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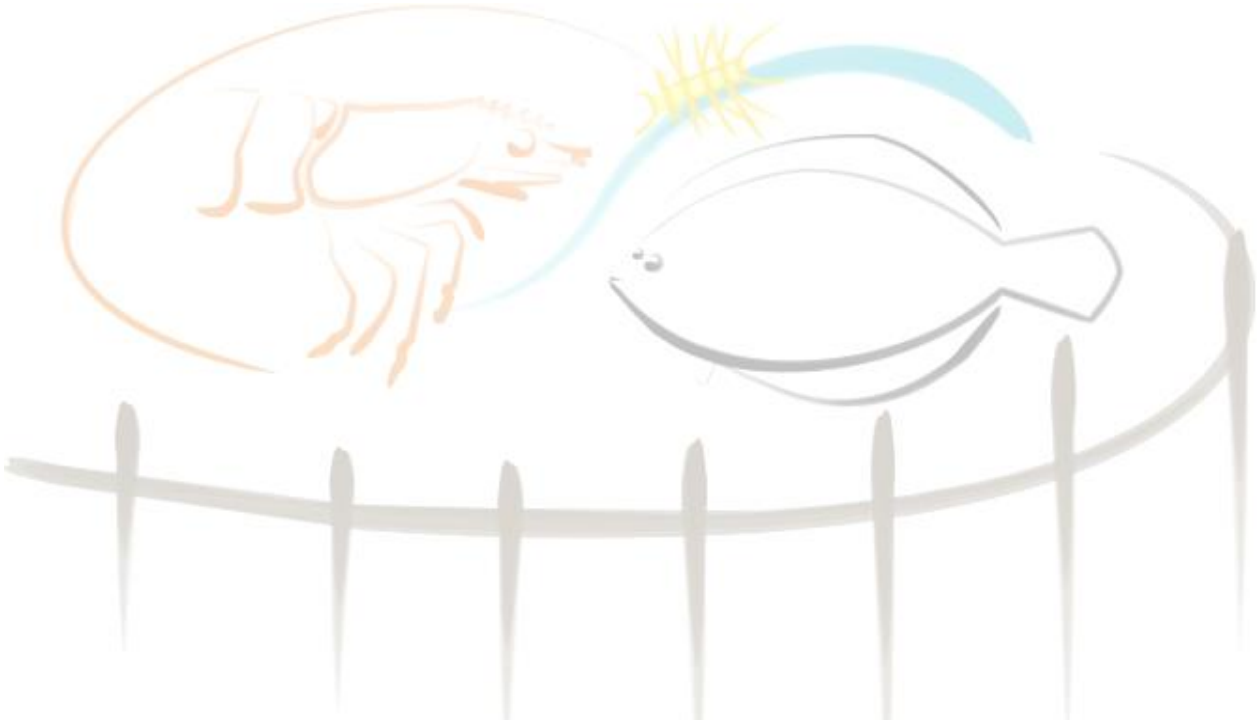

Caracterização da microbiota bacteriana associada à formação de flocos e no processo de nitrificação em sistemas de produção intensiva (Biofloc Technology - BFT) do camarão-branco *Litopenaeus vannamei.*

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Tese de Doutorado

Caracterização da microbiota bacteriana associada à formação de flocos e no processo de nitrificação em sistemas de produção intensiva (Biofloc Technology - BFT) do camarão-branco *Litopenaeus vannamei*.

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Pelos caminhos que ando

um dia vai ser

só não sei quando...

(Paulo Leminski)

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Resumo Geral

As bactérias têm diversas funções cruciais para o sucesso da produção de organismos aquáticos com a tecnologia de bioflocos (Biofloc Technology – BFT), entretanto, o nosso conhecimento sobre a diversidade e atividade das bactérias tem sido limitado principalmente pela falta de técnicas mais eficientes para caracterização dessas comunidades bacterianas. Portanto, o objetivo desse trabalho foi analisar a diversidade da comunidade bacteriana envolvida na formação de bioflocos e no processo de nitrificação em sistemas de aquacultura utilizando o sistema BFT e a adição de substratos artificiais para a formação de biofilme durante a produção intensiva do camarão-branco *Litopenaeus vannamei*. Para isto foi necessário implementar uma técnica de analise de diversidade bacteriana para sistemas de aquacultura, no caso, a técnica de impressão digital ("fingerprinting") Temporal Temperature Tradiente Electrophoresis (TTGE). Nesse trabalho foi possível observar que o biofilme serve como complemento alimentar e diminuiu o estresse causado pela alta densidade de estocagem, melhorando o desempenho zootécnico dos camarões, entretanto, a adição dos substratos artificiais no ambiente de produção não influenciou na nitrificação. Esta foi mais influenciada pela reutilização da água com bioflocos, acelerando a oxidação de amônia a nitrito. Outro fator que influenciou no processo de nitrificação foi a intensidade de mistura da água (turbulência), controlando o tamanho dos bioflocos. Foi observado que o uso de pedras porosas apresentou menor intensidade de mistura e permitiu a formação de bioflocos de maior tamanho e maior eficiência na nitrificação do que com o uso de aerotubes nos tanques de produção, que apresentaram maior intensidade de mistura, mas bioflocos menores e nitrificação menos eficiente, demonstrando a importância da turbulência no tamanho dos bioflocos no sistema BFT. A técnica de TTGE foi eficaz para analise da diversidade e sucessão da comunidade bacteriana do bioflocos e do biofilme em sistema BFT, podendo ser aplicada nos diversos sistemas de aquicultura. O uso dessa técnica, aliada aos dados ambientais durante o ciclo de produção de *L. vannamei*, permitiu inferir ribotipos bacterianos relacionados a formação dos bioflocos e ao processo de nitrificação. Além disso, foi observado que as comunidades bacterianas do biofloco e biofilme são semelhantes entre si.

Abstract

Bacteria have crucial functions for the successful production of aquatic organisms using biofloc technology (BFT), however, our knowledge about bacterial diversity and activity has been limited by the lack of efficient techniques for characterization of these of bacterial communities. Therefore, the aim of this study was to verify the diversity of the bacterial community involved in the biofloc formation and nitrification in aquaculture systems using BFT and the addition of artificial substrates for biofilm formation during intensive production of the white shrimp *Litopenaeus vannamei*. To do this, it was necessary to implement a technique to analyze bacterial diversity in aquaculture systems, in this case, the "fingerprinting" technique Temporal Temperature Gradient Electrophoresis (TTGE). In this study it was observed that biofilm serves as a food supplement, reduced the stress caused by high stoking density, improving growth performance of shrimp, however, the addition of artificial substrates had no effect on nitrification in the production environment. This was more influenced by the reuse of water with bioflocs, accelerating the oxidation of ammonia to nitrite. Another factor that influenced the nitrification process was the intensity of water mixing (turbulence), controlling the bioflocs size. It has been observed that the use of air stones showed a lower mixing intensity, allowing the formation of larger bioflocs and more efficient nitrification process than using aerotubes, which generate greater mixing intensity, but smaller bioflocs and less efficient nitrification, demonstrating the importance of the turbulence and size on bioflocos in the BFT system. The TTGE technique was effective for analysis of the diversity and sucession of the bacterial communities in the biofilm and bioflocs in BFT system and can be applied in various aquaculture systems. Using this technique allowed inferring bacterial ribotypes related to environmental conditions during a production cycle of *L. vannamei,* such as the biofloc formation and nitrification process. Furthermore, it was observed that the bacterial communities of biofilm and biofloc are similar.

Introdução Geral

A aquicultura tem como objetivo primordial o aumento da produção sem afetar substancialmente o uso dos recursos naturais (água e terra), aliando o desenvolvimento de sistemas de produção sustentáveis e uma relação positiva de custo-benefício, ou seja, que esses sistemas sejam economicamente viáveis (Crab et al.,2012). A intensificação dos sistemas de produção de organismos aquáticos está associada ao acúmulo de amônia e outros compostos nitrogenados na água, oriundos de restos de ração não consumida e da excreção dos animais aquáticos produzidos que utilizam grandes quantidades de proteína em sua ração (Hepher, 1988; Burford et al., 2003). Peixes e camarões liberam amônia como principal produto de excreção resultante do consumo da ração. Entretanto, a alta concentração de compostos nitrogenados na água de produção pode se tornar tóxica para os organismos produzidos (Boyd, 2007). Portanto ocorre a necessidade de troca de água dos viveiros e tanques, com altas taxas de renovações de água, o que pode causar eutrofização dos corpos d'água que recebem os efluentes da aquicultura, muitas vezes ricos em nitrogênio e fósforo.

Nos anos 90 foram desenvolvidos alguns sistemas de produção de organismos aquáticos sem renovação de água. Um dos mais efetivos é a tecnologia de bioflocos ("Biofloc Technology System - BFT"), que tem se difundido pelo mundo, unindo alta produtividade ao menor dano ao meio ambiente. Os sistemas BFT oferecem diversas vantagens em relação aos sistemas convencionais de produção de camarões (McAbee et al., 2003; Haslun et al., 2012). Dentre estas podemos citar: o menor uso de espaço físico, a possibilidade de construção desses sistemas em locais distantes das zonas litorâneas, o uso de elevadas densidades de estocagem e troca de água zero, ou mínima. Nesses sistemas os micro-organismos presentes nos bioflocos, ou agregados microbianos, mantêm a qualidade da água e servem como fonte alimentar suplementar para os organismos produzidos (Chamberlaim et al., 2001; Burford et al., 2003; Avnimelech, 2006; Wasielesky et al., 2006; Nootong et al., 2011). Esses bioflocos são compostos por EPS (substancias poliméricas extracelulares), colonizado por bactérias, fitoplâncton, flagelados, ciliados dentre outros micro-organismos, além de restos de exoesqueleto, ração e fezes dos animais produzidos. A formação dos bioflocos em tanques e viveiros de produção de peixes e crustáceos pode levar até seis semanas para

formar bioflocos maduros, isto é, capazes de manter a qualidade da água e ainda servir de fonte alimentar complementar (Ballester et al., 2007; McAbee et al., 2003, Ferreira, 2008).

Bactérias e protozoários presentes nos flocos servem como fonte suplementar de alimento, permitindo a diminuição dos níveis de proteína bruta na ração, além de melhorar as taxas de conversão alimentar tanto para peixes, como para camarões (Van Wyk, 1999; Burford et al., 2004; Avnimelech, 2006; Ray et al., 2010a). Além disto, as bactérias realizam a ciclagem de nutrientes dentro do próprio sistema de produção, proporcionando a melhoria da qualidade de água devido à rápida assimilação de elementos nitrogenados tóxicos como a amônia e o nitrito (Avnimelech, 1999, Van Wyk, 1999; Avnimelech, 2006; Ebeling et al., 2006; De Schryver et al., 2008; Krummenauer et al., 2010; Silva et al., 2011; Ray et al., 2012). Entretanto, pouco se sabe sobre a composição e ação dos micro-organismos, especialmente bactérias, em relação aos fatores abióticos durante a formação do biofloco. Desta forma, são necessários estudos para caracterizar a comunidade microbiana dos bioflocos e as interações entre esses organismos com os diferentes fatores ambientais em sistema BFT.

A formação dos agregados microbianos (bioflocos) depende de interações biológicas, físicas e químicas que acontecem simultaneamente no meio. Biddanda e Pomeroy (1988) descreveram que o processo de formação dos agregados é iniciado pelo aumento na abundância de bactérias e pela produção de EPS (substancias poliméricas extracelulares) por estes micro-organismos. Posteriormente há a proliferação de flagelados, ciliados e amebas. A presença de protozoários leva à redução da comunidade bacteriana, provocando a ruptura dos agregados, levando à diminuição do tamanho do biofloco. Esses eventos podem ocorrer repetidamente e essa relação entre bactérias e protozoários é a causa mais comum da desagregação e formação dos agregados.

Além disso, Ferreira (2008) demonstrou que o processo de formação dos bioflocos é influenciado pelo hábito alimentar do organismo produzido. Isto provavelmente ocorre porque camarões como o *Litopenaeus vannamei* excretam menos fósforo, o que beneficia as bactérias cocóides. Foi observado que o aumento na quantidade de material em suspensão se deu juntamente com o aumento da abundância de bactérias cocóides produtoras de muco (EPS). Este muco produzido pelas bactérias

deve ter auxiliado na agregação entre as partículas, causando um aumento do tamanho dos flocos microbianos.

Dentre os fatores físicos e químicos relacionados com a formação do biofloco estão o movimento browniano, número de Reynolds (turbulência), as forças de van der Waals, diferença de potencial elétrico entre as partículas e diferencial de sedimentação entre as partículas (Hietanen, 1998; De Schryver et al., 2008). A aeração dos tanques em sistemas BFT é um dos principais fatores que podem influenciar físico e quimicamente na formação dos bioflocos, não só pelo fornecimento de oxigênio para os organismos produzidos, mas também circulação da água e intensidade de mistura do material particulado na coluna d'água, que afeta os processos de agregação e desagregação do biofloco (Simon et al., 2002; Serra e Casamitjana, 1998). Este fato foi comprovado por Lara et al.,(*in press*) que observaram que diferentes tipos de aeradores (blower, propulsor e chafariz) influenciaram na formação dos bioflocos, no tamanho dos agregados e na eficiência de nitrificação nos tanques de produção de camarões em sistema BFT.

A remoção dos produtos nitrogenados, como amônia e nitrito, pode ser feita por bactérias heterotróficas que assimilam a amônia dissolvida em conjunto com carbono orgânico e estes são utilizados na formação de novas células. Outra possibilidade é a nitrificação, onde as bactérias nitrificantes oxidam amônia a nitrito e depois, nitrito a nitrato, usando carbono inorgânico como fonte de carbono. A diminuição nas concentrações de amônio também pode se dar pela fotossíntese do fitoplâncton, que competem com as bactérias pelos nutrientes dissolvidos na água (Chamberlaim et al., 2001; Ray et al., 2012).

O uso de fertilizações orgânicas em sistemas BFT, pela a adição de carbono, por exemplo, melaço ou dextrose, é realizado a fim de controlar os níveis de amônia pelo crescimento de bactérias heterotróficas, que absorvem o carbono adicionado juntamente com a amônia presente em alta concentração (Hargreaves, 2006). As bactérias heterotróficas tem um tempo de geração bem menor do que as bactérias nitrificantes (Michaud et al., 2006) e, por isso, o processo de remoção de nitrogenados por bactérias heterotróficas e fitoplâncton é mais rápido do que a nitrificação, sendo empregado como uma ferramenta emergencial para diminuir os níveis de elementos nitrogenados tóxicos (Burford et al., 2003).

Uma estratégia utilizada para otimizar a remoção de compostos nitrogenados do sistema é o reuso da água com bioflocos "maduros". O inóculo da água de produção com bioflocos provenientes de outros tanques e viveiros pode acelerar a formação de novos bioflocos e encurtar o tempo no processo de estabilização da comunidade microbiana nitrificante (McAbee et al., 2003). Este fato foi verificado por Krummenauer et al. (2012, 2014), onde a reutilização entre 25 a 100% de água de produção com bioflocos acelerou o processo a formação dos bioflocos e o estabelecimento de bactérias nitrificantes, resultando na rápida remoção de amônia e nitrito dissolvidos na água.

A adição de substratos artificiais para colonização do biofilme também é usado em sistemas de aquicultura para aumentar a eficiência da produção de camarões. Diversos estudos demonstram a importância da comunidade microbiana presente no biofilme no controle da qualidade da água do ambiente de produção, pela remoção de compostos nitrogenados da água (Thompson et al., 2002; Crab et al., 2007), principalmente amônia e nitrito e também como fonte de alimento para os organismos produzidos, com alta qualidade nutricional, fornecendo nutrientes essenciais, como ácidos graxos poli-insaturados, esteroides, aminoácidos e vitaminas proporcionando maior sobrevivência e crescimento dos camarões (Thompson et al., 2002; Preto et al., 2005; Abreu et al., 2007; Ballester et al., 2007; Silva et al., 2008).

O biofilme é definido como uma comunidade microbiana auto- e heterotrófica embebida por uma matriz orgânica e aderida a substratos naturais ou artificiais submersos. Essa comunidade microbiana é composta por bactérias, fungos, microalgas, flagelados, ciliados, zooplâncton, detritos dentre outros (Wetzel, 1983). Além disso, os substratos atuam como um refúgio para os camarões aliviando os efeitos do estresse causado pelas altas densidades de estocagem (Zhang et al., 2011).

Seja participando nas comunidades microbianas do biofloco, ou do biofilme, fica clara a importância dos micro-organismos no ambiente de produção de organismos aquáticos. Entretanto, o nosso conhecimento sobre a diversidade microbiana e atividade das bactérias tem sido limitado principalmente pela falta de técnicas mais eficientes para a identificação das bactérias, ou caracterização da composição destas comunidades. O isolamento, cultura e aplicação de testes fisiológicos para identificação das bactérias pode ser aplicado a um pequeno número de espécies, uma vez que a grande maioria das

bactérias aquáticas não são cultiváveis nos meios atualmente existentes (Hugenholtz et al., 1998; Muyzer, 1999; Farnleitner et al., 2000). Para contornar estes problemas, estudos em biologia molecular têm sido amplamente empregados, uma vez que estes têm se mostrado de grande importância para análise da diversidade microbiana, especialmente para espécies não cultiváveis (Pernthaler and Amann, 2005; Luo et al., 2007; Park et al., 2008).

