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IMPORTANCIA DE LOS MICROORGANISMOS EN LA PRODUCCIÓN DE LANGOSTINOS MARINOS EN SISTEMAS DE BIOFLÓCULOS Y SIN RENOVACIÓN DE AGUA

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Resumen

Los microorganismos son esenciales para poder llevar a cabo la producción intensiva de langostinos marinos, ya que son los encargados de mantener el agua con una calidad óptima para los animales. Además, los microorganismos permiten minimizar o eliminar las necesidades de realizar cambios de agua. Entre todos los microorganismos presentes en los sistemas intensivos, sin renovación de agua, las bacterias y las microalgas son los más abundantes y los que tienen un papel más importante en el sistema de producción.

En esta tesis se profundiza en el conocimiento de la dinámica de las microalgas presentes en un sistema de preengorde y en un sistema de bioflóculos, para el cultivo intensivo de langostinos marinos, utilizando la metodología HPLC/CHEMTAX. Además, se testa si la bacteria *Bacillus amyloliquefaciens* puede tener un papel probiótico en los sistemas de bioflóculos y si es capaz de mejorar el proceso de maduración del sistema.

Los resultados obtenidos han determinado que la metodología HPLC/CHEMTAX es una forma rápida y eficiente para analizar el fitoplancton y el perifiton en los sistemas intensivos de langostinos, siempre que predominen los procesos autotróficos. Esta técnica, ha permitido detectar por primera vez prasinofíceas y primnesiofíceas en el perifiton que crece sobre los tanques de policloruro de vinilo (PVC). También se ha observado la influencia del estado trófico del sistema y la exposición a la luz sobre la dinámica de las microalgas en los sistemas de bioflóculos.

Por lo que respecta a la aplicación de *Bacillus amyloliquefacien* a los sistemas de bioflóculos, esta implica un refuerzo del sistema inmunológico de los langostinos, sumando sus efectos a los producidos por los bioflóculos. Estos beneficios, ocurren incluso al aplicar dosis del orden de 10^3 ufc/mL, siendo esta menor a la recomendada para otras bacterias probióticas. A pesar de que los resultados de otras investigaciones apuntaban que *Bacillus amyloliquefaciens* podría participar en la formación de bioflóculos, la mejora de la calidad del agua y el crecimiento de los langostinos, la aplicación de esporas sobre el agua no mostró efectos estadísticamente significativos sobre la dinámica del sistema, la calidad del agua o los parámetros zootécnicos.

Resum

Els microorganismes són essencials per a poder dur a terme la producció intensiva de llagostins marins, ja que són els encarregats de mantenir l'aigua amb una qualitat òptima per als animals. A més a més, els microorganismes permeten minimitzar o eliminar les necessitats de realitzar canvis d'aigua. Entre tots els microorganismes presents en els sistemes intensius, sense renovació d'aigua, les bactèries i les microalgues són les més abundants i les que tenen un paper mes important en el sistema de producció.

En aquesta tesis es profunditza en el coneixement de la dinàmica de les microalgues presents als sistemes de preengreixament i en un sistema de bioflòculs, per al cultiu intensiu de llagostins marins, utilitzant la metodologia HPLC/CHEMTAX. A més a més, es prova si la bactèria *Bacillus amyloliquefaciens* pot tenir un paper probiòtic en els sistemes de bioflòculs i si es capaç de millorar el procés de maduració del sistema.

Els resultats obtinguts han determinat que la metodologia HPLC/CHEMTAX és una forma ràpida i eficient per a analitzar el fitoplàncton i el perifíton als sistemes intensius de llagostins, sempre que predominen els processos autotròfics. Aquesta tècnica, ha permès detectar per primera vegada prasinofícies i primnesiofícies al perifíton que creix sobre els tancs de policlorur de vinil (PVC). També s'ha observat l'influencia del estat tròfic del sistema i l'exposició a la llum sobre la dinàmica de les microalgues als sistemes de bioflòculs.

Pel que respecta a l'aplicació de *Bacillus amyloliquefaciens* en els sistemes de bioflòculs, aquesta implica un reforç del sistema immunològic dels llagostins, sumant els seus efectes als produïts pels bioflòculs. Aquestos beneficis, permaneixer al aplicar dosis de l'ordre de 10^3 ufc/mL, sent aquesta menor a la recomanada per a altres bactèries probiòtiques. Encara que els resultats d'altres investigacions apunten que *Bacillus amyloliquefaciens* podria participar en la formació de bioflòculs, la millora de la qualitat de l'aigua i el creixement dels llagostins, l'aplicació d'espores a la columna d'aigua no va mostrar ningún efecte estadísticament significant sobre la dinàmica del sistema, la qualitat de l'aigua o els paràmetres zootècnics.

Abstract

Microorganisms are essential for the intensive production of marine shrimps, since they are responsible for maintaining the water with an optimum quality for them. Also, these microorganisms minimize or eliminate water renewal. Among all the microorganisms present in intensive culture systems, without water renewal, microalgae and bacteria play the most important role and are the most abundant ones.

In this thesis we analyze the microalgae dynamics using HPLC/CHEMTAX methodology in two intensive culture systems of marine shrimp, a shrimp nursery and a biofloc system. We also test the role of the bacteria *Bacillus amyloliquefaciens* as a probiotic and its capacity to improve the maturation process of the biofloc system.

The results showed that the HPLC/CHEMTAX methodology is a fast and efficient way to analyze phytoplankton and periphyton in intensive culture systems of shrimp, provided that autotrophic processes are predominant. This technique allowed to detect prasinophytes and prymnesiophytes for the first time in the periphyton that grows on the polyvinyl chloride (PVC) tanks. Also, it has allowed to study the influence of trophic state and light exposition on microalgae dynamic in the biofloc systems.

The application of *Bacillus amyloliquefaciens* in biofloc systems strengthens the immune system of shrimp and adds its probiotic effects to that produced by bioflocs. These benefits happen even with doses of 10^3 cfu/mL, lower than recommended doses for other probiotic bacteria. Other researches indicated that *Bacillus amyloliquefaciens* might have other effects such as participating in the biofloc system formation, improving water quality and shrimp growth; however, the application of spores in the culture water did not show any statistically significant effect on the system dynamic, water quality or zootechnic parameters.

Índice

Capítulo 1. Introducción **1**

1.1. Estado general de la acuicultura 2

1.2. La acuicultura en la Unión Europea y España 3

1.3. Tipología de los sistemas de cultivo 5

1.4. Sistemas de bioflóculos 6

1.5. Las microalgas en los cultivos de langostinos 8

1.6. Las bacterias probióticas en los cultivos de langostinos 10

1.7. Bibliografía 12

Capítulo 2. Objetivos generales y organización de la memoria **19**

Capítulo 3. Periphyton and phytoplankton assessment in a shrimp nursery: signature pigments analysis 24

3.1. Abstract 25

3.2. Introduction 25

3.*3. Material and methods* *27*

3.3.1. Location and shrimp cultura system *27*

3.3.2. Water quality *28*

3.3.3. Biological parameters *28*

3.3.4. Statical analysis *29*

3.4. Results and discussion 30

3.5. Conclusion 39

3.6. Acknowledgements 40

3.7. References 40

Capítulo 4. Phytoplankton evolution during the creation of a biofloc system for shrimp cultura **46**

4.1. Abstract 47

4.2. Introduction 48

4.3. Material and methods 50

4.3.1. Shrimp *50*

4.3.2. Environmental parameters *51*

4.3.3. Biological parameters *51*

4.3.4. Statical analysis *54*

4.4. Results 54

4.5. Discussion 62
4.6. Conclusion 65
4.7. Acknowledgements 66
4.8. References 66

Capítulo 5. Application of *Bacillus amyloliquefaciens* as probiotic for *Litopenaeus vannamei* (Boone) cultivated in a biofloc system 73

5.1. Abstract 74
5.2. Introduction 75
5.3. Material and methods 77
5.3.1. Shrimp 77
5.3.2. Water quality 78
5.3.3. Growth parameters 78
5.3.4. Immunologic parameters 79
5.3.5. Statical analysis 80
5.4. Results 80
5.4.1. Water quality 80
5.4.2. Growth parameters 83
5.4.3. Immunological system 84
5.5. Discussion 85
5.6. Conclusion 88
5.7. Acknowledgements 88
5.8. References 88

Capítulo 6. The role of *Bacillus amyloliquefaciens* on *Litopenaeus vannamei* during the maturation of a biofloc system 93

6.1. Abstract 94
6.2. Introduction 94
6.3. Material and methods 96
6.3.1. Location and shrimp cultura system 96
6.3.2. Probiotic treatments 98
6.3.3. Chlorophyll a and microbial avtivity 98
6.3.4. Zootechnical performance 99
6.3.5. Immunologic parameters 99
6.3.6. Statistical analysis 100

6.4. Results 100
6.4.1. Water quality *100*
6.4.2. Microbial activity *106*
6.4.3. Zootechnical performance *108*
6.4.4. Immune system parameters *109*
6.5. Discussion 110
6.5.1. Effects on the biofloc system *110*
6.5.2. Effects on shrimps *113*
6.5. Conclusion 115
6.6. Acknowledgements 115
6.7. References 116

Capítulo 7. Discusión general 122

7.1. El rol del fitoplancton en los cultivos de langostinos sin renovación de agua 123
7.2. El rol del perifiton en los cultivos de langostinos sin renovaicón de agua 125
7.3. El HPLC/CHEMTAX como metodología para el estudio de las microalgas 126
7.4. El rol de *Bacillus amyloliquefaciens* en los sistemas de bioflóculos 127
7.4.1. Efecto de Bacillus amyloliquefaciens sobre el sistema de bioflóculos *127*
7.4.2. Implicaciones de Bacillus amyloliquefaciens para los langostinos *127*
7.4.1. Manejo de Bacillus amyloliquefaciens en los sistemas de bioflóculos *129*
7.5. Bibliografía 129

Capítulo 8. Conclusiones generales y futuras líneas de investigación 135
8.1. Conclusiones generales 136
8.2. Futuras líneas de investigación 138

CAPÍTULO 1

Introducción

1.1. ESTADO GENERAL DE LA ACUICULTURA

Desde la mitad del siglo XX, los mercados mundiales ponen a la disposición de los consumidores una mayor cantidad de pescados y mariscos procedentes de la pesca y la acuicultura (FAO 2016). El incremento progresivo de la producción de pescados y mariscos ha permitido satisfacer las necesidades de los mercados internacionales, derivadas del aumento del consumo por habitante a nivel mundial y la demanda producida por el incremento de la población (FAO 2016). Hasta finales del siglo XX, el aumento de la presión pesquera era la forma en la que el mercado satisfacía la demanda de pescados y mariscos, pero desde finales de siglo, se observa un estancamiento en las capturas y un aumento progresivo de la producción acuícola para satisfacer esta demanda mundial (Luchini y Panné-Huidobro 2008; APROMAR 2017).

A nivel mundial, los organismos acuáticos mas cultivados son los peces, seguidos por las algas y los crustáceos (FAO 2016). Aunque la acuicultura esta presente en todos los continentes habitados, Asia lidera la producción mundial (Luchini y Panné-Huidobro 2008; FAO 2016). Por lo que respecta a la acuicultura de los crustáceos (o carcinocultura), esta se basa principalemente en el cultivo de *Litopenaeus vannamei*, y se localiza principalmente en el continente Americano y el Asiático (Anderson et al. 2017), desde donde se exporta al resto del mundo.

Actualmente, existe una tendencia mundial a mejorar la sostenibilidad de los sistemas de producción de los alimentos, entre los que se encuentra la acuicultura (FAO 2016). Por ello, los sistemas acuícolas están siendo adaptados mediante el desarrollo de nuevas técnicas de producción y de manejo, con el objetivo de reducir el impacto de estas actividades sobre el medio ambiente y aumentar su productividad. En cuanto a la carcinocultura existe una apuesta clara, por parte de los productores, para establecer sistemas de producción de langostinos más respetuosos con el medio ambiente y más bioseguros, mediante la minimización de la renovación de agua y el aumento de las densidades de siembra (Wasielesky et al. 2006; Crab et al. 2012). Dentro de este marco, los sistemas de biofióculos pueden jugar un papel muy importante, debido a que permiten aumentar la densidad de cultivo, mejoraran la bioseguridad y reducen o eliminan la necesidad de realizar cambios de agua, minimizando los impactos ambientales respecto a los sistemas de cultivo convencionales (Crab et al. 2012).

1.2. LA ACUICULTURA EN LA UNIÓN EUROPEA Y ESPAÑA

Los habitantes de la Unión Europea tienen una de las tasas de consumo, de pescados y mariscos, más altas a nivel mundial. Además, son los que realizan el mayor gasto por habitante en estos productos. Estas características hacen de la Unión Europea uno de los principales mercados mundiales de pescados y mariscos (Román y Castillo 2012; EUMOFA 2017). Actualmente, la Unión Europea no tiene la capacidad de producir todo el pescado y marisco que consume, por ese motivo necesita importar en torno al 54% del pescado y marisco que demanda (Anderson et al. 2017; EUMOFA 2017). Entre las importaciones que realizaron los estados miembros de la Unión Europea en el año 2016, los langostinos fueron el producto acuícola más importado con 614.733 toneladas, por un valor de 4.700 millones de euros (EUMOFA 2017). Las importaciones de langostinos, proceden en su mayoría de América, ya que la pesca y la acuicultura europeas, solo proporcionan el 8% de los langostinos que consumen sus habitantes (EUMOFA 2017). Del total de las importaciones de langostinos, una cuarta parte se destinaron para satisfacer la demanda del mercado español, siendo este el principal consumidor de langostinos de la Unión Europea (Román y Castillo 2012; EUMOFA 2017).

El sector de la acuicultura en España está dedicado fundamentalmente a la producción de peces, al igual que en el resto de la Unión Europea, siendo los cultivos de langostinos poco representativos respecto al total (APROMAR 2017; EUMOFA 2017). Los primeros cultivos de langostinos llegaron a España de la mano del investigador Sant Feliu durante los años 60, y se realizaron en el Centro Superior de Investigaciones Científicas (CSIC) de Torre la Sal (Cabanes, Castelló, Comunitat Valenciana), donde se estudió la viabilidad de criar langostinos autóctonos *Melicertus kerathurus* (Mas-Álvarez y Tiana-Mariscal 1986). Las primeras producciones comerciales se establecieron fundamentalmente en Andalucía durante los años posteriores, donde se cultivo el langostino tigre *Marsupenaeus japonicus,* el cual cuenta con una apariencia similar al *M. kerathurus*, pero su cultivo es más productivo (Mas-Álvarez y Tiana-Mariscal 1986; Rodríguez 1999). La mayor parte de las producciones de langostinos se realizaban en estanques de tierra, situados en las marismas, donde se les alimentaba con piensos comerciales (Rodríguez 1999). La expansión de los cultivos de langostinos incentivó la producción de post larvas por parte de los laboratorios españoles, cerrando el ciclo productivo del langostino tigre (Rodríguez 1999). La mayoría de las granjas tenían pequeñas producciones de langostinos, los cuales se comercializaban a nivel local (Comunicación

personal). Solamente la empresa ACUINOVA (Ayamonte, Huelva, Andalucía), llegó a establecer una granja con una producción elevada de langostinos *M. japonicus*, mediante su cultivo en grandes tanques interiores (Rodríguez 1999). La posibilidad de cultivar especies más rentables en las marismas y el cierre de la granja de Ayamonte, han provocado la caída de la producción de *M. japonicus* y el cese de la producción de post larvas en territorio español (Zurita et al. 2014). Durante los años 2014 y 2015, solamente se registró una única empresa que cultivó *M. japonicus*, situada en la provincia de Cádiz (Andalucía), la cual sembró post larvas procedentes de laboratorios de otros estados de la Unión Europea. La producción de langostino tigre durante los últimos años es tan escasa, que solamente se produjeron 434 y 610 kg durante los años 2014 y 2015 respectivamente (Zurita et al. 2015, 2016).

Los sistemas de cultivo tradicionales en el resto de estados de la Unión Europea, tenían las mismas características que los cultivos españoles (baja densidad, con alimentación natural o parcialmente suplementada con piensos). En países como Chipre, Italia, Francia y Grecia también existían cultivos de langostinos para el mercado local, donde se cultivaban tanto *M. japonicus* como *M. kerathurus* (Comunicación personal).

En la actualidad, se han establecido dos empresas dedicadas a la producción de langostinos blancos *L. vannamei* en territorio español (APROMAR 2017). Estas empresas utilizan los sistemas de bioflóculos para la producción intensiva de langostinos blancos, procedentes de laboratorios en Estados Unidos de América. Los sistemas de bioflóculos les permiten reutilizar el agua y no depender de una entrada de agua salada constante, por ese motivo ninguna de las dos instalaciones dispone de puntos de captación de agua salada.

La aparición de nuevos proyectos para la producción de langostinos *L. vannamei* no es exclusiva de España. En otros lugares de la Unión Europea también proliferan proyectos para cultivar langostinos de forma intensiva, mediante sistemas de biofloculos o sistemas de recirculación. Hoy en día, existen diferentes granjas en Alemania, Bélgica y Letonia, dedicadas a la producción intensiva de langostino blanco. Además, existen proyectos en Italia, Bulgaria, España y Grecia para el desarrollo de nuevas granjas, todas ellas basadas en el cultivo intensivo de *L. vannamei.*

1.3. TIPOLOGÍA DE LOS SISTEMAS DE CULTIVO

Las granjas de langostinos pueden desarrollar diferentes sistemas y técnicas de manejo para optimizar su producción. El proceso de producción de langostinos puede dividirse en tres etapas: larvicultura, preengorde y engorde (Valverde-Moya y Alfaro-Montoya 2015). La larvicultura, suele realizarse en laboratorios específicos dedicados a la producción de poslarvas. En estas instalaciones los animales pasan sus primeros días de vida, para finalmente ser trasladadas a las granjas de engorde cuando tienen entre 20 y 45 días (FAO 2004). Las granjas de engorde pueden realizar el cultivo en una etapa o en dos (preengorde y engorde). Si se utiliza una única etapa de cultivo, las post larvas se siembran directamente sobre los tanques de cultivo, donde permanecen hasta el momento de la despesca. En cambio, si se opta por realizar un preengorde, se utilizan unos tanques intermedios donde se cultivan las post larvas en altas densidades, hasta que alcanzan entorno a 1 gramo, momento en el que son trasladados a los tanques de engorde (Barbieri y Ostrensky 2002). La producción por etapas permite aumentar la productividad y optimizar tanton los espacios como los recursos (Valverde-Moya y Alfaro-Montoya 2015).

Los sistemas de producción también pueden ser clasificados en función de la densidad de cultivo en extensivos, semintensivos, intensivos y superintensivos (Crespi y New 2009). Para cada especie de langostinos, se utiliza una desnsidad de siembra diferente en función de su biología y su comportamiento. Los requerimientos de espacio, aireación, alimentación, etc. cambian en función de la densidad y la especie con la que se trabaje. La tabla 1.3 muestra las densidades de siembra de los sistemas extensivos, semiintensivos, intensivos y superintensivos para el cultivo de *L. vannamei* y *M. japonicus* (Coman et al. 2004; Crespi y New 2009).

Tabla 1.3. Densidades cultivo para *L. vannamei* y *M. japonicus* en los sistemas extensivos, semiintensivos, intensivos y superintensivos.

Tipo de cultivo	*Litopenaeus vannamei*	*Marsupenaeus japonicus*
Extensivo	4 – 10 langostinos/m^2	1 – 3 langostinos/m^2
Semiintensivo	10 – 30 langostinos/m^2	10 langostinos/m^2
Intensivo	30 – 300 langostinos/m^2	50 langostinos/m^2
Superintensivo	300 – 450 langostinos/m^2	160 langostinos/m^2

Fuente: Tabla elaborada con los datos de Coman et al. (2004) y Crespi y New (2009).

1.4. SISTEMAS DE BIOFLÓCULOS

Entre los sistemas utilizados para la producción de langostinos, el sistema convencional es el más utilizado y el que causa un mayor impacto ambiental. Estos sistemas utilizan estanques de grandes dimensiones, donde se cultiuvan langostinos en densidades de 5 a 30 langostinos/m^2 (Barbiery y Ostrensky 2002). Para mantener la calidad de agua en unos valores óptimos, los productores necesitan abastecerse de agua limpia para poder realizar cambios periódicos del agua de cultivo (Barbiery y Ostrensky 2002). Por el contrario, los sistemas de bioflóculos facilitan la producción intensiva y superintensiva (200 – 500 langostinos/m^2), minimizando o eliminando las necesidades de realizar recambios de agua durante todo el cultivo (Crab et al. 2012; Emerenciano et al. 2013b). Mientras en los sistemas convencionales se pueden obtener entre 1 y 3 toneladas por hectárea en cada ciclo (Whetstone et al. 2002), los sistemas de bioflóculos pueden producir entre 20 y 50 toneladas por hectárea (Krummenauer et al. 2011). También existe una gran diferencia en cuanto al consumo de agua de ambos sistemas. Los cultivos convencionales requieren entre 20000 y 64000 L de agua para producir 1 kg de langostinos, mientras que los sistemas de bioflóculos necesitan entre 100 y 169 L para obtener la misma cantidad de langostinos (Krummenauer et al. 2014). Además, el sistema de bioflóculos permite utilizar el agua de un cultivo para el siguiente (Crab et al. 2012).

En los sistemas de bioflóculos se favorece la proliferación de una compleja red de microorganismos dominada por bacterias heterotróficas y microalgas, con el objetivo de mantener la calidad del agua. Estos microorganismos, son los encargados de controlar los compuestos nitrogenados procedentes de las excreciones, restos de alimentación y materia orgánica en descomposición, manteniendo así la calidad del agua en unos valores óptimos para los langostinos (Crab et al. 2012; Emerenciano et al. 2013b). Para garantizar el correcto desarrollo de las bacterias heterotróficas, se requiere una relación adecuada de carbono y nitrógeno (Avnimelech 1999, 2009), puesto que existe un exceso de nitrógeno en el sistema, procedente del amonio generado por los animales, este se debe reequilibrar mediante la adición de carbono (Avnimelech 1999, 2009). La gran cantidad de microorganismos que se desarrollan en la columna de agua demandan una gran cantidad de oxígeno, que, junto a los requerimientos de los langostinos cultivados, hace imprescindible el uso de una fuente de aireación constante (Avnimelech 2009; Serra et al. 2015).

Otro de los beneficios de los sistemas de bioflóculos, es que la proteína microbiana generada en la columna del agua puede ser consumida por los langostinos como suplemento a su dieta, mejorando así el crecimiento de los langostinos y permitiendo un mejor aprovechamiento de los nutrientes aportados al sistema en forma de pienso (Schryver et al. 2008; Avinmelech 2009; Kuhn et al. 2009; Xu y Pan 2013). Además, los sistemas de bioflóculos son mas bioseguros que los sistemas de cultivo tradicionales, ya que los microorganismos presentes en los bioflóculos compiten por los recursos (nutrientes y espacio) con los patógenos (Emerenciano et al. 2013b), llegando incluso a inhibir su crecimiento y dificultando así la aprición de enfermedades (Crab et al. 2012; Emerenciano et al. 2013b).

Durante el proceso de formación del sistema de bioflóculos podemos diferenciar dos estados de maduración, el primer estado es el inmaduro y está caracterizado por la acumulación de amonio y/o nitrito, debido a que no existe un gran número de micrograni smos capaces de eliminar estos compuestos (Jatobá et al. 2014; Xu et al. 2016). El segundo estado es el maduro, el cual se caracteriza por poseer una comunidad microbiana totalmente desarrollada. Esta comunidad mantiene el amonio y el nitrito en concentraciones muy bajas, debido a que estos son oxidados rápidamente por las bacterias y transformados en nitratos que se acumulan en el agua (Jatobá et al. 2014; Xu et al. 2016). Durante el proceso de maduración del sistema de bioflóculos, se somete a los langostinos a niveles altos de amonio y nitrito, los cuales pueden causarles estrés y incluso la muerte (Lin y Chen 2001, 2003). Los niveles de toxicidad

del nitrito y el amonio varian en función de la salinidad utilizada, sieno estos compuestos mas toxicos para los langostinos en aguas con baja salinidad (Lin y Chen 2001, 2003). Para reducir el tiempo de maduración del sistema de bioflóculos, se pueden utilizar inóculos de bioflóculos procedentes de cultivos anteriores, agilizando el proceso de maduración y reduciendo los riesgos para los langostinos (Krummenauer et al. 2014; Emerenciano et al. 2017).

1.5. LAS MICROALGAS EN LOS CULTIVOS DE LANGOSTINOS

Las bacterias y las microalgas, son los microorganismos más importantes en los sistemas intensivos sin renovación de agua, debido a sus efectos sobre la calidad del agua y los langostinos. Las diferentes condiciones de cultivo propician la dominancia de un tipo u otro de microorganismos, lo que afecta al estado trófico del sistema (Ebeling et al. 2006). En los sistemas convencionales realizados en viveros, de baja densidad y expuestos a la luz natural, los microorganismos autrotróficos dominan el sistema (Brune et al. 2003). Por el contrario, en los cultivos intensivos con bioflóculos en viveros o tanques expuestos a la luz natural, como el utilizado en los experimentos de esta tesis, la proliferación de bacterias hetrotróficas junto a las microalgas y las bacterias autoróficas, conforma una comunidad diversa que evoluciona en función de las condiciones ambientales (Ebeling et al. 2006; Zhao et al. 2014). Finalmente, en los sistemas superintensivos, que se realizan en tanques interiores con control de la iluminación, las bacterias heterotróficas son los microorganismos más abundantes, ya que las condiciones de cultivo no permiten el desarrollo microorganismos autótrofos (Ray y Lotz 2014).

La gestión que se realiza de las microalgas en los sistemas de bioflóculos es muy diversa y varía en función de cada productor. Aunque las microalgas pueden ayudar a mantener la calidad del agua y pueden tener un alto valor nutricional (Khatoon et al. 2007b; Emerenciano et al. 2017), algunos productores prefieren controlar su proliferación en la columna de agua. Para ello, utilzan toldos de sombreo que reducen o impiden la incidencia de la luz sobre la columna del agua (Emerenciano et al. 2013a). La motivación principal para minimizar el crecimiento de las microalgas, es el temor a que aparezcan floraciones de alguna especie no deseadas como las cianobacterias, ya que estas pueden producir toxinas o aportar malos sabores a la carne de los langostinos (Alonso-Rodríguez y Páez-Osuna 2003).

Mientras algunos productores evitan el crecimiento de microalgas en sus sistemas de cultivo, otros han desarrollado diferentes estrategias de manejo para aprovechar el potencial de estos microorganismos. La primera estrategia de manejo, es colocar estructuras en los tanques que facilite el crecimiento de perifiton (Khatoon et al. 2007a), el qual servirá como alimento para los langostinos, complementando su dieta (Ballester et al. 2007). Otra estrategia de manejo, es colocar estructuras ya recubiertas de perifiton procedente de monocultivos (Khatoon et al. 2007b), con lo que se seleccionan que microalgas ramonean los langostinos. La tercera estrategia de manejo es la siembra de fitoplancton procedente de monocultivos. Al aportar grandes cantidades de microalgas de la misma especie, esta consigue ser la especie dominante en los bioflóculos (Brito et al. 2016). Además, la elevada densidad de estas microalgas en los bioflóculos garantiza que estas se incorporen a la dieta de los langostinos, cuando estos consumen los bioflóculos, mejorando su valor nutricional (Brito et al. 2016).

La acumulación de nutrientes y las elevadas temperaturas que se dan en los sistemas intensivos sin renovación de agua, propician el crecimiento natural de las microalgas. La dinámica de las microalgas en estos sistemas se ve influenciada por una gran cantidad de factores como la presencia de diferentes nutrientes, la temperatura y la incidencia de la luz (Yusoff et al. 2002; Alonso-Rodríguez y Páez-Osuna 2003; Zhang et al. 2009; Baloi et al. 2013; Pandey et al. 2014) y en el caso concreto del perifiton, también por el tipo de sustrato del que dispone (Khatoon et al. 2007a; Anand et al. 2013; Schveitzer et al. 2013). La dinámica compleja de estos organismos, dificulta su gestión y un mayor aprovechamiento de sus beneficios, siendo vistos generalmente como un problema por los productores de langostinos. Un mayor conocimiento de la dinámica de las microalgas en los sistemas intensvos, podría suponer el desarrollo de mejores estrategias de gestión, permitiendo tanto aprovechar sus beneficios como evitar la proliferación de las especies no deseadas.

