated in petri dishes containing Thiosulphate Citrate Bile Sucrose (TCBS) agar, in triplicate. The dishes were incubated at 35 $^\circ\text{C}$ for 24 h for further quantification (CFU mL^{-1} or CFU g^{-1}), classification according to the use of sucrose in the medium (negative sucrose - green colony and positive sucrose - yellow colony) and counted between 30 and 300 colonies using a colony counter.

2.9. Total hemocyte count for shrimp and oysters

The oyster's hemolymph (100 μL) was withdrawn from the posterior adductor muscle at the end of the experiment using a 1 mL syringe. After collecting the hemolymph, the cell density of hemocytes was determined using a hemocytometer.

Shrimp hemolymph collection (100 μL) was conducted at the end of the experiment from the ventral sinus with a 1 mL sterile syringe, with 200 μL of precooled anticoagulant solution (modified Alsever solution (MAS) (336 mmol L^{-1} NaCl, 115 mmol L^{-1} glucose, 27 mmol L^{-1} sodium citrate, 9 mmol L^{-1} EDTA, pH 7.2) in the proportion of 1:2 (v:v). To perform total hemocyte count, triplicates of 0.8 mL of diluted hemolymph were counted for the number of hemocytes using a hemocytometer under a light microscope (Guertler et al., 2013).

2.10. Statistical analysis

A one-way ANOVA was used to analyze production parameters, proximate composition and total hemocyte count, after confirming homoscedasticity (Cochran $p < 0.05$) and normality (Shapiro-Wilk $p < 0.05$). Tukey's test ($p < 0.05$) was performed to compare and rank mean results from the three treatments and the control. Water

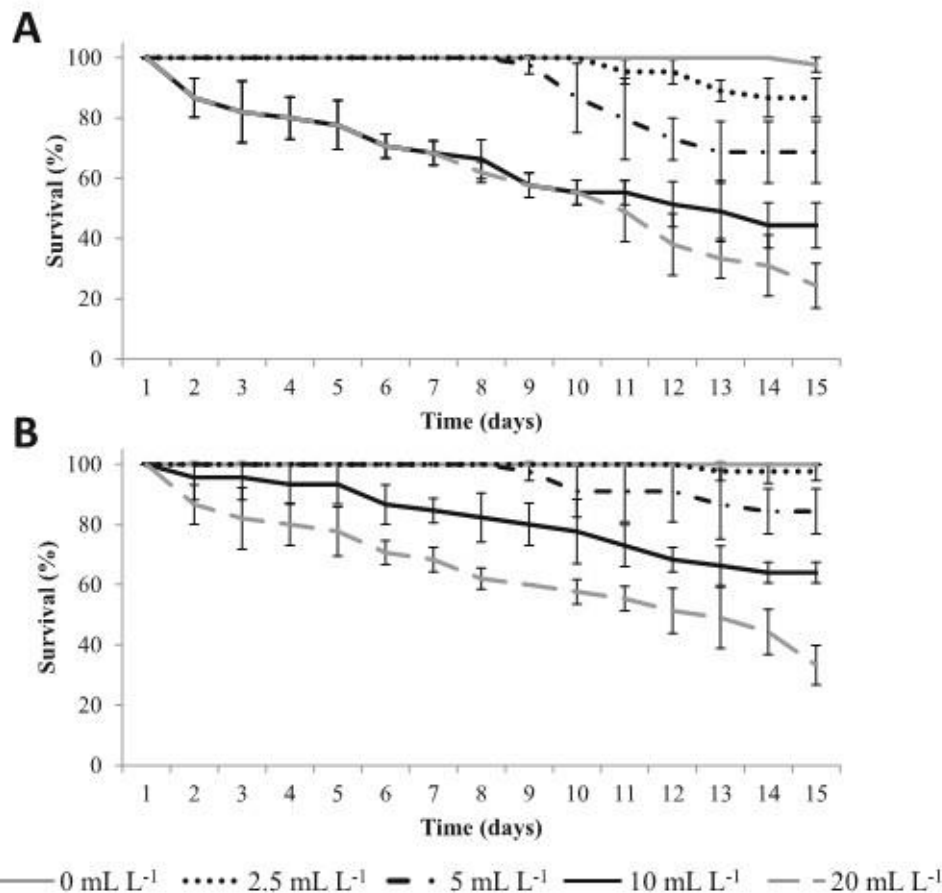


Fig. 2. Survival of seeds (A) and juveniles (B) oysters of *Crassostrea* sp. at different concentrations of settleable solids, during 15 days. Error bars indicate the standard deviation.

quality parameters were analyzed by performing repeated measures ANOVA and Tukey's test ($p < 0.05$). For non-parametric statistical data (seed and juvenile oyster survival test and *Vibrio* count), the Kruskal-Wallis ($\alpha < 0.05$) and Dunn tests ($\alpha < 0.05$) were used to compare and rank median results from the three treatments and the control. The Spearman correlation (ρ) was also applied for the survival test of oyster seeds and juveniles at different levels of settleable solids. Data analyses were performed using Statistica 10 software.