A clonagem e sequenciamento da região 16S do RNA ribossomal é uma poderosa ferramenta. No entanto, esta é muito laboriosa e demanda muito tempo e dinheiro (Pernthaler and Amann, 2005). Por outro lado, técnicas de DNA recombinante estão proporcionando meios para obtenção dos perfis das comunidades microbianas (Hugenholtz et al., 1998; Hallin et al., 2006; Leddy, 2007), auxiliando na determinação da dinâmica e estrutura das comunidades microbianas em diferentes ambientes, em condições naturais ou de estresse (Muyzer et al., 1993).

Outra técnica que vem sendo bastante utilizada em ecologia microbiana é a hibridização in situ fluorescente (FISH), que permitem a visualização, identificação e enumeração de células microbianas individuais através do uso de oligonucleotideos de DNA gerais ou específicos marcados com um fluorocromo. Esta técnica combina a precisão da biologia molecular com a informação visual da microscopia (Moter e Gobel, 2000). Já as técnicas de análise da impressão digital (fingerprinting), fornecem um padrão ou o perfil de diversidade microbiana, permitindo a comparação entre comunidades microbianas de diferentes ambientes assim como seu desenvolvimento ao longo do tempo. A eletroforese em gel de poliacrilamida com desnaturação constante (CDGE) foi desenvolvida visando à detecção de mutações, especialmente em pesquisas na área da saúde (Wu et al., 1999). Entretanto, estas e outras técnicas similares utilizando a eletroforese em gel de poliacrilamida como DGGE, TGGE e TTGE, têm demonstrado ser bastante efetiva para o estudo da ecologia microbiana (Muyzer, 1999). Estas técnicas conseguem detectar pequenas mudanças nas sequências de nucleotídeos, pois elas provocam alterações no ponto de desnaturação (“melting point”) do fragmento de DNA (Farnleitner et al., 2000; Dorigo et al., 2005), sendo capazes de fornecer estimativas qualitativas e semi-quantitativas de biodiversidade microbiana.

Diferentes gradientes de saturação do gel de poliacrilamida podem ser aplicados para separação de sequencias de DNA no gel. A Eletroforese em gel de poliacrilamida

pode ser realizada com desnaturação constante ("Constant Desnaturing Gel Electrophoresis – CDGE" - Børrensen-Dale et al., 1997), por gradiente de temperatura ("Temperature Gradiente Gel Electrophoresis – TGGE" - Muyzer, 1999), por gradiente químico ("Denaturing Gradient Gel Electrophoresis – DGGE" – Muyzer, 1999; Marzorati et al., 2008) em geral utilizando uréia e formamida, em concentrações que variam de 0% a 100% para produzir a desnaturação. O maior problema das técnicas de TGGE e DGGE está relacionado com a pouca reprodutibilidade dos géis, isto porque nenhum gel é preparado exatamente igual ao outro. Desta forma, quando se utilizam muitos géis para se determinar a composição de diferentes amostras, a comparação entre estas fica comprometida (Farnleitner et al., 2000). Para solucionar a o problema da falta de reprodutibilidade dos géis e das condições de corrida, foi desenvolvida a técnica de eletroforese em gel de poliacrilamida com gradiente de temperatura ao longo do tempo ("Temporal Temperature Gradiente Gel Electrophoresis – TTGE"), nessa técnica o gel tem uma taxa de desnaturação constante pela adição de uréia, assim como no CDGE e TGGE, e a separação das diferentes sequências de DNA em bandas se dá por gradiente de temperatura produzido ao longo do tempo, que é uniforme em todo o gel (Børrensen-Dale et al., 2008).

Na aquicultura, grande parte dos estudos sobre ecologia microbiana têm sido realizados por meio de microscopia e utilização das técnicas básicas de microbiologia como isolamento, cultivo e identificação (Leonard et al., 2000; Moss et al., 2000; Yanbo et al., 2005; Ray et al., 2010b). Mais recentemente, a técnica de hibridização fluorescente *in situ* (Fluorescent *in situ* hybridization – FISH) foi aplicada com grande sucesso em estudos de ecologia microbiana e probióticos (Oliveira et al., 2006; Del Duca et al., 2013). No entanto, devido às limitações anteriormente abordadas, surgiu a necessidade de estudos mais aprofundados para investigação dos processos ecológicos que ocorrem no ambiente de produção, como ciclagem de nutrientes, especialmente de compostos nitrogenados. Nesta Tese, foi aplicada a técnica de "Temporal Temperature Gradiente Gel Electrophoresis – TTGE" para analise das comunidades bacterianas presentes nos bioflocos e no biofilme.

Objetivo Geral:

O objetivo geral desse trabalho foi analisar a comunidade bacteriana envolvida na formação de bioflocos e no processo de nitrificação em sistemas de aquicultura utilizando a tecnologia de bioflocos (Biofloc Technology – BFT) e substratos artificiais para a formação de biofilme durante a produção intensiva do camarão branco *Litopenaeus vannamei*.

Objetivos Específicos

- ✓ Avaliar o uso do biofilme, pela adição de substratos artificiais, em sistema BFT durante a produção intensiva do camarão-branco *Litopenaeus vannamei.*
- ✓ Verificar a diversidade das comunidades bacterianas dos bioflocos e do biofilme durante a produção do camarão branco *Litopenaeus vannamei* em sistema BFT.
- ✓ Verificar o efeito de diferentes tipos de aeração (aerotube e pedra porosa) sobre a formação de bioflocos e nitrificação durante a produção de *L. vannamei* em sistema BFT.

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Capítulo I

Biofilme vs. Bioflocos: Substratos artificiais para formação de biofilme são necessários em sistemas BFT?

Biofilm vs. Biofloc: Are artificial substrates for biofilm production necessary in the BFT system?

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Resumo

O biofilme e o biofloco têm sido amplamente utilizados na Aquicultura como uma ferramenta para melhorar as condições de produção de organismos aquáticos. A característica comum a estes dois elementos é a presença de micro-organismos. O biofilme é importante no controle de qualidade da água e como alimento suplementar com alta qualidade nutricional. Na aquicultura o uso do biofilme também pode contribuir como uma importante fonte de carbono e nitrogênio para a biomassa de camarões. A tecnologia de bioflocos ("Biofloc Technology – BFT) surgiu como uma alternativa ambientalmente amigável para o desenvolvimento da aquicultura intensiva. Assim como o biofilme, o biofloco também mantem a qualidade da água condições adequadas, além de fornecerem alimento extra, para peixes e crustáceos que estão sendo produzidos. O objetivo deste estudo foi avaliar se a maior disponibilidade biofilme devido a adição de substratos artificiais em sistemas BFT resultaria em uma maior produção do camarão-branco *Litopenaeus vannamei*. Tanques com volume útil de 800 L foram abastecidos com água contendo bioflocos (25% do volume total) e água do mar filtrada. Três tratamentos, com três repetições cada, foram comparados: 1) Controle (Ctrl), sem adição de substrato artificial e os tratamentos 2) 200 e 3) 400, com adição de substrato artificial equivalente a 200% e 400% da área lateral dos tanques, respectivamente. Os camarões foram estocados com peso médio inicial de 0.40 ± 0.15 g em uma densidade de 300 camarões/m^2. A alimentação dos camarões foi feita com ração (Guabi/38 Active, Brasil), fornecida 2 vezes ao dia, com uma taxa de alimentação variando entre 12% e 4% da biomassa total de camarões. O experimento teve duração de 35 dias. As medidas de temperatura, salinidade, pH e elementos nitrogenados estavam dentro dos valores favoráveis ao crescimento de *L. vannamei*. A quantidade de sólidos em suspensão (SS) foi maior no tratamento Ctrl (82.1 ±19 mL L^{-1}), mas este somente foi estatisticamente diferente do tratamento 400. A média de SS nos tratamentos 200 e 400 foi abaixo de 10 mL L^{-1}. A reutilização de água com bioflocos acelerou a nitrificação nos tanques, no entanto, não houve diferenças significativas entre tratamentos. A presença de biofilme não interferiu nos níveis de amônia e nitrito, mas os maiores valores de sobrevivência e de crescimento nos tratamentos com mais substratos indicam que estes forneceram alimento extra e maior proteção aumentando os índices de crescimento dos camarões.

Capítulo II

Caracterização da comunidade bacteriana do biofloco e do biofilme em sistemas de aquicultura usando a técnica de Temporal Temperature Gradient Gel Electrophoresis (TTGE).

Characterization of the Bacterial Communities of Bioflocs and Biofilms in Aquaculture Systems Using Temporal Temperature Gradient Gel Electrophoresis (TTGE)

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O artigo encontra-se no anexo 2.

Resumo

O objetivo deste trabalho foi o de analisar a composição da comunidade bacteriana presente no biofloco e no biofilme em sistemas de produção intensiva do camarão-branco *Litopenaeus vannamei*, através da técnica de "Temporal Temperature Gradient Electrophoresis" (TTGE). As análises foram feitas em tanques com reutilização de água com bioflocos ("R", inoculação de 25% do volume útil do tanque) e tanques com formação de bioflocos a partir de água clara ("CW"). O experimento teve duração de 35 dias e constou de quatro tratamentos, com três repetições cada: 1)Tratamento R+0, com reuso de água com biofloco e sem adição de substratos artificiais; 2) R+400, com reuso e adição de substrato artificial; 3) CW+0, água clara e sem adição de substrato artificial; e 4) CW+400, água clara e adição de substrato artificial. A adição de substrato (tela de nylon) foi equivalente a 400% da área lateral dos tanques. Foram estocados juvenis de *L. vannamei*, com peso médio inicial de 0,40 ±0,15 g, densidade de 300 camarões/m², em tanques com 800 L de volume útil. Os camarões foram alimentados com ração (Guabi/38 Active, Brasil) duas vezes ao dia. Os parâmetros de qualidade da água estavam de acordo com os valores recomendados para produção de *L. vannamei*. O tratamento R+0 apresentou índices zootécnicos inferiores aos demais tratamentos. Onde houve a adição de substratos o peso médio, crescimento (TCE) e biomassa de camarões foram maiores do que nos tratamentos sem substratos. O reuso da água com bioflocos acelerou o processo de nitrificação. Os tratamentos "R" apresentaram baixas concentrações de amônio, provavelmente devido ao inóculo de bactérias amonio-oxidantes presentes no biofloco. Os resultados do TTGE mostraram valores de similaridade das comunidade bacteriana do biofloco e biofilme variando entre 41,6% e 50%. Uma banda de DNA presente somente nos tratamentos "R", onde houve maior transformação de amônio em nitrito, pode ser proveniente de uma espécie de bactéria amônio-oxidante. O TTGE se mostrou uma ferramenta eficaz no estudo da comunidade bacteriana em sistemas BFT, devendo ser empregada em futuros estudos de ecologia microbiana em sistemas de aquacultura.

Capítulo III

Bactérias responsáveis pela formação do biofloco e nitrificação durante a produção de *Litopenaeus vannamei* em sistema BFT.

Bacterial species responsible for biofloc formation and nitrification during the production of *Litopenaeus vannamei* in BFT systems.

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Artigo submetido para Aquaculture.

O artigo submetido encontra-se no Anexo 3.

Resumo

O objetivo desse trabalho foi o de avaliar a influência de diferentes difusores de ar na formação dos bioflocos e no processo de nitrificação durante a produção intensiva do camarão-branco *Litopenaeus vannamei* com bioflocos ("Biofloc Technology – BFT"). O experimento foi realizado com dois tratamentos, em triplicata: 1) tratamento AS, que utilizou pedras porosas como distribuidor de ar, na proporção uma pedra porosa/m^2 e 2) tratamento AT, que utilizou aerotubes, na proporção uma peça de aerotube/1,5 m^2. Camarões com peso médio inicial de 0,032g (± 0,016g) foram estocados em uma densidade de 400 camarões/m^2. A alimentação dos camarões foi realizada com ração (Guabi/38 Active, Brasil) fornecida 3x ao dia. O experimento teve a duração de 104 dias Os dois tratamentos apresentaram diferenças de salinidade, sólidos sedimentáveis, compostos nitrogenados e comunidade microbiana no período entre 30 e 60 dias. Verificou-se que o tipo de aeração influenciou a intensidade de mistura (turbulência) da água de produção, salinidade e tamanho dos bioflocos. Estes fatores afetaram o processo de nitrificação no ambiente de produção. O tratamento AS apresentou menor turbulência, salinidade e bioflocos de maior tamanho. Este tratamento também apresentou maior eficiência no processo de nitrificação. A análise da comunidade microbiana mostrou quatro ribotipos de bactérias presentes nos dois tratamentos, provavelmente de bactérias que atuam na formação dos bioflocos. Por outro lado, apenas no tratamento AS, que apresentou maior eficiência de nitrificação, foram encontrados três ribotipos exclusivos, provavelmente pertencentes a bactérias nitrificantes.

Discussão Geral

Diversos trabalhos já relatam os benefícios do biofilme em sistemas de aquacultura convencionais na manutenção da qualidade da água de produção (Thompson et al., 2002; Crab et al., 2007) e como fonte de alimento suplementar de alto valor nutricional aos organismos produzidos (Abreu et al., 2007; Ballester et al., 2007; Silva et al., 2008). Esses substratos também amenizam o efeito da alta densidade de estocagem, aliviando o estresse no ambiente de produção, conforme verificado por Zhang et al. (2011). Nesse estudo, a adição de substratos artificiais em sistemas BFT aumentou a disponibilidade de alimento natural aumentando o crescimento dos camarões, e ainda, proporcionou a redução da concentração de sólidos suspensos (bioflocos) na coluna d'água, provavelmente por que os substratos podem ter alterado a circulação da água nos tanques de produção formando bioflocos de maior tamanho ou pela agregação desses flocos ao substrato, podendo ser utilizado para controlar o excesso de sólidos suspensos. Entretanto, a adição de substratos artificiais não influenciou no processo de nitrificação (anexo 1).

Esse processo foi influenciado pela reutilização de água de um ciclo de produção anterior em sistema BFT, usado como inóculo de bioflocos, proporcionando o inóculo de bactérias nitrificantes aumentando a eficiência da nitrificação e adiantando a oxidação de amônia no sistema.

As bactérias atuam como produtores primários em sistemas BFT e possuem funções extremamente importantes para o bom funcionamento desses sistemas. A remoção de compostos nitrogenados é realizada principalmente pelas bactérias, heterotróficas e nitrificantes, que compõem os bioflocos. Já que a fotossíntese realizada pelas microalgas é reduzida pelo sombreamento devido a elevada concentração de material em suspensão na coluna d'água. Tanto a formação de agregados microbianos (bioflocos) quanto a nitrificação são influenciados por fatores físicos, químicos e biológicos, sendo de grande importância descobrir e compreender como esses fatores influenciam as comunidades bacterianas responsáveis pelos processos ciclagem de nutrientes no ambiente de produção.

A técnica TTGE proporcionou a analise da comunidade bacteriana do biofloco e do biofilme e nos permitiu relacionar os diferentes perfis dessas comunidades com as

diferentes consdições ambientais das unidades de produção de camarões. Primeiramente foi verificado que as comunidade bacterianas do biofloco e do biofilme são semelhantes entre si, independente do tratamento, com reuso da água de produção ou pelo bioflocos formatos a partir de água clara, indicando um padrão de formação dos bioflocos, onde as bandas 6, 7 e 8 podem estar relacionadas a esse processo. Também foi possível observar um único ribotipo (banda 15), nos tratamentos com reuso, que pode estar relacionado à oxidação de amônia. No entanto seria necessário o sequenciamento e identificação dessa banda para confirmar essa hipótese (anexo 2).

Também foi observado que diferentes difusores de ar (Aerotube e pedra porosa) influenciaram na intensidade de mistura da água nos tanques de produção. Sabe-se que a turbulência pode controlar o tamanho dos flocos. E que a dinâmica de agregação e desagregação de partículas, ex. bioflocos, dependem da intensidade de mistura da água (Simon et al., 2002; Serra et al., 1997; Serra e Casamitjana, 1998). Onde havia pedra porosa, a intensidade de mistura foi menor (menor turbulência) permitindo a formação de bioflocos de maior tamanho. E, consequentemente, o tamanho das partículas são influenciam no processo de nitrificação, como observado por Lara et al., 2014 (*in press*). Portanto essa diferença na intensidade de mistura da água acabou interferindo no processo de nitrificação e na comunidade bacteriana (anexo 3).

A análise da sucessão bacteriana dos bioflocos formados, analisada pela técnica TTGE, em tanques com aerotube e pedra porosa permitiu a visualização de ribotipos de bactérias presentes em ambos os tratamentos com alta intensidade de banda coincidindo com o período de aumento do material em suspensão, indicando que essas bandas podem estar relacionadas à formação dos bioflocos (bandas 40 e 45). Nesse mesmo período (de formação), a nitrificação ocorreu de forma diferente entre os tratamentos, e foram observadas bandas que só estavam presentes no tratamento com pedra porosa, onde houve maior eficiência na nitrificação atribuída ao maior tamanho dos bioflocos, indicando que essas bandas (12, 44 e 56) estão relacionadas ao processo de nitrificação.

Conclusões Gerais

Os substratos artificiais (biofilme) no sistema BFT aumentaram a disponibilidade de alimento natural aumentando o crescimento dos camarões. No entanto, a adição de substratos artificiais não influenciou no processo de nitrificação. A presença de substrato artificial pareceu amenizr o efeito da alta densidade de estocagem, aliviando o estresse no ambiente de produção. E ainda, serviram para controlar o excesso de sólidos suspensos na coluna d'água.

O processo de nitrificação foi influenciado pela reutilização de água com biofloco, serviu como inóculo de bactérias nitrificantes aumentando a eficiência da nitrificação e adiantando a oxidação de amônia no sistema.