La mayoría de los estudios realizados sobre el desarrollo natural de microalgas en sistemas de cultivo de langostinos han centrado su interés sobre el fitoplancton (Ju et al. 2008; Becerra-Dorame et al. 2011; Brito et al. 2016), desarrollando muestreos puntuales (Ju et al. 2008) o durante períodos cortos de tiempo (Lukwambe et al. 2015). Generalmente los estudios de las microalgas se realizan mediante su identificación con microscopios ópticos (Thompson et al. 2002; Khatoon et al. 2007a, Anand et al. 2013). Esta técnica requiere de un trabajo arduo y puede subestimar grupos de microalgas de pequeño tamaño o poco abundante en las muestras (Devilla et al. 2005; Silva et al. 2008). El arduo trabajo y el grado de especialización, a la hora

de identificar el fitoplancton, dificulta la realización de estudios que profundicen en las interacciones entre las microalgas y el medio (Ju et al. 2008). La técnica de muestreo compuesta por el análisis de los pigmentos firma, mediante la cromatografía liquida de alta resolución (HPLC), y su posterior interpretación con el programa CHEMTAX, permite identificar los taxones de las microalgas y su contribución a la comunidad fitoplanctónica (Latasa et al. 2010). Para utilizar el programa CHEMTAX el primer paso es realizar un análisis de conglomerado de las muestras, con el fin de crear grupos homogéneos en función de los pigmentos presentes (Latasa et al. 2010). Posteriormente, a partir de una matriz de ratios de pigmentos inicial, para cada conglomerado, el programa estadístico CHEMTAX realiza diferentes simulaciones, con el objetivo de minimizar el error y converger en una matriz de ratios final (Latasa 2007). La matriz de ratios final permite establecer la contribución de cada grupo de microalgas al total de clorifila *a* detectada en la muestra (Latasa et al. 2010). Mientras que el HPLC, ya ha sido utilizada para la detección de pigmentos en muestras puntuales procedentes de diferentes sistemas acuícolas, con resultados positivos (Ju et al. 2008; Jiang et al. 2016; Lemonnier et al. 2016). La interpretación de los pigmentos firma con el programa CHEMTAX, ha sido realizada con éxito para el monitoreo de las microalgas en diferentes ecosistemas (Sebastiá et al. 2012; Ahmed et al. 2016). La metodología combinada HPLC/CHEMTAX puede aportar un diagnóstico rápido, reproducible y más rentable que el análisis microscópico (Schlüter et al. 2006, Ju et al. 2008; Schlüter et al. 2016), por lo tanto podría ser una técnica apta para estudiar la dinámica de las microalgas, de los sistemas intensivos, en mayor profundidad.

1.6. LAS BACTERIAS PROBIÓTICAS EN LOS CULTIVOS DE LANGOSTINOS

Durante las últimas décadas, la aparición de diferentes enfermedades ha causado estragos en algunas de las principales zonas productoras de langostinos del mundo, donde predominan los cultivos tradicionales. Algunos de los peores episodios se han producido en países como Ecuador, donde el virus de la mancha blanca redujo la producción nacional un 60% desde 1999 a 2001 (Notarianni 2006), en Taiwán, el virus del síndrome de la taura acabo con más del 80% de la produccion de langostinos en el año 1999 (Yu y Song 2000), la bacteria *Vibrio parahemolyticus*, redujo al 20% la producción China en el 2011, así como el 40% de la tailandesa y el 60% de la mexicana entre 2012 y 2013 (Sanchez-Paz et al. 2014; Zorriehzahra y Banaederakhshan 2015).

Uno de los principales factores de propagación de las enfermedades en las granjas de langostinos es el acopio y descarga de aguas, que realizan los criaderos en los entornos naturales, ya que facilitan el contagio de enfermedades entre las granjas (Anh et al. 2010). Este hecho ha llevado a los productores a utilizar sistemas cerrados, como el de los bioflóculos, ya que reduce el riesgo de infección (Emerenciano et al 2013b). Además del aislamiento de los cultivos frente a posibles aguas contaminadas, los sistemas de bioflóculos también tienen un efecto probiótico sobre los langostinos (Crab et al. 2012; Emerenciano et al. 2013b). La adición de algunas bacterias en los sistemas de bioflóculos puede mejorar los efectos probióticos de los sistemas de bioflóculos (Souza et al. 2012). La utilización de bacterias probióticas ha estado ampliamente estudiada en los cultivos tradicionales y con recirculación de agua, donde sus efectos pueden ser diversos: refuerzo del sistema inmunológico, disminución de las afecciones por enfermedades, mejoras en la calidad del agua o un mayor engorde (Rengpipat et al. 2000; Dalmin et al. 2001; Zhou et al. 2009; Pandiyan et al. 2013). Los efectos de los probióticos pueden ser diferentes en función de la concentración, la forma de administración, el sistema al que se aplican y las especies de bacterias utilizadas (Van Hai y Fotedar 2010). Para poder aplicar probióticos en los sistemas de bioflóculos de forma eficiente es necesario conocer el efecto de las diferentes bacterias probióticas bajo las condiciones de estos sistemas.

En Asia y América la industria del langostino tiene a su alcance una gran cantidad de piensos, muchos de los cuales incorporan bacterias probióticas a sus fórmulas, con el objetivo de mejorar la productividad de los cultivos (Van Hai y Fotedar 2010). En la Unión Europea la escasa demanda de piensos específicos para langostinos, provoca que la oferta de estos productos sea muy reducida. Por lo tanto, la mejor forma de aplicar probióticos a los cultivos es directamente sobre el agua, permitiendo que cada productor decida qué tipo de bacteria probiótica es más adecuada en función de las condiciones de su cultivo.

La bacteria *Bacillus amyloliquefaciens,* se comercializa en la Unión Europea como probiótico para su aplicación en pollos bajo la marca Ecobiol Plus®. Esta bacteria, también parece indicada para su uso como probiótico en la acuicultura, ya que actualmente ha sido testada con éxito en sistemas de recirculación en cultivo de peces y langostinos (Camacho 2012; Nuez-Ortín et al. 2013; Huang et al. 2015; Saputra et al. 2016). Entre los beneficios de su aplicación en sistemas de reciculación para la producción de langostinos, se encuentra el refuerzo del sistema inmunológico, la mejora de la supervivencia y del crecimiento de los

animales (Camacho 2012; Nuez-Ortín et al. 2013). Además, Xie et al. (2013) indica que *B. amyloliquefaciens* es capaz de eliminar los nitritos presentes en el agua. *B. amyloliquefaciens* tiene un rápido crecimiento y un alto valor nutricional, características que pueden mejorar el rendimiento y las propiedades nutricionales de los bioflóculos, por lo que Bao (2014) determinó que podría jugar un papel importante en el desarrollo de estos sistemas. Para poder utilizar *B. amyloliquefaciens* como probiótico en los sistemas de bioflóculos se requieren estudios previos, puesto que las condiciones de los ensayos en laboratorio y los ensayos en sistemas de recirculación son diferentes a las condiciones de los sistemas de bioflóculos, haciéndose indispensable el estudio de esta bacteria en los sistemas de bioflóculos para determinar su eficacia como probiótico.

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CAPÍTULO 2

Objetivos

y

Organización de la memoria

En esta investigación se han marcado dos objetivos generales, el primero estudiar la dinámica de las microalgas que se desarrollan en los cultivos intensivos de langostinos sin renovación de agua. El segundo objetivo general, es determinar si la bacteria *Bacillus amyloliquefaciens* tiene efectos probióticos en los sistemas de bioflóculos, utilizados en la producción intensiva de langostinos marinos. Además de los objetivos generales, se establecen una serie de objetivos específicos que se detallan a continuación:

1. Estudiar la dinámica de los grupos taxonómicos del fitoplancton y perifiton en un sistema de preengorde de *Marsupenaeus japonicus,* utilizando la metodología HPLC/CHEMTAX.
2. Caracterizar y analizar la evolución del fitoplancton, durante la formación de un sistema de bioflóculos para el engorde de *Litopenaeus vannamei,* utilizando la metodología HPLC/CHEMTAX.
3. Determinar el papel probiótico de la bacteria *B. amyloliquefaciens*, aplicada en el agua, en un sistema de bioflóculos.
4. Analizar la implicación de la bacteria *B. amyloliquefaciens* en la maduración de los sistemas de bioflóculos sin inóculo y el efecto sobre los langostinos de una reducción de la dosis aplicada.

Para alcanzar estos objetivos, se realizaron tres experimentos cuyos resultados se estructuran en cuatro capítulos (3, 4, 5 y 6), que imitan la estructura de los artículos científicos. Estos capítulos están escritos en inglés, ya que es esta la lengua principal de divulgación científica. En cada uno de estos capítulos, se contextualizan los objetivos específicos que se abordan, se describe la metodología utilizada y se exponen y discuten los resultados obtenidos. A continuación se resumen el contenido de cada capítulo.

- **Capítulo 3.** Periphyton and phytoplankton assessment in a shrimp nursery: signature pigments analysis. En este capítulo se estudió la dinámica de las microalgas que se desarrollan en un cultivo de preengorde, sin renovación de agua, de *M. japonicus*, utilizando la metodología combinada HPLC/CHEMTAX. El capítulo, detalla la composición del fitoplancton y del perifiton, mediante muestreos semanales, durante 45 días, permitiendo observar las sucesiones de las microalgas. Además, se caracteriza el agua de cultivo con la finalidad de detectar la influencia de las variables ambientales sobre la composición y abundancia de las diferentes microalgas. Este experimento, se realizó en las instalaciones de la Universitat Politècncia de València,

en Gandia (Comunitat Valenciana). Una versión previa de los resultados del capítulo 3 fueron presentados a la revista *Aquaculture international* y actualmente se encuentra bajo revisión (Tabla 2.1).

- **Capítulo 4.** Phytoplankton evolution during the creation of a biofloc system for shrimp culture. En el capítulo 4, se caracteriza el fitoplancton durante la maduración de un sistema de bioflóculos, durante el engorde de langostinos *L. vannamei*. El estudio de la composición y abundancia del fitoplancton se realiza mediante la detección de muestras semanales de los pigmentos con el HPLC, y su posterior interpretación con el programa CHEMTAX. Además, se analizan las variables ambientales de los sistemas de bioflóculos para determinar que variables influyen sobre la estructura de la comunidad fitoplanctónica. Este experimento tuvo una duración de 164 días y se llevo a cabo en las instalaciones de la Universitat Politècncia de València (Gandia, Comunitat Valenciana). Los resultados de los capítulos 4 y 6 se obtuvieron de forma simultánea, al provenir del mismo experimento. Los resultados del capítulo 4, han sido publicados en la revista científica *International Journal of Environmental Science and Technology*, como se detalla en la tabla 2.1.
- **Capítulo 5.** Application of *B. amyloliquefaciens* as probiotic for *L. vannamei* (Boone) cultivated in a biofloc system. En este capítulo se determina el papel probiótico de la bacteria *B. amyloliquefaciens* en un sistema de bioflóculos, mediante su aplicación en el agua. El capítulo profundiza en los efectos de esta bacteria sobre la calidad del agua, el crecimiento y el sistema inmunológico de los langostinos, para determinar si puede ser utilizado como probiótico en un sistema de bioflóculos. Este experimento duró 42 días, y fue realizado en las instalaciones de la Universidade Federal do Rio Grande (Rio Grande, Rio Grande do Sul, Brasil) y sus resultados han sido enviados a la revista internacional *Iranian Journal of Fisheries Sciences*, donde se encuentran en proceso de revisión (Tabla 2.1).
- **Capítulo 6.** The role of *B. amyloliquefaciens* on *L. vannamei* during the maturation of a biofloc system. Este capítulo, determina la implicación de la bacteria *B. amyloliquefaciens* en la maduración de los sistemas de bioflóculos sin inóculo y el efecto sobre los langostinos de una reducción de la dosis aplicada. Para ello, se analiza el efecto de la bacteria probiótica sobre la calidad del agua y la actividad microbiana del sistema bioflóculos, así como sus afecciones al crecimiento y el sistema inmunológico de los langostinos, tanto en las fases inmadura y madura del sistema de bioflóculos. Este experimento tuvo una duración de 164 días y se llevo a cabo en las

instalaciones de la Universitat Politècncia de València (Gandia, Comunitat Valenciana). Sus resultados se obtuvieron de forma simultánea a los expuestos en el capítulo 4. Los resultados previos presentados en el capítulo 6, han sido enviados a la revista *Fish and Shellfish Immunology,* para que esta considere su publicación. Como se muestra en la tabla 2.1, actualmente se encuentran bajo revisión.

Siguiendo a los capítulos 3, 4, 5 y 6, se realiza una discusión conjunta de los resultados obtenidos (capítulos 7), que permite tener una visión más amplia tanto del papel de las microalgas y la bacteria probiótica *B. amyloliquefaciens* en los sistemas sin renovación de agua. Finalmente, en el capítulo 8, se resumen las diferentes conclusiones obtenidas y se plantean futuras líneas de investigación, que permitirán seguir avanzando en el conocimiento de los sistemas sin renovación de agua, para la producción intensiva de langostinos.

Tabla 2.1. Relación de publicaciones científicas y capítulos de la presente memoria.

Nº Capítulo	Título	Autores	Revista	Indexación	Estado
Capítulo 3	Periphyton and phytoplankton assessment in a shrimp nursery: signature pigments analysis.	Ferran Llario, Miguel Rodilla, Silvia Falco, Julia Escrivá, Maria Teresa Sebastiá-Frasquet.	Aquaculture International, ISSN 0967-6120.	JCR Science Edition, Factor de Impacto en 2016 de 1.095	Bajo revisión.
Capítulo 4	Phytoplankton evolution during the creation of a biofloc system for shrimp culture.	Ferran Llario, Miguel Rodilla, Julia Escrivá, Silvia Falco, Maria Teresa Sebastiá-Frasquet.	International Journal of Environmental Science and Technology, ISSN 1735-1472.	JCR Science Edition, Factor de Impacto en 2016 de 1.915	Aceptado. Doi:10.1007/s13762-018-1655-5
Capítulo 5	Application of *Bacillus amyloliquefaciens* as probiotic for *Litopenaeus vannamei* (Boone) cultivated in a biofloc system.	Ferran Llario, Luís Alberto Romano, Miguel Rodilla, Maria Teresa Sebastiá-Frasquet, Luis Henrique Poersch.	Iranian Journal of Fisheries Sciences, ISSN 1562-2916.	JCR Science Edition, Factor de Impacto en 2016: 0.285	Aceptado.
Capítulo 6	The role of *Bacillus amyloliquefaciens* on *Litopenaeus vannamei* during the maturation of a biofloc system.	Ferran Llario, Silvia Falco, Maria Teresa Sebastiá-Frasquet, Julia Escricá, Miguel Rodilla, Luis Henrique Poersch.	Helgoland Marine Research, ISSN 1438-387X.	JCR Science Edition, Factor de Impacto en 2016: 1.013	Bajo revisión.

CAPÍTULO 3

Periphyton and phytoplankton assessment in a shrimp nursery: signature pigments

Llario, F., Rodilla, M., Falco, S., Escrivá, J., Sebastiá-Frasquet, M. T. (2018) Periphyton and phytoplankton assessment in a shrimp nursery: signature pigments analysis. *Aquaculture International,* under review process.

3.1. ABSTRACT

Microalgae play a fundamental role in aquaculture systems, especially in nurseries. They can supplement the nutrition of cultured species, and also contribute to keep good water quality. Their value depends on the groups present in major abundance. Thus, being able to understand the factors that influence microalgae composition is key for an improved system management. In this study, the microalgae community structure in a *Marsupenaeus japonicus* nursery is studied. Instead of the classic microscopy approach, the composition and abundance of periphyton and phytoplankton are analyzed using signature pigments analysis by high pressure liquid chromatography coupled with the software CHEMTAX. The environmental parameters, which can affect periphyton and phytoplankton groups, are analyzed statistically. The results showed that diatoms were the dominant group both in phytoplankton and periphyton. The analysis of signature pigments, allowed to report the presence of previously undetected groups on periphyton, prasinophytes and prymnesiophytes, which are characterized by a high nutritional value. This is especially important in nurseries because of shrimp grazing on periphyton can increase post-larvae survival. Nutrients played a key role on phytoplankton evolution, but had a minor effect on periphyton, which was more affected by colonization processes and other environmental variables.

Keyword: biofilm; CHEMTAX; *Marsupenaeus japonicus*; microalgae; high-performance liquid chromatography

3.2. INTRODUCTION

Microalgae play a crucial role in aquaculture tanks, both as a feed and for its ability to maintain good water quality (Ballester et al. 2007; Khatoon et al. 2007a; Anand et al. 2013). These microalgae can be phytoplankton, the autotrophic component of plankton that drifts in the water column (Harris 2012); or periphyton, which grows fixed on the substrate (Azim et al. 2005). By origin, they can come from specific cultures or develop naturally in the environment. Cultured microalgae usually come from intensive monocultures and are provided to the cultured species (e.g. shrimps) in different ways: lyophilized and included in the feed (Macias-Sancho et al. 2014), as live feed (Barbieri and Ostrensky 2001; Brito et al. 2016) or fixing them on a substrate that is later supplied to the cultured species (Khatoon et al. 2007b). The effects of microalgae vary according to their abundance and taxonomic

composition. Previous studies have shown that there are groups of microalgae, such as diatoms, able to improve water quality, and with high nutritional value (Khatoon et al. 2007b). In contrast, other groups, such as cyanobacteria, are highly toxic, and have a negative effect on survival and growth of cultured species (Alonso-Rodríguez and Páez-Osuna 2003).

The dynamics of microalgae in aquaculture systems respond to a complex relationship between different environmental and biological variables (Alonso-Rodríguez and Páez-Osuna 2003; Pandey et al. 2014; Llario et al. 2018). Being able to understand these dynamics can help shrimp farmers to manipulate aquaculture systems for keeping the most desired microalgae composition. However, most studies have focused on phytoplankton dynamics, while periphyton studies are scarcer. Temperature, light and nutrients are environmental variables that have a key effect on microalgae abundance and composition (Yusoff et al. 2002; Zhang et al.2009). In general, low N:P ratios facilitate undesired species such as dinoflagellates and cyanobacteria blooms in aquaculture tanks (Alonso-Rodríguez and Páez-Osuna 2003), while high silica levels favors diatoms (Llario et al. 2018). For periphyton, is also of paramount importance the effect of substrate. Substrates such as bamboo, plastic sheet, polyvinylchloride, polyethylene, fibrous scrubber, ceramic, paddy straw and coconut fronts have been tested by some authors (Thompson et al. 2002; Khatoon et al. 2007a; Anand et al. 2013; Schveitzer et al. 2013; Sruthisree et al. 2015). Also, shrimp graze on periphyton (Ballester et al. 2007) and the substrate colonizing potential of some microalgae species can be relevant factors (Khatton et al. 2007a; Zhang et al. 2012).

Periphyton studies are scarce mainly due to sampling and analysis complexity. In general, the recollection of periphyton, from different aquaculture systems, is based on the suspension of the periphyton in water and its subsequent analysis following the techniques described for phytoplankton (Kahoon et al. 2007a; Anand et al. 2013). Some studies scrape the periphyton from the substrate and add preservatives such as formaldehyde for its preservation (Azim et al. 2001). Others, fix the periphyton on the substrate with lugol or formaldehyde and then resuspend the periphyton with the help of an ultrasonic homogenizer or a vortex (Anand et al. 2013; Viau et al. 2013). Taxonomic groups are usually identified by optic microscopy (Azim et al. 2001; Thompson et al. 2002; Kahoon et al. 2007a; Anand et al. 2013; Viau et al. 2013). However, this technique involves hard work, and its results generally underestimate the groups with smaller size or those with lower abundance (Gocke et al. 2003; Devilla et al. 2005; Silva et al. 2008). In the last decades, the analysis of signature pigments by high-

performance liquid chromatography (HPLC) has arisen as a reliable technique to supplement microscope counts (Schlüter et al. 2006). This technique has already been successfully used for the analysis of phytoplankton from aquaculture tanks, both for analysis of point samples (Jiang et al. 2016; Lemonnier et al. 2016) and for complete monitoring of a culture (Llario et al. 2018). In this study, we aim to prove that it is also useful for periphyton analysis.

The primary objective of this research was to estimate microalgae community structure in a *Marsupenaeus japonicus* nursery. Periphyton and phytoplankton composition and abundance were determined using HPLC signature pigment analysis coupled with CHEMTAX software. The secondary objectives were to analyze the environmental parameters that can affect the abundance, composition and evolution of microalgae in a shrimp nursery.

3.3. MATERIAL AND METHODS

3.3.1. Location and shrimp culture system

The experiment was developed in the Universitat Politècnica de València facilities (Gandia, Valencia, Spain), between June 2 and July 17, 2015. Postlarvae shrimps (PLs) of *M. japonicus* were purchased from La Petite Canau (Leucate, Aude, France). The PLs had an initial size of 0.020 ± 0.008 g. The nursery facilities comprised 9 concrete tanks located inside a greenhouse, and constantly individually aerated. The greenhouse had natural lighting (15 hours every day) and the light intensity was between 3000 and 6000 lux. The tanks were covered with polyvinyl chloride (PVC) canvas for waterproofing. Each tank had a surface of 3.2 m^2, and was filled with 2250 L of seawater (36.5 salinity). The water was disinfected with 10 mg/L of chlorine, which was subsequently removed by adding ascorbic acid to the tanks (Krummenauer et al. 2014). The shrimp were distributed in three densities per triplicate 25 (tanks 5, 7 and 8), 50 (tanks 1, 3 and 9) and 75 (tanks 2, 4 and 6) shrimp/m^2. Feeding was provided twice a day, with commercial feed specifically designed, according to Barbieri and Ostrensky (2001).

3.3.2. Water quality

During the experiment, dissolved oxygen (DO), salinity and temperature (T) were monitored in-situ, using a multi-parameter probe (YSI ProODO and WTW Multi 340i respectively) twice a day. Freshwater was added to maintain salinity around 36.5, when the salinity value exceeded 37. The pH was measured once a day using pH-Meter BASIC 20^{+} Crison.

An initial fertilization of the system was done with sucrose, with a theoretical 15:1 carbon/nitrogen ratio, with the aim of guaranteeing the initial development of heterotrophic bacteria. During the rest of the experiment a C:N ratio of 15:1 was maintained, through the addition of a carbon source (sucrose) when N-TA values higher than 1 mg/L were detected (Ebeling et al. 2006).

Every 2 days an aliquot of water was collected to determine the concentration of total dissolved ammonia (N-TA mg/L), nitrites ($N\text{-}NO_2^-$ mg/L) and phosphates ($P\text{-}PO_4^{3-}$ mg/L) using the methodology described by Baumgarten et al. (2010). The nitrates ($N\text{-}NO_3^-$ mg/L) were analyzed twice a week, by means of the difference between nitrites plus nitrates using the methodology described by Grasshoff (1976).

3.3.3. Biological parameters

Before the experiment, pieces of 200 cm^2 of PVC canvas were submerged at a depth of 30 cm from the surface. Pieces of canvas of about 20 cm^2 were cut for the analysis of periphyton once a week (Hagerthey et al. 2006). Water samples for phytoplankton pigment analysis were filtered on GF/F fiberglass filters (25 mm diameter) once a week. Photosynthetic pigments were extracted using acetone (100% HPLC grade) and were measured using reverse-phase high-performance liquid chromatography (HPLC). The HPLC method employed was that proposed by Wright et al. (1991) slightly modified as per Hooker et al. (2001). The system was calibrated with external standards obtained commercially from the DHI Water and Environment Institute (Hørsholm, Denmark). For more details on the benefits of this analytical procedure see Sebastiá et al. (2012).

In order to identify the phytoplankton and periphyton groups present in the tanks, we observed a sample per tank in the microscopy some weeks, the aim of these samples was supplementing information on group presence for CHEMTAX. Phytoplankton samples were fixed with formaldehyde, concentrated according to UNE EN15204:2006, based on Utermohl

(1985), and qualitatively examined under a LEICA DM IL inverted microscope. Periphyton samples were scraped and suspended in filtered seawater (Azim et al. 2001), for subsequent treatment in the same way as phytoplankton.

Once the concentration of important photosynthetic pigments was determined, the phytoplankton community was studied using the CHEMTAX software (Mackey et al. 1996) version 1.95 (S. Wright, pers. comm.) to obtain the contribution to total chlorophyll *a* (Chl*a*) from the phytoplankton and periphyton groups identified with microscopy as described in Sebastiá et al. (2012) and Sebastiá and Rodilla (2013). In order to identify groups of samples with similar characteristics, a cluster analysis was performed using STATGRAPHICS Centurion XVI.I to group samples according to pigments concentration. CHEMTAX was applied independently to obtain the contribution of eight phytoplankton and periphyton groups to the Chl*a* stock: diatoms, dinoflagellates, euglenophytes, chlorophytes, cryptophytes, prymnesiophytes, prasinophytes, and cyanobacteria. The final matrix used to estimate the contribution of the different groups to Chl*a* stock is published in Llario et al. (2018).

3.3.4. Statistical analysis

Previously to statistical analysis, we calculated weekly average of environmental parameters to be able to compare with phytoplankton and periphyton data, which were sampled weekly. Normality and homocedasticity of all variables were tested before multivariate analysis. As all the variables were not normally distributed, a non-parametric one-way analysis of variance (Kruskal–Wallis) was performed to statistically assess variations in the median fraction of all monitored variables within the experimental tanks. Spearman rank correlation analyses were performed on environmental and biological variables (salinity, pH, T, DO, N-TA, N-NO_2^-, N-NO_3^-, P-PO_4^{3-}, total Chl*a* in phytoplankton and total Chl*a* in periphyton) with phytoplankton or periphyton groups in order to examine significant relationship. The significant relationship between phytoplankton and periphyton groups was also assessed.

3.4. RESULTS AND DISCUSSION

During the experiment, no statistically significant differences were observed on physicochemical variables within the experimental tanks according to Kruskal–Wallis analysis results ($P > 0.05$). Salinity, pH and dissolved oxygen were kept stable under conditions suitable for the culture of *M. japonicus* (Blachier 1998; Barbieri and Ostrensky 2001). The average recorded salinity was 36.5; this value increased to 37.2 due to water evaporation, but freshwater was added to avoid exceeding this limit (minimum salinities of 35.6 were due to freshwater supply). The pH was stable during all the experiment with values between 8.41 and 8.68. Dissolved oxygen increased slightly from 5.86 to 6.75 mg/L by the end of the experiment. The temperature was generally maintained at optimum levels for the culture of *M. japonicus* (24 to 30 °C) according to Preston et al. (1995) and Hewitt and Duncan (2001), with an average of 30.2 °C. Occasionally, water temperature reached 32.1°C, which is the maximum temperature recommended.

The levels of ammonia and nitrites were maintained within the limits of safety determined by Lin and Chen (2001), to avoid toxic effects on shrimp. N-TA average was 0.53 mg/L, but occasionally reached 3.70 mg/L; while $N\text{-}NO_2^-$ varied between non detected and 0.58 mg/L, and $N\text{-}NO_3^-$ between non detected and 0.69 mg/L. Both $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ levels increased along the study period. $P\text{-}PO_4^{3-}$ also increased, with maximum values close to 1 mg/L during the last culture week.

The following signature pigments were detected in water samples: peridinin, fucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, diadinoxanthin, alloxanthin, lutein and zeaxanthin. According to pigment analysis and microscope observations, these seven phytoplankton groups were present: diatoms, dinoflagellates, cryptophytes, euglenophytes, prasinophytes, prymnesiophytes and cyanobacteria. A Kruskal–Wallis analysis was performed to detect statistically significant differences in phytoplankton groups abundance between tanks. In Table 3.1, we summarize phytoplankton statistics both for absolute composition (absolute contribution to chlorophyll *a*, µg/L) and relative composition (percentage of total chlorophyll *a*, %). In this table, statistically significant differences are assessed according to Kruskal-Wallis results. There were no significant differences ($P > 0.05$) in phytoplankton abundances (Table 3.1), except for dinoflagellates relative contribution which was higher in tank 4 than in the others.

Table 3.1. Phytoplankton statistics (average and maximum values) both for absolute and relative contribution to total chlorophyll *a*. Kruskal–Wallis analysis significance results are shown in *P* value column.

	Phytoplankton absolute contribution to chlorophyll *a* (µg/L)			**Phytoplankton relative contribution to chlorophyll *a* (%)**		
Groups	**Average**	**Maximum**	***P***	**Average**	**Maximum**	***P***
Diatoms	6.57	57.85	0.48	93.03	100.00	0.91
Dinoflagellates	0.08	4.25	0.13	0.37	16.12	0.01
Euglenophytes	0.01	0.10	0.10	0.12	2.86	0.07
Cryptophytes	0.02	1.26	0.13	1.03	53.74	0.07
Prasinophytes	0.12	4.72	0.73	2.89	100.00	0.73
Prymnesiophytes	8.62×10^{-4}	0.02	0.31	0.02	0.48	0.26
Cyanobacteria	0.07	0.79	0.06	2.55	52.25	0.15

The average phytoplankton biomass was 6.87 µg/L of Chl*a*, but it varied between non detected and 58.01 µg/L during study period. Diatoms were the dominant group of phytoplankton, on average they accounted a 93% of total Chl*a*, and at specific moments they reached 100% (Table 3.1). In figure 3.1, we represented the temporal evolution of phytoplankton abundance by groups. In this figure, the clear predominance of diatoms along the entire experiment is observed. The mean abundance of other phytoplankton groups was on average lower than 3%. However, both cryptophytes and cyanobacteria arrived to maximum percentages above 50% (Table 3.1). Moreover, prasinophytes even get to 100% of total Chl*a* (Table 3.1). The incidence of these high percentages was limited since they were isolated cases, that happened on one tank only and disappeared in the next analysis. Prasinophytes bloom was only observed in tank 7 on day 31, cyanobacteria bloom was detected in tank 1 on day 38, and cryptophytes bloom happened in tank 5 on day 45.

