JOÃO RIBEIRO RITO

Effect of probiotic microorganisms on growth and health status of rearing species under biofloc technology (BFT)

University of Algarve, Faculty of Science and Technology

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Master's degree in Aquaculture and Fisheries
(Specialization in Aquaculture)

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I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and included in the reference list.

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(João Ribeiro Rito)

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Abstract

Environmental impacts associated with aquaculture productions are mainly related to the discharge of nutrient- rich wastewater, mainly N (Nitrogen) and P (Phosphorous). Biofloc technology (BFT), a microbial- based system, is seen as a promising sustainable production system. It operates as a zero-water exchange system, where nutrients and organic particles in the culture water are recycled by autotrophic and heterotrophic bacteria into protein- rich flocs, providing a complement food source to fish diets and ultimately improving circular economy. Biological control strategies, including bacterial probiotics or microalgae products, have proven to enhance the health of reared animals and improve water quality in aquaculture. This project aimed to deepen the knowledge of the ecology of microalgae for their cultivation in conditions that allow interaction with floccules, generating a food alternative that combines the benefits of both bacteria and microalgae. The effects of microalgae addition in the cultured species, *Penaeus vannamei* (Pacific white shrimp), were analyzed and compared with bacterial probiotic addition in a BFT production. Four different treatments were analyzed: biofloc (control), bacterial probiotic inclusion, microalgae inclusion, and a combination of both. This work was conducted in two phases, with the same experimental design but under distinct production conditions (density, tank volume, experimental period). Furthermore, shrimp resilience was assessed through three environmental challenges: 1) hypoxia, 2) density and 3) combined hypoxia and density, whereby shrimp survival was analyzed for each challenge. The results indicated that both bacterial and microalgae populations remained stable, providing both water quality and health benefits for the shrimps. Microalgae community evolution under biofloc conditions showed stability and capability to proliferate, even though nutrients overload on the culture water negatively impacted survival and growth performance of the reared shrimps. However, shrimp in microalgae-groups exhibited enhanced resilience when subjected to environmental stress challenges.

Key words

Biofloc, Probiotics, Microalgae, *Penaeus vannamei*, Sustainability.

Resumo

A indústria da aquacultura tem vindo a aumentar drasticamente a sua produção nas últimas décadas. No decorrer deste desenvolvimento esteve presente uma ambição de promover uma produção mais sustentável, recorrendo a um mínimo de recursos naturais, desenvolvendo sistemas de produção mais eficientes e sustentáveis, e consequentemente, aumentar a economia circular. Ainda assim, os impactos ambientais associados às produções aquícolas estão principalmente relacionados com a descarga de águas residuais ricas em nutrientes em águas adjacentes, especificamente azoto (N) e fósforo (P). Relativamente à produção de camarão, os impactos ambientais são diversos, estando estes associados às técnicas e sistemas de produção em prática. Tradicionalmente, sistemas de produção extensivos e semi-intensivos são os mais utilizados, sendo representados por uma produção de baixa densidade animal (10-30 animais/m^2) que por norma requer o uso de grandes áreas naturais (1 e 5 hectares). Estas áreas estão normalmente associadas a zonas costeiras intertidais onde a renovação de água é realizada de uma forma natural pela entrada e saída da maré nestas áreas. No entanto, estes sistemas de produção apresentam repercussões associadas como a destruição de ecossistemas aquáticos devido à necessidade de construção de tanques-terra. Por outro lado, sistemas de produção intensiva como RAS (Sistemas de recirculação aquícola) acabam por ser uma versão mais sustentável sem necessidade de uso de grandes áreas de terreno (sistemas fechados). No entanto, os custos associados ao investimento neste sistema podem revelar-se elevados. A tecnologia Biofloc (BFT) trata-se de um sistema de base microbiana de produção intensiva, sendo esta representada como uma promissora produção sustentável de peixe e camarão, com baixos custos de produção associados. É um sistema sem necessidade de trocas de água, no qual os nutrientes e as partículas orgânicas acumulados na água da cultura são reciclados por bactérias autotróficas e heterotróficas, gerando flóculos ricos em proteínas, proporcionando assim uma fonte de alimento complementar para as rações de peixe. Além disso, a reciclagem de nutrientes no sistema biofloc não só aumentará a economia circular deste sistema, como também será uma grande oportunidade para se tornar uma fonte alternativa à farinha de peixe, de alta qualidade nutricional. Um conjunto de estratégias de controlo biológico podem ser usadas em produções animais na aquacultura, incluindo probióticos bacterianos e produtos derivados de microalgas. A aplicação dos mesmos resulta numa série de benefícios, tais como a melhoria da saúde dos animais produzidos, prevenindo o aparecimento de possíveis doenças e o crescimento de patógenos na água de cultura, e uma melhoria de qualidade de água associado ao controlo da concentração de nutrientes, tais como amónia (NH_3), nitritos (NO_2^-), nitratos (NO_3^-) e fosfatos (PO_4^{3-}), que se podem revelar tóxicos em concentrações altas. Este estudo visa aprofundar o conhecimento da ecologia de microalgas no seu cultivo em condições que lhes permitam interagir com os flóculos gerados no sistema biofloc, gerando assim um complemento alimentar que combina benefícios bacterianos e de microalgas. Os efeitos da adição de microalgas na produção da espécie cultivada, *Penaeus vannamei* (camarão branco do Pacífico), foram avaliados. Neste sentido, quatro tratamentos foram realizados e analisados, sendo um primeiro tratamento de controlo somente com água de biofloc (C), um segundo que incluiu a adição de um probiótico bacteriano (P), um terceiro com adição de microalga (M) e finalmente um último tratamento que incluiu um probiótico bacteriano e microalgas (MP). Este estudo dividiu-se em duas fases consecutivas com a mesma estrutura experimental, considerando os mesmos tratamentos. Respetivamente às condições de produção, na primeira fase, foi utilizada uma densidade animal de 150 camarões/m^2, num tanque com um volume de 200 litros por um período experimental de 28 dias. No caso da segunda fase, foi considerada uma maior densidade de animais (285 camarões/m^2), assim como um maior volume de tanque (600 litros) e período experimental (68 dias). No sentido de analisar o impacto dos diferentes tratamentos na qualidade da água assim como no crescimento dos camarões, diversos

parâmetros foram analisados. Especificamente, de modo a analisar o impacto e evolução das populações de microalga nos respetivos tanques, diversos parâmetros de qualidade de água (amónia (mg/L), nitritos (mg/L), nitratos (mg/L) e fosfatos (mg/L)) e clorofila total (mg/L), foram medidos regularmente. Relativamente ao crescimento dos animais, parâmetros de crescimento incluindo ganho de peso (g), taxa de crescimento (g/dia), taxa de conversão alimentar (FCR), produtividade (g/m^3) e sobrevivência (%) foram calculados e analisados. No fim da segunda fase deste estudo, os animais criados na mesma foram submetidos a três desafios ambientais, de modo a testar a resiliência dos mesmo entre os diferentes tratamentos testados, com o intuito de inferir qual dos probióticos adicionados impactou da melhor forma os animais. Neste sentido, três desafios ambientais foram realizados, incluindo um de hipoxia (baixo nível de oxigénio dissolvido (2-2.5 mg/L)), um desafio de alta densidade (2205 camarões/m^3), e finalmente um desafio que combinava hipoxia e altas densidades. Estes desafios foram aplicados por 6 horas no caso dos dois primeiros, e 3 horas no caso do último desafio, sendo que, após este período os animais foram transferidos para aquários e observados até 24 horas após os desafios. Neste caso, o parâmetro de análise foi a sobrevivência dos camarões, sendo registado em dois momentos, ao fim das 6 (ou 3) e 24 horas. Tendo em conta os resultados deste estudo, foi possível concluir que as populações bacterianas e de microalga se mantiveram estáveis no decorrer de ambas as fases. Além disso, a evolução da comunidade de microalgas sob condições de BFT mostrou estabilidade e capacidade de proliferar, até um período máximo de 30 dias, onde se verificou a necessidade de reforçar a população. Ainda assim, foram verificados benefícios em relação à qualidade da água de cultura, onde se observou um controlo na carga de nutrientes ao longo do tempo. Contudo, especificamente na segunda fase deste estudo, uma sobrecarga de nutrientes na água da cultura dos camarões, devido à alta concentração dos mesmos no inóculo de microalga utilizado, impactou negativamente a sobrevivência e o crescimento dos mesmos. No entanto, a resiliência dos camarões na submissão aos diversos desafios ambientais mostrou os melhores resultados nos animais criados com a adição de microalgas, porém, devido à falta de uma análise estatística dos resultados não foi possível retirar qualquer conclusão. De modo a aprofundar o conhecimento sobre os diversos impactos tanto do probiótico bacteriano e da microalga, seria ideal a realização de uma análise metagenómica da água de biofloc de modo a compreender a comunidade bacteriana existente, assim como uma análise proximal do biofloc e dos camarões, com o intuído de entender as diferenças na composição nutricional entre os diferentes tratamentos testados.