Outro fator que também influenciou na nitrificação foi o tamanho dos bioflocos. Os distintos difusores de ar influenciaram na intensidade de mistura da água, nos tanques com pedras porosas apresentou menor turbulencia, permitindo a formação de flocos de maior tamanho e nitrificação mais eficiente em relação a onde havia maior turbulência e flocos menores.

O TTGE foi uma ferramenta importante no estudo da composição e sucessão microbiana em sistemas de aquicultura. Indicando que há um padrão de formação dos bioflocos em sistemas BFT. Além disso, foi observado que as comunidades bacterianas do biofloco e biofilme são semelhantes entre si.

Considerações Finais

Esse trabalho aponta para a necessidade de novos estudos em relação à intensidade de mistura e circulação de água da água dos tanques de produção. Como também a determinação do tamanho mínimo dos bioflocos para permitir o desenvolvimento da comunidade nitrificante.

Essa ferramenta possibilitará um entendimento dos principais fatores ecológicos que influenciam na formação e composição dos bioflocos, principalmente para estimular a eficiência dessas bactérias nitrificantes, que demoram mais a se estabelecer nos flocos.

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Anexos:

Anexo 1 – Biofilm vs. Biofloc: Are artificial substrates for biofilm production necessary in the BFT system?

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Abstract

Biofilm is important in the control of water quality and as high nutritional quality food sources. Biofilm usage in aquaculture can contribute large amounts of carbon and especially nitrogen to shrimp biomass. Currently, biofloc technology (BFT) systems are considered environmentally friendly alternatives to intensive aquaculture development because they maintain water quality and provide extra food sources, such as biofilms, for the produced organisms. The aim of this study was to evaluate the addition of artificial substrates in BFT systems that are used for the intensive production of *Litopenaeus vannamei*. The experiment lasted 35 days. Tanks with a system volume of 800 L were filled with water containing bioflocs (25% of the total volume) and filtered seawater. Three treatments with three replicates each were compared: 1) Ctrl (Control), i.e., without the addition of artificial substrate, 2) 200 and 3) 400, with added artificial substrate equivalent to 200% and 400% of the lateral area of the tanks, respectively. The shrimp with average initial weight of 0.40 ± 0.15 g were stocked at a density of 300 shrimp/m^2. Feed (Guabi/38 Active, Brazil) was supplied 2 times per day, and the feeding rate ranged from 12% to 4% of the total shrimp biomass. The water quality parameters were suitable for the production of *L. vannamei*. The settleable solids (SS) were higher in the Ctrl treatment (82.1 ±19 mL L^{-1}) but only differed statistically with the 400 treatment; it is noteworthy that the 200 and 400 treatments showed average SS values below 10 mL L^{-1}. The reuse of water with bioflocs accelerated nitrification in the tanks, and no significant differences were shown between tanks. The presence of biofilm did not interfere with ammonium or nitrite levels, but it did serve as a food source that optimized shrimp performance, as shown by higher final weights. More studies are needed to evaluate the circulation and mixing intensity effects of the substrates on water throughout the production cycle.

Key words: Biofloc, biofilm, suspended solid, food sources, *Litopenaeus vannamei*.

1. Introduction

Biofilms are defined as autotrophic and heterotrophic microbial communities that are embedded in an organic matrix and adhere to submerged natural or artificial substrates. This microbial community includes bacteria, fungi, algae, flagellates, ciliates, zooplankton and detritus (Wetzel, 1983). Several studies have demonstrated the importance of this microbial community to the control of water quality and as a complementary food source for produced organisms.

Regarding water quality maintenance, it has been demonstrated that the use of biofilms is a suitable means to remove nitrogenous compounds from water, especially ammonium and nitrite, which are highly toxic (Crab et al., 2007). Thompson et al. (2002) demonstrated the importance of biofilms in maintaining water quality: nitrifying bacteria and microalgae assimilate ammonia, which allows the cycling of nutrients within the farming environment. Moreover, biofilms also uptake phosphate and produce oxygen in the system.

The addition of artificial substrates provides greater survival and growth rates among the shrimp; the biofilms formed act as additional food sources with high nutritional quality, providing essential nutrients such as polyunsaturated fatty acids, steroids, amino acids and vitamins (Abreu et al., 2007; Ballester et al., 2007; Preto et al., 2005; Silva et al., 2008).

Ballester et al. (2007) demonstrated that the consumption of biofilms increases survival and growth rates of the pink shrimp *Farfantepenaeus paulensis* in floating cages, and Abreu et al. (2007) demonstrated through the use of the stable isotope technique that biofilms contribute 49% of the carbon and 70% of the nitrogen content of the *F. paulensis* biomass. According to these results, the authors showed that commercial feeds with high protein content are ingested but not fully assimilated by the shrimp, which represents extra costs for the shrimp production.

It was also demonstrated that the microbial community composition influences the nutritional quality of biofilms. Silva et al. (2008) observed a strong correlation between centric diatoms and biofilm protein content, while heterotrophic nematodes were most responsible for biofilm lipid content. It was further observed that the substrates act as a refuge for shrimp, relieving the effects of stress caused by high

stocking densities, as demonstrated by Zhang et al. (2011), who obtained higher growth and survival rates of *Litopenaeus vannamei* with the increased addition of substrate to the tanks.

Currently, biofloc technology (BFT) is considered an environmentally friendly alternative to the development of intensive aquaculture because it offers several advantages over conventional systems, such as high rates of water renewal (Haslun et al., 2012; McAbee et al., 2003; Wasielesky et al., 2006). Similar to biofilms, bioflocs (microbial aggregates) are composed of algae, fungi, bacteria, flagellates, ciliates and other micro-organisms that adhere to an organic matrix and are present as floating flocs in the water column (De Schryver et al., 2008). In this technology, microorganisms maintain water quality and serve as food for the produced organisms (Burford et al., 2003; Chamberlain et al., 2001).

Many studies have demonstrated the importance of bioflocs in the feeding and water quality maintenance of BFT systems, especially because bioflocs perform nutrient cycling of nitrogenous compounds (Avnimelech, 2006; McIntosh, 2000a, 2000b; Nootong et al., 2011; Wasielesky et al., 2006). Moreover, in our laboratory, the effectiveness of the BFT system has been tested in studies on stocking density (Krummenauer et al., 2011), effect of depth of the production tanks (Krummenauer et al., Submitted), re-use of water with bioflocs (Krummernauer et al., 2012), carbon sources (Serra, 2012; Suita, 2009), correction of pH and alkalinity (Furtado et al., 2011), different types of aerators (Lara et al., 2014 in press), formation of bioflocs (Ferreira, 2008) and control of suspended solids in the water column (Gaona et al., 2011), among others.

Considering the benefits of biofilms and BFT, we hypothesized that the integration of these two production methods (biofilms and BFT) could generate better shrimp growth rates and water quality control in intensive shrimp production systems than using these techniques separately. Therefore, the aim of this study was to evaluate the effect of the addition of artificial substrates to BFT systems on the water quality and production of the white shrimp *L. vannamei*.

2. Methods

The experiment was conducted at the Marine Aquaculture Station of the Institute of Oceanography - Federal University of Rio Grande (FURG), southern Brazil. To evaluate the effect of adding artificial substrates (nylon nets) to BFT systems, three treatments were compared with three replicates each: 1) Control (Ctrl), without the addition of artificial substrate, 2) 200, with added artificial substrate equivalent to 200% of the lateral surface area of the tanks, and 3) 400, with added artificial substrate equivalent to 400% of the lateral surface area of the tanks.

Tanks with system volume of 800 L with bottom area of 1.76 m^2 and 0.45 m depth were used. Each tank was filled to 25% of its total volume with water containing bioflocs, which originated from a production tank of *L. vannamei* in the BFT system (Krummenauer et al., 2012). The remaining tank volume was filled with filtered seawater that had been previously chlorinated (10 ppm) and later dechlorinated with ascorbic acid (1 mg L^{-1}). The artificial substrates used for biofilm attachment were composed of nylon nets with a mesh size of 1.0 mm. The addition of substrate was calculated according to the lateral area of the tanks, representing 4.8 m^2 and 9.68 m^2 of the artificial substrates in the 200 and 400 treatments, respectively. The substrates were added in the first day of study and remained submerged throughout the experimental period.

The experiment lasted 35 days. The shrimps were stocked at a density of 300 shrimp/m^2 with an average initial weight of 0.40 ±0.15 g. For biofloc formation, organic fertilization was performed according to the methodology proposed by Avnimelech (1999). This method uses molasses from sugar cane as a carbon source, maintaining a C:N ratio between 15 – 20:1. Feed (i.e., a commercial diet with 38% crude protein) (Guabi/38 Active, Brazil) was supplied 2 times per day, with a feeding rate ranging from 12% to 4% of the shrimp biomass, according to the methodology proposed by Jory et al. (2001).

Measurements of dissolved oxygen (DO) and water temperature were conducted twice daily using a oximeter (WTW OXI-315i, Germany). The pH and salinity were measured daily in the morning using a bench meter (Mettler Toledo ®, FE20 model) and a refractometer (Atago, 103, Japan), respectively. The amount of settleable solids

(SS) was measured using Imhoff cones, and the transparency was measured using a Secchi disk; both measurements were performed 2 times per week. The concentrations of ammonia (UNESCO, 1983) and nitrite (Strickland & Parsons, 1972) were measured daily. The nitrate concentration (Strickland & Parsons, 1972) was measured 1 time per week.

Weekly samplings were performed to monitor shrimp growth. At the end of the experiment, all shrimps were weighed and quantified for late survival and growth analysis, as final weight, biomass in grams and productivity in g m^{-2} dia^{-1}. Apparent feed conversion ratio (FCR) and specific growth rate (SGR) were analyzed according to the following formula:

FCR = PF/ GW

Where FCR = apparent feed conversion ratio, PF = amount of provided feed and GW = Gain weight total (Van Wyk and Scarpa, 1999).

The SGR of the shrimp was expressed in %/day and determined according to the formula:

SGR (%/day) = ($\ln W_f - \ln W_i$ / t) x 100

Where ln = neperian logarithm, W_f = final weight, W_i = initial weight, and t = time (days) (Bagenal & Tesch, 1978).

2.1. Statistics

Possible differences between treatments (Ctrl, 200, 400) were analyzed using an ANOVA analysis. When significant differences among treatments occurred, a post-hoc Tukey's test was applied. If the data followed the appropriate assumptions, a nonparametric Kruskal-Wallis test was performed (Sokal and Rohlf, 1969).

3. Results

The water quality parameters (Table 1) were suitable for the production of *L. vannamei*. The temperature showed little variation between treatments, remaining within the range of tolerance of *L. vannamei*. The average temperature was kept at approximately 28 °C. The DO and pH were within the range of optimal growth for the species in all treatments.

The SS was higher in the Ctrl treatment, while a smaller concentration of bioflocs occurred in the water column of the 200 and 400 treatments. The Ctrl treatment was only significantly different from the 400 treatment. The 200 and 400 treatments showed average values below 10 mL L^{-1} of SS (Fig. 1 and Table 1).

Table 1: Mean values (± SD - standard deviation), minimum and maximum of water quality parameters in treatments during the study period [1].

	Ctrl	**200**	**400**
Temperature (°C)	27.4 ±2.2 [a]	26.8 ±1.9 [a]	26.9 ±1.8 [a]
DO (mg O_2 L^{-1})	6.8 ±0.5 [a]	6.8 ±0.5 [a]	6.6 ±0.5 [a]
pH	8.1 ±0.2 [a]	8.0 ±0.2 [ab]	7.9 ±0.2 [b]
Salinity	35.8 ±0.6 [a]	35.7 ±0.4 [a]	34.7 ±0.5 [a]
Secchi Disc (cm)	11.1 ±1.4 [a]	13.6 ±0.9[ab]	16.6 ±2.0 [b]
SS (mL L^{-1})	82.1 ±19 [a]	7.5 ±2.9 [b]	4.8 ±1.1 [b]
Turbidity (NTU)	171.8 ±43 [a]	88.6 ±6.8 [ab]	69.6 ±15 [b]

[1] DO = dissolved oxygen, SS = settleable solids, NTU = nephelometric turbidity units. Different letters = significant difference.

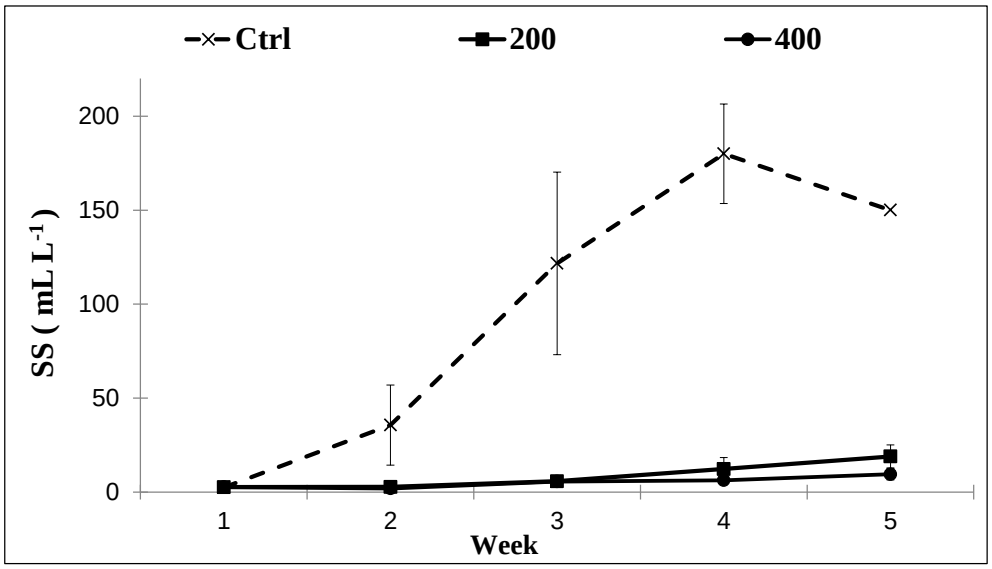

Fig. 1: Mean values (± standard deviation) of settleable solids (SS) in treatments.

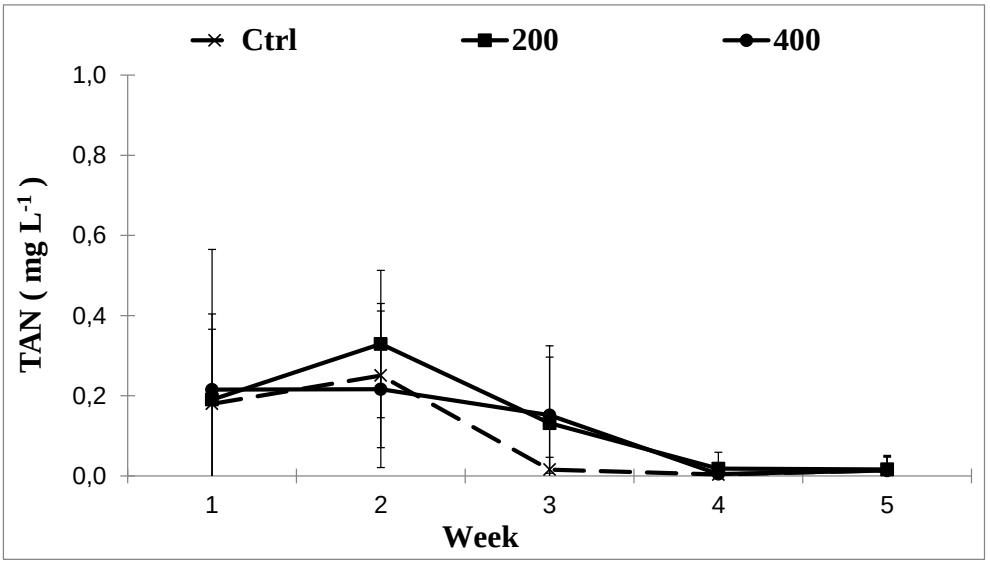

Fig. 2: Mean values (± standard deviation) of total ammonia nitrogen (TAN) in the treatments during the trial period.

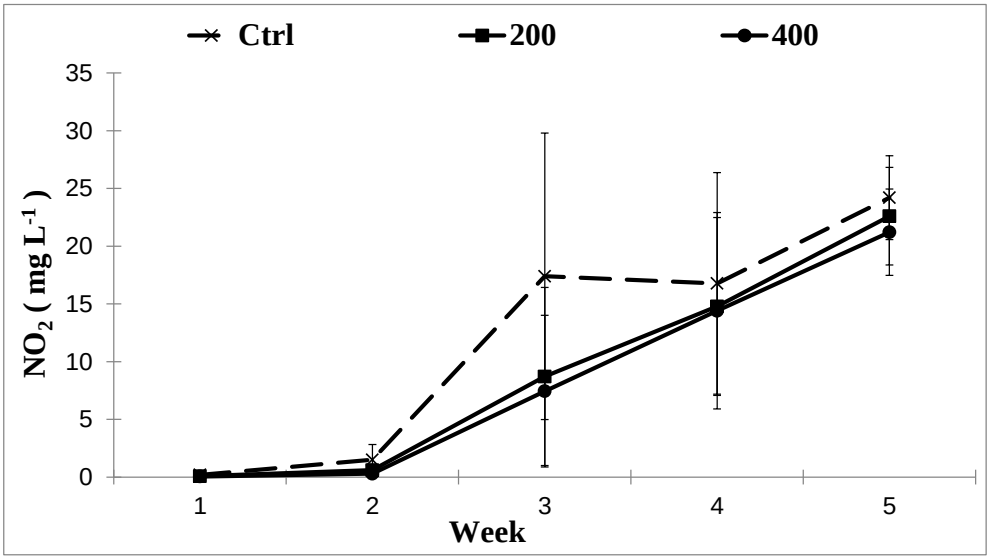

Fig. 3: Mean values (± standard deviation) of nitrite (NO_2) in the treatments during the trial period.