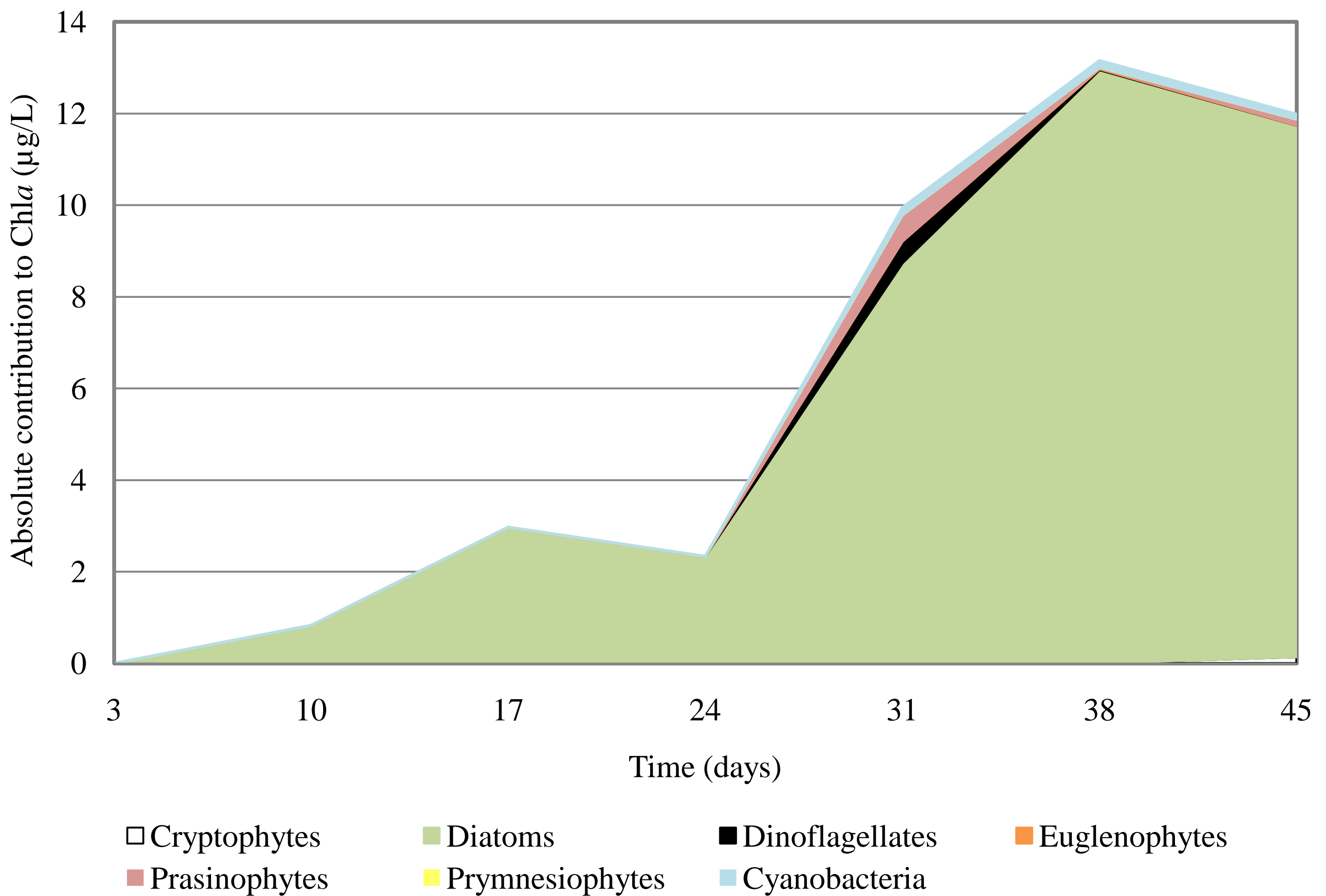

Figure 3.1. Phytoplankton groups mean contribution to chlorophyll *a* concentration (µg/L) temporal evolution.

Chl*a* concentration in water is used as a proxy for phytoplankton biomass (Gaona et al. 2011). In this experiment, phytoplankton was first detected on day 10, pigments concentration was below detection level on day 3. Before, no Chl*a* was detected due to the initial disinfection of culture water. Water samples from day 10 to day 24 had Chl*a* concentrations around 3 µg/L. During this first phase, only diatoms were observed. Since day 31, an increase in Chl*a* was observed, with concentrations around 12 µg/L by the end of the study period. During this second phase, diatoms were dominant but other phytoplankton groups appeared, such as dinoflagellates (0.4 µg/L on average) and prasinophytes (0.5 µg/L on average). A maximum Chl*a* value of 57.85 µg/L was detected in tank 9 on day 38; however, no statistical differences were detected among tanks when considering the entire period Chl*a* abundance. Chl*a* concentration in aquaculture waters is highly variable, because it depends on a large number of factors (Burford 1997). The Chl*a* values quantified in this experiment in phytoplankton are

similar to those observed by other authors (Guerrero-Galvá et al. 1999; Yusoff et al. 2002 and Lemonnier et al. 2016).

In general, diatoms are the dominant group in most shrimp cultures developed in saltwater tanks (Yusoff et al. 2002; Casé et al. 2008; Lemonnier et al. 2016). Environmental variables directly influence phytoplankton and can help us understand phytoplankton dynamics and the dominance of one or another group (Sanders et al. 1987). In this study, salinity and pH had great stability throughout the culture period, for that reason, these variables do not have a significant correlation with any taxonomic group (Table 3.2). In contrast, temperature showed a positive correlation with different groups, and dissolved oxygen a negative correlation (Table 3.2); these correlations were previously observed by Casé et al. (2008) and Llario et al. (2018) in shrimp aquaculture. The maximum growth of diatoms is usually observed between 25 and 30ºC (Renaud et al. 2002), which is the temperature range used in *M. japonicus* cultures. The proliferation of phytoplankton in aquaculture tanks is accompanied by the increase of bacteria, which decompose the organic matter present in the system and consume oxygen (Paerl and Tucker 1995).

The highest correlations were observed between phytoplankton groups and nutrients (N-TA, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$). Minor phytoplankton groups correlated significantly with a single nutrient each, while diatoms showed a significant positive correlation with the four nutrients analyzed (Table 3.2). Diatoms dominance in phytoplankton (93% relative contribution to Chl*a*) is explained by their rapid use of nutrients and their high growth rate (Ryther and Officer 1981). While other groups of phytoplankton have a preference for some forms of nitrogen (Paerl and Tucker 1995; Šupraha et al. 2014; Reed et al. 2016), diatoms rapidly consumes nitrogen in all its forms (Ryther and Officer 1981). The decrease or depletion of silica usually causes the decline of diatoms and proliferations of cyanobacteria (Casé et al. 2008). In this experiment, the tanks were filled with coastal waters from Gandia Harbor characterized by high silica levels (Sebastiá et al. 2012; Sebastiá and Rodilla 2013). This high initial silica content can maintain important diatom abundances in aquaculture tanks longer periods than usual (Llario et al. 2018), which explains why their decrease was not observed. The presence of diatoms in shrimp nurseries is highly desired due to its high nutritional value (Kent et al. 2011), and its efficiency in the removal of nutrients helps to maintain water quality (Khatoon et al. 2007b).

Table 3.2. Rank correlation matrix (Spearman's) between environmental and biological variables (Salinity, T –temperature, DO – dissolved oxygen, N-TA - total dissolved ammonia, $N\text{-}NO_2^-$ -nitrites, $N\text{-}NO_3^-$ - nitrates, $P\text{-}PO_4^{3-}$ - phosphates, and Pe-Chl*a* – total chlorophyll *a* in the periphyton) and phytoplankton groups.

	Salinity	**pH**	**DO**	**T**	**N-TA**	**$N\text{-}NO_2^-$**	**$N\text{-}NO_3^-$**	**$P\text{-}PO_4^{3-}$**	**Pe-Chl*a***
Diatoms	-0.051	-0.200	**-0.464**[a]	0.127	**0.317**[b]	**0.275**[b]	**0.437**[a]	**0.577**[a]	**0.354**[a]
Dinoflagellates	0.072	-0.023	**-0.285**[b]	**0.410**[a]	0.221	**0.265**[b]	0.237	0.227	0.118
Euglenophytes	0.111	-0.013	**-0.294**[b]	**0.444**[a]	0.197	0.197	**0.273**[b]	0.194	0.088
Cryptophytes	0.123	0.006	**-0.292**[b]	**0.446**[a]	0.186	0.179	**0.273**[b]	0.189	0.101
Prasinophytes	-0.093	-0.096	**-0.249**[b]	0.069	0.236	0.222	0.129	**0.297**[b]	-0.081
Prymnesiophytes	0.043	0.019	-0.247	**0.403**[a]	0.173	0.152	0.197	0.166	0.025
Cyanobacteria	0.117	0.050	**-0.307**[b]	**0.470**[a]	0.206	0.214	**0.293**[b]	0.201	0.218

Bold numbers are those that show significant correlation: [a] $P<0.01$, [b] $P<0.05$

The appearance of phytoplankton blooms during the culture period, such as prasinophytes, cyanobacteria and cryptophytes blooms, can have detrimental effects on shrimp and water quality. All the phytoplankton blooms observed in this experiment, had a short duration and were produced in different tanks during the final phase of the culture period (days 31, 38 and 45). In this period, $P\text{-}PO_4^{3-}$ y $N\text{-}NO_3^-$ accumulated from shrimp excretions and from the nitrification of nitrogen compounds (N-TA and $N\text{-}NO_2^-$). According to several authors, cyanobacteria, cryptophytes and prasinophytes, grow better with a supply of $N\text{-}NO_3^-$ y $P\text{-}PO_4^{3-}$ (Paerl and Tucker 1995, Šupraha et al. 2014, and Reed et al. 2016). In this experiment, this is corroborated by the significant positive correlation between $N\text{-}NO_3^-$ and both cryptophytes and cyanobacteria, and the positive correlation of $P\text{-}PO_4^{3-}$ and prasinophytes (Table 3.2). Cyanobacteria blooms are common in shrimp farming, and are more common in low light conditions (Smith et al. 2008).

The following signature pigments were detected in periphyton samples: peridinin, fucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, diadinoxanthin, alloxanthin, zeaxanthin and chlorophyll *b*. According to pigment analysis and microscope observations, these eight periphyton groups were present: diatoms, dinoflagellates, chlorophytes, cryptophytes, euglenophytes, prasinophytes, prymnesiophytes and cyanobacteria. Like

phytoplankton, a Kruskal–Wallis analysis was performed to detect statistically significant differences in groups abundance between tanks. In Table 3.3, we summarize periphyton statistics both for absolute composition (absolute contribution to chlorophyll *a*, µg/m^2) and relative composition (percentage of total chlorophyll *a*, %). In this table, statistically significant differences are assessed according to Kruskal-Wallis results. There were no significant differences ($P > 0.05$) in periphyton abundances, except for chlorophytes absolute contribution to Chl*a* (Table 3.3). This minor group had higher abundances in tanks 3, 4, 5 and 8, than in the others.

Table 3.3. Periphyton statistics (average and maximum values) both for absolute and relative contribution to total chlorophyll *a*. Kruskal–Wallis analysis significance results are shown in *P* value column.

	Periphyton absolute contribution to chlorophyll *a* (µg/m^2)			**Periphyton relative contribution to chlorophyll *a* (%)**		
Groups	**Average**	**Maximum**	***P***	**Average**	**Maximum**	***P***
Diatoms	72.57	415.43	0.19	67.92	100.00	0.69
Dinoflagellates	0.04	2.16	0.30	0.20	10.81	0.62
Euglenophytes	3.07	60.58	0.52	4.84	56.82	0.45
Chlorophytes	1.97	19.68	0.03	2.70	44.94	0.06
Cryptophytes	0.07	3.07	0.30	0.04	1.68	0.73
Prasinophytes	10.41	107.58	0.63	15.84	100.00	0.75
Prymnesiophytes	5.23	213.62	0.26	1.99	66.51	0.23
Cyanobacteria	7.71	108.29	0.31	6.47	65.38	0.28

Chl*a* concentration in periphyton had mean values of 101.07 µg/m^2, but maximum values of 441.42 µg/m^2 were detected in tank 1 on day 45. Mean Chl*a* values are similar to those observed by Khatoon et al. (2007a) on the same type of surface (PVC) after 45 days of culture, although other authors observed superior values in this substrate (Thompson et al. 2002; Sruthisree et al. 2015). Shrimps graze on periphyton (Ballester et al. 2007), so the increase of available surfaces, through the placement of PVC sheets in the tank, may increase of periphyton biomass and improve shrimp productivity. But, the substrate is not the only variable that affects periphyton development, other environmental variables have an important influence (Khatoon et al. 2007a; Anand et al. 2013; Sruthisree et al. 2015).

Although periphyton was dominated by diatoms (67.92% of Chl*a*) from the beginning of the culture, a higher percentage of other taxonomic groups was detected than in phytoplankton. The most abundant groups, apart from diatoms, were prasinophytes (15.84%), cyanobacteria (6.47%) and euglenophytes (4.84%) as shown in table 3.3. The rest of groups had a mean contribution lower than 3% of Chl*a*. Although most of the samples were dominated by diatoms, the punctual dominance of other periphyton groups was observed, such as euglenophytes on day 10 (tank 8), day 17 (tank 1) and day 45 (tank 8), prasinophytes on day 10 (tanks 1, 6 and 7), day 17 (tanks 2 and 6) and day 31 (tank 2), prymnesiophytes on day 38 (tank 4) and cyanobacteria on day 31 (tank 8).

Chl*a* concentration evolution in periphyton samples by group is represented in figure 3.2. In this experiment, the same as with phytoplankton, no Chl*a* was detected before day 10 in periphyton samples. On day 10, periphyton absolute abundance was 97 µg/m^2 of Chl*a*, mainly due to diatoms and prasinophytes. These two groups abundance decreased on day 17, but later recovered. Since day 24 until the end of the experiment, a progressive increase in total Chl*a* was observed, reaching a maximum of 129 µg/m^2 on day 38. This increase was mainly due to the growth of cyanobacteria and prymnesiophytes (14 and 9 µg/m^2 of Chl*a* respectively), while diatoms and prasinophytes maintained constant levels around 84 and 13 µg/m^2 of Chl*a* respectively.

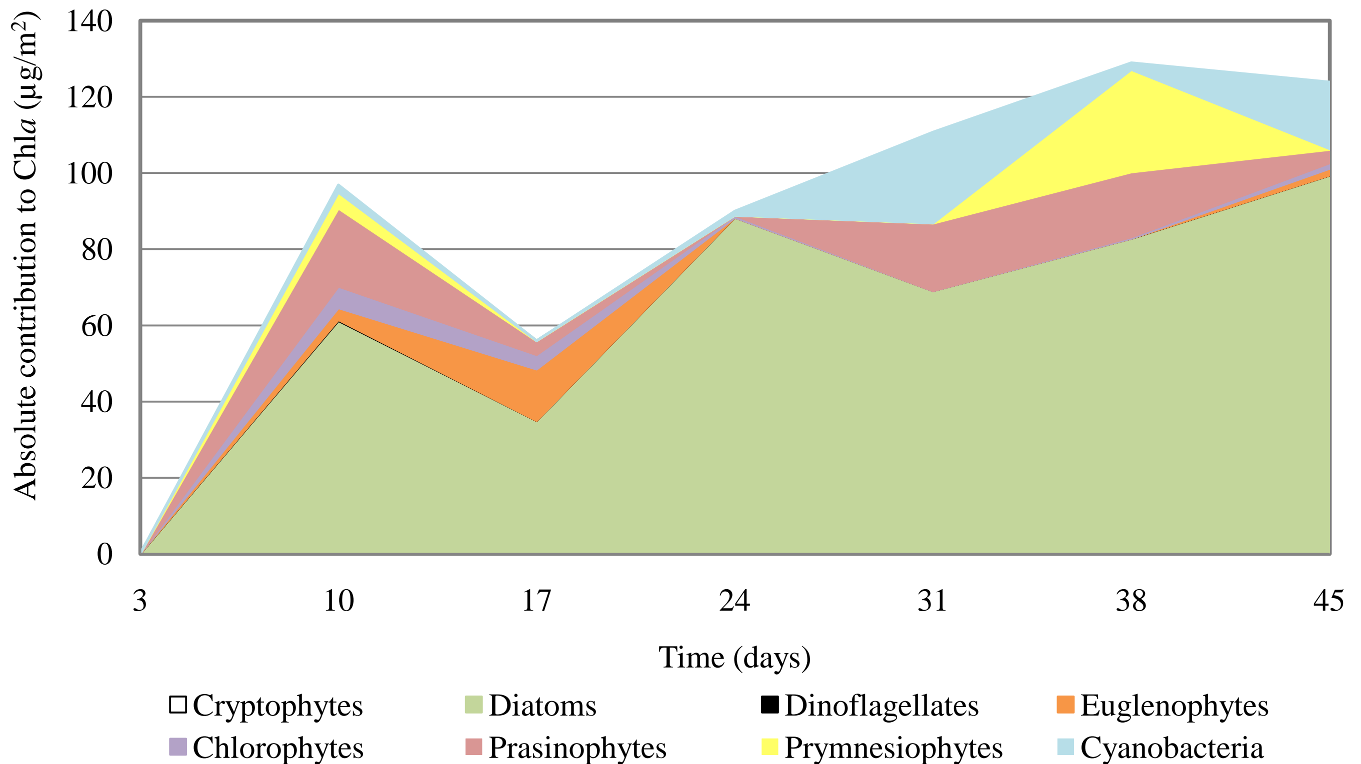

Figure 3.2. Periphyton groups mean contribution to chlorophyll *a* concentration (µg/m^2) temporal evolution.

Table 3.4 shows rank correlation matrix (Spearman's) between environmental and biological variables with periphyton groups, less significant correlations were observed than with phytoplankton groups. The pH did not have significant correlation with any group. Salinity was positively correlated with two of the three major groups (diatoms and prasinophytes). Some authors have observed that high salinity favors the proliferation of periphyton on aquaculture tanks, fundamentally diatoms (Khandeparker et al. 2017). Dissolved oxygen was directly correlated with chlorophytes, which were more abundant at the beginning of the experiment, and inversely correlated with dinoflagellates, which were more abundant at the end when oxygen was lower. Temperature was in general positively correlated with some periphyton groups, this positive effect of high temperatures has also been observed by other authors (Phinney and McIntire 1965; Zhang et al. 2009). Nutrient significant correlation with periphyton groups was minor as compared with phytoplankton. Only N-TA was positively correlated with chlorophytes, and N-NO_3^- with dinoflagellates, cryptophytes and prymnesiophytes. Chen (2001) also observed that chlorophytes easily assimilate N-TA. The scarcely significant correlations suggest that environmental variables played a minor role in determining peryphyton evolution. The major role must have been played by the different

colonization capacity, typical of each microalgae group. The colonization process is highly dependent on phytoplankton composition (Khandeparker et al. 2017). Diatoms are one of the first colonizers because they generate a large amount of extracellular polymeric substance, which helps them to bind to the substrate (Hanlon et al. 2006, Chouldhary et al. 2017). Also, diatoms with apical pads adhere easily to the substratum and usually form the first periphyton layer, that serves as anchorage to other groups such as stalk forming species (Ács et al. 2000). As shown in Table 3.4, there is a significant correlation between periphyton diatoms and phytoplankton biomass, which main contributor are diatoms. During the first phase of surface colonization, a smaller variety of groups is observed in periphyton. Over time the number of groups and their abundance increases (Ács et al. 2000; Jöbgen et al. 2004; Khatton et al. 2007a; Zhang et al. 2012). The main groups present in phytoplankton (diatoms, euglenophytes, prasinophytes, prymnesiophytes and cyanobacteria) have species that generate extracellular polymeric substance that can facilitate their adhesion to the periphyton (Claquin et al. 2008; Brake and Hasiotis 2012; Eldridge et al. 2012; Chouldhary et al. 2017). The thick periphyton cover could be responsible of stabilizing the diatoms growth at the end of the study period.

Table 3.4. Rank correlation matrix (Spearman's) between environmental and biological variables (Salinity, T –temperature, DO – dissolved oxygen, N-TA - total dissolved ammonia, $N\text{-}NO_2^-$ -nitrites, $N\text{-}NO_3^-$ - nitrates, $P\text{-}PO_4^{3-}$ - phosphates, and Ph-Chl*a* – total chlorophyll *a* in the phytoplankton) and periphyton groups.

	Salinity	**pH**	**DO**	**T**	**N-TA**	**$N\text{-}NO_2^-$**	**$N\text{-}NO_3^-$**	**$P\text{-}PO_4^{3-}$**	**Ph-Chl*a***
Diatoms	**0.264[b]**	0.012	-0.223	0.055	0.040	-0.143	0.139	0.026	**0.383[a]**
Dinoflagellates	0.062	0.102	**-0.258[b]**	**0.262[b]**	0.141	0.018	**0.281[b]**	0.227	0.169
Euglenophytes	0.080	-0.078	-0.002	-0.103	-0.100	0.038	0.054	0.083	0.113
Chlorophytes	**0.254[b]**	0.200	**0.273[b]**	-0.122	**0.405[a]**	-0.085	-0.043	-0.189	0.007
Cryptophytes	0.043	0.070	-0.198	0.217	0.175	0.065	**0.281[b]**	0.172	0.193
Prasinophytes	**0.265[b]**	0.013	0.074	-0.0114	-0.143	0.053	-0.019	0.032	0.117
Prymnesiophytes	**0.261[b]**	0.003	-0.220	**0.253[b]**	0.135	0.184	**0.357[a]**	0.221	**0.295[b]**
Cyanobacteria	-0.037	-0.030	-0.237	**0.396[a]**	0.170	0.093	0.158	0.011	0.212

Bold numbers are those that show significant correlation: [a] $P < 0.01$, [b] $P < 0.05$

The dominance of diatoms and cyanobacteria to a lesser extent, on periphyton, coincides with observations by other authors (Thompson et al. 2002; Ballester et al. 2007; Khatoon et al. 2007a; Anand et al. 2013). The presence of prasinophytes and prymnesiophytes in the periphyton has not been reported in most previous studies on aquaculture (Ballester et al. 2007; Kahoon et al. 2007a; Anand et al. 2013; Betancur-González et al. 2016). However, these studies analyzed periphyton composition by optic microscopy. In phytoplankton studies, carried out with optical microscopy, a large presence of these three groups is not reported either (Ballester et al. 2007; Kahoon et al. 2007a; Anand et al. 2013; Sruthisree et al. 2015; Betancur-González et al. 2016). On the other hand, the analysis of photosynthetic pigments by HPLC in aquaculture tanks has allowed to report the presence of these groups in phytoplankton (Jiang et al. 2016; Lemonnier et al. 2016; and Llario et al. 2018).

Due to the small size of these groups, optical microscopy often underestimates the abundance of these groups or even does not detect them. But even small concentrations of signature pigments are detected by HPLC, and this makes possible to quantify their abundance more accurately (Gocke et al. 2003; Devilla et al. 2005; Schlüter et al. 2006; Silva et al. 2008). The coexistence of diatoms, prasinophytes and prymnesiophytes in the periphyton increases its nutritional potential, since these groups are the most used as a source of food for shrimps (Benemann 1992; Jaime-Ceballos et al. 2006).

3.5. CONCLUSION

The use of HPLC coupled with CHEMTAX software is consolidated as an adequate tool for the determination of microalgae in aquaculture tanks. The analysis of signature pigments, has allowed to report the presence of previously undetected groups on periphyton, prasinophytes and prymnesiophytes, characterized by their high nutritional value. This is especially important in nurseries because shrimp grazing on periphyton can increase post-larvae survival, as periphyton is a supplementary feed source. In this experiment, diatoms dominated the phytoplankton due to its high yield in the use of all nutrients. However, the accumulation of nitrate and phosphate, could have caused the timely appearance of blooms of other phytoplankton groups. Diatoms also dominated periphyton. Nevertheless, nutrients had a minor effect on the evolution and composition of the periphyton, which was more affected by colonization processes.

3.6. ACKNOWLEDGEMENTS

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CAPÍTULO 4

Phytoplankton evolution during the creation of a biofloc system for shrimp culture

Llario, F., Rodilla, M., Escrivá, J., Falco, S., Sebastiá-Frasquet, M. T. (2018) Phytoplankton evolution during the creation of a biofloc system for shrimp culture. *International Journal of Environmental Science and Technology,* 1-12. doi: 10.1007/s13762-018-1655-5

4.1. ABSTRACT

Microalgae play a key role in the dynamics of biofloc technology aquaculture systems. Some phytoplankton groups, such as diatoms, are desired for their high nutritional value and contribution to water quality. Other groups, such as cyanobacteria, are undesired because of their low nutritional value and capacity of producing toxins. So, monitoring the phytoplankton community structure and succession is key for managing biofloc systems. However, research on phytoplankton in these systems is scarce and mostly done by microscopy. The primary objective of this research was to estimate phytoplankton community structure in shrimp biofloc system water samples, using high-performance liquid chromatography methods and CHEMTAX software. The major groups present in our system were diatoms, euglenophytes, cyanobacteria and chlorophytes, while dinoflagellates were only remarkable at the initial period. We observed a clear dominance of diatoms all along the 5 months that comprised a complete biofloc system culture. The characteristic succession of autotrophic processes by heterotrophs of the biofloc systems, was observed by the reduction of net primary production. Light intensity played a key role in determining the phytoplankton composition and abundance. Algal pigment analyses using high-performance liquid chromatography and subsequent CHEMTAX analysis in water samples was useful for estimating the phytoplankton community structure in the biofloc systems. However, we found some limitations when the biofloc system was in heterotrophic mode. Under these conditions, some dinoflagellates and cyanobacteria behaved as heterotrophs and lost or decreased their biomarkers pigments. So, further research is needed to increase knowledge on the accuracy of high-performance liquid chromatography /CHEMTAX under these conditions.

Keywords: CHEMTAX; high-performance liquid chromatography; *Litopenaeus vannamei*; pigments

4.2. INTRODUCTION

Biofloc technology (BFT) has been defined as an environmentally friendly aquaculture technique based on *in situ* microorganism production, and it is considered the "blue revolution" in aquaculture (Emerenciano et al. 2017). In BFT systems, physicochemical variables of the culture are modified to favor the proliferation of particular biotic communities, for both, improving the recirculation of nutrients (maintaining the water quality), and as direct food source for the cultured organisms (Avnimelech 2007). Biofloc systems are highly dynamic, and the physical, chemical, and biological interactions that occur into these systems are complex (Natrah et al. 2014; Emerenciano et al. 2017). Ju et al. (2008) pointed out that the relationship between environmental factors and the microbial community (bacteria and microalgae) present in the floc of aquatic culture ecosystems is one of the least understood areas of crustacean aquaculture. Knowledge of the abundance, composition and succession of the phytoplankton is a prerequisite for the successful management of BFT system (Lukwambe et al. 2015). Microalgae have an important nutritional value, that depends on its size and shape, digestibility, biochemical composition, and bioactive compounds as enzymes, vitamins, antioxidants, etc. (Emerenciano et al. 2017). We can classify phytoplankton into desired groups, because of their nutritional value and their positive effect on water quality, and not desired groups, because of their low nutritional value and bad effect on water quality (toxin production). In this sense, diatoms are one of the most desired groups, because they can enhance the contents of essential amino acids and highly unsaturated fatty acids in shrimp tissue, and increase shrimp production (Becerra-Dórame et al. 2011; Lukwambe et al. 2015; Brito et al. 2016). On the other side, cyanobacteria are generally considered an undesired group, which is favored by excessive concentrations of nitrogen and phosphorus. Cyanobacteria may produce uncontrolled blooms, that produce toxic compounds to aquatic animals, and can cause unpleasant flavors in cultured species (Sinden and Sinang 2016; Emerenciano et al. 2017). Their dominance in shrimp ponds has caused heavy economic losses (Ju et al. 2008).

Scarce studies on phytoplankton community structure have been reported on crustacean aquaculture in BFT (Casé et al. 2008; Ju et al. 2008; Becerra-Dórame et al. 2011; Schrader et al. 2011; Brito et al. 2016). Moreover, the majority focus on a short time period and do not study the full culture period. For instance, Lukwambe et al. (2015) studied the effect in shrimp production of the application of probiotics during one month; and both Becerra-

Dórame et al. (2011) (microcosms experiment) and Brito et al. (2016) (indoor trial) conducted experiments during 28 days to evaluate the productive response of the Pacific white shrimp (*Litopenaeus vannamei*). According to Ju et al. (2008), a major reason for this scarce information is the lack of rapid analytical techniques to monitor changes in the community structure of microorganisms. Almost all the BFT research used microscope methodology for studying phytoplankton composition and abundance. Only Ju et al. (2008) used an alternative methodology. They used high-performance liquid chromatography (HPLC) for detecting photopigments in samples, these pigments are algal biomarkers that allow to estimate the phytoplankton community structure. Diagnostic photopigment analyses are able to detect significant changes in phytoplankton and are routinely used for monitoring programs designed to observe trends in water quality in response to nutrient enrichment (Niemi et al. 2004; Sebastiá et al. 2012). Conventional microscope methodology has some disadvantages that can be overwhelmed using HPLC. Microscopy is time-consuming, labor intensive, potentially vulnerable to subjective judgements, and requires advanced taxonomic skills and expertise; while HPLC has proved to be rapid, reproducible, and cost-effective (Duarte et al. 1990; Schlüter et al. 2006; Ju et al. 2008; Schlüter et al. 2016). Moreover, algal groups with potentially harmful effects, such as cyanobacteria and dinoflagellates, could also be present at a low abundance in the phytoplankton community so microscope analysis could not detect them, while the high accuracy of the pigment method is able to detect all the functional groups present (Duarte et al. 1990; Schlüter et al. 2006; Schlüter et al. 2016). Ju et al. (2008) tested HPLC methodology during one week and used a multiple regression model to estimate the contribution of each phytoplankton group to total chlorophyll *a*. Multiple regression models were a common methodology during their research, but in recent years CHEMTAX software is mostly applied (Latasa et al. 2010; Garrido et al. 2011; Higgins et al. 2011; Ahmed et al. 2016) and it is recommended by the SCOR (Scientific Committee on Oceanic Research) (Roy et al. 2011).