Palavras-chave

Biofloc, Probiótico, Microalga, *Penaeus vannamei*, Sustentabilidade.

Table of contents

Figure Index

Table Index

Appendix Index

1. Introduction

1.1. Crustacean aquaculture

Marine shrimp production dominates crustacean aquaculture (Bardera et al., 2019; Briggs et al., 2004; Tacon, 2003), contributing significantly to the overall aquatic protein production (Bondad-Reantaso et al., 2012). Regarding this sector's economic impact, in 2020 crustacean's aquaculture production reached 11.2 million tons, correspondent to a value of 81.5 billion USD, whereby this production represented 12.8% of the total aquaculture aquatic animals' production (87.5 million tons) (FAO, 2022). As well, this aquaculture sector represents a highly important source of foreign exchange earnings for several developing-countries in Asia (FAO, 2022; Tacon, 2003).

To date, most shrimp culture have developed in Asia and South/Central America. In the 1970's the achievement of controlled reproduction and larvae growth marked a pivotal shift, steering this sector towards a mode of development that was based on intensifying and specializing production (Raux & Bailly, 2002). Subsequently, a rapid growth was observed in Asia and South/Central America, leading world production from mid-1970's to 1988, comprising annual growth rates between 20 and 30% (Raux & Bailly, 2002). Over the following decade (1985-1995), farm- raised shrimp contribution to world shrimps' supply grew about 400% (Raux & Bailly, 2002). Furthermore, during the early 2000's, crustaceans aquaculture production kept on growing for all major species, comprising an average annual rate 15% faster than in the previous decade (Bondad-Reantaso et al., 2012). Crustacean production sector's fast development is largely a reflection of drastic increases in Pacific white leg shrimp (*Penaeus vannamei)* productions in China, Thailand, and Indonesia (Bondad-Reantaso et al., 2012). Nowadays, most shrimp are produced in Asia and Latin America, with Ecuador recently overtaking Thailand to become the world's fifth largest shrimp producer (FAO, 2020). In 2016, Europe's highest imported product was aquaculture produced shrimp, with 614.733 tons, reaching a value of 4700 million euros, since EU provided less than 1% of the global shrimp catches and only 8% of shrimp European consumption (EUFOMA, 2017).

Shrimp is a very popular product with significant consumption levels due to its high quality. Other advantages include short production cycle, good price and the possibility of new products. Its production is growing at a faster rate when compared to other aquaculture species (62% between 2008-2017) (EUFOMA, 2017). In 2002, over 75% of the total shrimp production included only three species, *Penaeus monodon* (giant tiger prawn), *Penaeus*

chinensis (fleshy prawn) and *Penaeus vannamei* (pacific white shrimp) (Briggs et al., 2004). Particularly, pacific white shrimp (*Penaeus vannamei*) is the most economically significant species in crustacean aquaculture. With a production of 4.966 million kg, it has the highest world total value, amounting to USD 28.782 million, with a unitary value of 5.80 USD/kg (APROMAR, 2020). In addition, it was the world's top-produced species in 2020, with a total production of 5.812 million tones, accounting for 51.7% of the total crustacean aquaculture (FAO, 2022). Traditionally, *P. vannamei* has been produced in semi-intensive systems, associated with large earth ponds (2-25 hectares) in wetlands from Asia and South/Central America and low densities systems (5-25 shrimp/m^2) (Boyd & Clay, 1998; Centre Oceanologique du Pacifique, 1984; Kungvankij, 1984). Nevertheless, intensive, and super intensive systems are increasing around the world, including RAS and Biofloc technology (BFT), with much higher density (250-500 shrimp/m^2) (Avnimelech et al., 2015; Kuhn et al., 2009).

1.2. *Penaeus vannamei* aquaculture and associated environmental issues

Globally, Penaeid shrimps are considered a high valued seafood commodity, being *Penaeus vannamei* the main cultured species from this family (Hussain et al., 2021). Commonly known as Pacific white shrimp *(Penaeus vannamei*) (Figure 1.1), this neo-tropical species is recognized by its production advantages, being an easy species to farm, characterized by its stable production (Kungvankij, 1984; Suwoyo & Hendrajat, 2021). This species possesses a high density tolerance, being able to undergo production densities of 400 animals per m^2 (Bardera et al., 2019; Briggs et al., 2004). Another advantage of *Penaeus vannamei* production is its euryhaline characteristics, being able to withstand and adapt to a wide range of salinities (Suwoyo & Hendrajat, 2021). The intensification of this species' production has enabled the gathering of substantial knowledge regarding its biology, genetics and zootechnics (Bardera et al., 2019).

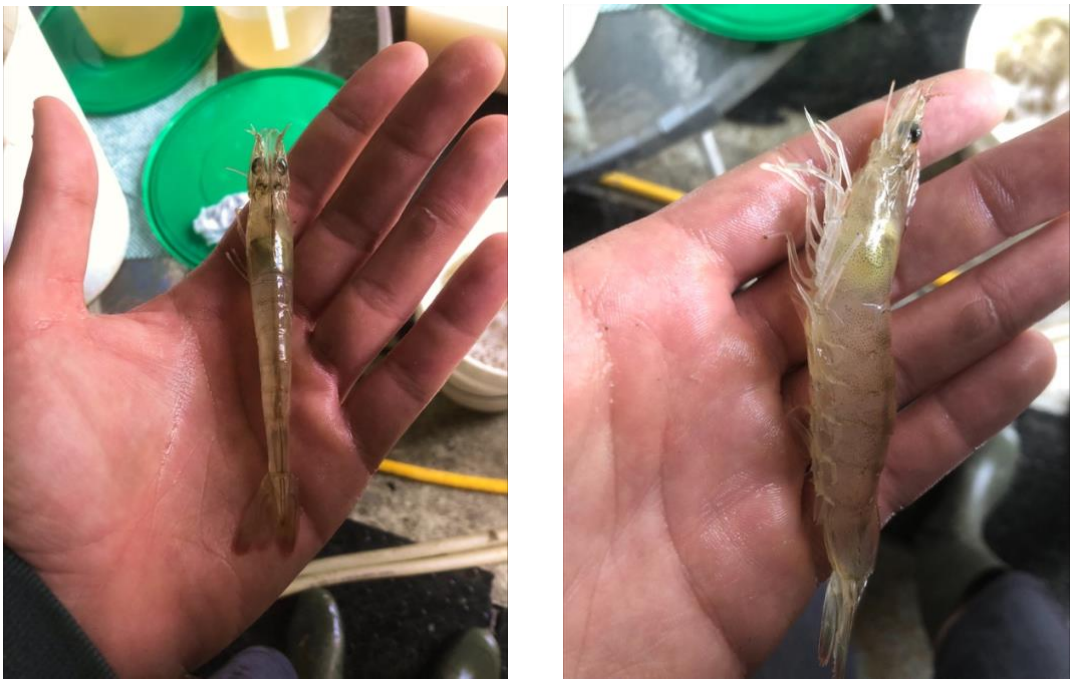

Figure 1.1. The Pacific white shrimp (Penaeus vannamei).

Traditionally, *P. vannamei* are produced in extensive, semi-intensive, intensive, and super-intensive systems, representing low, medium, high, and extremely high stocking densities, respectively (FAO, 2009). Extensive farming (Figure 1.2) is considered the most ecological technique as it does not require the use of aeration or pumping equipment, since it is usually conducted in tidal areas (FAO, 2009). It is carried out in earthen ponds that can span over 30 hectares, with the animals being stocked at a density of 2-10 individuals/m^2. The yield is influenced by the primary productivity of the system, reaching 50-500 kg/ha distributed over up to 2 harvests per year (FAO, 2009).

Figure 1.2. Extensive production system - shrimp farming facility (Empagran).