3. Results

3.1. Seed and juvenile oyster survival test at different levels of settleable solids

In the survival test, it was observed that the settleable solids have a negative ($\rho = -0.79$) and significant ($p < 0.05$) correlation with seed and juvenile oyster survival. Higher mortality was associated to higher amounts of settleable solids. For both sizes of oyster, the lowest mortalities were observed at 0, 2.5 and 5 mL L⁻¹ of settleable solids (Fig. 2). In addition, survival was also influenced by size, where juveniles were more resistant than seeds to settleable solids.

3.2. Water quality

The water quality parameters are described in Table 1. During the experiment, no significant differences were found between the treatments for temperature (29.58 ± 0.21 °C), dissolved oxygen (5.39 ± 0.15 mg L⁻¹), pH (7.98 ± 0.06), alkalinity (122.84 mg L⁻¹) and orthophosphate (27.53 ± 2.07 mg L⁻¹). Differences were found

for nitrogen compounds in the treatments with oysters at densities of 100 and 200 oysters m⁻², which had lower values than the control and MB-300. The MB-300 had the highest values of these compounds, reaching up to 4.01 and 4.63 mg L⁻¹ of TAN and nitrite-N respectively, in the last two weeks of culture (Fig. 3).

For solids, differences were observed between treatments with oysters compared to the control. The control had higher total suspended solids (431.13 mg L⁻¹), volatile suspended solids (217.49 mg L⁻¹) and settleable solids (13.19 mL L⁻¹). In addition, all treatments had an increase in solids over time, while in the control treatment, the total suspended solids exceeded 350 mg L⁻¹ in the fourth week of culture (Fig. 4).

The control treatment had the longest time of use of the settling chamber ($p < 0.05$) as well as the highest amount of water use per kilogram of shrimp produced (Table 1). Considering the total water replenishment, the control treatment also differs significantly from the others ($1.37 \pm 0.02\%$ day⁻¹).

3.3. Shrimp and oyster zootechnical performance

At 45 days, significant differences ($p < 0.05$) were observed for the shrimp zootechnical performance, between MB-300 and the other treatments for final weight, FCR and yield (Table 2). For the oysters, the survival rate was higher in the MB-100 and MB-200 than in the MB-300. The total shell length and total weight increased and mollusk soft parts did not differ significantly ($p > 0.05$) among the treatments. The oysters had continuous growth during the culture, from 3.21 cm to 3.82 cm, for an average growth of $18.91 \pm 5.24\%$.

Table 1
Water quality in multitrophic biofloc culture of *Litopenaeus vannamei* and *Crassostrea* sp., during 45 days.

Variables	Treatments			
	Control	MB -100	MB - 200	MB - 300
Temperature (°C)	29.84 ± 0.12 ^a	29.83 ± 0.09 ^a	29.96 ± 0.10 ^a	29.70 ± 0.15 ^a
DO (mg L ⁻¹)	5.40 ± 0.14 ^b	5.35 ± 0.09 ^b	5.33 ± 0.05 ^b	5.37 ± 0.11 ^b
Salinity (g L ⁻¹)	28.86 ± 0.51 ^a	28.19 ± 0.19 ^a	28.59 ± 0.44 ^a	28.55 ± 0.21 ^a
pH	7.94 ± 0.11 ^a	7.89 ± 0.14 ^a	7.99 ± 0.09 ^a	7.87 ± 0.14 ^a
Alkalinity (mg L ⁻¹)	127.33 ± 7.19 ^a	122.61 ± 8.19 ^a	120.67 ± 8.86 ^a	120.75 ± 7.89 ^a
TAN (mg L ⁻¹)	0.66 ± 0.07 ^b	0.35 ± 0.06 ^c	0.44 ± 0.06 ^c	1.50 ± 0.02 ^a
Nitrite-N (mg L ⁻¹)	1.37 ± 0.21 ^b	1.14 ± 0.37 ^c	1.15 ± 0.15 ^c	1.58 ± 0.04 ^a
Nitrate-N (mg L ⁻¹)	11.11 ± 2.24 ^{ab}	7.17 ± 1.31 ^b	8.45 ± 2.49 ^b	22.91 ± 6.60 ^a
Orthophosphate (mg L ⁻¹)	24.00 ± 2.57 ^a	29.02 ± 1.97 ^a	25.35 ± 2.14 ^a	28.12 ± 2.04 ^a
SS (mL L ⁻¹)	13.19 ± 0.55 ^a	6.36 ± 0.44 ^b	6.90 ± 0.53 ^b	6.95 ± 0.52 ^b
TSS (mg L ⁻¹)	431.13 ± 69.87 ^a	309.22 ± 60.67 ^b	312.77 ± 59.10 ^b	306.98 ± 59.10 ^b
VSS (mg L ⁻¹)	217.49 ± 28.37 ^a	107.71 ± 24.22 ^b	108.92 ± 25.67 ^b	83.76 ± 10.09 ^c
Settling chamber use (h Kg shrimp ⁻¹)	190.21 ± 10.73 ^a	79.01 ± 11.27 ^b	78.86 ± 9.29 ^b	82.08 ± 2.30 ^b
Water consumption (L Kg shrimp ⁻¹)	235.42 ± 8.34 ^a	171.08 ± 8.26 ^c	167.77 ± 11.33 ^c	203.38 ± 7.66 ^b
Water replenishment (% day ⁻¹) ¹	1.37 ± 0.02 ^a	1.07 ± 0.05 ^b	1.00 ± 0.10 ^b	1.02 ± 0.11 ^b

The data correspond to the mean of thirty replicates ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly ($p < 0.05$). Results from one-way ANOVA and Tukey's test. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m⁻²); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m⁻²) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m⁻²).