The primary objective of this research was to estimate phytoplankton community structure in shrimp BFT water samples, using HPLC analysis and CHEMTAX software. The secondary objectives were to study the phytoplankton changes in the culture system during a complete biofloc culture cycle with high sampling frequency (weekly), and to analyze the environmental parameters that can affect the phytoplankton population. This research was developed in Gandia (València, Spain) from May to October 2016.

4.3. MATERIALS AND METHODS

4.3.1. Shrimp

Postlarvae white shrimp (PL) were purchased from a commercial laboratory (Shrimp Improvement Systems, SIS, Florida, USA), they were certificated as free of pathogen. PLs were moved to Universitat Politècnica de València (UPV) – Spain for BFT system experiment development. The *L. vannamei* shrimp were transferred to a nursery laboratory, where the PLs grow up to 0.0675 ± 0.0433 g, at which time the experiment started.

The shrimp were distributed in 9 square tanks filled with 2,250 L of water and with a surface of 3.2 m^2 each tank. Each tank was filled with disinfected seawater which had a salinity level of 22.5. The tanks were located in a greenhouse and constantly individually aerated. Shrimp density was 200 shrimp/m^2. The greenhouse system is very useful, in Mediterranean area, for shrimp culture during the cold and temperate seasons. During the hot season, the greenhouses need to be covered with different awnings to keep water temperature between 28 - 32ºC. Temperatures higher than 32ºC are critical for shrimp culture (Van Wyk and Scarpa 1999). In our study, water temperature was kept to optimum values with the following system: 1) at the beginning of the study period (May, 5) the greenhouse roof was covered with a white awning, 2) on day 59 (July, 8) the awning was substituted by a black one, and 3) on day 136 (September, 29) the black awning was removed, due to lower environmental temperature at the end of summer.

Every day the shrimp were fed with commercial feed (Le Gouessant) specifically designed for *L. vannamei*. Feed amount was calculated according to the shrimp biomass, according to Jory et al. (2001). Feeding was provided twice a day, 40% in the morning and 60% in the afternoon, and distributing the feed in feed trays.

Biofloc development was achieved following the methodology proposed by Avnimelech (1999) and Ebeling et al. (2006). The initial fertilization of the system was done with sucrose, with a theoretical 15:1 carbon/nitrogen ratio. During the experiment, sucrose was added when the ammonia reached a concentration greater than 1 mg/L. Renewal of the water during the experiment was minimal and was performed when the nitrite level reached 15 mg/L. Higher nitrite levels could cause mortality in the shrimp *L. vannamei*, as indicated by Lin and Chen (2003).

4.3.2. Environmental parameters

Dissolved oxygen (DO), salinity and temperature were monitored in situ, using a multi-parameter probe (YSI ProODO and WTW Multi 340i respectively) twice a day. pH was measured once a day using pH-Meter BASIC 20^{+} the Crison.

Every two days an aliquot of water was collected to determine the concentration of total dissolved ammonia (N-TA mg/L) using the methodology described by Baumgarten et al. (2010), nitrites ($N\text{-}NO_2^-$ mg/L), using the methodology of Bendschneider and Robinson described in Baumgarten et al. (2010), the nitrates ($N\text{-}NO_3^-$ mg/L) were analyzed by means of the difference between nitrites plus nitrates using the methodology described by Grasshoff (1976) and phosphates ($P\text{-}PO_4^{3-}$ mg/L) were analyzed following the colorimetric reaction described by Murphy and Riley (1962).

The biofloc volume (mL/L) and light intensity (lux) were measured weekly. The biofloc volume (BV) was determined by placing one litre of water in an Imhoff cone, following the methodology described by Avnimelech (2007). The light intensity was measured with a luxometer (Delta OHM HD9221).

4.3.3. Biological parameters

Samples for phytoplankton pigment analysis were filtered on GF/F fiberglass filters (25 mm diameter) once a week. Pigments were extracted using acetone (100% HPLC grade) and were measured using reverse-phase high-performance liquid chromatography (HPLC). The HPLC method employed was that proposed by Wright et al. (1991) slightly modified as per Hooker et al. (2001). The system was calibrated with external standards obtained commercially from the DHI Water and Environment Institute (Hørsholm, Denmark). Phytoplankton signature pigments analyses are able to detect significant changes in phytoplankton community composition over a broad range of time scales (Sebastiá et al. 2012).

In order to identify the phytoplankton groups present in the biofloc system, we observed a sample each tank in the microscopy some weeks, the aim of these samples is supplementing information on group presence for CHEMTAX. Utermohl (1985) was used for micro and macroplanktonic cell size. Phytoplankton samples were fixed with formaldehyde, concentrated according to UNE EN15204:2006, based on Utermohl (1985), and qualitatively examined under a LEICA DM IL inverted microscope.

Once the concentration of important photosynthetic pigments was determined, the phytoplankton community was studied using the CHEMTAX program (Mackey et al. 1996) version 1.95 (S. Wright, pers. comm.) to obtain the contribution to total chlorophyll *a* from the phytoplankton groups identified with microscopy. In order to identify groups of samples with similar characteristics, a cluster analysis was performed using STATGRAPHICS Centurion XVI.I to group samples according to pigments concentration. City block distances were calculated and samples clustered according to Ward's method. Pigment samples were separated into two subsets because it is highly recommended to apply CHEMTAX to dataset where pigment ratios within the different groups do not change (Latasa et al. 2010). CHEMTAX was applied independently to each subset to obtain the contribution of eight phytoplankton groups to the Chl*a* stock: diatoms, dinoflagellates, euglenophytes, chlorophytes, cryptophytes, prymnesiophytes, prasinophytes, and cyanobacteria. The procedure was described in Latasa et al. (2010) and a complete description can be found in Sebastiá and Rodilla (2013). The final matrix used to estimate the contribution of the different groups to Chl*a* stock is presented in Table 4.1.

During all the experiment net primary production of the water column (mgO_2 / (L·h)) was measured once a week, using the equation proposed by Strickland (1960). Three transparent bottles were filled with water culture, and were left dangling to 3 cm under water surface. In each tank, average net primary productivity was calculated 8 hours after. Net primary productivity informs about the trophic state of the biofloc system, a positive net primary production indicates that the system is autotrophic, while a negative one indicates that the system is heterotrophic.

$$\text{Net primary production}\left(\frac{\text{mgO}_2}{\text{L}\cdot\text{h}}\right)=\left(\frac{\text{finalO}_2\text{ light bottle}-\text{initialO}_2\text{light bottle}}{\text{time}}\right)$$

Table 4.1. Matrix of pigment to Chl*a* ratios obtained from CHEMTAX for the samples of both clusters. Per correspond to peridinin, 19’But to 19'butanoyloxyfucoxanthin , Fuc to fucoxanthin, 19’Hex to 19’hexanoyloxyfucoxanthin, Neo to neoxanthin, Pras to prasinoxanthin, Viol to violaxanthin, Allo to alloxanthin, Lut to lutein, Zea to zeaxanthin and Chl*b* to chlorophyll *b* .

Class / Pigment	Per	19'But	Fuc	19'Hex	Neo	Pras	Viol	Allo	Lut	Zea	Chl*b*
Diatoms											
Cluster 1	-	-	0.290	-	0.001	-	-	-	-	-	-
Cluster 2	-	-	0.387	-	0.001	-	-	-	-	-	-
Dinoflagellates											
Cluster 1	0.569	-	-	0.018	-	-	-	-	-	-	-
Cluster 2	0.333	-	-	0.025	-	-	-	-	-	-	-
Euglenophytes											
Cluster 1	-	-	-	-	0.017	-	-	-	-	-	0.427
Cluster 2	-	-	-	-	0.030	-	-	-	-	-	0.587
Chlorophytes											
Cluster 1	-	-	-	-	0.021	-	0.018	-	0.087	0.040	0.183
Cluster 2	-	-	-	-	0.050	-	0.046	-	0.022	0.067	0.272
Cryptophytes											
Cluster 1	-	-	-	-	-	-	-	0.121	-	-	-
Cluster 2	-	-	-	-	-	-	-	0.127	-	-	-
Prasinophytes											
Cluster 1	-	-	-	-	0.065	0.017	0.109	0.000	0.018	0.082	0.421
Cluster 2	-	-	-	-	0.047	0.305	0.053	0.000	0.021	0.072	0.236
Prymnesiophytes											
Cluster 1	-	0.011	0.236	0.278	-	-	-	-	-	-	-
Cluster 2	-	0.012	0.243	0.257	-	-	-	-	-	-	-
Cyanobacteria											
Cluster 1	-	-	-	-	-	-	-	-	-	0.592	-
Cluster 2	-	-	-	-	-	-	-	-	-	0.260	-

4.3.4. Statistical analysis

Previously to statistical analysis, we calculated weekly average of environmental parameters to be able to compare with phytoplankton pigments data, collected weekly. Normality and homoscedasticity of all variables were tested before multivariate analysis. As all the variables were not normally distributed, a non-parametric one-way analysis of variance (Kruskal–Wallis) was performed to statistically assess variations in the median fraction of all monitored variables within the experimental tanks. This analysis was also used for comparing chemical parameters, biofloc volume and phytoplankton absolute composition in different lighting conditions (white awning, black awning and no awning). Spearman rank correlation analyses were performed on environmental parameters (pH, temperature, DO, N-TA, N-NO_2^-, N-NO_3^-, P-PO_4^{3-} and biofloc volume) and phytoplankton groups, Chl*a* and net primary production in order to examine significant relationship.

Complementarily, the redundancy analysis (RDA) was selected from among the different multivariate ordination methods available (Braak and Smilauer 2002). Phytoplankton pigments, shrimp weight and net primary production were included in CANOCO 4.5 as dependent variables and environmental variables were included as independent variables. The statistical significance of the relationships was evaluated using Monte Carlo permutation tests with a manual forward selection procedure, under 499 permutations (Seoane et al. 2011).

4.4. RESULTS

During the experiment no statistically significant differences were observed on physicochemical parameters within the experimental tanks according to Kruskal–Wallis analysis results ($P > 0.05$). The average recorded pH was 7.79 ± 0.37, whereas the average temperature and dissolved oxygen in the water were 27.6 ± 1.8 ºC and 5.96 ± 0.40 mg/L, respectively. The salinity was stable during all the experiment with an average of 22.5 ± 0.0. The values of light intensity, chemical parameters and biofloc volume, varied along the study period, showing statistically significant differences. The mean, standard deviation (SD) and Kruskal-Wallis analysis *P*-value are presented in Table 4.2, for three periods: white awning (beginning), black awning (middle) and no awning (end). N-NO_3^-, P-PO_4^{3-} and biofloc volume showed a clear increasing trend.

Table 4.2. Average of light intensity, chemical parameters and biofloc volume for the three periods studied: white awning (beginning), black awning (middle) and no awning (end). Kruskal-Wallis analysis significance results are shown in *P*-value column.

Variables	White awning	Black awning	No awning	*P*- value
Light intensity (lux)	3,106 ± 932	1,477 ± 1546	4,346 ± 1787	0.000
N-TA (mg/L)	0.24 ± 0.45	0.07 ± 0.06	0.17 ± 0.05	$7.7x10^{-11}$
N-NO_2^- (mg/L)	3.55 ± 4.88	10.31 ± 7.58	0.26 ± 0.13	0.000
N-NO_3^- (mg/L)	0.35 ± 0.27	12.32 ± 14.33	54.02 ± 9.52	0.000
P-PO_4^{3-} (mg/L)	0.48 ± 0.59	2.83 ± 2.12	9.57 ± 2.81	0.000
BV (mL/L)	1.9 ± 3.0	9.6 ± 7.3	15.8 ±6.8	0.000

The following signature pigments were detected in water samples: peridinin, fucoxanthin, neoxanthin, prasinoxanthin, violaxanthin, diadinoxanthin, alloxanthin, lutein, zeaxanthin and chlorophyll *b*. According to pigment analysis and microscope observations these phytoplankton groups were present: diatoms, dinoflagellates, chlorophytes, cryptophytes, euglenophytes, prasinophytes, prymnesiophytes and cyanobacteria. A Kruskal-Wallis analysis was performed to detect statistically significant differences in phytoplankton groups abundance (absolute contribution to chlorophyll *a*, µg/L) between tanks (statistically significant differences *P*-value < 0.01), the analyses are presented in Table 4.3. No statistically significant differences were found in any group abundance between experimental tanks, except for diatoms ($P = 1 \cdot 10^{-8}$). Diatom absolute abundance was significantly higher in tanks 1, 2 and 3. The contribution to Chl*a* of each one of the observed phytoplankton groups is represented in figure 4.1 along the study period. But, due to the observed differences, we calculated the mean concentration for tanks 1, 2 and 3 (n = 3), figure 4.1a, and, for the rest of tanks (n = 6), figure 4.1b.

A progressive Chl*a* increase is observed during the first weeks (day 1 to day 52) (Figure 4.1). The first two weeks Chl*a* concentration was below the detection limit due to the initial disinfection process. On day 52 (week 8) an absolute maximum concentration of 496 ± 236

μg/L was observed. After day 59 Chl*a* concentration started to decrease, and remained below 200 μg/L until day 143. Later the Chl*a* concentration increased until 411 μg/L. The Kruskal-Wallis analysis showed statistically significant differences among periods (*P*-value), with significantly lower light intensity during black awning coverage. Thus, the temporal variation in Chl*a* concentration could be related to lighting conditions.

Table 4.3. Average of phytoplankton groups abundance (μg/L), chlorophyll *a* (μg/L) and net primary production (mgO_2 / (L·h)) and standard deviation for the three periods studied: white awning (beginning), black awning (middle) and no awning (end). Kruskal-Wallis analysis significance results are shown in *P*-value column.

Variables	White awning	Black awning	No awning	*P*- value
Diatoms	70.20 ± 64.54	91.48 ± 76.79	99.32 ± 73.69	0.087
Dinoflagellates	15.03 ± 29.50	0.04 ± 0.15	0.10 ± 0.14	$2.8x10^{-11}$
Euglenophytes	32.86 ± 35.82	21.82 ± 22.25	84.16 ± 47.14	$3.3x10^{-11}$
Chlorophytes	67.38 ± 93.00	3.68 ± 14.77	29.04 ± 40.69	$3.7x10^{-10}$
Cryptophytes	1.29 ± 1.94	0.59 ± 1.98	0.89 ± 1.27	0.001
Prasinophytes	4.49 ± 6.37	4.14 ± 3.54	1.39 ± 2.43	$2.8x10^{-4}$
Prymnesiophytes	0.12 ± 0.25	0.03 ± 0.09	0.17 ± 0.24	0.003
Cyanobacteria	99.94 ± 142.45	9.43 ± 22.16	75.55 ± 65.55	$8.4x10^{-7}$
Chlorophyll *a*	226.57 ± 247.40	131.23 ± 95.48	290.62 ± 166.12	$2.8x10^{-3}$
Net primary production	0.32± 0.36	-0.05± 0.34	-0.29 ± 0.10	0.000

According to net primary production values, autotrophic processes predominate during the first weeks, a period characterized by the absence of nitrifying bacteria and high levels of T-NA. This period coincides with the presence of white awning (Figure 4.1). Subsequently, nitrifying bacteria develop, and net photosynthesis decreases until reaching negative values when heterotrophy predominates in the system.

The same temporal trend was observed in all the tanks for diatoms, however, concentration values differ (Figure 4.1a). Tanks 1, 2 and 3 showed the higher diatoms abundance along all the study period, the average value was 138 ± 78 µg/L Chl*a*. The other tanks showed an average value of 60 ± 54 µg/L Chl*a*. Dinoflagellates were present only from day 17 to day 31 with an average value of 35 ± 37 µg/L Chl*a*. The rest of the study period their presence were minimal (<1.8 µg/L Chl*a*). Chlorophytes were mainly abundant during day 31 to day 59 with an average value of 94 ± 98 µg/L Chl*a*. They were also present from day 87 to 94, and from day 143 to 164, but their abundance was lower and average value was 29 ± 41 µg/L Chl*a*. Euglenophytes temporal trend was similar to that observed for Chl*a* (phytoplankton biomass) with two peaks. Their average abundance was 37 ± 40 µg/L Chl*a*. Cyanobacteria also show a similar trend to Chl*a* characterized by two peaks, however, they reduced their abundance to minimums from day 66 to 136. Their average abundance was 100 ± 142 µg/L Chl*a* during the first peak, and 76 ± 66 µg/L Chl*a* during the second peak. Prasinophytes were a low abundant group, that appears on day 17 and has an average of 4 ± 5 µg/L Chl*a*. Cryptophytes and prymnesiophytes are the groups less abundant with average values of 0.87 ± 1.88 µg/L Chl*a* and 0.08 ± 0.20 µg/L Chl*a* respectively.

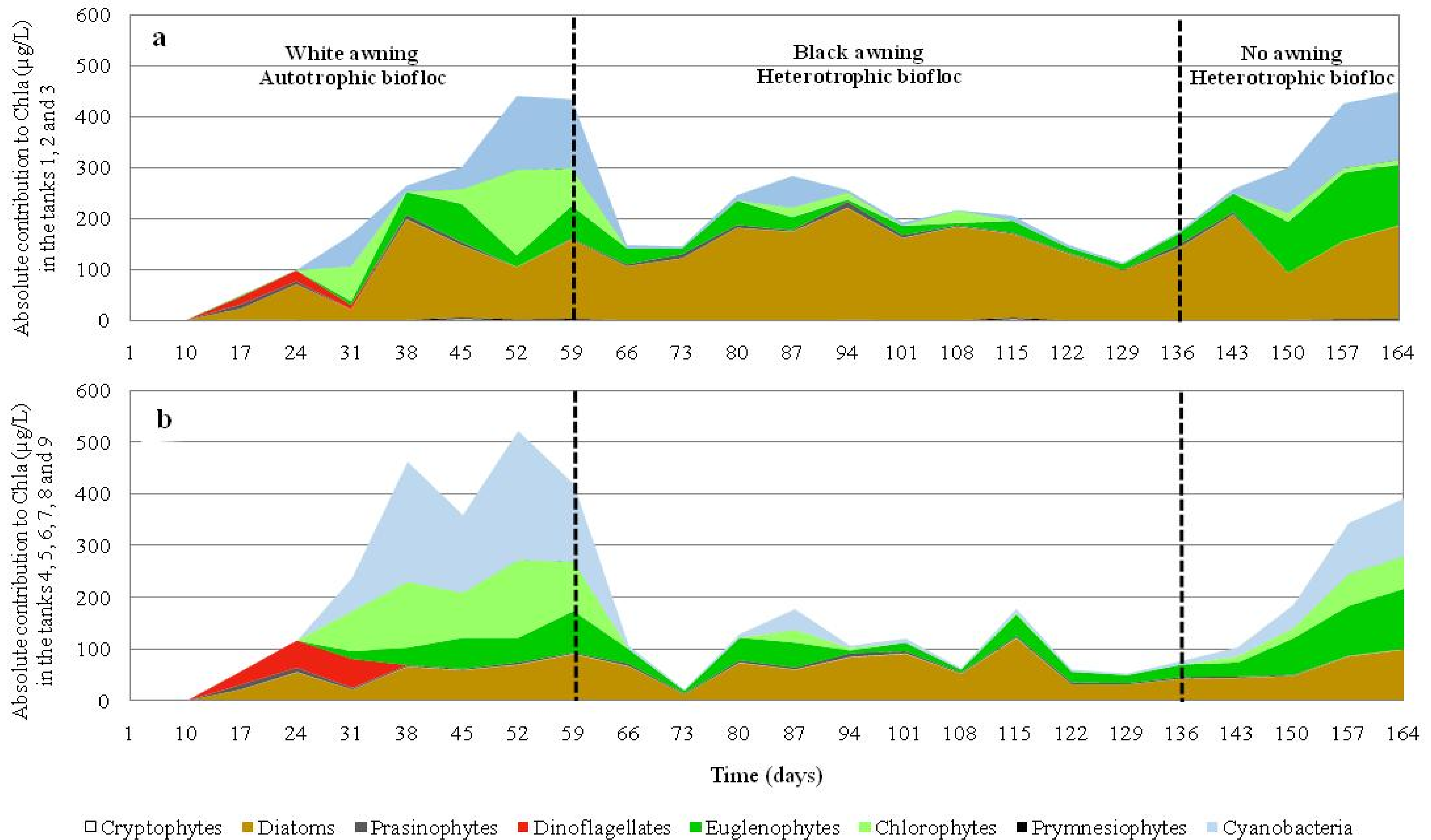

Figure 4.1. Phytoplankton groups mean contribution to chlorophyll a concentration (µg/L) temporal evolution. a) Tanks 1, 2 and 3 b) Tanks 4, 5, 6 and 7.

Spearman rank correlation analyses were performed on environmental parameters (pH, temperature, DO, N-TA, N-NO_2^-, N-NO_3^-, P-PO_4^{3-} and biofloc volume) and phytoplankton groups, Chl*a* and net primary production in order to examine significant relationship. Table 4.4 shows the correlation results.

Table 4.4. Rank correlation matrix (Spearman's) between environmental (T^a –temperature, DO – dissolved oxygen, N-TA - total dissolved ammonia, N-NO_2^- -nitrites, N-NO_3^- - nitrates, P-PO_4^{3-} - phosphates and BV – biofloc volume) and biological variables (phytoplankton groups).

	T^a	**DO**	**pH**	**N-TA**	**N-NO_2^-**	**N-NO_3^-**	**P-PO_4^{3-}**	**BV**
Diatoms	**0.210**[a]	**-0.321**[a]	**-0.329**[a]	-0.078	**0.282**[a]	0.119	**0.348**[a]	**0.299**[a]
Dinoflagellates	**0.192**[a]	**0.191**[a]	**0.212**[a]	0.132	-0.032	-0.034	**-0.135**[b]	-0.115
Euglenophytes	-0.006	**-0.510**[a]	**-0.492**[a]	**0.191**[a]	0.132	**0.185**[a]	**0.549**[a]	**0.509**[a]
Chlorophytes	**0.219**[a]	-0.114	-0.049	**0.186**[a]	0.077	0.009	0.115	**0.150**[b]
Cryptophytes	0.126	-0.053	-0.026	-0.064	0.118	0.018	0.084	0.121
Prasinophytes	0.131	-0.110	-0.043	-0.101	**0.258**[a]	-0.089	0.054	0.055
Prymnesiophytes	-0.064	**-0.194**[a]	**-0.260**[a]	-0.091	**0.151**[b]	**0.223**[a]	**0.345**[a]	**0.386**[a]
Cyanobacteria	**0.179**[a]	**-0.427**[a]	**-0.351**[a]	**0.190**[a]	**0.239**[a]	0.086	**0.433**[a]	**0.445**[a]
Chlorophyll *a*	**0.302**[a]	**-0.295**[a]	**-0.235**[a]	**0.159**[b]	**0.208**[a]	0.052	**0.311**[a]	**0.288**[a]
Net primary production	**0.547**[a]	**0.532**[a]	**0.735**[a]	0.032	0.008	**-0.504**[a]	**-0.743**[a]	**-0.650**[a]

[a] $P < 0.01$, [b] $P < 0.05$

Complementarily, the redundancy analysis (RDA) performed with CANOCO 4.5 is showed in Figure 4.2. For a detailed interpretation of the graphs, see Ter Braak (1994). The RDA retained five variables: DO, N-TA, N-NO_3^-, P-$PO_4^{3-}$and biofloc volume. These variables together explained 39% of the variance in the biological variables (phytoplankton composition, phytoplankton biomass (Chl*a*) and net primary production). The biological variables were classified according to their association with the environmental variables. Distance between sample points symbols in the diagram approximates the dissimilarity of their pigment composition, measured by their Euclidean distance (Ter Braak 1994). We observed that samples taken from day 1 to day 38 are grouped by days in the triplot chart (Figure 4.2), where the samples of the same day are located very close. That shows that phytoplankton composition is very similar in all the tanks for the same day, while between different days is more diverse. Samples from the rest of the study period are not grouped daily. Environmental variable arrows point in the expected direction of the steepest increase of values of environmental variable. Axis 1 shows a gradient of the variables: DO increasing to the right side, and an opposite gradient of P-PO_4^{3-} and biofloc volume. The acute angle between variable arrows indicates high correlations between individual environmental variables. For example, P-PO_4^{3-} and biofloc volume show a positive correlation, which is confirmed by the Spearman rank analysis. High values of these variables are highly correlated with euglenophytes and cyanobacteria. In the opposite side, high DO is strongly correlated with dinoflagellates, and to a minor extent to net primary production (see also Spearman Rank Table 4.4). Axis 2 shows a gradient of N-TA and N-NO_3^-. High concentration of N-TA is strongly correlated with chlorophytes and prasinophytes abundance (see also Spearman Rank Table 4.4). High concentration of N-NO_3^- is strongly correlated with prymnesiophytes abundance (see also Spearman Rank, Table 4.4). The sample symbols can be projected perpendicularly onto the line overlaying the arrow of particular environmental variable. The sample points are in the order of predicted increase of values of the particular environmental variable, so sample points projecting onto the coordinate origin are predicted to correspond to samples with an average value of that environmental variable. For instance, at the beginning of the study period, samples from day 1 to day 31, we have high DO levels. Samples are grouped temporally in three groups. In the first group, we find samples from day 1 to day 10. This first two weeks all pigments concentration was below the detection limit. In the second group, we find sample from day 17 to 31. These samples are characterized by high increasing N-TA concentrations. On day 31 the nitrification processes started and N-NO_2^- was detected. From day 1 to day 24 the highest DO were observed. In the third group, we include all other

samples, these samples do not show a temporal gradient. During this period a point cloud is observed in the left side of the Figure 4.2, Canoco graph.

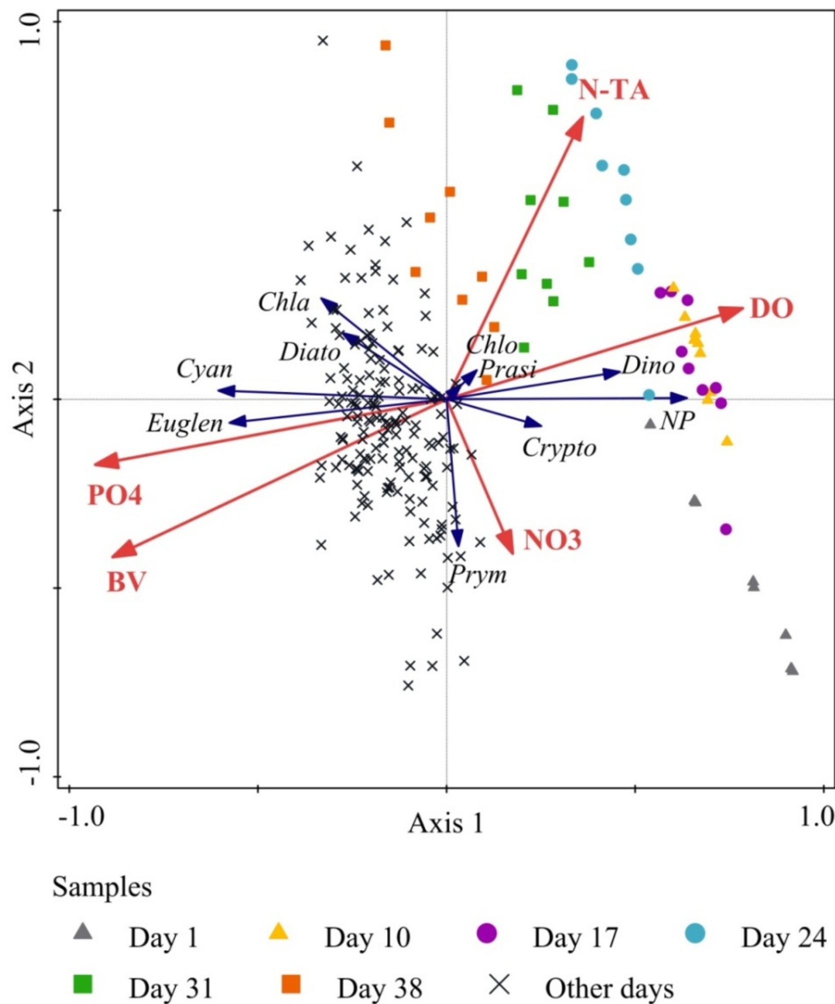

Figure 4.2. Correlation plots of the RDA, on the relationship between the environmental variables (gray arrows), the biomass of the phytoplankton groups, the net primary production and total chlorophyll *a* (black arrows) and samples. Sample symbol corresponds to the sampling day detailed in the legend. Labels in black arrows mean: Diato-diatoms, dino-dinoflagellates, chloro-chlorophytes, crypto-cryptophytes, euglen-euglenophytes, prasi-prasinophytes, prym- prymnesiophytes, cyan-cyanobacteria, NP-net primary production and Chl*a*-Chlorophyll *a*. Labels in gray arrows mean: DO–dissolved oxygen, BV–biofloc volum, PO4–phosphates, NO3–nitrates, N-TA–total dissolved ammonia.

