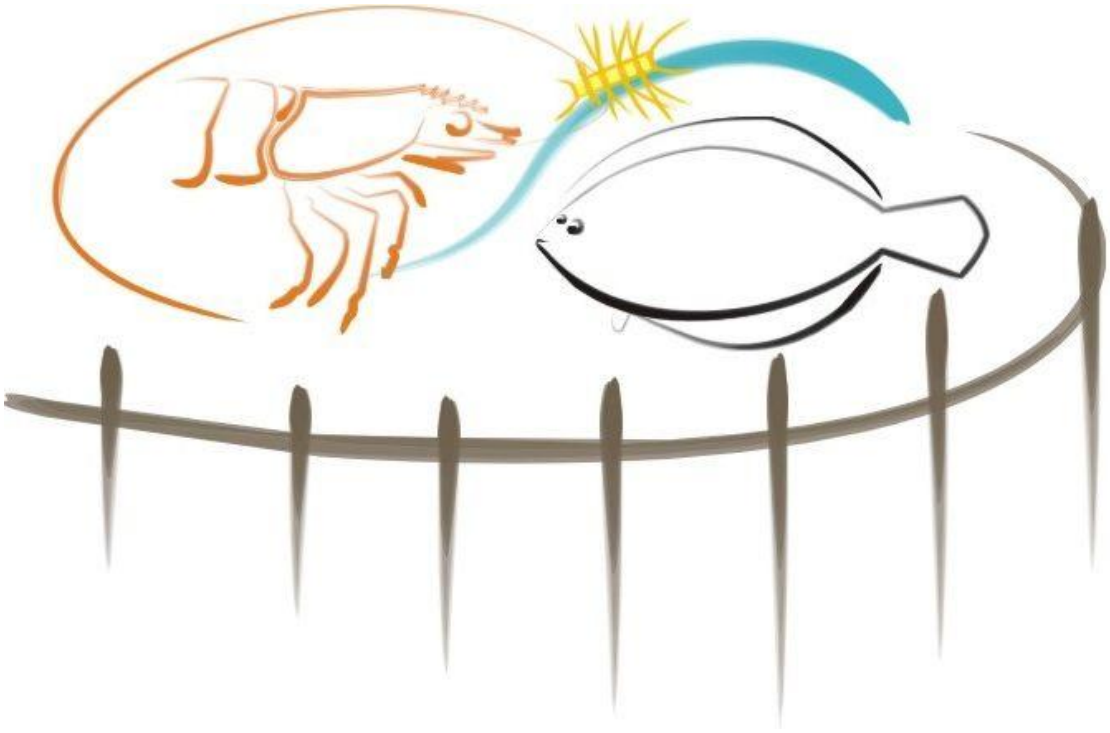
UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS- GRADUAÇÃO EM AQUICULTURA



CONTRIBUIÇÃO DAS DIATOMÁCEAS E SUBSTRATOS ARTIFICIAIS NO DESEMPENHO DOS CAMARÕES *Litopenaeus vannamei* CULTIVADOS EM SISTEMAS COM BIOFLOCOS

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TESE DE DOUTORADO

CONTRIBUIÇÃO DAS DIATOMÁCEAS E SUBSTRATOS ARTIFICIAIS NO DESEMPENHO DOS CAMARÕES *Litopenaeus vannamei* CULTIVADOS EM SISTEMAS COM BIOFLOCOS

TATIANA GERMANO MARTINS MACHADO

Tese apresentada ao Programa de Pós-graduação em Aquicultura da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de DOUTOR.

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Coorientador: Wilson Wasielesky Jr. (FURG)

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A minha amada filha Ana Luísa

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RESUMO

A aquicultura é a atividade de produção animal com maior taxa de crescimento no mundo e a carcinocultura movimenta alto valor comercial. O avanço tecnológico permitiu o desenvolvimento de sistema de cultivo super-intensivo sem renovação de água na presença de bioflocos, denominado de sistema BFT (*Biofloc Technology*). Esse sistema apresenta grandes vantagens, pois reduz a emissão de efluentes pelo reuso da água por vários ciclos de produção, diminuindo os riscos de disseminação de doenças e provendo benefícios nutricionais. A microbiota contribui na manutenção da qualidade de água e serve de alimento suplementar para os animais cultivados. Neste trabalho foi avaliada a influência da presença de diatomáceas (*Amphora coffeaeformis Cylindrotheca closterium, Conticribra weissflogii*) e substrato artificial na formação e qualidade nutricional dos bioflocos e no desempenho de camarões *Litopeneaus vannamei* (Boone,1931) cultivados em BFT. Os experimentos foram realizados em tanques retangulares (0,5 m^2; 200 L) no interior de estufa com os objetivos de (1) avaliar a contribuição das diatomáceas para a formação dos bioflocos e desempenho do crescimento de juvenis do camarão em BFT; (2) testar se a seleção das espécies de diatomáceas pode melhorar o desempenho de crescimento de juvenis do camarão em BFT; e (3) comparar o efeito da adição de diatomáceas e de substrato artificial em BFT no desempenho de crescimento deste camarão. Foi constatado que a manutenção de diatomáceas em alta densidade celular requer a adição de sílica e contribui para o desempenho dos camarões. A adição de *Amphora coffeaeformis* e *Conticribra weissflogii* resultou no melhor crescimento dos camarões, que apresentaram elevadas taxas de sobrevivência, ganho de peso, biomassa média final e eficiente conversão alimentar. Os níveis de lipídeos (2,64 a 5,11 %) incluem os ácidos graxos essenciais, destacando-se o ácido linoleico (C 18:02) e o ácido eicosapentanóico (EPA) (C 20:05 n3). A presença das diatomáceas foi mais importante do que o uso de substratos artificiais para a melhoria do desempenho do crescimento do camarão *L. vannamei*. Em resumo, os resultados obtidos auxiliam no aprimoramento do BFT, e indicam que a adição de silica é necessária para manter a presença de diatomáceas em alta densidade. A seleção de espécies de diatomáceas é importante para o seu próprio melhor crescimento e desempenho do camarão. Por último, destaca-se que a adição de diatomáceas é mais importante do que o substrato artificial, visando uma melhor eficiência e desempenho em sistema de cultivo com bioflocos.

ABSTRACT

Aquaculture is the activity of animal production with highest growth rate in the world and carcinoculture drives high commercial value. The technological development of super-intensive farming without water renewal in the presence of biofloc (called Biofloc Technology, BFT) effectively reduces the emission of effluents for water reuse for several production cycles, reduces the risk of disease and provides nutritional benefits. The microbiota contributes to the maintenance of water quality and serves as supplementary food for farmed animals. This study aims to evaluate the influence of the presence of diatoms (*Amphora coffeaeformis Cylindrotheca closterium, Conticribra weissflogii*) and artificial substrate in the formation and nutritional quality of the bioflocs and the performance of the shrimp *Litopeneaus vannamei* (Boone) grown under BFT. The assays were performed in rectangular tanks (0,5 m2, 200 L) in a greenhouse, with the objectives of (1) evaluating the contribution of diatoms to the formation of bioflocos and the growth performance of shrimp juveniles; (2) testing whether the selection of diatoms is important to improve the growth performance of juvenile shrimp and (3) comparing the effect of adding diatoms and artificial substrate on the growth performance of juvenile shrimp in BFT system. It was found that the maintenance of diatoms in high cell density requires the addition of silica and contributes to better results on the performance of prawns. Among the tested diatoms, *Amphora coffeaeformis* and *Conticribra weissflogii*, were those that contributed most to the growth of shrimp, which showed high rates of survival, weight gain, final average biomass and efficient feed conversion. Lipids level (2,64 to 64, 5, 11) included the essential fatty acids linoleic acid (C 18:02) and eicosapentaenoic acid (EPA) (n3 C 20:05). Moreover, the presence of these diatoms was more important than the use of artificial substrates to improve the growth performance of the shrimp *L. vannamei*. In summary, the results enable the improvement of the BFT culture system, and suggest that sílica addition is necessary to maintain diatoms in high density. Furthermore, the selection of diatom species is important for there own best growth and performance of the shrimp. Finally, it is noteworthy that the addition of diatoms is more important than the artificial substrate, aiming at better efficiency and performance of the bioflocs culture system.

INTRODUÇÃO GERAL

A aquicultura é a atividade de produção animal com maior taxa de crescimento no mundo (8, 3% ao ano), atingindo em 2008 uma produção total de 68, 3 milhões de toneladas e um valor financeiro estimado em US$ 105,8 bilhões (FAO, 2010). As previsões indicam que no ano 2030 a produção gerada pela aquicultura será de 83 milhões de toneladas, tornando-se importante ferramenta para a geração de empregos, representando uma nova opção sócia- econômica para comunidades litorâneas (FAO, 2009). Entre as diversas atividades aquícolas, a carcinocultura destaca-se no setor devido ao alto valor comercial que os crustáceos atingem no mercado, cuja produção no ano de 2008 gerou US$ 22,7 bilhões (FAO, 2010).

A carcinocultura, até os anos 80, utilizava práticas extensivas de cultivo, assim fazendas expandiam suas áreas, como estratégia para aumentar a produção (Hargreaves, 2006). No entanto, com o avanço da tecnologia, principalmente através do uso de aeradores, dietas comerciais e alta taxa de renovação de água (10 – 15% diária), muitos produtores aumentaram a densidade de estocagem, passando a operar em sistemas semi-intensivos e intensivos (Mishra et al., 2008).

Porém, os sistemas intensivos na aquicultura são uma fonte potencial de poluição aos corpos hidricos, pois os seus efluentes são caracterizados por altas concentrações de nitrogênio (N), fósforo (P) e material em suspensão, consequentemente altos valores de demanda bioquímica de oxigênio (DBO) (Jackson et al., 2003; Cohen et al., 2005).

Além da descarga de nutrientes,os cultivos intensivos aumentam os riscos de invasão de espécies exóticas, a disseminação de patógenos e a dependência de farinha de pescado como fonte de proteína (Naylor et al., 2000; Boyd, 2003). Esses problemas exigem a aplicação de novas técnicas para a manutenção da qualidade da água e

sustentabilidade dos recursos hídricos, conjugado a rentabilidade do cultivo por meio de densidades de estocagem e alimentação adequadas (Gaona, 2011), além de mínima ou nehuma taxa de renovação de água ao longo do período de cultivo (Hopkins et al., 1995; Wasielesky et al., 2006).

Para minimizar ou evitar as emissões de efluentes, foi desenvolvido um sistema de cultivo super-intensivo mas sem renovação de água na presença de bioflocos, denominado de sistema BFT (*Biofloc Technology*). Neste sistema, a mesma água do cultivo é utilizada por vários ciclos de produção, onde a microbiota contribui na manutenção da qualidade de água e serve de alimento suplementar para animais cultivados diminuindo os riscos de disseminação de doenças e provendo benefícios nutricionais. (Wasielesky et al., 2006; Avnimelech et al., 2007; Ballester et al., 2010). O BFT necessita intensa aeração, mínima troca de água e a fertilização com fontes de carbono para permitir a formação de bioflocos, os quais são constituídos basicamente por bactérias, microalgas, protozoários, larvas de invertebrados, exoesqueletos e restos de animais mortos, predominando uma biota aeróbica e heterotrófica (De Schryver et al., 2008).

A utilização do sistema BFT tem mostrado eficientes resultados na produção de camarões. Silva et al. (2013) observaram que camarões incorporam 35% do total de fósforo do sistema em biomassa. Burford et al. (2004) relataram que até 29% do alimento consumido pelo camarão branco *Litopenaeus vannamei* pode ser proveniente dos bioflocos presentes no meio de cultivo. McAbee et al. (2003) verificaram sobrevivências superiores a 90% em cultivo super-intensivo de juvenis de *L. vannamei* em sistema de raceway encoberto. Wasielesky et al. (2006) confirmaram o benefício da produtividade natural desse tipo de sistema em relação à sobrevivência, crescimento,

ganho de peso, consumo alimentar e na taxa de conversão alimentar para juvenis de *L.vannamei.*

O sistema BFT têm a vantagem de incrementar a dieta através da produtividade natural presente nos viveiros (Moss et al., 2001; Burford et al., 2003), propiciando a utilização de rações com menor teor de proteína bruta (Moss et al., 2001, 2002; Samocha et al., 2004; Ballester et al., 2007). Uma importante fonte de alimento nos bioflocos são as microalgas, que são imprescindíveis para uma boa produção e manejo alimentar em larviculturas de crustáceos, moluscos e peixes (Barbieri & Ostrensky, 2001). As microalgas são fontes de macronutrientes, vitaminas e elementos-traço, pois são ricas em proteínas, carboidratos, e especialmente ácidos graxos poliinsaturados que são essenciais para o crescimento e metamorfose de larvas de peixes (Koven et al., 1989), camarões e moluscos (De Pauwn et al., 1988; Valenzuela-Espinoza et al., 2002; Silva et al., 2009). Algumas espécies contêm grande quantidade de ácidos graxos poliinsaturados de alta qualidade (Guschima & Harwood, 2006) e proteínas (Silva et al., 2009).

As microalgas têm sido empregadas na alimentação direta e indiretamente, como na produção de zooplâncton, manutenção da qualidade da água em cultivos usando a "técnica de água verde" ou mesocosmos (Lavens & Sorgeloos, 1996; Amjad & Jones, 1994; Reitan et al., 1997), no balanço do pH por removerem o excesso de dióxido de carbono (Hoff & Snell, 1987). Além disto, algumas espécies possuem uma função bacteriostática e outras combatem bactérias patogênicas pela produção de substâncias antibióticas (Reitam et al., 1997). Dietas artificiais geralmente carecem de pigmentos que dão aos organismos, como o salmão e truta, a sua coloração típica. Esses pigmentos são produzidos por microalgas e os carotenoides como a astaxantina podem ser oferecidos na dieta para intensificar a coloração dos animais. Rações contendo 5 a 20%

de *Arthrospira* (cianobactéria rica em carotenos) intensificam os padrões de vermelho e amarelo em carpas bem como o brilho das partes brancas. Essa definição de cor aumenta o valor de venda (Spolaore et al., 2006)

Entre as microalgas, as diatomáceas destacam-se como o grupo mais representativo no plâncton marinho. São reconhecidos 285 gêneros de diatomáceas de hábitos planctônico ou bentônico, envolvendo entre 10.000 e 12.000 espécies (Round et al., 1990). As diatomáceas apresentam parede celular (frústulas) compostas de sílica amorfa hidratada, seus principais pigmentos fotossintéticos são clorofilas *a* e *c*, *β* caroteno, fucoxantina, diatoxantina e diadinoxantina, e seus produtos de reserva são crisolaminarina e gotículas de óleo. O crescimento das diatomáceas e o seu conteúdo de silício são determinados por vários fatores, como (1) o suprimento de nutrientes, destacando-se o nitrogênio, fósforo e sílica, (2) variações nas condições de luz, temperatura e pH, (3) variações bióticas como a densidade da cultura, diâmetro de suas valvas e em conseqüência o volume e superfície da célula, e estágio de vida (Werner, 1977). Na água do mar, as formas mais abundantes e estáveis do silício são o silicato (SiO_3^{2-}), dióxido de silício (SiO_2) e ácido ortossilícico (H_4SiO_4). A demanda das diatomáceas por silício é tão alta quanto à de nitrogênio (Brzezinski, 1985), de forma que os dois elementos devem ser oferecidos em proporção igual (N:Si 1:1). Contudo na maioria dos meios de cultura, o silício é adicionado em concentração mais baixa do que a de nitrogênio, eventualmente equivalente a apenas 1/3 ou 1/4 das concentrações de nitrogênio (Lourenço, 2006).

As diatomáceas são tradicionalmente classificadas em dois grandes grupos: diatomáceas cêntricas, com frústulas de simetria radial, e diatomáceas penadas, com frústulas de simetria bilateral. As diatomáceas cêntricas são predominantemente

planctônicas, e a maioria habita ambientes com baixa concentração de matéria orgânica dissolvida, enquanto que as diatomáceas penadas são predominantemente bentônicas ou epífitas, habitando ambientes ricos em matéria orgânica dissolvida (Werner, 1977; Round et al., 1990). As diatomáceas bentônicas apresentam uma fenda longitudinal, designada de rafe, através da qual são liberadas substâncias extracelulares poliméricas (EPS) (Smith & Underwood, 1998), que formam uma mucilagem que se liga aos filamentos de actina, gerando a força necessária ao movimento de deslizamento e a consequente adesão ao substrato. Os EPS são liberados paralelamente a rafe como resultado das contrações dos microfilamentos do citoesqueleto (Edgar & Pickett – Heaps, 1984). Algumas espécies de diatomáceas cêntricas também produzem EPS, como os gêneros *Thalassiosira* e *Conticriba*. Os EPS são moléculas de polissacarídeos cadeia longa (95%) compostas por açúcares neutros, ácido úrico e/ou açúcares sulfatados (Salt, 2003). Os hidratos de carbono dividem-se normalmente em duas frações, as de baixo peso molecular (solúveis em álcool; "soluble EPS') e as de elevado peso molecular (não solúveis em álcool; "bound EPS") (Orvain et al., 2003). As diatomáceas bentônicas produzem diferentes tipos de EPS quanto a sua estrutura e composição dos hidratos de crabono, cuja produção varia com as condições ambientais (Staats et al., 2000; Smith & Underwood, 2000; De Brouwer & Stal, 2002). Os principais fatores que infuenciam a quantidade e a composição química dos EPS são a radiação, disponibilidade de nutrientes, fase de crescimento e os ritmos de migração vertical associados à fotossíntese. Segundo Underwood et al. (2004) as diatomáceas bentônicas produzem maior concentração de EPS quando em situações de limitação de nutrientes. Assim, ao produzirem a matriz de EPS, as células criam um microambiente estável com condições ótimas para seu crescimento (Decho, 2000), importante na

adesão das células aos substratos (Daniel et al., 1987), na locomoção (Edgar & Pickett – Heaps, 1984) e na resistência a toxinas (Decho, 1990).

As diatomáceas bentônicas são os primeiros colonizadores autotróficos e os constituintes mais importantes na comunidade microfitobêntica do ambiente marinho (Patil & Anil, 2005). Além disto, a capacidade heterotrófica facultativa é bem difundida entre as diatomáceas penadas, mas raramente em diatomáceas cêntricas (Lylis & Trainor, 1973; Lee et al., 1975).

Em viveiros, Moss et al. (1992) relataram que as diatomáceas freqüentemente dominam sob condição de alta concentração de matéria orgânica em suspensão. Juvenis de *Farfantepenaeus paulensis* cultivados em substratos artificiais contendo biofilme demonstraram uma preferência por forragear diatomáceas cêntricas (Ballester et al., 2007; Silva et al., 2009). Por outro lado, Godoy et al. (2012) demonstraram que os juvenis de camarões cultivados em ambiente contendo diatomáceas como suplemento, ganharam 17% mais peso e apresentaram conversão alimentar excelente (0,47), comparados àqueles cultivados em bioflocos, e de mistura entre os dois (conversão alimentar 0,76 e 0,80, respectivamente). Os camarões peneídeos são consumidores de diatomáceas em viveiros de cultivo, nos quais as mesmas provêem adequada nutrição por curto período de tempo, propiciando o crescimento de camarões juvenis, quando em falta de alimento peletizado (Moss, 1994). Jaime–Ceballos et al. (2006) utilizaram hepatopancreatina como reagente e observaram uma alta taxa (94%) de digestibilidade *in vitro* da proteína de *Chaetoceros muelleri* em pós-larvas de camarões. Moss (1994) mostrou que juvenis de *Litopenaeus vannamei* sobrevivem em monocultivo de diatomácea *Chaetoceros* sp. como única fonte de alimento, crescendo mais e exibindo concentração de ácidos nucléicos e razão RNA:DNA praticamente idêntica aos valores

encontrados nos camarões não alimentados com diatomáceas, mas cultivados em água proveniente de um cultivo intensivo rico em bioflocos.

Esses resultados apontam para a importância das diatomáceas como fonte de nutrientes essenciais e sua contribuição significativa para o melhor desempenho no crescimento dos camarões. Além disto, a presença de diatomáceas pode levar a uma redução de custos pelo menor uso de ração, mas também pela menor necessidade de aeração durante o dia, devido à produção de oxigênio pelo processo de fotossíntese (Godoy, 2008). No entanto, é difícil manter o crescimento das diatomáceas em sistemas de bioflocos (Godoy, 2008), possivelmente devido à elevada concentração de material em suspensão, que reduz a penetração da luz e inibe a sua fotossíntese ou devido ao requerimento de algum nutriente (sílica) não disponível em alta concentração no sistema.

O uso de substratos artificiais ainda foi pouco testado em cultivos de *L.vannamei* com bioflocos, embora tenha sido observada a sua eficiência (Browdy & Moss, 2005;). Substratos artificiais submersos são usados em cultivos intensivos de camarões marinhos e tilápias com o objetivo de mitigar os efeitos negativos do aumento da densidade de estocagem (Arnold et al., 2006) e fornecer uma superfície adicional para o camarão, minimizando a competição e interações comportamentais negativas, tais como canibalismo (Abdussamad & Thampy, 1994).

Uma comunidade complexa de organismos aquáticos vive aderida ao substrato submerso, denominada de perífiton ou biofilme (van Dam et al., 2002). Esta comunidade contém bactérias, fungos, protozoários, microalgas, zooplâncton, organismos bentônicos e detritos (Azim & Asaeda, 2005) e ajuda a controlar a qualidade da água (Arnold et al., 2009; Asaduzzaman et al., 2009), reduzindo a

ocorrência de bactérias patogénicas quer pela absorção do nitrogênio (Austin & Austin, 1999), pela produção de antibióticos por microalgas (Alabi et al., 1999) ou como fonte de alimento natural para a espécie de cultivo (Thompson et al., 2002; Abreu et al., 2007; Ballester et al., 2007; Silva et al., 2009). O uso de substrato artificial é freqüentemente associado com melhor desempenho de pós-larvas e juvenis de camarão marinho (Thompson et al., 2002; Arnold et al., 2006, 2009; Ballester et al., 2007).

O sistema BFT envolve a produção de densa comunidade microbiana que controla o teor de amônia liberada principalmente pelos organismos (De Schryver et al., 2008; Avnimelech, 2009), através da absorção por microalgas e bactérias heterotróficas ou transformação por bactérias nitrificantes (Ebeling et al., 2006). Ambos os bioflocos na coluna de água e no perifíton presente no substrato, cumprem funções similares no controle de qualidade de água, acelerando a remoção biológica de resíduos orgânicos e inorgânicos (Crab et al., 2007). A eficácia dos substratos artificiais em sistemas de cultivo de *Litopenaeus vannamei* com bioflocos foi somente testada por Schveitzer et al. (2013). Neste estudo, a presença de substrato artificial no BFT coincidiu com valores mais elevados de biomassa final, ganho de peso e taxa de sobrevivência, indicando que o substrato serviu para aumentar a superfície do tanque, reduzir a densidade relativa bem como o nível de stress dos camarões. Embora a utilização de substrato, em geral, seja considerada benéfica em culturas de camarão, em alguns estudos, a sua presença não afetou o desempenho dos animais (Sandifer et al., 1987; Samocha et al., 1993; Kumlu et al., 2001), nem a qualidade da água (Samocha et al., 1993). Portanto, existe a necessidade de aprofundar o conhecimento sobre o efeito da adição de diatomáceas e substrato em sistemas de produção BFT.

HIPÓTESES

Com a finalidade de responder às questões relacionadas as contribuições das diatomáceas e substrato artificial nos sistema BFT de cultivo de camarão branco *Litopennaeus vannamei*, serão testadas as seguintes hipóteses:

1) A permanência de diatomáceas melhora o desempenho dos camarões em sistema BFT;

2) As diatomáceas penadas apresentam um maior potencial para melhorar o desempenho dos camarões cultivados em sistema BFT do que as diatomáceas cêntricas;

3) A presença de substrato artificial melhora significativamente o desempenho dos camarões cultivados em sistema BFT;

Com base nessas hipóteses, o presente trabalho tem os seguintes objetivos geral e específicos:

OBJETIVO GERAL

Avaliar a influência da presença de diatomáceas e substrato artificial na formação e qualidade nutricional dos bioflocos e no desempenho de juvenis de camarão branco *Litopenaeus vannamei* cultivados em sistema BFT.

OBJETIVOS ESPECÍFICOS

a) Isolar, cultivar e identificar espécies nativas de diatomáceas adaptadas ao sistema com bioflocos;

b) Produzir camarões em sistema com bioflocos contendo as diatomáceas selecionadas e comparar com sistema padrão com bioflocos, usado na EMA;

c) Quantificar e caracterizar os microorganismos presentes nos bioflocos formados;

d) Avaliar a sobrevivência, ganho de peso, taxa de crescimento específico e taxa de conversão alimentar dos juvenis cultivados nos bioflocos formados;

e) Avaliar a utilização de substratos artificiais para a fixação de biofilme e no desempenho de juvenis de *L. vannamei* em sistema com bioflocos.

METODOLOGIA GERAL

1) Local da realização dos experimentos:

Os experimentos foram conduzidos em estufa localizada na Estação Marinha de Aquicultura, Instituto de Oceanografia da FURG (Fig. 1), em detalhe das unidades experimentais (Fig. 2) localizada na praia do Cassino, município de Rio Grande – RS.

Figura 1. Interior da estufa na Estação Marinha de Aquicultura, Instituto de Oceanografia da FURG.

Figura 2. Detalhe do microscosmo formado no interior da estufa para realização dos experimentos dessa Tese.

2) Obtenção dos animais experimentais:

Naúplios de *Litopenaues vannamei* foram adquiridos de laboratório comercial (Aquatec), e mantidos até a fase de pós-larvas no setor de larvicultura do do laboratório de Carcinocultura da Estação Marinha de Aquicultura, IO/ FURG. Ao atingir o tamanho utilizado nos experimentos, os juvenis foram transferidos para as respectivas unidades experimentais.

3) Contagem de microorganismos:

As contagens de microorganismos foram realizadas no Laboratório de Ecologia de Fitoplâncton e Microorganismos Marinhos da FURG. Para a caracterização e contagem das diatomáceas e microorganismos, subamostras (2,1 ml) fixadas com solução de lugol (2 %), foram levadas à câmara de sedimentação e contados no mínimo 10 a 15 campos por câmara, escolhidos aleatoriamente, utilizando microscópio invertido

Zeiss Axiovert equipado com contraste de fase e magnificação final de 400x, seguindo-se metodologia clássica (Utermöhl, 1958).

O biofilme das amostras de substrato artificial foi removido com auxílio de um aparelho de ultrasom (Ultrasonic Homogenizer 4710 Series, ColeParmer Instrument Co.) na amplitude de 20 Khz de 6-8 vezes em intervalos de 15 a 20 segundos seguidos, ou com 15 a 20 segundos de descanso para evitar aquecimento da amostra (Thompson et al., 2002).

4) Conteúdo de lipídeos e perfil de ácidos graxos dos bioflocos:

O conteúdo de lipídeos e perfil de ácidos graxos para os experimentos do Capítulo 2 e 3 desta Tese foram realizados nos Laboratórios Kolbe e LACON, ambos da Escola de Química e Alimentos da FURG. Todas as amostras de bioflocos foram coletadas, lavadas, concentradas e secas a 60 °C até atingir peso constante, e levadas para os laboratórios citados acima para a realização das análises.

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CAPÍTULO I

Seleção, isolamento, cultivo e identificação de duas espécies nativas de diatomáceas adaptadas ao sistema com bioflocos

Resumo

As diatomáceas geralmente ocorrem em baixa concentração em sistemas de cultivo com bioflocos. Com o objetivo de elucidar aspectos da seleção, isolamento, cultivo e identificação de diatomáceas nativas adaptadas aos bioflocos, foram isoladas duas espécies de amostras de bioflocos de um cultivo de juvenis de *L. vannamei* em andamento. As diatomáceas foram isoladas conforme metodologia de isolamento por pipetagem e diluições sucessivas. As células foram transferidas uma em cada tubo de ensaio (10 mL) contendo meio nutritivo F/2, salinidade de 30, sob condições controladas de temperatura (20°C), fotoperíodo (12 h claro:12 h escuro), irradiância de 163 $\mu mol.m^{-2}.s^{-1}$ e mantidas em câmara de germinação. Os cultivos iniciais foram monitorados semanalmente para verificar o crescimento das células, e aquelas que apresentaram crescimento foram transferidas para frascos do tipo Erlenmeyer (250 mL) e mantidas em câmara germinadora. Os cultivos iniciais foram usados como inóculo para cultivos em fase intermediária (recipientes de 20 L), e esses foram repicados para cultivos em fase final (tanques 200 L) e inoculação nos experimentos. A identificação de *Cylindrotheca closterium* (Ehrenberg) Lewin & Reimann (1964) foi efetuada com base em sua morfologia em microscopia optica; a identificação de *Amphora coffeaeformis* (Agardh) Kützing (1844) foi realizada com base na observação de ultra-estruturas com técnica de microscopia eletrônica de varredura e foi confirmada por especialista. Os resultados mostraram a possibilidade de selecionar, isolar, cultivar e identificar diatomáceas adaptadas ao crescimento em sistema de bioflocos e contribuir de forma efetiva para melhorar o desempenho dos organismos cultivados.