Abbreviations: DO = Dissolved oxygen; TAN = Total ammonia nitrogen; SS = Settleable solids; TSS = Total suspended solids; VSS = Volatile suspended solids.
¹Water replenishment (% day⁻¹) is the percentage of freshwater used to compensate the evaporation losses.

3.4. Proximate composition

The average values of the proximate composition of the microbial flocs are summarized in Table 3. The moisture results ranged from 87.71 to 88.96%, protein from 13.32 to 20.15%, lipid from 3.43 to 6.33%, fiber from 4.09 to 4.42%, and ash from 22.87 to 26.53%. There was a significant difference ($p < 0.05$) between treatments and the lowest levels of protein and lipid were observed in the treatment with the highest density of oysters (MB-300), as well as the highest ash level.

3.5. *Vibrio* count

The initial mean concentration (CFU mL⁻¹) of the *Vibrio* spp. in the water was 0.79×10^3 CFU mL⁻¹, with 83.5% negative sucrose colonies (green), however, at the end of the culture there was an increase in the total concentration ($p < 0.05$), to between 0.85 and 1.42×10^3 CFU mL⁻¹, but the number of negative sucrose colonies declined to 0.00–27.77% (Table 4). The same occurred for mean concentration of *Vibrio* spp. (CFU g⁻¹) in shrimp hepatopancreas and oyster. In addition, at the end of the culture there was a significant difference ($p < 0.05$) between shrimp monoculture and the multitrophic biofloc treatments in the water and shrimp samples, where the treatments with *Crassostrea* sp. had a lower concentration of *Vibrio*, but they did not differ from each other ($p > 0.05$).

3.6. Total hemocyte count for shrimp and oysters

No significant differences were observed for the total hemocyte count, with an average of 33.73 and 2.65×10^6 cells mL⁻¹, for shrimp and oysters, respectively (Fig. 5).

4. Discussion

Even though oysters can feed in turbid waters, it was observed that the level of solids influences the survival of these bivalves, regardless of size, which was confirmed by the negative correlation found between the survival of oysters and the amount of settleable solids. From the results obtained in the survival test at different levels of solids, juvenile oysters (3.5 cm in length) were selected to conduct the experiment in a multitrophic biofloc system.

The water quality variables remained within the ideal range for the shrimp culture during the experimental period (Samocho et al., 2017). Due to the application of sodium bicarbonate, fluctuations in pH and alkalinity were not observed during culture and they remained in the ideal range for nitrification (100–150 mg CaCO₃ L⁻¹), while these variables tend to decline in intensive systems (Ebeling et al., 2006; Samocho et al., 2017) because of the microbial biomass consumption.

The nitrogen compounds remained within the ideal range for the shrimp culture (Samocho et al., 2017), indicating their efficient transformation (Ebeling et al., 2006), using organic fertilization (anaerobic and aerobic) and added bacteria. But in the treatment at a density of 300 oysters m⁻², the TAN values in the last weeks were above the ideal range recommended by Lin and Chen (2001) and Samocho (2019), and the nitrite-N and nitrate-N values were also higher. This increase may be the result of the intense filtration rate of oysters in the treatment with greater density, where the higher removal of solids, and consequently of bacteria present in the flocs can harm the nitrification processes, increasing ammonia and nitrite levels (Ebeling et al., 2006; Samocho et al., 2017). The higher density also contributed to an increase in the amount of oyster excreta in the system.

Thus, the treatments with lower densities more effectively maintained water quality, and had lower levels of TAN and nitrite-N than the monoculture (Control). Control of these variables is extremely important, especially TAN, as it is toxic and can damage shrimp gills, reduce growth and lead to mortality (Samocho et al., 2017). Ramos et al. (2010) also found that oysters (*Crassostrea rhizophorae*), were able to reduce nitrogen compounds in the effluent from shrimp culture.