The production of *Penaeus* spp. shrimp in semi-intensive systems is the most commonly used technique in the majority of developing countries (Centre Oceanologique du Pacifique, 1984). In addition to pond fertilization, this involves the use of compound feed, daily water exchanges, and moderate aeration in some cases (Apud, 1984; Centre Oceanologique du Pacifique, 1984). Stocking densities can range from 10-30 individuals/m^2, and the shrimps are typically raised in ponds ranging from 1 to 5 hectares (FAO, 2009). Production yields may vary between 500 and 2000 kg/ha in 2 harvests per year (FAO, 2009). On the other hand, intensive farming conditions involves working with high stocking densities, approximately between 40 and 300 individuals/m^2 (FAO, 2009). Continuous aeration and water exchanges are essential, and the feeding relies solely on manufactured feed (FAO, 2009). This method incurs higher production costs but leads to higher profits (FAO, 2009). Finally, thanks to the implementation of advanced technology, it is possible to reach a production level known as super-intensive, where, to maintain the temperature, the tanks are placed in greenhouses (FAO, 2009). This allows for densities of up to 500 shrimp/m^2 and average productions of 100,000 kg/ha per year (FAO, 2009). In 2018, *Penaeus vannamei* accounted for an annual global production of 4966.2 thousand tones, representing 52.9% of the total aquaculture production of crustaceans (FAO, 2020).

Nevertheless, the strong development of shrimp sector has generated adverse effects on the natural environment such as the reduction of natural areas due to the construction of facilities dedicated to farming, along with the salinization of farm soils and considerable contributions of organic matter to the coastal marine environment (Ocampo Héctor & Ximhai Ra, 2010). Examples of some impacts associated with shrimp farming include the destruction of aquatic ecosystems due to ponds constructions, or the eutrophication of adjacent water caused by the discharge of nutrient and organic matter -rich pond wastewater (Table 1.1) (Clay, 1996).

Table 1.1. Summary of main environmental problems derived from shrimp production. Describes the activities, the impacts and its results (Clay J, 1996).

SHRIMP PRODUCTION ENVIRONMENTL IMPACTS		
Activities	Impacts	Results
Pond Constructions	Destruction of aquatic ecosystems	Habitat lost and reduced ecosystem productivity
		Microclimate alteration
Groundwater extraction	Saline water intrusion and salinization of aquafers	Decrease in water supply for agriculture
		Land sinking
Sea water intake	Extraction of larvae and juveniles of fish and shellfish	Lower catches for fishermen's subsistence
Pond effluent discharge	Eutrophication of adjacent waters due to organic matter	Transmission of diseases to wild animals
	Chemical contamination of waters due to the use of antibiotics	Proliferation of antibiotic-resistant pathogens
Overfishing of post- larvae and ovate females	Decline in wild shrimp population	Low catches for fishermen
		Larvae shortage for shrimp farmers
Disease spread	Introduction of diseases to existing shrimp farms and local ecosystems	Loss of aquatic life and changes in species diversity

Finally, it is also worth highlighting the considerable carbon footprint derived from the production, processing, and distribution of shrimp due to the significant amount of greenhouse gases generated and emitted. Moreover, given that shrimp production occurs mainly in Asia and Latin America, and European consumption depends almost exclusively on exports, the environmental impact derived from transportation is inevitable (Pinargote Segura Andrea Antonella, 2021). According to the results obtained by Pinargote Segura Andrea Antonella, (2021), for every kg of raw whole shrimp, from cultivation to distribution to the national and international market, 6.04 ± 0.04 kg of CO_2 are produced. In the cultivation stage, 4.39 kg of CO_2 was generated, mainly due to the generation of organic waste together with the consumption of diesel; in the processing stage, 163 kg of CO_2 was produced mainly as a result of the high consumption of electrical energy; and finally in the distribution stage 0.006 kg of CO_2 was generated, with land transportation generating most of the emissions (Pinargote

Segura Andrea Antonella, 2021). Additionally, shrimp farms may be responsible for 25 to 50% of the global mangrove clearance that has occurred since 1960 (Gowing & Ocampo-Thomason, 2007). One possible solution to alleviate the great environmental impact of shrimp production and export is to restrict the location of ponds in mangrove areas (Primavera, 1994). Additionally, consumer regions like Europe could lower their carbon footprint by starting local shrimp production, shortening the supply chain (Cappel & Huttington, 2023). A promising solution could be the production of shrimp using Biofloc technology, which increases the efficiency of the system, reduces water and energy consumption, minimizes environmental pollution, and improves production biosafety (Avnimelech et al., 2015).

1.3. Biofloc technology (BFT)

Aquaculture production has been rapidly increasing and evolving over the last decades, aiming to address three major constraints (Avnimelech et al., 2015). These challenges include 1) increasing production without raising the use of basic natural resources such as water and land; 2) developing more sustainable and environmentally friendly production systems; and finally, 3) enhancing the circular economy by developing systems with a better cost/benefit ratio (Avnimelech et al., 2015). Originated in the 1990s, biofloc technology (BFT) has capture the attention of the scientific community as an exciting and cost-effective food production alternative. Initially developed to facilitate intensive culture, BFT provides low maintenance costs and the potential to recycle feed (Avnimelech et al., 2015). It functions as a closed system with artificial aeration, where the ongoing suspension of organic rich particles promotes the development of a heterotrophic microbial community (Avnimelech et al., 2015; Khanjani & Sharifinia, 2020). The presence of a dense diverse heterotrophic microbial biomass also helps to reduce microbial disease outbreaks (Avnimelech et al., 2015). Ekasari et al., (2014) referred to an increased activity of the immune system on reared animals in biofloc system, which enhanced survival rate post viral challenge. Furthermore, according to Bossier & Ekasari, (2017), regarding a study on the productivity of cultured animals under different rearing systems, it was concluded that biofloc technology may improve the productivity of reared species by 8 to 43% in comparison with other non-biofloc systems. These include semi-intensive systems with required water exchange or intensive and super-intensive systems such as RAS (Recirculating Aquaculture Systems) technology.

BFT is regarded as a sustainable and environmentally friendly approach with the potential to contribute to several United Nations (UN) Sustainable Development Goals (SDGs) (United Nations General Assembly, 2015). It is a production system which recycles nutrients in the

water body, reducing feed input and increasing profits, without the need of extensive land use, addressing SDG15 (life on land) (Islam et al., 2022; Khanjani & Sharifinia, 2020). In Bangladesh, the low cost of equipment and maintenance allows people to cultivate shrimp at home using household labor, harvesting up to 400-500 kg of fish per year with a profit margin of 30 - 40% (Islam et al., 2022). In addition, BFT offers a valuable livelihood in regions of Asia and Africa that face water shortages or challenges with pond aquaculture. This system is less affected by climate hazards compared to conventional aquaculture methods, provides income and job opportunities to marginalized and female household members, and can increase fish consumption among those with insufficient nutrition (Islam et al., 2022). Given these benefits, biofloc technology (BFT) can contribute to several SDGs, including SDG 1 (no poverty), SDG 2 (zero hunger), SDG 3 (good health and wellbeing), SDG 5 (gender equality), SDG 8 (decent work and economic growth), SDG 12 (responsible production and consumption), SDG 13 (climate action), SDG 14 (life below water), and lastly, SDG 15 (life on land) (Islam et al., 2022; Khanjani & Sharifinia, 2020).

Specifically, the benefits of using BFT are well-documented, including 1) zero or minimal water exchange, thereby avoiding the need of water input, maximizing biosecurity, minimizing possible external environmental effects on the culture, and avoiding possible environmental impacts associated with the discharge of nutrient-rich wastewater; 2) reduction of artificial feed input, reducing production costs; and lastly, 3) the occurrence of a natural established microbiota which enhances growth performance, as well as their immune system of the reared animals (Avnimelech et al., 2015; Khanjani & Sharifinia, 2020).

Given the zero water exchange and decreased feed input benefits, this technology relies primarily on the principle of waste nutrients recycling, more specifically, nitrogen into microbial biomass, presented in the form of protein- rich flocs (Ogello et al., 2021). More in depth, for this nitrogen conversion to take place, heterotrophic microbiota undergoes growth stimulation by steering the C/N ratio in the water, being performed through the addition of an external carbon source or even by modifying the carbohydrate content in the animal's feed (Figure 1.3) (Ogello et al., 2021). The carbon sources used can be several, including molasses, cane sugar, dextrose or even rice bran (Jiménez-Ordaz et al., 2021). The most widely used carbon source is molasses, since it provides the best nutritional input on the biofloc (Jiménez-Ordaz et al., 2021). Furthermore, chemoautotrophic bacteria will oxidize ammonia into nitrite-nitrogen (NO_2^--N) through the process of nitrification. Consequently, nitrite will then be

oxidized to nitrate-nitrogen (NO_3^--N), being the latter the less toxic nitrogen form for the cultured animals. Regarding heterotrophic bacteria, these will assimilate ammonia nitrogen into the building of bacterial protein (without producing NO_2^- or NO_3^-), using organic carbon as energy source, and owning a high nutritional content (Ferreira et al., 2021; Luo et al., 2020). In addition, heterotrophic bacteria are capable of colonizing dead organisms, unconsumed feed and feces, and produce bacterial biomass (Khanjani & Sharifinia, 2020). The produced microbial flocs, able to reach sizes over 1000 µm, are basically a heterogeneous mixture of microorganisms, particles, colloids, cations and dead cells, being lightly bound with bacterial mucus, forming visible nutritious floating flocs, also known as single cell proteins (SCP) (De Schryver et al., 2008; Ogello et al., 2021). The formation of these floccules is of high importance since these aggregates behave as indicators of the system conditions (Jiménez-Ordaz et al., 2021).