4.5. DISCUSSION

All the water quality parameters in the tanks remained within the recommended rate suitable for growing *L. vannamei*, specially the values of pH, temperature, dissolved oxygen and salinity (Van Wyk and Scarpa 1999). The biofloc systems are characterized by a peak of N-TA, followed by peak of N-NO_2^- and finally an accumulation of N-NO_3^- in the system, what coincides with the observed evolution of these variables (Table 4.2) (Azim and Little 2008; Avnimelech 2009). Also, in all biofloc system we can observe an accumulation of PO_4^{3-} and biofloc volume (Ray et al. 2011; Correia et al. 2014). The levels of ammonia and nitrites can be toxic, but, during the experiment, these levels were maintained within the limits of safety determined by Lin and Chen (2001) and (2003). The accumulation of P-PO_4^{3-} and biofloc volume started from the beginning of experiment and followed during all the time. P-PO_4^{3-} and N-NO_3^- do not have any negative effect on shrimp. The biofloc volume values were lower than maximum recommended by Avnimelech (2009). The succession of autotrophic processes by heterotrophs, observed by the reduction of net primary production (Table 4.3 and Figure 4.1), is characteristic of the biofloc systems (Vinatea et al. 2010; Marinho et al. 2016). The significant inverse correlation of net primary production with BV and P-PO_4^{3-} (Table 4.4 and Figure 4.2), is explained because these variables values increase during heterotrophic culture phase (Ray et al. 2011; Correia et al. 2014).

Chlorophyll *a* (Chl*a*) is commonly used as a proxy of phytoplankton biomass, also in BFT systems (Gaona et al. 2011). The range of Chl*a* concentration measured is similar to the one observed in other *L. vannamei* BFT systems (Gaona et al. 2011; Baloi et al. 2013; Martins et al. 2016). At the beginning of the study period, from day 1 to 10, no pigments were detected. This is due to the process of initial chlorination of the seawater to eliminate bacteria, which also reduces the amount of phytoplankton (Yusoff et al. 2002). The temporal variation in Chl*a* concentration is related to lighting conditions, as revealed by Kruskal-Wallis analysis (Table 4.3), so it is the evolution of all the phytoplankton groups except diatoms (Table 4.3). Other authors have observed a direct relationship between reduced light intensity and phytoplankton decrease (Gaona et al. 2011; Baloi et al. 2013; Martins et al. 2016). All phytoplankton groups presented significantly lower abundances (µg/L Chl*a*) with black awning, while diatoms abundance showed no statistically significant differences (Figure 4.1).

Four major groups of phytoplankton, including diatoms, dinoflagellates, cyanobacteria and chlorophytes are usually observed in *L. vannamei* biofloc systems (Lukwambe et al. 2015; Martins et al. 2016). In our study, the major groups were diatoms, euglenophytes, cyanobacteria and chlorophytes, similar to the observed by Schrader et al. (2011) in other biofloc systems, while dinoflagellates were only remarkable at the initial period. The most abundant phytoplankton group was diatoms all along the study period (Figure 4.1). The diatom predominance is commonly observed in BFT (Schrader et al. 2011 (in some tanks); Godoy et al. 2012; Martins et al. 2016). However, other authors remark an abundance decrease at the end of their studies, and a replacement by undesired cyanobacteria (Yusoff et al. 2002; Schrader et al. 2011 (in some tanks)). This decrease has been related to a silica limitation and to a phosphorus enrichment. Martins et al. (2016) observed that silica addition was essential for the growth and maintenance of high diatom cell density in the biofloc system. Coastal waters used to fill the aquaculture tanks in our study are characterized by high silica levels. This is due to groundwater discharges rich in silica (Sospedra et al. 2017), because of the lixiviation of biogenic silica from the wetland species of Gramineae, which are characterized by high silica content (Sebastiá et al. 2012; Sebastiá and Rodilla 2013). This high initial concentration of silica can explain the maintenance of diatom levels all time long. The predominance of diatoms is highly desired because of their nutritional properties, they can enhance the contents of essential amino acids and highly unsaturated fatty acids in shrimp tissue, and their consumption improve shrimp growth (Godoy et al. 2012; Brito et al. 2016; Martins et al. 2016). On the contrary, cyanobacteria are undesired because their nutritional value is low, are commonly responsible of noxious blooms, impart unpleasant flavors to cultured animals and negatively affect water quality (Paerl and Tucker 1995; Yusoff et al. 2002; Ju et al. 2008; Schrader et al. 2011).

The Kruskal-Wallis analysis results revealed that cyanobacteria biomass was lower during low light intensity conditions (black awning). However, cyanobacteria are myxotroph organisms that can take advantage of different environmental conditions, by changing their trophic mode. Thus, in low light conditions, they can adopt heterotrophy mode and reduce their pigment content (Chl*a* and zeaxanthin) (Yu et al. 2009; Lohscheider et al. 2011; Gris et al. 2017). These can explain the lower pigment concentrations measured by HPLC, and the lower cyanobacteria biomass estimated by CHEMTAX. But, microscope controls demonstrated high abundance of filamentous cyanobacteria also during low light conditions. Positive correlation between cyanobacteria and phosphate and biofloc volume (Table 4.4 and

Figure 4.2) has also been observed in other BFT studies (Yusoff et al. 2002; Green et al. 2014). Green et al. (2014) explained that filamentous cyanobacteria help to cohesion the different components of the floc, obtaining larger aggregates.

Dinoflagellates are usually one of the dominant phytoplankton groups in biofloc cultures (Ju et al. 2008; Ballester et al. 2010; Manan et al. 2016; Marinho et al. 2016), which sometimes persist throughout the study period (Yusoff et al. 2002). In our study, peridinin, which is the signature pigment of dinoflagellates, was only present from day 17 to 31, when the biofloc system was not mature. This period was characterized by autotrophic processes, and dinoflagellates showed significant positive correlation with net primary production (Table 4.4). Although no peridinin was detected in later stages, dinoflagellates were observed under the microscope. Dinoflagellates are mixotrophic organisms, capable of feeding on various prey species, including bacteria, flagellates, diatoms, heterotrophic protists and metazoans (Ismael 2003; Jeong et al. 2010). At the same time, they are able to perform photosynthesis, thus increasing their rate of growth (Li et al. 1999). It is possible that, while the system was autotrophic, the dominant dinoflagellates in the water were autotrophic or mixotrophic dinoflagellates, with a high rate of peridinin (Jeffrey et al. 1975). As the biofloc system matures, the light and net primary production decrease, so the dinoflagellates present show a heterotrophic behavior. This would cause a decrease in the synthesis of peridinin, which is no longer detected by HPLC (Li et al. 1996). Note that, depending on the species, dinoflagellates presence can adversely affect the immune system of shrimp, due to production of toxins (Pérez-Linares et al. 2008; Campa-Córdova et al. 2009; Pérez-Morales et al. 2017). Although not all species are harmful, as some are used as a nutrient source in carcinoculture (Ge et al. 2016).

Chlorophytes are usually present in biofloc systems (Yusoff et al. 2002; Manan et al. 2011; Schrader et al. 2011). They are a desirable group as they remove ammonium (Chen 2001), and improve shrimp yield and survival (Ge et al. 2016). Maicá et al. (2012) observed, in their microscopic counts, that chlorophytes dominated biofloc systems at low salinities (2-4), but were replaced by diatoms at salinities of 25. Ju et al. (2008) also observed a predominance of lutein, signature pigment of chlorophytes (Schlüter et al. 2006), in samples with a low salinity (5-18), and an increase in fucoxanthin, signature pigment of diatoms (Schlüter et al. 2006), with increasing salinity. Only Martins et al. (2016) observed that chlorophytes dominated their biofloc culture at high salinity (37) and absence of diatoms. Our tanks have an

intermediate salinity (22.5) in which the two groups coexist, although the diatoms are more abundant as shown in figure 4.1.

Euglenophytes are one of the major groups in most biofloc systems, although they are not the most abundant (Green et al. 2014; El-Dahhar et al. 2015; Marinho et al. 2016). This group is able to adapt to waters with a wide spectrum of salinity (Figueroa et al. 1998), but they have been found in greater quantity in studies with freshwaters (Schrader et al. 2011) or low salinity waters (Ju et al. 2008). In our study, their abundance is highly correlated with biofloc volume (Figure 4.2), a relationship already observed by Green et al (2014), and with phosphates (Horabun 1997). Kingston (1999) attributed this relation to their inhibition by high light intensities, showing greater abundances in highly turbid environments, as the one characteristic of a mature biofloc system.

4.6. CONCLUSION

Algal pigment analyses using HPLC and subsequent CHEMTAX analysis in water samples can provide useful information for estimating the phytoplankton community structure in the BFT systems. This technique is very useful for monitoring the abundance variation of beneficial as well as potentially harmful algae. However, we found some limitations when the BFT systems are in heterotrophic mode. Under these conditions, some dinoflagellates and cyanobacteria behave as heterotrophs and lose or decrease their biomarkers pigments. The HPLC/CHEMTAX methodology is widely applied for monitoring nutrient enriched waters, in both continental and marine ecosystems. But, little research has been developed in heterotrophic systems. So, further research is needed to increase knowledge on the accuracy of HPLC/CHEMTAX under these conditions. The analysis of phytoplankton evolution allowed us to observe the key role played by light intensity on abundance and composition of phytoplankton during the creation of a biofloc system. In general, a major light intensity caused an increase in phytoplankton biomass as indicated by Chl*a* concentration. This increase is mainly due to higher abundances of euglenophytes, chlorophytes and cyanobacteria. However, diatoms and net primary production were not significantly affected by different light intensity. Diatoms abundance was constant all along the study period, while primary production followed the normal trend in biofloc system. Coastal waters used to fill the aquaculture tanks in our study were characterized by high silica levels, that allowed to

maintain diatom population. According to our results, and in agreement with other authors, light intensity and dissolved silica concentration are key parameters for controlling phytoplankton composition and abundance.

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4.8. REFERENCES

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CAPÍTULO 5

Application of *Bacillus amyloliquefaciens* as probiotic for *Litopenaeus vannamei* (Boone) cultivated in a biofloc system

Llario, F., Romano, L. A., Rodilla, M., Sebastiá-Frasquet, M. T., Poersch, L. H. (2018) Application of *Bacillus amyloliquefaciens* as probiotic for *Litopenaeus vannamei* (Boone) cultivated in a biofloc system. *Iranian Journal of Fisheries Science.* Accepted.

5.1. ABSTRACT

Several studies have shown that the application of probiotics in aquaculture systems with water recirculation is beneficial. Probiotics can improve growth, survival and resistance to pathogenic organisms of the cultivated species. However, their possible benefits on biofloc systems have been less studied. In this study, the benefits of *Bacillus amyloliquefaciens* bacterium, on a biofloc culture of *Litopenaeus vannamei* were evaluated. *B. amyloliquefaciens* was applied as dissolved in water. To our knowledge, no previous assays on biofloc systems have been published, and on recirculation systems it has only been tested mixed with feed. The objective of the present study was to evaluate the effect of *B. amyloliquefaciens* on water quality, growth parameters and the immune system of shrimp. Three concentrations of probiotic were tested in triplicate (9.48×10^4, 1.90×10^5, and 3.79×10^5 cfu/mL) and were compared with the control (without probiotic). Water quality parameters such as nitrogenous compounds, phosphates and suspended solids were monitored. In *L. vannamei*, growth, survival and their immune system parameters (total protein concentration, cell number with apoptosis and percentage of granular and hyaline hemocytes) were studied. The results show that the application of *B. amyloliquefaciens* did not produce significant differences in water quality or shrimp growth as compared to the control. However, it showed significant improvements in the immune system. As compared with the control treatment, an increase in the total protein concentration and granular hemocytes, and a decrease in the cell number with apoptosis in the hempolymph were observed. Thus, we can conclude that *B. amyloliquefaciens* provides greater response capacity to shrimp against the attack of pathogens in biofloc systems.

Keywords: growth parameters; immunological parameters; water quality; white shrimp

5.2. INTRODUCTION

Biofloc technology systems (BFT) is based on the development of macroaggregates composed of heterotrophic bacteria, phytoplankton, food debris, organic matter and other organisms, capable of maintaining water quality at adequate values for shrimp and fish farming (Crab et al. 2012; Emerenciano et al. 2013). When heterotrophic bacteria present in the BFT have an appropriate C:N ratio, they oxidize the total ammonia nitrogen, excreted by cultivated species (Crab et al. 2012; Emerenciano et al. 2013). This nitrification process leads to an increase in microbial biomass (Schyrve et al. 2008; Kuhn et al. 2009), which is rich in protein and is consumed as a food supplement by cultivated species, improving its growth (Schyrve et al. 2008; Kuhn et al. 2009).

BFT can have a probiotic effect on shrimp and fishes (Crab et al. 2012; Emerenciano et al. 2013). However, it has been observed that the extra addition of probiotics can enhance the beneficial effects of BFT (Souza et al. 2012). Probiotics are live microorganisms, usually bacteria, which effects vary depending on method of application, dosage and bacteria species (Van Hai and Fotedar 2010). There are three application methods: through larvae inmersion in the probiotic culture (Van Hai and Fotedar 2010), mixed with feed or dissolved in water. Immersion in probiotic cultures is only feasible for very small specimens due to its difficult handling (Van Hai and Fotedar 2010). The application in feed is the most usual and is usually done during the manufacturing process (Wang et al. 2008). However, the administration of probiotics in commercial feeds prevents producers from selecting separately the most appropriate feed and probiotic for their cultures. Probiotics dissolved in water are used in a wide dosage range, depending on the species and culture conditions, doses from 10^3 to 10^8 cfu mL^{-1} being the most usual (Zhou et al. 2009; Van Hai and Fotedar 2010; Souza et al. 2012; Ramezani-Fard et al. 2014).

In aquaculture systems, with water recirculation, it has been observed that the application of probiotics can produce the following positive effects: (i) decrease occurrence of diseases, by direct competition with pathogenic organisms or by secretion of bactericidal substances (Pandiyan et al. 2013), (ii) consumption of nitrogenous compounds in water, which produces an improvement in water quality (Dalmin et al. 2001), (iii) enzyme secretion in the intestine of the cultivated species that help food digestion (Zhou et al. 2009), (iv) stimulation of the immune system, increasing the protection of the host against pathogens (Rengpipat et al. 2000).

In BFT, some studies have been conducted on the effect of probiotics composed of a combination of different genera or species of bacteria. Krummenauer et al. (2014) observed that by applying probiotics composed of different genera of bacteria (*Bacillus* sp., *Enterococcus* sp., *Thiobacillus* sp., *Paracoccus* sp. and *Lactobacillus* sp.) the effects of a *Vibrio parahemolyticus* infection on *L. vannamei* were reduced. Rengpipat et al. (2000) also observed, the benefitial effects of *Bacillus* sp. against *Vibrio* sp. Vita (2008) observed improvements in the size of white shrimp *L. vannamei* by using a probiotic composed by two species of *Bacillus* sp. (*Bacillus licheniformis* and *Bacillus subtilis*). Souza et al. (2012) tested three probiotics, a *Bacillus* sp. mix (*Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus), a m*ultistrain probiotic (*Bacillus* sp., *Enterococcus* sp. and *Lactobacillus* sp.) and a monoespecific probiotic with *Bacillus cereus* var. *toyoi*, and observed that different probiotic bacteria improved growth, survival and the immune system of *Farfantepenaeus brasiliensis*. In this experiment, Souza et al. (2012), observed that a combination of *Bacillus* sp., had better results than other bacteria combinations. However, the common technique of studying the effect of combined probiotics prevents the determination of the role of each genus or species of probiotic in aquaculture systems in general, and in BFT in particular. *Bacillus amyloliquefaciens* is a probiotic bacterium, which has been applied in feed to *Litopenaeus vannamei*, *Cyprinus carpio* and *Oreochromis niloticus,* cultured in water recirculation systems, by Camacho (2012), Nuez-Ortín (2013), Huang et al. (2015) and Saputra et al. (2016). Their results showed the following advantages of applying *B. amyloliquefaciens* as a probiotic in water recirculation systems: 1) it produces digestive enzymes which improve the growth of the cultured species (Nuez-Ortín 2013); 2) it has bactericidal character produced by the secretion of barnase and lactic acid (Cao et al. 2011; Nuéz-Ortín 2013); 3) it improves the immune system and survival of guests (Huang et al. 2015; Saputra et al. 2016); 4) it has bactericidal character against *Vibrio alguinolyticus*. Camacho (2012); and 5) it has good properties to promote biofloc creation because it has higher protein levels and grow faster than other bacteria (Bao 2014). In spite of these characteristics, there are no references to the application of *B. amyloliquefaciens* in biofloc systems and to its application directly in water.

In this study, we assessed the effect of a monospecific probiotic, *B. amyloliquefaciens*, applied dissolved in water. The objective is to evaluate the effect of *B. amyloliquefaciens* on water quality, zootechnical development and the immune system of white shrimp in a biofloc system.

5.3. MATERIAL AND METHODS

5.3.1. Shrimp

The shrimp post larvae (PLs) were bought from a comercial laboratory (Aquatec), which certificated that PLs were free of phatogens, and moved to Universidade Federal do Rio Grande (FURG) instalations. After acclimation, the PLs underwent an intermediate nursery phase. This phase was carried out in a greenhouse, in 35 m^3 tank, provided with mature bioflocs at a temperature of 28ºC and a salinity of 30. The larvae were cultivated at a density of 1500 shrimps/m^2 and were fed 4 times a day with feed (38% protein), they remained in the nursery until reaching 2.0. ± 0.7 g of weight. Then, the shrimp were moved to experimental tanks to begin the experimen, which lasted 42 days.

The shrimp were distributed in twelve 500 L square tanks (1,25 m^2 each one). Each tank was inoculated with 50 L of heterotrophic biofloc from a previous culture of *L. vannamei*, and filled with 450 L of disinfected marine water (final salinity 17.33 ± 0.59 g). The tanks were constantly individually aerated. Shrimp density was 300 shrimp per cubic metre.

Ecobiol Plus®, a probiotic made up of *B. amyloliquefaciens* was tested as follows. Before applying Ecobiol Plus® to culture water, five samples of probiotic were seeded on soy agar, during 24 hours at 30°C, to determine the accurate concentration of *B. amyloliquefaciens* CECT-5940. Four treatments were essayed in triplicate: 1) control treatment (CO) without probiotic; 2) treatment A (TA) with Ecobiol Plus® in a dose of 9.48×10^4 cfu/mL; 3) treatment B (TB) with Ecobiol Plus® in a dose of 1.90×10^5 cfu/mL (twice the recommended dose of probiotics); and 4) treatment C (TC) with Ecobiol Plus® in a dose of 3.79×10^5 cfu/mL (four times the recommended dose). The recommended dose is the average recommended dose for other probiotics applied in water, since there are no previous studies of the application of *B. amyloliquefaciens*.

The shrimp were fed daily with commercial feed (Guabi – Active 38) specifically designed to encourage growth in *L. vannamei*. The quantity of feed was calculated according to shrimp biomass (Jory et al. 2001). The feed was provided twice per day, 40% in the morning and 60% in the afternoon, and distributed on feeding trays. Water renewal during the experiment was minimal, and limited to avoid surpass of 8 mg/L nitrite. Levels above that threshold value can cause mortality in the shrimp *L. vannamei*, as indicated by Lin and Chen (2003).

The maintenance of the biofloc system was carried out following the methodology proposed by Avnimelech (1999) and Ebeling et al. (2006). The system was fertilized with molasses of sugar cane. Molasses were administered when total ammonia nitrogen reached a concentration greater than 1 mg/L, to mantain a carbon:nitrogen relationship of 15:1.

5.3.2. Water quality

pH, dissolved oxygen, salinity and temperature were monitored in situ, using a multi-parameter probe (YSI Professional Plus), twice a day (morning and afternoon).

Every two days, a water alliquot was collected to determine the concentration of the following nutrients: 1) total ammonia nitrogen (N-TA) using the methodology described by UNESCO (1983); 2) nitrites ($N\text{-}NO_2^-$) using the methodology of Bendschneider and Robinson (1952) described in Baumgarten et al. (2010); 3) nitrates ($N\text{-}NO_3^-$) were analyzed using the methodology described by Grasshoff (1976); and 4) phosphates ($P\text{-}PO_4^{3-}$) were analyzed following Murphy and Riley (1962).

The biofloc volume (BV) was monitored weekly by placing one liter of water in an Inhoff cone, following the methodology described by Avinmelech (2009). Total suspended solids (TSS) were determined as described by Baumgarten et al. (2010), an aliquot of 50 mL from each tank was filtered (0.45 µm) and filters were dried for approximately 24 hours at 105ºC. Then, non volatile suspended solids (NVSS) and volatile suspended solids (VSS), were calculated according to Baumgarten et al. (2010) after calcination in a muffle. Water alkalinity was monitored at the beginning, in the middle and at the end of the experiment, using the trimetric method of APHA (1998).

5.3.3. Growth parameters

30 shrimp per tank were measured using a 0.1 g precision digital scale (Marte Slim). These measurements were done at the beginning of the experiment and every 10 days during the study period, to monitor weight growth of the shrimp (g) and to re-adjust the feed amount. Once the experiment finished survival, weight gain (WG), weekly weight gain (WWG), biomass gain (BG), feed conversion rate (FCR) and productivity (P) were determined following the equations described by Furtado et al. (2011) and Macias-Sancho et al. (2014).

$$\text{Survival} = \frac{\text{final shrimps amount}}{\text{initial shrimps amount}} \text{x } 100$$

$$\text{WG} = \text{final wet weight} - \text{initial wet weight}$$

$$\text{WWG} = \frac{\text{final wet weight} - \text{initial wet weight}}{\text{week number}}$$

$$\text{BG} = \text{final biomass} - \text{initial biomass}$$

$$\text{FCR} = \frac{\text{dry feed consumption}}{\text{biomass gain}}$$

$$\text{P} = \frac{\text{biomass gain}}{\text{m3}}$$

5.3.4. Immunologic parameters

To study the shrimp immunological system the following parameters were determined, after 42 days: granular hemocyte (GH) and hyaline hemocyte (HH) percentage, total protein concentration in hemolymph (TPC), and the apoptotic cell number in hemolymph. A hemolymph sample was extracted from the hearts of 5 shrimp per tank, using a 50 µL Hamilton syringe. The samples were transferred to polyethylene tubes containing heparin to avoid the coagulation of the samples (Wang and Zhang 2008).

The percentage of granulate and hyaline hemocyte present was determined by microscope observation following Weibel (1980) from one drop of hemolymph spread on a microscope slide. A microscope lens with integration Disc.1- @5 points G49 (Carl Zeis) connected to a Zeiss Primo Star microscope was used. The TPC in the shrimp serum, was determined according to the Bradford (1976) method, using a 10 µL hemolymph aliquot.

The apoptotic cell number in hemolymph was evaluated by the TUNEL method using the ApopTag® Plus Peroxidase In Situ Apoptose Detection kit (Millipore) according to Charriaut-Marlangue and Ben-Ari (1995) and Wang and Zhang (2008). A 5 µL hemolymph aliquot was placed in histological sheets positively charged to enable the identification and counting of cells with apoptose using an optic microscope (Carl Zeis).

5.3.5. Statistic analysis

A non parametric one-way analysis of variance (Kruskal-Wallis) was used to test differences in physico-chemical variables between probiotic treatments (CO, TA, TB and TC). An analysis of variance (ANOVA) was used to test differences in growth parameters and immunologic parameters between probiotic treatments (CO, TA, TB and TC). The software Statgraphics® Centurion XVII was used.

5.4. RESULTS

5.4.1. Water quality

Temperature remained stable in the greenhouse, although small variations were observed between 23.6 and 33.0ºC. Dissolved oxygen values were kept above 5 mg/L in all treatments. pH range had small variations in all treatments between 6.9 and 9.1. Salinity was kept between 16.5 and 18.6. The alkalinity decreased during the experiment in all treatments, it decreased from 255 (day 1) to 45 (day 42) mg $CaCO_3$/L.

The evolution of N-TA was similar in all treatments (Figure 5.1a), N-TA values were not statistically different between treatments ($P > 0.05$). N-TA concentration was under 0.20 mg/L during all the study period. For the first 20 days, the maximum values of N-TA detected reached maximum values of 0.84, 0.70, 0.71 and 1.19 mg/L in CO, TA, TB and TC treatments respectively. After the first 20 days, N-TA values dropped to nearly 0 mg/L in all the treatments. The maximum N-NO_2^- concentration was 5.88, 6.33, 6.83 and 6.49 mg/L in CO, TA, TB and TC treatments respectively, these values were detected between days 11 and 26. To avoid toxic levels, water was renewed depending on each tank needs, 30%, 8.33% 21.67% and 38.33% of total water volume in CO, TA, TB and TC treatments respectively. Nitrite concentration for the rest of the study period was always below 5 mg L^{-1} (Figure 5.1b). There was no significant differences ($P > 0.05$) in nitrite concentration among the treatments.

N-NO_3^- evolution was different to the observed for N-NO_2^- and N-TA (Figure 5.1c). N-NO_3^- remained below 10 mg/L during the first 19 days, after that N-NO_3^- started increasing. The maximum nitrates values were reached on the last study day, these values were 53.02, 51.89, 53.00, and 45.27 mg/L in treatments CO, TA, TB and TC (Figure 5.1c). No statistical differences were observed in nitrates levels between treatments ($P > 0.05$).

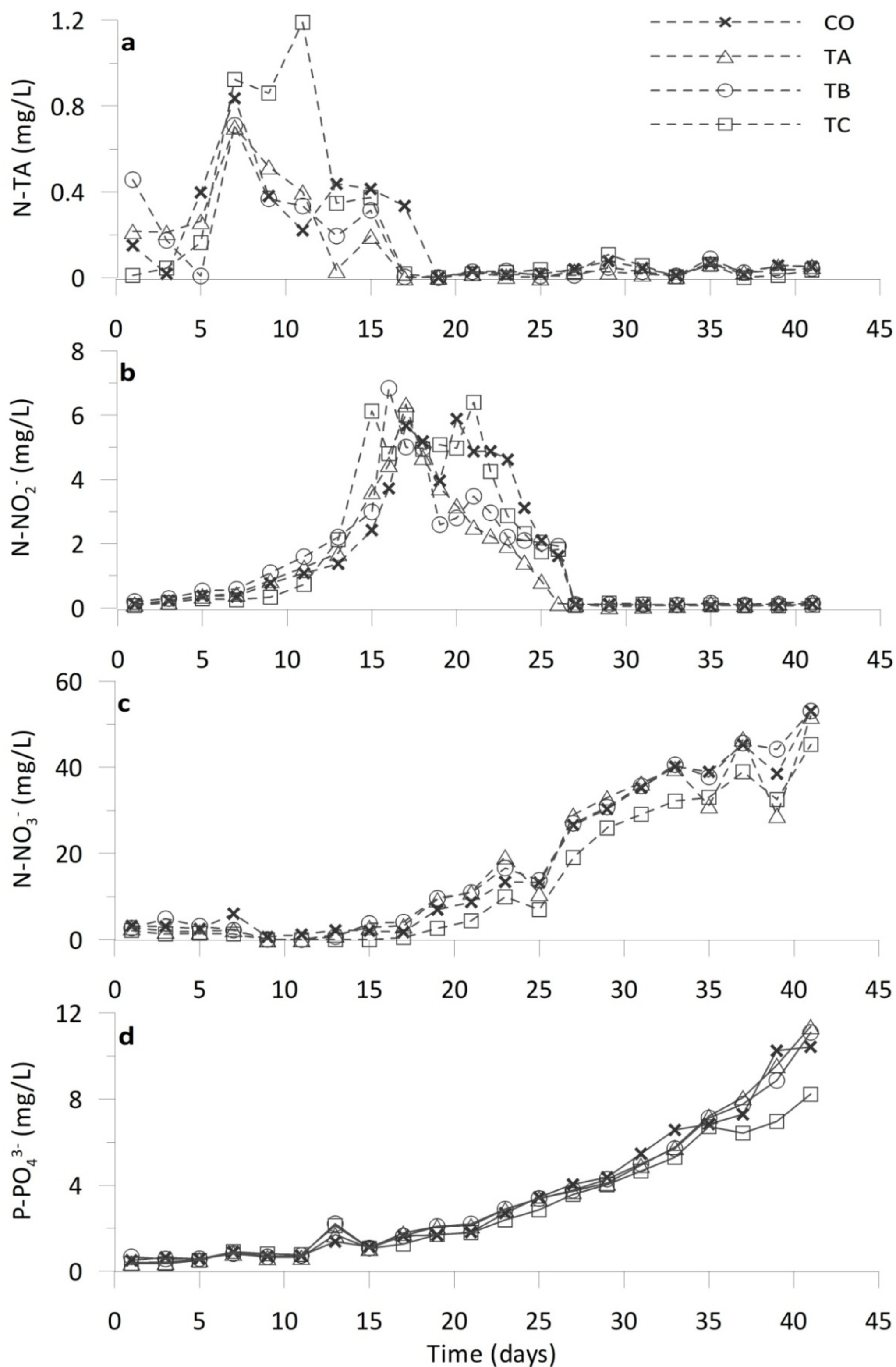

Figure 5.1. Evolution of total ammonia nitrogen, nitrite, nitrate and phosphate concentration. Control treatment (CO) contains 0 cfu/mL, treatment A (TA) 9.48×10^4 cfu/mL, treatment B (TB) 1.90×10^5 cfu/mL, and treatment C (TC) 3.79×10^5 cfu/mL. Kruskal-Wallis analysis was applied for comparison of treatments. No statistical differences between treatments were observed ($P > 0.05$).

A rising trend in $P\text{-}PO_4^{3-}$ was observed in all treatments. Maximum $P\text{-}PO_4^{3-}$ values were 10.43, 11.38, 11.10 and 8.23 mg/L in treatments CO, TA, TB and TC. No statistical difference was observed between treatments ($P > 0.05$). However, TC showed lower values of phosphates in the last days of the experiment, as shown in Figure 5.1d.