Intensive systems tend to accumulate solids in their environment due to reduced water exchange, addition of organic carbon, uneaten feed, and fecal matter (Samocho, 2019). All treatments showed this increase, but in multitrophic biofloc treatments with *Crassostrea* sp. the total suspended solids (TSS) and settleable solids (SS) were lower than in the shrimp monoculture ($P < 0.05$). In multitrophic biofloc treatments, the solids remained controlled by the filtering action of the mollusks, which can reduce the suspended particles. Oysters have the ability to improve water quality by transforming suspended particulate matter, coagulating small particles into larger, more complex particles, through the aggregation of expelled material (Smaal et al., 2019; Kautsky and Evans, 1987). Furthermore, as observed in this study, Omont et al. (2020), in a multitrophic system, observed that the

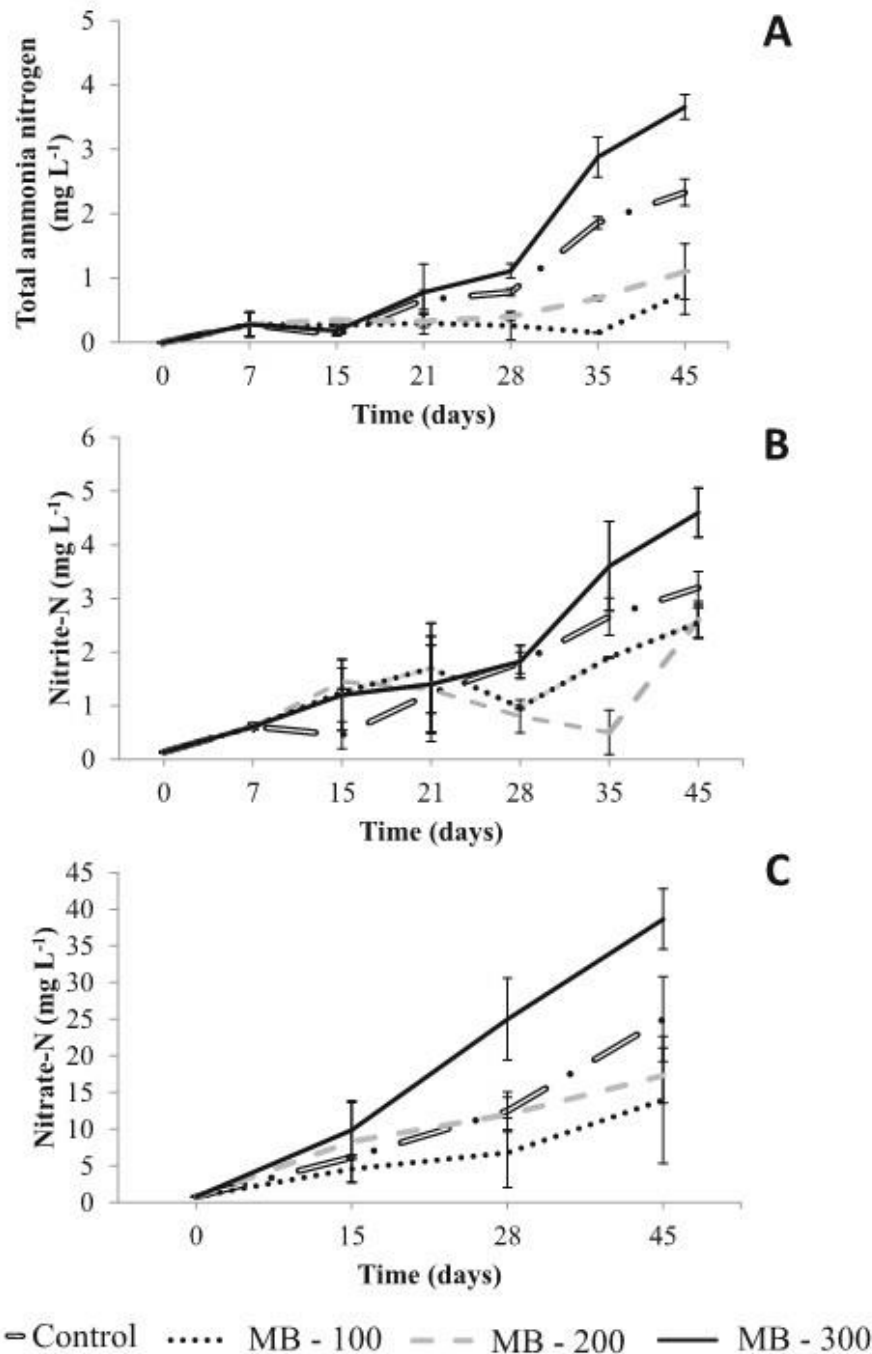


Fig. 3. Variations in the average concentrations of total ammonia nitrogen (A), nitrite-N (B) and nitrate-N (C) in multitrophic biofloc system during 45 days. Error bars indicate the standard deviation. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

presence of oysters (*Crassostrea gigas*) decreased settleable solids by half the level found in a traditional shrimp monoculture system. In an intensive system, [Ekasari et al. \(2014\)](#) found that the mussel (*Perna viridis*) can consume 36.0 to 57.2 g of TSS per kilogram of animal (wet weight), regardless of the floc size.

Particles suspended in water are ingested as food, passing through the gills, which act as a filter, concentrating organic particles, microalgae and planktonic organisms ([Ward, 1996](#); [Smaal et al., 2019](#)). For this reason, the lowest value of VSS (organic suspended solids) was found in the MB-300 treatment ($83.76 \pm 10.09 \text{ mg L}^{-1}$), while the

highest was found in the control treatment ($217.49 \pm 28.37 \text{ mg L}^{-1}$).