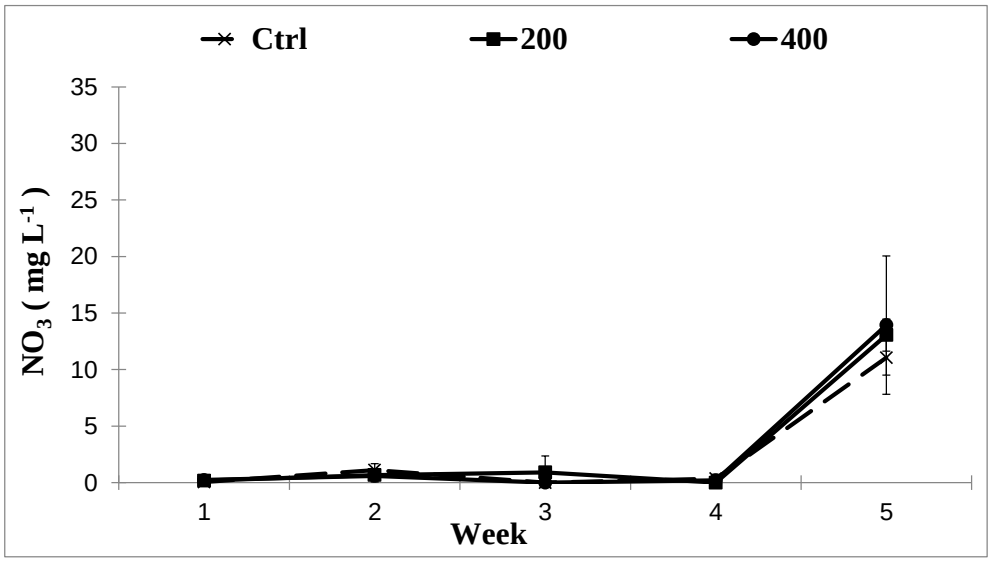

Fig. 4: Mean values (± standard deviation) of nitrate (NO_3) in the treatments during the trial period.

Regarding nitrogen compounds (Figs. 2, 3 and 4), there were no significant differences among the treatments, although the complete removal of ammonia was observed first in the Ctrl treatment. The nitrite concentration was higher in this same treatment, although the differences among the treatments were not statistically significant.

The total ammonia nitrogen (TAN) was below 1 mg L^{-1} in all treatments. The oxidation of ammonia to nitrite occurred from the 2nd week in all treatments (Figs. 2 and 3).The second step of nitrification, i.e., the oxidation of nitrite to nitrate, began in 4th week of the experiment (Figs. 3 and 4). It was not possible to observe whether the addition of substrate influenced in this step of nitrification.

The zootechnical performance of shrimp in the Ctrl treatment was inferior to the other treatments. No significant differences were observed between treatments with the artificial substrate. Compared to the final weight, final biomass, productivity and SGR in the Ctrl treatment were significantly lower than those in the 400 treatment. Additionally, survival was not significantly different among treatments, but it was slightly higher in the Ctrl (Table 2).

Table 2: Zootechnical performance of shrimp in the treatments (± SD - standard deviation) [1].

	Ctrl	**200**	**400**
W_i (g)	0.40 ±0.15	0.40 ±0.15	0.40 ±0.15
W_f (g)	2.34 [a] ±0.92	3.15 [ab] ±0.81	3.43 [b] ±0.95
Survival (%)	90.0 ±7.0	89.1 ±8.2	85.6 ±6.9
Biomass (g)	878.1 (± 31.4) [a]	1172.4 (± 19,2) [ab]	1234.8 (±197.5) [b]
Producitivity ($g/m^2/day$)	14.0 (±0.5) [a]	18.7 (±0.3) [ab]	19.7 (±3.2) [b]
SGR (%/day)	5.05 (±0.2) [a]	5.89 (±0.2) [b]	6.12 (±0.4) [b]
FCR	1.13	0.97	0.96

[1] W_i = initial weight, W_f = final weight, SGR = specific growth rate and FCR = feed conversion factor. Different letters = significant difference.

4. Discussion

In this study, we observed a positive effect of adding substrate, especially in shrimp development, which is most likely due to the increased food availability and possibly due to the control of suspended solids in the water column. Regarding the water quality, however, the influence of biofilms was not marked.

The excess of total suspended solids (bioflocs) can cause negative effects in BFT systems. The accumulation of particles in the water column increases the biochemical oxygen demand and causes the clogging of gills, which may reduce shrimp growth (Ray et al., 2010). Studies demonstrate that the excess of suspended material can be detrimental to the production of *L. vannamei*, thereby slowing its growth and reducing its biomass and FCR. Therefore, the control of total suspended solids (TSS) is recommended (Almeida, 2012; Gaona et al., 2011, Ray et al., 2010).

In this study, the treatments with the addition of substrate showed SS and turbidity values smaller than those in the Ctrl treatment. An explanation for this finding is that the addition of artificial substrates may have influenced the circulation of the water in the tanks by reducing turbulence, which may have facilitated sedimentation or increased the floc size.

Several processes contribute to the breakdown of aggregates particles, such as mechanical disruption, consumption by detritivores and microbial decomposition (Simom et al., 2002). Thus, it is also likely that the presence of the artificial substrate may have led to lower shear forces, leading to an increase of aggregates (Sharma et al., 2005).

Aeration is a crucial factor in BFT systems because it influences the oxygen supply to the produced organisms and microbial community and maintains the flocs in suspension, aiding in in the circulation of the water and preventing thermal stratification (Boyd and Clay, 2002; Van Wyk, 1999). A large amount of leftover food and feces generates an excess of material in suspension that may form sediments and cause dead zones at the bottom of the tank.

In our study, the concentrations of dissolved oxygen exhibited no significant differences among treatments, but a difference was observed in the intensity of water mixing and the amount of flocs in the water column. Lara et al. (2014, in press) found that the mixing intensity has great influence on biofloc formation. They also found different patterns of nitrification in tanks with different water mixing rates. This fact was related to the larger floc sizes, which allow the establishment of colony-forming nitrifying bacteria (Delatolla et al., 2009; Vlaeminck et al., 2010).

Regarding nitrogen compounds, we observed the occurrence of nitrification in all treatments, which maintained the ammonia and nitrite at safe levels for *L. vannamei* (Lin and Chen, 2001, 2003). However, the addition of substrate did not affect the oxidation of ammonia that kept their levels below 1 mg L^{-1} in all treatments. Indeed, the Ctrl treatment tended to be more effective in the oxidation of ammonia to nitrite than were other treatments with the addition of substrate. However, the higher nitrate concentration in the 200 and 400 treatments indicates that these treatments were apparently more efficient in the oxidation of nitrite.

The reuse of water with bioflocs accelerates the nitrification process (Krummenauer et al., 2014). Thus, it is more likely that effective nitrification in all treatments was influenced by inoculation of bioflocs that already had established nitrifying communities. Since, it is the water with bioflocs that acts as an inoculum for the formation of new bioflocs and the spread of nitrifying bacteria.

The removal of nitrogen compounds in BFT systems can be performed by controlling the TSS in the water column. Almeida (2012) found that in treatments with less TSS, between 100 – 400 mg L^{-1}, the process of solid removal was also responsible for removing nitrogen compounds from the aquaculture environment. Additionally, treatments with greater amounts of TSS showed higher accumulations of nitrite and nitrate dissolved in water, originating from nitrification (Almeida, 2012).

Studies using biofilms normally show increased shrimp survival (Audelo-Naranjo et al., 2011; Bratvold and Browdy, 2001; Zhang et al., 2011). In this study, the survival of the shrimp in all treatments was satisfactory compared with other studies in BFT systems; however, in the treatments with addition of substrate, the survival was slightly lower. It must be highlighted that other studies with less TSS showed better performance of shrimps (FCR, final weight and weekly growth) without affecting survival (Almeida, 2012; Gaona et al., 2011; Ray et al., 2010).

It is very likely that the biofilms that formed in the 200 and 400 treatments remained as a supplementary food source for the shrimp, thereby improving their production performance (final weight, biomass, SGR, FCR and productivity). Studies have confirmed the benefits of biofilms as a food source, resulting in increased growth, survival and feed conversion rates of the produced shrimp (Abreu et al., 2007; Ballester et al., 2007; Silva et al., 2008; Thompson et al., 2002). More recently, Audelo-Naranjo et al. (2011) evaluated the effect of adding artificial substrates in the production of *L. vannamei* in a system without water exchange and found that the formed biofilm was an additional food source that improved shrimp growth performance without affecting the water quality. Moreover, the substrates act as a refuge due to the additional space created by substrate addition, as verified by Zhang et al. (2011), who obtained higher growth rates and survival of shrimp along with increased number of substrates.

5. Conclusion

The addition of artificial substrates in BFT systems served to optimize the food source availability, aside from the commercial food. Moreover, the substrates promoted the control of suspended solids (bioflocs) in the water column. However, the presence of more biofilms seemed to have no significant effect on the nitrogen cycle in the system. Therefore, further studies are necessary to evaluate the possible effects of biofilms during the entire production cycle.

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Anexo 2 – Characterization of the Bacterial Communities of Bioflocs and Biofilms in Aquaculture Systems Using Temporal Temperature Gradient Gel Electrophoresis (TTGE)

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Abstract

In this study we have evaluated the bacteria composition of biofloc and biofilm in treatments with the reuse of water with bioflocs ("R") and with biofloc formed from clear water ("CW"). The experiment lasted 35 days and consisted of four treatments with three replicates: 1) R+0 treatment, with reuse and no added artificial substrate; 2) R+400, with reuse and with the addition of artificial substrate; 3) CW+0, clear water and no added artificial substrate; and 4) CW+400, clear water and added artificial substrate. The added substrate (nylon net) was equivalent to 400% of the lateral area of the tanks. Shrimps (0.40 ± 0.15 g) were stocked with a density of 300 shrimps/m² in 800 L tanks. The shrimps were fed with commercial feed with 38% protein content twice daily. The growth performance of the shrimp was higher in the tanks with substrate. However, the reuse of water with bioflocs accelerated the process of nitrification. Bacterial composition was determined by the molecular biology technique Temporal Temperature Gradient Electrophoresis (TTGE). This technique indicated that bacterial communities of the biofilms and bioflocs have levels of similarity ranging from 41.6% to 50%. Moreover, the TTGE indicated the presence of only one species of ammonium-oxidizing bacteria.

Introduction

Our knowledge of microbial diversity and its role in nature has primarily been limited by traditional microbiology techniques, such as microscopy and culture, which show limited capabilities to sort and identify bacteria (Hugenholtz et al. 1998; Muyzer 1999). In addition, estimates indicate that 99% of the bacteria in natural environments are not easily cultivable, hampering the use of metabolic tests for their identification (Streit and Schmitz 2004). Most eukaryotic organisms can be classified based on their phenotypic characteristics. However, for prokaryotic organisms, classification is limited by the small size of bacteria and their lack of distinguishable features (Muyzer 1999; Farnleitner et al. 2000; Dorigo et al. 2005).

To overcome these problems, molecular biology methods have been used in the analysis of microbial diversity, especially for non-cultivated species of bacteria

(Pernthaler and Amann 2005; Luo et al. 2007; Park et al. 2008). The cloning and sequencing of 16S ribosomal RNA gene is a powerful tool, although it can be highly laborious, time-consuming and expensive (Pernthaler and Amann, 2005). Recombinant DNA techniques and molecular methods provide means for obtaining profiles of microbial communities and their identification (Hugenholtz et al. 1998; Hallin et al. 2006; Leddy 2007), allowing investigation of the dynamics, composition and structure of microbial communities in different environments under natural or stressful conditions (Muyzer et al. 1993; Hastings et al. 2006).

"Fingerprinting" techniques, such as constant denaturing gel electrophoresis (CDGE), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and temperature temporal gradient electrophoresis (TTGE), reveal patterns of microbial diversity, allowing comparisons of microbial communities in different environments as well as their development over time. These techniques all employ electrophoresis in polyacrylamide gels for the separation of DNA obtained through polymerase chain reaction (PCR) amplification (Nicholson et al. 2007).

These techniques allow the separation of DNA fragments over a denaturing gel gradient. DNA fragments of the same size but with different nucleotide sequences are easily separated using these methods because the denaturing conditions are different for each sequence, according to the number of hydrogen bonds between complementary base pairs (Dorigo et al. 2005). In general, sequences composed of adenine-thymine (AT) pairs present more hydrogen bridges with lower melting points than sequences with a large number of cytosine-guanine (CG) pairs, which present triple bonds that are more difficult to break. In general, these techniques exhibit high sensitivity, showing the ability to differentiate bands with only a single different base pair (Dorigo et al. 2005). However, techniques based on gel electrophoresis are very much dependent on the type of sample, DNA extraction method, and running conditions, which can be quite variable.

Different gradients of saturation can be applied in the separation of DNA sequences in a polyacrylamide gel. Constant denaturing gel electrophoresis (CDGE) is a technique involving uniform denaturing conditions and is used for the partial denaturation of one fragment, or multiple fragments with the same domain of denaturation. For example, this technique allows the separation of wild-type and mutant

fragments with sequences that differ slightly from each other (Børrensen-Dale et al. 1997).

Denaturing gradient gel electrophoresis (DGGE) allows the separation of DNA bands through a chemical denaturant gradient, usually generated via adding formamide and urea to the gel at concentrations ranging from 0% to 100%. The main problem with this technique is related to reproducibility of the results since two gels are never exactly the same. Thus, when you have many samples, the ability to compare samples from different gels is limited (Farnleitner et al. 2000).

In temperature gradient gel electrophoresis (TGGE), the gel contains a uniform concentration of denaturant, and band separation occurs through a temperature gradient that increases along the gel during electrophoresis (Muyzer 1999). In this case, sequences with lower denaturation temperatures cease migration earlier in the gel, while DNA fragments with higher melting temperatures migrate farther along the gel, forming a pattern of bands for the sample. Similar to DGGE, this technique presents problems with reproducibility due to variations in the temperature gradient of the heating plate in which the gel is placed (Børrensen-Dale et al. 1997).

To solve the problem of the reproducibility of results, temperature temporal gradient electrophoresis (TTGE) was developed. This technique uses a constant denaturing gel, similar to CDGE and TGGE, but separation of the different fragments (bands) occurs due to changes in temperature over time that is uniform across the gel (Børrensen-Dale et al. 1997). During electrophoresis, the temperature increases over time, resulting in a linear temperature gradient during the run, referred to as the temperature ramp, which is given in ° C / hour (Zoller et al. 2008). This gradient allows the separation of fragments with different domains of denaturation. Thus, it allows the separation of quite variable DNA sequences (Hastings et al. 2006; Børrensen-Dale et al. 1997; Zoller et al. 2008). Although this technique was proposed several years ago, there have been few studies applying this methodology in aquatic environments. We can only cite a single study by Kosntanjsek et al. (2004), who used the TTGE technique to assess the bacterial community in the sediment of Lake Shkodra / Skadar. To our knowledge, there are no reports of this technique being employed to characterize the bacterial community in aquaculture systems.

The use of biofilms and bioflocs allows intensive production of aquatic organisms such as shrimp and fish with reduced water renewal and effluent production. This is only possible because bacteria and other microorganisms present in the biofilms and bioflocs help to maintain the water quality, especially through the recycling of nitrogen compounds, in addition to functioning as a food supplement (Preto et al. 2005; Avnimelech 2006; Wasielesky et al. 2006; Abreu et al. 2007; Ballester et al. 2007; Silva et al. 2008; Krummenauer et al. 2011, 2012). However, little is known about the composition and dynamics of the bacterial communities in aquaculture systems containing biofilms and bioflocs.

Therefore, the aim of this work was to implement the TTGE technique for the analysis of bacterial diversity in the biofilms and bioflocs used in the intensive production (> 300 shrimps / m^2) of the white shrimp *Litopenaeus vannamei* to evaluate the degree of similarity of the bacterial communities and the diversity of bacteria in the biofilms and bioflocs.

Materials and Methods

Experimental Design

The experiment was conducted at the Marine Aquaculture Station of the Institute of Oceanography - Federal University of Rio Grande (FURG), southern Brazil. Four treatments were tested in triplicate. The treatments were divided into two groups: "R" treatments, involving the reuse of water with bioflocs, and “CW” treatments, where bioflocs were formed from clear water (seawater). The treatments were as follows: 1) an R+0 treatment, involving reuse of water with bioflocs, without the addition of an artificial substrate; 2) R+400, involving reuse of water with bioflocs and the addition of a substrate (nylon net; 1 mm mesh size), equivalent to 400% of the lateral surface of the tank; 3) CW+0, with clear water and without the addition of an artificial substrate; and CW+400, with clear water and the addition of a substrate equivalent to 400% of the lateral surface of the tank. The addition of the nylon net resulted in the formation of a greater amount of biofilm in the tanks.

The water used in the R treatments filled 25% of the useful volume of the tanks (800 L). The water with bioflocs came from a tank in which *Litopenaeus vannamei* were raised using biofloc technology (BFT). These bioflocs were formed over 90 days.