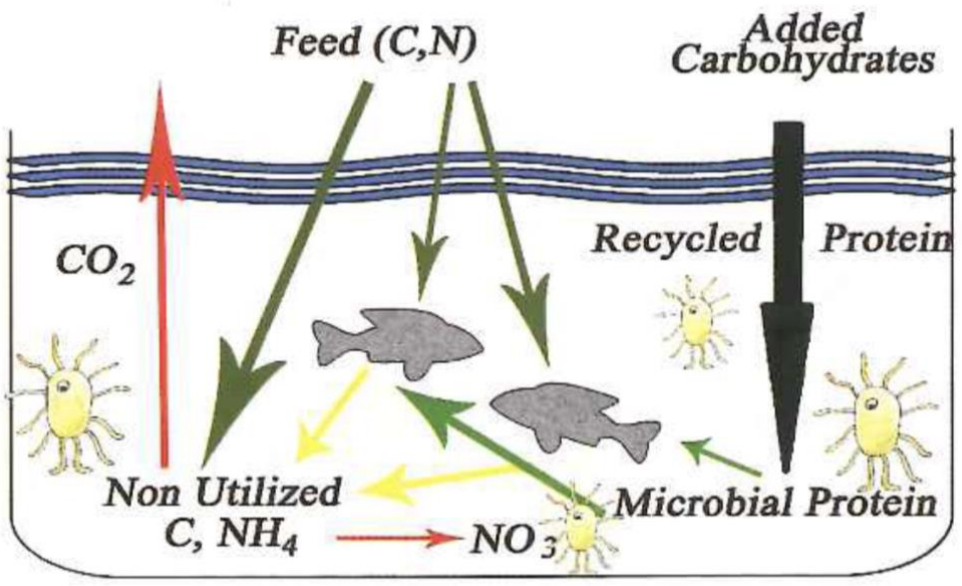

Figure 1.3. Biofloc technology system (BFT) scheme. Representation of the principle of waste nutrients recycling. Source: (Avnimelech et al., 2015).

The produced microbial protein can either be utilized by the reared animal *in situ,* appearing as suspended flocs in the pond, or harvested for processing into feed ingredients (Avnimelech et al., 2015; Ogello et al., 2021). In BFT systems, protein utilization by the reared animals is twice as high when compared to conventional ponds (Avnimelech et al., 2015). In shrimp produced in BFT, up to 30% of the given feed can be lowered due to the consumption of the biofloc (Emerenciano et al., 2013). Hence, biofloc as a complementary food source may lead to a

decrease of the required input of feed protein, as well as maintaining an optimal water quality for the reared animals (Bossier & Ekasari, 2017; Ekasari et al., 2014).

In regard to the naturally established microbial community, it is composed of chemoautotrophic and heterotrophic bacteria, which are able to improve water quality by controlling toxic inorganic nitrogen in the pond (Avnimelech et al., 2015). The microbiota's composition in the BFT is defined by many factors, such as temperature, salinity, light intensity, photoperiod, nutrient availability or even water quality (Jiménez-Ordaz et al., 2021). Furthermore, according to Hussain et al. (2021) an improvement on shrimps' growth performance and immune responses was observed by comparing a biofloc and a non- biofloc shrimp production, whereby the addition of prebiotics (carbon source) and probiotics increased feed conversion, growth rate, weight gain, immune system, and disease resistance. Heterotrophic bacteria tends to grow more favorably than autotrophic bacteria, suggesting that ammonia is likely to be assimilated first by heterotrophic bacteria (Luo et al., 2020). This would prevent the accumulation of nitrogen compounds in the water body, which is directly associated with the autotrophic bacteria nitrifying processes (Luo et al., 2020). However, several studies focused on biofloc technology, have encountered high accumulations of nitrates (NO_3^-) and phosphates (PO_4^{3-}) (Luo et al., 2020; Rios Da Silva et al., 2013). Despite this, no extensive studies have been performed, offering little or no information regarding this problematic.

1.4. Effect of probiotic microorganism's addition in BFT

Several biological control strategies can be used in closed systems. In shrimps' culture, microalgae products, prebiotics, probiotics, and synbiotics are commonly used (Hussain et al., 2021). Specifically, prebiotics are considered non-digestible food that is resistant to gastric acidity. They can initiate numerous enzymatic processes, enhance gastrointestinal digestion, provide protection against non-beneficial bacteria, or even stimulate activity of beneficial microbes (Butt et al., 2021). Probiotics are basically live microorganisms capable of preventing diseases or the growth of pathogens in the water, as well as improving immunity and survival of the reared shrimps (Butt et al., 2021). Examples of probiotics normally used in this sector are diverse bacteria, phages, microalgae, and yeast (Butt et al., 2021). Regarding synbiotics, these include a mix of both prebiotics and probiotics in a synergistic relationship (Butt et al., 2021). In a general sense, the addition of these as dietary supplements may provide nonspecific disease protection and the improvement of growth indicators (Hussain et al., 2021).

Bacterial probiotics have been implemented for the last 20 years in fish and crustacean's aquaculture due to their benefits associated with gut health and environmental bioremediation of soil and water in culture systems. These can be generically described as "Live organisms which added in adequate quantities provide benefit actions on host and environmental wellness that surrounds them" (Cienfuegos Martínez Kathia et al., 2017). According to Jiménez-Ordaz et al. (2021), the use of probiotics can increase shrimps' nutrient absorption, increasing the growth rate and subsequently the biomass of the culture. The addition of probiotics such as *Bacillus* bacteria in shrimp culture was shown to improve its immunity as well as enhance disease resistance (He et al., 2023). These bacteria will compete for nutrients, thus inhibiting the rapid growth of bacteria such as *Vibrio* (Sadat Hoseini Madani et al., 2018). Additionally, according to He et al., (2023), the addition of *Bacillus subtilis* as a probiotic to a shrimp- based biofloc system, helped to control water parameters, having impact on the ongoing nitrification processes and increasing the survival of cultured shrimps. Regarding another study by Sadat Hoseini Madani et al., (2018), comprehending the effect of the administration of *Bacillus subtilis* and *Bacillus licheniformis* in *Penaeus vannamei* culture, it was revealed that the body composition of shrimps was altered with the addition of *Bacillus* spp. in the diet. More specifically, higher levels of dry matter, crude protein and ash were found in the treatment that included *Bacillus* spp. Additionally, on this same study, through an induced stress test, lower levels of plasma cortisol and glucose, which are stress indicators, were found in the probiotic-fed shrimp.

Visually, BFT systems can be defined as "brown-water" biofloc systems when the dominant populations comprise chemolithoautotrophic and heterotrophic bacteria, and "green-water" biofloc systems when both algal and bacterial processes help to control the water quality (Figure 1.4) (Khoa et al., 2020; Vergara et al., 2012).

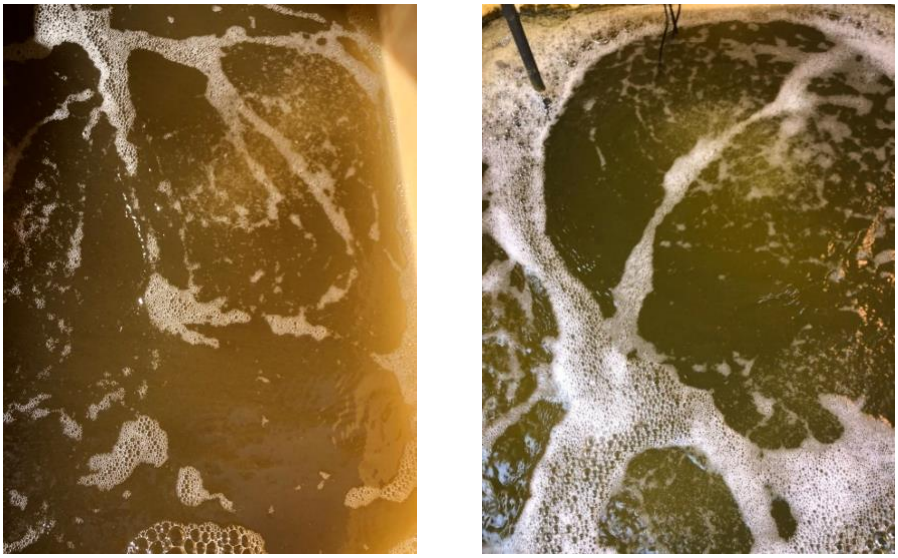

Figure 1.4. "Brown- water" biofloc (Left) (Bacterial probiotics inclusion) and "Green- water" biofloc (Right) (Microalgae and bacterial probiotics inclusion).