Settling chambers were used to control solids, however, there was nonetheless an increase in solids throughout the culture period in all of the treatments ([Fig. 4](#)). In the shrimp monoculture (Control), these solids accumulated faster than the others, reaching up to 20 mL L^{-1} in the last week of culture. Consequently, the control treatments had a longer settling chamber use ($190.21 \pm 10.73 \text{ h Kg shrimp}^{-1}$) and water consumption ($235.42 \pm 8.34 \text{ L Kg shrimp}^{-1}$). Strategies to reduce suspended particles can efficiently control solids, providing a substantial improvement in the concentration of the other water quality

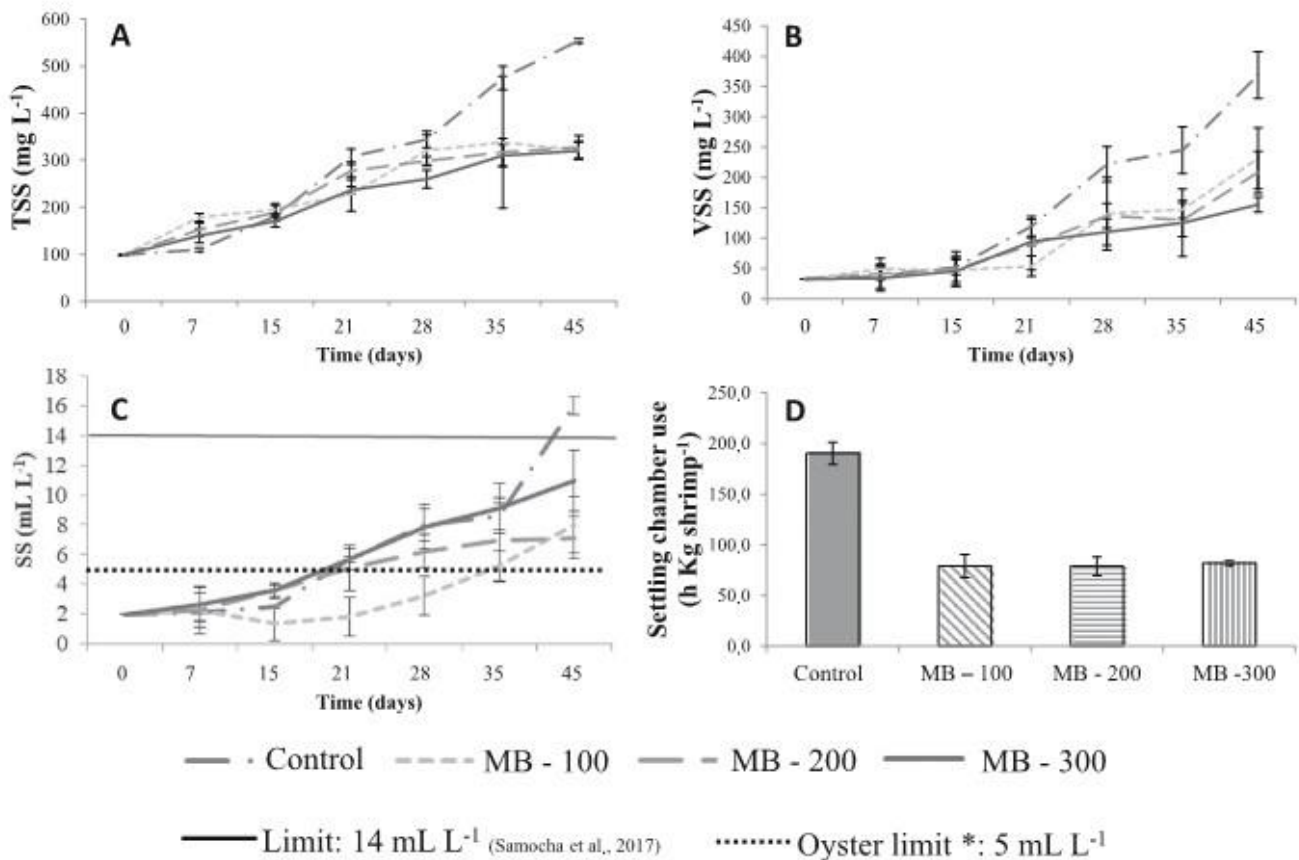


Fig. 4. Total suspended solids (A), volatile suspended solids (B), settleable solids (C) and settling chamber use (D) in a multitrophic biofloc system over the 45 days. ANOVA followed by Tukey's means comparison test, with a significance level of p of 0.05. Error bars indicate the standard deviation. * - Limit found in the juvenile survival test (5 mL L^{-1}). Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

parameters, and consequent improvement in shrimp production (Arantes et al., 2017).

The efficiency in conserving culture water can be measured by the amount of water used to produce shrimp biomass (Browdy and Moss, 2005). During culture, there was no water replacement, water was only added to compensate for evaporation losses, resulting in low water use

rates (approximately $1\% \text{ day}^{-1}$). In this study, due to the lower level of solids found in the oyster treatments and the final weights, the water consumption in the treatments with 100 and 200 oysters m^{-2} was lower than control and MB-300. Which were also lower than reported by Hargreaves (2013) and Samocha (2019) for shrimp nursery in non-multitrophic biofloc system (200 to 400 L Kg shrimp $^{-1}$).