The remainder of the tank volume was filled with filtered seawater that had previously been chlorinated with 10 ppm chlorine and then dechlorinated with ascorbic acid (1 mg L^{-1}). In the clear water treatments, only chlorinated and dechlorinated filtered seawater was used. The substrates were placed in a subsurface position and remained submerged throughout the experimental period. The biofloc was formed according to the methods proposed by Avnimelech (1999), using sugar cane liquid molasses and rice bran as carbon sources to maintain a C:N ratio of 15 – 20:1.

All of the tanks were stocked with *L. vannamei* white shrimp with an average initial weight of 0.40 ±0.15 g at a density of 300 shrimps/m^2. Feed (commercial diet with 38% crude protein; Guabi/38 Active, Brazil) was supplied twice a day at a feeding rate ranging from 12% to 4% of the shrimp biomass, according to the methodology proposed by Jory et al. (2001). The experiment lasted 35 days.

Parameters of Water Quality

Measurements of dissolved oxygen and water temperature were conducted twice per day using an oxymeter (WTW, Oxi 315i, Germany). The pH and salinity were measured daily in the morning using a pH meter (Mettler Toledo ®, FE20 model) and a refractometer (Atago, 103, Japan), respectively. Daily water samplings were also conducted to verify the concentrations of total ammonium (UNESCO 1983) and nitrite (Strickland & Parsons 1972). The nitrate concentration (Strickland & Parsons 1972) was measured once per week. The content of settleable solids (SS) was measured using Imhoff cones, while transparency was measured with a Secchi disk, and water turbidity was measured using a Turbidimeter (Hach 2100P, Hack Company, Loveland, Colorado, United States); these measurements were performed one time per week.

Shrimp Monitoring:

An initial biometric analysis was performed to estimate the mean weight of the shrimps (n = 100) to be stocked in each experimental unit. During the experiment, shrimps (n=30) were collected every 10 days to evaluate their weight gain and to adjust the amount of feed to be offered. Weight gain, biomass, productivity and survival were assessed at the end of the experiment by counting and weight the animals in each

experimental unit. The apparent feed conversion ratio (FCR) and specific growth rate (SGR) were determined according to the following formulas:

FCR = PF/ WG

Where FCR = apparent feed conversion ratio; PF = amount of provided feed; and WG = total weight gain (Van Wyk 1999); and

SGR (%/day) = $(\ln W_f - \ln W_i / t) \times 100$

Where ln = neperian logarithm; W_f = final weight; W_i = initial weight; and t = time (days) (Bagenal & Tesch 1978).

Statistical Analysis:

The water quality and shrimp monitoring data were subjected to one-way ANOVA. When the test indicated a significant difference between treatments, the post-hoc Tukey test was further employed to identify which treatments presented differences. The data were assessed for normality and homoscedasticity. For non-normal and not homoscedastic data, significant differences between treatments were determined with the nonparametric Kruskal-Wallis test (Sokal and Rohlf 1969).

Bacterial DNA Extraction

Water samples of different volumes (30 - 50 ml) were collected from all tanks on the first (day 1) and last (day 35) days of experiment. Biofilm collection was performed by scraping pieces of the artificial substrates at day 35. The biofilm samples were transferred to Eppendorf tubes and then frozen at -20 ° C until further DNA extraction.

Water samples with bioflocs were concentrated onto polycarbonate membrane filters (Nuclepore, 0.2 µm). After filtration, each filter was placed in a sterile Eppendorf tube and frozen at -20 °C until subsequent DNA extraction. The DNA samples were extracted using the QIAmp DNA Stool commercial kit according to the manufacturer's instructions (Qiagen).

Polymerase Chain Reaction (PCR)

Nested PCR was performed using the *Bacteria* domain primers 11F (5′-GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TACC TTG TTA CGA CTT-3′) (Siripong

and Rittman 2007), which amplify nearly the entire bacterial 16S gene, for the first step and the primers GC-338F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Henriques et al. 2006), which amplify approximately 180 bp of the V3 hypervariable region within the amplicons from the first PCR step, for the second step. The first step was performed in a 12.5 μL reaction using the following final concentrations: 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer, 20 ng μL^{-1} BSA, 0.1 μL of Platinum® Taq DNA polymerase (5 U μL^{-1}, Invitrogen), and 1.0 μL of the DNA template. The PCR conditions were as follows: 5 min at 94 °C, followed by 30 cycles of 5 min at 94 °C, 1 min at 52 °C and 1 min and 30 s at 72 °C, with a final extension step of 10 min at 72 °C. The second step was performed in a total volume of 50 μL using the following final concentrations: 1× reaction buffer, 0.3 mM each dNTP, 0.3 μM each primer, 1.0 μL of Pfx 50™ DNA polymerase (5 U μL^{-1}, Invitrogen), and 2.0 μL of the DNA product containing the first-step amplicons. The PCR conditions were as follows: 5 min at 94 °C, followed by 20 touchdown cycles (van der Gucht et al. 2007) of 1 min at 94 °C, 1 min starting at 65 °C and ending at 55 °C (decreasing 0.5 °C each cycle), and 1 minute at 68 °C, then 20 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 68 °C, and a final extension step of 30 min at 68 °C to minimize the artifactual bands obtained (Janse et al. 2004). The sizes of the PCR products were validated through 1 % agarose gel electrophoresis and quantified using the Quant-It™ dsDNA Broad-Range Assay Kit (Invitrogen).

Temperature Temporal Gradient Gel Electrophoresis (TTGE)

TTGE was performed with the DCode system (Bio-Rad) using 14 % polyacrylamide gels (37.5:1 acrylamide:bisacrylamide), 7 M urea, and 1.25× TAE buffer. The gels were polymerized with 50 μL of TEMED and 100 μL of 10 % APS for 2 – 3 h and subjected to a prerun at 200 V for approximately 30 min. Samples of PCR-amplified DNA were then loaded with 1:1 sample:gel loading dye (70 % glycerol, 0.05 % bromophenol blue, and 0.05% xylenocyanol). The run was set to 66.0–69.7 °C at 68 V for 18.5 h with a 0.2 °C h^{-1} ramp. The gels were stained for 15 min in 1.25× TAE buffer containing 1 μg μL^{-1} ethidium bromide and destained for 15 min in 1.25× TAE buffer. The gels were then photographed using UV transillumination with T1201

Sigma/UltraLum UltraCam Digital Imaging equipment coupled to a PowerShot A620 Canon Camera (They et al. 2013).

Analysis of TTGE Images.

The gel images were saved in TIFF format in the Zoom Browser program (Lab. Molecular Biology, ICB) and edited in the D-Scan program to obtain an image with a white background and black bands in grayscale, and the brightness and contrast were adjusted. Then, the images were analyzed in the free program Gel Analyser 2010a (www.gelanalyser.com/download.html), through the following steps: automatic detection of columns and manual adjustment of the column position, followed by detection and manual adjustment of the bands. The band position data (area and raw volume) were registered to establish a distance matrix, according to Ishii et al. (2009). The matrix was constructed based on a ± 1 % position error. Prior to the analysis, bands with a frequency below 10 % were excluded so that the data could be transformed using Hellinger transformation (Ramette 2007). Subsequently, the Euclidean dissimilarity test was performed to determine the similarity and dissimilarity between treatments (Clarke 1993).

Results

Shrimp Growth Parameters:

Regarding the growth parameters of shrimps, only the R+0 treatment showed poorer results than the other treatments (Table 1). Survival tended to be higher in the CW treatments, although there were no significant differences between treatments. In general, the treatments with an added artificial substrate led to a greater weight gain, SGR, biomass and productivity of the shrimp than the treatments without an added substrate (Table 1).

Table 1: Growth parameters for the shrimp in the treatments (±SD, standard deviation) throughout the experimental period[1].

	R+0	**R+400**	**CW+0**	**CW+400**
W_i (g)	0.40 ±0.15	0.40 ±0.15	0.40 ±0.15	0.40 ±0.15
W_f (g)	2.34 ^{a} ±0.92	3.43 ^{c} ±0.95	3.10 ^{b} ±0.94b	3.20 ^{bc} ±1.02
Survival (%)	90.0 ±7.0	85.6 ±6.9	90.6 ±7.4	94.2 ±3.6
Biomass (g)	878.1 ±31.4	1234.8 ±197.5	1185.1 ±224.9	1265.7 ±69.0
Productivity ($g/m^2/day$)	14.0 ± 0.5	19,7 ±3.2	18.9 ±3.6	20.0 ±1.1
SGR (%/dia)	5.05 ± ^{a}	6.12 ± ^{b}	5.84 ± ^{b}	5.94 ± ^{b}
FCR	1.13	0.96	0.94	0.90

[1] W_i = initial weight, W_f = final weight, SGR = specific growth rate and FCR = feed conversion ratio. Different letters indicate a significant difference.

Water Quality

The recorded water quality parameters were suitable for the production of *L. vannamei* (Table 2). The temperature was maintained within the recommended range for the species, with no significant difference being observed between treatments. The concentration of dissolved oxygen (DO) and pH showed little variation among the treatments, although there were significant differences between treatments. The DO concentration was higher than 5 mg L^{-1}, and the pH was maintained between 7.0 and 9.0. In the CW treatments, salinity was significantly higher than in the R treatments.

The transparency of the water in the R treatments tended to be lower than in the CW treatments (Table 2). As expected, the formation of bioflocs was faster in the R+0 treatment compared with the other treatments (Table 2).

Table 2: Mean values (±SD, standard deviation) of water quality parameters in the treatments during the study period [1].

	R+0	R+400	CW+0	CW+400
Temperature (°C)	27.4 ±2.2	26.9 ±1.8	26.7 ±1.8	27.0 ±1.7
DO ($mgO_2\ L^{-1}$)	6.8 ±0.5 [a]	6.6 ±0.5 [ab]	6.8 ±0.5 [ab]	6.5 ±0.5 [b]
pH	8.1 ±0.2 [a]	7.9 ±0.2 [b]	8.0 ±0.1 [a]	7.9 ±0.1 [ab]
Salinity	35.8 ±0.6 [a]	34.7 ±0.5 [a]	38.6 ±1.6 [b]	39.0 ±0.2 [b]
transparency (cm)	11.1 ±1.4 [a]	16.6 ±2.0 [ab]	17.5 ±2.5 [ab]	19.6 ±1.7 [b]
SS ($mL\ L^{-1}$)	82.1 ±19 [a]	4.8 ±1.1 [ab]	7.6 ±3.4 [ab]	3.1 ±1.1 [b]
Turbidity (NTU)	171.8 ±43 [a]	69.6 ±15 [ab]	71.3 ±23 [ab]	47.3 ±13 [b]

[1] DO = dissolved oxygen, SS = settleable solids, NTU = nephelometric turbidity units. Different letters indicate a significant difference.

The reuse of water with bioflocs influenced the nitrification process. In the CW treatments, accumulation of total ammonium was observed until the 4th week, while in the treatments involving water reuse (R), the total ammonium concentration was below 1 mg L^{-1} throughout the experimental period (Fig. 1). Oxidation of ammonium to nitrite occurred from the 2nd week onward in the treatments with inoculated bioflocs (R+0 and R+400). In the treatments without water reuse (CW), this process was only detected after the 3rd week of the experiment. However, only the R+0 treatment presented nitrite values that were significantly higher than the CW+400 treatment (Fig. 2). Oxidation of nitrite to nitrate began in the fourth week, regardless of the treatment, but the R treatments still exhibited higher nitrate concentrations, indicating a greater efficiency in the oxidation of nitrite. However, the only significant difference was found between the R+400 and the CW+400 treatments (Fig. 3).

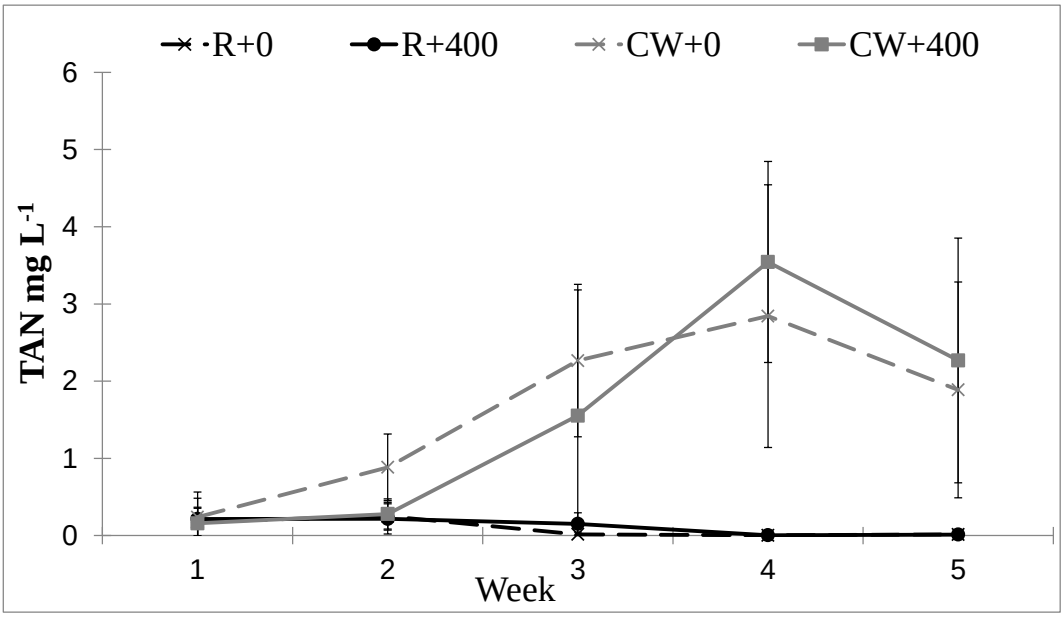

Figure 1: Mean contents (± standard deviation) of total ammonium (TAN) in the treatments during the trial period.

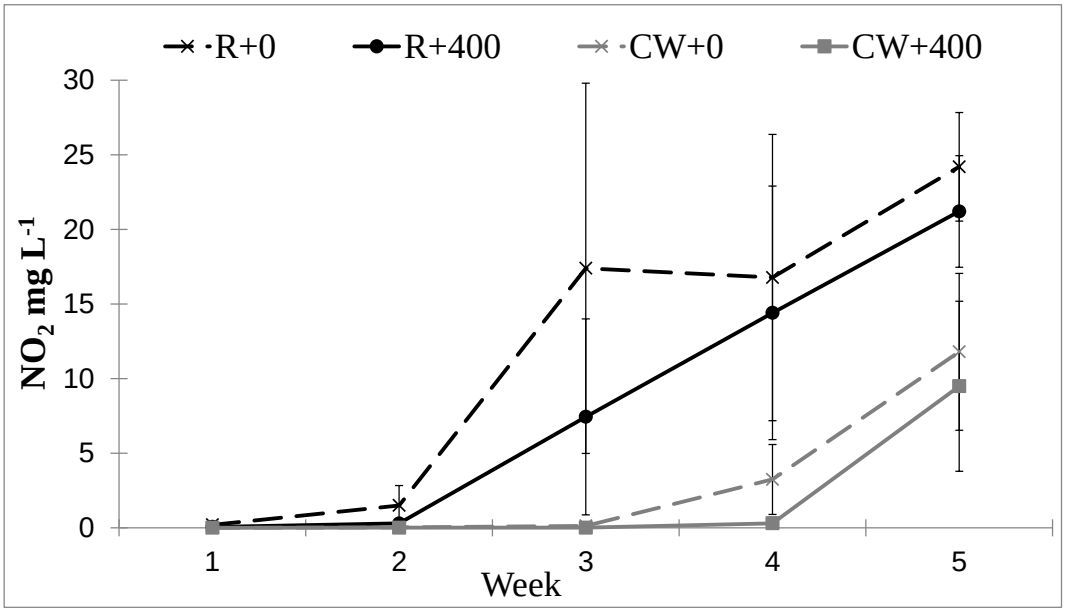

Figure 2: Mean contents (± standard deviation) of nitrite (NO_2) in the treatments during the trial period.

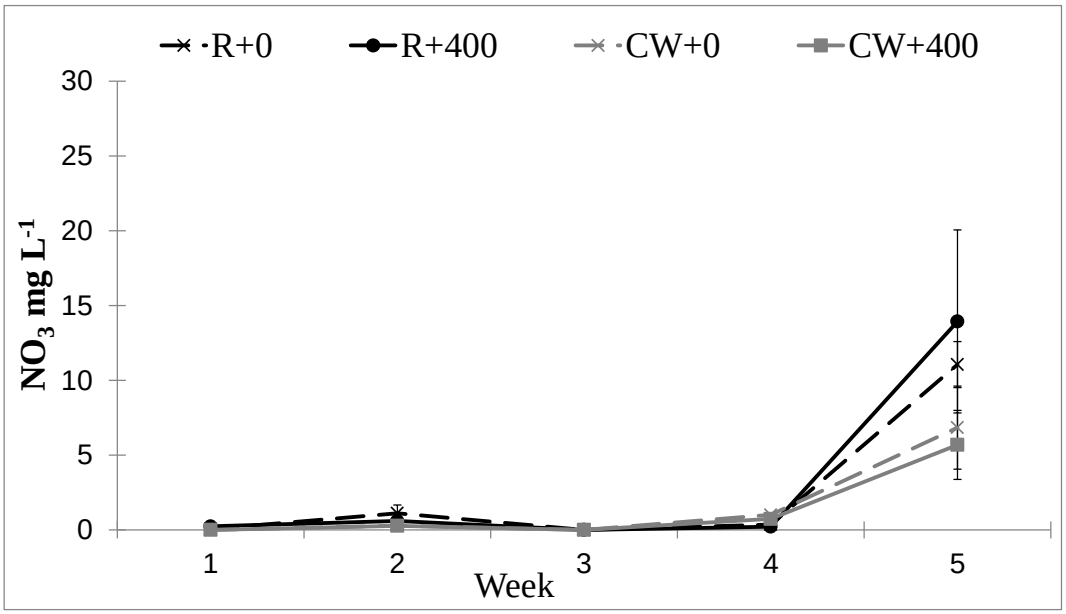

Figure 3: Mean contents (± standard deviation) of nitrate (NO_3) in the treatments during the trial period.