TSS and BV showed an increasing trend as shown in Figure 5.2. Table 5.1 shows the mean and range of biofloc volume, total suspended solids, volatile suspended solids and non-volatile suspended solids for each treatment. According to Kruskal-Wallis test, there was no significant difference between treatments in these parameters ($P > 0.05$).

Table 5.1. Values of total suspended solids (TSS), volatile suspended solids (VSS) and non-volatile suspended solids (NVSS) and biofloc volume (BV) (mean and range). Control treatment (CO) contains 0 cfu/mL, treatment A (TA) 9.48×10^4 cfu/mL, treatment B (TB) 1.90×10^5 cfu/mL, and treatment C (TC) 3.79×10^5 cfu/mL.

	CO	**TA**	**TB**	**TC**
BV	16.0	15.0	14.3	21.2
(ml/L)	(0.0 – 40.0)	(0.0 – 34.3)	(0.0 – 35.0)	(0.0 – 69.0)
TSS	0.4321	0.4585	0.4512	0.5064
(g/L)	(0.2247 – 0.7073)	(0.2687 – 0.7780)	(0.2547 – 0.7440)	(0.2567 – 1.0113)
VSS	0.2193	0.2265	0.2164	0.1958
(g/L)	(0.0694 – 0.4902)	(0.0947 – 0.4984)	(0.0977 – 0.4207)	(0.0609 – 0.5066)
NVSS	0.2088	0,2320	0.2348	0.3106
(g/L)	(0.1358 – 0.3961)	(0.1591 – 0.3262)	(0.1214 – 0.3397)	(0.1742 – 0.5048)

Kruskal-Wallis analysis was applied for comparison of treatments. No statistical differences between treatments were observed ($P > 0.05$).

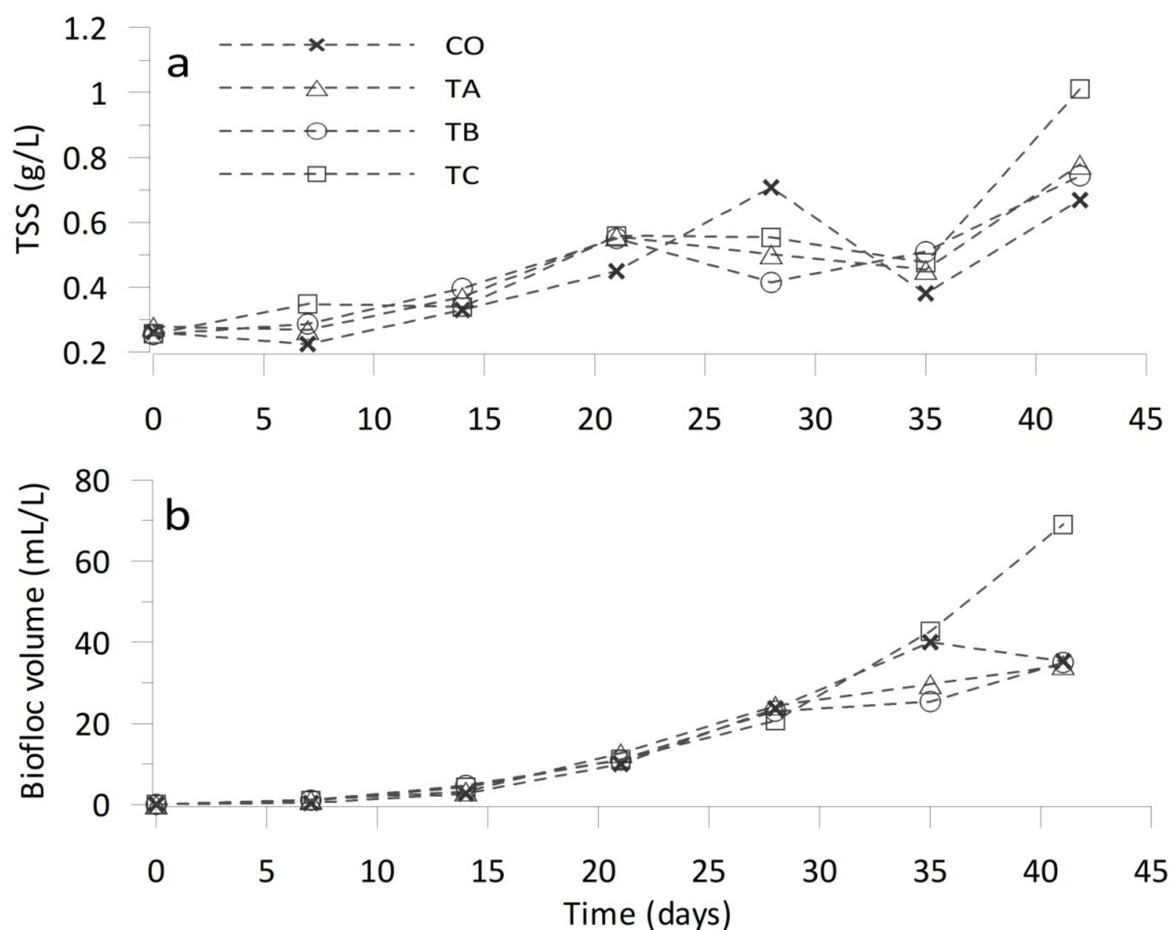

Figure 5.2. Evolution of total suspended solids and biofloc volume. Control treatment (CO) contains 0 cfu/mL, treatment A (TA) 9.48×10^4 cfu/mL, treatment B (TB) 1.90×10^5 cfu/mL, and treatment C (TC) 3.79×10^5 cfu/mL. Kruskal-Wallis analysis was applied for comparison of treatments. No statistical differences between treatments were observed ($P > 0.05$).

5.4.2. Growth parameters

The results of survival, final weight, weight gain, weekly weight gain, biomass gain, FCR and productivity can be seen in table 5.2. According to the ANOVA analysis, there was no significant difference between treatments in growth parameters ($P > 0.05$). At the end of the experiment, average shrimp weight ranged between 9.2 and 10.2 g, average survival was 99.30, 99.56, 98.45 and 96.21% in treatments CO, TA, TB and TC respectively, and average feed conversion rate ranged between 1.2 (treatments CO, TA and TB) and 1.3 (treatment TC). During the experiment, the shrimp grew around 1.3 g per week and the average productivity was 2.315, 2.383, 2.055 and 2.118 g m^{-3} in treatments CO, TA, TB and TC respectively.

Table 5.2. Probiotic effect on growth parameters as survival, final weight, weight gain, weekly weight gain (WWG), biomass gain (BG), feed conversion rate (FCR) and productivity (P). Control treatment (CO) contains 0 cfu/mL, treatment A (TA) 9.48×10^4 cfu/mL, treatment B (TB) 1.90×10^5 cfu/mL, and treatment C (TC) 3.79×10^5 cfu/mL. The table shows the average and standard deviation.

	CO	TA	TB	TC
Survival (%)	99.33 ± 1.15	99.56 ± 0.77	98.45 ± 1.93	96.21 ± 3.35
Final weight (g)	9.9 ± 0.5	10.1 ± 0.9	9.2 ± 1.2	10.2 ± 0.6
Weight gain (g)	8.1 ± 0.6	8. 3 ± 0.6	7.3 ± 1.1	7.8 ± 0.6
WWG (g)	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
BG (kg)	1.199 ± 0.083	1.235 ± 0.106	1.074 ± 0.170	1.111 ± 0.033
FCR	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
P (kg/m^3)	2.351 ± 0.166	2.383 ± 0.217	2.055 ± 0.328	2.118 ± 0.062

ANOVA analysis was applied for comparison of treatments. No statistical differences between treatments were observed ($P > 0.05$).

5.4.3. Immunological system

The results of TPC, HG, HH and number of cells with apopotose can be seen in table 5.3. The TPC analysis indicated that TA, TB and TC had significantly higher levels of TPC in the hemolymph (128 and 124 mg/mL) than CO (104 mg/mL respectively) ($P < 0.05$) (Table 5.3). The percentage of GH was also significantly higher in TA, TB and TC shrimps (79, 81 and 77 % respectively) than CO (51 %) ($P < 0.05$). Conversely, HH had significantly lower percentage in TA, TB and TC (21, 19, 23 %) than CO (49 %) ($P < 0.05$). The number of cells with apoptose in the hemolymph was 3 in CO treatment which was significantly higher than in probiotic treatments (only 1 or 2 cells with apoptose) ($P < 0.05$).

Table 5.3. Total protein concentration, percentage of granular hemocytes, hyaline hemocytes (HH) and number of the cells with apoptose. Control treatment (CO) contains 0 cfu/mL, treatment A (TA) 9.48×10^4 cfu/mL, treatment B (TB) 1.90×10^5 cfu/mL, and treatment C (TC) 3.79×10^5 cfu/mL. The table shows the average and standard deviation.

	CO	**TA**	**TB**	**TC**
TPC (mg/mL)	104 ± 7^a	128 ± 4^b	128 ± 4^b	124 ± 6^b
GH (%)	51 ± 7^a	79 ± 5^b	81 ± 5^b	77 ± 5^b
HH (%)	49 ± 7^a	21 ± 5^b	19 ± 5^b	23 ± 5^b
Cell number with apoptose	3 ± 1^a	1 ± 1^b	1 ± 1^b	2 ± 1^b

Means with the same letter in the row are not significantly different as showed by ANOVA analysis ($P < 0.05$).

5.5. DISCUSSION

The physical parameters such as temperature, dissolved oxygen, pH, salinity and alkalinity were maintained during all the study period at the optimum value for shrimp cultivation (Van Wyk and Scarpa 1999). The levels of N-TA and N-NO_2^- were maintained within the limits of safety determined by Li and Chen (2001, 2003). For this reason, it can be stated that the water quality was at optimal values for white shrimp production. Concentrations of N-TA, N-NO_2^- and N-NO_3^- in all the treatments during the experiment followed the dynamic observed by Avnimelech (2009). During the first two weeks, the N-TA was accumulated in the system. The N-TA peak was replaced by a second peak of N-NO_2^- when the oxidation processes by the heterotrophic bacteria began. Two weeks later, nitrification was completed, the N-NO_2^- peak disappeared and an accumulation of N-NO_3^- was observed in the system during the rest of the experiment. Some authors have observed that probiotics are able to eliminate nitrogen compounds from traditional aquaculture systems, helping to maintain water quality (Rengpipat et al. 1998; Vaseeharan and Ramasamy 2003; Balcázar et al. 2007). However, in the biofloc system of our study, no significant differences in water quality were observed between the control treatment and the treatments with *B. amyloliquefaciens*. This may be due

to the high efficiency of the heterotrophic bacteria of the BFT in the elimination of nitrogen compounds. Due to the high transformation rate by heterotrophic bacteria, the addition of probiotic bacteria does not produce a significant enhancement in this process. Other authors observed no improvement in water quality with the addition of probiotics applied in water (Vita 2008; Souza et al. 2012; Krummenauer et al. 2014), but no studies had been done with *B. amyloliquefaciens* dissolved in water.

TSS and BV were inside the range recommended by Avimelech (2009) and Ray et al. (2010). It has been demonstrated that *B. amyloliquefaciens* shows a high growth rate in vitro (Bao 2014). The average value of total bacteria in a BFT according to Emerenciano et al. (2012) and Kim et al. (2014) is of the order of 10^7 cfu/mL. However, the daily addition of 9.48×10^4, 1.90×10^5 or 3.79×10^5 cfu/mL of *B. amyloliquefaciens* in the BFT did not produce an increase in suspended solids, neither in weight or in volume (Table 5.3). This indicates that the probiotic bacteria did not colonized water.

In this experiment, survival rate and growth of shrimp were those characteristic of BFT (Krummenauer et al. 2011; Baloi et al. 2013). Our results showed no improvement in the probiotic treatments as compared to the control. Other authors observed the benefitial effects of *B. amyloliquefaciens* probiotics in recirculation systems (Nuez-Ortín 2013), which include improved survival rates, weekly gains in weight and FCR. The nutritional benefits of BFT as compared to recirculation systems have been already studied (Crab et al. 2012; Emerenciano et al. 2013). It seems that the addition of *B. amyloliquefaciens* did not produce a significant enhancement of the BFT benefits. However, other authors observed growth benefits in BFT with other probiotic bacteria appplied in feed (Vita 2008; Souza et al. 2012). Then, the positive effect of probiotics on growth parameters in BFT must not be disregarded, but should be better studied to unveil the specific role of each species.

The TPC levels in the hemolymph obtained were within the range detected by Cheng et al. (2002), Li et al. (2008), Macias-Sancho et al. (2014) and Souza et al. (2016) in white shrimp. Our results showed that *B. amyloliquefaciens* increased the TPC of the hemolymph (TA, TB and TC were higher than CO). Previous studies in BFT with other probiotics did not show this effect on shrimp (Souza et al. 2012). The proteins in the hemolymph are the mechanism of the shrimp to identify pathogens and their morphology, furthermore the proteins regulate the union of pathogens with the hemocytes (Johansson et al. 1999) and the phagocytosis capacity

of hemocytes (Wang and Zhang 2008). Then, an increase in TPC is key for an enhance immunological system.

The results of our experiment showed that all treatments had a high percentage of GH to the detriment of HH, similar to that observed by Macias-Sancho et al. (2014) and Souza et al. (2016) in white shrimp, cultivated in biofloc systems. The percentage of GH was significantly higher in the treatments with *B. amyloliquefaciens* (TA, TB and TC) than in the control (CO). The effect of *B. amyloliquefaciens* on the percentage of GH, has already been observed by Camacho (2012) in a recirculation system. However, other probiotic tested in shrimp in BFT did not produced this effect (Souza et al. 2012). The higher percentage of GH increases the response capacity against pathogens (Xu and Pan 2013). The GH have different ways to counter pathogens, such as phagocytosis, encapsulation, cytotoxicity, storage and release into the proenoloxidase system; while the hyaline hemocytes only can fight against pathogens through phagocytosis (Johansson et al. 2000).

The number of cells with apoptose observed in our control treatment was similar to the observed by Macias-Sancho et al. (2014) in white shrimp cultivated in biofloc system. But, in our experiment the number of cells with apoptose was significantly lower for all tested doses (TA, TB and TC) than in the control. Apoptose, also known as programmed cell death, is a mechanism that normally occurs in cells of all tissues in normal physiological situations. In pathological situations, apoptose is produced to avoid the replication or dispersion of pathogens which are fundamentally viruses (Everett and McFadden 1999). This mechanism is used by shrimp to avoid the replication of white spot syndrome virus (Khanobdee et al. 2002) and the yellow head virus among others (Wongprasert et al. 2003). The smaller number of cells with apoptosis in treatments with probiotic is related with an increase in the percentage of GH and TPC. That means that *B. amyloliquefaciens* reinforces the immune system so it can fight pathogens through other mechanisms without resorting to cell death. (Khanobdee et al. 2002; Wongprasert et al. 2003; Wang and Zhang 2008).

5.6. CONCLUSION

To conclude, the application of *B. amyloliquefaciens* dissolved in water in a biofloc system strengthened the immune system of shrimp: increased the percentage of granular hemocytes and the concentration of total protein in the hemolymph, and decreased the number of cells with apoptosis. Thus, *B. amyloliquefaciens* improved the ability of detection and performance of the immune system to combat pathogens. No positive effect of *B. amyloliquefaciens* on growth parameters in BFT was observed, but it must not be disregarded if applied combined with other probiotic species. Future studies should better study effects on shrimp growth to unveil the specific role of each species, as well as the minimal dose for observing effects.

5.7. ACKNOWLEDGEMENTS

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CAPÍTULO 6

The role of *Bacillus amyloliquefaciens* on *Litopenaeus vannamei* during the maturation of a biofloc system

Llario, F., Falco, S., Sebastiá-Frasquet, M. T., Escrivá, J., Rodilla, M., Poersch L. H. (2018) The role of *Bacillus amyloliquefaciens* on *Litopenaeus vannamei* during the maturation of a biofloc system. *Helgoland Marine Research,* under review process.

6.1. ABSTRACT

Probiotics play an important role in biofloc systems, since they allow to improve the effects of these systems on the cultured species, and on shrimps in particular. The objective of this study is to study the role of the probiotic bacterium *Bacillus amyloliquefaciens* during the formation of a biofloc system for the culture of *Litopenaeus vannamei*. Water quality, microbial activity, zootechnical development and immune system state of shrimps were monitored throughout the maturation of the biofloc system. We studied a control without probiotic and two daily doses of 10^3 and 10^4 cfu/mL of *Bacillus amyloliquefaciens*, applied directly on water. The results indicate that microbial activity and water quality were not altered by the application of *Bacillus amyloliquefaciens*. According to other authors, this probiotic improves feed digestibility, but this did not cause an observable improvement in the growth of *Litopenaeus vannamei*. However, a strengthening of the immune system was observed, thanks to an increase in the percentage of granular hemocytes present in the shrimp hemolymph. This positive effect on the shrimp immune system was observed throughout the study period, as compared with control treatment. Both, during the maturation process (characterized by a process of incomplete nitrification, with accumulation of nitrites, which causes great stress to shrimps) and during the mature phase (characterized by low concentrations of total ammonia nitrogen and nitrites).

Keywords: growth parameters, immunological system, microbiological activity, probiotic, shrimp, water quality

6.2. INTRODUCTION

Biofloc technology (BFT) is characterized by a high amount of microorganisms in the water column (Emerenciano et al. 2013). Among the microorganisms present in BFT systems, bacteria are the most important for the maintenance of the system. These bacteria, under intense aeration and organic fertilization, consume the nitrogen compounds dissolved in water and turn them into microbial protein which can be consumed by the cultured species, such as shrimps; this way the water quality is maintained (Avnimelech 2009; Crab et al. 2012; Emerenciano et al. 2013). This reduces or eliminates water renewal during the farming cycle (Avnimelech 2009). This is the main reason why BFT systems have been considered as an environmentally friendly aquaculture technique (Emerenciano et al. 2013). In addition, these

bacteria compete for resources and space with pathogens, and have a probiotic effect on shrimps (Emerenciano et al. 2013). BFT systems also allow to increase the farming density, being more productive than traditional aquaculture systems (Wasielesky et al. 2006).

Biofloc technology is being developed mostly in farms in North America, South America and Asia (Emerenciano et al. 2013), where there is an important tradition in shrimp farming. In Europe, this technology has started to develop recently. One of the main disadvantages of shrimp farms in the European Union is the limited choice of products marketed, specific to this activity, such as feed and probiotics. In addition, importing these products is difficult, due to the strict legislation of the European Union.

Probiotics are live microorganisms, whose administration to water allows to eliminate the medicines and antibiotics that are usually used in aquaculture (Van Hai and Fotedar 2010). These microorganisms can improve the inherent probiotic activity of BFT systems (Crab et al. 2012; Souza et al. 2012). Generally, they are used preventively (Van Hai and Fotedar 2010), but they can also be used for the treatment of pathogens like *Vibrio parahemolitycus* (Krummenauer et al. 2014). The addition of probiotics can help the cultivated species to better resist the stress produced by adverse conditions, such as those produced during the maturation of a biofloc system (Farzanfar 2006; Crab et al. 2012; Jatobá et al. 2014). Probiotics are characterized by distinctive action mechanisms (Van Hai and Fotedar 2010): 1) enzyme production in the host intestine, which increases the digestibility of the food and improves the growth of the host (Zhou et al. 2009; Nuez-Ortín et al. 2013); 2) production of bactericidal compounds inside the host (Pandiyan et al. 2013); 3) stimulation of the host immune system, which makes it more resistant to pathogens (Rengpipat et al. 2000, Tseng et al. 2009); 4) competition with the rest of bacteria for the resources, which can diminish the presence of pathogens (Paiva-Maia et al. 2013; Vargas- Albores et al. 2017); and 5) maintenance of water quality, probiotics consume nitrogen compounds, such as nitrites and ammonia, which are toxic to cultured species (Dalmin et al. 2001; Nimrat et al. 2012).

Currently, there are different probiotics in the market, most of them are included in the feed composition (Van Hai and Fotedar 2010). However, in areas with little tradition in shrimp farming, such as the European Union, the supply of feed with probiotics is low, and it is more feasible to apply probiotics directly to water. The direct application to water allows greater flexibility in the choice of the probiotic, allowing to choose the one that best suits the growing conditions and the cultured species.

The probiotic Ecobiol Plus® is composed of the bacterium *Bacillus amyloliquefaciens*, and is marketed in the European Union, for use in chickens and pigs (Jerzsele et al. 2012; Ahmed et al. 2014; Larsen et al. 2014; Lei et al. 2015). Different experiences have shown that *B. amyloliquefaciens* can have a high potential as a probiotic in systems with water recirculation (Camacho 2012; Nuez-Ortín et al. 2013; Xie et al. 2013; Huang et al. 2015; Saputra et al. 2016). Xie et al. (2013) showed the potential of *B. amyloliquefaciens* in the treatment of wastewater from aquaculture, reducing nitrite levels. Huang et al. (2015) and Saputra et al. (2016) demonstrated that the bacterium *B. amyloliquefaciens* produced a reinforcement of the immune system in *Cyprinus carpio* and *Oreochromis niloticus*. Nuez-Ortín et al. (2013) observed improvements in the digestibility of feeds for shrimps containing Ecobiol Plus®. The results of these studies in recirculation systems, encourage us to consider this bacterium as probiotic for the shrimp *Litopenaeus vannamei* produced in biofloc systems.

The objective of this research is to study the role of the probiotic bacterium *B. amyloliquefaciens* during the maturation process of a biofloc system. We studied its effect on the biofloc system (water quality dynamics, trophic state development and microbial activity evolution), and on cultured shrimps (zootechnical performace and immune system).

6.3. MATERIAL AND METHODS

6.3.1. Location and shrimp culture system

The experiment was developed in the Universitat Politècnica de València facilities (Grau de Gandia, Spain), during 169 days, between May, 11 (end of spring) and October, 16 (early autumn) 2016. Postlarvae white shrimp (PL) were purchased from a commercial laboratory (Shrimp Improvement Systems, Florida, USA), and they were certificated as free of pathogen. PLs of 0.07 ± 0.04 g weight were distributed in 9 square tanks filled with 2,250 L of water and with a surface of 3.2 m^2 each tank. Shrimp density was 200 shrimp/m^2. Each tank was filled with a mixture of seawater and freshwater which had a salinity level of 22.5. The water was disinfected with 10 mg/L of chlorine, which was subsequently eliminated by adding ascorbic acid to the tanks (Krummenauer et al. 2014). The tanks were located in a greenhouse and constantly individually aerated.Every day the shrimp were fed with commercial feed (Le Gouessant) specifically designed for *L. vannamei*. Feed amount was calculated according to the shrimp biomass and water temperature, according to Jory et al. (2001).

The following water quality parameters were monitored: salinity, temperature, pH, alkalinity, dissolved oxygen, nutrients and total suspended solids (TSS). Dissolved oxygen (DO), salinity and temperature were monitored in situ, using a multi-parameter probe (YSI ProODO and WTW Multi 340i respectively) twice a day. The pH was measured once a day using pHMeter BASIC 20+ the Crison. Alkalinity was controlled after the first month of culture, every two weeks, by titration with HCl (Baumgarten et al. 2010). Every two days an aliquot of water was collected to determine the concentration of total dissolved ammonia (N-TA mg/L) using the methodology described by Baumgarten et al. (2010), nitrites (N-NO_2^- mg/L), using the methodology of Bendschneider and Robinson described in Baumgarten et al. (2010), the nitrates (N-NO_3^- mg/L) were analyzed by means of the difference between nitrites plus nitrates using the methodology described by Grasshof (1976). Also, phosphates (P-PO_4^{3-} mg/L) were analyzed following the colorimetric reaction described by Murphy and Riley (1962). TSS were analyzed once a week as described by Baumgarten et al. (2010).

Due to evaporation salinity can increase above to 22.5, in this case freshwater was added to keep salinity under this threshold. Different shade awnings were used to control the greenhouse temperature, in order to keep the water temperature in the optimal range for shrimp culture according to Van Wyk and Scarpa (1999). At the beginning of the study period (May, 5) the greenhouse roof was covered with a white awning. On day 59 (July, 8) the awning was substituted by a black one. Finally, on day 136 (September, 23) the black awning was removed, due to lower environmental temperature at the end of summer and beginning of autumn. The evolution of maximum and minimum daily environmental temperature was recorded from the data of the weather station of the Reial Club Nàutic de Gandia (AVAMET 2017). The pH was maintained between 7 and 9 (Van Wyk and Scarpa 1999). We added 0.15 mg/L of calcium hydroxide when pH values below 7.50 were detected (Furtado et al. 2014). Also, we added 0.20 g/L of sodium bicarbonate when alkalinity below 120 mg $CaCO_3$/L were observed (Furtado et al. 2011).

The initial fertilization of the system was done with sucrose, with a theoretical 15:1 carbon/nitrogen ratio, to facilitate the initial development of heterotrophic bacteria. During the experiment, sucrose was added when the ammonia reached a concentration greater than 1 mg/L (Ebeling et al. 2006; Avnimelech et al. 2009). Eventual renewal of the water during the experiment was minimal and was performed when the nitrite level reached 15 mg/L to avoid

toxic effects of nitrites (Lin and Chen 2003). 25% of the water in the tank was changed and the feed was reduced by 40%, to minimize the contribution of nitrogen to the system.

6.3.2. Probiotic treatments

During the experiment, the probiotic Ecobiol Plus® was used, with a certified content of 1.3×10^{10} cfu/g of viable spores of *B. amyloliquefaciens*.

Two treatments were tested with different doses of probiotic and a control treatment without probiotic, each treatment had three experimental units (tanks). The treatment dose (A) was 2.8×10^{4} cfu/mL, treatment (B) was 2.8×10^{3} cfu/mL and treatment (C) was control. Doses were fixed based on previously unpublished research by F. Llario.

6.3.3. Chlorophyll *a* and microbial activity

To determine chlorophyll *a* (Chl*a*) an aliquot of water was filtered on a glass fiber filter (25mm Ø) and ultra-frozen (-86°C). Subsequently, the pigments were extracted with acetone HPLC grade. Chlorophyll *a* was measured by high-performance liquid chromatography (HPLC), according to the method of Wright et al. (1991), modified by Hooker et al. (2001). Microbial activity was analyzed once a week during the whole experiment. The method of dark and transparent bottles of Strickland adapted by Schveitzer et al. (2013) was used. Six bottles of 100 mL (3 dark and 3 transparent) were placed in each tank five centimeters below the water surface. To keep the flocs in suspension inside the bottles, they were shaken manually every 20 minutes, following the methodology of Schveitzer et al. (2013). The bottles were incubated approximately 8 hours. Dissolved oxygen was measured at the beginning and end of each incubation. Water column respiration (WCR), net primary productivity (NPP) and gross primary productivity (GPP) were calculated following Dodds and Cole (2007).

$$\mathrm{WCR}\ (\mathrm{mg}\ \mathrm{O_2}/(\mathrm{L} \cdot \mathrm{h})) = \frac{\text{initial } \mathrm{O_2} \text{ of dark bottle} - \text{final } \mathrm{O_2} \text{ of dark bottle}}{\text{time (h)}}$$

$$\mathrm{NPP}\ (\mathrm{mg}\ \mathrm{O_2}/(\mathrm{L} \cdot \mathrm{h})) = \frac{\text{final } \mathrm{O_2} \text{ of light bottle} - \text{initial } \mathrm{O_2} \text{ of light bottle}}{\text{time (h)}}$$

$$\mathrm{GPP}\ (\mathrm{mg}\ \mathrm{O_2}/(\mathrm{L} \cdot \mathrm{h})) = \mathrm{WCR} + \mathrm{NPP}$$

6.3.4. Zootechnical performance

An initial biometry was performed on 100 PLs with a balance (Kern ABT 220-4M; ± 0.0001). Biometry of 30 shrimps per tank was done every two weeks with a balance (Kern EW600-2M; ± 0.01), to monitor growth and adjust the dose of feed required. At the end of the experiment, the number of shrimps was counted and a biometry was performed on 50 shrimps per tank. We calculated weight gain, weekly weight gain, final biomass, biomass increase, feed conversion rate (FCR) and survival with the following equations.

$$\text{Weight gain (g)} = \text{final wet weight} - \text{initial wet weight}$$

$$\text{Weekly weight gain (g/week)} = \frac{\text{weight gain}}{\text{number of weeks}}$$

$$\text{Biomass } \left(\frac{\text{g}}{\text{m}^2}\right) = \frac{\text{wet weight shrimp x number of shrimp}}{\text{m}^2}$$

$$\text{Biomass gain (g /m}^2\text{)} = \text{final biomass} - \text{initial biomass}$$

$$\text{Feed conversion rate} = \frac{\text{dry feed consumption}}{\text{weight gain}}$$

$$\text{Survival (\%)} = \frac{\text{final shrimp amount}}{\text{initial shrimp amount}} \text{ x } 100$$

6.3.5. Immunological parameters

The state of the shrimp immune system was determined in the middle of the experiment (day 86), when the system was immature, and at the end of the experiment (day 169), when the system had completed its maturation process. For the analysis of the immune system, hemolymph was extracted to 5 shrimps from each tank with a sterile syringe BD Plastipak®, the sample was divided into two aliquots. The first aliquot of 20 µL was mixed with 80 µL of anticoagulant Alsever solution to count the hemocytes (Maggioni et al. 2004) and determine the percentage of granular (GH) and hyaline (HH) hemocytes, using a Bürker chamber and a Leica DM 2500 microscope. The second aliquot of 500 µL was allowed to coagulate for 2 h at 4°C. Then it was centrifuged at 2000 *g* to extract the serum, which was frozen (Maggioni et al. 2004). Subsequently, the total protein concentration (TPC) was analyzed using the method described by Lowry et al. (1951).