Table 2
Shrimp and oyster performance cultured in nursery multitrophic biofloc system after a 45-day trial.

Parameters	Control	MB -100	MB - 200	MB - 300
Shrimp				
Initial weight (mg)	9.64 ± 0.23 ^a	9.64 ± 0.23 ^a	9.64 ± 0.23 ^a	9.64 ± 0.23 ^a
Final weight (g)	1.13 ± 0.05 ^a	1.18 ± 0.04 ^a	1.17 ± 0.04 ^a	0.94 ± 0.03 ^b
Survival (%)	92.67 ± 2.85 ^a	89.00 ± 1.53 ^a	90.67 ± 1.86 ^a	93.33 ± 1.76 ^a
FCR	1.00 ± 0.05 ^a	1.05 ± 0.03 ^a	1.01 ± 0.04 ^a	1.17 ± 0.03 ^b
SGR (% day $^{-1}$)	10.59 ± 0.11 ^a	10.69 ± 0.08 ^a	10.56 ± 0.07 ^a	10.18 ± 0.17 ^a
Yield(Kg m^{-3})	2.59 ± 0.13 ^a	2.60 ± 0.08 ^a	2.65 ± 0.09 ^a	2.26 ± 0.05 ^b
Oyster				
Survival (%)	-	93.94 ± 3.03 ^a	96.43 ± 3.57 ^a	83.33 ± 5.52 ^b
Final weight(g)	-	18.69 ± 0.59 ^a	12.66 ± 2.03 ^a	17.35 ± 1.20 ^a
Oyster meat weight (g)	-	1.30 ± 0.09 ^a	0.99 ± 0.41 ^a	1.11 ± 0.03 ^a
Final length (cm)	-	3.81 ± 0.17 ^a	3.77 ± 0.29 ^a	3.87 ± 0.07 ^a
Cumulative growth (%)	-	18.58 ± 5.41 ^a	17.65 ± 1.44 ^a	20.50 ± 2.17 ^a

The data correspond to the mean of thirty replicates ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly ($p < 0.05$). Results from one-way ANOVA and Tukey's test. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

Abbreviations: FCR = feed conversion ratio; SGR = specific growth rate.

Table 3

Proximate composition (% dry weight) of microbial floc from multitrophic biofloc system culture of *Litopenaeus vannamei* and *Crassostrea* sp. oyster.

Proximate composition ¹ (%)	Treatments			
	Control	MB -100	MB - 200	MB - 300
Moisture	88.40 ± 0.34 ^a	88.88 ± 0.02 ^a	88.46 ± 0.12 ^a	88.46 ± 0.23 ^a
Crude protein	19.67 ± 0.29 ^a	18.88 ± 0.55 ^a	17.99 ± 0.51 ^b	14.96 ± 0.94 ^c
Lipid	6.14 ± 0.51 ^a	6.06 ± 0.37 ^a	5.68 ± 0.26 ^a	3.71 ± 0.22 ^b
Fiber	4.12 ± 0.94 ^a	4.22 ± 0.55 ^a	4.35 ± 0.47 ^a	4.39 ± 0.95 ^a
Ash	22.03 ± 0.32 ^b	22.01 ± 0.11 ^b	22.97 ± 0.31 ^b	26.15 ± 0.22 ^a

The data correspond to the mean of thirty replicates ± standard deviation by treatments. Mean values in the same row with different superscript letters differ significantly ($p < 0.05$). Results from one-way ANOVA and Tukey's test. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

¹ Except for moisture (%), the other values are expressed in % of dry matter.

Table 4

Vibrio count of water, shrimp and oyster samples from multitrophic biofloc system.

	Initial	Final			
		Control	MB - 100	MB - 200	MB - 300
Water (10^3 CFU mL^{-1})					
Positive sucrose	0.13 (16.46%)	1.42 (100%)	0.85 (100%)	0.92 (100%)	0.65 (72.22%)
Negative sucrose	0.66 (83.54%)	0.00 (0.00%)	0.00 (0.00%)	0.00 (0.00%)	0.25 (27.77%)
Total	0.79	1.42 ^a	0.85 ^b	0.92 ^b	0.90 ^b
Shrimp (10^3 CFU g^{-1})					
Positive sucrose	0.13 (35.14%)	3.95 (78.06%)	1.21 (93.08%)	1.47 (96.71%)	0.84 (95.45%)
Negative sucrose	0.24 (64.86%)	1.11 (21.94%)	0.09 (6.92%)	0.05 (3.29%)	0.04 (4.55%)
Total	0.37	5.06 ^a	1.30 ^b	1.52 ^b	0.88 ^b
Oyster (10^3 CFU g^{-1})					
Positive sucrose	1.23 (40.07%)	-	6.87 (62.97%)	6.30 (71.03%)	9.54 (68.00%)
Negative sucrose	1.84 (59.93%)	-	4.04 (37.03%)	2.57 (28.97%)	4.49 (32.00%)
Total	3.07	-	10.91 ^a	8.87 ^a	14.03 ^a

The data correspond to the mean ± standard deviation of triplicate determinations by treatments. Results from Kruskal-Wallis and Dunn's test. Mean values in the same row with different superscript letters differ significantly ($\alpha < 0.05$).