Bacterial Community Composition of Biofilms and Bioflocs, Characterized by TTGE.

Regarding the bacterial community of the bioflocs, the profiles obtained in the treatments without an added substrate showed clear differences between the CW and R treatments at the beginning of the experiment (Fig. 4, columns 1 and 3). However, when the substrate was added, the initial bacterial communities in the bioflocs were similar in the R+400 and CW+400 treatments (columns 5 and 8). At the end of the experiment (D35), it was found that the profiles of the bacterial communities of the bioflocs were similar for all treatments (Fig. 4, columns 2, 4, 6 and 9), as were those of the biofilms (columns 7 and 10), independent of the CW or R treatments.

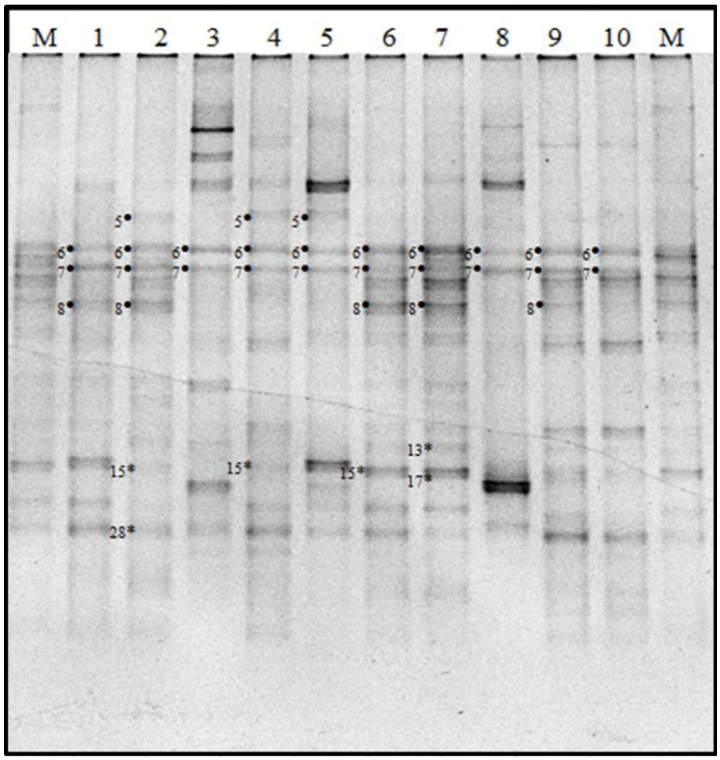

Figure 4: Bacterial communities of the bioflocs and biofilms in the treatments by temporal temperature gradient gel electrophoresis (TTGE) using samples of water with bioflocs collected on days 1 and 35 (D1 and D35) and samples obtained through scraping the biofilm (D35) that developed during the study period. Treatments without substrates: (1) R+0 treatment, D1; (2) R+0, D35; (3) CW+0, D1; (4) CW+0, D35; and treatments with the addition of artificial substrate equivalent to 400% of the lateral area of the tanks: (5) R+400, D1, (6) R+400, D35, biofloc; and (7) R+400, D35, biofilm; (8) CW+400, D1; (9) CW+400, D35, biofloc; (10) CW+400, D35 biofilm; and (11) ladder for aligning the marker bands. Bands 6, 7 and 8 were present in all treatments and were assigned to biofloc formation. Already bands 5, 15 and 28 were unique to biofloc and bands 13 and 17 exclusive of biofilm.

The bacterial compositions of bioflocs and biofilms showed a similar richness, with 12 and 10 ribotypes being detected in the R+400 and CW+400 treatments, respectively, among which 5 ribotypes were similar in the two treatments (Table 3). In the R+400 treatment, the bacterial composition of the biofilm and biofloc showed a similar richness, although with a higher percentage of dissimilarity being observed between the biofilm and biofloc compositions. In the CW+400 treatment, the richness

of the bacterial community of the biofloc was higher than that of the biofilm. However, the detected dissimilarity was 50% for both the biofilm and biofloc (Table 3).

Bands 6, 7 and 8 were the most common ribotypes among the treatments from the beginning of the experiment. Band 8 was more abundant in the treatments with water reuse and may have been related to the maturation of the bioflocs and/or biofilms. Bands 5, 15 and 28 were found exclusively in the bioflocs, while bands 13 and 17 were exclusively present in the biofilms.

Table 3: Number of bands, both exclusive and shared, in the bacterial communities of the biofloc (BF) and biofilm (BM) at day 35 (D35) of the study period, obtained through TTGE.

	Number of bands					
	BF	**BM**	**BF ∩ BM**	**BF ≠ BM**	**Similarity**	**Dissimilarity**
R+400	8	9	5	7	41.66 %	58.33 %
CW+400	8	6	5	5	50 %	50 %

Discussion

Several studies have demonstrated the benefits of adding artificial substrates for the development of biofilms. Microorganisms present in biofilms represent a source of supplementary food, resulting in better growth, survival and feed conversion ratios in shrimp farming (Thompson et al. 2002; Wasielesky et al. 2006; Abreu et al. 2007; Ballester et al. 2007; Sharma et al. 2008). In this study, the clear water treatment without added substrate (CW+0) showed contents of settleable solids and turbidity similar to the R+400 treatment. However, the growth performance of the shrimp in this treatment was not as favorable as in the treatments with added substrates. It is likely that in addition to furnishing extra food, the added substrates can represent a refuge for shrimps, contributing to decreasing the stress of raised shrimps, as suggested by Zhang et al. (2010).

The lower growth of shrimps in the R+0 treatment was most likely related to the excess suspended matter in the water column. Large amounts of bioflocs have negative effects on shrimp in BFT systems due to clogging their gills and increasing their oxygen

demand, leading to lower shrimp growth (Ray et al. 2010; Gaona et al. 2011). However, this excess of bioflocs did not appear to influence shrimp survival. The removal of suspended solids through the use of settling tanks improves the growth performance of *L. vannamei* in BFT systems (Ray et al. 2010; Gaona et al. 2011), and the same effect was obtained from the presence of the artificial substrates in this study.

Rink et al. (2003) observed differences in the community composition of free-living and attached (flocs) bacteria. They also found that some bacterial ribotypes occur independent of environmental conditions (generalists), while others appear or disappear under certain circumstances (specialists). In the present study, the TTGE results indicated that the obtained bioflocs and biofilms presented a similar bacterial composition, showing 5 similar bands, which corresponds to 41.6% and 50% similarities of the biofilm and biofloc bacteria in the R+400 and CW+400 treatments, respectively. Bands 6, 7 and 8 were present in the biofilms and bioflocs in both the CW and R treatments from the beginning of the experiment. On the other hand, bands 5, 15 and 28 were found exclusively in bioflocs, while bands 13 and 17 were only present in biofilms. It is very likely that these biofloc-exclusive bands are related to the nitrification process, as the R treatments always presented low levels of ammonium.

In a similar study conducted by Zhao et al. (2012) with the shrimp *Macrobrachium japonicus*, the composition of the bacterial communities in clear water and water with bioflocs showed a similar richness of ribotypes. These authors conducted a comparison of the predominant bacterial communities present in the bioflocs and clear water and found a low diversity of both bacterial communities.

Rochex et al. (2008) described the pattern of biofilm formation in the following way: (1) initial adhesion phase of the biofilm, with a high diversity of planktonic bacteria that adhere to the surface, during which there is low selection pressure on the bacteria; (2) biofilm growth phase, with reduced bacterial diversity due to competition between microorganisms; and (3) maturation phase of the biofilm, with increased bacterial diversity due to the complex architecture of the biofilm, involving various microhabitats. The low number of bands found in TTGE analysis in the biofilms of the R+400 and CW+400 treatments indicates that both systems were in phase 2 of bacterial succession.

The accumulation of ammonium and other nitrogen compounds in water is associated with intensification of aquaculture systems (Crab et al. 2012) and the use of diets with a high protein content (Hepher 1988). Thus, the microbial decomposition of the remaining feed and the excretion of the produced organisms lead to the accumulation of nitrogen elements, such as ammonium and nitrite, in the aquaculture environment. These nitrogen compounds are extremely toxic to produced fish and shrimp (Boyd 2007; Azim et al. 2008). To reduce ammonium and nitrite concentrations, water renewal is employed. However, this process leads to eutrophication of the water bodies that receive aquaculture effluents. Moreover, other elements, such as phosphorus and organic matter, also contribute to the deterioration of the water quality of adjacent coastal waters (Piedrahita 2003; Crab et al. 2012).

Current techniques used for the removal of nitrogen in aquaculture systems operate either inside or outside of aquaculture systems. The microorganisms present in biofilters located outside of tanks are able to remove nitrogenous compounds. These biofilters are composed of different substrates that are submersed in water reservoirs connected to the tanks where shrimp or fish are raised. These substrates allow the formation of a biofilm and its colonization by nitrifying bacteria (Avnimelech 1999). In BFT systems, the absorption of nitrogenous elements by heterotrophic and nitrifying bacteria present in bioflocs occurs inside the tanks. Heterotrophic bacteria are efficient in the uptake of ammonium after the addition of a carbon source (e.g., molasses or dextrose), resulting in an immediate decrease in ammonium and increase in bacterial density (De Schryver et al., 2008; Chamberlain et al. 2001).

Nitrifying bacteria are also present in bioflocs (Avnimelech 2012) but require a longer time to remove ammonium and nitrite, especially in saltwater (Thompson et al. 2002; Avnimelech 2006; Nootong et al. 2011). Heterotrophic bacteria exhibit a much shorter generation time than nitrifying bacteria (Michaud et al. 2006). Therefore, the removal of toxic nitrogen compounds by heterotrophic bacteria is faster compared with nitrifying bacteria (Burford et al. 2003).

Throughout the experiment, we verified an increased nitrification efficiency in the treatments involving the reuse of water with pre-formed bioflocs (R treatments), indicating that the reused water serves as an inoculum of nitrifying bacteria. Krummenauer et al. (2014) reported that reuse of 25 to 100% of water containing

bioflocs accelerates biofloc formation and the establishment of nitrifying bacteria, resulting in the rapid removal of ammonium and nitrite dissolved in water.

However, it appears that only ammonium-oxidant nitrifying bacteria were present in our experimental treatments, as the TTGE technique indicated the presence of only a single band in bioflocs of the R treatments. It is likely that these nitrifying bacteria belong to the ammonium-oxidizing group, but to confirm this would be required sequencing and identification of this band.

In conclusion, we demonstrated that the Temporal Temperature Gel Electrophoresis (TTGE) technique is a powerful tool to be used in the study of bacterial dynamics and processes in aquaculture systems.

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Anexo 3 – Bacterial Microbiota responsible for biofloc formation and nitrification during the production of Litopenaeus vannamei in BFT systems.

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Abstract

This study aimed to evaluate the influence of different air diffusers on the bacterial species responsible for biofloc formation and nitrification during the production of *Litopenaeus vannamei* in biofloc (BFT) systems. An experiment was conducted using two treatments: aerotube (AT) and air-stone (AS). The experiment lasted for 104 days, and different patterns of salinity, settling solids, nitrogenous compounds and microbial community were observed in the AT and AS tanks between days 30 and 60. The type of aeration affected the mixing intensity (i.e., turbulence) and the evaporation patterns, which caused differences in salinity and bioflocs size between the treatments. These factors also appeared to affect the nitrification process in the BFT system. The AS treatment tanks exhibited increased nitrification compared to AT treatment tanks, this observation is likely due to the formation of larger bioflocs in less saline water in the AS treatment. Temporal temperature gradient gel electrophoresis (TTGE), which was employed to characterize the bacterial community composition, identified three specific ribotypes that were found only in the AS treatment. These ribotypes probably represented nitrifying bacteria during the period of smaller salinity. In contrast, two bacterial ribotypes that were found in both treatments are likely to be related to biofloc formation.

Key words: aeration, biofloc formation, nitrification, shrimp, TTGE.

1. Introduction

Biofloc Technology (BFT) was developed simultaneously in the USA and Israel in the 1990s (Avnimelech, 1993, 1994; Sandifer and Hopkins, 1996). BFT provides several advantages, such as the reduction of water and land use, high biosecurity and the reduction of effluent discharges, compared to conventional systems (Boyd and Clay, 2002; Ray et al., 2010, 2012; Wasielesky et al., 2006). The high productivity of this system is achieved via the action of microorganisms that improve the water quality and function as a complementary food source (De Schryver et al., 2008).

The consumption of bacteria and protozoa that are present in bioflocs reduces the need for high levels of crude protein in the diets of the produced organisms and improves the feed conversion rates (Avnimelech, 2006; Burford et al., 2004; Ray et al., 2010; Van Wyk, 2006). Further, these microorganisms perform nutrient cycling within the production system, improving water quality, particularly due to the rapid assimilation and transformation of nitrogen compounds (Avnimelech, 1999, 2006; De Schryver et al., 2008; Ebeling et al., 2006; Krummenauer et al., 2010; Silva et al., 2013). However, despite the recognized importance of biofloc microorganisms, few studies have characterized the composition and activity of these microorganisms, particularly bacteria, during biofloc formation.

Aeration is a crucial factor in BFT systems because it influences the oxygen supply to the organisms and maintains the flocs in suspension. This intensive aquaculture demands higher oxygen levels to meet the requirements of the aquatic organisms that are present in high densities and support the decomposition of organic matter by bacteria (Vinatea et al., 2010). Further, aeration keeps particles in suspension and prevents thermal stratification (Boyd and Clay, 2002; Van Wyk, 2006). Aeration also influences in aggregation and disaggregation of the bioflocs depending on the mixing intensity and bubbles size (Simon et al., 2002; Serra et al., 1997; Serra and Casamitjana, 1998). However, microorganisms, especially bacteria, are the main driving force for biofloc formation. In contrast, trophic interactions between bacteria and protozoa may contribute to the disaggregation of flocs (Biddanda and Pomeroy, 1988).

In addition to these factors, the produced organisms also play a role in biofloc formation. For example, Ferreira (2008) demonstrated in a recent study that the increase in the amount of flocs was higher in tanks with the white shrimp *Litopenaeus vannamei*

than in tanks with the pink shrimp *Farfantepenaeus paulensis*. The authors observed that increase in biofloc concentration was related to a greater abundance of coccoid bacteria that producing EPS (extracellular polysaccharides substances) in the *L. vannamei* tanks. They also observed that the presence of these bacteria is likely related to the lower phosphate concentrations measured in the *L. vannamei* tanks; these reduced concentrations were generated by increased incorporation of phosphate by the white shrimp (Silva et al., 2013). Similarly, Lara et al. (in press) observed that the nitrification process exhibited contrasting patterns in tanks with different aeration systems; nitrification was more effective in the presence of the larger flocs that were found in tanks aerated by air-stones. This difference was related to the fact that nitrifying bacteria are more efficient in colonies, which can only be established in large aggregates.

Considering these previous results, we investigated bacterial microbiota responsible for biofloc formation and nitrification in a BFT system. To achieve our objective, temperature temporal gradient gel electrophoresis (TGGE) was used to characterize bacterial composition during the production of the white shrimp *L. vannamei* in a BFT system in tanks with different air diffusers (i.e., aerotubes and air-stones).

2. Methods

The experiment was conducted for 104 days at the Marine Station of Aquaculture in the Institute of Oceanography at the Federal University of Rio Grande (FURG) in a greenhouse with six rectangular 35 m^2 tanks with a useful volume of 35,000 L that were made of high density polyethylene (HDPE). The tanks were filled with sand-filtered sea water. After filling the tanks, the water was chlorinated (10 ppm) and subsequently dechlorinated with ascorbic acid (1 mg L^{-1}). The experimental design consisted of two treatments: 1) AS treatment, which used air-stones as an air distributor in the proportion of one 10 cm air-stone for each m^2, and 2) AT treatment, which used microperforated Aerotube™ air hoses as air distributors. The hoses were cut into 10 cm pieces and connected to a PVC pipe. The aerotubes were distributed in the proportion of one piece of aerotube for every 1.5 m^2 in the tanks. The treatments were performed in triplicate. Aeration was provided by a 7 HP blower for both treatments.

L. vannamei post larvae were purchased from the Aquatec^{TM} commercial laboratory (Canguaretama - Rio Grande do Norte, Brazil). One day prior to stocking the shrimp, a diatomaceous inoculation with 33×10^4 cells L^{-1} of *Conticribra weissflogii* was performed. The shrimps with an average initial weight of 0.032 g (± 0.016) were stocked at a density of 400 shrimps/m^2. Feed (i.e., a commercial diet with 38% crude protein (Guabi/Potimar 38 Active, Brazil) was supplied 3 times per day. The feeding rate ranged from 60% of the shrimp biomass at the start of the experiment to 3% of the biomass at the end of the production cycle according to the methodology proposed by Jory et al. (2001). Feeding trays were used to monitor feed consumption.