INTRODUÇÃO

Os estudos sobre os cultivos de microalgas iniciaram há cerca de 140 anos, acompanhando os progressos das ciências ambientais, fisiologia e da microbiologia. Historicamente, são considerados dois períodos fundamentais nesses estudos: o primeiro foi iniciado com o próprio advento da atividade de cultivo de microalgas e estendeu-se até meados do século XX; o segundo período estende-se até a atualidade. O primeiro foi marcado pelas descobertas fundamentais que permitiram melhor compreensão das algas e seu cultivo, já o segundo é marcado pela grande diversificação dos estudos, sobretudo as aplicações biotecnológicas. Não há um marco histórico concreto separando os dois períodos, mas a mudança ocorreu entre meados dos anos 1940 (no pós-guerra) e o início dos anos 1950 (Lourenço 2006).

Embora o advento do alimento artificialmente formulado tenha colaborado para suprir o requerimento nutricional dos animais cultivados em sistemas de berçário e engorda, as microalgas ‘’in natura’’ cultivadas em laboratório continuam sendo importantes e indispensáveis em larviculturas de camarões marinhos e moluscos (Barbieri & Ostrensky, 2001). As microalgas têm sido empregadas na alimentação direta, mas também indiretamente, como na produção de zooplâncton, manutenção da qualidade da água em cultivos usando a “técnica de água verde” ou mesocosmos (Lavens & Sorgeloos, 1996; Amjad & Jones, 1994; Reitan et al. 1997) e no balanço do pH por removerem o excesso de dióxido de carbono (Hoff & Snell, 1987). Algumas espécies combatem bactérias patogênicas pela produção de substâncias antibióticas (Reitam et al. 1997). As dietas artificiais geralmente carecem de pigmentos que dão aos organismos, como o salmão e truta, sua coloração típica e pigmentos carotenoides como a astaxantina podem ser oferecidos na dieta para intensificar a coloração dos animais. Rações contendo 5 a 20% de *Arthrospira* (cianobactéria rica em carotenos) aumentam

os padrões de vermelho e amarelo em carpas e intensificam o brilho das partes brancas. Essa definição de cor aumenta o valor de venda (Spolaore et al. 2006)

As microalgas são fontes de macronutrientes, vitaminas e elementos - traço, pois são ricas em proteínas, carboidratos, e especialmente ácidos graxos poliinsaturados que são essenciais para o crescimento e metamorfose de larvas larvas de peixes (Koven et al. 1989), camarões e moluscos (De Pauwn et al. 1988; Valenzuela-Espinoza et al. 2002; Silva et al. 2009). Algumas espécies contêm grande quantidade de ácidos graxos poliinsaturados de alta qualidade (Guschima & Harwood 2006) e proteínas (Silva et al. 2009).

Nas últimas décadas mais de cem espécies de microalgas foram testadas e avaliadas para o uso na aquicultura, mas dentre estas, apenas aproximadamente 20 tiveram seu uso amplamente difundido (Brown et al. 1997; Olaizola 2003), por apresentarem atributos fundamentais para a aquicultura (Laing 2001). Entre estes atributos destacam-se a alta taxa de crescimento, ser de fácil cultivo, ser atóxica, apresentar tamanho e forma adequados para ingestão pelos organismos de interesse, bem como apresentar alta qualidade nutritiva (Brown et al 1997; Lourenço 2006).

Entre as microalgas, as diatomáceas destacam-se como o grupo mais representativo no plâncton marinho. São reconhecidos 285 gêneros de diatomáceas de hábitos planctônico ou bentônico, envolvendo entre 10.000 e 12.000 espécies (Round et al. 1990). As diatomáceas apresentam parede celular (frústulas) compostas de sílica amorfa hidratada, seus principais pigmentos fotossintéticos são clorofilas *a* e *c*, β caroteno, fucoxantina, diatoxantina e diadinoxantina, e seus produtos de reserva são crisolaminarina e gotículas de óleo. O crescimento das diatomáceas bem como o seu conteúdo de silício é determinado por vários fatores, como (1) o suprimento de nutrientes, destacando-se o Nitrogênio, Fósforo e Sílício, (2) variações nas condições de

luz, temperatura e pH, (3) variações bióticas como a densidade da cultura, diâmetro de suas valvas e em conseqüência o volume e superfície da célula, e estágio de vida (Werner, 1977). Na água do mar, as formas mais abundantes e estáveis do Silício são o silicato (SiO_3^{2-}), dióxido de Silício (SiO_2) e ácido ortossilícico (H_4SiO_4). A demanda das diatomáceas por Silício é tão alta quanto à de Nitrogênio (Brzezinski 1985), de forma que os dois elementos devem ser oferecidos em proporção igual (N:Si 1:1). Contudo na maioria dos meios de cultura, o Silício é adicionado em concentração mais baixa do que a de Nitrogênio (1/3 ou 1/4; Lourenço, 2006).

As diatomáceas são tradicionalmente classificadas em dois grandes grupos: diatomáceas cêntricas, com frústulas de simetria radial; e diatomáceas penadas, com frústulas de simetria bilateral. As diatomáceas cêntricas são predominantemente planctônicas, e a maioria habita ambientes com baixa concentração de matéria orgânica dissolvida, enquanto que as diatomáceas penadas são predominantemente bentônicas ou epífitas, habitando ambientes ricos em matéria orgânica dissolvida (Werner, 1977; Round et al. 1990). O hábito bentônico de algumas espécies é favorecido pela presença de um sulco (rafe) ao longo de seu eixo longitudinal, no qual é liberada a mucilagem com capacidade adesiva, propiciando o deslizamento das células sobre o substrato e contribuindo na estabilização do mesmo (Lourenço, 2006). As diatomáceas penadas são os primeiros colonizadores autotróficos e os constituintes mais importantes na comunidade microfitobêntica do ambiente marinho (Patil & Anil, 2005). Além disto, a capacidade heterotrófica facultativa é bem difundida entre as diatomáceas penadas, mas raramente em diatomáceas cêntricas (Lylis & Trainor, 1973; Lee et al. 1975). As diatomáceas são facilmente digeridas, por apresentarem baixo conteúdo de fibras (Moss 2000).

Em viveiros, Moss et al. (1992) relataram que condições com alta concentração de matéria orgânica em suspensão são freqüentemente dominadas por diatomáceas. Juvenis de *Farfantepenaeus paulensis* cultivados em substratos artificiais contendo biofilme demonstraram uma preferência por forragear diatomáceas cêntricas (Ballester et al., 2007; Silva et al. 2009). Os camarões peneídeos são consumidores de diatomáceas em viveiros de cultivo, nos quais eles provêem adequada nutrição por curto período de tempo, propiciando o crescimento de camarões juvenis quando em falta de alimento peletizado (Moss, 1994). Jaime–Ceballos et al. (2006) utilizando hepatopancreatina como reagente, determinaram uma alta taxa (94%) de digestibilidade (*in vitro*) da proteína de *Chaetoceros muelleri* para pós-larvas de camarões.

Dentre os sistema de cultivo, o BFT funciona com trocas mínimas de água e fertilização com fontes de carbono na formação de bioflocos, os quais são constituídos por bactérias, microalgas, protozoários, larvas de invertebrados, exoesqueletos e restos de animais mortos, predominando uma biota aeróbica e heterotrófica (De Schryver et al., 2008). As microalgas presentes no sistema BFT contribuem para os eficientes resultados na produção de camarões marinhos. Burford et al. (2004) relataram que até 29% do alimento consumido pelo camarão branco *Litopenaeus vannamei* pode ser proveniente dos bioflocos presentes no meio de cultivo. Wasielesky et al. (2006) confirmaram o benefício da produtividade natural desse tipo de sistema em relação à sobrevivência, crescimento, ganho de peso, consumo alimentar e na taxa de conversão alimentar para juvenis de *L.vannamei.* Por outro lado, Godoy et al. (2012) demonstraram que os juvenis de camarões cultivados em ambiente contendo diatomáceas como suplemento, ganharam 17% mais peso e apresentaram conversão alimentar excelente (0,47), comparados àqueles cultivados em bioflocos, e de mistura entre os dois (conversão alimentar 0,76 e 0,80, respectivamente). O sistema BFT têm a

vantagem de incrementar a dieta através da produtividade natural presente nos viveiros (Moss et al. 2001; Burford et al. 2003), possibilitando assim a utilização de rações com menor teor de proteína bruta (Moss et al. 2001, 2002; Samocha et al. 2004; Ballester et al. 2007).

Os resultados apontam para a importância das diatomáceas, como fonte de nutrientes essenciais e sua contribuição significativa para o melhor desempenho no crescimento dos camarões. Além disto, a presença de diatomáceas pode levar a uma redução de custos pelo menor uso de ração, mas também pela menor necessidade de aeração durante o dia, devido à produção de oxigênio pelo processo de fotossíntese (Godoy, 2008). No entanto, é difícil manter o crescimento das diatomáceas em sistemas de bioflocos, possivelmente devido à elevada concentração de material em suspensão, o que reduz a penetração da luz e inibe a fotossíntese das microalgas ou devido ao seu requerimento de nutrientes não disponíveis em alta concentração no sistema, como a sílica. Em diversos estudos, foi observada a dificuldade de manter as diatomáceas em sistema BFT (Ray et al., 2010; Vinatea et al., 2010).

O presente estudo teve como objetivo elucidar aspectos da seleção, isolamento, cultivo e identificação de diatomáceas bentônicas nativas adaptadas ao sistema com bioflocos.

MATERIAL E MÉTODOS

1) Local do estudo: Estação Marinha de Aqüicultura (EMA) e Laboratório de Ecologia do Fitoplâncton e Microorganismos Marinhos da FURG.

2) Seleção e isolamento: Espécies de diatomáceas bentônicas foram isoladas de amostras de bioflocos em cultivo de juvenis de *L.vannamei* em um raceway, formados com água de oriunda da Praia do Cassino (32°12 S; 51°50 W) em Rio Grande, Rio

Grande do Sul, Brasil. Alíquotas dessas amostras foram observadas e quando detectadas diatomáceas, as mesmas foram isoladas conforme metodologia de isolamento por pipetagem e diluições sucessivas (Andersen et al., 2005), amplamente utilizada para gerar culturas de microalgas. A técnica consiste no isolamento da microalga de interesse numa pequena porção de líquido, usando-se uma pipeta Pasteur com ponta fina ou capilar de vidro e transferência para porções frescas e filtradas de água do mar. Transferências sucessivas são necessárias para que se atinja o isolamento e limpeza adequada das células da alíquota original. São necessárias várias transferências cuidadosas de uma alíquota para outra de meio líquido, até que apenas uma única célula da espécie-alvo esteja presente. Nesse momento, a espécie- alvo é transferida para uma pequena fração de meio de cultura. Usando-se esta técnica, as células de diatomáceas de interesse foram colocadas uma em cada tubo de ensaio (10 mL) contendo meio nutritivo Guillard F/2 (Guillard, 1975), preparado com água do mar previamente filtrada (filtro GF/F 0,45 μm, 47 mm, Whatman), posteriormente esterilizado em autoclave (120 °C, 30 minutos), e mantidas em câmara de germinação (modelo 347-CDG, Fanem). Os tubos de ensaio foram monitorados semanalmente para verificar o crescimento das células, e aquelas que apresentaram crescimento foram transferidas para frascos de tipo Erlenmeyer (250 mL), contendo 200 mL de meio de cultura com salinidade de 30, acondicionados em câmara germinadora (Figura 1), sob condições controladas de temperatura (20°C), fotoperíodo (12 horas claro: 12 horas escuro) e irradiância PAR (400-700 nm) de 163 $\mu mol.m^{-2}.s^{-1}$ medido com sensor esférico Li-Cor. As diatomáceas selecionadas foram mantidas nessas condições até atingida à fase Log de crescimento. Os cultivos foram utilizados como inóculo para os tratamentos a serem testados nos trabalhos posteriores desta tese.

Figura 1: Cepas de microalgas na Câmara Ambiente de Germinação – FANEM Modelo 347.

3) Cultivo:

3a) Preparação do material utilizado no cultivo:

A vidraria e instrumental utilizados nos cultivos foram previamente lavados com agitação e escovação com detergente neutro, enxaguados em água doce encanada (clorada), mantidos imersos em solução de ácido clorídrico 3% por no mínimo 2 h. Após novo enxágue com água doce (clorada), foram enxaguados com água destilada e secos em estufa (marca Biopar - modelo S1506 D 2) para posterior autoclavagem com meio de cultura. A autoclavagem do meio de cultura foi realizada somente para recipientes de 250 mL e 2L. Os recipientes (carboys, 20 L; tanques, 200 L) foram

esterilizados com cloro puro a 13%, adicionado 1 mL por litro de água do mar filtrada em filtro tipo Cuno (1μm) e mantidos por no mínimo 2h, e máxima 12h., após neutralização com tiossulfato de sódio ($Na_2S_2O_3.5\ H_2O$) (Kawachi & Noël, 2005).

3b) Condições de cultivo:

O cultivo inicial foi realizado em frascos de tipo Erlenmeyer (250 mL), contendo meio nutritivo Guillard F/2 (Guillard 1975), acondicionados em câmara germinadora (Figura 1), sob condições controladas ditas acima.

O cultivo em fase intermediária (recipientes de 20 L), contendo meio nutritivo Guillard F/2 (Guillard 1975) foi realizado em sala climatizada, sobre bancada, com iluminação proveniente de 4 lâmpadas fluorescentes do tipo luz do dia, posicionadas atrás dos recipientes de 20 L. A intensidade de irradiação na sala foi de 163 $\mu mol.m^{-2}.s^{-1}$, como mantido nos inóculos em câmara germinadora, e em 24 h luz. A temperatura da sala foi mantida em 24°C ± 1°C por um aparelho de ar condicionado do tipo *splitter* (marca Migrare). A fase final (tanques 200 L) (Figura 2) foi realizada em sala com temperatura ambiente e iluminação proveniente de 2 lâmpadas fluorescentes posicionadas atrás dos tanques. A intensidade de irradiação na sala foi de 163 $\mu mol.m^{-2}.s^{-1}$, como nos inóculos em câmara germinadora, e em 24 h luz. Quando era necessário elevar a temperatura (25–26°C), utilizou-se aquecedores com termostato. Para o aumento de biomassa, o volume inicial de 250 mL foi, repicado para 2000 mL, após repicado para 20.000 mL (carboys) e 200.000 mL (tanques).

Figura 2: Tanques de cultivo das diatomáceas isoladas, em fase final para utilização nos experimentos posteriores. Tanque de *Cylindrotheca closterium* (foto à esquerda) e o tanque de *Amphora coffeaeformis* (foto à direita).

4) Identificação das espécies isoladas:

Para a identificação taxonômica, as frústulas das diatomáceas foram limpas e oxidadas para a confecção de lâminas permanentes, segundo Christensen (1988). A identificação de *Cylindrotheca closterium* (Ehrenberg) Lewin & Reimann, 1964 foi efetuada com base nessas lâminas e bibliografia (Round et al. 1990, Tomas et al. 1996). Para a identificação da outra diatomácea isolada foi necessária observação de ultra-estruturas (Figura 3) com técnica de microscopia eletrônica de varredura (microscópio JEOL 30 KV, Centro de Microscopia Eletrônica da FURG), com base nos trabalho de Kaczmarska et al. (2005). A identificação de *Amphora coffeaeformis* (Agardh) Kützing, 1844 foi confirmada pela especialista Dra. Marines Garcia.

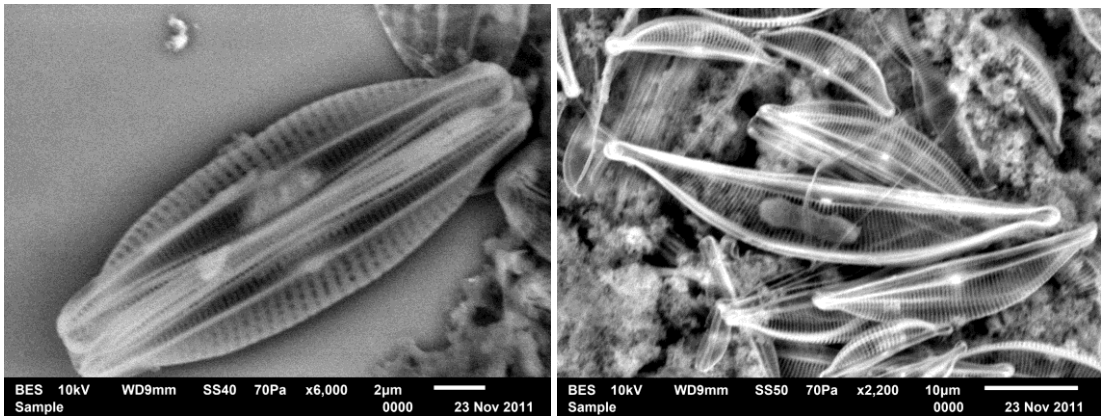

Figura 3: Fotos de microscopia eletrônica de varredura da diatomácea bentônica *Amphora coffeaeformis.* Uma frútula, evidenciando as estrias (foto à esquerda) e várias frústulas evidenciando o seu contorno (foto à direita).

RESULTADOS

Nas amostras de bioflocos foram detectadas microalgas clorofíceas (*Planctonema* sp. e *Oocystis* sp.), cianobactérias (colônias cocoides e tricomas da Família Pseudonabaenoidae), e algumas diatomáceas, entre elas uma diminuta diatomácea cêntrica (diâmetro 5–8 µm) e diatomáceas penadas, entre elas, uma espécie não identificada (tamanho 15–20 µm) e outras que foram isoladas e identificadas como *Amphora coffeaeformis*, (25–35 µm) (Figura 3 e 4) e *Cylindrotheca closterium* (4-5 x 30–35 µm) (Figura 5).

DISCUSSÃO

Entre as espécies de diatomácaes presentes no biofloco, destacamos os gêneros *Amphora* e *Cylindrotheca*, pela sua maior ($3,5 \times 10^4$ e $1,5 \times 10^4$, respectivamente) Segundo Tomas et al. (1996), o gênero *Amphora* pertence à divisão Bacillariophyta, classe Bacillariophyceae, ordem Thalassiophysales e família Catenulaceae. A espécie *Amphora coffeaformis* (Agardh) Kützing (1844), tem como sinônimos *Frustulia*

coffeaeformis Agardh (1827), *Amphora salina* Smith (1853) e *A. lineata* Gregory (1857) e caracteriza-se por suas valvas de 30-53µm de comprimento, 5–6.5 µm de largura, estrias dorsais em número de 17–26 µm em 10 µm até o centro, e 20–24 em 10 µm próximo das extremidades. A estria ventral não é finalizada (Archibald & Schoemann, 1984). É uma microalga de característica mixohalina e hábito bentônico (Brockmann, 1950; Archibald, 1983), foi citada como potencialmente tóxica (Sala et al., 1998; Ferrario et al., 2002), mas a sua toxicidade não foi confirmada (Fryxell & Hasle, 2004).

A presença do gênero *Amphora* em biofilme e bioflocos foi observada em outros estudos (Thompson et al., 1999; Godoy, 2008). O gênero foi usado como fonte alimentar de juvenis de *Mithraculus forceps* (Penha-Lopes et al., 2006), e pós-larvas de *Penaeus monodon* (Khatoon et al., 2009) e a espécie *A. luciae* foi usada como alimento de pós-larvas de abalone *Haliotis discus* (Gordon et al., 2006). Apresenta ampla distribuição, desde a Europa (Cantoral-Uriza & Aboal, 2008), Ilhas Atlântica (Ojeda Rodríguez, Gil-Rodriguez, & Moreira-Reyes, 2005), USA (Wachnicka & Gaiser, 2007), Argentina (Sala et al., 1998) e no Brasil nos estados desde a região Nordeste até o Sul do Brasil (Eskinazi-Leça et al., 2010).

O gênero *Cylindrotheca* pertence à divisão Bacillariophyta, classe Bacillariophyceae, ordem Bacillariales e família Bacillariaceae (Tomas et al., 1996). *C. closterium* (Ehrenberg) Reimar & J. C. Lewin (1964) tem por sinônimo *Ceratoneis closterium* e caracteriza-se por suas células solitárias ou formando colônias estreladas ou lineares, que podem estar incluídas em tubos de mucilagem. As valvas são retas ou sigmóides, estreitas, lineares, lanceoladas ou elípticas e às vezes expandidas centralmente, mais ou menos simétricas em relação ao plano apical. As extremidades são geralmente rostradas ou capitadas e as estrias unisseriadas não são interrompidas por esterno lateral, contendo aréolas arredondadas. Canópio ou costelas às vezes

presentes. Sistema de rafe de reto a fortemente excêntrico, fibulado e com disposição diagonalmente oposta na epivalva e na hipovalva da célula. Terminações distais da rafe simples ou curvadas para a margem distal (Lange–Bertalot & Simonsen, 1978; Kramer & Lange–Bertalot, 1988). Apresenta eixo apical 30–400 µm, eixo transapical entre 2,5–8 µm, com 10–12 fibulas em 10 µm e 70–100 interestrias em 10 µm próximo das extremidades (Lange–Bertalot & Simonsen, 1978; Kramer & Lange–Bertalot, 1988). Este é uma diatomácea marinha de hábito epipélico ou planctônico (Lange–Bertalot & Simonsen, 1978; Kramer & Lange–Bertalot, 1988), que também está presente em biofilme (Thompson et al., 1999) e bioflocos (Godoy, 2008). Esta espécie pode causar injúrias mecânicas em organismos filtradores e foi relacionada com produção de agregados mucilaginosos no Mar Adriático, fenômeno conhecido como "*Mare sporco*"(mar sujo), que efetou o turismo e a pesca (Fanuko et al., 1989; Stachowitsch et al. 1990). A distribuição geográfica de *C. closterium* é ampla, desde a Europa (Vanormelingen et al., 2013), Ilhas Altânticas (Ojeda Rodríguez et al., 2005), América do Norte (Kim et al., 2004) e no Brasil, desde a região Nordeste até Sul (Eskimazi-Leça et al., 2010), onde também ocorre na Praia do Cassino (Odebrech, et al. 2010).

CONCLUSÃO

Os resultados mostraram a possibilidade de selecionar, isolar, cultivar e identificar diatomáceas que já estão adaptadas as condições do sistema de bioflocos, com reduzida intensidade luminosa e elevada concentração de material em suspensão, e dessa forma contribuirem para melhorar o desempenho dos organismos cultivados.

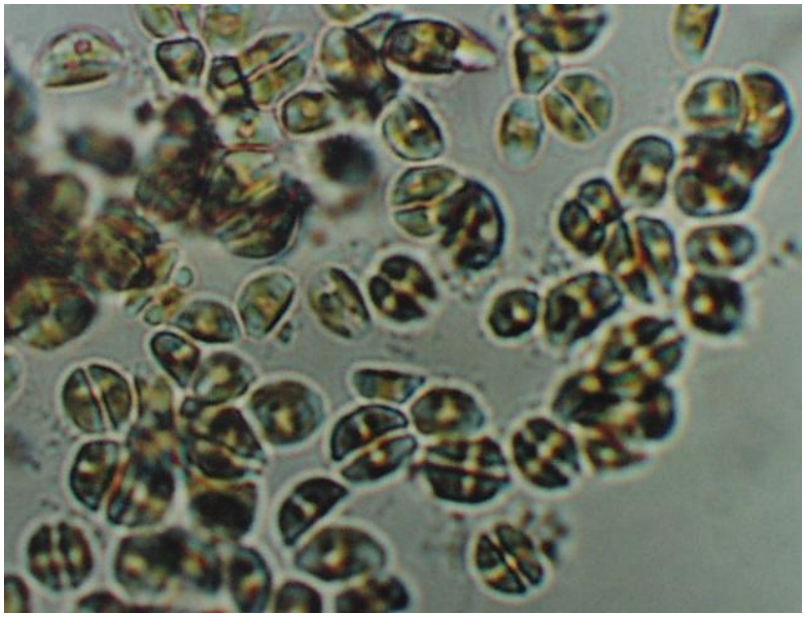

Figura 4: Foto do cultivo da *Amphora coffeaeformis.*

Figura 5: Foto do cultivo da *Cylindrotheca closterium*.

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CAPÍTULO II

A contribuição das diatomáceas no desempenho de juvenis *Litopenaeus vannamei* (Boone, 1931) em sistema com bioflocos.

Artigo submetido a revista Aquaculture Research

Manuscrito completo encontra-se no Anexo I

RESUMO

Juvenis de *Litopenaeus vannamei* foram criados (30 dias, três repetições por tratamento), na densidade de 390 camarões/m^2, em tanques (0, 5 m^2; 200 l) inoculados com diatomáceas e adição de silicato: *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T), ou apenas biofloco (BF, rico em clorofíceas). Parâmetros de qualidade da água foram monitorados diariamente e a microbiota ao 1°, 10°, 20° e 30° dias. O conteúdo de lípidos e o perfil de ácidos graxos dos bioflocos foram analisados ao fim do experimento. O biofloco de A apresentou o maior conteúdo de lipídios, diferindo significativamente de BF e T. O conteúdo de ácido eicosapentanóico (C 20:05) foi significativamente maior em A e menor em BF, enquanto que o ácido linoléico (C 18:02) foi significativamente maior em BF (Tabela 1). A sobrevivência dos camarões foi excelente no tratamento A, e significativamente menor em BF (Tabela 2). Os resultados indicam que a alta densidade de células de diatomáceas pode ser mantida com sucesso, com adição de silica em sistemas biofloco e que a penada *A. coffeaeformis* e cêntrica *T. weissflogii* são potencialmente mais adequadas do que a penada *C. closterium* como suplemento alimentar para dietas de camarão em berçário com bioflocos.

Tabela 1. Valores médios (± DP) do conteúdo de lipídeos e perfil de ácidos graxos em relação a biomassa seca dos bioflocos nos tratamentos *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) e bioflocos (BF). Diferentes letras na mesma linha indicam diferença significativa ($p < 0,05$).

	TRATAMENTOS			
	A	**C**	**T**	**BF**
Conteúdo de lipídeos (%)	5.11 ± 1.02^{a}	4.49 ± 0.70^{ab}	2.64 ± 0.15^{c}	3.79 ± 0.29^{b}
Perfil ácidos graxos				
C 14: 0	6.50 ± 4.24^{a}	7.6 ± 0.14^{b}	8.95 ± 2.47^{b}	3.10 ± 0.85^{a}
C 15: 0	0.95 ± 0.21^{a}	1.1 ± 0.00^{a}	1.55 ± 0.35^{b}	0.85 ± 0.07^{a}
C 16: 1	17.76 ± 5.18	19.15 ± 0.42	18.35 ± 3.04	7.80 ± 0.14
C 16: 0	33.90 ± 5.65	39.5 ± 1.41	36.60 ± 3.81	35.50 ± 1.48
C 17: 0	0.30 ± 0.10	0.7 ± 0.30	0.65 ± 0.07	0.60 ± 1.20
C 18: 2	4.35 ± 0.49^{b}	3.5 ± 0.14^{b}	4.60 ± 2.68^{b}	12.60 ± 0.56^{a}
C 18: 1C	18.95 ± 8.69^{a}	7.4 ± 0.84^{b}	8.55 ± 3.60^{b}	21.40 ± 0.84^{a}
C 18: 1T	5.00 ± 2.12^{a}	6.05 ± 1.30^{b}	7.35 ± 1.20^{b}	7.80 ± 0.28^{b}
C 18: 0	6.65 ± 5.44	6.05 ± 0.77	6.35 ± 2.05	4.25 ± 0.21
C 20: 5	4.00 ± 2.40^{a}	2.45 ± 0.07^{b}	1.85 ± 0.49^{b}	1.00 ± 0.28^{c}
C 24: 0	0.30 ± 0.03	0.6 ± 0.04	0.65 ± 0.07	0.70 ± 0.00

Tabela 2. Valores médios (±DP) dos parâmetros de desempenho de juvenis de *L. vannamei* criados em sistema superintensivo nos tratamentos *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and (BF) biofloco. Diferentes letras na mesma linha indicam diferença significativa ($p < 0,05$).