-: There are no oysters in this treatment.

Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

In relation to shrimp zootechnical performance, all treatments were similar to the results of other culture studies of *L. vannamei* in intensive systems, with final weights between 0.69 and 1.08 g, productivity between 1.21 and 2.42 $Kg m^{-3}$, FCR up to 1.37 and survival between 71.3 and 95.6% (Brito et al., 2016; Abreu et al., 2019), with density from 2500 to 3000 shrimp m^{-3} . The multitrophic biofloc system with *Crassostrea* sp. at stocking densities of 100 and 200 oysters m^{-2} did not affect the performance of the shrimp, however, the treatment with 300 oysters m^{-2} had the lowest values for final weight (0.94 g) and productivity (2.26 $kg m^{-3}$), and the highest feed conversion factor (1.17), probably, due to the high values of nitrogen compounds.

Oyster survival was greater than 80% in all treatments, even when the settleable solids exceeded the limit established in the pre-test (5 $mL L^{-1}$). However, density influenced survival and the MB-300 treatment had the highest mortality. Modesto et al. (2010), when culturing oysters (*C. rhizophorae*) in shrimp pond effluents, also found that an increase in stocking density reduced survival, there highest density (750 oysters m^{-2}) had 83.4% mortality, compared to 55.4% at 250

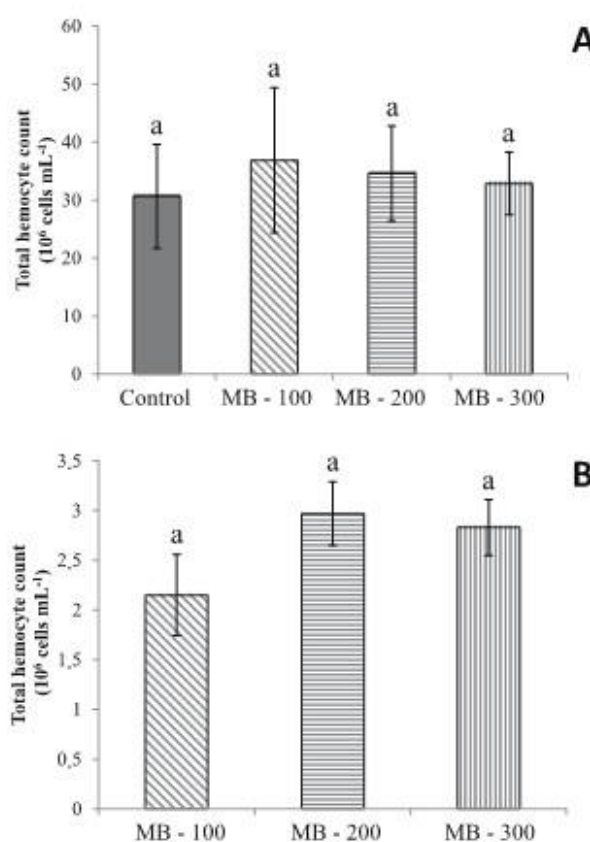


Fig. 5. Total hemocyte count of shrimp (A) and oysters (B), at 45 days in a multitrophic biofloc system. ANOVA followed by Tukey's means comparison test, with a significance level of p of 0.05. Control (Shrimp monoculture); MB-100 (Multitrophic biofloc with oysters in the stocking density of 100 oysters m^{-2}); MB-200 (Multitrophic biofloc with oysters in the stocking density of 200 oysters m^{-2}) and; MB-300 (Multitrophic biofloc with oysters in the stocking density of 300 oysters m^{-2}).

oysters m^{-2} after three months. As for the growth of oysters, proximate composition of particles is important during filtration, thus, the microbial flocs, as the only food source for the oysters in the multitrophic system, could be a limiting factor, as reported by Omont et al. (2020). In this study, there was no influence of stocking density and the average growth in length was 6.1 mm over 45 days, similar to that reported by Modesto et al. (2010) for *C. rhizophorae* culture (4.3–4.4 mm month⁻¹).

In terms of the proximate composition of microbial flocs, it was observed that the stocking density of oysters influenced the protein, lipid and ash contents. The lowest levels of protein and lipid were found

in the treatment with a density of 300 oysters m^{-2} , while for ash content the opposite was observed, where the highest value was found in this treatment. The filtration of particles captured, ingested or rejected by mollusks, can affect the quantity and quality of material that is recycled in the water column (Deslous-Paoli et al., 1992; Smaal et al., 2019). This is because the nutritional value of particles from solubilized pseudofeces is lower than that of the original particles, because the organic content is lower than the mineral content (Deslous-Paoli et al., 1992).