6.3.6. Statistical analysis

The statistical analysis was carried out using Statgraphics® Centurion XVI.I. First, the normality and homoscedasticity of all the parameters were analyzed. A multivariate analysis was performed to detect significant differences according to the probiotic treatment (A, B or C, Table 6.1 and 6.2) in all the variables studied. ANOVA test used was for variables that followed a normal distribution, or were transformed to a normal distribution through a $\log_{10}$ or $\sqrt[2]{x}$ conversion (WCR, Chl*a*, zootechnical performance and immune system parameters). The non-parametric Kruskal-Wallis test was applied to the variables with non-normal distribution (nutrients, TSS, GPP and NPP). Also, significant differences according to the sampling day were tested on immune system parameters (Table 6.3).

6.4. RESULTS

6.4.1. Water quality

In Figure 6.1, average water temperature, minimum and maximum ambient temperature values are shown along the study period. Water temperature ranged between an average maximum temperature of 31.0°C (day 35) and a minimum average of 22.5°C (day 165). The change in water temperature was due to ambient temperature variation. Thanks to handling the awnings that covered the greenhouse, water temperature was kept on average on 27.6 ± 1.8ºC. Table 6.1 shows pH, alkalinity, salinity and dissolved oxygen (concentration and saturation percentage) average values and range for each treatment. The maximum variation range of pH and alkalinity was 2.23 and 97.61 mg $CaCO_3$/L respectively. Dissolved oxygen and salinity were stable with a maximum variation range of 1.9 mg/L and 0.4 respectively.

Table 6.1. pH, alkalinity, salinity, dissolved oxygen concentration and dissolved oxygen saturation percentage, average and range for each treatment (A, B and C).

	Treatment A	Treatment B	Treatment C
pH	7.77 (6.83-8.61)	7.74 (6.35-8.58)	7.74 (6.82-8.62)
Alkalinity (mg $CaCO_3$/L)	108.89 (46.39-144.00)	96.70 (44.42-140.83)	101.56 (62.46-143.67)
Dissolved oxygen (mg/L)	5.88 (5.10-6.92)	5.97 (5.02-6.95)	5.95 (5.19-6.93)
Dissolved oxygen (%)	91.4 (76.7-105.0)	93.0 (70.7-104.8)	93.7 (76.3-104.4)
Salinity	22.5 (22.3-22.7)	22.5 (22.3-22.7)	22.5 (22.4-22.7)

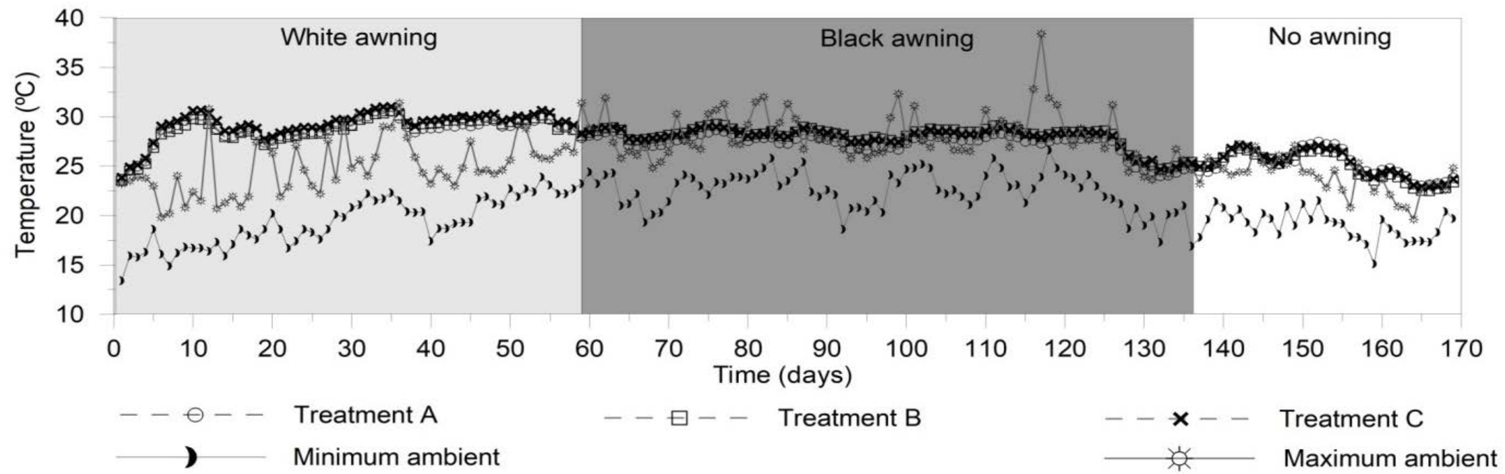

Figure 6.1. Evolution of water temperature, minimum and maximum ambient temperature values are shown along the study period. Each data represents the average of the three experimental units for treatment A ($2.8x10^4$ cfu/mL), treatment B ($2.8x10^3$ cfu/mL) and treatment C (without probiotic).

The N-TA accumulated in the water during the first 45 days (Figure 6.2a), registering maximum values of 1.44 mg/L in treatment B and 0.89 mg/L in treatments A and C. $N\text{-}NO_2^-$ remained at very low values until day 35, when it began to increase until reaching a maximum of 19.08mg/L in treatment A, 20.39 mg/L in treatment B and 21.10 mg/L in treatment C. Around day 100, the concentrations of $N\text{-}NO_2^-$ were progressively reduced reaching very low levels from day 118 (Figure 6.2b). $N\text{-}NO_3^-$ was detected for the first time on day 90 and increased progressively reaching its maximum value at the end of the culture (Figure 6.2c), when concentrations of 65.18, 77.72 and 74.54 mg/L were measured in treatments A, B and C respectively. $P\text{-}PO_4^{3-}$ accumulated in the water during the entire culture (Figure 6.2d). The highest values were observed at the end of the culture, being 17.91, 15.34 and 13.45 mg/L in treatments A, B and C respectively. No statistically different levels of nutrients were observed between treatments (N-TA, $P = 0.11$; $N\text{-}NO_2^-$, $P = 0.06$; $N\text{-}NO_3^-$, $P = 0.53$; $P\text{-}PO_4^{3-}$, $P = 0.95$). However, a trend close to significance ($P = 0.06$) was observed for $N\text{-}NO_2^-$ with higher values in the control treatment.

TSS accumulated in the system during the culture period (Figure 6.3). The maximum average values obtained were 1.19, 0.73 and 0.67 g/L in treatments A, B and C. Between day 60 and 130, small decreases were observed in TSS concentration, which were produced due to water renewal, to control $N\text{-}NO_2^-$ levels in the system. No statistically different levels of total solid suspenses were observed between treatments ($P = 0.06$).

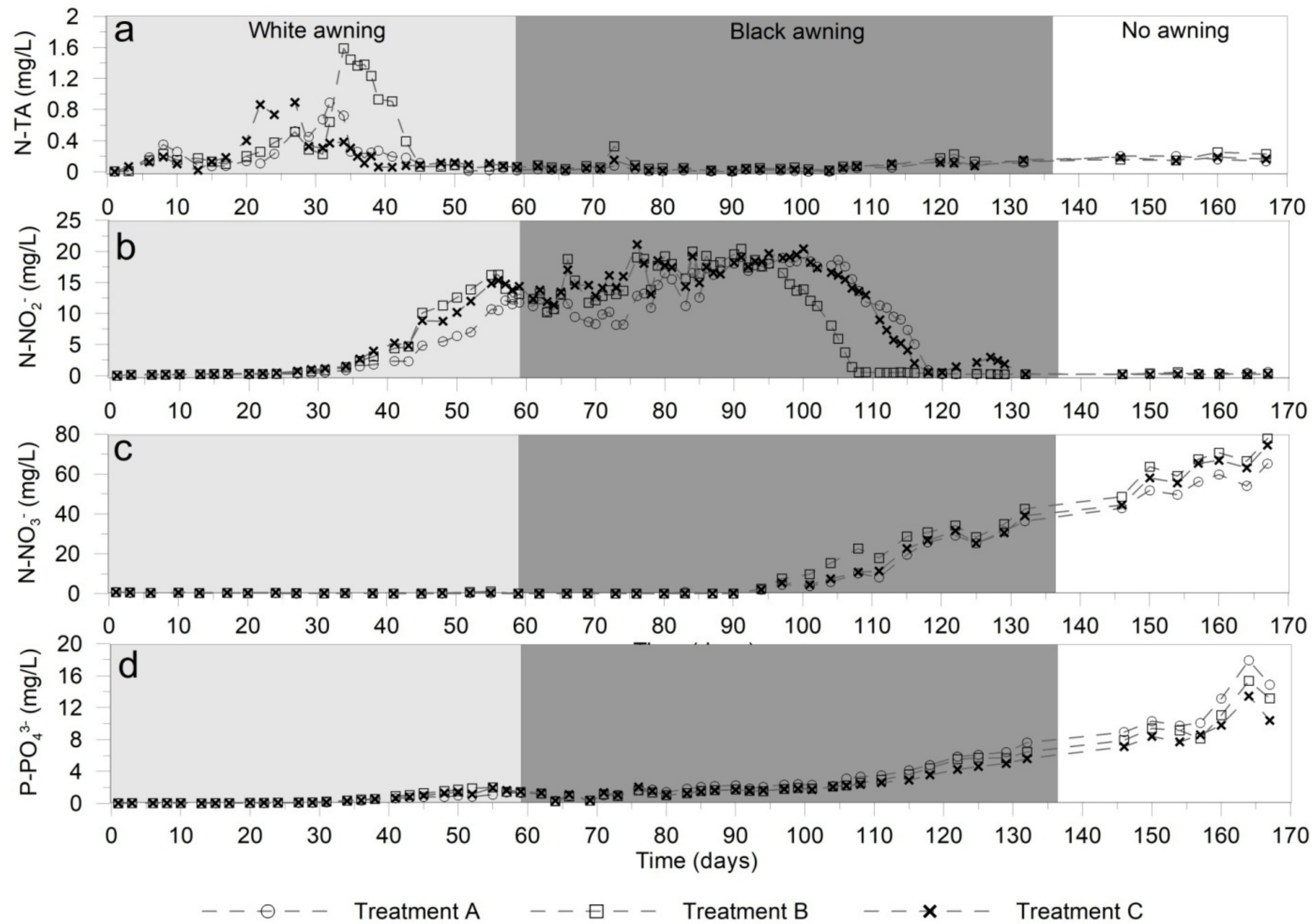

Figure 6.2. Evolution of nitrogen total amoniacal, nitrites, nitrates and phosphates. Each data represents the average of the three experimental units for treatment A (2.8x10^4 cfu/mL), treatment B (2.8x10^3 cfu/mL) and treatment C (without probiotic).

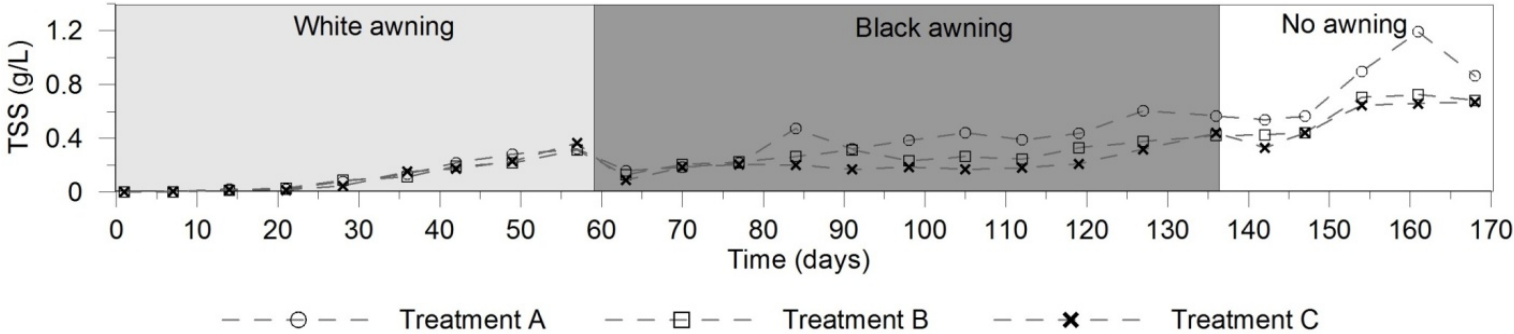

Figure 6.3. Evolution of total solids suspends. Each data represents the average of the three experimental units for treatment A ($2.8x10^4$ cfu/mL), treatment B ($2.8x10^3$ cfu/mL) and treatment C (without probiotic).

6.4.2. Microbial activity

Figure 6.4 shows the variation of NPP, GPP, WCR and Chl*a* concentration throughout the experiment. GPP was maximum during the first culture phase, where values of 1.15, 0.93 and 0.74 mg O_2/(L·h) were reached in treatments A, B and C respectively; after day 58 it remained below 0.5 mg O_2/(L·h) in all treatments (Figure 6.4a). WCR increased during the first weeks until day 35, then remained stable throughout the experiment, it reached its maximum on day 135 with values of 0.44, 0.45 and 0.51 mg O_2/(L·h) in treatments A, B and C respectively (Figure 6.4b). The NPP was positive during the first weeks, observing a maximum of 0.82, 0.72 and 0.61 mg O_2/(L·h) in treatments A, B and C respectively. On day 58, the first NPP negative values were recorded, which slowly decreased until the end of the experiment (Figure 6.4c). No statistically significant differences were observed between the different treatments for NPP (P = 0.55), GPP (P = 0.29) nor WCR (P = 0.30). Chl*a* (Figure 6.4d) was below detection limit in the first two weeks, increasing later to reach a maximum of 573.82, 460.16 and 703.58 µg/L in treatments A, B and C (between days 38 and 52). When the greenhouse was covered with black awning the concentration of Chl*a* was stabilized around 150 µg/L. When the black awning was removed, day 136, a second Chl*a* rise was observed. There were no statistically significant differences between treatments (P = 0.07) for this variable.

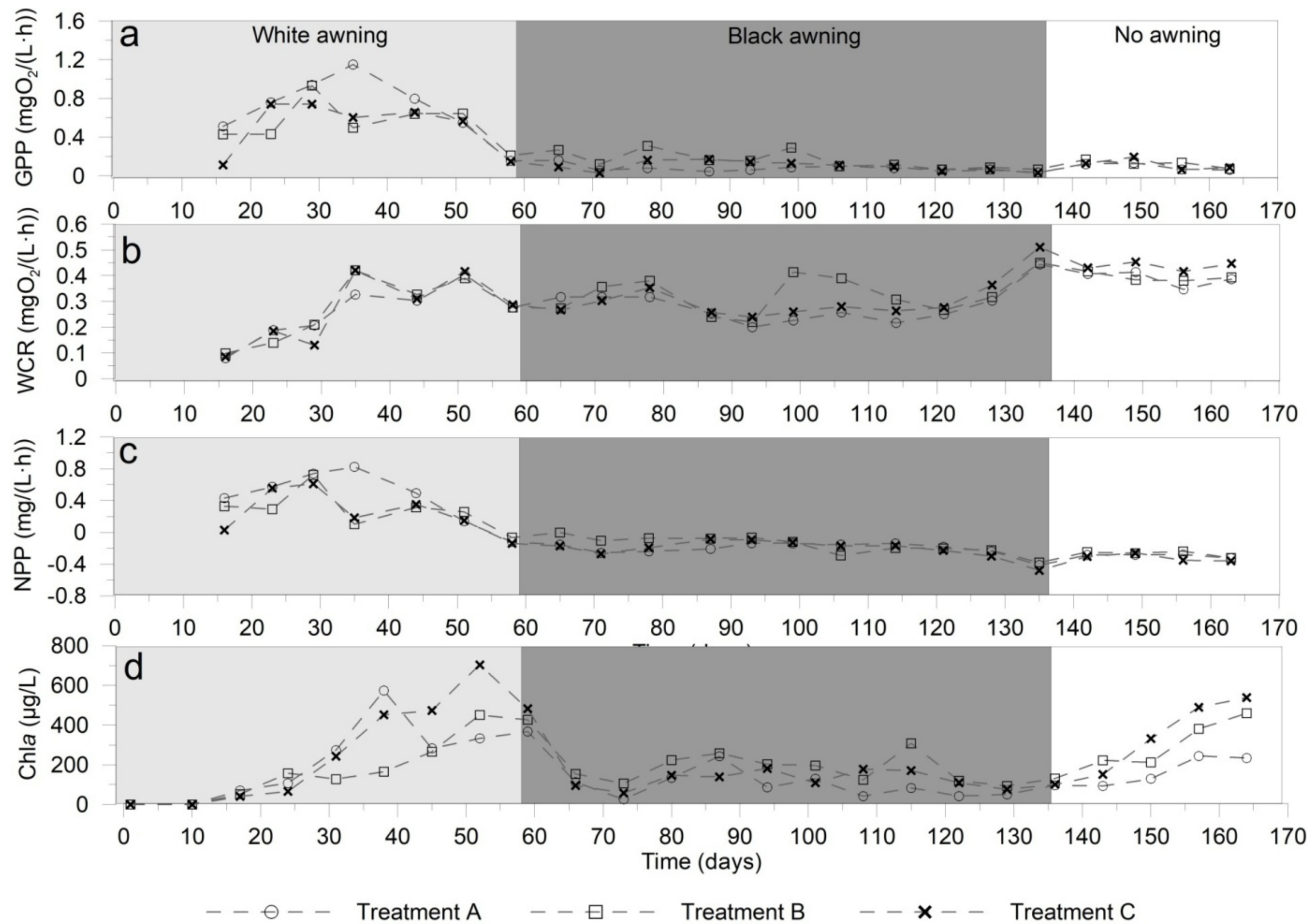

Figure 6.4. Evolution of gross primary production, water column respiration, net primary production and chlorophyll *a*. Each data represents the average of the three experimental units for treatment A ($2.8x10^4$ cfu/mL), treatment B ($2.8x10^3$ cfu/mL) and treatment C (without probiotic).

6.4.3. Zootechnical performance

In figure 6.5, we observe that shrimp growth was equal for all the treatments. At the beginning of the experiment the shrimps weighed 0.0675 ± 0.0433 g (initial biomass 13.5074 g/m^2). They grew up to 16.76 ± 0.15, 17.58 ± 1.41 and 17.94 ± 0.65 g in treatments A, B and C respectively.

Weight gain, weekly weight gain, biomass gain, FCR and survival results are included in table 6.2. ANOVA analysis showed no statistically significant differences between the different treatments in any zootechnical parameters.

Table 6.2. Mean and standard deviation of the zootechnical performance according to treatment (A, B or C).

	Treatment A	**Treatment B**	**Treatment C**	***P***
Weight gain (g)	16.59 ± 0.15	17.41 ± 1.42	17.76 ± 0.65	0.33
Weekly weight gain (g/week)	0.69 ± 0.01	0.72 ± 0.06	0.73 ± 0.03	0.33
Biomass production (ton/hectarea)	21.74 ± 1.83	23.00 ± 2.67	21.07 ± 2.03	0.58
FCR	1.91 ± 0.11	1.81 ± 0.15	1.96 ± 0.21	0.53
Survival (%)	65.77 ± 4.97	66.19 ± 3.48	59.53 ± 4.92	0.21

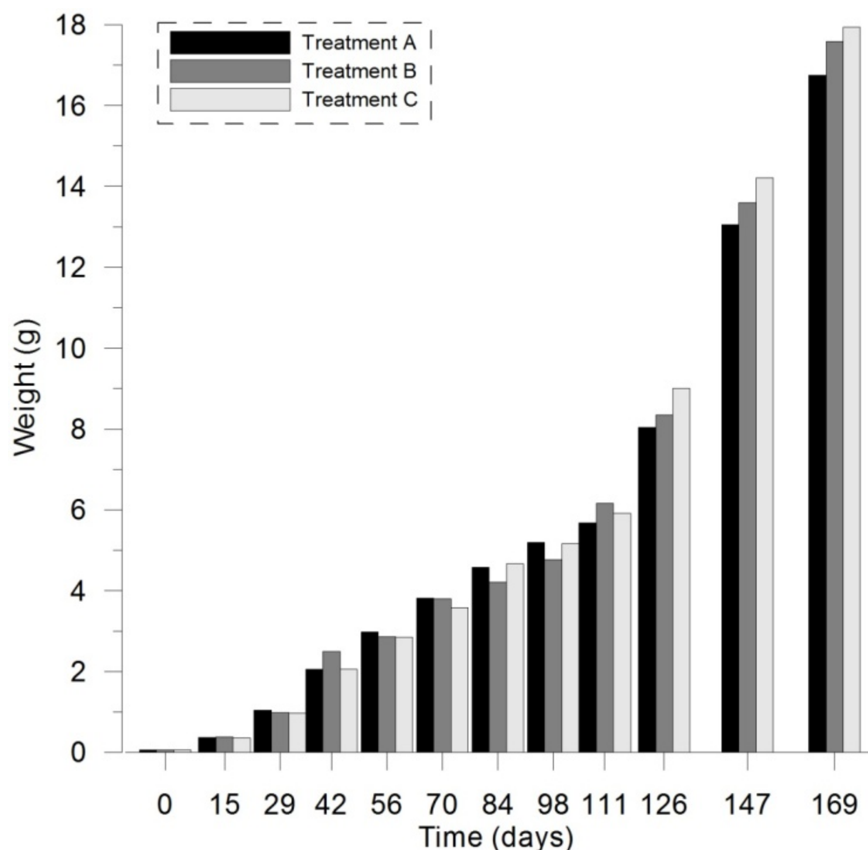

Figure 6.5. The shrimp weight gain. Each bar represents the average of the three experimental units for treatment A ($2.8x10^4$ cfu/mL), treatment B ($2.8x10^3$ cfu/mL) and treatment C (without probiotic).

6.4.4. Immune System Parameters

Table 6.3 shows immune system parameters in different stages of BFT evolution (immature and mature BFT). On day 86, immature system, TPC was around 80-90 mg/mL in the shrimp hemolymph. Statistical analysis showed no significant differences between treatments (P = 0.54). GH percentage was around 30 and HH was around 70% in the treatments with *B. amyloliquefaciens* (A and B). In the control treatment (C) values were close to 25 and 75% for GH and HH respectively. This difference (5%) between control and treatments with probiotic is statistically significant (P = 0.02).

On day 169, mature system, TPC in the hemolymph was around 120 mg/mL in all treatments (P = 0.94). GH percentage was around 46 and HH was around 54% in the treatments with *B. amyloliquefaciens* (A and B). In the control treatment (C) values were close to 39 and 61% for GH and HH respectively. This difference (7%) between control and treatments with probiotic is statistically significant (P = 0.00).

Table 6.3. Immune system parameters in different stages of BFT evolution (immature and mature BFT).

Immature biofloc system (day 86)			
	Treatment A	**Treatment B**	**Treatment C**
TPC (mg/mL)	93.18 ± 23.99^{a}	89.49 ± 26.18^{a}	82.58 ± 28.32^{a}
GH (%)	30.00 ± 6.64^{a}	31.33 ± 6.77^{a}	25.13 ± 4.78^{b}
HH (%)	70.00 ± 6.64^{a}	68.67 ± 6.77^{a}	74.87 ± 4.78^{b}
Mature biofloc system (day 169)			
	Treatment A	**Treatment B**	**Treatment C**
TPC (mg/mL)	123.14 ± 26.87^{a}	120.62 ± 32.92^{a}	117.75 ± 47.08^{a}
GH (%)	46.66 ± 5.10^{a}	46.28 ± 5.41^{a}	39.03 ± 7.44^{b}
HH (%)	53.34 ± 5.10^{a}	53.72 ± 5.41^{a}	60.97 ± 7.44^{b}

^{a} and ^{b} superscript indicate ANOVA test results. Those treatments that do not share the same letter show statistically significant differences for the variable shown ($P < 0.05$).

6.5. DISCUSSION

6.5.1. Effects on the biofloc system

The experiment was carried out between spring and autumn in a Mediterranean climate area. In temperate climates, greenhouses are a good strategy to maintain water temperature, facilitating shrimp culture during the coldest months (Gaona et al. 2011; Ray 2012; Toledo et al. 2016). During the experiment the high environmental temperatures forced to use a white awning in spring and a black awning in summer for temperature control inside the greenhouse. At the end of the experiment (autumn), the awnings were removed to avoid water cooling. Using different awnings is usual to control the effect of environmental temperatures inside the greenhouse (Montero 2009), thanks to them the water temperature was maintained

within the adequate range for the cultivation of *L. vannamei* (Van Wyk and Scarpa 1999) in this experiment.

Dissolved oxygen remained above 5 mg/L and 85% saturation, recommended values according to Cheng et al. (2003), throughout the experiment. The pH and alkalinity decreased throughout the culture, this trend is common in BFT, due to the high metabolic rate of bacteria present in the system (Ebeling et al. 2006). The addition of sodium bicarbonate and calcium hydroxide managed to maintain pH and alkalinity respectively within the optimal values determined by Van Wyk and Scarpa (1999).

The nitrogen compounds (N-TA, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$) followed the usual evolution in BFT described by Avnimelech (2009). At the beginning of the experiment, N-TA accumulated, which is typical of the immature BFT phase. When the bacterial nitrification started, N-TA was oxidized to $N\text{-}NO_2^-$, then, N-TA descended and a $N\text{-}NO_2^-$ peak appeared. From day 115, the peak of $N\text{-}NO_2^-$ disappeared and $N\text{-}NO_3^-$ accumulated in water, indicating a mature BFT characterized by low levels of N-TA and $N\text{-}NO_2^-$ (Jatobá et al. 2014). N-TA did not exceed the safety level recommended by Lin and Chen (2001) for the cultivation of *L. vannamei*. On the contrary, $N\text{-}NO_2^-$ levels did exceed safety limits. According to Lin and Chen (2003), exceeding the safety level (15 mg/L $N\text{-}NO_2^-$) does not cause shrimp mortality, but prolonged exposure to high levels could cause it. In this experiment, the 15 mg/L limit of $N\text{-}NO_2^-$ was exceeded for approximately 30-40 days, with a maximum value of 21.10 mg/L. Xie et al. (2013) observed, under laboratory conditions, that *B. amyloliquefaciens* germinated could eliminate up to 10 mg/L of $N\text{-}NO_2^-$ in 24 hours. Previous studies with mature biofloc systems did not found significant nitrite reductions, when the probiotic was applied directly to the water column. These studies attributed it to the important microorganism's community, which displaced *B. amyloliquefaciens* (Emerenciano et al. 2013). However, the addition of *B. amyloliquefaciens* on the water column, during the maturation process of the biofloc, that is, when there is not microorganism's community in it (dominated by heterotrophic bacteria responsible for removing the N-TA pick), also has not produced a significant reduction of nitrites with respect to control treatment (P = 0.05). $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ accumulated in the water throughout the culture, following normal BFT dynamics and reaching levels similar to those observed in other experiments (Ray et al. 2010; Furtado et al. 2011; Correia et al. 2014).

The evolution of the TSS was similar to that observed by Ray et al. (2010) and Gaona et al. (2011). In the final two weeks of the experiment, TSS levels were slightly higher than the optimal value of 0.5 g/L stablished by Samocha et al. (2007) for the cultivation of *L. vannamei.* TSS reduction would reduce the amount of bacteria present in the system and therefore reduce the consumption of dissolved oxygen (Gaona et al. 2011). In this experiment, since dissolved oxygen concentrations did not drop below 5 mg/L, it was not necessary to use solids removal techniques, allowing to simplify culture management. The results obtained show that, as other probiotics (Souza et al. 2012), the application of spores of *B. amyloliquefaciens* does not affect the dynamics of N-TA, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$ and TSS in biofloc systems.

Average Chl*a* values were similar to those observed by other authors such as Liu et al. (2014) and Martins et al. (2016) around 150 µg/L. Maximum Chl*a* levels recorded (703.58 µg/L) were higher than those found by Gaona et al. (2011) and Emerenciano et al. (2014) (500 µg/L), although they were well below those observed by Schrader et al. (2011). Chl*a* concentration in BFT is highly variable and depending on environmental conditions and changes that occur in the BFT itself (Schrader et al. 2011). In this experiment, two Chl*a* peaks were observed at the beginning and at the end of the experiment due to awnings management. The first peak was observed with the white awning and the second when the awnings were removed. NPP, WCR and GPP dynamics are in agreement with those observed by Vinatea et al. (2010), where the WCR increased and the GPP decreased, mainly during the first two culture months. WCR values were similar to those observed by Vilani et al. (2016), around 0.45 mg O_2/(L·h) and lower than those observed by Vinatea et al. (2010) and Schveitzer et al. (2013), who obtained values above 1 mg O_2/(L·h). NPP evolution shows the predominance of autotrophic processes in the BFT during the first culture weeks, characterized by lower oxygen consumption than production. From day 58 a change of trophic state to a dominance of heterotrophic processes was observed. Chl*a* evolution shows a second peak at the end of the experiment. This increase in Chl*a* did not lead to an increase in GPP and NPP. Thus, the change in trophic status of the BFT is attributed not to the evolution of Chl*a* but to the evolution of the bacterial population. The beginning of nitrification processes by bacteria, begins to generate a significant bacterial mass that causes heterotrophic processes to predominate in the culture system. WCR increases slowly over the weeks, at the same time as the TSS increases, which consume water dissolved oxygen (Gaona et al. 2011).