	TRATAMENTOS			
Parâmetros	**A**	**C**	**T**	**BF**
Peso médio inicial (g)	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03
Peso médio final (g)	2.43 ± 0.89	1.75 ± 0.45	2.58 ± 0.92	2.15 ± 0.59
Sobrevivência (%)	99.5 ± 0.89^{a}	92.8 ± 4.2^{ab}	94.8 ± 1.4^{ab}	88.0 ± 11.4^{b}
Ganho de peso (g)	2.23 ± 0.89	1.55 ± 0.45	2.38 ± 0.92	1.95 ± 0.59
Biomassa média final ($g\ m^{-2}$)	944.1 ± 349.7	643.2 ± 187.9	955.8 ± 348.6	744.2 ± 263.0
Taxa de crescimento específico (%)	5.15 ± 0.01	5.14 ± 0.01	5.15 ± 0.01	5.15 ± 0.01
Taxa de conversão alimentar (%)	0.84 ± 0.37	1.08 ± 0.54	0.96 ± 0.35	1.03 ± 0.63

CAPÍTULO III

A seleção de espécies de diatomáceas é importante para promover crescimento juvenis (*Litopenaeus vannamei*) (Boone, 1931) em sistema com bioflocos ?

Artigo submetido a revista Journal of the World Aquaculture Society

Manuscrito completo encontra-se no Anexo II

RESUMO

Juvenis de *Litopenaeus vannamei* foram cultivados, em densidade de estocagem de 390 camarões/m^2, em tanques (0, 5 m^2; 200 l) no interior de uma estufa por 30 dias. As unidades experimentais foram inoculados com três espécies de diatomáceas (três repetições por tratamento): biofloco maduro (BF); biofloco maduro + *Amphora coffeaeformis* (BFA), biofloco maduro + *Cylindrotheca closterium* (BFC), biofloco maduro + *Conticribra* (*Thalassiosira*) *weissflogii* (BFT). Durante este período, os parâmetros de qualidade de água foram monitorizados diariamente, os nutrientes inorgânicos dissolvidos no mínimo a cada dois dias e a microbiota nos dias 1, 15 e 30. O conteúdo e o perfil dos ácidos graxos dos lípidos nos bioflocos foram analisadas ao fim do experimento. O biofloco BFA apresentou maior teor de lipídios diferindo dos tratamentos BF e BFC, e o conteúdo de ácido linoléico (C 18:02) foi significativamente maior no BF (Tabela 1). Os tratamentos com adição das diatomáceas penada (*A. coffeaeformis*) e da cêntrica (*C. weissflogii*) apresentaram os melhores valores médios de sobrevivência, ganho de peso, biomassa final e taxa de conversão alimentar (Tabela 2). Os resultados indicam que *A. coffeaeformis* e *C. weissflogii* apresentam um maior potencial para enriquecer e suplementar os bioflocos na produção de camarão em comparação com a *C. closterium* e que a adição do silicato é necessária para a manutenção das diatomáceas no sistema de cultura BFT.

Tabela 1. Valores médios (±DP) do conteúdo de lipídeos e perfil de ácidos graxos da biomassa seca dos bioflocos nos tratamentos: biofloco maturo (BF), biofloco maturo + *Amphora coffeaeformis* (BFA), biofloco maturo + *Cylintrotheca closterium* (BFC) e biofloco maturo + *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

	TRATAMENTOS			
	BF	**BFA**	**BFC**	**BFT**
Conteúdo de lipídeos (%)	3,79 ± 0,29^{a}	5,00 ± 1,33^{b}	3,03 ± 0,25^{ac}	2,93 ± 0,13^{ab}
Perfil de ácidos graxos				
C 14: 0	3,1 ± 0,85^{a}	4,55 ± 0,92^{ac}	7,05 ± 2,61^{bc}	5,65 ± 0,21^{c}
C 15: 0	0,87 ± 0,07	1,35 ± 0,21	2,35 ± 2,47	0,95 ± 0,21
C 16: 1	7,8 ± 0,14^{a}	8,00 ± 0,56^{a}	16,65 ± 5,58^{bc}	14,95 ± 3,18^{c}
C 16: 0	35,55 ± 1,48	43,75 ± 3,32	43,2 ± 9,33	43,7 ± 0,42
C 17: 0	0,6 ± 1,20	1,2 ± 0,84	*	*
C 18: 2	12,6 ± 0,56^{a}	7,4 ± 0,70^{b}	4,50 ± 2,40^{b}	6,2 ± 0,42^{b}
C 18: 1C	21,40 ± 0,84^{a}	14,15 ± 0,92^{b}	13,10 ± 0,00^{b}	13,15 ± 0,49^{b}
C 18: 1T	7,8 ± 0,28	6,85 ± 0,07	5,15 ± 3,32	5,95 ± 1,20
C 18: 0	4,25 ± 0,21^{a}	5,8 ± 0,00^{ab}	3,95 ± 1,62^{ac}	4,50± 0,28^{abc}
C 20: 5	1,0 ± 0,28	1,1 ± 0,77	1,1 ± 0,77	0,7 ± 0,98
C 24: 0	0,7 ± 0,00	1,0 ± 0,77	*	*

*não detectado.

Diferentes letras na mesma linha indicam diferenças significantes ($p < 0.05$).

Table 2. Valores médios (±DP) dos parâmentros de desempenho de juvenis *L. vannamei* criados em sistema superintensivo nos tratamentos: biofloco maduro (BF), biofloco maduro + *Amphora coffeaeformis* (BFA), biofloco maduro + *Cylintrotheca closterium* (BFC) and biofloco maduro + *Conticribra (Thalassiosira*) *weissflogii* (BFT).

PARÂMETROS	**TRATAMENTOS**			
	BF	**BFA**	**BFC**	**BFT**
Peso médio inicial (g)	0.215 ± 0.03	0.215 ± 0.03	0.215 ± 0.03	0.215 ± 0.03
Peso médio final (g)	2.15 ± 0.59	2.45 ± 0.49	1.63 ± 0.43	2.48 ± 0.14
Sobrevivência (%)	88.03 ± 11.40	95.89 ± 1.36	88.71 ± 0.52	93.33 ± 2.23
Ganho de peso (g)	1.95 ± 0.59	2.25 ± 0.49	1.43 ± 0.43	2.28 ± 0.14
Biomassa média final (g/ m^2)	744.22 ± 263.08^b	915.18 ± 182.40^a	563.40 ± 145.68^b	901.38 ± 51.56^a
Taxa crescimento espécifico (%)	4.80 ± 0.00	4.80 ± 0.00	4.70 ± 0.00	4.80 ± 0.00
Taxa de conversão alimentar (%)	1.03 ± 0.63	0.76 ± 0.22	1.22 ± 0.29	0.81 ± 0.07

Letras diferentes na mesma linha indicam diferenças significativas ($p < 0.05$).

CAPÍTULO IV

Comparação da adição de diatomáceas e de substratos artificiais
para o crescimento de *Litopenaeus vannamei* (Boone, 1931)
em sistema com bioflocos

Artigo submetido a revista Atlântica

Manuscrito completo encontra-se no Anexo III

RESUMO

Juvenis de *Litopenaeus vannamei* (0,07 ± 0,04 g) foram criados no interior de uma estufa em tanques (0,5 m^2, 200 L) com densidade de estocagem (400/ m^2) em sistema de bioflocos por 30 dias. Foram analisadas os seguintes tratamentos (três repetições): biofloco maduro (BW), biofloco maduro com substrato artificial (BWSA), inoculação da diatomácea *Amphora coffeaeformis* (AW), inoculação da diatomácea *Amphora coffeaeformis* (AWSA) com substrato artificial, inoculação da diatomácea *Conticribra* (*Thalassiosira*) *weissflogii* (CW) e inoculação da *Conticribra weissflogii* (CWSA) com substrato artificial. Os parâmetros de qualidade de água foram monitorizados diariamente e a microbiota de água e dos substratos artificiais nos dias 1, 15 e 30. A taxa de sobrevivência foi elevada em todos os tratamentos (91-95%), no entanto, os camarões atingiram maior peso final, ganho de peso, biomassa final, taxa de crescimento específico e melhor conversão alimentar nos tratamentos com a inoculação de diatomáceas independentemente da adição de substrato (AW, AWSA, CW, CWSA) em comparação com os tratamentos com apenas biofloco (BW, BWSA) (Fig 1). Estes resultados destacam os benefícios da adição de diatomáceas em sistema com bioflocos. Embora o substrato artificial aumentasse a área para o crescimento de diatomáceas, a sua presença não alterou substancialmente o desempenho do crescimento de juvenis de camarão, nas condições testadas.

Tabela 1. Mean values (± SD) of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments with of mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *A. coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticriba weissflogii* (CW) and inoculation the diatom *C. weissflogii* with artificial substrate (CWAS).

	TRATAMENTOS					
Parâmetros	**BW**	**BWSA**	**AW**	**AWSA**	**CW**	**CWSA**
Peso médio inicial (g)	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04
Peso médio final (g)	0.85 ± 0.05^{b}	1.02 ± 0.04^{b}	1.65 ± 0.07^{a}	1.71 ± 0.14^{a}	1.85 ± 0.14^{a}	1.57 ± 0.18^{a}
Sobrevivência (%)	93.33 ± 1.65	94.92 ± 1.10	94.92 ± 1.92	94.13 ± 3.17	91.11 ± 7.02	91.13 ± 2.20
Ganho de peso (g)	0.78 ± 0.05b	0.95 ± 0.04^{b}	1.58 ± 0.07^{a}	1.64 ± 0.14^{a}	1.78 ± 0.14^{a}	1.50 ± 0.18^{a}
Biomassa final (g/ m^2)	333.55 ± 16.37^{b}	405.36 ± 16.95^{b}	659.16 ± 21.94^{a}	676.16 ± 54.52^{a}	709.78 ± 105.86^{a}	621.35 ± 84.79^{a}
Taxa crescimento específico (%)	8.32 ± 0.18^{b}	8.92 ± 0.12^{b}	10.54 ± 0.13^{a}	10.65 ± 0.28^{a}	10.91 ± 0.24^{a}	10.35 ± 0.39^{a}
Taxa conversão alimentar (%)	2.19 ± 0.26^{b}	2.12 ± 0.37^{b}	1.5 4 ± 0.24^{a}	1.49 ± 0.07^{a}	1.33 ± 0.11^{a}	1.61 ± 0.09^{a}

Letras diferentes na mesma linha indicam diferença significativas ($p < 0.05$).

DISCUSSÃO GERAL

Entre os vários grupos de microalgas, mais notadamente as diatomáceas são nutritivas e benéficas para a produção de camarões, devido a facil digestibilidade pelo seu baixo conteudo de fibras (Moss, 2001) e pela quantidade de aminoácidos essenciais e ácidos graxos altamente insaturados (Ju et al., 2008; Moss et al., 2001). Os camarões peneídeos são consumidores de diatomáceas em viveiros de produção, nos quais elas provêem adequada nutrição por curto período de tempo, para o crescimento de camarões juvenis quando em falta de alimento peletizado (Moss, 1994). Jaime-Ceballos et al. (2006) determinaram que a digestibilidade (*in vitro*) das proteínas de *Chaetoceros muelleri* chega a 94% para pós-larvas de camarões. Moss (1994) observou que juvenis de *Litopenaeus vannamei* sobrevivem e crescem em monocultivo de diatomácea como única fonte de alimento. Neste estudo, os camarões alimentados com *Chaetoceros* sp. cresceram mais e exibiram concentração de ácidos nucléicos e razão RNA:DNA praticamente idênticos aos valores encontrados nos camarões não alimentados e cultivados em água proveniente de um cultivo intensivo rico em agregados microbianos.

Entre os desafios do aprimoramento do sistema BFT, está à seleção de microorganismos e o manejo das condições de produção visando à manutenção destas comunidades neste meio junto aos bioflocos de modo a enriquecer o seu valor nutricional e garantir a qualidade da água (Ballester, 2008). O baixo número de diatomáceas em sistemas BFT, parece ser um padrão regular (Burford et al., 2003; Ray et al., 2010; Vinatea et al., 2010). Em geral, existe uma grande dificuldade para manter diatomáceas em meios com bioflocos, o que poderia ser devido à elevada concentração de material em suspensão, que pela redução da penetração da luz, inibiria a fotossíntese das microalgas ou devido à insuficiência de algum nutriente, possivelemtne a sílica, no

sistema. O presente estudo fornece informações sobre a contribuição das diatomáceas no desempenho dos juvenis de camarão *L. vannamei* cultivados em sistema com bioflocos. Até o presente estudo, inexistiam informações sobre as espécies de diatomáceas melhor adaptadas a crescer nesse tipo de sistema, nem sobre o seu efeito na qualidade nutricional dos bioflocos, e existem poucos dados disponíveis sobre a contribuição de diatomáceas no desempenho de juvenis cultivados no sistema BFT. Diante da dificuldade de manutenção de diatomáceas neste sistema, foram testadas três espécies, duas de penadas - *Amphora coffeaeformis* e *Cylindrotheca closterium* - isoladas de amostras de bioflocos de um cultivo de *L.vannamei*, provenientes da EMA-FURG, e uma cêntrica - *Conticribra weissflogii*, que faz parte do banco de microalgas do Laboratório de Fitoplâncton e Microrganismos Aquáticos da Furg.

Os resultados obtidos indicam que a alta densidade de células de diatomáceas contribuiu para um melhor desempenho no crescimento de camarão, independentemente de estarem somente na coluna d'agua ou na presença de substrato artificial e que a escolha das espécies de diatomáceas é um fator relevante. Observamos que as diatomáceas *A. coffeaeformis* e C. *weissflogii* foram mais adequadas do que *C. closterium* como suplemento alimentar para a dieta de camarão cultivado em meio aos bioflocos, o qual apresentou elevadas taxas de sobrevivência, ganho de peso, biomassa média final e eficiente conversão alimentar. Os resultados do presente estudo mostram que diatomáceas podem ser mantidas com sucesso em sistemas de bioflocos ($> 10^6$ células de L^{-1}), mas requerem após a sua inoculação, o monitoramento periódico da concentração de sílica no meio, e se necessário, a adição deste nutriente essencial para o seu crescimento. Os tratamentos com inoculação das diatomáceas bentônica *A. coffeaeformis* e cêntrica *C. weissflogii* apresentaram maior abundância de

microorganismos, o que vai de encontro com a característica dessas especies, de liberar grande quantidade de substâncias extracelulares poliméricas (SEP), formando uma mucilagem.

Além disso, foi observeda participação de *A. coffeaeformis* nos agregados, onde muitas células unidas estavam presentes, ou em agregado formado por muitas células e microorganismos, contribuindo para a formação dos bioflocos. Já *C. weissflogii* raramente participou da formação de agregados e sim estavam em maior quantidade livre na coluna de água, o que também foi observado para *C. closterium*.

As SEP são compostos por polissacarídeos (95%), que podem ser constituídos por açúcares neutros, ácido úrico e/ou açúcares sulfatados (Stal, 2003). A quantidade e a composição química dos EPS varia com a radiação, disponibilidade de nutrients, fase de crescimento e os ritmos de migração vertical associados à fotossíntese. Segundo Underwood et al. (2004) as diatomáceas bentônicas produzem maiores concentrações de EPS quando em situações de limitação de nutrients. Assim, ao produzirem a matriz de EPS, as células criam um microambiente estável e condições ótimas para seu crescimento (Decho, 2000), importantes na adesão das células aos substratos (Daniel et al., 1987), na locomoção (Edgar & Pickett–Heaps, 1984) e na resistência a toxinas (Decho, 1990).

Na formação dos bioflocos foi possível observar uma grande concentração e diversidade de protistas heterotróficos, incluindo ciliados, dinoflagelados e demais organismos flagelados. Esses micoorganismos na coluna de água e no perifíton presente em substratos artificiais, aceleram a remoção biológica de resíduos orgânicos e inorgânicos (Crab et al., 2007), cumprindo funções similares no controle de qualidade da água. Os microorganismos em suspensão, no sistema de bioflocos, são responsáveis

pela ciclagem de nutrientes, mas a biomassa de alta densidade populacional pode influenciar a eficiência de substratos artificiais. Os ciliados desempenham um papel importante no fluxo de energia nos ecossistemas aquáticos como predadores de microalgas, bactérias e fungos e como fonte de alimento para os metazoários, peixes e larvas de camarão (Nagano & Decamp, 2004). A abundância e diversidade de ciliados são bons indicadores de qualidade da água (Decamp et al., 1999). Decamp et al. (2007) relataram que os ciliados atingem concentrações extremamente elevadas (6000 células/ml) em sistemas BFT, dependendo da salinidade da água e de interações dinâmicas entre os próprios ciliados. Esses protistas se destacam como bioindicadores atuando como filtro externo (Decamp et al., 2003), predam bactérias e aumentam a absorção de partículas pelo biofilme (Decamp et al., 1999; Eisenmann et al., 2001). Observa-se em geral, uma sucessão de grupos ecológicos no sistema BFT com uma tendência para o domínio de ciliados de vida livre nos estágios iniciais e uma transição para raspadores após a segunda semana de produção. Os flagelados são organismos taxonomicamente diversos, incluindo espécies autotróficas e heterotróficas e estavam presentes em alta densidade, como observado por outros autores (Maicá et al., 2011). Flagelados são uma fonte de ácidos graxos altamente insaturados (HUFAs) e esteróis (Decamp & Nagano, 2001; Thompson et al., 1999); trabalhos anteriores indicaram a sua importância junto com ciliados na dieta de larvas de *Farfantepenaeus paulensis*.

O nível de lípidos recomendado para crustáceos é inferior a 10% (D'Abramo, 1997), valor não ultrapassado no presente estudo (2,64–5,11). Entre os ácidos graxos essenciais, destaca-se o ácido linoleico (C 18:02) e o ácido eicosapentanóico (EPA) (C 20:05 n3). Os crustáceos são incapazes de elongar eficientemente os ácidos graxos C18 e, portanto, necessitam a inclusão na sua dieta, de ácidos graxos essenciais de cadeia

longa 20 (n-3) ou 22 (n-3) (Sargent et al., 1989). A dieta deveria incluir 1-2% ácidos graxos altamente insaturados de cadeia longa. Neste estudo, todos os tratamentos com bioflocos foram capazes de fornecer o nível recomendado de ácidos graxos de cadeia longa particularmente ácidos eicosapentanóico (EPA) (20: 5-C HUFA), essenciais para o crescimento ótimo de organismos.

Em resumo, os resultados obtidos nesta tese auxiliam no aprimoramento da tecnologia de bioflocos, e sugerem que a redução de sílica é um importante fator que limita a presença de diatomáceas em alta densidade. Além disto, a seleção de espécies de diatomáceas é importante para um melhor crescimento dos camarões. Preferencialmente, devem ser utilizadas espécies que produzam substâncias extracelulares poliméricas (SEP) que contribuem na formação dos bioflocos, aumentam o número de microorganismos e consequentemente geram melhores resultados de desempenho dos camarões em cultivo. Por último, destaca-se que a adição de diatomáceas em meio de bioflocos é mais importante do que o uso de substrato artificial, para camarões com tamanho igual ao utilizado para esse estudo, visando um melhor eficiência e desempenho da tecnologia de cultivo em meio com bioflocos.

CONCLUSÃO GERAL

O presente estudo teve como objetivo principal avaliar a influência de diatomáceas e substrato artificial no desempenho de juvenis do camarão *L. vannamei* produzidos em sistema de bioflocos, visando o aprimoramento deste sistema.

As informações disponíveis antes desse estudo sobre a dificuldade de manter diatomáceas em sistema de bioflocos referiam-se principalmente à elevada concentração de material em suspensão, que reduz a penetração da luz e inibe a fotossíntese das microalgas e à uma baixa concentração de sílica no sistema. Os resultados obtidos neste trabalho comprovam que as diatomáceas são essenciais em diversos aspectos relacionados ao cultivo dessa espécie, que a elevada concentração de material em suspensão não afeta a manutenção das mesmas nesse sistema, mas que as mesmas são afetadas principalmente pela indisponibilidade de sílica. Sendo assim, para manutenção de altas concentrações de diatomáceas em sistemas de bioflocos é necessária adição periódica de sílica, propiciando um melhor desempenho dos camarões. Destaca-se que a diatomácea penada *Amphora coffeaeformis*, isolada de amostras de biofloco em cultivo da Estação Marinha de Aquicultura da FURG, mostrou-se muito bem adaptada e apresentou os melhores resultados de desempenho dos camarões.

A utilização de substratos artificias em sistema de bioflocos não influenciou o desempenho de juvenis de *L. vannamei*, mas a presença de diatomáceas foi mais importante. No entanto, observou-se que os camarões estavam constantemente sob os susbstratos artificiais, provavelmente alimentando-se dos micoorganismos aderidos, compostos principalmente de diatomáceas, alguns ciliados e flagelados, todos conhecidos por fazerem parte da dieta natural de camarões. Apesar disto, no presente

estudo não foi obtida diferença estatística no desempenho dos camarões, entre os tratamentos com e sem substrato.

Os resultados obtidos neste trabalho auxiliam no aprimoramento da tecnologia de bioflocos, que vem sendo utilizada em todo mundo para criar camarões marinhos, mas nos quais as diatomáceas estão geralmente em pequena densidade ou presentes apenas nos primeiros dias de cultivo.

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ANEXO I

The contributions of diatoms to biofloc and the growth performance of juvenile *Litopenaeus vannamei* (Boone, 1931) in a biofloc bculture system

The contributions of diatoms to biofloc and the growth performance of juvenile *Litopenaeus vannamei* (Boone, 1931) in a biofloc culture system

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Key words: microbiota, nursery, rearing, *Litopenaeus vannamei*

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Abstract

This study aimed to analyze the permanence of diatoms in biofloc culture systems and their contribution on the growth performance of juvenile shrimp. Juveniles of *Litopenaeus vannamei* were reared (30 days; three replicates per treatment) in biofloc systems inoculated with diatoms and silicate addition: *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T), or biofloc only (BF, chlorophycean rich). Water quality parameters were monitored daily and the microbiota 1, 10, 20 and 30 days. The lipid content and fatty acid profiles of the bioflocs were analyzed at the end of the experiment. Shrimp survival rate at treatment A was significantly lower at BF. The biofloc of A presented the highest lipid content, differing significantly from BF and T. The content of eicosapentanoic acid (C 20:5) was significantly higher in A and lower in BF, while linoleic acid (C 18:2) was significantly higher in BF. The results indicate that high cell density of diatoms can be successfully maintained with silicate addition in biofloc systems and that the pennate *A. coffeaeformis* and the centric *T. weissflogii* are potentially better suited than the pennate *C. closterium* as food supplements for shrimp diets in biofloc nurseries system.

Introduction

Biofloc Technology Culture Systems (BFT) represent a new paradigm in marine shrimp farming (De Schryver, Crab, Defoirdt, Boon & Verstraete 2008). They reduce the emission of effluents by efficiently reusing water for several cycles. In addition, microbiota can improve water quality and serve as extra food source for the cultured animals (Wasielesky, Atwood, Stoks, McIntosh, Bauman & Pearson 2006). Microalgae are widely used in hatcheries to maintain water quality (Hangreaves 2006) and as a food source due to their nutritional value and ability to synthesize and accumulate polyunsaturated fatty acids (PUFAs) including omega-3 series. Juvenile *Litopenaeus vannamei* can be fed with *Chaetoceros* spp. (Moss 1994), easily digested due to their low fiber content (Moss 2000). The presence of diatoms improves the growth of *Litopenaeus vannamei* in intensive ponds (Moss & Pruder 1995). In a study where shrimp were produced with bioflocs or with a mixture of bioflocs and diatoms (*Thalassiosira weissflogii* and *Chaetoceros muelleri*) the shrimp consumed less feed, presented a better feed conversion ratio and higher weight gain (Godoy, Odebrecht, Ballester, Martins & Wasielesky 2012). Most benthic or epiphytic diatoms (Class Bacillariophyceae) inhabit dissolved organic matter-rich environments, whereas planktonic species are also successful in environments with relatively low concentrations of dissolved organic matter (Round, Crawford & Mann 1990). The benthic diatoms contains a longitudinal slit so-called raphe. It is through this slit that is the release of extracellular polymeric substances (EPS) (Smith & Underwood 1998), which forms a mucilage which binds to actin filaments, leading the force necessary for movement, also called "gliding" and consequent adhesion to the substrate. The EPS are released alongside the raphe as a result of the contraction of the microfilaments of the

cytoskeleton (Edgar & Pickett - Heaps 1984), but some diatoms centric of the genus Thalasiosira also produce EPS.

The benthic diatoms produce different types of EPS, which vary in structure and composition of hydrates crabono whose output varies with the environmental conditions (Staats, Stal, De Winder & Mur. 2000; Smith & Underwood 2000; De Brouwer & Stal 2002). The quantity and chemical composition of EPS varies with radiation, availability of nutrients, growth phase and rhythms of vertical migration associated with photosynthesis. According to Underwood et al. (2004) benthic diatoms produce higher concentrations of EPS in situations when limiting nutrients. Thus, to produce the EPS matrix, cells create a microenvironment stable and optimal conditions for their growth (Decho 2000), important in cell adhesion to substrates (Daniel, Chamberlain & Jones 1987), locomotion (Edgar & Pickett - Heaps 1984) and resistance to toxins (Decho 1990).

Culture ponds with a high concentration of suspended organic matter are often dominated by pennate diatoms (Bacillariophycidae, Fragillariophycidae), with benthic habits (Moss 1994). However, it is difficult to maintain the diatoms in the biofloc, possibly due to the high concentration of suspended material, which reduces light penetration and inhibits microalgae photosynthesis or due to low silicate in the system. The present study aimed to evaluate the permanence in biofloc of two pennate and a centric diatom and their contribution to the growth performance of early shrimp juveniles cultured.

Materials and methods

Selection, isolation and cultivation of microalgae and formation of bioflocs

The pennate diatoms *Amphora coffeaeformis* (25 – 35 µm) and *Cylindrotheca closterium* (4-5 (30 – 35 µm)) were isolated using a capillary micropipette in successive dilutions from samples of bioflocs from raceway in Marine Aquaculture Station of the Federal University of Rio Grande - FURG formatted with water of Cassino Beach (32°12 S and 51°50 W) in Rio Grande, Rio Grande do Sul states, Brazil. Taxonomic identifications were performed using clean frustules mounted on slides (Christensen 1988). Specimens of the diatom *Thalassiosira weissflogii* (12- 15µm) were obtained from the strains bank of the Microalgae and Microorganisms Laboratory (Federal University Rio Grande -FURG). All of the diatoms were kept in a germination chamber (BOD type) in Guillard F/2 medium (Guillard 1975), which was prepared with previously filtered sea water (glass fiber filter Whatman GF/F pore size 0.45 µm; diam. 47 mm) and sterilized in an autoclave at 120° C for 30 minutes. Three tanks (1,000 L) containing filtered water (5 µm pore filter, Cuno) were inoculated with the diatoms (after they had reached the exponential growth phase) to develop bioflocs.

The initial nominal C:N ratio was approximately 15:1, was necessary to add organic fertization to the tanks, during the experiment no water exchange was performed. The addition of sugar cane molasses (37.46 % C and 0.57 % N) into carbon source was based on (Avnimelech 1999) and (Ebeling, Timmons & Bisogni 2006) who determined that 6 g is needed to convert 1g total ammonium nitrogen (TA-N) into bacterial biomass. The addition of sugar cane molasses was made when the concentration of (TA-N) were equal or higher than 0.5 mg/L. Every four days, the silicate (SiO_2) concentration was measured and if the concentration was lower than 1mg/L, it was added, in equal proportions (1N:1Si) (Brzezinski 1985). On the first

experimental day, the experimental units were inoculated with diatoms and biofloc (initial cell density of $3x10^7$ cells/L) and shrimp were stocked.

The Experimental System

Inside a greenhouse, 12 plastic rectangular tanks (0.5 m^2; 200 L) were equipped with aeration systems and airstones. The inocula prepared, the diatoms and biofloc were pumped with a submerged pump for their respective experimental units. The treatment BF was prepared from biofloc mature system in a raceway (TA- N = 0.17 mg/L, NO_2 = 0.02 mg/L , NO_3 = 0.86 mg/L, PO^3_4 = 0.26 mg/L, Alcalinity = 127 mg/L, Silicate = 0,4 mg/L, Chl *a* = 6,3 μg/L e TSS = 138 mg/L).

The experimental design was random with three replicates for each treatment: *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and Biofloc (BF). Diatom and microorganisms counts were performed on specimens fixed in Lugol's iodine solution (2%) in sedimentation chambers on days 1, 10, 20 and 30. At least 10 fields were chosen at random using a Zeiss Axiovert microscope equipped with phase contrast at a 400x final magnification (Utermöhl 1958).