The microbial flocs in an intensive system have greater fluctuations in their proximate composition, varying according to the carbon source, proximal feed composition, environmental conditions, culture time, and other factors (Emerenciano et al., 2013; Ekasari et al., 2014). *L. vannamei* juveniles are able to satisfy up to 30% of their needs with microbial flocs (Burford et al., 2004), however, these are insufficient for meeting the entire nutritional requirements of shrimps (Marinho et al., 2014). In this study, the values found in treatments, except in MB-300, were close to those found by Abreu et al. (2019) in the *L. vannamei* culture under biofloc system with the addition of *Navicula* sp. and molasses fertilization (17.2 to 22% crude protein and 6.3 to 9.8% lipid).

In the intensive system, high densities and quantities of organic matter favor the survival and spread of pathogens, such as *Vibrio* and *Aeromonas* (Yanong and Erlacher-Reid, 2012). The genus *Vibrio* spp. occurs naturally in marine environments, and has also been reported as one of the bacteria present in the intestinal and hepatopancreatic bacterial community of shrimp (Huang et al., 2016; Aguilera-Rivera et al., 2019) and to be predominant in microbial aggregates (floc) (Manan et al., 2017). However, these microorganisms can reduce shrimp growth and survival (Tran et al., 2013). Some species of this genus, microbiologically characterized as negative sucrose, are associated with lethal bacterial infections in aquatic organisms, such as *Vibrio vulnificus*, *V. harveyi* and *V. parahaemolyticus* (Thompson et al., 2006; Chatterjee and Haldar, 2013). Colony coloration is due to the ability to consume sucrose from the medium, where *Vibrio* that are able to use sucrose will form yellow colonies (positive sucrose), while strains that are unable will form green colonies (negative sucrose) (Thompson et al., 2006).

In this study, the amount of *Vibrio* spp. increased throughout the culture period, due to the increase in solids, however, there was a change in the proportion of groups. In the beginning, most were composed of negative sucrose colonies (59.93 to 83.54%), while in the end, positive sucrose colonies were predominant (62.97 to 100%), this was observed for water, shrimp and oysters. Organic fertilized (anaerobic and aerobic) and the addition of beneficial microorganisms, such as *Bacillus* and *Saccharomyces*, may have suppressed the development of negative sucrose bacteria (*Vibrio* spp.) by specific competitions. The bacteria used in the formulation of probiotics and bioremediators can help control outbreaks of *Vibrio* spp. (Aguilera-Rivera et al., 2014; Hostins et al., 2017), because the addition of beneficial species increases competition for nutrients and substrate (Zorriehzahra et al., 2016).

At 45 days of culture, the colony-forming unit in oysters had grown 188.92% to 357.00% above the level at the beginning of culture. The microbiota of oyster meat may be directly associated with the environment in which it originates. Since mollusks, as filtering organisms, have the ability to retain particles and concentrate bacteria present in water in their intestine (Vieira et al., 2007), they function as a reservoir of innumerable pathogens (Burge et al., 2016). For this reason, in multitrophic biofloc culture with oysters the concentration of colony-forming units was lower than the control, in both water and the shrimp hepatopancreas, with a reduction of 37.32 and 75.69%, respectively. The *Vibrio* genus includes more than 30 species, and at least 12 are pathogenic to humans (Thompson et al., 2006), however, there is no legislation that regulates the tolerable levels of *Vibrio* spp. in raw bivalve mollusks, only for *V. parahaemolyticus* (1×10^4 per gram), *V. cholerae* (presence of organism) and *V. vulnificus* (30 Most Probable Number per gram) (FDA, 2019).

Crustaceans and mollusks have only innate immune systems and hemocytes are the main immunocompetent cell in these organisms (Pipe and Coles, 1995; Jiravanichpaisal et al., 2006). In an intensive culture, the microbial community and the bioactive compounds in the flocs can have a positive effect on shrimp health (Xu and Pan, 2013), stimulating the immune system (Kim et al., 2014) and increasing the total hemocyte count.

In this study, the multitrophic biofloc system with oysters did not influence the hemocyte count of shrimp, and the average values found in this study were close to those found by Abreu et al. (2019), from 22.84 to 41.25×10^6 cells mL^{-1} , and higher than those found by other authors in the traditional system (12.4×10^6 cells mL^{-1}) (Javahery et al., 2019). Meanwhile, for oysters, there is a high inter-individual variability in the number of circulating hemocytes. In this study the average found was 2.65×10^6 cells mL^{-1} , which is close to that found by other authors for healthy oysters of the genus *Crassostrea* sp., 2.50 and 2.52×10^6 cells mL^{-1} , to *C. gigas* and *C. hongkongensis*, respectively (Simões et al., 2015; Li et al., 2018).

5. Conclusions

This study shows that in the shrimp and oyster multitrophic biofloc system, stocking densities of 100 and 200 oysters m^{-2} can be used without compromising shrimp growth, survival and yield. In addition, this is a viable alternative for maintaining nitrogen compounds and controlling solids in a shrimp nursery with a biofloc system. However, high oyster density (300 oysters m^{-2}) can reduce water quality and flocs nutritional value due to intense filtration rate. The technology developed in this study creates possibilities for use of multitrophic biofloc shrimp and oyster cultures. Further research is needed on multitrophic biofloc system with oysters at other phases of culture, as well as the commercial application of this system and an economic evaluation.

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Credit author statement

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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 paper.

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