Microalgae can be used to increase biofloc's nutritional value, improving animal production due to the high-quality nutritional value (Roy & Pal, 2015). Additionally, other than being of highly importance in water quality control by the assimilation of inorganic nitrogen and phosphorus, they are responsible for segregating mucilaginous substances as well as a variety of beneficial antioxidants, hence enhancing the biofloc formation and increasing microorganisms' diversity comprised in the biofloc (Y. Chen et al., 2023; Dong et al., 2022; Jiménez-Ordaz et al., 2021). Studies reported a better growth of the cultured shrimps in BFT systems when mixed with phytoplankton flora rather than systems with only heterotrophic bacteria (Jiang et al., 2020). Concordantly, according to (Fleckenstein et al., 2019), it was reported a lower lipid and protein content in the biofloc, when using only bacterial probiotic, without microalgae input. Furthermore, in a study done by Reis et al. (2019), this author stated that biofloc should contain autotrophic microorganisms such as microalgae, and that in order to improve the nitrification process, the systems should be under a photoperiod regime of 12:12h natural light-dark.

Microalgae applications in aquaculture have gained significant importance due to their remarkable added nutritional value and antioxidant benefits to the culture (Gui et al., 2022; Lim et al., 2023). These microorganisms are responsible for producing a variety of valuable natural products, which include proteins, carbohydrates, lipids, polyunsaturated fatty acids or carotenoids (Gui et al., 2022). In addition, carotenoids can be synthesized by photosynthetic bacteria, plants, algae, and even by some non-photosynthetic bacteria and some fungi (Borowitzka et al., 2016). According to Lim et al., (2023), these compounds comprise essential bioactive molecules recognized by their antioxidant capacities which mitigates oxidative stress.

Generically, these are known for enhancing the host defense system and disease tolerance in a wide range of aquatic animals, which ultimately improves stress tolerance as well as survival (Lim et al., 2023). Furthermore, microalgae help to maintain the concentration of nitrogen-derived compounds in the water column, and hence, the maintenance of a good water quality, preventing pathogenic bacteria such as *Vibrio*, that may lead to the death of the reared animals (Jiménez-Ordaz et al., 2021). V*ibrio* bacteria are opportunists especially in marine or estuarine environments, hence can cause negative economic impacts, associated with high mortalities, necrosis or even growth impairment (Silva et al., 2022). In addition, regarding *Penaeus vannamei* immune stimulation, various studies mention a positive impact of microalgae addition on the survival rate, immune responses towards possible pathogens appearance, capacity to resist diseases, as well as on the reinforcement of shrimp's enzymatic antioxidant system (J. Chen et al., 2024; Eissa et al., 2023; Medina Félix et al., 2017). Microalgae species belonging to *Chlorella*, *Acutodesmus* or *Chlamydomonas* have already proven to be a stable biofloc composition (Y. Chen et al., 2023). Specifically, *Chlorella sp.* is known by its capability of improving the immune system of the culture animals and, once applied in a BFT it promotes a reduction in nutrients concentration on the culture water (Silva et al., 2022).

1.5. Justification

Traditional aquaculture production systems such as flow- through systems or even more recent ones as RAS (Recirculating Aquaculture Systems) have downsides, such as the output of nutrient- rich wastewater or the cost-efficiency relation. Biofloc technology (BFT) is seen as a recent technology, which presents solutions for both mentioned downsides. Additionally, this technology also provides both nutritional and immunological benefits to reared species, while being a sustainable approach and profitable opportunity. Furthermore, by using microalgae as probiotics, it is expected to observe additional improvements associated with nutritional and health benefits in reared animals. In addition, in BFT systems, nutrients such as nitrates and phosphates have been showing a tendency for its accumulation throughout time (Luo et al., 2020; Rios Da Silva et al., 2013). In this sense, microalgae addition to this system could be seen as a possible solution for this problem.

1.6. Objectives

This study focuses on understanding the effect of probiotic microorganisms, specifically bacterial versus microalgal probiotics, on shrimp (*P. vannamei*) reared under Biofloc Technology (BFT) conditions. To accomplish this general objective, the first step was to establish the best environmental conditions to achieve a stable microalgae population within BFT system. Following this, a comparative analysis was conducted to assess the impact of bacterial probiotics and/or microalgae on shrimp growth and health, as well as to evaluate the effects of adding probiotics on water quality.

The specific objectives were the following:

- Achieve a stable microalgae population under Biofloc Technology (BFT) conditions.
- Assess the effect of microalgae population on water quality.
- Evaluate the effect of adding bacterial probiotics or microalgae to biofloc on shrimp growth and survival.

2. Material and Methods

To achieve the mentioned objectives, this work was divided in two sequential phases. Phase 1 focus was directed on understanding how the addition of bacterial probiotics or/and microalgae would affect the water quality and how the different microalgae communities would evolve throughout the trial period. Phase 2, comprising the same treatments, consisted in a growth trial, whereby the focus also included the understanding of how shrimps' growth performance and immune system was affected by each treatment.

2.1. Phase 1

2.1.1. Experimental design

In order to understand the effect of adding probiotic bacteria or microalgae on *P. vannamei* production under BFT, four different treatments were tested in triplicate (Figure 2.1). Specifically, a) a control group only with biofloc water (C1), b) a second group with bacterial probiotic inclusion (P1), c) a third group with microalgae inclusion (M1), and finally, d) a fourth group comprising a combination of bacterial probiotics and microalgae (MP1). The microalgae used in this study, *Chlorella vulgaris*, was previously selected as described in Appendix I.

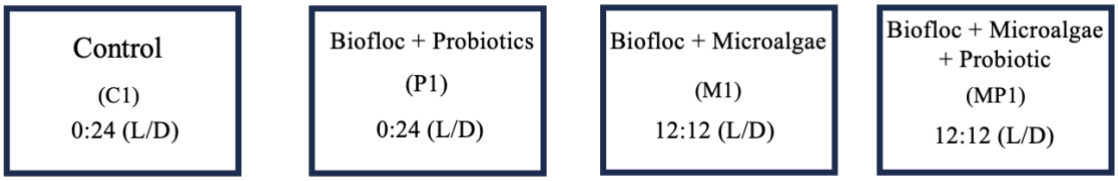

Figure 2.1. Phase 1: Experimental design of 4 treatments in triplicates: Control (Biofloc) (C1); Biofloc + Probiotic (P1); Biofloc + Microalgae (M1); Biofloc + Microalgae + Probiotic (MP1). The respective photoperiods (Light/Dark) are presented bellow in each treatment.

For the experimental setup, 12 tanks of 200L were filled by mixing a mature biofloc system and seawater to achieve 150 mg/L of total suspended solids (TSS). Afterwards, in microalgae experimental groups, *Chlorella vulgaris* was inoculated from a previous culture (see Appendix II) to obtain a concentration of 0.3 AU (Absorbance at 680 nm), which corresponds to a concentration of $7x10^6$ cells/mL. Both the inoculation of microalgae initial tanks and the routinary microalgae cells counting was done according to the method described by Alejos-Cabrera et al. (2023), which directly correlates absorbance at wavelength of 680 nm with cell concentration. The commercial brand PRO- W, Sano-Life (INVE, England) was used as bacterial probiotic at a concentration of $5x10^{10}$ cfu/g (colony- forming unit per gram) and

constituted mainly by *Bacillus subtilis* and *Bacillus licheniformis*. The probiotic inoculation followed the manufacturer's guidelines with an initial dose of 3 g/m³, followed by a weekly maintenance dose of 1 g/m³ (Hostins et al., 2017). The bacteria were previously activated by the suspension of the bacteria powder and 1 hour aeration.

The dimension of the tanks in this phase was 92 x 58 x 42 cm, comprehending a surface area of 0.53 m^2 and a total volume of 220 L (0.22 m^3). Each tank was supplied with an aeration system and a heater set to 28 ºC. Tank lids were covered with a black opaque plastic, so that it was possible to control the photoperiod in each experimental treatment (Figure 2.2).