Groups of 195 juveniles of *L. vannamei* (390/m^2; initial weight 0.215 ± 0.03 g) from hatcheries of the Marine Aquaculture Station of the Federal University of Rio Grande were stocked in the experimental units. Shrimp were fed twice a day (08:00 and 17:00 hours) with a commercial feed Potimar Active 38 (38% CP, 1.6 mm, Guabi ®, Campinas, SP, Brazil) offered on feeding trays (∅ = 15 cm, 5 mm mesh size, one per tank). The initial feeding rate was set at 10% of the total biomass and adjusted to 5% at the end of the 30 days experiment (Jory, Cabreras, Durwood, Fegan, Lawrence, Jackson, McIntosh & Castañeda 2001).

Thirty shrimp from each experimental unit were individually weighed and returned to the tanks on days 1, 10 and 20, to adjust rate of feeding and all shrimp were

measured at the end of the experiment. The growth performance was evaluated according to the following parameters: survival (S, %) = [(initial n - final n)/ initial n x 100], where n = number of shrimp; final weight (FW, g) = ∑ final weight of live shrimp/ total shrimp; weight gain (WG, g) = ∑ FW- initial weight (g); specific growth rate (SGR; %/day) = [(ln final weight (g) - ln initial weight (g)) / time in days) x 100]; final biomass (FB, g) = ∑ FW of live shrimp; feed conversion ratio (FCR, %) = [(total feed consumed (g) / WG (g)) x 100 (assuming that all feed offered was consumed)].

Water quality

Temperature, pH, salinity and dissolved oxygen were measured daily twice (08:00 and 17:00) in each experimental unit (YSI model ® 556 MPS-USA). Water samples were collected filtrated in glass-fiber filters (GF 50-A), to quantify: total ammonia nitrogen ($N\text{-}NH_3 + NH_4$; UNESCO 1983) and nitrite ($N\text{-}NO_2$) were measured every two days, nitrate ($NO_3\text{-}N$; Aminot & Chaussepied 1983) and phosphate ($P\text{-}PO_4$; Aminot & Chaussepied 1983) were measured once a week, and the concentration of silicate (SiO_2; Strickland & Parsons 1972) was measured every four days using a digital Micronal B342 II spectrophotometer. The concentration of total suspended solids (TSS) were measured every two days by filtering water samples (50 ml) through glass-fiber filters (Whatman GF/F pore size 0.45 μm; diam. 47 mm), values were estimated by difference between final and initial weight of each filter, following (AOAC 2000) using a Sartorius balance (0,001 g). The concentration of chlorophyll *a* (Chla) fluorescence was measured every two days using a Turner Design fluorimeter, following (Welschmeyer 1994). Alkalinity was monitored weekly by titration with hydrochloric acid to the methyl orange endpoint, following (APHA 1998).

Lipid content and fatty acid profile of biofloc

At the end of the experiment, biofloc samples were collected from each treatment, filtered through strainer (50 µm), washed with 20 mL (0.5 M ammonium formate)(Zhu & Lee 1997) concentrated and dried in an oven (60°C) to constant weight.

Lipids were extracted according to (Folch, Lees & Stanley 1957), and fatty acids were saponified with KOH (50%). Fatty acid methyl esters (FAMEs) were prepared by esterification with BF_3 10% in methanol, following (Metcalfe & Schmitz 1961). The FAME profile was separated by gas chromatography (Shimadzu GCMS-QP2010Plus, Japan) equipped with a split/splitless injector and mass detector with an operation temperature of 280°C and inlet temperature of 230°C. The detection occurred as a complete scan of 0.2 seconds (*m/z* 30 to *m/z* 500). The column used was Crossbond 5% diphenyl/95% dimethyl polysiloxane (30 m x 0.25 mm x 0.25 µm, Restek). The electron ionization occurred at 70 eV. The conditions of operation of the chromatograph were as follows: injector at 250ºC; column at 80ºC (initial temperature, 0 min), followed by an increase of 10°C/min up to 180°C and 7°C min^{-1} to the final temperature of 330°C; helium flow of 1.3 mL min^{-1}; pressure of 88.5 kPa; mean linear velocity of 42 cm s^{-1}; 1 µL injection volume with a split ratio of 1:100. The compounds were characterized by the retention time and confirmed by mass spectrometry.

Statistical analysis

The growth performance data were analyzed using a one-way ANOVA (α = 0.05) after verification of the homoscedasticity (Levene's test) and normality of the data (Kolmogorov-Smirnov test). The specific growth rate, survival and fatty acid concentrations were arcsine transformed before analysis. Differences among the treatments were tested with Tukey's multi-comparison test (Sokal & Rolf 1969). The

abiotic parameters of water quality, concentration of Chl *a* and TSS were analyzed by nonparameric Kruskal- Wallis test.

Results

Water quality parameters

Water quality values are shown in Table 1. Total ammonia nitrogen (TAN) showed significant differences between treatments, whith higher values in T and C and lower in BF and A. The values of silicate were significantly higher in A, C and T and lower in the BF tanks. Nitrite concentrations were significantly lower in treatments T and BF and higher in the C tanks. The mean values of temperature, dissolved oxygen, pH, salinity, alkalinity, total suspended solids (TSS), Chl *a* nitrate and phosphate exhibited no significant differences between treatments.

INSERT TABLE 1

INSERT FIGURE 1

INSERT FIGURE 2

INSERT FIGURE 3

INSERT FIGURE 4

Microbiota

The variety of organisms reflects the diversity of trophic levels present in the water (Table 2). Exceept in BF, the cell density of diatoms remained high throughout the experiment (Figs. 1, and 2, and 3 and 4). BF presented centric (5-8 µm; 3.4 x 10^5 cells L^{-1}) and pennate (15- 20 µm; 1 x 10^7 cells L^{-1}) diatoms on the first day of experiment only. Cyanobacteria were present in all treatments: coccoid cyanobacteria were more abundant in treatment A (2. 87 x 10^4 indiv L^{-1}) and less abundant in T (0.10 x 10^4 indiv L^{-1}), and filamentous cyanobacteria were more abundant in treatment T

(52.96 x 10^4 indiv L^{-1}) and less so in C (8.14 x 10^4 indiv/L^{-1}). The filamentous cyanobacteria belonged to the subfamily Pseudanabaenoideae (Pseudanabaenaceae) (Komárek & Anagnostidis 2005). The Chlorophyceae *Planctonema* sp. (17.8 x 10^7 indivL^{-1}) and *Ocystis* sp. (0.3 x 10^7 indiv L^{-1}) were denser in treatment BF.

Heterotrophic protists were also observed: ciliates in three size ranges (C1 < 25 μm; C2 25–50 μm; C3 > 50 μm), flagellates in two size ranges (F1 < 10 μm; F2 10–20 μm), dinoflagellates in two size ranges (D1 < 15 μm; D2 > 25 μm). In treatment A, ciliates, dinoflagellates of both size ranges, flagellates F1 and spherical unidentified cells (UN A 5-7μm; UN B 17- 22 μm) were observed. Treatment T also included flagellates, ciliates, unidentified cells of both sizes and size D1 dinoflagellates. Flagellates, unidentified of both sizes, C1 and C2 ciliates and D1 dinoflagellates were observed in treatment C. Ciliates, unidentified of all size classes, F1 flagellates and D1 dinoflagellates were present in the treatment BF tanks.

Lipid content and fatty acid profile of biofloc

The lipid content varied from 2.64 ± 0.15 to 5.11 ± 1.02 (Table 3). Treatment A exhibited the highest lipid content of biofloc (5.11%), differing significantly from treatments BF and T. The concentration of the fatty acids considered essential for farmed organisms eicosapentanoic acid (EPA) (C 20:5) was significantly higher in treatment A (4%) and lower in BF (1%), while the linoleic acid (C 18:2) was significantly higher in treatment BF (12.6%).

Growth performance of shrimp

The mean survival rate of the shrimp (88 to 99%) exhibited differences among treatments: it was significantly higher value in the A and lowest in treatment BF (Table 4). The shrimp consumed less feed in treatment A, but the apparent feed conversion rate did not differ significantly among treatments. The lowest mean values of final weight

and weight gain and the highest feed conversion rates were recorded for treatment C, although there were no significant differences for each index.

Discussion

Water quality

The water temperature and salinity levels measured in the tanks favored the growth of shrimp (Ponce- Palafox, Martinez-Palacios & Ross 1997) and did not differ among treatments. The dissolved oxygen concentration, the alkalinity and pH values were the recommended range for the cultivation of the studied species (Van Wyk & Scarpa 1999). In this study, the alkalinity values were within the recommended range for *L. vannamei* (≥ 100 mg/L) (Van Wyk & Scarpa 1999). The biofloc technology culture system used clarification was effective for the maintenance of total suspended solids particulate at 500 mg/ L, (Gaona, Poersch, Krummenauer, Foes & Wasielesky 2011).

In the present study, the concentration of suspended solids was similar to that found by (McIntosh, Samocha, Jones, Lawrencw, Mckee, Horowitz & Horowitz 2000) and lower than the values found by (Avnimelech 2007) and (Godoy *et al.* 2012). The addition of an organic carbon source raises the C: N ratio and promotes the absorption of nitrogen by microbiota and a rapid oxidation of toxic ammonia, nitrite and nitrate (Avnimelech 1999) and (Boyd 2007). In this experiment, the concentration of total ammonia nitrogen remained within the safe range of up to 3.95 mg/L for a salinity of 35 for juvenile *L. vannamei* (Lin & Chen 2001). The mean nitrite concentration remained within the safety level for juveniles in marine waters 25.7 mg/L, (Lin & Chen 2003). The nitrate concentration in all treatments was also acceptable ≤ 60 mg/L, (Van Wyk & Scarpa 1999). Phosphorus is an important nutrient for the growth of microalgae, and phosphate fertilizers increase the availability of microalgae as a natural food source for

cultured organisms. (Silva, Wasielesky & Abreu 2013) observed that shrimp biomass incorporated 35% of the total phosphorus input. The phosphate concentrations were similar among treatments and comparable to those found in other studies (Casillas-Hernándes, Nolasco-Soria, Garcia-Galano, Carrillo-Farnes & Páez-Osuna 2007). Silicate, required by the diatoms for building their siliceous frustules, was added to avoid nutrient limitation for their growth and permanence in the experimental units. The silicate values remained relatively high in treatments A, C and T (SiO_2; 0.45–0.65 mg/L). In the BF treatment without silicate addition, the concentration of this nutrient (0.20 mg/L) was slightly higher than growth-limiting concentrations in most lacustrine instances, which are mainly below about 0.1 mg/L, (Reynolds 2006). We may conclude that silicate addition was important for the growth and maintenance of high diatom cell densities and that the lower silicate concentration in BF explains the drastic diatom reduction observed in this treatment.

Bioflocs: microbiota, lipid content and fatty acid profile

Biofloc provides packed microbial protein and nutrients for cultivated animals (Avnimelech 2009), being constituted by bacteria, microalgae, protozoa and metazoa, undigested food, feces and suspended debris under strong aeration (De Schryer *et al.* 2008). Several taxonomic groups of microalgae occur in aquaculture environments, and they are an important food source for zooplankton, transferring their nutrients to higher levels of the food chain (Ray, Seaborn, Leffler, Wilde, Lawson & Browdy 2010). Microalgae use toxic TAN, nitrate and phosphate for the construction of cellular structures such as proteins and carbohydrates Ray *et al.* (2010). The biomass of microalgae is higher in BFT systems and is usually expressed as chlorophyll *a* content (*Chla*) due to the presence of this pigment in all groups (Ju, Forster, Conquest, Dominy,

Kuo & Horgen 2008). In this study, the mean concentration of *Chla* range (175–282.5 μg/L) was relatively low when compared to other experiments in biofloc system (Burford, Thompson, McIntosch, Bauman & Pearson 2003, 134.29–435.10 μg/L) and (Decamp, Conquest, Cody & Forster 2007, 373–509 μg/L).

Among the microalgae, diatoms present excellent nutritional value and contribute essential amino acids and polyunsaturated fatty acids (PUFAs) from the omega-3 series (Ju *et al.* 2008). Aside from this, their digestion by shrimp is facilitated by their low fiber content (Moss 2000). Low number of diatoms in BFT systems as observed in our experiment seems to be a regular pattern found also by (Ray *et al.* (2010) and (Vinatea, Gávez, Browdy, Stokes, Venero, Havemann, Lewis, Lawson, Shuler & Leffler 2010).

Throughout the experiment, *A. coffeaeformis* remained at high density (3.0, 13.2, 3.2 and 2.2 x 10^7 cells L^{-1}) and the cells mostly formed aggregates or were attached to other households, epiphyton or being preyed upon by other organism. *T.weissflogii* showed similar cell density (3.0, 14.2, 2.5 and 1.5 x 10^7 cells L^{-1}) to *A. coffeaeformis* but the vast majority of their cells grew free, with little participation in the bioflocs similarly to that found in *C. closterium* (3.0, 3.5, 2.0 and 0.3 x 10^7 cells L^{-1}). The latter showed cell density far below the previous ones. In the treatments with *A. coffeaeformis* and *T.weissflogii* a large amount of protozoans were present. This may be due the production of extracellular polymeric substance (EPS) produced by the diatoms.

The benthic diatoms produce different types of EPS, which vary in structure and composition of carbon hydrates, according to the environmental conditions (Staats *et al.*, 2000; Smith & Underwood, 2000; De Brouwer & Stal, 2002). The quantity and chemical composition of EPS varies with irradiation, availability of nutrients, growth phase and rhythms of vertical migration associated with photosynthesis. According to

Underwood *et al*. (2004) benthic diatoms produce higher concentration of EPS in limiting nutrients situations. The EPS matrix provides a stable microenvironment and optimal conditions for cell growth (Decho, 2000), important in the adhesion to substrates (Daniel *et al*. 1987), locomotion (Edgar & Pickett-Heaps, 1984) and in the resistance to toxins (Decho, 1990).

A. coffeaeformis has twice the biovolume of *T.weissflogii* but even this smaller size and not participating in the formation of very bioflocs, contributed to the good performance of the shrimp. Already *C. closterium* with biovolume eight times smaller than *A. coffeaeformis* participated bit of training bioflocos and contributed to the worst values in the performance of shrimp. We show that diatoms may be successfully maintained in BF systems following their inoculation and silicate addition ($>10^{6}$ cells L^{-1}).

Cyanobacteria and Chlorophyceae are commonly found in marine and brackish systems with biofloc, respectively (Vinatea *et al.* 2010) and (Ray *et al.* 2010). The BF treatment supported high densities of filamentous cyanobacteria, although the latter was abundant in all treatments. Toxicity and mortality were not observed in the presence of these organisms (Alonso-Rodriguez & Paez-Osuna 2003) and (Zimba, Camus, Allen & Burkholder 2006). Blooms of cyanobacteria also form aggregates (Hoppe 1981), which are rapidly colonized by bacteria and protozoa, becoming sites of intense microbial activity (Young 2006). These aggregates are considered an important link in the food chain and may also contribute to the transfer of carbon to zooplankton and shrimp, considering that the efficiency of this pathway is better than that of the microbial loop (Grossart, Berman, Simon & Browdy 1998).

Among the heterotrophic protists, the ciliates play an important role in energy flow in aquatic ecosystems as predators of microalgae, bacteria and fungi and as a food

source for metazoans, fish and shrimp larvae (Nagano & Decamp 2004). The abundance and diversity of ciliates are good indicators of water quality and ecosystem dynamics (Decamp, Warren & Sánchez 1999). The ciliates stand out as bioindicators in external filter systems (Decamp *et al.* 2003), as they predate bacteria and increase the absorption of particles by the biofilm (Eisenmann, Letsiou, Feuchtinger, Beisker, Mannweiller, Hutzler & Arnz 2001).

These authors observed a succession of ecological groups in the BFT system with a tendency towards dominance by free-living ciliates in the early stages and a transition to scrapers after the second week of production. The flagellates are taxonomically diverse organisms including autotrophic and heterotrophic species and were present in this study. Flagellates are source of highly unsaturated fatty acids (HUFAs) and sterols (Decamp & Nagano 2001). In previous work has indicated their importance together with ciliates in the diet of *Farfantepenaeus paulensis* larvae (Thompson, Abreu & Cavalli 1999).

The recommended lipid level for crustaceans is less than 10% (D'Abramo 1997). Therefore, in the present work, the lipid content of the biofloc was within level recommended (A, 5.11%; C, 4.49%; BF, 3.79% and T, 2.64%). Other studies have found lower values (Tacon, Cody, Conquest, Diyakaran, Forster & Decamp 2002), 0.61%;(Wasielesky *et al.* 2006), 0.49%; (Ju *et al.* 2008), 1.2 –2.3%; (Maicá, Borba & Wasielesky 2011), 2.1–3.6%) but also higher values (McInctosh *et al.* 2000), 12.5%. Among the essential fatty acids, the concentration of eicosapentanoic acid (EPA) (C 20:5) was significantly higher in treatment A (4%), and the concentration of linoleic acid (C 18:2) was significantly higher in treatment BF (12.6%). Penaeid shrimps lack de novo synthesis of n-3 and n-6 fatty acids and exhibit a low rate of phospholipid

biosynthesis, thus requiring these nutrients in a supplemented diet (Tacon 1987). In this experiment, fatty acids (20:5n3) were supplied in the biofloc, especially in treatment A.

Growth performance of shrimp

The growth and survival rates of shrimp are usually elevated in the presence of biofloc in intensive and super-intensive systems (Hari, Kurup, Varghese, Schrama & Verdegem 2006); (Samocha *et al.* 2007); (Godoy *et al.* 2012). The survival rate in this study was outstanding high in the treatment with the pennate diatom *Amphora coffeaeformis* (99%). This diatom was likely maintained in the biofloc system due to its epiphytic benthic habit in organic matter-rich environments. Throughout the experiment, the density of *Amphora* sp. remained high (10^{7} cells L^{-1}), and the cells formed aggregates or were epiphyticon other aggregates.

Despite the differences in survival rate, the growth rates of juveniles in all treatments (4.70 % / day) were higher (Tacon *et al.* 2002), 3.35–4.43 % / day), similar (Godoy *et al*. 2012), 4.49–4.75 % / day) or lower than (Maicá *et al.* 2011), 5.13–5.37 % / day) to those found in systems without water renewal. The high growth rates reinforce the importance of food quality in systems without an exchange of water, which can be observed in the results of this study, where the shrimp were fed with a 38% CP commercial feed at a low rate of only 10% of total biomass. The natural food available in extensive cultivation ponds can contribute to meeting the nutritional requirements of shrimp (Nunes, Gesteira & Goddard 1997). (Wasielesky *et al.* 2006) observed a significantly higher final biomass in biofloc water compared to clear water, confirming the benefit of natural productivity in farming systems without water exchange. The mean final biomass of juveniles was high in all treatments (T, 955.86 g/m^2; A, 944.14 g/m^2; BF 744.23 g/m^2 and C 643.28 g/m^2), which is comparable to values observed by

(Decamp *et al.* 2003) with salinity values of 25 (630 g/m^2) and 18 (600 g/m^2) but different from the salinity value of 9 (350 g/m^2). Maicá *et al.* (2011) also report significantly higher values at a salinity of 25 (220.5 g/m^2), compared to salinities of 4 (154.6 g/m^2) and 2 (91.2 g/m^2).

The feed conversion ratio (FCR) is an important index in aquaculture, with values lower than or equal to 1.00 being considered very good (Wasielesky *et al.* 2006). As the cost of food represents up to 60% of total production costs, a strict technical control becomes necessary to efficiently convert commercial feed into biomass. The average feed conversion rates were higher in the treatments with *Cylindrotheca* (1.08), biofloc (1.03), and lower in the treatments *Thalassiosira* (0.96) and *Amphora* (0.84). Similar results of FCR 0.86, 0.87 and 0.81 were found at salinities of 2, 4 and 36, respectively (Maicá *et al.* 2011), but higher values are very common (FCR 1.8 and 1.6 at salinity 18 and 36, respectively, Decamp *et al.* 2003); (FCR 2.3 and 2.1 at salinity of 2 and 20, respectively, Sowers, Tomasso, Browdy & Atwood 2006). The choice of a benthic diatom species exhibited good results in terms of maintaining the presence of the diatoms in the bioflocs. The centric diatom *T. weissflogii* also exhibited a high cell density throughout the experiment in the same way as *Amphora*, but the great majority of cells were free with little participation in the biofloc. The survival, final weight, weight gain and feed conversion rates were better in the treatment with *Amphora coffeaeformis* inocula and the centric diatom *T. weissflogii* compared to *Cylindrotheca closterium* and biofloc.

The biofloc from the *Amphora coffeaeformis* treatment exhibited high lipid levels, especially C 20:5-HUFA. The highly unsaturated fatty acids (HUFAs) have been regarded as critical for high rates of survival, growth and reproduction and a low rate of feed conversion for a wide variety of species of marine and freshwater organisms (Brett

& Müller- Navarra 1997). HUFAs are essential components of the formation of cell membranes, osmoregulation and the synthesis of prostaglandins that activate the immune system (Léger & Sorgeloos 1992). The benthic diatom *C. closterium* may not be attractive for shrimp considering that this treatment produced the worst results for mean final weight, weight gain and apparent feed conversion rate together with elevated feed consumption. This microalgae exhibited a lower cell density (10^6–10^7 cells/L) throughout the experiment, with free cells that did not participate much in the development of biofloc.

Conclusions

The results highlight the great potential of the diatoms *Amphora coffeaeformis* and *T. weissflogii* as triggers of biofloc formation and as food supplement for shrimp diet in biofloc nurseries system. The populations of these diatoms increased and remained in high cell density in the biofloc treatment with the addition of silicate. Significant difference was found for the survival rate, and all parameters of juveniles performance showed higher values in the treatments with *Amphora coffeaeformis* and the centric diatom *T. weissflogii* compared to *Cylindrotheca closterium* and biofloc only. Shrimp in those treatments exhibited the best survival rates, weight gain, average final biomass and feed conversion ratio, respectively.

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Table 1. Mean values ± standard deviation of the water quality parameters in the rearing of juvenile *L. vannamei* in treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and biofloc only (BF).

Table 2. Mean, minimum and maximum density of the microbiota in treatments *Amphora coffeaeformis* (A), *Cylintrotheca closterium* (C), *Thalassiosira weissflogii* (T) and (BF) Biofloc.

Table 3. Mean percentage ± standard deviation of lipid content in relation to the dry biomass and fatty acids of the biofloc in treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and biofloc (BF).

Table 4. Mean values ± standard deviation of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and (BF) biofloc.

Figure Legend

Figure 1. Cell concentration of diatoms and other microalgae *Planctonema* sp., *Oocystes* sp. in the treatment biofloc (BF).

Figure 2. Cell concentration of *Amphora coffeaeformis* in the treatment (A).

Figure 3. Cell concentration of *Cylindrotheca closterium* in the treatment (C).

Figure 4. Cell concentration of *Thalassiosira weissflogii* in the treatment (T).

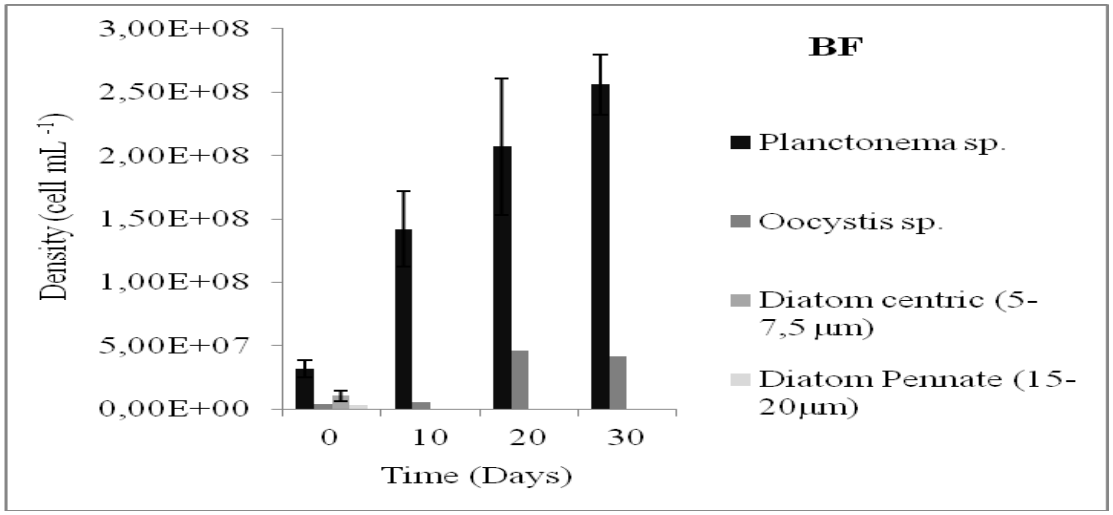

Figure 1.

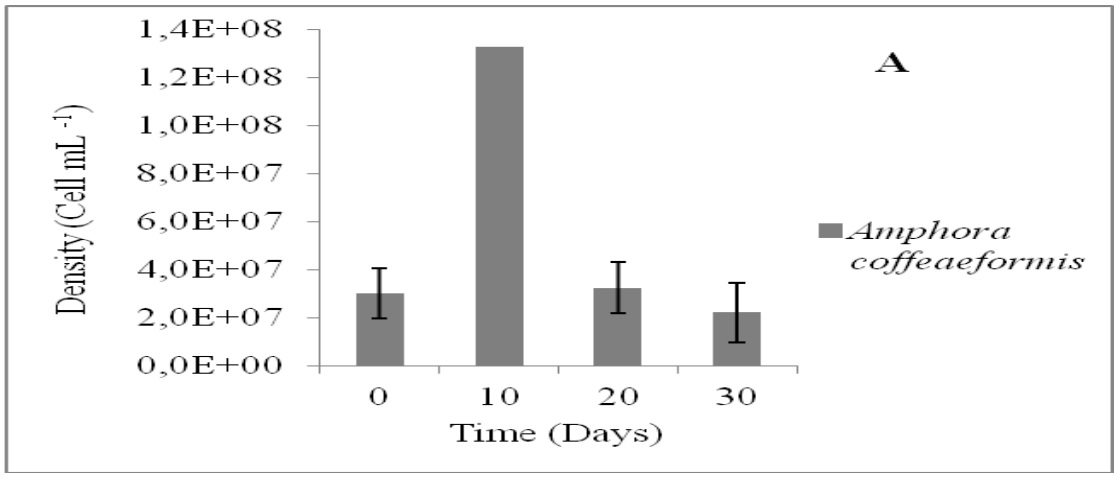

Figure 2.

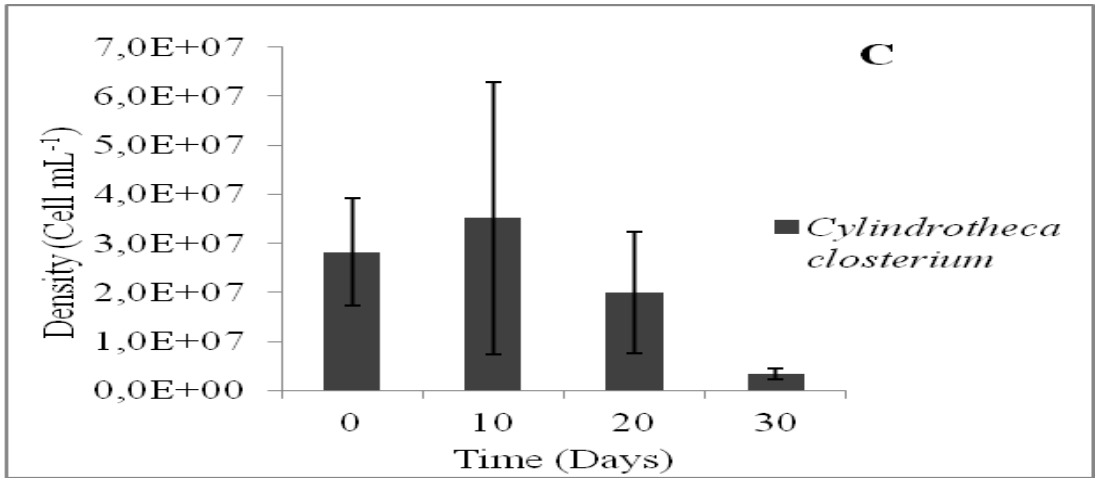

Figure.3

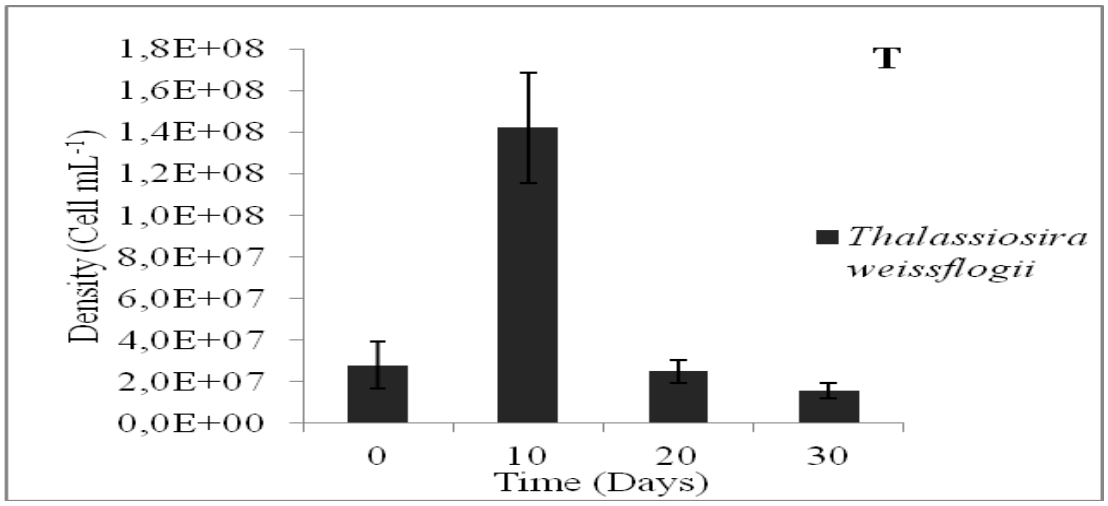

Figure 4.