Organic fertilization was performed according to the methodology proposed by Avnimelech (1999) and Ebeling et al. (2006), which employs molasses from sugar cane as a carbon source. Weekly samplings were performed to monitor shrimp growth. At the end of the experiment, all shrimps were weighed and quantified for late survival and growth analysis. In addition, a commercial probiotic (INVE®) was applied weekly to assist in the maintenance of water quality in all treatments.

2.1. Water quality monitoring

Measurements of temperature, dissolved oxygen (DO), pH, and salinity were collected daily using an YSI 556 MPS ® multiparameter probe (YSI Incorporated, Yellow Springs, Ohio, USA). The pH and alkalinity were corrected with calcium hydroxide whenever the pH was < 7.0 and the alkalinity was ≤ 100 mg $CaCO_3$ L^{-1}. The alkalinity was measured by titration once per week, according to the method proposed by APHA (1998).

The amount of settling solids (SS) was measured using Imhoff cones, and the transparency was measured using a Secchi disk; both measurements were performed 3 times per week. The total suspended solids (TSS) were determined by gravimetry using a protocol adapted from AOAC (2000). The concentrations of ammonia (UNESCO, 1983), nitrite and nitrate (Strickland & Parsons, 1972) were measured 5, 3 and 1 time per week, respectively. Between the 57th day and the end of culture, 7 and 8 water renewals consisting 50% of the useful volume of the tank were performed in the AS and AT treatments, respectively, due to high nitrite concentrations.

2.2. Bacterial DNA extraction

The water from the tanks was sampled every 5 days in the first 2 months and every 10 days thereafter. Volumes between 30 and 50 mL of culture water were filtered through a previously sterilized and autoclaved Millipore filtration apparatus. The cells were concentrated in polycarbonate membrane filters (Nuclepore, 0.2 mm of porosity and a 2.5 mm of diameter). After filtration, each filter was placed in a sterile Eppendorf tube and frozen at -20 °C for subsequent DNA extraction and additional procedures. The DNA samples were extracted using the QIAmp DNA Stool commercial kit according to the manufacturer's instructions (Qiagen).

2.3. Polymerase chain reaction (PCR)

Two tanks from each treatment (i.e., AT tanks 7 and 10 and AS tanks 5 and 8) were sampled. PCRs were performed in duplicate for each sample to ensure the reproducibility of the results.

Nested PCR was performed using the primers 11F (5′-GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-TACC TTG TTA CGA CTT-3′) (Siripong and Rittman, 2007), which amplify nearly the entire bacterial 16S gene, in the first step and the primers GC-338F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Henriques et al., 2006), which amplify approximately 180 bp of the V3 hypervariable region within the amplicons from the first step of the PCR, in the second step. The first step was performed in a 12.5 μL reaction using the following final concentrations: 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, 20 ng μL^{-1} of BSA, 0.1 μL of 5 U μL^{-1} Platinum® Taq DNA polymerase (Invitrogen), and 1.0 μL of the DNA template. The PCR conditions were as follows: 5 min at 94 °C; 30 cycles of 5 min at 94 °C, 1 min at 52 °C and 1 min and 30 s at 72 °C; and a final extension step of 10 min at 72 °C. The second step was performed in a total volume of 50 μL using the following final concentrations: 1× reaction buffer, 0.3 mM of each dNTP, 0.3 μM of each primer, 1.0 μL of 5 U μL^{-1} Pfx 50™ DNA polymerase (Invitrogen), and 2.0 μL of DNA product containing the first-step amplicons. The PCR was conducted as follows: 5 min at 94 °C; 20 touchdown cycles (van der Gucht et al., 2007) of 1 min at 94 °C, 1 min starting at 65 °C and ending at 55 °C (decreasing 0.5 °C

each cycle), and 1 minute at 68 °C; 20 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 68 °C; and 30 min at 68 °C as a final extension step to minimize artifactual bands (Janse et al., 2004). The sizes of the PCR products were validated using 1 % agarose gel electrophoresis and quantified using the Quant-It™ dsDNA Broad-Range Assay Kit (Invitrogen).

2.4. Temperature Temporal Gradient Gel Electrophoresis (TTGE)

TTGE was performed using a DCode system (BioRad) with 14 % polyacrylamide gels (37.5:1 acrylamide:bisacrylamide), 7 M urea, and 1.25× TAE buffer. The gels were polymerized with 50 μL of TEMED and 100 μL of 10 % APS for 2 – 3 h and submitted to a prerun at 200 V for approximately 30 min. Samples with approximately 600 ng of PCR amplified DNA were loaded in a proportion of 1:1 sample:gel loading dye (70 % glycerol, 0.05 % bromophenol blue, and 0.05% xylenocyanol). The run was set to 66.0–69.7 °C at 68 V for 18.5 h with a 0.2 °C h^{-1} ramp. The gels were stained for 15 min in 1.25× TAE buffer containing 1 μg μL^{-1} of ethidium bromide and destained for 15 min in 1.25× TAE buffer. The gels were then photographed using UV transillumination with T1201 Sigma/UltraLum UltraCam Digital Imaging equipment coupled to a PowerShot A620 Canon Camera (They et al., 2013).

2.5. Analysis of TTGE images

The gel images were first edited using the D-Scan program and subsequently merged side by side into a single image for further analysis using the Gel Analyzer 2010a freeware (www.gelanalyser.com/download.html). A distance matrix was created from the raw volume obtained for each DNA band according to the methodology applied by They et al. (2013).

The construction of the distance matrix was based on Ishii et al. (2009), who established a routine for a cluster peak-based alignment algorithm for the analysis of many types of fingerprints, including data from denaturing gradient gel electrophoresis (DGGE). The routine used in this study is attached and available in the supplementary material on the electronic magazine website. The matrix was constructed based on ± 1

% position error. Prior to analysis, bands with a frequency below 10 % were excluded so the data could be transformed using Hellinger transformation (Ramette, 2007).

2.6. Statistics

For water quality parameters, such as temperature, DO, pH, salinity, nitrogenous compounds and suspended matter (i.e., TSS and Secchi Disk), the t-test was applied to verify significant differences between two independent groups. The assumptions of normality and homoscedasticity that are required for the test were verified. When these assumptions were not met, as in the case of SS, a nonparametric Mann-Whitney test was performed (Sokal and Rohlf, 1969).

3. Results

Most of the time, the water quality parameters monitored in this study exhibited no significant differences between the AT and AS treatments (Table 1). However, in the period between 30 and 70 days, the salinity was higher ($p < 0.05$) in the AT treatment tanks than the AS treatment tanks (Figure 1).

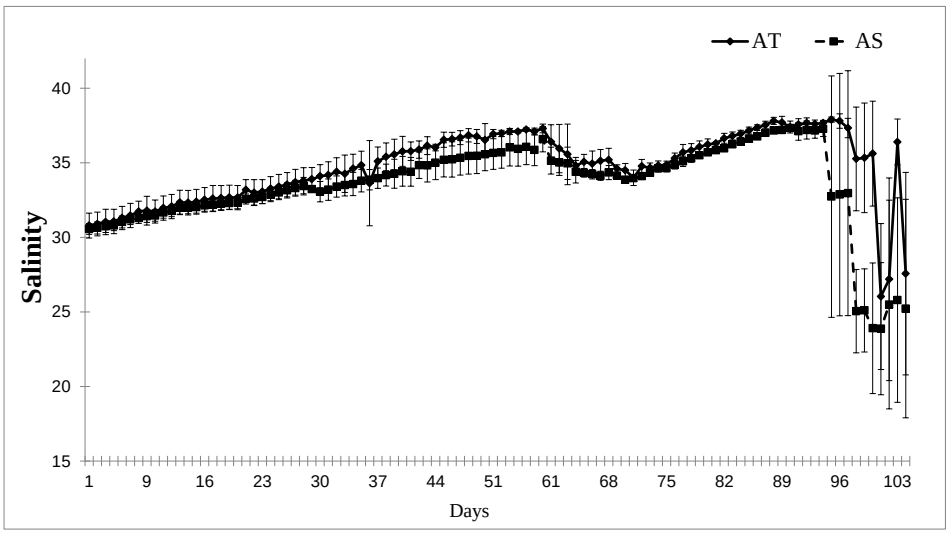

Figure 1: Mean values (± standard deviation) of salinity in the two treatments during the trial period.

Table 1: Mean values (± SD - standard deviation) and maximum and minimum values of quality water parameters in both treatments throughout the study period. DO = Dissolved oxygen, TSS = Total solids suspended, SS = Settling solids. No significant differences were observed between treatments.

	AEROTUBE (AT)			**AIR-STONE (AS)**		
	MEAN	MAX	MIN	MEAN	MAX	MIN
Temperature (°C)	27.8 ±1.81	32.8	22.8	27.7 ±1.85	32.6	22.8
DO (mg O_2 L^{-1})	5.6 ±0.69	7.1	1.9	5.4 ±0.76	7.8	1.9
pH	7.7 ±0.28	8.9	6.7	7.6 ±0.27	9.0	6.5
Alkalinity (mg L^{-1} $CaCO_3$**)**	132.9 ±34.8	235	70	136.6 ±41.2	225	70
SS (mL L^{-1})	13.9 ±8.8	40.0	0.0	16.7 ±18.3	110	0.0
TSS (mg L^{-1})	216.0 ±141	665	54.7	204.9 ±145	730	18.7
Secchi Disc (cm)	20.2 ±10.4	10.4	7.0	20.2 ±13.7	13.7	7.0

The TSS exhibited an increasing trend from the beginning of the experiment until day 42 in both treatments. There was a subsequent decrease in TSS on day 69, but after day 77, the amount of suspended material increased again and peaked on day 93 (Figure 2). The amount of suspended solids was similar in the two treatments throughout the production cycle, with no significant differences between the treatments. However, the SS exhibited different behavior between the two treatments in the period between days 35 and 60, with the AS treatment tanks exhibiting higher SS values than the AT treatment; however, this difference was not statistically significant (Figure 3).

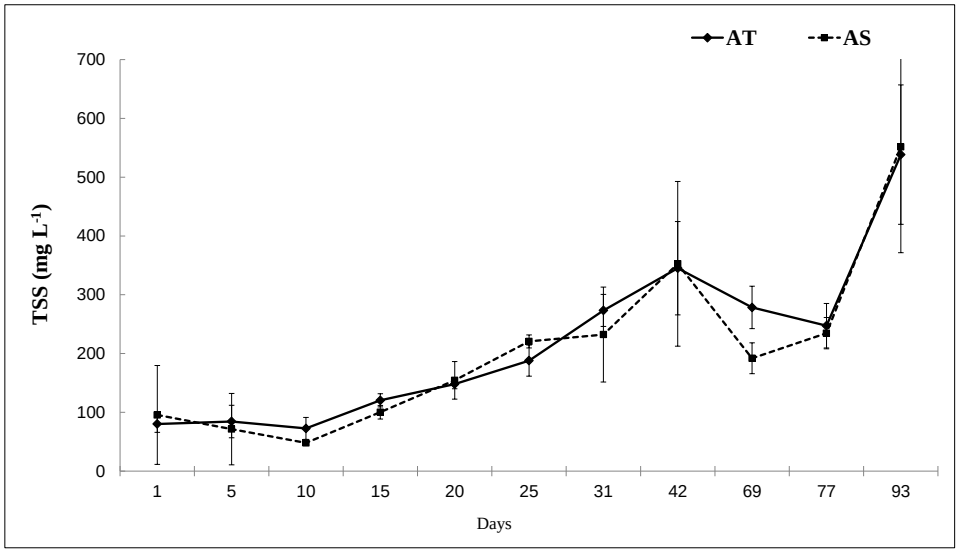

Figure 2: Mean values (± standard deviation) of TSS in the two treatments during the trial period.

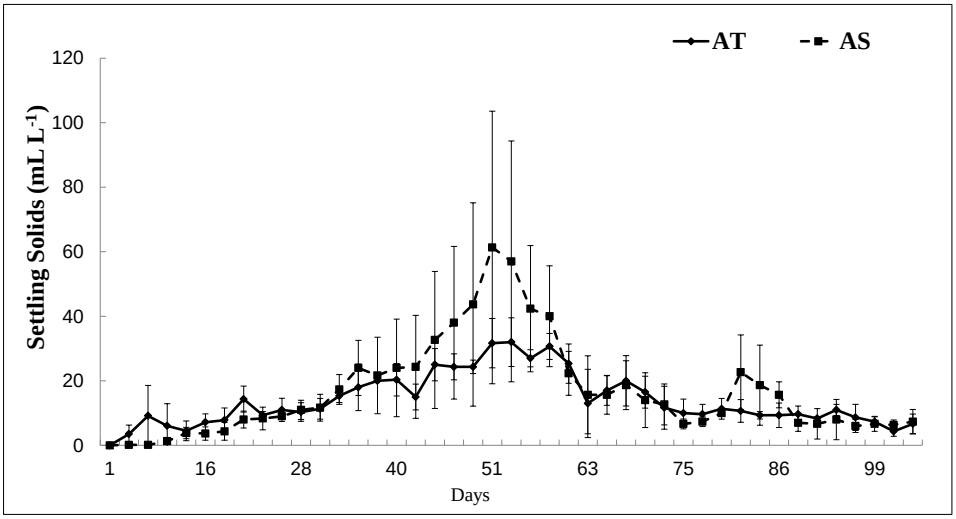

Figure 3: Mean values (± standard deviation) of settling solids in the two treatments during the trial period.

The total ammonia nitrogen (TAN) exhibited similar behavior in both treatments, with the exception of the period between days 37 and 56, when the TAN values were lower in the AS treatment than in the AT treatment. There were two peaks of TAN on days 15 and 37 of the experimental period. The TAN levels began increasing after the 8th day and reached a peak of ca. 2 mg L^{-1} near the 15th day of

study. After peaking, the levels dropped. After day 20, the TAN concentrations increased continuously, peaking at 5 mg L^{-1} on day 37. Immediately after this period, TAN decreased and after the 57th day, the concentrations remained below 1 mg L^{-1} (Figure 4).

Similar to TAN, nitrite and nitrate concentrations were similar between the two treatments. However, the AS treatment had a higher nitrite concentration in the period between days 36 and 64; this difference was not statistically significant. The nitrite concentration began to increase after 35 days of cultivation in the AS treatment tanks, coinciding with the time that the TAN began to decrease. In the AT treatment, the amount of nitrite only increased after 41 days, exhibiting a delay in the onset of nitrification. Due to the high concentrations of nitrite and the low growth of the shrimps, water renewals were needed from day 57 until the end of the crop, with a total of eight renewals in the AT treatment and seven renewals in the AS treatment. After day 57, the nitrite concentration began to decrease and subsequently oscillated without a definite pattern (Figure 5). Likewise, nitrate concentrations were similar in the two treatments, and the AS treatment had higher nitrate concentrations between days 41 and 69 of the experiment. The nitrate concentrations subsequently decreased in both treatments and remained below 10 mg L^{-1} (Figure 6).

The average weight of the shrimps after 104 days was 10 g in both treatments. The shrimps grew at an average rate of 0.63 g $week^{-1}$ in the first 35 days of cultivation. After this period, there was a decline in growth between days 42 and 63, when the rate decreased to 0.44 and 0.53 g $week^{-1}$ in the AT and AS treatments, respectively, due to high concentrations of nitrogenous compounds. After water renewals on day 70, the shrimps resumed growth of approximately 1 g $week^{-1}$ until the end of cultivation (Table 2).

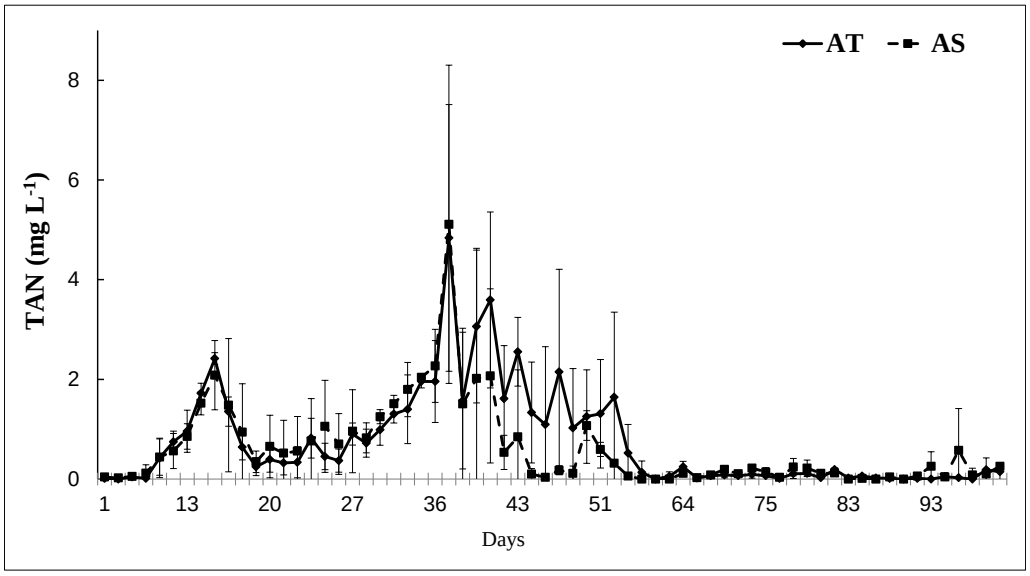

Figure 4: Mean values (± standard deviation) of total ammonia concentration (TAN) in the two treatments during the trial period.