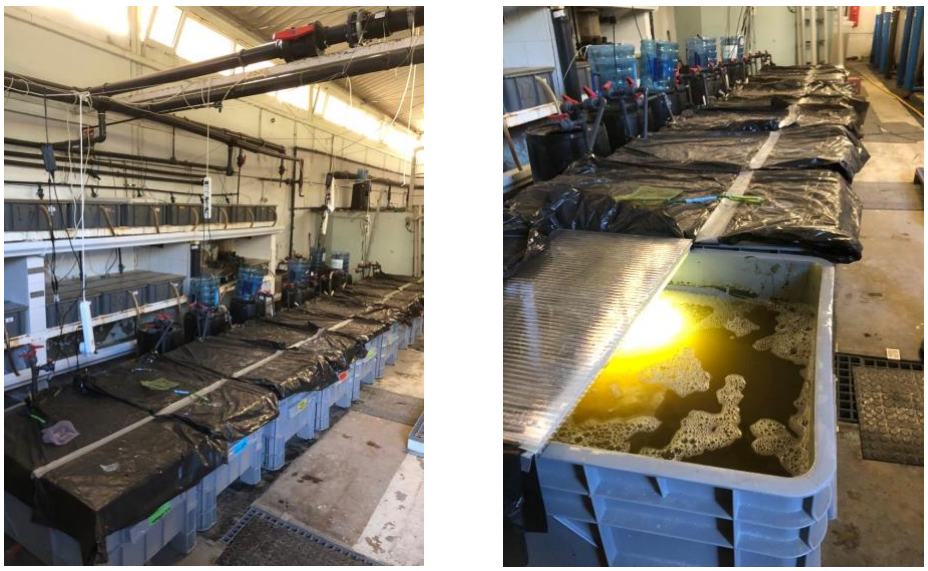

Figure 2.2 - Experimental setup – Phase 1. Twelve tanks, comprising the four assessed treatments (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion) in triplicates.

In each tank, 80 shrimps with an initial mean weight of 3.15 ± 0.23g were stocked at a density of 150 shrimps/m^2. Throughout the totality of the trial period, the shrimps were fed with two different commercial feeds (Le Gouessant, France) according to their weight (g). Feed doses were calculated according to Jory et al. (2001) (Table 2.1).

Table 2.1. Feed rate table (culture water temperature < 28.5 ºC). Comprises the given feed (% Biomass) according to duration (weekly) and size of the reared shrimp (Source (Adapted). Jory et al. (2001)).

Week	Size (g)	% Biomass
0	0.01	200
1	0.04	60
2	0.14	30
3	0.40	12
4	0.9	8.0
5	1.7	6.5
6	2.6	4.5
7	3.4	3.5
8	4.2	3.3
9	5.0	3.1
10	5.8	2.9
11	6.6	2.8
12	7.4	2.6
13	8.2	2.5
14	9.0	2.4
15	9.8	2.4
16	10.6	2.4
17	11.4	2.3

Pre- grower feed was given until shrimps reach 4.5 grams, followed by a mix of pre- grower and grower (50/50 %) between 4.5-5.5 grams, and finally, once shrimps reach 5.5 grams, only grower feed was given. The trial lasted 27 days with a photoperiod of 12h:12h (Light/Dark). The proximate composition of the feeds is provided in the Table 2.2 bellow.

Table 2.2. Feeds proximate composition (%). Pre- Grower and Grower feeds' components percentage.

Components (%)	Feeds	
	Pre- Grower	Grower
Crude Protein	38 %	35 %
Crude Fat	7 %	7 %
Crude Fiber	1.70 %	2 %
Crude Ash	8.70 %	9.10 %
Calcium	1.28 %	1.35 %
Phosphorus	0.87 %	0.91 %
Sodium	0.26 %	0.27 %

2.1.2. Water parameters

Temperature, salinity, pH and dissolved oxygen were checked and registered daily using a HI98194 multi-parameter meter (HANNA, Romania). Dissolved oxygen was maintained at a level higher than 5 mg/L. Phosphates, ammonia (Koroleff method (1969)) described in Grassof et al., 1983), nitrites (Bendschneider & Robinson, 1952), nitrates and TSS were measured twice per week. Below, in Table 2.3, are presented the different parameters measured, their range values, and the frequency of measurements. The protocols used for these analyses are described in Appendix III.

Table 2.3. Water parameters description for Penaeus vannamei culture, range values, and frequency of measurements.

Parameters	Values	Frequency
Temperature	28 – 32 ºC	Daily
Salinity	28 – 31 ppt	Daily
Dissolved Oxygen	5 – 9 mg/L	Daily
pH	7 – 8.3	Daily
Phosphates	0.3 – 3 mg/L	Twice per week
Ammonia	< 0.03 mg/L	Twice per week
Nitrite	< 1 mg/L	Twice per week
Nitrate	< 60 mg/L	Twice per week
Total Suspended Solids (TSS)	< 500 mg/L	Twice per week

The control and maintenance of water parameters optimal values was done according to the results obtained in each sampling moment, being done post measurement. Temperature and dissolved oxygen were regulated directly in the heater and aeration system settings, respectively, and salinity, by the addition of either fresh or seawater. Alkalinity and pH were regulated using sodium bicarbonate. Regarding the nitrogen compounds (ammonia, nitrites and nitrates) and phosphates, these were regulated only in extreme cases, being done by the renewal of roughly 10-15% of the water in the tank. Finally, TSS was regulated using a hand manufactured decanter, which would be left working for at least 2 hours.

2.1.3. Microalgae evolution – Total Chlorophyll analysis

Chlorophyll was measured twice a week. For this analysis, water samples from all 12 tanks were collected and filtrated using glass microfiber filters. Once filtered, falcon tubes were prepared with 10 mL of 90% acetone- water solution, whereby the filters (post-filtration) were placed in the specific tube, mixed by vortex, and left in the fridge for a period of 24 hours. Afterwards, the solution was carefully pipetted into microtubes and centrifuged at 6000 rpm (rotations per minute) for 10 minutes to remove possible filters remaining debris. Finally, the absorbance of samples was measured by a spectrophotometer (T60UV, PG Instruments, United Kingdom) using four different wave lengths: 630, 647, 664 and 750 nm. The obtained values in these readings were used to calculate Total Chlorophyll levels (mg/L), using the following formulas:

$$\text{Chlorophyll a} = (11.85 \times (A664-A750)) - (1.54 \times (A647-A750)) - (0.08 \times (A630-A750))$$

$$\text{Chlorophyll b} = (-5.47 \times (A664-A750)) - (21.03 \times (A647-A750)) - (2.66 \times (A630-A750))$$

$$\text{Chlorophyll c} = (-1.67 \times (A664-A750)) - (7.6 \times (A647-A750)) - (24.52 \times (A630-A750))$$

$$\text{Total Chlorophyll} = \text{Chlorophyll a} + \text{Chlorophyll b} + \text{Chlorophyll c}$$

2.1.4. Shrimps' growth performance and survival rate

By the end of the trial, in each experimental group the following productive parameters were measured: survival rate, weight gain (WG), growth rate, feed conversion rate (FCR) and productivity (P).

- Survival rate = (final shrimp number/initial shrimp number) x 100 (%)
- WG = (final wet weight – initial wet weight)/number of individuals (g)
- Growth rate = weight gain/days number (g day^{-1})
- FCR = dry feed consumption/biomass gain
- P = biomass gain/m^3 (g/m^3)

2.2. Phase 2

2.2.1. Experimental design

To determine if longer exposition to probiotic microorganisms affected productivity, the experimental design from Phase 1 was maintained for a total experimental period of 68 days, with some modifications. Specifically, higher tank volumes and production densities were assayed in this second phase (Figure 2.3). Twelve isothermal tanks (triplicates for each treatment) with a total volume of 1 m^3 and a surface area of 1.40 m^2 were divided in four experimental groups (three replicates/group), with photoperiod of 12h:12h (Light/dark) and

initial biofloc of 150 mg/L of TSS. The experimental groups were similar to those of Phase 1: C2, a control group only with biofloc water; P2 with bacterial probiotic inclusion; M2 with microalgae inclusion; and a fourth group comprising a combination of bacterial probiotics and microalgae (MP2). Similar to Phase 1, an inoculation with previously cultured *Chlorella vulgaris* (see Appendix II) was done, reaching an initial absorbance level of 0.3 AU ($7x10^6$ cells/mL), and the initial conditions were based in reaching 150 mg/L TSS.

Figure 2.3. Experimental setup - Phase 2. Twelve tanks, comprising the four assessed treatments (C2 (control), P2 (Bacterial probiotic), M2 (Microalgae) and MP2 (Microalgae and bacterial probiotic) in triplicates.