Table 1. Mean values ± standard deviation of the water quality parameters in the rearing of juvenile *L. vannamei* in treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and biofloc only (BF).

Parameters	**Treatments**			
	A	**C**	**T**	**BF**
Temperature a.m. (°C)	25.99 ± 1.45	25.89 ± 1.43	25.89 ± 1.46	25.64 ± 1.35
Temperature p.m. (°C)	28.67 ± 2.59	28.76 ± 2.36	28.46 ± 2.39	28.91 ± 2.76
DO a.m. (mg L^{-1})	5.80 ± 1.18	5.84 ± 1.25	5.67 ± 1.26	5.76 ± 1.55
DO p.m. (mg L^{-1})	5.09 ± 1.61	5.17 ± 1.56	5.08 ± 1.58	5.03 ± 1.594
pH a.m.	8.08 ± 0.16	8.08 ± 0.19	8.06 ± 0.13	8.05 ± 0.20
pH p.m.	8.17 ± 0.23	8.14 ± 0.24	8.12 ± 0.22	8.22 ± 0.34
Salinity (g L^{-1})	37.20 ± 1.36	37.61 ± 1.01	37.36 ± 1.28	37.00 ± 1.34
Chl *a* (µg L^{-1})	219.9 ± 120.76	175.0 ± 137.82	199.5 ± 84.31	282.5 ± 297.99

Alkalinity (mg L^{-1} $CaCO_3$)	191.5 ± 58.83	172.5 ± 45.57	221.6 ± 72.37	189.9 ± 41.70
TSS (mg L^{-1})	260.1 ± 183.95	261.7 ± 197.28	270.4 ± 205.63	245.2 ± 152.76
TA-N (mg L^{-1})	1.1 ± 1.18[a]	1.2 ± 1.04 [b]	2.0 ± 2.64[b]	0.8 ± 1.01[a]
NO_2- N (mg L^{-1})	1.3 ± 2.97[a]	3.4 ± 5.47[b]	0.6 ± 2.01[a]	1.3 ± 2.98[a]
NO_3- N (mg L^{-1})	4.2 ± 6.65	3.4 ± 4.98	1.5 ± 2.37	2.5 ± 4.84
PO^3_4 – P (mg L^{-1})	1.0 ± 0.69	1.1 ± 0.71	1.0 ± 0.65	1.0 ± 0.73
Silicate (mg L^{-1})	0.5 ± 0.30[a]	0.6 ± 0.71[a]	0.4 ± 0.40[a]	0.2 ± 0.28[b]

Different letters in the same row indicate significant differences ($p < 0.05$).
DO, dissolved oxygen; TSS, total suspended solids; Chl *a*, chlorophyll *a*; TA-N, total ammonium nitrogen; NO_2-N, dissolved inorganic nitrite; NO_3-N, dissolved inorganic nitrate; PO_4^3-P, phosphate.

Table 2. Mean, minimum and maximum density of the microbiota in treatments *Amphora coffeaeformis* (A), *Cylintrotheca closterium* (C), *Thalassiosira weissflogii* (T) and (BF) Biofloc.

Taxon	Treatments			
	A	**C**	**T**	**BF**
Chlorophyceae (10^7 indiv L^{-1})				
Planctonema sp.	*	*	*	17.8 (6.5 – 24.1)
Oocystis sp.	*	*	*	0.3 (0 - 1.0)
Cyanobacteria (10^4 indiv L^{-1})				
Coccoid colonies	2.87	0.44	0.10	0.51
Trichomes Pseudanabaenoidae (25–100 μm)	12.20	8.14	52.96	41.59
Heterotrophic protists (10^6 cells L^{-1})				
Ciliates I (< 25 μm)	2.3 (0.9 – 6.3)	0.8 (0 – 2.5)	1.6 (0 – 8.2)	0.6 (0 - 0.9)
Ciliates II (25–50 μm)	95.2 (0.4 – 1.1)	0.1(0 – 0.4)	0.5 (0 – 2.0)	0.1 (0 - 0.1)
Ciliates III (> 50 μm)	0.5 (0 – 1.5)	*	0.1 (0 - 2.8)	1.6 (0.2 - 4.4)

Flagellates I (< 10 μm)	184.8 (0 – 290)	14.2 (0 – 30.7)	54.4 (0 - 68.5)	11.9 (2.0 - 22.6)
Flagellates II (10–20 μm)	*	1.5 (0 – 2.2)	1.6 (0 - 2.5)	*
Dinoflagellates I (< 15 μm)	3.6 (0.2 – 6.4)	1.0 (0 – 2.3)	3.6 (0 - 6. 1)	5.2 (0.1 - 16.8)
Dinoflagellates II (>25 μm)	0.8 (0.6 – 1.2)	*	*	*
Unidentified (10 ^{6} cells L^{-1})				
UN A (<10 μm)	4.4 (2.0 – 7.7)	8.5 (0 – 19.8)	0.7 (0 – 1.1)	10.3 (4.5 – 16.1)
UN B (>10 μm)	6.4 (0.2 – 22.4)	2.6 (0 – 11.5)	13.3 (0 – 17.8)	4.2 (0.9 – 11.7)

* cell density lower than 10^{5} cells L^{-1}.

Table 3. Mean percentage ± standard deviation of lipid content in relation to the dry biomass and fatty acids of the biofloc in treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and biofloc (BF).

	TREATMENTS			
	A	**C**	**T**	**BF**
Lipid content (%)	5.11 ± 1.02 [a]	4.49 ± 0.70 [ab]	2.64 ± 0.15 [c]	3.79 ± 0.29 [b]
Fatty acid profile				
C 14: 0	6.50 ± 4.24 [a]	7.6 ± 0.14 [b]	8.95 ± 2.47 [b]	3.10 ± 0.85 [a]
C 15: 0	0.95 ± 0.21 [a]	1.1 ± 0.00 [a]	1.55 ± 0.35 [b]	0.85 ± 0.07 [a]
C 16: 1	17.76 ± 5.18	19.15 ± 0.42	18.35 ± 3.04	7.80 ± 0.14
C 16: 0	33.90 ± 5.65	39.5 ± 1.41	36.60 ± 3.81	35.50 ± 1.48
C 17: 0	0.30 ± 0.10	0.7 ± 0.30	0.65 ± 0.07	0.60 ± 1.20
C 18: 2	4.35 ± 0.49 [b]	3.5 ± 0.14 [b]	4.60 ± 2.68 [b]	12.60 ± 0.56 [a]
C 18: 1C	18.95 ± 8.69 [a]	7.4 ± 0.84 [b]	8.55 ± 3.60 [b]	21.40 ± 0.84 [a]
C 18: 1T	5.00 ± 2.12 [a]	6.05 ± 1.30 [b]	7.35 ± 1.20 [b]	7.80 ± 0.28 [b]
C 18: 0	6.65 ± 5.44	6.05 ± 0.77	6.35 ± 2.05	4.25 ± 0.21
C 20: 5	4.00 ± 2.40 [a]	2.45 ± 0.07 [b]	1.85 ± 0.49 [b]	1.00 ± 0.28 [c]

C 24: 0	0.30 ± 0.03	0.6 ± 0.04	0.65 ± 0.07	0.70 ± 0.00

Different letters in the same row indicate significant differences ($p < 0.05$).

Table 4. Mean values ± standard deviation of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments *Amphora coffeaeformis* (A), *Cylindrotheca closterium* (C), *Thalassiosira weissflogii* (T) and (BF) biofloc.

	TREATMENTS			
Parameters	**A**	**C**	**T**	**BF**
Initial mean weight (g)	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03	0.21 ± 0.03
Final mean weight (g)	2.43 ± 0.89	1.75 ± 0.45	2.58 ± 0.92	2.15 ± 0.59
Mean survival (%)	99.5 ± 0.89 ^{a}	92.8 ± 4.2 ^{ab}	94.8 ± 1.4 ^{ab}	88.0 ± 11.4 ^{b}
Weight gain (g)	2.23 ± 0.89	1.55 ± 0.45	2.38 ± 0.92	1.95 ± 0.59
Final mean biomass ($g\ m^{-2}$)	944.1 ± 349.7	643.2 ± 187.9	955.8 ± 348.6	744.2 ± 263.0
Specific growth rate (%)	5.15 ± 0.01	5.14 ± 0.01	5.15 ± 0.01	5.15 ± 0.01
Feed Conversion Ratio (%)	0.84 ± 0.37	1.08 ± 0.54	0.96 ± 0.35	1.03 ± 0.63

Different letters in the same row indicate significant differences ($p < 0.05$).

ANEXO II

Is the Selection of the Diatom Species Important to Improve the Growth Performance of Juvenile Shrimps (*Litopenaeus vannamei)* (Boone, 1931) in Biofloc Technology System?

Is the Selection of the Diatom Species Important to Improve the Growth Performance of Juvenile Shrimps (*Litopenaeus vannamei*) (Boone, 1931) in Biofloc Technology System?

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Abstract

This study aimed to analyze the nutritional contribution of biofloc culture systems and their effects on growth performance of juvenile shrimps. Biofloc systems with juveniles of *Litopenaeus vannamei* were inoculated with three diatom species (three replicates per treatment): mature biofloc (BF); mature biofloc + *Amphora coffeaeformis* (BFA), mature biofloc + *Cylindrotheca closterium* (BFC), mature biofloc + *Conticribra* (*Thalassiosira) weissflogii* (BFT). The experiment lasted for 30 days. During this period, water quality parameters were monitored daily, nutrients every two days and the microbiota on the days 1, 15 and 30. The lipid content and fatty acid profile of the bioflocs were analyzed at the end of the experiment. The addition of the pennate (*A. coffeaeformis*) and centric (*C. weissflogii*) diatoms showed the best mean values of survival, weight gain, final biomass and feed conversion rate. The biofloc BFA presented highest lipid content differing from treatments BF and BFC, and the content of linoleic acid (C 18:2) was significantly higher in BF. The results indicate that *A. coffeaeformis* and *C. weissflogii* present a larger potential in enriching biofloc and supplementing food in shrimp production compared to *C. closterium* and that silicate additions are required for the maintenance of the diatoms in the BFT culture system.

Introdution

In the last decades, advanced technologies like water recirculation and the use of microbial bioflocs (Biofloc Technology System) emerged as alternatives to increase productivity without water renewal and emissions of effluents to the adjacent environment (De Schryver et al. 2008). The Biofloc Technology Culture System allows high stocking densities in the production of shrimp and tilapia, effectively reduces the emission of effluents by reusing the water for several cycles, reduces the risk of diseases and provides nutritional benefits. The microbiota can also maintain the water quality and serves as additional food source for the cultured animals (Avnimelech et al. 2007; Ballester et al. 2010).

Biofloc Technology Culture System permits the use of diets with lower crude protein content, which is supplied in part by the natural food associated with the formation of bioflocs. Burford et al. (2004) reported that up to 29% of the food consumed by the shrimp *Litopenaeus vannamei* (Boone, 1931) was derived from bioflocs. Among the microbiota of bioflocs, diatoms are widely used in the hatcheries to maintain water quality in the tanks (Hangreaves 2006) and as a food source due to their nutritional value and ability to synthesize and accumulate polyunsaturated fatty acids (PUFAs), including omega-3 series. The benthic diatoms produce extracellular polymeric substances (EPS) (Smith and Underwood 1998) and centric for Genus Thalassiosira. The quantity and chemical composition of EPS varies with radiation, availability of nutrients, growth phase and rhythms of vertical migration associated with photosynthesis. Diatoms are important in conventional systems (Patil et al. 2007), but possibly due to the high concentration of suspended material, which reduces light penetration and inhibits microalgae photosynthesis (Godoy et al. 2012) or due to low silicate in the system, it is difficult to maintain the diatoms in biofloc system.

The present study aimed to evaluate the addition of three species of diatoms to identify differences in their nutritional contribution of bioflocs and to the growth performance of juvenile cultured shrimp. The pennate diatoms *Amphora coffeaeformis* and *Cylindrotheca closterium* and the centric diatom *Conticribra weissflogii* previously classified as *Thalassiosira weissflogii*, were compared with a treatment only with biofloc.

Materials and Methods

Experimental System

In a greenhouse, 12 plastic rectangular tanks (0.5 m^2; 200 L; equipped with aeration systems and airstones) were filled with seawater and inoculated with diatoms and mature biofloc rich in Chlorophyceae (10 %) available in a biofloc raceway system and pumped into their respective experimental units using a submerged pump. Two pennate diatoms, *A. coffeaeformis* and *C. closterium*, and the centric diatom *C. weissflogii* were tested (initial cell density of $1x10^7$ cells/L). The experimental design was random with three replicates for each treatment: (BF) mature biofloc, (BFA) mature biofloc + *Amphora coffeaeformis*, (BFC) mature biofloc + *Cylindrotheca closterium* and (BFT) mature biofloc + *Conticribra* (*Thalassiosira) weissflogii*.

Diatoms and microorganisms were counted in Lugol's iodine solution (2%) fixed samples in sedimentation chambers on days 1, 15 and 30 of the experiment using a Zeiss Axiovert microscope equipped with phase contrast at the final magnification of 400x (Utermöhl 1958) in at least 10 fields chosen at random or the count of 200 diatoms.

Groups of 195 juveniles of *L. vannamei* (390/m^2) with initial weight of 0.215 ± 0.030 g, from the hatcheries of the Marine Aquaculture Station of the Federal University of Rio Grande, were stocked in the experimental units. Shrimp were fed twice a day (08:00 and 17:00 hours) with a commercial feed Potimar Active 38 (38% CP, 1.6 mm, Guabi ®, Campinas, SP, Brazil) offered on feeding trays (∅ = 15 cm, 5 mm mesh size, one per tank). The initial feeding rate

was set at 10% of the total biomass and adjusted to 5% at the end of the 30 days experiment (Jory et al. 2001).

Thirty shrimp from each experimental unit were individually weighed and returned to the tanks on days 1, 10 and 20, to adjust rate of feeding and all shrimp were measured at the end of the experiment. The growth performance was evaluated according to the following parameters: survival (S , %) = [(initial n - final n) / initial n x 100], where n = number of shrimp; final weight (FW, g) = ∑ final weight / total shrimp; weight gain (WG, g) = ∑ FW - initial weight (g); specific growth rate (SGR, % day) = [(ln final weight (g) - ln initial weight (g))/ time in days) x 100]; feed conversion ratio (FCR, %) = [(total feed consumed (g) / WG (g)) x 100] (assuming that all feed offered was consumed) and final biomass (FB, g) = ∑ FW of live shrimp.

Diatom Culture and Biofloc Source

Two pennate diatoms were tested: *A. coffeaeformis*, with a larger size (25-35 µm, biovolume 2826 μm^3), and the small-sized *C. closterium* (4-5 x 30-35 µm, biovolume 350 μm^3), and the centric diatom *C. weissflogii* (12-15 µm, biovolume 1327 μm^3). The morphology of these diatoms differs from lemmon wedge type (*A. coffeaeformis*), elongated rotational ellipsoid (C. *closterium*) to cylindrical (*C. weissflogii*). Their habitats are also different: *A. coffeaeformis* is epiphytic, epilithic or epipelic, *C. closterium* is epipelic but regularly stirred up in the plankton and *C. weissflogii* is planktonic (Round et al. 1990).

Strains of *A. coffeaeformis, C. closterium* and *C. weissflogii* were obtained from the Marine Microalgae Culture Collection, Institute of Oceanography-FURG. The diatoms were kept in a germination chamber in Guillard F/2 medium (Guillard 1975) prepared with previously filtered sea water (glass fiber filter Whatman GF/F pore size 0.7 µm) and sterilized in an autoclave at 120° C for 30 minutes. When the diatoms reached the exponential growth

phase, they were transferred to tanks containing filtered seawater (5 µm pore filter, Cuno) preparing for inoculation. The mature biofloc from a raceway system was used in the four treatments (TA- N = 0.17 mg/L , NO_2 = 0.02 mg/L , NO_3 = 0.86 mg/L, PO^3_4 = 0.26 mg/L, Alcalinity = 127 mg/L, Silicate = 0,4 mg/L, Chl *a* = 6,3 µg/L e TSS = 138 mg/L).

The initial nominal C:N ratio was approximately 15:1, was necessary to add organic fertization to the tanks, during the experiment no water exchange was performad. The addition of sugar cane molasses (37.46 % C and 0.57 % N) into carbon source was based on Avnimelech (1999) and Ebeling et al. (2006) who determined that 6 g is needed to convert 1g total ammonium nitrogen (TA-N) into bacterial biomass. The addition of sugar cane molasses was made when the concentration of (TA-N) were equal or higher than 0.5 mg/L.

Every four days, the silicate (SiO_2) concentration was measured and if the concentration was lower than 1 mg/L, it was added, in equal proportions (1N:1Si) (Brzezinski 1985). On the first experimental day, the experimental units were inoculated with diatoms (initial cell density of $1x10^7$ cells/L) and biofloc and shrimp were stocked to aid in biofloc development.

Water Quality

Temperature, pH, salinity and dissolved oxygen were measured twice daily (8:00; 17:00) in each experimental unit (YSI model ® 556 MPS-USA). Every two days, water samples were filtrated in glass-fiber filters (GF 50-A) and the total ammonia nitrogen ($N\text{-}NH_3$ + NH_4), (UNESCO 1983) and nitrite ($N\text{-}NO_2$) were measured. Once a week, nitrate (NO_3-N) and phosphate ($P\text{-}PO_4$) were measured (Aminot and Chaussepied 1983), and the concentration of silicate (SiO_2), was estimated (Strickland and Parsons 1972) every four days, using a digital Micronal B342 II spectrophotometer. The concentration of chlorophyll *a* (Chla) and total suspended solids (TSS) were measured every two days by filtering water samples (50 ml)

through glass-fiber filters (Whatman GF/F pore size 0.70 µm; diam. 47 mm). The Chl *a* fluorescence was measured using a Turner Design fluorimeter, following (Welschmeyer 1994) and TSS values were estimated by difference between final and initial weight of each filter, following (AOAC 1995) using a Sartorius balance (0,001 g). Alkalinity was monitored weekly by titration with hydrochloric acid to the methyl orange endpoint, following (APHA 1998).

Lipid Content and Fatty Acid Profile of Biofloc

At the end of the experiment, biofloc samples were sampled, filtered through strainer (50 µm), washed with 20 mL (0.5 M ammonium formate) (Zhu & Lee 1997), concentrated and dried in an oven (60 C) to constant weight.

Lipids were extracted according to (Folch et al. 1957), and fatty acids were saponified with KOH (50%). Fatty acid methyl esters (FAMEs) were prepared by esterification with BF_3 10% in methanol, following (Metcalfe and Schmitz 1961). The FAME profile was separated by gas chromatography (Shimadzu GCMS-QP2010 Plus, Japan) equipped with a split/splitless injector and mass detector with an operation temperature of 280 C and inlet temperature of 230 C. The detection occurred as a complete scan of 0.2 seconds (mz^{-1} 30 to mz^{-1} 500). The column used was Crossbond 5% diphenyl/95% dimethyl polysiloxane (30 m x 0.25 mm x 0.25 µm, Restek). The electron ionization occurred at 70 eV. The conditions of operation of the chromatograph were as follows: injector at 250 C; column at 80 C (initial temperature, 0 min), followed by an increase of 10 C/min up to 180 C and 7 C/min to the final temperature of 330 C; helium flow of 1.3 mL/min; pressure of 88.5 kPa; mean linear velocity of 42 cm/s; 1 µL injection volume with a split ratio of 1:100. The compounds were characterized by the retention time and confirmed by mass spectrometry.

Statistical Analysis

The growth performance data were analyzed using a one-way ANOVA ($\alpha = 0.05$) after verification of the homoscedasticity (Levene's test) and normality of the data (Kolmogorov-Smirnov test). The specific growth rate, survival and fatty acid concentrations were arcsine transformed before analysis. Differences among the treatments were tested with Tukey's multi-comparison test (Sokal and Rolf 1995). The abiotic parameters of water quality, concentration of Chla and TSS were compared by nonparameric Kruskal-Wallis test.

Results

Water Quality Parameters

Water quality values are shown in Table 1. Only the silicate concentrations data showed significant differences ($P<0.05$), with the lowest values in the BF treatment, which has not received addition of silicate during the trial period. The mean values of temperature, dissolved oxygen, pH, salinity, Chla, alkalinity, total suspended solids (TSS), total ammoniacal nitrogen (TAN), nitrite, nitrate and phosphate showed no significant differences between treatments.

Microbiota

The variety of organisms reflects the diversity of trophic levels present in the water (Table 2). Except in BF, the cell density of diatoms remained throughout the experiment, however, BF presented centric (5-8 µm; 3.4×10^5 cells/L) and pennate (15-20 µm; 1×10^7 cells/L) diatoms on the first day of experiment only (Figs. 1, 2, 3, 4). Cyanobacteria were present in all treatments; coccoid cyanobacteria were more abundant in the treatment BFC (4.21×10^4 cells/L) and less abundant in BF (0.75×10^4 cells/L), and filamentous cyanobacteria

of the Subfamily Pseudanabaenoideae (Pseudanabaenaceae) were more abundant in the treatment BF (41.59 x 10^4 cells/L) and less abundant in BFT (9.35 x 10^4 cells/L). The chlorophytes *Planctonema* sp. and *Ocystis* sp. were present in all treatments but more abundant in BF (17.8 x 10^4 and 3.01 x 10^4 cells/L, respectively) and less abundant in BFT (4.80 x 10^4 and 0.06 x 10^4 cells/L, respectively).

Heterotrophic protists were also observed: ciliates in three size ranges (C1 < 25 μm; C2 25-50 μm; C3 > 50 μm), two size ranges of flagellates (F1 < 10 μm; F2 10-20 μm) and heterotrophic dinoflagellates (D1 < 15 μm; D2 > 25 μm). In treatment BF, ciliates of both size ranges, dinoflagellates D1, flagellates F1 and spherical unidentified cells (UN A 5-7 μm; UN B 17- 22 μm) were observed. Treatment BFA also included ciliates, dinoflagellates, flagellates F1, and spherical unidentified cells (UN A 5-7 μm; UN B 17- 22 μm). In BFC ciliates of both size ranges, flagellates F1, dinoflagellates D1, and spherical unidentified cells (UN B 17- 22 μm) were present. Ciliates, flagellates and dinoflagellates of all classes and spherical unidentified cells (UN B 17- 22 μm) were present in the treatment BFT tanks.

Lipid Content and Fatty Acid Profile of Biofloc

The lipid content varied from 2.54 ± 0.46 to 5.00 ± 1.33 (Table 3). The biofloc in the treatment BFA exhibited the highest lipid content (5%), differing significantly from the other treatments. The concentration of the fatty acids considered essential for farmed organisms, linoleic acid (C 18:2) was significantly higher in treatment BF (12.6%), while the eicosapentanoic acid (EPA) (C 20:5) was not significantly different among the treatments.

Growth Performance of Shrimp

The mean survival rate of the shrimp (88 to 95%) was high in all treatments and no significant differences were observed for the growth performance indicators at $P < 0.05$ (Table

4, Figure 1). The final mean biomass was higher in BFA and BFT treatments significant difference with at $P < 0.05$ (Table 4, Figure 1). However, there was a clear tendency of higher mean values of final weight, weight gain, in the treatments with the diatoms *Amphora coffeaeformis* and *Conticribra weissflogii* compared to the treatments with the diatom *Cylindrotheca closterium* and biofloc only. The mean feed conversion rate also showed better performance in the former (0.7-0.8) than in the latter (1.0-1.2).

Discussion

Water Quality

The temperature, salinity and the concentration of dissolved oxygen during this experiment were within the recommended ranges for the best growth and survival of juvenile *L. vannamei* (Ponce-Palafox et al. 1997; Van Wyk and Scarpa 1999). Alkalinity and pH were also appropriate, according to Van Wyk and Scarpa (1999). The concentration of total ammonia nitrogen and nitrite remained within the safe level of up to respectively 3.95 mg/L (Lin and Chen 2001) and 25.7 mg/L (Lin and Chen 2003) for juvenile *L. vannamei*. The nitrate concentration in all treatments was also acceptable (≤ 60 mg/L; Van Wyk and Scarpa 1999). Phosphorus is an important nutrient for the growth of microalgae and is accumulated over the cultivation period, as observed in this experiment, with values similar to those found by (McIntosh et al. 2000; Burford et al. 2003; Casillas-Hernándes et al. 2007). The Biofloc Technology culture system is designed to use minima or no water exchange, hampering the elimination of phosphorus through water renewal. However, high concentration of phosphate should be avoided because this may lead to the occurrence of harmful cyanobacteria blooms in culture systems (Smith 1983; Anderson et al. 2002). The mean concentration of total suspended solids (201-288 mg/L) was similar to that found by (McIntosh et al. 2000), but lower than the values found by (Avnimelech 2007; Vinatea et al. 2010; Godoy et al. 2012). Silicate was added

in the experimental units containing diatoms, as these microalgae require this element to build their cell covering, the siliceous frustules. Therefore, silicate values were relatively high due to its addition every four days in treatments BFA, BFC and BFT, in contrast with the treatment BF without silicate additions. In the latter, the mean concentration was slightly higher than growth-limiting concentration (≤0.1 mg/L; Reynolds 2006) but minimum values (0.01-0.05 mg/L) clearly limited the growth and maintenance of diatoms in the biofloc system during the experiment. Therefore, the drastic reduction of diatoms observed in the treatment BF can be explained by the low silicate concentration and highlights the necessity of adding this element to propitiate their growth in BF systems. Normally, there are few diatoms in biofloc culture systems (Burford et al. 2003; Ray et al. 2010; Vinatea et al. 2010) and silicate limitation seems to be an important factor hampering their growth.

Bioflocs: Microbiota, Lipid Content and Fatty Acid Profile

Biofloc provide extra microbial protein and nutrients for reared animals (Burford et al. 2004; Avnimelech 2009). In shrimp culture with bioflocs, chlorophytes and cyanobacteria attain high Chla values and are generally co-dominants in brackish (Ray et al. 2010) and marine water (Vinatea et al. 2010). In this study, even with diatoms inoculation at the beginning of the experiment, the mean concentration of Chla was lower than in other experiments with (Godoy et al. 2012) or without (Burford et al. 2003; Decamp et al. 2007) the addition of diatoms. The BFT system is still recent and further studies are necessary for a better understanding of the microbiota dynamics and to improve its efficiency.

Diatoms used in this study have excellent nutritional value, contributing with essential amino acids and polyunsaturated fatty acids (PUFAs) from the omega-3 series (Moss 2002; Ju et al. 2008), and their digestion by shrimp is facilitated by low fiber content (Moss 2002). The benthic diatoms produce extracellular polymeric substances (EPS) (Smith and Underwood

1998), which forms a mucilage which binds to actin filaments, leading the force necessary for movement, also called "gliding" and consequent adhesion to the substrate, but diatoms centric to the genus Thalassiosira also produced EPS. The quantity and chemical composition of EPS varies with radiation, availability of nutrients, growth phase and rhythms of vertical migration associated with photosynthesis. According to Underwood et al. (2004) benthic diatoms produce higher concentrations of EPS in situations when limiting nutrients. Thus, to produce the EPS matrix, cells create a microenvironment stable and optimal conditions for their growth (Decho 2000), important in cell adhesion to substrates (Daniel et al. 1987), locomotion (Edgar and Pickett - Heaps 1984) and resistance to toxins (Decho 1990). Despite their great importance in conventional systems (Patil et al. 2007), little is known about the metabolism and behavior of diatoms in the Biofloc system. The high concentration of suspended material in these systems significantly reduces the light penetration and may inhibit photosynthesis. In addition, the competition for nutrients by diatoms and heterotrophic bacteria and cyanobacteria seems to further difficult the maintenance of high densities of diatoms in this system (Godoy et al. 2012).