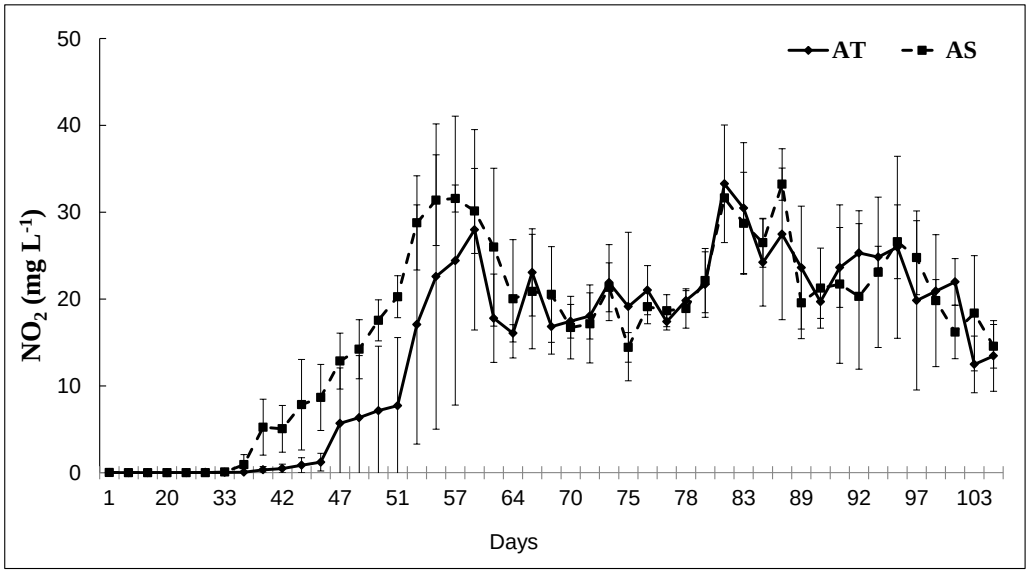

Figure 5: Mean values (± standard deviation) of nitrite concentration in the two treatments during the trial period.

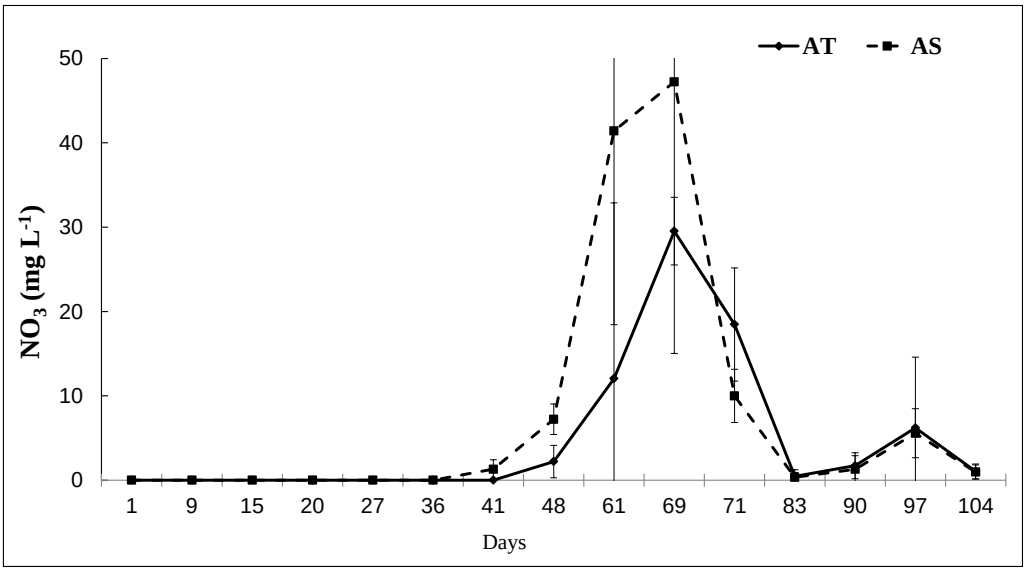

Figure 6: Mean values (± standard deviation) of nitrate concentration in the two treatments during the trial period.

Table 2: Mean values (± SD - standard deviation) of initial and final weight, survival, weekly weight gain, specific growth rate (SGR), feed conversion ratio (FCR) and productivity. No significant differences were observed between treatments.

	AEROTUBE (AT)	AIR-STONE (AS)
Initial weight (g)	0.032 (±0.02)	0.032 (±0.02)
Final weight (g)	10.50 (± 2.2)	10.96 (±2.3)
Survival (%)	89.7 (± 2.08)	86.7 (± 2.31)
Weight Gain (g $week^{-1}$)	0.75	0.78
SGR (% day^{-1})	5.56 (± 0.02)	5.60 (± 0.05)
FCR	1.25 (± 0.02)	1.32 (± 0.05)
Productivity (g m^{-2} day^{-1})	36.22 (± 0.51)	36.36 (± 1.85)
Productivity (kg $tank^{-1}$ day^{-1})	1.27 (± 0.02)	1.27 (± 0.06)

3.1. Bacterial diversity characterized by TTGE

The bacterial community composition changed during the trial period in both treatments. For the sake of simplicity, we present the TTGE results for water collected at the beginning of the experiment (i.e., day 5), during maximum biofloc formation (i.e., days 31 and 41) and near the end of the experiment (i.e., day 78) (Figures 7 and 8). The bands found in the samples collected on days 31, 41 and 78 also represent the period when the biggest differences in nitrogen compounds were observed between the treatments.

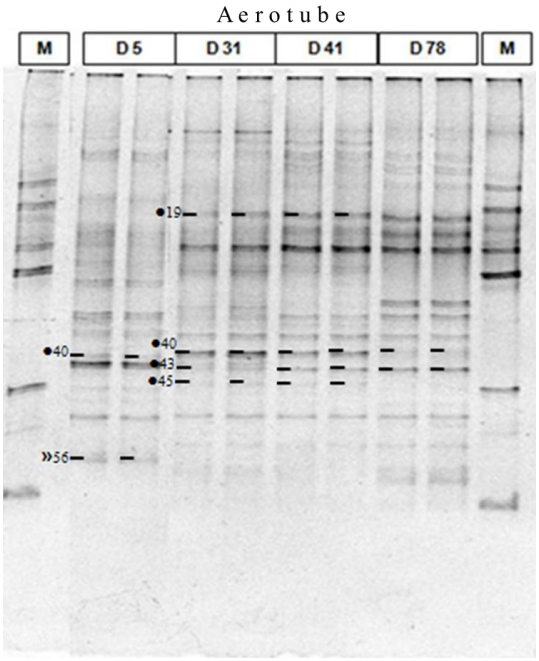

Figure 7: Composition of the bacterial community, as indicated by TTGE, in the AT treatment (Aerotube). (M) Mix of isolates. Samples from days 5 (D5), 31 (D31), 41 (D41) and 78 (D78) are displayed.

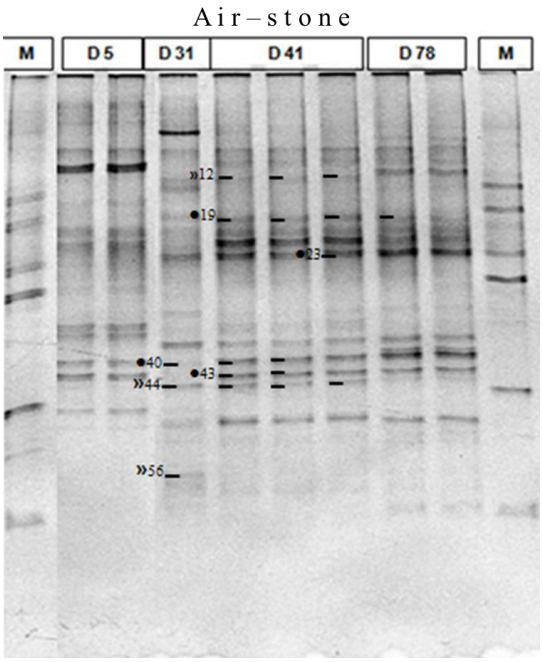

Figure 8: Composition of the bacterial community, as indicated by TTGE, in the AS treatment (Air-stone). (M) Mix of isolates. Samples from days 5 (D5), 31 (D31), 41 (D41) and 78 (D78) are displayed.

With respect to band composition, both treatments had a similar number of exclusive bands in the initial and biofloc formation periods. However, during biofloc formation, the two treatments had the highest number of shared bands. This result demonstrates that ribotype richness increased along with TSS in both treatments and suggests that the shared bands observed on days 31 and 41 may be responsible for biofloc production. At 78 days of culture, there was a reduction in the richness of bacterial ribotypes. The number of exclusive bands decreased from the initial period in the AT treatment but remained constant at 8 bands in the AS treatment. In addition, the number of bands shared between the treatments dropped to 8. Among the shared bands that were present in both treatments during the period of biofloc formation, bands 40 and 45 attained a high intensity in this period and a lower intensity on day 78 after water renewals and the subsequent reduction of TSS.

In the AT treatment, the raw volume of band 40 followed the behavior of the suspended material. It increased by 2.2 times compared to the initial period between days 31 and 41, which coincides with the observed increase in suspended material, and decreased after the water renewal, when a drop in suspended material was also observed. In the AS treatment, band 40 was only present during biofloc formation and exhibited a high raw volume. Band 45 also only appeared during biofloc formation in both treatments and disappeared on day 78 (Figures 7 and 8).

Between days 31 and 78, when the greatest difference in nitrogen compounds was observed between the two treatments, bands 12, 44 and 56 were only present in the AS treatment and absent in the AT treatment.

Table 3: The principal bands that had a frequency of at least 10 % and contributed to biofloc formation and nitrification during the production cycle. The band intensity values obtained after Hellinger transformation are presented. In bold, bands related to the biofloc formation. Underline, bands related to the nitrification process. Cloud, shared bands between the AT and AS treatments in each experimental period (D5, D31 and D41, D78).

	D5		D31		D41		D78	
Bands	**AT**	**AS**	**AT**	**AS**	**AT**	**AS**	**AT**	**AS**
3	0.1519	0.2682	0	0.2444	0	0	0	0
7	0.1885	0	0.1392	0	0.1557	0.1432	0	0.1912
8	0	0.1523	0.1297	0.1435	0.0408	0.1470	0.0594	0.1641
9	0.2470	0	0.1288	0	0.0849	0	0.2258	0.1356
10	0.0698	0.2009	0	0	0.2014	0.1343	0	0.1297
12	0	0	0	0.1576	0	0.3804	0	0
19	0	0	0.1409	0	0.1639	0.1785	0.0800	0.0899
22	0	0.2670	0	0.0490	0	0.2671	0.0574	0.2421
23	0	0	0	0	**0.2399**	**0.3123**	0	0
30	0.2480	0	0	0	0.2089	0983	0	0
33	0	0	0.1606	0.2026	0	0	0.2038	0.1904
35	0.1793	0.1480	0.1502	0	0.1559	0	0.1540	0
39	0	0.2420	0.1674	0	0.0568	0.1698	0	0.2040
40	0.1038	0	**0.1573**	**0.2644**	**0.1874**	**0.0812**	0.1040	0
42	0.1918	0.1515	0.0715	0	0.1170	0.0775	0	0.2060
43	0	0	0.0636	0.1368	0.2721	0.1302	0.1359	0.0650
44	0	0	0	0.2321	0	0.2351	0	0
45	0.1839	0	**0.1437**	**0.1571**	**0.1304**	**0.1692**	0	0
49	0	0.1321	0	0	0	0.1893	0.0815	0.2001
50	0.1525	0.1560	0.1193	0.1704	0.1224	0.0577	0.1357	0.0648
56	0.2292	0	0	0.2793	0	0.1816	0	0

4. Discussion

In this study, the water quality parameters and zootechnical indices measured in the two treatments were similar and satisfactory for the production of *L. vannamei* in BFT systems (Van Wyk and Scarpa, 1999; Cohen et al., 2005) (Table 2). In addition, both treatments exhibited similar patterns of biofloc formation, with an increase in TSS starting on day 10, an initial peak on day 42, a subsequent decrease due to water renewal, and a second peak on day 93 (Figure 2).

Floc formation depends on biological, physical and chemical interactions that occur simultaneously in the aquatic environment. Among the physical and chemical factors related to microbial aggregation processes, Brownian motion, turbulence, van der Waals forces, electric potential difference between the particles and differential sedimentation of particles play an important role (Hietanen, 1998; De Schryver et al., 2008). However, biofloc formation is dependent on the action of microorganisms, particularly bacteria. According to Biddanda and Pomeroy (1988), aggregate formation is initiated by an increase in bacterial abundance and mucus (i.e., extracellular polysaccharide) production by these microorganisms. In contrast, disaggregation is caused by the consumption of mucus-producing bacteria by protozoa, which shifts the aggregate structure.

In this study, the results for total suspended solids (TSS) demonstrated that biofloc formation was similar in both treatments, indicating that the different aeration systems employed in the two treatments did not affect particle aggregation in different ways. In addition, the TTGE results clearly demonstrated that a number of bacterial species were present in both treatments, particularly during the period of higher biofloc formation.

The occurrence of mucus-producing bacteria is recognized as the main factor responsible for floc formation. For example, Grossart et al. (2006) used DGGE to demonstrate that bacteria attached to aggregates were phylogenetically different from free-living bacteria and could be characterized into two different functional groups. In addition, Gardes et al. (2010) demonstrated the importance of attached bacteria to phytoplankton aggregation. The production of transparent exopolymer particles (TEP) by bacteria is a basic requirement for aggregation, and these particles act as a biogenic glue that immobilizes cells in multicellular aggregates (Vlaeminck et al., 2010).

Moreover, Ferreira (2008) demonstrated that mucus-producing bacteria, which are present in greater abundance in tanks with *L. vannamei*, were responsible for higher loads of flocs in these systems than in tanks in which *F. paulensis* was grown.

It is worth noting that ribotypes 40 and 45 were detected with increased intensity during the period of high biofloc formation (i.e., on days 31 and 41) by the TTGE analysis in both treatments, indicating an increased abundance of these species during this period. These bands nearly disappeared on day 78, coinciding with the decreased TSS values observed after a small water renewal.

To confirm the identity and functionality of these biofloc-forming bacteria, a genome sequence analysis of these specific bands will be conducted in the near future. In addition to species identification, genome characterization will facilitate the creation of probes for fluorescent *in situ* hybridization (FISH) analysis. This method will make it possible to visualize and enumerate these specific bacteria and conduct further autecology studies of these species, in a manner similar to that employed in the study of probiotics present in the tilapia gut that was conducted by Del Duca et al. (2013). This genome analysis will also allow further isolation, identification and culture of these bacterial species, which will be used to accelerate biofloc formation in BFT systems.

Despite the similarity of biofloc formation in both treatments, a difference in settling solids was observed between the AT and AS tanks from days 30 to 60. During this period, the treatment with aerotubes (AT) exhibited higher salinity. This observation is likely due to the higher evaporation caused by the small air bubbles. On the other hand the AS treatment exhibited lower salinity and higher settling solids indicating an increase in the size of the bioflocs. Pronk et al. (2013) evaluated the effects of salinity on aerobic granular sludge and found that the structure of flocs was stable but that an increase in salt concentrations (20 g Cl^{-} L^{-1}) resulted in flocs of smaller size. The authors attributed this effect to the fragility of the mucus produced by the attached bacteria under high salinity. Similar results were also reported by Ismail et al. (2010), who associated the small flocs with the lack of bacterial extracellular polymers in conditions of high salinity.

Besides this difference in biofloc size during the period from 30 and 60 days the AS treatment also exhibited a lower ammonium concentration and higher levels of nitrite and nitrate, indicating a superior nitrification process. Nitrifying bacteria from

marine environments require more time to complete the transformation of ammonium into nitrate because these bacteria have lower rates of nitrification than freshwater bacteria. In environments with changing salinity, the ammonia-oxidizing bacteria are affected but can be reestablished after a period of adaptation to the new salinity conditions. Nitrite-oxidizing bacteria are more sensitive and take longer to become established in marine waters (Nijhof and Bovendeur, 1990).

In addition, ammonium- and nitrite-oxidizing bacteria are present in aggregates that form colonies (Delatolla et al., 2009; Vlaeminck et al., 2010), and the establishment of these colonies may be influenced by particle size. Larsen et al. (2008) demonstrated that the increase in mixing rate (i.e., shear) in systems with activated sludge generated smaller particles, leading to lower rates of ammonium oxidation. Thus, more nitrification occurred in the AS treatment between days 30 and 60 of the experiment. This result is likely due to the presence of larger flocs in this treatment, which facilitated the establishment of colonial nitrifying bacteria in the larger flocs, similar to the observations of Lara et al. (in press) in another study testing the effect of different aerators.

The presence of specific bands (i.e., bands 12, 44 and 56) only in the air-stone (AS) treatment during the phase of higher nitrification is an indication that colony-forming nitrifying bacteria were present in this treatment due to the presence of a larger floc size.

As described for biofloc-forming bacteria, the bands found in the AS treatment that are likely to be related to nitrifying bacteria will be sequenced and identified. In the future, the isolation and culture of these bacterial species will generate important probiotic microorganisms that can be used in the control of ammonium and nitrite concentrations in BFT systems.

5. Conclusions

Aeration generated different mixing intensities (i.e., turbulence) in the water but failed to interfere in biofloc formation in treatments with aerotubes and air-stones. In both cases, aggregate-forming bacteria appear to act in biofloc formation in similar ways. However, tanks with air-stones had lower salinity and larger flocs. The observed 1 ppm difference in salt content was sufficient to cause differences in nitrification, with higher nitrogen transformation in tanks with larger flocs. The use of the fingerprint molecular biology technique (TTGE) proved to be a powerful tool in the characterization of bacterial composition in the BFT system.

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