2.2.2. Water parameters

Temperature, salinity, pH, and dissolved oxygen were measured daily, ammonia and Total Suspended Solids (TSS) twice a week, and alkalinity, nitrite, nitrate, and phosphates were measured once a week. These analyses were performed using the protocols provided in Appendix III. The parameters were maintained at optimal levels, according to the values expressed in Table 2.3. In this second phase, Imhoff cones were used as another routine control measure to check the Settleable Solids (SS) in each tank, as a direct measure of solids in the

water (ml/L). This procedure involved collecting a sample of 1 L of water into the cone and measure the expressed concentration after 30 minutes (Figure 2.4).

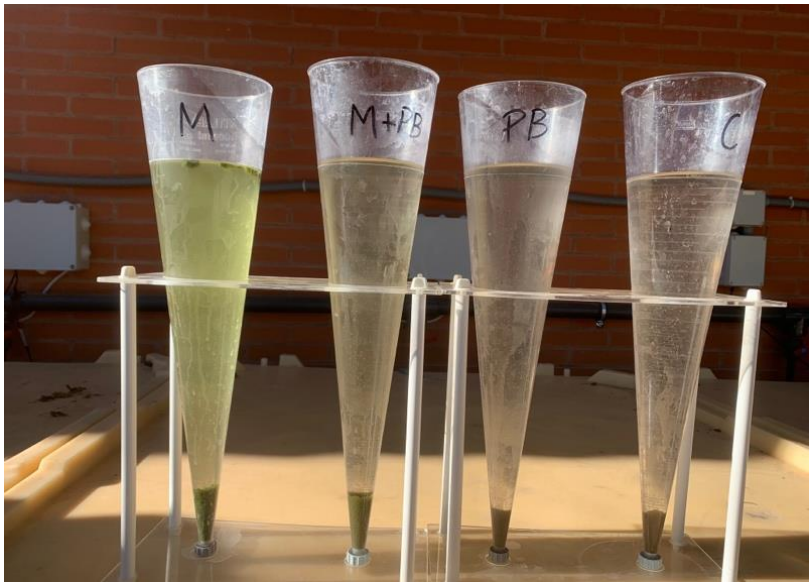

Figure 2.4. Settleable Solids (SS) quantification using Imhoff cones.

The regulation and maintenance of water parameters optimal values was done using the same methodology as in Phase 1.

2.2.3. Microalgae evolution – Total Chlorophyll analysis

Chlorophyll analyses were conducted and prepared as previously described in Phase 1. The absorbance of samples was measured by a spectrophotometer (T60UV, PG Instruments, United Kingdom) at four different wave lengths: 630, 647, 664 and 750 nm. The obtained values were used to calculate Total Chlorophyll levels (mg/L), using the formulas presented in phase 1 methodology.

2.2.4. Shrimps' growth performance and survival rate

In this phase, 285 shrimps with an initial body weight of 2.48 ± 0.07g were stocked in each of the 12 tanks, comprising a density of 200 shrimps/m^2. Shrimps were weighed weekly to determine growth performance and productive parameters for each experimental group. The assessed parameters were as described in phase 1 methodology: survival rate, weight gain (WG), growth rate, feed conversion rate (FCR) and productivity (P).

2.3. Environmental challenges

At the end of the growth assay, environmental challenges were set up and applied to evaluate the possible effect of probiotic or microalgae addition on shrimps' survival (Figure 2.5). Therefore, after finishing Phase 2 trial, the reared animals from each experimental treatment were divided in 3 groups. Each group was submitted to one of the three stress challenges: 1) hypoxia, 2) high-density, and 3) a combination of hypoxia and high-density. The challenges consisted of submitting the animals for 6 (1st and 2nd stress test) and 3 (3rd stress test) hours to the stressful condition, followed by an observation period until completing a total challenge period of 24 hours.

Figure 2.5. Environmental challenges experiment set up. Aquariums (above) for post-observation; Buckets (bellow) for stress challenges.

For the hypoxia challenge, four small tanks, one for each treatment, with a volume of 0.085 m^3 were prepared. The level of dissolved oxygen in the water was decreased by injecting nitrogen gas (N_2) in the water until it reached a value between 2 and 2.5 mg/L, which was then

maintained throughout the 6h period. In these tanks, 30 shrimps were stocked at a density of 350 animals/m^3. The shrimps remained in the tanks for a 6-hour stress test before being transferred to the aquarium for observation. The density challenge was conducted by placing 30 shrimps in each bucket with a volume of 0.013 m^3, achieving a density of 2205 shrimps/m^3. The shrimps were left in the buckets for a 6-hour stress test period before being moved to the aquarium for subsequent observation. As for the combined hypoxia and density challenge, 30 shrimps were stocked in each bucket with a volume of 0.013 m^3. The dissolved oxygen level was maintained at a low level (2-2.5 mg/L), and the same density of 2205 shrimps/m^3 was achieved. The shrimps were kept under these conditions for 3 hours before being transferred to the observation aquariums.

Throughout these challenges, the control of water parameters (pH, temperature, dissolved oxygen, and salinity) was done hourly, from 8:00 AM to 2:00 PM. Mortality was assessed after the completion of the stress tests period and 24 hours of the challenge event. Survival rate after the stress test period was calculated taking into consideration the initial number of shrimps (n=30), and at 24h the number of shrimps that survived from the stress test, and not the totality.

2.4. Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics (version 29.0.2.0). Data was verified for normality of distribution and homogeneity of variances using the Shapiro-Wilk and Levene's tests, respectively. Differences among groups were performed by one-way analysis of variance (ANOVA) followed by Tukey´s multi-comparison test. Repeated measures ANOVA was performed to analyze the results of time series. All statistical differences were considered significant at $P < 0.05$.

3. Results

3.1. Phase 1

3.1.1. Water parameters

Temperature, salinity, pH, alkalinity, and dissolved oxygen remained in the optimal levels for shrimp cultured (Table 3.1). Total suspended solids (TSS) ranged from 285 to 1001 mg/L, showing no significant differences between groups ($p > 0.05$). Along the trial, ammonia (NH_3) and nitrites (NO_2^-) levels were below 0.4 mg/L and never reached toxic levels, ranging from 0.02 to 0.37 mg/L and from 0.03 to 3.22 mg/L, respectively (Appendix IV).

Table 3.1. Temperature, salinity, pH, alkalinity and dissolved oxygen mean values – Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

	Temperature (℃)	*Salinity (PSU)*	*pH*	*Alkalinity (mg/L)*	*Diss. Oxygen (mg/L)*
C1	27.43±4.03	21.92±3.37	7.84±1.15	129.0±22.0	5.86±0.88
P1	27.12±3.97	21.76±3.31	7.86±1.15	123.4±19.4	5.93±0.89
M1	27.29±4.02	21.13±4.44	7.76±1.14	111.8±16.2	5.82±0.94
MP1	27.23±3.99	21.47±3.19	7.76±1.14	113.7±15.5	5.85±0.89

Data correspond to the average values of three replicates ± SD.

Throughout the experimental period, nitrate (NO_3^-) and phosphate (PO_4^{3-}) levels showed significant differences ($p < 0.05$) between microalgae (M1 and MP1) and non-microalgae treatments (P1 and C1) (Table 3.2).

Table 3.2. Nitrates (NO_3^-) and Phosphates (PO_4^{3-}) parameters' mean and increment values - Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

	NO_3^- (mg/L)	*PO_4^{3-} (mg/L)*	*Δ [NO_3^-] (mg/L)*	*Δ [PO_4^{3-}] (mg/L)*
C1	67.19±23.65^{b}	7.50±3.22^{b}	1.75±0.68	2.15±0.75^{a}
P1	74.94±25.94^{b}	8.00±3.11^{b}	1.88±0.69	1.78±0.51^{ab}
M1	167.41±24.97^{a}	16.41±3.61^{a}	1.08±0.15	1.59±0.29^{ab}
MP1	180.56±25.12^{a}	16.32±3.44^{a}	1.08±0.16	1.15±0.23^{b}

Data correspond to the average values of three replicates ± SD (mg/L). Different letters in the same column indicate the averages differ significantly among treatments ($p < 0.05$). Δ [NO_3^-] and Δ [PO_4^{3-}] represent the increase of NO_3^- and PO_4^{3-} throughout time.

On the other hand, the increase of PO_4^{3-} ($\Delta [PO_4^{3-}]$) in the water was higher in non-microalgae groups, showing significative differences between C1 and MP1 treatments ($p < 0.05$). It was observed a similar tendency for NO_3^-, however without significant differences ($p > 0.05$) (see Appendix V and VIII).

3.1.2. Total chlorophyll

As expected, the mean concentration of chlorophyll was significantly higher ($p < 0.05$) in microalgae groups (M1 and MP1) compared with the groups C1 and P1, which did not include microalgae (Table 3.3). On the other hand, for each treatment, no significant differences were found along the experimental period ($p > 0.05$) (Figure 3.1).