High-water-quality in our experiment was probably due to the uptake of dissolved inorganic nutrients (ammonium, phosphate) as observed in the presence of biofilm composed mainly of pennate diatoms (*Amphora*, *Campylopyxis*, *Navicula*, *Synedra*, *Hantzschia* and *Cylindrotheca*; Thompson et al. 1999). In addition, except for BFT, the treatments in this study supported high density of filamentous cyanobacteria, but toxicity and mortality were not observed, as they were in other studies (Alonso-Rodriguez and Paez-Osuna 2003; Zimba et al. 2006). Among heterotrophic protists, ciliates play an important role in the energy flow in aquatic ecosystems (Nagano and Decamp 2004) and occurred in a high concentration in treatments BFA (C1 - 0.89 x 10^6 cells/L and C2 - 0.30 x 10^6 cells/L) and BF (C3 - 1.63 x 10^6 cells/L). The abundance and diversity of ciliates are good indicators of water quality and

ecosystem dynamics (Decamp et al. 1999). In addition, they contain high intracellular concentrations of free amino acids (Decamp et al. 2001). Decamp et al. (2007) reported that ciliates reach extremely high concentrations (6,000 cells/mL) in biofloc systems depending on the salinity of the water and dynamic interactions between the ciliates. The flagellates are taxonomically diverse organisms including hardly identifiable autotrophic and heterotrophic species and were present in high densities in this study. Flagellates are a source of highly unsaturated fatty acid (HUFAs) and sterols (Decamp et al. 2001). In a BFT system, Maicá et al. (2011) found lower mean values (400 cells/mL) at low salinity (2–4) and highest (1,515 cells/mL) at salinity 25. The treatment A shown the higher values in all protozoan observed, can be related to the production of large quantities of EPS, which is a carbon hydrate is an attractive source for these organisms.

The lipid content of the biofloc in the present experiment was appropriate (BFA, 5.00%; BF, 3.79%; BFC, 3.03% and BFT, 2.93%), considering that the recommended level for crustaceans is less than 10% (D'Abramo 1997). Our results are similar to those found by Maicá et al. (2011) (2.1-3.6%) but other studies have found lower values (Tacon et al. 2002, 0.61%; Wasielesky et al. 2006, 0.49%; Ju et al. 2008, 1.2 -2.3%) but also higher values (McInctosh et al. 2000, 12.5%).

Among the essential fatty acids, the concentration of linoleic acid (C 18:2-EFA) was significantly higher in treatment BF (12.6%), although eicosapentanoic acid (EPA) (C 20:5-HUFA) values (0.07 to 1.1%) did not differ significantly among treatments. Penaeid shrimps lack *de novo* synthesis of n-3 and n-6 fatty acids and exhibit a low rate of phospholipid biosynthesis, thus requiring these nutrients in a supplemented diet (Tacon 1987). Crustaceans are unable to efficiently or sufficiently elongate C_{18} fatty acids and thus require long-chain 20(n-3) or 22(n-3) fatty acids as essential inclusions in their diet (Sargent et al. 1989). In these cases, diets should include 1-2% long-chain PUFAs. In this experiment, all treatments with

bioflocs were able to supply the recommended level of fatty acids of long-chain particularly eicosapentanoic acids (EPA) (20: 5 C-HUFA), essential to the optimum growth of organisms.

Growth Performance of Shrimp

The Biofloc Technology culture system provided high survival and growth rates for shrimp. The survival rate was highest in the treatments with addition of the pennate diatoms *A. coffeaeformis* (BFA 95.9%) and centric *C. weissflogii* (BFT 93.3%) when compared to the treatments with *C. closterium* (BFC 88.7%) and BF (88.0%). The mean values of final biomass and feed conversion ratio of juvenile *L. vannamei* were also better in the treatments inoculated with the epiphytic pennate *A. coffeaeformis* (BFA) and the centric diatom *C. weissflogii* (BFT) than in the treatment with the pennate diatom *C. closterium* (BFC). The cell volume of *A. coffeaeformis* is twice the value of *C. weissflogii* and it is better adapted to bioflocs aggregates, but the latter also contributed to an excellent shrimp performance. On the other hand, the eight times smaller *C. closterium*, compared with *A. coffeaeformis*, did not participate in the formation of biofloc and coincided with lower mean performance of shrimp growth. The diatom *C. closterium* is probably not attractive for the enrichment of bioflocs, considering that this treatment produced the lower results for mean final weight and apparent feed conversion rate (1.22), together with an elevated feed consumption. *C. closterium* was considered a good choice as food for aquaculture (Matos et al. 2007), since this species presented the highest amount of chlorophyll *a*, soluble protein, total amino acids, nitrate, sodium and phosphorus compared to *Chaetoceros gracilis* and *Tetraselmis gracilis*. According to our results *C. closterium* was a poor option for shrimps juveniles aquaculture, which can be related with the cell form (needle-like), low nutritional content or palatability, or with low production of EPS, which is an attractive source for various microorganisms. It may be successfully used in other industrial applications as a settlement agent in abalone hatcheries, bioassay for the detection of

heavy metals in surface sediments, and as remediation tool to remove nutrients from aquaculture effluents (Kingston 2009).

The specific growth rates in this study (4.70 to 4.80% per day) exhibited values higher than those observed for juveniles of *L. vannamei* cultured in most BFT systems (Tacon et al. 2002; 3.35-4.43%), similar (Godoy et al. 2012, 4.49-4.75%) or lower than found by (Maicá et al. 2011; 5.13-5.37% per day). The high specific growth rate values reinforce the importance of the quality of natural food in BFT systems. In this experiment, the shrimp were fed with the amount of 10% of the total biomass of a commercial feed with 38% of crude protein and adjusted to 5% at the end of the experiment. Juvenile shrimp reared with diatoms consumed even less feed, exhibited best feed conversion rates (0.47) and highest weight gain (17%) compared to BF treatment and diatoms with bioflocs, at the end of a 30 day experiment (Godoy et al. 2012).

The final biomass in our experiment in the treatments BFA (915.20 g/m^2), BFT (901.38 g/m^2) and BF (744.23 g/m^2) was higher than observed by (Decamp et al. 2003) at the salinity of 25 (630 g/m^2) and 18 (600 g/m^2) and by (Maicá et al. 2011) at the salinity of 25 (220.5 g/m^2), of 4 (154.6 g/m^2) and 2 (91.2 g/m^2). The feed conversion ratio is an important index in aquaculture, with values lower than or equal to 1 (one) being considered good (Wasielesky et al. 2006). The FCR obtained in the experiments can be classified as lower for treatments BFA (0.76) and BFT (0.81), and higher for treatments BF (1.03) and BFC (1.22). (Maicá et al. 2011) obtained similar values in biofloc systems initially inoculated with *C.* (*Thalassiosira) weissflogii* (0.86, 0.87 and 0.81).

Conclusion

Natural food had great importance for the growth performance of shrimp in all treatments. The treatment BFA exhibited the greatest lipid content of bioflocs (5%), differing

significantly from the treatments BF and BFC. The content of linoleic acid (C 18:2-EFA) was significantly higher in BF (12.6%), and that of eicosapentanoic acid (EPA) (C 20:5-HUFA) did not differ significantly among treatments. The addition of the pennate diatom *A. coffeaeformis* and the centric diatom *C. weissflogii* resulted in an excellent growth performance of the shrimp cultivated with bioflocs with the best survival values, weight gain, mean final biomass and feed conversion rate, respectively, differently from what happened with the pennate diatom *C. closterium*, with the lowest values of survival, weight gain , mean final biomass and feed conversion rate. These results highlight the need for selecting the diatom species and the great potential of the pennate diatom *Amphora coffeaeformis* and the centric diatom *Conticribra weissflogii* to enriching biofloc and supplementing food in shrimp cultivations with Biofloc Technology culture systems.

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Figure Legend

Figure 1. Cell concentration of diatoms and other microalgae *Planctonema* sp., *Oocystes* sp.in the treatments (BF).

Figure 2. Cell concentration of *Thalassiosira weissflogii* and other microalgae *Planctonema* sp. and *Oocystes* sp. in the treatment (BFT).

Figure 3. Cell concentration of *Amphora coffeaeformis* and other microalgae *Planctonema* sp. and *Oocystes* sp. in the treatment (BFA).

Figure 4. Cell concentration of *Cylindrotheca closterium* and other microalgae *Planctonema* sp. and *Oocystes* sp. in the treatment (BFC).

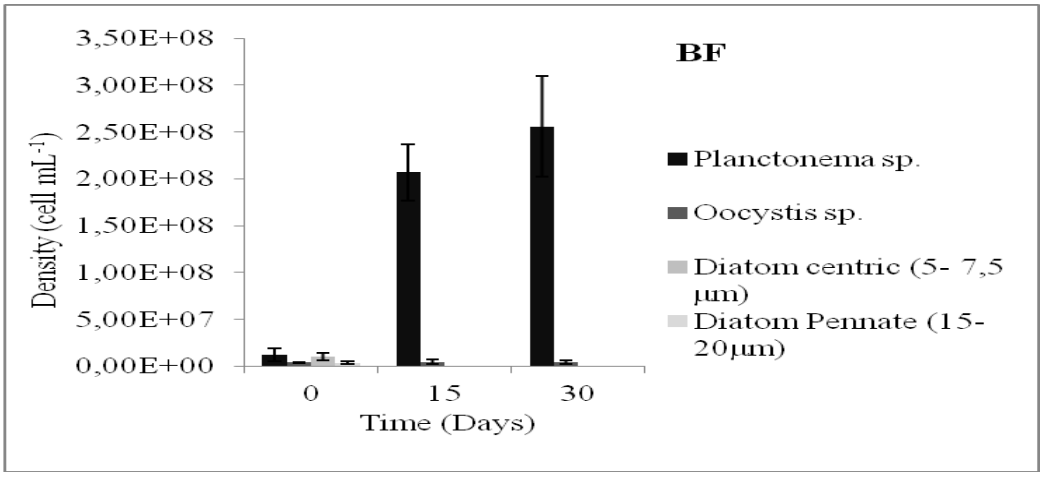

Figure. 1.

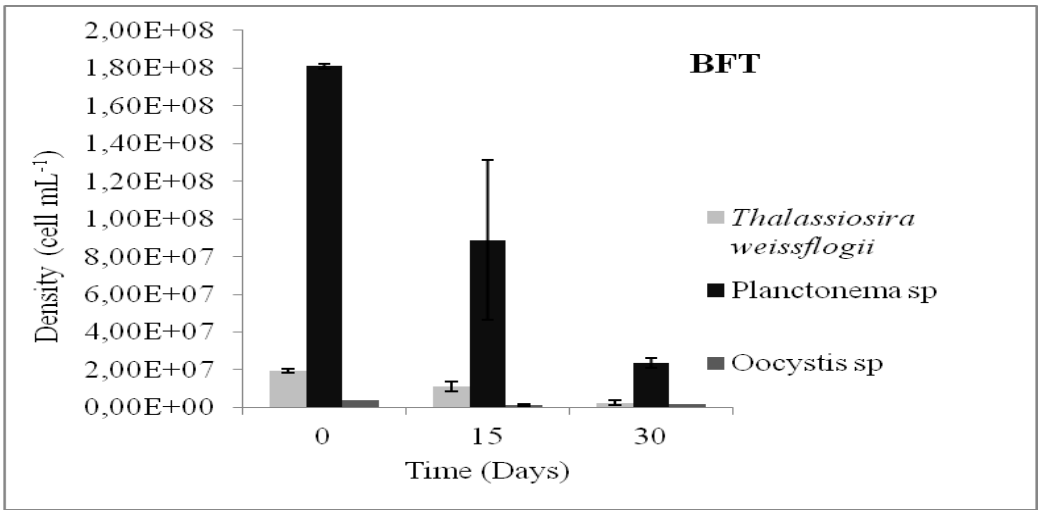

Figure. 2

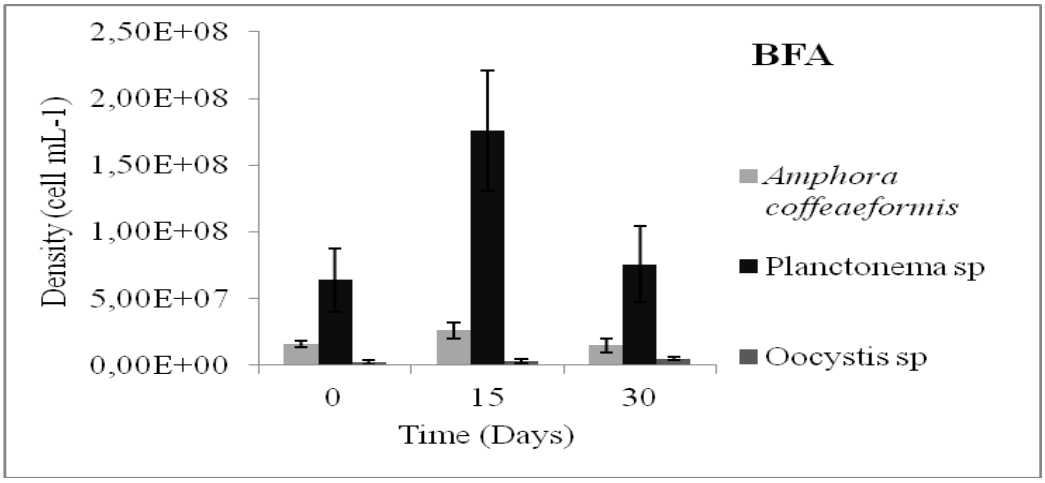

Figure. 3.

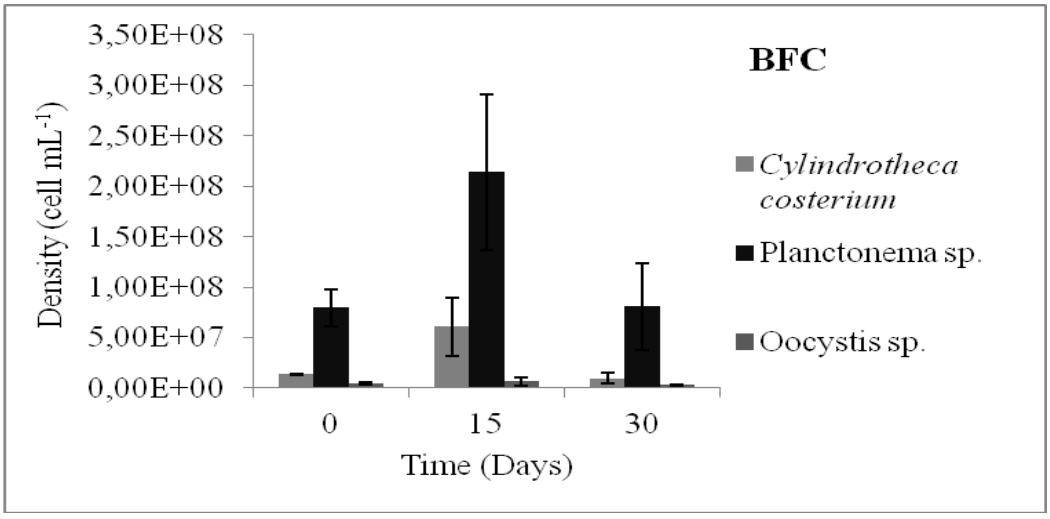

Figure. 4.

Table 1. Mean values (± SD) of the water quality parameters in the rearing of juvenile *L. vannamei* in treatments, mature biofloc only (BF), mature biofloc + *Amphora coffeaeformis* (BFA), mature biofloc + *Cylintrotheca closterium* (BFC) and mature biofloc + *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

Table 2. Mean, minimum and maximum density of the microbiota in the treatments: mature biofloc (BF), mature + *Amphora coffeaeformis* (BFA), mature + *Cylintrotheca closterium* (BFC) and mature + *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

Table 3. Mean percentage (±SD) of lipid content in relation to the dry biomass and fatty acids of the biofloc in treatments: mature biofloc (BF), mature + *Amphora coffeaeformis* (BFA), mature + *Cylintrotheca closterium* (BFC) and mature+ *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

Table 4. Mean values (± SD) of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments: mature biofloc (BF), mature + *Amphora coffeaeformis* (BFA), mature + *Cylintrotheca closterium* (BFC) and mature + *Conticribra (Thalassiosira*) *weissflogii* (BFT).

Table 1. Mean values (± SD) of the water quality parameters in the rearing of juvenile *L. vannamei* in treatments, mature biofloc only (BF), mature biofloc + *Amphora coffeaeformis* (BFA), mature biofloc + *Cylintrotheca closterium* (BFC) and mature biofloc + *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

Parameters	**Treatments**			
	BF	**BFA**	**BFC**	**BFT**
Temperature a.m. (C)	25.64 ± 1.35	25.72 ± 1.41	25.68 ± 1.46	26.00 ± 1.30
Temperature p.m. (C)	28.91 ± 2.76	28.53 ± 2.62	28.97 ± 2.86	28.57 ± 2.58
DO a.m. (mg/L)	6.50 ± 2.55	6.27 ± 1.04	6.34 ± 0.95	6.31 ± 1.05
DO p.m. (mg/L)	5.03 ± 1.59	5.08 ± 1.58	5.07 ± 1.61	5.00 ± 1.60
pH a.m.	8.05 ± 0.20	8.08 ± 0.19	8.07 ± 0.18	8.08 ± 0.16
pH p.m.	8.22 ± 0.34	8.20 ± 0.27	8.26 ± 0.31	8.25 ± 0.26
Salinity (ppt)	37.00 ± 1.00	37.41 ± 1.74	37.48 ± 1.51	37.40 ± 1.51
Chl *a* (µg/L)	282.51 ± 297.99	143.43 ± 89.93	242.60 ± 291.91	157.52 ± 135.55
Alkalinity (mg/L $CaCO_3$)	189.95 ± 41.70	200.83± 48.71	199.16 ± 46.85	205.41 ± 59.97
TSS (mg/L)	245.23 ± 152.76	201.36 ± 99.16	200.69 ± 102.94	288.87 ± 144.41
TA-N (mg/L)	0.81 ± 1.01	0.72 ± 0.56	0.70 ± 0.59	0.87 ± 1.14
NO_2-N (mg/L)	1.35 ± 2.98	2.88 ± 4.79	1.74 ± 3.12	1.36 ± 3.20
NO_3-N (mg/L)	2.50 ± 4.84	3.67 ± 4.68	3.25 ± 2.51	3.25 ± 4.14
PO^3_4-P (mg/L)	1.1 ± 0.71	1.0 ± 0.72	1.0 ± 0.53	1.0 ± 0.59
Silicate (mg/L)	0.20 ± 0.28^a	0.47 ± 0.20^b	0.46 ± 0.27^b	0.45 ± 0.31^b

Different letters in the same row indicate significant differences ($p < 0.05$).

DO, dissolved oxygen; TSS, total suspended solids; Chl *a*, chlorophyll *a*; TA-N, total ammonium nitrogen; NO_2-N, dissolved inorganic nitrite; NO_3-N, dissolved inorganic nitrate; PO_4^3-P, phosphate.

Table 2. Mean, minimum and maximum density of the microbiota in the treatments: mature biofloc (BF), mature + *Amphora coffeaeformis* (BFA), mature + *Cylintrotheca closterium* (BFC) and mature + *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

TAXON	**TREATMENTS**			
	BF	**BFA**	**BFC**	**BFT**
Chlorophyceae (10^7 cells/ L)				
Planctonema sp.	17.8 (6.57 - 31.5)	10.5 (4.70 - 41.6)	12.5 (3.21- 30.0)	4.8 (0.05 - 18.6)
Oocystis sp.	3.01 (0.025 - 1.08)	1.35 (0.34 - 4.64)	0.25 (0.03 - 0.21)	0.06(0.05 - 1.93
Cyanobacteria (10^4 cells/ L)				
Coccoid colonies	0.75	2.45	4.21	1.65
Trichomes Pseudanabaenoidae (25 - 100 μm)	41.59	21.23	24.86	9.35
Heterotrophic protists (10^6 cells/ L)				
Ciliates I (< 25 μm)	0.66 (0 - 1.16)	0.89 (0 - 1.93)	0.54 (0.03 - 1.42)	0.24 (0 - 1.41)
Ciliates II (25–50 μm)	0.06 (0 - 12.90)	0.30 (0 - 1.03)	0.23 (0 - 0.64)	0.13 (0 - 0.28)
Ciliates III (> 50 μm)	1.63 (0.26 - 4.39)	0.51 (0 - 1.77)	0.44 (0 - 0.89)	0.06 (0 - 0.27)
Flagellates I (< 10 μm)	12.00 (2.06 - 22.60)	24.93 (0 - 120.21)	1.66 (0 - 4.20)	6.06 (0 - 8.25)
Flagellates II (10–20 μm)	*	*	*	0.57 (0 - 1.39)
Dinoflagellates I (< 15 μm)	5.28 (1.55 - 11.0)	1.17 (0 - 3.33)	0.68 (0 - 1.93)	3.42 (0 - 5.10)
Dinoflagellates II (>25 μm)	*	0.28 (0 - 0.72)	*	0.39 (0.21 - 0.58)
Unidentified (10^6 cells/ L)				
UN A (5- 7 μm)	10.31 (4.51 - 16.1)	2.19 (0 - 4.77)	*	*
UN B (17-22 μm)	4.26 (0.90 - 11.70)	1.61 (0.20 - 5.03)	1.14 (0.09 - 5.77)	0.68 (0 - 3.76)

* cell density lower than 10^5 cells/ L.

Table 3. Mean percentage (±SD) of lipid content in relation to the dry biomass and fatty acids of the biofloc in treatments: mature biofloc (BF), mature + *Amphora coffeaeformis* (BFA), mature + *Cylintrotheca closterium* (BFC) and mature+ *Conticribra* (*Thalassiosira*) *weissflogii* (BFT).

	TREATMENTS			
	BF	**BFA**	**BFC**	**BFT**
Lipid content (%)	$3,79 \pm 0,29^{a}$	$5,00 \pm 1,33^{b}$	$3,03 \pm 0,25^{ac}$	$2,93 \pm 0,13^{ab}$
Fatty acid profile				
C 14: 0	$3,1 \pm 0,85^{a}$	$4,55 \pm 0,92^{ac}$	$7,05 \pm 2,61^{bc}$	$5,65 \pm 0,21^{c}$
C 15: 0	$0,87 \pm 0,07$	$1,35 \pm 0,21$	$2,35 \pm 2,47$	$0,95 \pm 0,21$
C 16: 1	$7,8 \pm 0,14^{a}$	$8,00 \pm 0,56^{a}$	$16,65 \pm 5,58^{bc}$	$14,95 \pm 3,18^{c}$
C 16: 0	$35,55 \pm 1,48$	$43,75 \pm 3,32$	$43,2 \pm 9,33$	$43,7 \pm 0,42$
C 17: 0	$0,6 \pm 1,20$	$1,2 \pm 0,84$	*	*
C 18: 2	$12,6 \pm 0,56^{a}$	$7,4 \pm 0,70^{b}$	$4,50 \pm 2,40^{b}$	$6,2 \pm 0,42^{b}$
C 18: 1C	$21,40 \pm 0,84^{a}$	$14,15 \pm 0,92^{b}$	$13,10 \pm 0,00^{b}$	$13,15 \pm 0,49^{b}$
C 18: 1T	$7,8 \pm 0,28$	$6,85 \pm 0,07$	$5,15 \pm 3,32$	$5,95 \pm 1,20$
C 18: 0	$4,25 \pm 0,21^{a}$	$5,8 \pm 0,00^{ab}$	$3,95 \pm 1,62^{ac}$	$4,50 \pm 0,28^{abc}$
C 20: 5	$1,0 \pm 0,28$	$1,1 \pm 0,77$	$1,1 \pm 0,77$	$0,7 \pm 0,98$
C 24: 0	$0,7 \pm 0,00$	$1,0 \pm 0,77$	*	*

*not detected.

Different letters in the same row indicate significant differences ($p < 0.05$).

Table 4. Mean values (± SD) of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments: mature biofloc (BF), mature + *Amphora coffeaeformis* (BFA), mature + *Cylintrotheca closterium* (BFC) and mature + *Conticribra (Thalassiosira) weissflogii* (BFT).

PARAMETERS	**TREATMENTS**			
	BF	**BFA**	**BFC**	**BFT**
Initial mean weight (g)	0.215 ± 0.03	0.215 ± 0.03	0.215 ± 0.03	0.215 ± 0.03
Final mean weight (g)	2.15 ± 0.59	2.45 ± 0.49	1.63 ± 0.43	2.48 ± 0.14
Mean survival (%)	88.03 ± 11.40	95.89 ± 1.36	88.71 ± 0.52	93.33 ± 2.23
Weight gain (g)	1.95 ± 0.59	2.25 ± 0.49	1.43 ± 0.43	2.28 ± 0.14
Final mean biomass (g/ m^2)	744.22 ± 263.08 [b]	915.18 ± 182.40 [a]	563.40 ± 145.68 [b]	901.38 ± 51.56 [a]
Specific growth rate (%)	4.80 ± 0.00	4.80 ± 0.00	4.70 ± 0.00	4.80 ± 0.00
Feed Conversion Ratio (%)	1.03 ± 0.63	0.76 ± 0.22	1.22 ± 0.29	0.81 ± 0.07

Different letters in the same row indicate significant differences

ANEXO III

Comparing the addition of diatoms and artificial substrates for the growth performance of *Litopenaeus vannamei* (Boone, 1931) in biofloc culture system

Comparing the addition of diatoms and artificial substrates for the growth performance of *Litopenaeus vannamei* (Boone, 1931) in biofloc bulture system

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ABSTRACT

This study aimed to evaluate the influence of adding artificial substrates and diatoms on the growth performance of juveniles of the shrimp *Litopenaeus vannamei* in biofloc system reared for 30 days. The following treatments (three replicates) were analyzed: mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *Amphora coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticribra* (*Thalassiosira) weissflogii* (CW) and inoculation the diatom *Conticribra weissflogii* with artificial substrate inoculated (CWAS). Water quality parameters were monitored daily and the microbiota of water and artificial substrates on the days 1, 15 and 30. Survival rates were high in all treatments (91-95%), however, the shrimp reached higher final weight, weight gain, final biomass, specific growth rate and best feed conversion rate in treatments with the addition of diatoms independently of the substrate addition (AW, AWAS, CW, CWAS) compared to the treatments with biofloc only (BW, BWAS). These results highlight the benefits of adding diatoms in biofloc systems. Although artificial substrates increase the area for the growth of diatoms they did not substantially change growth performance of the juvenile shrimp.

Key worlds: microbiota, nursery, rearing, *Litopenaeus vannamei*

Comparação da adição de diatomáceas e substratos artificiais para o melhor desempenho no crescimento de *Litopenaeus vannamei* (Boone, 1931) em sistema de bioflocos

RESUMO

Este estudo teve como objetivo avaliar a influência da adição de substratos artificiais e diatomáceas no desempenho do crescimento de juvenis do camarão *Litopenaeus vannamei* criados em sistema de bioflocos por 30 dias. Foram analisadas os seguintes tratamentos (três repetições): biofloco maduro (BW), biofloco maduro com substrato artificial (BWSA), inoculação da diatomácea *Amphora coffeaeformis* (AW), inoculação da diatomácea *Amphora coffeaeformis* com substrato artificial (AWSA), inoculação da diatomácea *Conticribra* (*Thalassiosira*) *weissflogii* (CW) e inoculação da diatomácea *Conticribra weissflogii* com substrato artificial (CWSA). Parâmetros de qualidade de água foram monitorizados diariamente e a microbiota de água e substratos artificiais nos dias 1, 15 e 30. A taxa de sobrevivência foi elevada em todos os tratamentos (91-95%), no entanto, o camarão atingiram maior peso final, ganho de peso, biomassa final, taxa de crescimento específico e melhor conversão alimentar nos tratamentos com a adição de diatomáceas independentemente da adição de substrato (AW, AWSA, CW, CWSA) em comparação com os tratamentos com apenas biofloco (B, BWAS). Estes resultados destacam os benefícios da adição de diatomáceas em sistema com bioflocos. Embora substratos artificiais aumentem a área para o crescimento de diatomáceas, esses não alteraram substancialmente o desempenho do crescimento do camarão juvenil.