6.5.2. Effects on shrimps

The results in zootechnical perfomance, showed a survival and a weekly weight increase around 60% and 0.7 g/week respectively, in all the treatments, and these values are low, they were similar to those observed by other authors (Krummenauer et al. 2011; Ray et al. 2011; Baloi et al. 2013; Schveitzer et al. 2013). These low survival values are related to the increase of N-NO_2^- during an important phase of the culture. The long exposure of shrimps to high nitrites values forced to reduce feed to 60%, which decreased shrimp growth between days 70 and 110, as observed in figure 6.5. During the period of peak N-NO_2^-, a few dead shrimps were observed in the feeders. This mortality could be a consequence of prolonged exposure to high levels of N -NO_2^- (Lin and Chen 2003), which affected the final survival in all treatments equally. Authors such as Camacho (2012) and Reda and Selim (2015), applied *B. amyloliquefaciens* in a recirculation system, and observed improvements in the zootechnical perfomance, due to the production of enzymes in the shrimp digestive system (Nuez-Ortín et al. 2013). However, we have not observed this effect when applying this probiotic in BFT.

During the experiment the state of the immune system was analyzed when the BFT was in two different states, immature (day 86) and mature (day 169) biofloc. The percentages of granular and hyaline hemocytes in the hemolymph were within the range observed by different authors for *L. vannamei* (Li et al. 2007; Camacho 2012; Macias-Sancho et al. 2014; Souza et al. 2016). In the first sampling, it was observed that the percentage of GH was 5% higher in treatments with *B. amyloliquefaciens*. In the second sampling, the difference between treatments and control probiotic was 7%. These results show that the application of *B. amyloliquefaciens* increases the percentage of GH with respect to that of HH. GH have different mechanisms of action against pathogens such as phagocytosis, encapsulation, cytotoxicity and storage and release of prophenoloxidase in the system (Johansson et al. 2000; Cuéllar-Anjel 2008). HH only fight pathogens by phagocytosis, making them less effective against pathogens (Johansson et al. 2000; Cuéllar-Anjel 2008). It should be noted that *B. amyloliquefaciens* especially increased the percentage of GH when the BFT was in an immature state, which subjected the shrimps to great stress. As the shrimp immune system is sensitive to environmental conditions and may suffer under stress situations (Johansson et al. 2000, Guo et al. 2013), the application of *B. amyloliquefaciens* could alleviate the negative effect of culture conditions.

TPC observed was similar to that observed in *L. vannamei* by other researchers (Li et al. 2007; Camacho 2012; Macias-Sancho et al. 2014; Souza et al. 2016). In this variable no statistically significant differences were observed between treatments, in any of the samples. Previously unpublished research by F. Llario observed an improvement in TPC when applying *B. amyloliquefaciens* in a mature BFT. The stress produced during the maturation of the BFT could have affected to a greater extent to the TPC than the probiotic, masking the effects of *B. amyloliquefaciens* on this variable. Among the functions of the proteins in the hemolymph is the recognition of pathogens, their inhibition and their agglutination so that they can be eliminated by the hemocytes (Cuéllar-Anjel 2008), being a variable widely used to monitor the state of the immune system (Macias-Sancho et al. 2014; Souza et al. 2016).

If we compare the results in the immune system between the mature and immature state of BFT (obviating the dose of probiotic received), it is appreciated that the GH values increased 15% with the maturation of the BFT ($P = 0.00$). Also, TPC increased 34 mg/mL ($P = 0.00$) from day 86 to 169. Although shrimp growth could positively affect TPC levels (Chen and Cheng 1993), also the maturation of BFT, and the elimination of N-NO_2^- by BFT bacteria, causes improvements in the shrimp immune system (Guo et al. 2013; Ekasari et al. 2014; Xu and Pan 2014). In this experiment it is shown that the effect of probiotics complements that of BFT. The role of *B. amyloliquefaciens* is of great importance mainly during the maturation of BFT, because it provides a reinforcement of the immune system that helps the shrimps to fight against possible pathogens.

The different doses of *B. amyloliquefaciens* tested (treatments A ($2.8x10^4$ cfu/mL) and B ($2.8x10^3$ cfu/mL)), did not produce significant differences. This shows that *B. amyloliquefaciens* is effective, when applied to water, at a dose of at least 10^3 cfu/mL, doses clearly lower than those used by other authors in shrimp culture (Van Hai and Fotedar 2010; Souza et al. 2012).

6.6. CONCLUSION

Despite the good characteristics of *B. amyloliquefaciens* as a biofloc promoter bacterium, its direct application in water column did not have a significant effect on water quality. The addition of *B. amyloliquefaciens* to the water column, during the maturation process of the biofloc, did not produced significant changes on nutrient dynamics, nor a significant reduction of nitrites with respect to control treatment. Further studies are necessary to analyze if improving the application technique in water can enhance their effect. The addition of the probiotic also failed to alter the trophic state of the system or influence Chl*a* levels.

There was also no significant effect on the zootechnical performance when applying the probiotic bacteria. These results seem to indicate that the nutritional improvements of the biofloc system surpass those that *B. amyloliquefaciens* could have. However, where this bacterium can play a very important role, it is in the reinforcement of the shrimp immune system, mainly under unfavorable culture conditions, such as an immature biofloc system. *B. amyloliquefaciens* manages to increase the percentage of GH, both in mature and immature BFT, although the increase in GH that a mature BFT provides is more important than that produced by the probiotic. In addition, it has been observed that *B. amyloliquefaciens* is effective at a dose of 10^3 cfu/mL, a dose lower than that recommended for other probiotics, reducing costs and maintaining the benefits on the system.

6.7. ACKNOWLEDGEMENTS

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CAPÍTULO 7

Discusión general

7.1. EL ROL DEL FITOPLANCTON EN LOS CULTIVOS DE LANGOSTINOS SIN RENOVACIÓN DE AGUA

La medición de la clorofila *a* (Chl*a*), es utilizada comúnmente como indicador de la biomasa de fitoplancton en los sistemas acuícolas (Gaona et al. 2011). Los niveles de Chl*a* observados en los diferentes experimentos (capítulos 3 y 4), variaron en función de las condiciones de cada uno de los sistemas. La principal diferencia entre los dos sistemas utilizados, se encuentra en la biomasa de langostinos sembrada (entre 0.5 y 1.5 g/m^2 en el experimento desarrollado en el capítulo 3 y 13.5 g/m^2 en el experimento del capítulo 4). Una mayor biomasa de langostinos, implica un mayor aporte de nutrientes procedentes de las excreciones de los langostinos, restos de alimentación y materia orgánica acumulada en el sistema (Crab et al. 2012; Emerenciano et al. 2013). Las diferentes densidades utilizadas, se tradujeron en que mientras en el experimento del capítulo 3 a los 45 días de cultivo se registraron en torno a 12 µg/L de Chl*a*, en el del capítulo 4 se observaron entorno a 340 µg/L de Chl*a*.

La dinámica del fitoplancton no solo se vió afectada por la biomasa de los langostinos, otros factores como la dinámica de nutrientes y la luz tuvieron un claro impacto sobre ella. En los sistemas sin recambio de agua, el proceso de nitrificación del amonio a nitrito y su posterior oxidación a nitrato (Crab et al. 2012; Emerenciano et al. 2013), junto a la acumulación de fosfatos, afecta directamente a la dinámica del fitoplancton. En el experimento del capítulo 3, se observó una clara dominancia de las diatomeas en el cultivo de preengorde, representando el 93% del fitoplancton. Con la acumulación del nitrato y fosfatos en el sistema, aparecieron floraciones puntuales de otros grupos como las cianobacterias, prasinofíceas y primnesiofíceas. Puesto que el cultivo de engorde (capítulo 4), duró 164 días (respecto a los 45 días del experimento del capítulo 3), se observó una mayor acumulación de nutrientes, que la observada en el capítulo 3. La acumulación de nutrientes en el sistema de bioflóculos (capítulo 4), permitió el desarrollo de una comunidad fitoplanctónica mucho más diversa. Aunque las diatomeas fueron el grupo taxonómico dominante durante el cultivo de engorde, se observó una mayor proporción de otros grupos fitoplanctónicos presentes en el agua.

Como observaron Yusoff et al. (2002), Lemonnier et al. (2016) y Martins et al. (2016), la utilización de agua salada o salobre permite que las diatomeas se desarrollen con facilidad y dominen los sistemas acuícolas. El predominio de las diatomeas sobre los otros grupos fitoplanctónicos, se debe a su rápido crecimiento y su eficiencia en la utilización de los nutrientes, ya que pueden consumir de forma eficiente todas las formas nitrogenadas (amonio,

nitrito y nitrato) (Ryther y Officer 1981), lo que no pueden hacer, de forma tan eficaz, otros grupos fitoplanctónicos (Šupraha et al. 2014; Reed et al. 2016). Yusoff et al. (2002) observó que, en los cultivos sin renovación de agua, la dominancia inicial de las diatomeas suele ser sustituida por la proliferación de cianobacterias, que terminan predominando en el sistema. Para mantener una población de diatomeas abundante, suelen realizase aportaciones de sílice en el sistema (Martins et al. 2016). En los experimetnos de los capítulos 3 y 4, la utilización de agua procedente del puerto de Gandia, con una gran riqueza en sílice (Sebastiá et al. 2012; Sebastiá y Rodilla 2013), facilitó el mantenimiento de altas biomasas de diatomeas, sin la necesidad de aportar más sílice al sistema.

Por lo que respecta al sistema de bioflóculos (capítulo 4), las cianobacterias, clorofíceas, euglenofíceas y los dinoflagelados tuvieron una carga de biomasa relevante junto con las diatomeas. La utilización de una salinidad inferior en el sistema de bioflóculos (22.5), permitió la coexistencia de clorofíceas y diatomeas, ya que las clorofíceas no se desarrollan adecuadamente en sistemas con una alta salinidad (Maicá et al. 2012), como la utilizada en el sistema de preengorde (36.5). La aparición de una cantidad considerable de cianobacterias y eulgenofíceas, puede ser explicada por las condiciones ambientales que generan los sistemas de bioflóculos, donde se acumulan nutrientes y sólidos suspendidos. Las euglenofíceas se desarrollan mejor en aguas dulces o salobres (Ju et al. 2008; Schrader et al. 2011), y su crecimiento responde a la presencia de fosfatos (Horabun 1997) y sólidos suspendidos (Green et al. 2014), como se observa en la tabla 4.4. Las cianobacterias, tan habituales en los sistemas acuícolas ricos en nutrientes, suelen suponer un problema para los acuicultores, ya que pueden provocar la mortalidad de los langostinos (Alonso-Rodríguez y Páez-Osuna 2003). Como se detalla en el capítulo 4, su comportamiento mixotrófico les facilita que puedan crecer en ambientes ricos en nutrientes (Yusoff et al. 2002; Casé et al. 2008) y en condiciones de baja luminosidad (Lohscheider et al. 2011; Gris et al. 2017). Por lo que respecta a los dinoflagelados, su presencia se detectó en ambos experimentos, ya que suelen estar presentes en muchos cultivos sin renovación de agua (Manan et al. 2016; Marinho et al. 2016). Sus toxinas pueden afectar negativamente al sistema inmunológico de los langostinos (Campa-Córdova et al. 2009; Pérez-Morales et al. 2017) y por eso su presencia no es deseada en los tanques de cultivo. Aunque no se observó una gran abundancia de estos organismos en los cultivos, su comportamiento mixotrófico (Ismael 2003; Jeong et al. 2010) les permite desarrollarse en diferentes condiciones de luminosidad.

7.2. EL ROL DEL PERIFITON EN LOS CULTIVOS DE LANGOSTINOS SIN RENOVACIÓN DE AGUA

El perifiton, fue monitoreado durante el cultivo de preengorde de *Marsupenaeus japonicus* (capítulo 3). Durante el engorde de *L. vannamei* en un sistema de bioflóculos (capítulo 4), no se monitoreó el perifiton porque la acumulación de sólidos suspendidos, reduce la penetración de la luz en la columnda del agua, y por consiguiente, dificulta el desarrollo de organismos autotróficos los biofilms que crecen en el fondo y los laterales de los tanques.

Mientras que la dinámica del fitoplancton tiene una gran influencia de los nutrientes (capítulos 3 y 4), el perifiton no se ve especialmente afectado por la presencia de nutrientes en el agua, más bien se vió afectado por la disponibilidad de un sustrato adecuado para cada uno de los grupos taxonómicos de microalgas. El proceso de colonización del perifiton empezó con las diatomeas, debido a que estas son capaces de generar una gran cantidad de polímeros extracelulares que les sirve como mecanismo de adhesión al sustrato (la lona de los tanques) (Hanlon et al. 2006; Chouldhary et al. 2017). El establecimiento de una primera capa de perifiton, conformada por diatomeas, crea las condiciones adecuadas para la fijación de otros grupos taxonómicos (Ács et al. 2000). Como ya observaron Ács et al. (2000), Khatoon et al. (2007) y Zhang et al. (2012) entre otros, con el paso del tiempo aumenta la biomasa del perifiton y el número de grupos que lo componen. El perifiton desarrollado durante el preengorde de *M. japonicus*, estuvo compuesto mayormente por diatomeas (67.92%), prasinofíceas (15.84%), cianobacterias (6.47%) y euglenofíceas (4.84%). Las primnesiofíceas aunque no fueron muy abundantes (1.99%) también tuvieron una especial relevancia en algunos de los tanques. Mientras que la presencia de las diatomeas y las cianobacterias han sido observadas frecuentemente en el perifiton de los tanques de acuicultura (Ballester et al. 2007; Khatoon et al. 2007, Anand et al. 2013), no se encontraron referéncias bibliográficas sobre la presencia de prasinofíceas y primnesiofíceas. Su pequeño tamaño pudo dificultar su identificación, con microscopios ópticos, entre los polímeros extracelulares del perifiton, por lo que su presencia no habría sido registrada. La presencia de estos grupos taxonómicos en el perifiton puede ayudar a entender el alto valor nutricional del perifiton para los langostinos (Benemann 1992; Jaime-Ceballos et al. 2006).

7.3. El HPLC/CHEMTAX COMO METODOLOGÍA PARA EL ESTUDIO DE LAS MICROALGAS.

La metodología HPLC/CHEMTAX, ha permitido determinar la evolución de los diferentes grupos taxonómicos que componen el perifiton (capítulo 3). Esta herramienta permitió la detección de grupos fitoplanctónicos de pequeño tamaño y/o poco abundantes, como es el caso de prasinofíceas y primnesiofíceas. La presencia de estos grupos no suele ser detectada cuando se utiliza el microscopio óptico para el estudio del fitoplancton en los sistemas acuícolas (Ballester et al. 2007; Kahoon et al. 2007a; Anand et al. 2013; Sruthisree et al. 2015; Betancur-González et al. 2016), pero sus pigmentos firma (prasinoxantina y 19'hexanolyfucoxantina respectivamente), sí han sido detectados en el experimento desarrollado en el capítulo 3.

Aunque la metodología HPLC/CHEMTAX agiliza el análisis del fitoplancton (Schlüter et al. 2006; Ju et al. 2008; Schlüter et al. 2016), puede tener algunas limitaciones en los sistemas de bioflóculos. Algunos organismos como los dinoflagelados y cianobacterias son mixotróficos, y se comportan de forma heterotrófica en condiciones de limitación de luz (Yu et al. 2009; Jeong et al. 2010; Gris et al. 2017). Por ese motivo en el capítulo 4, la aparición de las bacterias heterotróficas y la generación sólidos suspendidos provocó que no se detectaran dinoflagelados a partir del día 31. Al mismo tiempo, las cianobacterias detectadas por el HPLC/CHEMTAX, se redujeron cuando se cubrió el invernadero con un toldo negro, pero su presencia si fue abundante en las observaciones con el microscopio óptico.

Puesto que no se han encontrado referencias bibliográficas donde se registre la presencia de primnesiofíceas y prasinofíceas en el perifiton de los tanques destinados a la acuicultura, debido al pequeño tamaño de estos grupos taxonómicos y la gran cantidad de polímeros extracelulares que generan otros grupos taxonómicos (Hanlon et al. 2006; Chouldhary et al. 2017), que dificulta su identificación mediante microscopio óptico. La utilización de la metodología HPLC/CHEMTAX permite detectar las primnesiofíceas y prasinofíceas, aportando una ventaja frente a otras técnicas de análisis.

7.4. EL ROL DE *Bacillus amyloliquefaciens* EN LOS SISTEMAS DE BIOFLÓCULOS

7.4.1. Efecto de *Bacillus amyloliquefaciens* sobre el sistema de bioflóculos

Aunque algunas bacterias probióticas ayudan a mantener la calidad del agua en los sistemas tradicionales (Vaseeharan y Ramasamy 2003; Balcázar et al. 2007), cuando otros autores aplicaron diferentes probióticos en el agua de los sistemas de bioflóculos, no observaron cambios en la calidad del agua (Vita 2008; Souza et al. 2012; Krummenauer et al. 2014). La capacidad de *B. amyloliquefaciens* de eliminar nitritos en condiciones de laboratorio (Xie et al. 2013; Hui et al. 2018), le confiere un alto potencial para su utilización en los sistemas de bioflóculos. Los resultados observados en los experimentos desarrollados en los capítulos 5 y 6, muestran que la aplicación de esporas de esta bacteria en los sistemas de bioflóculos, no altera significativamente la dinámica de los nitritos. La presencia de otros microorganismos en el sistema de bioflóculos, podría ayudar a entender por que *B. amyloliquefaciens* no produjo cambios estadísticamente signficativosó en la calidad del agua, ya que las bacterias de los sistemas de bioflóculos pueden afectar al desarrollo de otros microorganismos (Emerenciano et al. 2013). La utilización de inoculos de cultivos anteriores, para facilitar la maduración del sistema de bioflóculos (capítulo 5), pudo afectar negativamente al *B. amyloliquefaciens*, impidiendo su actuación sobre la calidad del agua. Aunque en el capítulo 6 no se utilizó ningún inóculo, las bacterias heterotróficas que se desarrollaron gracias a la presencia del amonio y la adición de carbono, pudiendo afectar al desarrollo de las esporas de *B. amyloliquefaciens* en el sistema, impidiendo la remoción de nitritos. La aplicación de germinados de *B. amyloliquefaciens*, podría facilitar el establecimiento de una población de probiótico en la columna de agua y ayudar a la remoción de los nitritos acumulados durante la maduración del sistema de bioflóculos.

7.4.2. Implicaciones de *Bacillus amyloliquefaciens* para los langostinos

Nuéz-Ortín et al. (2013), observó que *B. amyloliqeufaciens* genera enzimas digestivas cuando es ingerido por los langostinos, mejorando así la asimilación del pienso y dando como resultado un mayor engorde. Este mismo efecto también fue observado por Camacho (2012) y Reda y Selim (2015), al aplicar *B. amyloliquefaciens* en los piensos suministrados a langostinos y peces cultivados en sistemas de recirculación. Por el contrario, al aplicar *B. amyloliquefaciens* a los sistemas de bioflóculo, no se observó una mejora en los parámetros

de crecimiento (capítulos 5 y 6). Estas diferencias, entre los resultados de aplicar *B. amyloliquefaciens* en sistemas de bioflóculos y sistemas de recirculación, puede deberse a los beneficios nutricionales de los sistemas de bioflóculos, entre los que destaca el aporte extra de proteína de origen microbiano (Emerenciano et al. 2013).

B. amyloliquefaciens podría haber jugado un papel importante durante la maduración del sistema de bioflóculos (capítulos 6), debido a que la germinación de esporas en el agua podría haber enriquecido nutricionalmente los bioflóculos, tal como apuntaban las conclusiones de Bao (2014). Además, el efecto positivo de las enzimas digestivas, podría haber supuesto una ventaja en los momentos donde se redujo la alimentación de los langostinos al 60% de la dosis recomendada por Jory et al. (2001) (con el objetivo de controlar la concentración de nitrito en el sistema). Desgraciadamente no se observó ninguna diferencia estadísticamente significativa entre tratamientos en ninguna de las biometrías realizadas, ni en los parámetros zootécnicos calculados al final del experimento. A diferencia de *B. amyloliquefaciens*, otras bacterias probióticas sí han conseguido mejorar estos parámetros al aplicarlas en los sistemas de bioflóculos (Vita 2008; Souza et al. 2012), confiriéndoles una ventaja competitiva frente a la aplicación de esporas de *B. amyloliquefaciens* en la columna del agua.

El efecto más importante de la aplicación de esporas de *B. amyloliquefaciens,* es el refuerzo del sistema inmunológico de los langostinos. En el experimetno desarrollado en el capítulo 5, ya se demostró que la aplicación de *B. amyloliquefaciens* provocó un aumento del porcentaje de hemocitos granulosos y de la concentración de proteína total de la hemolinfa, así como una disminución del número de células con apoptosis. Todos estos indicadores mostraron que *B. amyloliquefaciens* tiene un efecto probiótico, no solamente en los sistemas de recirculación (Camacho 2012; Reda y Selim 2015), sino que también es efectivo al aplicarse en sistemas de bioflóculos para el cultivo intensivo de langostinos. Los beneficios sobre el sistema inmunológico son de gran importancia durante el proceso de maduración de los bioflóculos, como se observó en el experimento expuesto en el capítulo 6. Durante la fase de maduración, se acumula amonio y nitrito en el sistema (Jatobá et al. 2014), lo que supone un estrés para los langostinos (Lin y Chen 2001, 2003). El estrés ambiental puede alterar negativamente el sistema inmunológico de los langostinos (Johansson et al. 2000; Guo et al. 2013), por lo tanto la aplicación de *B. amyloliquefaciens,* durante el proceso de maduración, refuerza los mecanismos de detección de patógenos y su posterior eliminación (Johansson et al. 2000; Cuéllar-Anjel 2008). En el capítulo 6, también se observó como el refuerzo del sistema

inmunológico, que aporta la aplicación de *B. amyloliquefaciens*, se suma al que realizan los sistemas de bioflóculos maduros (Emerenciano et al. 2013), por lo tanto, se puede afiramar que la aplicación de *B. amyloliquefaciens* aumenta la bioseguridad de los sistemas de bioflóculos.

7.4.3. Manejo de *Bacillus amyloliquefaciens* en los sistemas de bioflóculos

Las diferentes dosis de probiótico testadas ($3.79x10^5$, $1.9x10^5$, $9.48x10^4$ ufc/mL en el capítulo 5 y $2.8x10^4$, $2.8x10^3$ ufc/mL en el capítulo 6), dieron los mismos resultados sobre el sistema inmunológico de los langostinos. Por lo tanto, y a falta de testar dosis inferiores, la dosis mínima recomendada de esporas *B. amyloliquefaciens* es 10^3 cfu/mL, aplicadas diariamente en la columna de agua.

Comparando la dosis diaria mínima efectiva de *B. amyloliquefaciens*, aplicada directamente en el agua, con la recomendada por otros probióticos para su aplicación en sistemas de bioflóculos, se puede observar que la dosis diaria de 10^3 cfu/mL es menor que la recomendada por otros probióticos (Souza et al. 2012).

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CAPÍTULO 8

Conclusiones generales

y

Futuras líneas de investigación

8.1. CONCLUSIONES GENERALES

Una vez analizados y discutidos conjuntamente los resultados obtenidos durante la realización de esta tesis, se han obtenido las siguientes conclusiones generales:

- La utilización de la metodología HPLC/CHEMTAX, facilita la monitorización de las microalgas que crecen en los tanques de cultivo. Además, permite detectar, tanto en el fitoplancton como en el perifiton, grupos de pequeño tamaño y/o poco abundantes, difícilmente detectables con los microscopios ópticos.
- La técnica HPLC/CHEMTAX, puede subestimar los organismos mixotróficos cuando estos se encuentran en un sistema heterotrófico, puesto que dejan de producir pigmentos y no pueden ser detectados por el HPLC.
- Por primera vez se ha detectado la presencia de prasinofíceas y primnesiofíceas en el perifíton de tanques acuícolas. Su presencia puede ayudar a entender el alto valor nutricional del perifiton y su aportación a la nutrición de los langostinos.
- La dinámica del perifiton observada, estuvo influenciada por los procesos de colonización de las diferentes microalgas, así como por la capacidad de estas de crecer sobre la lona de PVC y sobre la capa de polímeros extracelulares generada por otras microalgas.
- La utilización de los toldos de sombreo durante la formación de un sistema de bioflóculos, puede limitar la proliferación de algunos grupos taxonómicos de microalgas, mientras que permite el desarrollo de otras microalgas como las diatomeas, de un alto valor nutricional. También provoca el cambio de comportamiento trófico de algunos grupos fitoplanctónicos como las cianobacterias.
- Las diatomeas son el grupo más abundante en los tanques de cultivo intensivo sin renovación de agua. Estas microalgas, que poseen un alto valor nutricional, son capaces de aprovechar rapidamente los nutrientes del sistema y toleran condiciones de baja luminosidad.
- Las bacterias autóctonas de los sistemas de bioflóculos, podrían afectar al desempeño de las esporas de *Bacillus amyloliquefaciens* en la columna de agua, dificultando que esta colonicen los bioflóculos, participen en la maduración del sistema o mejoren las propiedades nutricionales de los bioflóculos.

- La aportación nutricional que comporta el consumo de bioflóculos, enmascaró el efecto de las enzimas digestivas generadas por *B. amyloliquefaciens*.
- La aplicación de esporas de *B. amyloliquefaciens* en el agua, consigue reforzar el sistema inmunológico de los langostinos. Estas bacterias mejoran el porcentaje de hemocitos granulosos, la concentración de proteína total en la hemolinfa y reducen el número de células con apoptosis en la hemolinfa. Estos beneficios se mantienen tanto en condiciones de cultivo óptimas, como bajo condiciones de estrés.
- Los efectos probióticos de *B. amyloliquefaciens* sobre el sistema inmunológico de los langostinos, se suman a los que provocan los sistemas de bioflóculos. Su uso, puede ser de gran importancia durante los procesos de maduración de los sistemas de bioflóculos, para compensar el efecto negativo de las condiciones de cultivo adversas.
- Se necesita una dosis de 10^3 ufc/mL de *B. amyloliquefaciens* para obtener unos efectos positivos sobre los langostinos. Esta dosis es inferior a la recomendada para la aplicación en el agua de otros probióticos comerciales testados en sistemas de bioflóculos.

8.2. FUTURAS LÍNEAS DE INVESTIGACIÓN

Durante la realización de los diferentes experimentos, tanto en la Universitat Politècnica de València como en la Universidade Federal do Rio Grande, y el posterior análisis y discusión de los resultados, se han identificados diferentes aspectos que podrían ser considerados para futuras investigaciones y que se detallan a continuación.

Los muestreos semanales de microalgas, nos han permitido tener una visión más amplia de la composición del fitoplancton y del perifiton, así como conocer su dinámica en los sistemas intensivos de langostinos. Las futuras líneas de investigación, deben de profundizar en el papel de los nutrientes y los factores ambientales en la dinámica de las microalgas. También se debería reducir el período entre muestreos, para observar, con mayor claridad, la aparición de floraciones puntuales y determinar que factores los desencadenan. Conociendo los factores que provocan la aparición de floraciones puntuales, se pueden diseñar estrategias de manejo y gestión para favorecer la presencia de las microalgas deseadas y evitar las inapropiadas o perjudiciales para los cultivos de langostinos.

La acción de las esporas de *B. amyloliquefaciens* pudo verse reducida por los microorganismos presentes en los sistemas de bioflóculos, impidiendo que la adición del probiótico afectara a la calidad del agua del sistema. Para conocer en profundidad el potencial de *B. amyloliquefaciens* en los sistemas de bioflóculos, se debería estudiar los efectos de la aplicación de germinados de *B. amyloliquefaciens* sobre la columna de agua así como exponer a los langostinos al desafío de algún patógeno. Cabe la posibilidad, que la aplicación de germinados facilite al *B. amyloliquefaciens* colonizar los bioflóculos y reducir los niveles de nitritos, mejorar las propiedades nutricionales de los bioflóculos y mantener reforzando el sistema inmunológico de los langostinos, que provoca la aplicación de esporas. Otra de las cuestiones que deberían seguir siendo estudiada, es la reducción de la dosis de *B. amyloliquefaciens*, con el objetivo de determinar la dosis mínima efectiva, así como corroborar su efectividad mediante la realización de desafíos con algún patógeno. Todos estos aspectos, pueden ser de gran ayuda para su aplicación en los sistemas de bioflóculos, y la reducción de las condiciones adversas durante el proceso de maduración de los bioflóculos puesto que la inmadurez del sistema supone un gran estrés para los langostinos y una disminución de la productividad de los cultivos.

Durante la realización de la tesis doctoral, también se ha observado el potencial de los cultivos intensivos de langostinos en la Unión Europea, ya que actualmente hay un incremento del número de granjas intensivas de langostinos en suelo europeo. Este hecho, supone un nuevo reto para los investigadores de la Unión Europea, ya que las futuras investigaciones tienen que facilitar la instalación de nuevas granjas en territorio europeo siguiendo criterios de proximidad, sostenibilidad y seguridad alimentaria. El desarrollo de estudios sobre los sistemas de bioflóculos, es vital para que estos sistemas puedan adaptarse a la casuística europea y resulten un negocio viable para los inversores. Por todo ello los equipos investigadores deberían centrar sus esfuerzos en ayudar a los productores europeos a salvar los obstáculos con los que se encuentra el sector en estos momentos. Entre estos obstáculos, cabe destacar la falta de producción de poslarvas de *L. vannamei* en territorio europeo, la poca variedad de piensos disponibles o la eficiencia energética de las granjas. Además, es importante estudiar la aplicabilidad de los sistemas de bioflóculos para diferentes especies de peces y crustáceos, con el objetivo final de hacer una producción sostenible de los productos demandados por los mercados europeos.