Palavras Chaves: microbiota, berçario, criação, *Litopenaeus vannamei*

INTRODUCTION

In intensive production of juvenile of shrimp, substrates have been used in an effort to mitigate the negative effects of increasing the stocking density (Arnold et al. 2006) by providing an additional surface for the shrimp, reducing their competition for space, and negative behavioral interactions, such as cannibalism (Abdussamad & Thampy 1994). The presence of substrates allows the colonization with periphyton forming the biofilm, which is characterized as a complex community of aquatic organisms adhered to submerged substrates, including associated non-attached organisms and detritus (van Dam et al. 2002). The assembly contains bacteria, fungi, protozoans, microalgae, zooplankton and benthic organisms (Azim & Asaeda 2005), and helps to control water quality (Arnold et al. 2009) reducing pathogenic bacteria either by absorption of nitrogen (Austin & Austin 1999), producing antibiotics by microalgae (Alabi et al., 1999) and/or serving as natural food source for the cultured species (Abreu et al. 2007; Ballester et al. 2007; Silva et al. 2009) of marine shrimp post-larvae and juveniles in intensive production systems (Arnold et al. 2006, 2009; Ballester et al. 2007; Thompson et al. 2002). Although the use of substrates is in general considered beneficial for the shrimp cultures, some studies showed that their presence did not affect the performance of the cultured animals (Kumlu et al. 2001; Samocha et al. 1993); nor the water quality (Samocha et al. 1993).

The biofloc system involves the production of dense microbial communities that control the ammonia released from the cultivated organisms (De Schryver et al. 2008; Avnimelech 2009). Ammonia is absorbed by microalgae and heterotrophic bacteria or can be transformed by nitrifying bacteria (Ebeling et al. 2006).

Both the bioflocs in the water column and the periphyton present on the substrates, function to accelerate the biological removal of organic and inorganic wastes (Crab et al. 2007),

fulfilling similar functions in the control of water quality. In the superintensive biofloc culture system (BFT), suspended microorganisms are responsible for nutrients cycling, but the role of artificial substrates is poorly understood and studies with substrates and high shrimp biomass are still limited. The use of artificial substrate in BFT is cited in the literature (Browdy & Moss 2005; Krummenauer et al. 2011) but only recently it had been tested for *Litopenaeus vannamei* (Schveitzer et al. 2013). These authors found that higher final biomass, weight gain and survival rate were due to increased surface area of the tanks, which would reduce the relative stocking density and the level of shrimp stress.

Among the microorganism growing on substrates, most notably diatoms are nutritious and benefit shrimp production due to their low fiber content facilitating digestion by shrimp (Moss 2000) and by contributing qualities such as essential amino acids and highly unsaturated fatty acids (Ju et al. 2008). According to O'Brien (1994), the energetic value of diatoms is similar to that of copepods and polychaetes. The importance of diatoms in conventional systems is well known (Patil et al. 2007), but their metabolism and behavior in the BFT system were not studied in detail yet.

Considering the potential benefits of adding artificial substrates and diatoms to shrimp culture systems, the evaluation of these management tools in different production systems is important. The aim of this study was to compare the influence of artificial substrate and diatoms inoculation on the performance of juvenile shrimp *L. vanamei* in BFT system.

MATERIALS AND METHODS

Experimental design

Inside a greenhouse, 12 plastic rectangular tanks (0.5 m^2; 200 L) equipped with aeration systems and air stones were filled with filtered seawater (5 µm pore filter, Cuno, salinity 35) and inoculated with diatoms, artifical substrate, mature biofloc and stocked with

shrimp on the first experimental day. The biofloc used (TA- N = 0.0 mg/L, NO_2 = 0.0 mg/L, NO_3 = 64 mg/L, PO^3_4 = 0.70 mg/L, Alcalinity = 150 mg/L, Silicate = 0.3 mg/L, Chl *a* = 226.80 µg/L e TSS = 612.7 mg/L) was obtained from a raceway system, and pumped into the experimental units using a submerged pump. Strains of the pennate diatom *Amphora coffeaeformis* (C. Agardh) Kützing (1844) and the centric *Conticribra weissflogii* (Grunow) K. Stachura- Suchoples & D. M. Williams (2009) were obtained from the Marine Microalgae Culture Collection, of the Federal University of Rio Grande (FURG). The diatoms were kept in a germination chamber in Guillard F/2 medium (Guillard 1975) prepared with previously filtered sea water (glass fiber filter Whatman GF/F pore size 0.7 µm) and sterilized in an autoclave at 120° C for 30 minutes. When reaching the exponential growth phase, the diatoms were transferred to the tanks, with initial cell density of each, $2.5x10^7$ cells/L.

Artificial substrate (floating-type "Needlona ®" 17 x 23 cm) was fixed inside the tanks and 15 small pieces (2 x 2 cm) of this substrate were sewn at 10 cm depth. The area of the Needlona substrate added (150%) was calculated according to the lateral area of the tanks. Every two days small pieces were removed and stored in a freezer for chloropyll *a* and fixed with lugol's solution for microorganisms analysis.

Groups of 210 juveniles of *L. vannamei* ($400/m^2$) with initial weight of 0.07 ± 0.04 g, from the hatcheries of the Marine Aquaculture Station (FURG), were stocked in the experimental units. Shrimps were fed twice a day (08:00; 17:00 hours) with a commercial feed Potimar Active 38 (38% CP, 1.6 mm, Guabi ®, Campinas, SP, Brazil) offered on feeding trays (∅ = 15 cm, 5 mm mesh size, one per tank). The initial feeding rate was set at 50% of the total biomass and adjusted to 7% at the end of the 30 days experiment (Jory et al. 2001).

Three replicates were randomly assigned to the each treatment: juvenile shrimp *L. vannamei* stocked with mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom

Amphora coffeaeformis with artificial substrate (AWAS), inoculation the diatom *Conticribra weissflogii* (CW) and inoculation the diatom *Conticribra weissflogii* with artificial substrate inoculated (CWAS).

Water quality and biological analysis

The initial nominal C: N ratio was approximately 15:1, was necessary to add organic fertization to the tanks, during the experiment no water exchange was performad. The addition of sugar cane molasses (37.46 % C and 0.57 % N) into carbon source was based on Avnimelech (1999) and Ebeling et al. (2006) who determined that 6 g is needed to convert 1g total ammonium nitrogen (TA-N) into bacterial biomass. The addition of sugar cane molasses was made when the concentration of (TA-N) were equal or higher than 0.5 mg/L. Temperature, pH, salinity and dissolved oxygen were measured twice daily (8:00; 17:00) in each experimental unit (YSI model ® 556 MPS-USA). Every two days, water samples were filtrated in glass-fiber filters (GF 50-A) and the concentrations of total ammonia nitrogen ($N\text{-}NH_3$ + NH_4), (UNESCO 1983) and nitrite ($N\text{-}NO_2$) were measured. Once a week, nitrate ($NO_3\text{-}N$) and phosphate ($P\text{-}PO_4$) were measured (Aminot & Chaussepied 1983), and the concentration of silicate (SiO_2), was estimated every four days, using a digital Micronal B342 II spectrophotometer (Strickland & Parsons 1972). If the concentration of silicate (SiO_2) was lower than 1mg/L, silicate was added in equal proportion (1N:1Si) in the treatments with diatoms (Brzezinski 1985). The concentration of chlorophyll *a* (Chla) and total suspended solids (TSS) were measured every two days by filtering water samples (50 ml) through glass-fiber filters (Whatman GF/F pore size 0.70 μm; diam. 47 mm). The Chl *a* fluorescence in acetone extract was measured using a calibrated Turner Design fluorimeter, following (Welschmeyer 1994) and TSS values were estimated by difference between final and initial weight of each filter (AOAC 2000) using a Sartorius balance (0,001 g). Alkalinity was monitored weekly by titration with hydrochloric acid to the methyl orange endpoint (APHA

1998). Water and two small pieces of substrate samples were collected on days 1, 15 and 30 of experiment for analysis of chlorophyll a and microorganisms. Biofilm was detached from the artificial substrate with the aid of an ultrasonic homogenizer (470 Series, Cole Parmer Instrument, Chicago, Illinois). A 20- kHz frequency pulse was applied 6-8 times at equal intervals during 15-20 s time period preventing the sample overheating, and avoiding the disruption of microorganisms (Thompson et al., 2002).

Microorganisms in the water and artificial substrate were counted in Lugol's iodine solution (2%) fixed samples using sedimentation chambers and a Zeiss Axiovert microscope (Utermöhl 1958), equipped with phase contrast at the final magnification of 400x. At least 15 fields were chosen at random and the minimum of 200 cells were counted. Chlorophyll a present on the substrates was measured according to the method described above.

Thirty shrimps from each experimental unit were individually weighed and returned to the tanks on days 1, 10 and 20, to adjust rate of feeding, and all shrimps were measured at the end of the experiment. The growth performance was evaluated according to the following parameters: survival (S, %) = [(initial n - final n) / initial n x 100], where n = number of shrimps; final weight (FW, g) = ∑ final weight / total shrimp; weight gain (WG, g) = ∑ FW - initial weight (g); specific growth rate (SGR, % day) = [(ln final weight (g) - ln initial weight (g))/ time in days) x 100]; feed conversion ratio (FCR, %) = [(total feed consumed (g) / WG (g)) x 100] (assuming that all feed offered was consumed) and final biomass (FB, g) = ∑ FW of live shrimps.

Statistical analysis

The growth performance data were compared using a one-way ANOVA ($\alpha = 0.05$) after verification of the homoscedasticity (Levene's test) and normality of the data (Kolmogorov-Smirnov test). The specific growth rate, survival was arcsine transformed before analysis.

Differences among the treatments were tested with Tukey's multi-comparison test (Sokal & Rolf 1995). The abiotic parameters of water quality, concentration of Chla and TSS were compared by nonparameric Kruskal-Wallis test.

RESULTS

Water quality parameters

The mean values of temperature (a.m 27.03-27.34 ^{o}C; p.m. 30.37-30.96 ^{o}C), dissolved oxygen (a.m. 6.09-6.40 mg/L; p.m. 5.73-5.89 mg/L), pH (8.07-8.28), salinity (35.01-35.44), total ammonical nitrogen (0.54-2.22 mg/L) and substrate Chla (457.7-977.8 μg/cm) showed no significant differences between treatments (Table 1). The concentrations of water Chla and of TSS were different, with higher values in water samples from treatments without artificial substrate (BW, AW, CW) than in treatments with artificial substrate (BWAS, AWAS, CWAS). Alkalinity was significantly higher in the treatment CW (221.67 mg/L) while phosphate concentration was lowest (1.23 mg/L) in this treatment. The phosphate concentration was lower (1.23 mg/L) in CW. Nitrite concentration was significantly higher (4,1-6,2 mg/L) in treatments with diatoms and substrate (AWAS, CWAS) and biofloc only (BW, 2,8 mg/L) than in the treatments biofloc with substrate (BWAS; 1,8 mg/L) and diatoms (CW, AW; 0,7-2,8 mg/L). Nitrate was significantly higher (40, 6-48, 7 mg/L) in the treatments with biofloc only (BW, BWAS) compared to the diatom treatments (1, 4-5 mg/L; CW, AW, CWAS, AWAS). The values of silicate were significantly higher in the diatom tanks with this nutrient added (AW, AWAS, CW, CWAS) (Table 1).

Microbiota

The concentration of diatoms remained high throughout the experiment except in the biofloc treatments BW and BWAS (Table 2), with the following average values on days 1, 15 and 30 of the experiment, respectively: AW = 3.02, 3.49, 2.47 x 10^{7} cells/L; AWAS = 2.77,

1.92, 9.73 x 10^7 cells/L, CW = 3.20, 4.71, 7.00 x 10^7 ells/L; CWAS = 2.67, 1.25, 6.06 x 10^7 cells/L (Figures. 3, 4, 5, 6). In the treatments BW (1090 x 10^3 cells/L) and BWAS (287 x 10^3 cells/L) centric diatoms (2.5-5 μm) were observed on the first day of experiment only (Figures. 1, 2). Chlorophytes were present in the treatments BW (*Ocystis* sp., 3610 x 10^3 cells/L) and AW (3590 x 10^3 cells/L).

Heterotrophic protists were also observed: ciliates in three size classes (CI 10-20 μm; CII 20-60 μm; CIII > 60 μm), heterotrophic dinoflagellates in two size classes (DI 10-20 μm; DII > 20 μm) and flagellates (F 5–20 μm). In the treatment BW, ciliates CI and CII, dinoflagellates DI and DII and flagellates were found, while in BWAS the dinoflagellates DII were not observed. In the treatment AW, ciliates (CII, CIII), flagellates and in the former spherical unidentified cells (2.5-5μm) were found. In the treatments AWAS, larger ciliates (CII, CIII), flagellates and in the latter large dinoflagellates (DII) were also found. The treatment CW presented lowest diversity of heterotrophic protests (ciliates CII, flagellates), whereas CWAS presented in addition the ciliates CII, CIII, dinoflagellates DII and flagellates.

On the artificial substrates added (AAS, CAS) the diatoms increased throughout the first two weeks of experiment and decreased afterwards, with the following average values on days 1, 15 and 30 of the experiment, respectively: in AAS, *A. coffeaeformis* (0.0, 59.0, 45.30 x 10^3 cm^2) and *C. weissflogii* (0.0, 1.15, 0.74 x 10^3 cm^2); in CAS, *A. coffeaeformis* (0.0, 45.40, 32.90 x 10^3 cm^2), *C. weissflogii* (0.0, 3.50, 1.72 x 10^3 cm^2) and *Cylindrotheca closterium* (0.0, 14.80, 2.01 x 10^3 cm^2) (Figure. 8 and Figure. 9). It is important to note that *A. coffeaeformis* attained the highest cell density on the substrate, even in the CAS treatment, where it was not added, thus showing the best adaptation of this pennate diatom.

In the artifical substrate of biofloc (BAS), the pennate *A. coffeaeformis* was also found (*A. coffeaeformis* 0.0, 7.68, 3.24 x 10^3 cm^2) together with other small pennates (10-12 μm) (0.0, 7.25, 3.90 x 10^3 cm^2) (Figure. 7). The chlorophytes *Ocystis* sp. (0.0, 0.71, 0. 28 x 10^3 cm^2) and

others not identified (0.0, 0.97, 2.35 x 10^3 cm^2) were present in BAS only. The heterotrophic protists ciliates (CI, CII) and flagellates (5-20 μm) were observed in the treatment BAS; ciliates CII and flagellates in treatment AAS, while in CAS, the larger ciliates CII only.

Growth performance of shrimp

The mean survival rate of the shrimp (91-94%) was high in all treatments and no significant difference was observed for this growth performance indicators at $p < 0.05$ (Table 3). However, the shrimp reached higher final weight, weight gain, final biomass and specific growth rate in treatments with addition of the diatoms *Amphora coffeaeformis* (AW, AWAS) and *Conticribra weissflogii* (CW, CWAS) compared to the treatments with biofloc only (BW, BWAS). The mean feed conversion ratio was significantly lower in the treatments with diatoms AW, AWAS, CW and CWAS (1.33-1.61) than in BW and BWAS (2.12-2.19).

DISCUSSION

Water quality

The biofloc technology (BFT) is a culture system in which both the water quality is maintained by the bioflocs and *in situ* animal feed is simultaneously produced (Crab et al. 2007). Although bioflocs are beneficial, the concentration of total suspended solids must be controlled for optimal system performance, increased the availability of oxygen, improved water quality and resulted in the better growth performance of shrimp; used for clarification was effective for the maintenance of total suspended solids at 500 mg/ L (Gaona et al. 2011) as observed in the present study. The temperature, concentration of dissolved oxygen, pH and salinity during this experiment were within the recommended ranges for the best growth and survival of juvenile *L. vannamei* (Ponce-Palafox et al. 1997; Van Wyk & Scarpa 1999) as well as alkalinity (≥ 100 mg $CaCO_3$ /L; Van Wyk & Scarpa, 1999). The concentration of total ammonia nitrogen, nitrite and nitrate also remained within the safe range for juvenile *L.*

vannamei of up to respectively 3.95 mg/L (Lin & Chen 2001), 25.7 mg/L (Lin & Chen 2003) and acceptable (≤ 60 mg/L; Van Wyk & Scarpa 1999). Phosphorus is an important nutrient for the growth of microalgae and was accumulated over the cultivation period. It is known that *L. vannamei* incorporates only 39 and 35% of the nitrogen and phosphorus added to the system with feed and molasses (Silva et al. 2013).

Chlorophyll *a* and microbiota

Bioflocs provide packed microbial protein and nutrients for cultivated animals (Burford et al. 2004; Avnimelech 2009) being constituted by bacteria, diverse microalgae, protozoa and metazoa, undigested food, feces and suspended debris under strong aeration (De Schryer et al. 2008). Several groups of microalgae in intensive aquaculture systems are important food source for zooplankton, transferring their nutrients to higher levels of the food chain, while utilizing toxic ammonia and the less dangerous nitrate-nitrogen and phosphate compounds to construct cellular structures such as proteins and carbohydrates (Ray et al. 2010). In our study, mean water Chla concentrations in all treatments were higher compared to other studies in biofloc systems (Godoy et al. 2012; Maicá et al. 2011; Decamp et al. 2007).

Also the Chla concentration present on the artificial substrates (457–933 μg/cm) was relatively high. Studies with shrimp using diferent types of substrates have reported between 1 and 150 μg cm^{-2} of Chl-a on the periphyton (Thompson et al. 2002; Abreu et al. 2007; Ballester et al. 2007). The increased biomass of the periphyton explains the significant microbial activity in the tanks. According to van Dam et al. (2002), the predation performed by the cultured shrimp, the availability of nutrients and light and the type of substrate are among the factors that interfere with the formation of the periphyton. The screen used in this study was needlona®-needle felts made of a fibre layer and a supporting scrim. This screen is extremely compact, stable and mechanically robust, different from that used in other studies (Abreu et al. 2007; Ballester et al. 2007; Richard et al. 2009). The maturation time for the substrates can also

affect the periphyton biomass (Richard et al. 2009). In the present study, the substrates matured in the tanks for 3 days only. In superintensive biofloc systems, the high shirmp biomass and the environmental conditions in the culture tanks (low water transparency due to the excess suspended solids) can limit periphyton development on immobilized susbtrates and restrict using this mangement tool (Schveitzer et al. 2013). Our results do not confirm this and the periphyton increased even with low transparency in all treatments with and whithout addition of diatoms, suggesting that the needlona®-needle felts favored the development of the periphyton.

Among the various microalgae, most notably diatoms are nutritious and benefit shrimp production due to their low fiber content facilitating digestion by shrimp (Moss 2000). Diatoms contribute with essential amino acids and highly unsaturated fatty acids (Ju et al. 2008; Moss et al. 2001) and Sargent (1976) showed that benthic diatoms provide dietary essencial fatty acids as well.

The centric *Thalassiosira* and benthic diatoms produce extracellular polymeric substances (EPS) (Smith and Underwood 1998). The quantity and chemical composition of EPS varies with radiation, availability of nutrients, growth phase and rhythms of vertical migration associated with photosynthesis. According to Underwood et al. (2004) benthic diatoms produce higher concentrations of EPS in situations when limiting nutrients.

Many are the contributions in the production of EPS matrix such as create a microenvironment stable and optimal conditions for their growth (Decho 2000), important in cell adhesion to substrates (Daniel et al. 1987), locomotion (Edgar and Pickett - Heaps 1984) and resistance to toxins (Decho 1990).

The BFT system is still recent and further studies are necessary for a better understanding of the microbiota dynamics and to improve its efficiency. In our experiment,

diatoms remained in high concentration except in treatments where they were not inoculated, and no silicate was added, BW and BWAS. Diatoms are generally not been found in BFT (Burford et al. 2003; Ray et al. 2010; Vinatea et al. 2010), but we show that diatoms may be successfully maintained (>10^7 cells/L) in this system. Diatoms require silica to build their cell covering, the siliceous frustules, and the addition of silicate propitiated their growth in the tanks. In the treatments without silicate addition (BW, BWAS) the mean silicate was close to diatom growth-limiting concentration (≤0.1 mg/ L, Reynolds 2006) and the minimum values (0.01-0.05 mg/L) clearly limited the growth and maintenance of these microalgae.

It seems clear that, the shortage in silicate is the main cause of low concentrations of diatoms in biofloc production systems (Burford et al. 2003; Ray et al. 2010; Vinatea et al. 2010). Chlorophytes are commonly found in marine and brackish systems with bioflocs (Burford et al. 2003; Vinatea et al. 2010; Ray et al. 2010), but in our study this group was found in the treatments BW and AW only. Among heterotrophic protists, ciliates play an important role in the energy flow of aquatic ecosystems (Nagano & Decamp 2004) and their abundance and diversity are good indicators of water quality and ecosystem dynamics (Decamp et al. 1999). They predate bacteria and increase the absorption of particles by the biofilm acting as external filter systems (Decamp et al. 1999; Eisenmann et al. 2001). In addition, ciliates contain high intracellular concentrations of free amino acids (Decamp et al. 2001). The flagellates, present in high concentration in this study, are taxonomically diverse and include difficult to identify autotrophic and heterotrophic species. Flagellates are a source of highly unsaturated fatty acid (HUFAs) and sterols (Decamp et al. 2001).

The substrates were dominated by the inoculated diatoms *A. coffeaeformis* and *C. weissflogii* but other diatom pennates (12-25 µm) and *Cylindrotheca closterium*, chlorophytes, ciliates CI, CII and flagellates were also abundant. It must be noted that not only the species inoculated was growing on the respective substrate, indicating a contamination. But the high

concentration of *A. coffeaeformis* on the substrate of the treatment inoculated with *Conticribra* clearly indicates the preferential growth of the pennate diatom of epiphytic habitat rather than the latter inoculated species. The periphytic biota found in our study was a suitable natural food supplement for the shrimp, considering the nutritional composition of microorganisms, like nitrogen, phosphorus, essential polyunsaturated fatty acids, sterols, vitamins and carotenoids (Silva et al. 2009). Protozoans have a high protein to energy ratio and, due to their ability to synthesize long chain polynsaturated fatty acids they enrich the quality of microbial aggregates in the biofilm (Zhukova & Kharlamenko 1999). Comparing the isotopic signatures of periphyton grown on polyethylene substrates with that of *Penaeus esculentus* post-larvae, Burford et al. (2004) confirmed that the periphyton contributed up to fifty percent of shrimp carbon and nitrogen requirements. Abundant diatoms, filamentous algae and copepods, which were a diet component of the wild juvenile *Penaeus esculentus* from seagrass beds (O' Brien 1994), were found on artificial substrates as well (Arnold et al. 2006).

In the wild *L. vannamei* ingests a wide variety of microalgae, detritus, macrophytes, small molluscs, crustaceans and zooplankton (Senanan et al. 2009), however its feeding mechanisms is still unclear. It was suggested that penaeids (and specifically *L.vannamei*) present an unidentified method for consuming planktonic microalgae (Moss 1993), or that microalgal nutrition is obtained from intermediate prey (Stoner & Zimmermann 1988). The most cited method for *L.vannamei* food particle consumption is through grasping with chelate pereopods (Dall 1968), but it is unclear whether juvenile shrimp are able to filter particles from the water and artificial substrates. Physiology and behavior studies may assist in clarifying the complex nature of *L.vannamei* feeding mechanisms on suspended microrganisms in BFT. Burgett (1994) measured diatom digestion by quantifying photosynthetic pigments with high performance liquid chromatography finding that 63% of diatoms within detrital aggregates were digested by *L.vannamei* and concluded that diatoms in aggregates could support juvenile

penaeid growth. When feeding on diatoms in clumps bound by algin, an adhesive polysaccharide produced by algae, Moriaty (1976) reported a digestion efficiency of penaeids of 87%. Digestion and absorption take place before reaching the abdominal intestinal tract (Ceccaldi 1989) and the digestive anatomy and physiology of *L.vannamei*'s would contribute to the digestion of diatoms. Some studies suggested that penaeids are not able to digest cyanobacteria nor green algae (Burgett 1994; Kent et al. 2011). The sweeping behavior of the third maxillipeds may be the undefined mechanism which Moss (1993) suggested for consumption of small, suspended particles. The third maxillipeds have been shown to perform multiple functions, including manipulation of food particles, preening, and moving as a response to chemical stimuli (Alexander et al. 1980; Lee & Meyers 1995). The sweeping motion was suggested for sweeping aggregates towards the mouth in *L. vannamei* (Burgett 1994). The third maxillipeds are used for filter feeding by decapods (Bliss & Abele 1982), but these appendages are not commonly described as filter-feeding apparati in juvenile penaeid shrimp. Examination under the screening electron microscope revealed that the third maxillipeds have net-like setae which could potentially select for microorganism larger of 10µm in size, confirming the hypothesis that these appendages may be used by juvenile *L.vannamei* to filter food particles (Kent et al. 2011).

Growth performance of shrimp juveniles

The BFT system provided high survival for shrimp in all treatments, however, best values of final biomass, specific growth rate and feed conversion ratio of juvenile *L. vannamei* were obtained in the treatments inoculated with the pennate *A. coffeaeformis* (AW, AWAS) and the centric diatom *C. weissflogii* (CW, CWAS), regardless of substrate presence. The specific growth rates observed in this study (10.35-10.91% per day) were higher than those for juveniles of *L. vannamei* cultured in most BFT systems, with shrimp had larger than the present study (3.35-4.43%, Tacon et al. 2002; 4.49-4.75%, Godoy et al. 2012; 5.13-5.37%, Maicá et al.

2011), reinforcing the importance of the diatom addition and natural food in BFT systems. In this experiment, the shrimp were fed with 50% of the total biomass of a commercial feed with 38% of crude protein and adjusted to 7% at the end of the experiment. Juvenile shrimp reared with diatoms consumed even less feed and exhibited the best food conversion ratio (1.33 - 1.61). Other studies performed in biofloc systems inoculated with the diatom *Conticribra* (*Thalassiosira) weissflogii* reached feed conversion ratio even lower than 1.0 (Maicá et al. 2011 (0.24 ± 0.08 g); Godoy et al. 2012 (0.31 ± 0.10 g)), while studies conducted in different percentages of reuse of previous rearings with biofloc systems with shrimp (0.008 ± 0.003) only resulted in higher values (2.09-2.89, Krummenauer et al. 2012).

Several studies using artificial substrates were conducted with shrimp at diverse ontogenetic stages evaluating different substrate types under a variety of environmental conditions, with contrasting results. Some studies have reported that the periphyton adhered to substrates may be used as food source for the shrimp (Thompson et al. 2002; Abreu et al. 2007; Ballester et al. 2007; Silva et al. 2009) and that the addition of artificial substrates increases shrimp growth and reduces the feed conversion ratio (Arnold et al. 2006, 2009). Bratvold & Browdy (2001) observed that the presence of artificial substrates delayed negative effects of overcrowding, contributing to a better performance of *L. vannamei* reared in an intensive culture system. These authors reported higher shrimp production and lower feed conversion ratio (FCR 1.5) during the culture with artificial substrates (Aquamats ™) and that artificial substrates increased the nitrification in the tanks, which resulted in decreasing concentrations of NH3 nitrogen. Otoshi et al. (2006) grew juvenile shrimp in flow-through 110-L tanks in well water and in pumped water from an intensive shrimp culture pond with and without substrate (Aquamats ™) and showed significant increase in growth using pond water and substrate. In the only study conducted with BFT system, with shrimp (2.71 ± 0.1 g) did not appear to effect water quality since the concentrations of orthophosphate, ammonia and nitrite were not

significantly different in tanks with or without substates. The periphyton biomass was low and the biological activity on the substrates was not significant indicating that the microbial community association with the suspended bioflocs mainly controlled the water quality variables. The shrimp grow in the presence of the substrate exhibited greater weight gain, final biomass and survival than those without substrates, indicating that it served to increase the surface area of the tank and to reduce the relative stocking density, which appears to reduce the stress level of shrimp indicated by higher shrimp performance (Schveitzer et al. 2013). Enhanced production of *L. vannamei* postlarvae stocked at different densities in a flow-through system in the presence of substrate (Aquamats™) was also suggested to be due to the availability of attached particulate organic matter; and that the artificial substrate lessened the negative effects of high stocking density during the nursery phase (Moss & Moss 2004). Besides the nutritional contribution provided by the biofilm, the physical presence of artificial substrates within the culture units increases the area for shrimp distribution. Arnold et al. (2006) intensively grew post-larval tiger shrimp *Peneaus monodon* in tank systems at two densities with and without substrate (AquaMats™) and showed that the additional substrate enhanced survival, biomass, and feed conversion ratio.

In contrast, Kumlu et al. (2001) concluded that the presence of artificial substrates in tanks did not improve the growth or survival rates and moreover complicated the post-larval of *Metapenaeus monoceros* production management, not recommending their use in nursery culture systems of penaeid shrimp.

Our results indicate that artificial substrates did not influence the performance of juvenile *L. vannamei*, but rather the presence of diatoms was most important. However, it was observed that shrimps constantly were near the substrates and fed on the attached biofilm comprised mainly of diatoms and some ciliates and flagellates, which are all known as being part of the diet of penaeid shrimps in the natural environment (Allan & Maguire 1992; Allan et

al. 1995; Nunes et al. 1997). Notwithstanding, no statistical difference was observed in the treatments with and without substrates in the present study.

CONCLUSIONS

In biofloc system the addition of the diatoms *A. coffeaeformis* and *C. weissflogii* was more important than the presence of artificial substrate for the improvement of growth performance of the shrimp *L. vannamei* considering their final weight, weight gain, mean final biomass, specific growth rate and feed conversion rate.

The biofloc treatments with and without substrate presented lower values of final weight, weight gain, mean final biomass, specific growth rate and feed conversion rate.

These results highlight the benefits of adding diatoms in biofloc systems rather than artificial substrates. Considering that our results contrast with other studies showing the importance of substrate addition for the shrimp performance, we might conclude that microbial composition of the biofloc and substrate, are important factors influencing the final results.

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Figure Legend

Figure 1. Cell concentration of diatom and *Oocystes* sp.in the treatments (BW).

Figure 2. Cell concentration of diatom in the treatments (BWAS).

Figure 3. Cell concentration of diatoms and *Clorofícea* sp. in the treatments (AW).

Figure 4. Cell concentration of diatom in the treatments (AWAS).

Figure 5. Cell concentration of diatoms in the treatments (CW).

Figure 6. Cell concentration of diatoms in the treatments (CWAS).

Figure 7. Cell concentration of diatoms, *Clorofícea* sp and *Oocystes* sp on artificial substrates in the treatments (BAS).

Figure 8. Cell concentration of diatoms and *Nodularia* sp. on artificial substrates in the treatments (AAS).

Figure 9. Cell concentration of diatoms on artificial substrates in the treatments (CAS).

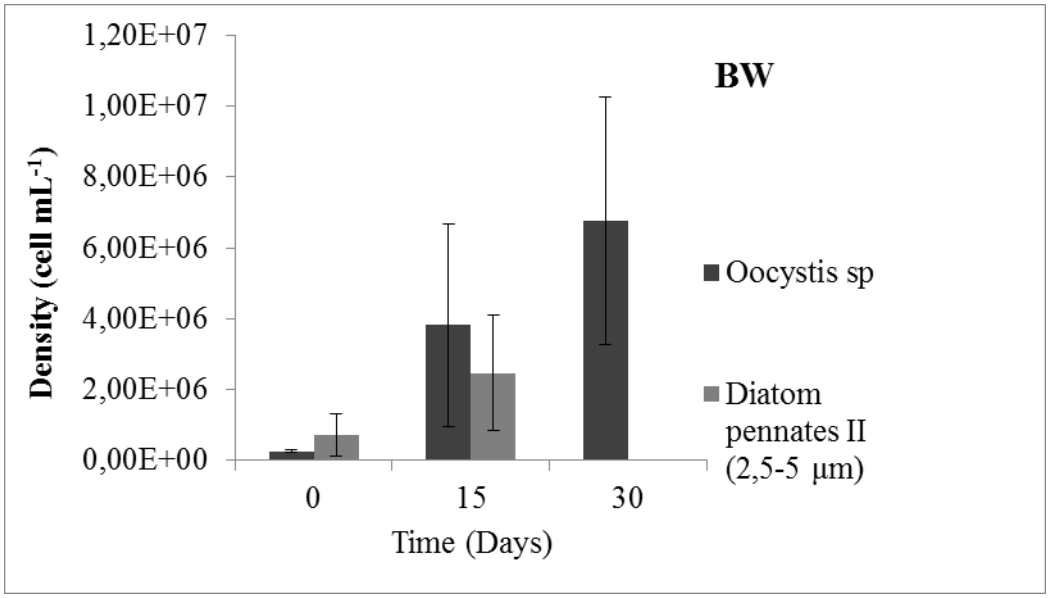
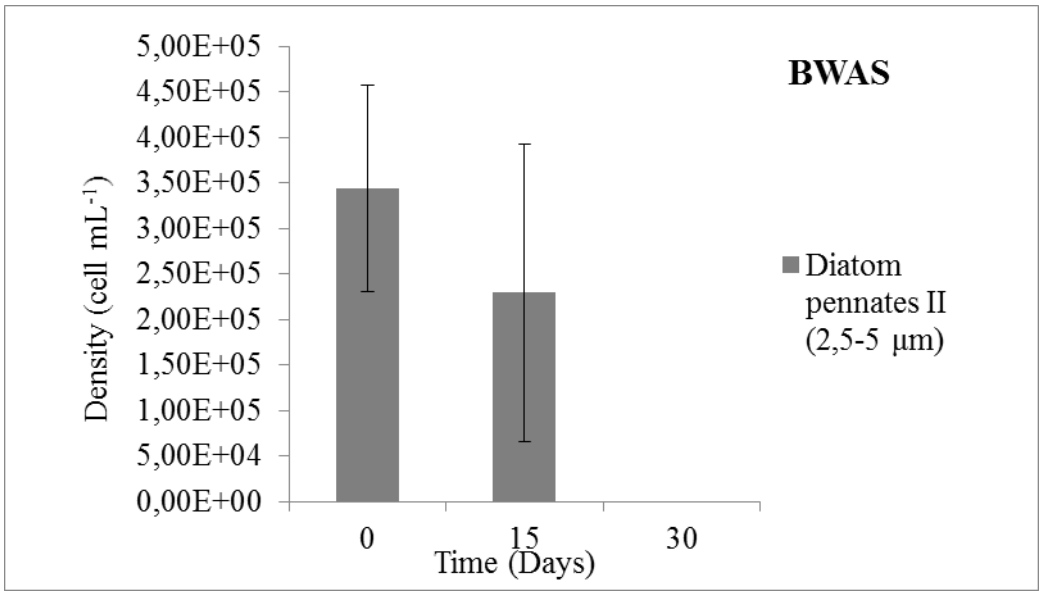


Figure 1.



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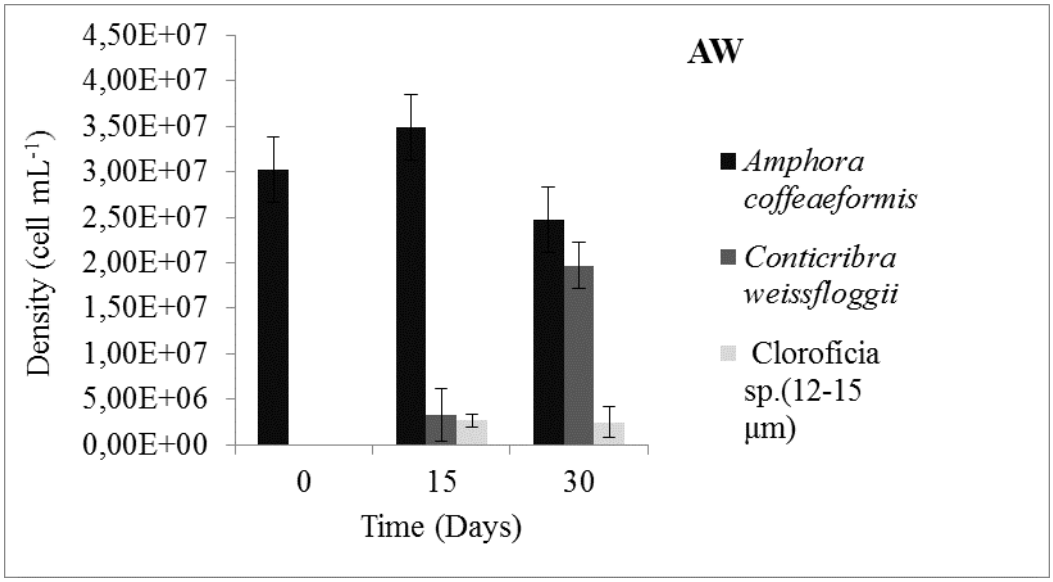

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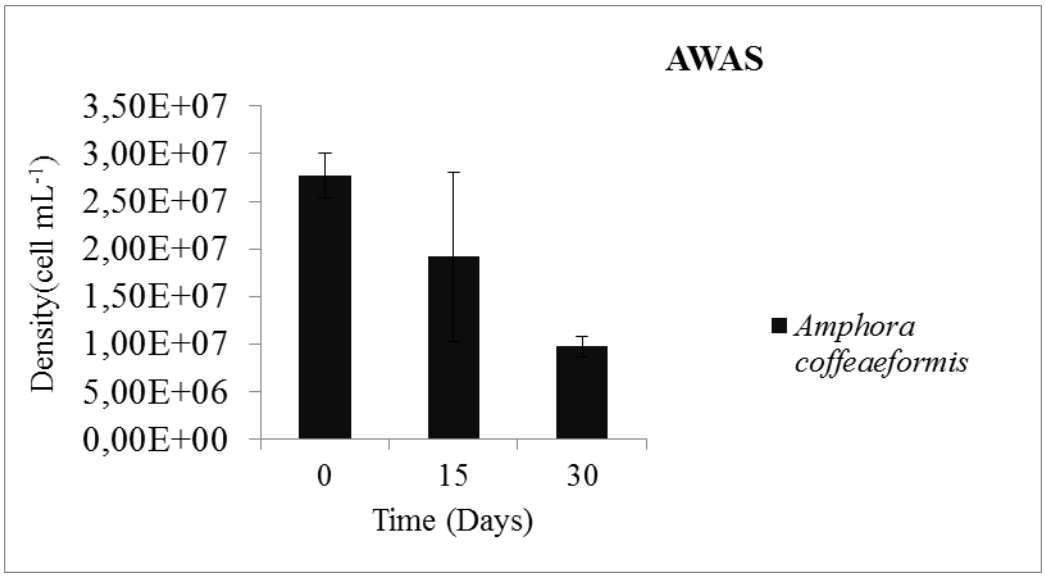

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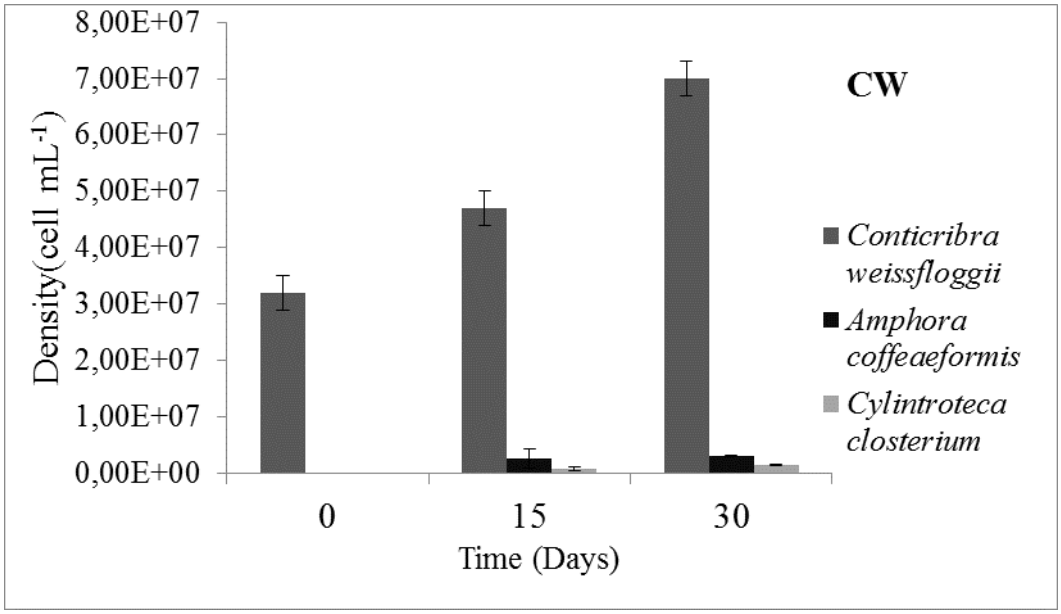
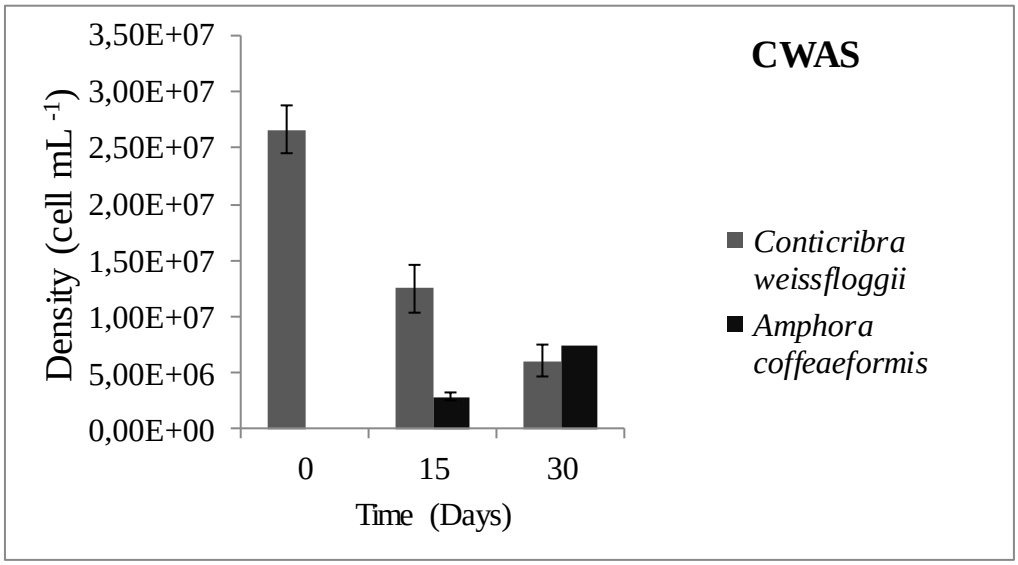


Figure 5



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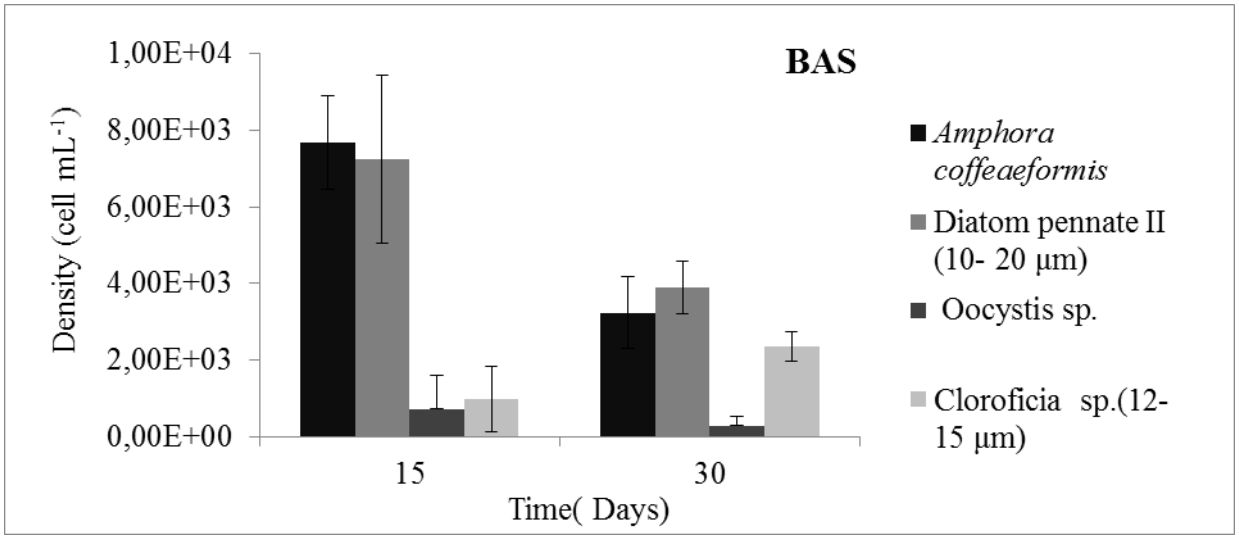

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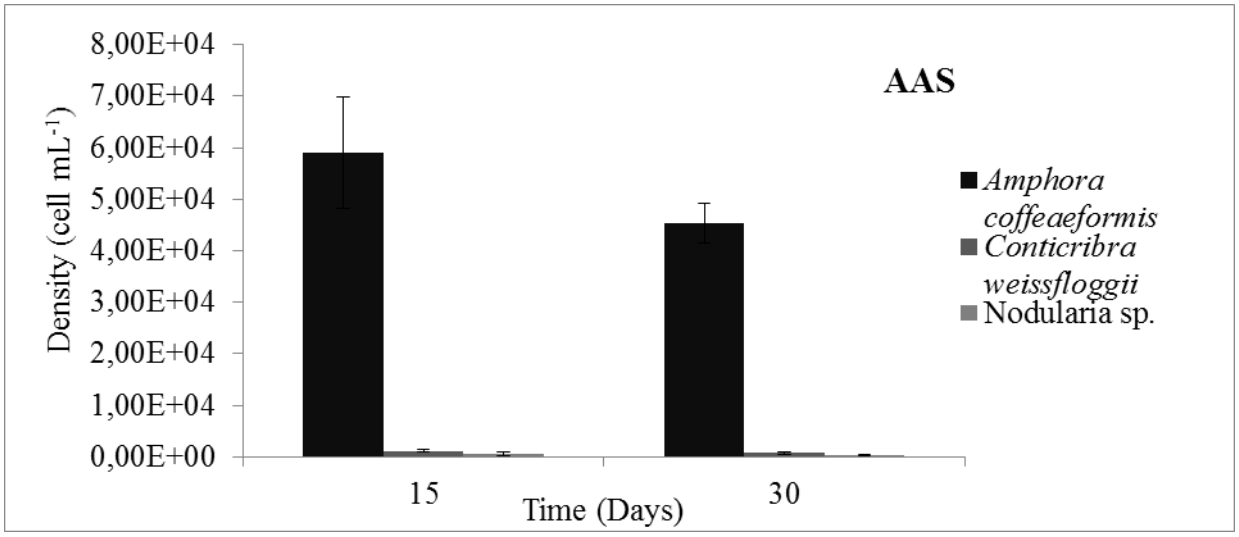
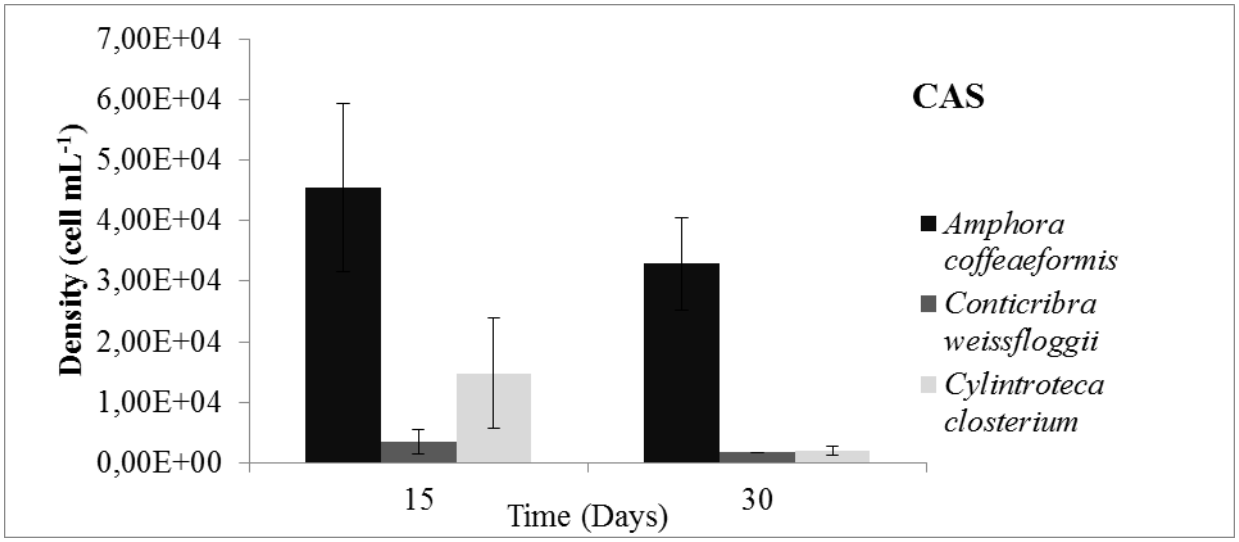


Figure 8


7,00E+04
6,00E+04
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0,00E+00
Density (cell mL-1)
15
30
Time (Days)
CAS
Amphora coffeaeformis
Conticribra weissfloggii
Cylintroteca closterium

Figure 9.

Table 1. Mean values (± SD) of the water quality parameters in the rearing of juvenile *L. vannamei* in treatments with of mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *A. coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticriba weissflogii* (CW) and inoculation the diatom *C. weissflogii* with artificial substrate (CWAS).

Table 2. Microbiota mean (minimum and maximum) concentrations in the water in the treatments of mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *A. coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticriba weissflogii* (CW) and inoculation the diatom *C. weissflogii* with artificial substrate (CWAS). (* $<10^5$cells/L; ND not detected).

Table 3. Mean values (± SD) of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments with of mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *A. coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticriba weissflogii* (CW) and inoculation the diatom *C. weissflogii* with artificial substrate (CWAS).

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Parameters	**BW**	**BWAS**	**AW**	**AWAS**	**CW**	**CWAS**
Temperature a.m. (°C)	27.05 ± 1.48	27.19 ± 1.32	27.03 ± 1.59	27.32 ± 0.92	27.25 ± 1.00	27.34 ± 1.05
Temperature p.m. (°C)	30.96 ± 2.02	30.37 ± 1.82	30.45 ± 1.56	30.55 ± 1.51	30.86 ± 1.88	30.44 ± 1.52
DO a.m. (mg L^{-1})	6.40 ± 0.59	6.26 ± 0.54	6.19 ± 0.57	6.09 ± 0.49	6.29 ± 0.56	6.10 ± 0.48
DO p.m. (mg L^{-1})	5.87 ± 0.42	5.89 ± 0.38	5.76 ± 0.42	5.73 ± 0.35	5.80 ± 0.45	5.73 ± 0.34
pH a.m.	8.09 ± 0.19	8.09 ± 0.21	8.11 ± 0.21	8.07 ± 0.14	8.28 ± 0.21	8.20 ± 0.09
Salinity (g L^{-1})	35.44 ± 2.77	35.01 ± 2.66	35.03 ± 1.32	35.03 ± 1.17	35.08 ± 1.23	35.03 ± 1.20
Water Chla (μg L^{-1})	787.19 ± 154.0 a	319.69± 140.5 b	1022.37 ± 704.6 a	240.20 ± 175.0 b	828.53± 98.9 a	176.16 ± 71.4 b
Substrate Chla (μg cm^{-2})	*	977.8 ± 1388.3 a	*	457.7 ± 593.6 a	*	933.19 ± 1164.0 a

Alkalinity ($mg\ L^{-1}\ CaCO_3$)	159.00 ±3.65 b	153.33 ± 3.12 b	176.00 ± 28.98 b	153.00 ± 6.71 b	221.67 ± 3.12 a	174.67 ±24.98 b
SST ($mg\ L^{-1}$)	426.86 ± 157.7	321.31 ±159.06	381.79 ± 117.88	236.33 ± 123.93	360.67 ± 114.29	253.62 ± 104.92
TA-N ($mg\ L^{-1}$)	0.61 ± 1.10	0.70 ± 1.17	2.22 ± 3.15	0.54 ± 0.68	1.37 ± 1.62	0.72 ± 0.82
NO_2- N ($mg\ L^{-1}$)	2.65 ± 2.74 b	1.76 ± 1.61 a	2.82 ± 5.32 a	6.23 ± 6.75 b	0.74 ± 1.36 a	4.07 ± 5.22 b
NO_3- N ($mg\ L^{-1}$)	48.72 ± 17.63 a	40.62 ± 12.99 a	1.56 ± 1.13 b	4.97 ± 3.09 b	1.38 ± 1.44 b	1.79 ± 1.95 b
PO^3_4 – P ($mg\ L^{-1}$)	2.07 ± 0.98 b	2.96 ± 1.53 b	2.32 ± 1.54 b	2.91 ± 1.90 b	1.23 ± 0.90 a	2.37 ± 1.69 b
Silicate ($mg\ L^{-1}$)	0.13 ± 0.06[b]	0.13 ± 0.07[b]	2.28 ± 1.93 [a]	2.69 ± 1.96 [a]	2.80 ± 1.53[a]	3.02 ± 1.50[a]

Different letters in the same row indicate significant differences ($p< 0.05$).

Table 2. Microbiota mean (minimum and maximum) concentrations in the water in the treatments of mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *A. coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticriba weissflogii* (CW) and inoculation the diatom *C. weissflogii* with artificial substrate (CWAS). (* $<10^5$cells/L; ND not detected).

	BW	BWAS	AW	AWAS	CW	CWAS
Diatoms 10^3 cells/L						
Amphora coffeaeformis	*	*	29900 (17500-45700)	18900 (8600-29500)	2810 (4.03-6620)	516 (ND-1720)
Conticribra weissfloggii	*	*	11500 (8600-28200)	*	49700 (66.10-86000)	15100 (4390-13900)
Cylindrotheca closterium	*	*	*	*	*	*
Pennates (12-25 μm)	1090 (ND-3220)	287 (ND-516)	*	*	104 (ND-215)	*
Chlorophytes 10^3 cells/L						
Ocystis sp.	3610 (215-7010)	*	*	*	*	*
Chlorophyte (12-15 μm)	*	*	3590 (ND-9070)	*	*	*
Other Protists 10^3 cells/L						
Ciliates CI (10-20 μm)	1070 (ND-2840)	322 (ND-1070)		*	*	*
Ciliates CII (20-60 μm)	451 (43-516)	57.30 (ND-215)	545 (86-1290)	631 (430-1330)	401 (129- 645)	330 (215- 473)
Ciliates CIII (> 60 μm)	*	*	363	251	*	365

			(215-903)	(ND-430)		(215-860)
Dinoflagellates DI (10 - 20 μm; *Gymnodinium* sp.)	1530 (43- 3650)	1400 (989- 2280)	*	*	*	*
Dinoflagellates DII (> 20 μm; *Gyrodinium* sp.)	105 (43-1070)	*	*	1480 (860- 3050)	*	1120 (ND-2410)
Flagellates FI (5-20 μm)	3320 (774-14700)	2760 (215- 3870)	2440 (430-6230)	2870 (1930- 5160)	16800 (430-38300)	3000 (258- 12100)
Unidentified A (10^6 cells/L)						
UNA (2,5-5 μm)	*	*	12600 (ND-26000)	*	*	*

Table 3. Mean values (± SD) of the development parameters of juvenile *L. vannamei* reared in a superintensive system in the treatments with of mature biofloc (BW), mature biofloc with artificial substrate (BWAS), inoculation the diatom *Amphora coffeaeformis* (AW), inoculation the diatom *A. coffeaeformis* with artificial substrate (AWAS), inoculation the diatom *Conticriba weissflogii* (CW) and inoculation the diatom *C. weissflogii* with artificial substrate (CWAS).

Parameters	TREATMENTS					
	BW	**BWAS**	**AW**	**AWAS**	**CW**	**CWAS**
Initial mean weight (g)	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04	0.07 ± 0.04
Final mean weight (g)	0.85 ± 0.05 [b]	1.02 ± 0.04 [b]	1.65 ± 0.07 [a]	1.71 ± 0.14 [a]	1.85 ± 0.14 [a]	1.57 ± 0.18 [a]
Survival (%)	93.33 ± 1.65	94.92 ± 1.10	94.92 ± 1.92	94.13 ± 3.17	91.11 ± 7.02	91.13 ± 2.20
Weight gain (g)	0.78 ± 0.05b	0.95 ± 0.04 [b]	1.58 ± 0.07 [a]	1.64 ± 0.14 [a]	1.78 ± 0.14 [a]	1.50 ± 0.18 [a]
Final biomass (g/ m^2)	333.55 ± 16.37 [b]	405.36 ± 16.95 [b]	659.16 ± 21.94 [a]	676.16 ± 54.52 [a]	709.78 ± 105.86 [a]	621.35 ± 84.79 [a]
Specific growth rate (%)	8.32 ± 0.18 [b]	8.92 ± 0.12 [b]	10.54 ± 0.13 [a]	10.65 ± 0.28 [a]	10.91 ± 0.24 [a]	10.35 ± 0.39 [a]
Feed Conversion Ratio (%)	2.19 ± 0.26 [b]	2.12 ± 0.37 [b]	1.5 4 ± 0.24 [a]	1.49 ± 0.07 [a]	1.33 ± 0.11 [a]	1.61 ± 0.09 [a]

Different letters in the same row indicate significant differences ($p < 0.05$).