Table 3.3. Total chlorophyll mean values - Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

	Total Chlorophyll (mg/L)
C1	0.05 ± 0.03^{b}
P1	0.06 ± 0.04^{b}
M1	0.14 ± 0.05^{a}
MP1	0.12 ± 0.05^{a}

Data correspond to the average values of three replicates ± SD. Different letters in the same column indicate the averages differ significantly among treatments ($p < 0.05$).

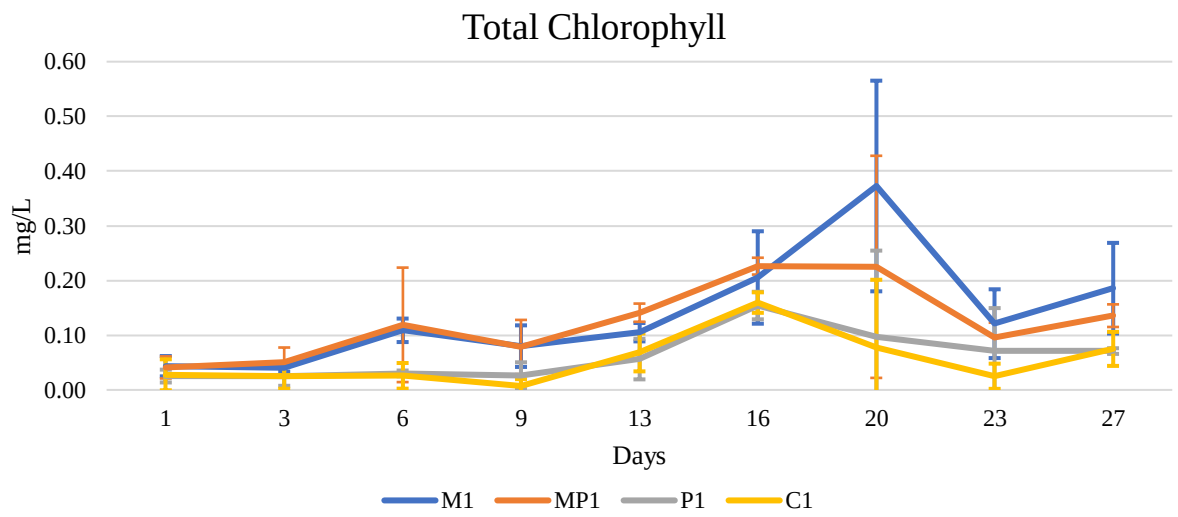

Figure 3.1. Total chlorophyll concentration (mg/L) throughout Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

3.1.3. Shrimp growth performance and survival rate

The experimental treatments did not significantly affect survival nor productive parameters ($p > 0.05$) (Table 3.4). Nevertheless, survival rates ranged from 84.16% in M1 and 94.16% in C1. At the end of the experimental period, shrimps reared under the different conditions gained approximately 4.2 g, being that shrimps grew from 0.15 g/day in treatments P1, M1 and MP1, to 0.16 g/day in the P1 treatment. Feed conversion ratios (FCR) and productivity values ranged from 1.60 in P1 to 1.73 in MP1, and from 1098.6 g/m^3 in M1 to 1343.3 g/m^3 in C1, respectively.

Table 3.4. Shrimps' survival rate (%), Weight gain (g), Feed conversion ratio (FCR), Growth rate and Productivity at Phase 1.

	Survival rate (%)	*WG (g)*	*FCR (Feed Conversion Ratio)*	*Growth rate (g/day)*	*Productivity (g/m^3)*
C1	94.2 ± 2.6	4.1 ± 0.1	1.61 ± 0.14	0.15 ± 0.00	1343.3 ± 28.8
P1	90.4 ± 4.0	4.4 ± 0.2	1.60 ± 0.03	0.16 ± 0.00	1329.6 ± 140.7
M1	84.2 ± 7.3	4.1 ± 0.4	1.57 ± 0.18	0.15 ± 0.01	1098.6 ± 302.4
MP1	85.4 ± 3.1	4.1 ± 0.2	1.73 ± 0.12	0.14 ± 0.00	1102.1 ± 72.1

Data correspond to the average values of three replicates ± SD. Data did not differ significantly among experimental treatments ($p > 0.05$).

3.2. Phase 2

3.2.1. Water parameters

Water parameters were measured throughout the experimental period. The levels were controlled during the experiment, remaining within the optimal levels for the cultured shrimps (Table 3.5).

Table 3.5. Temperature, salinity, pH, alkalinity and dissolved oxygen mean values – Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)).

	Temperature (℃)	*Salinity (PSU)*	*pH*	*Alkalinity (mg/L)*	*Dissolved Oxygen (mg/L)*
C2	27.51±1.18	20.70±1.07	8.03±0.21	149.37±9.28	6.22±0.40
P2	27.53±1.21	21.55±1.39	7.94±0.22	147.48±10.45	6.13±0.48
M2	27.59±1.46	21.83±1.06	8.00±0.26	147.30±12.46	6.17±0.47
MP2	27.70±1.19	21.82±1.23	7.94±0.26	156.53±11.67	6.22±0.39

Data correspond to the average values of three replicates ± SD.

No significant differences were found between experimental groups in terms of TSS mean values, starting from a common initial value of 420.6 mg/L up to 897.0 mg/L. Throughout the experimental period, TSS levels remained within the optimal range, due to the implementation of necessary measures.

Nitrogen compounds ammonia (NH_3) and nitrites (NO_2^-) started with high levels in all four treatments, but by day 5 they have decreased to normal levels, as indicated by the negative slope trendlines (see Appendix VI). However, the concentration of these compounds in the water was below 0.4 mg/L, never trespassing the threshold in terms of toxicity for the cultured animals. Ammonia levels remained between 0.01 and 0.36 mg/L and nitrites were kept between 0.02 and 0.73 mg/L.

Phosphate (PO_4^{3-}) levels in M2 and MP2 treatments were significantly higher compared to P2, and M2 showed statistically significant differences with C2 ($p < 0.05$). Furthermore, nitrate (NO_3^-) levels were significantly different ($p < 0.05$) between the microalgae including treatments (M2 and MP2) and the non-including treatments (P2 and C2) (Table 3.6).

Table 3.6. Nitrates (NO_3^-) and Phosphates (PO_4^{3-}) parameters' mean and increment values - Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)).

	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	Δ [NO_3^-] (mg/L)	Δ [PO_4^{3-}] (mg/L)
C2	180.10±11.24^{b}	13.87±1.33^{bc}	2.67±1.52^{a}	4.02±1.42^{a}
P2	181.32±13.12^{b}	12.82±2.34^{c}	2.99±1.73^{a}	3.18±1.12^{a}
M2	286.49±1.56^{a}	21.65±3.42^{a}	1.26±0.54^{b}	0.84±0.27^{b}
MP2	276.04±58.17^{a}	20.78±2.56^{ab}	1.34±0.56^{b}	0.72±0.29^{b}

Data correspond to the average values of three replicates ± SD (mg/L). Δ [NO_3^-] and Δ [PO_4^{3-}] represents the increase of NO_3^- and PO_4^{3-} throughout time. Different letters in the same column indicate the averages differ significantly among treatments ($p < 0.05$).

The increase of both nitrates and phosphates in the culture water showed significantly ($p < 0.05$) higher accumulation in the non-microalgae treatments than in the M2 and MP2 treatments, as seen in the increments (Δ [NO_3^-] and Δ [PO_4^{3-}]). The increase of these nutrients over the trial period is reflected in the slope values of the trendlines shown in the time evolution graphs for NO_3^- and PO_4^{3-} increments (see Appendix IX) and in the graphs of nutrients concentration over time (see Appendix VII).

3.2.2. Total chlorophyll

In phase 2, throughout most of the trial, significantly higher mean values of total chlorophyll were found in M2 and MP2 when compared to P2 and C2 groups ($p < 0.05$) (Table 3.7). Total chlorophyll concentration over time (Figure 3.8) showed greater differences between microalgae including (M2 and MP2) and non-including (P2 and C2) treatments, when compared to the previous phase 1.

Table 3.7. Total chlorophyll mean values at Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)).

	Total Chlorophyll (mg/L)
C2	0.03 ± 0.02^{b}
P2	0.04 ± 0.02^{b}
M2	0.17 ± 0.08^{a}
MP2	0.15 ± 0.09^{a}

Data correspond to the average values of three replicates ± SD. Different letters in the same column indicate the averages differ significantly among treatments ($p < 0.05$).

Nevertheless, a progressive decrease in this parameter was observed from day 12 onwards, reaching similar values to non-microalgae groups at day 47 (Figure 3.5).

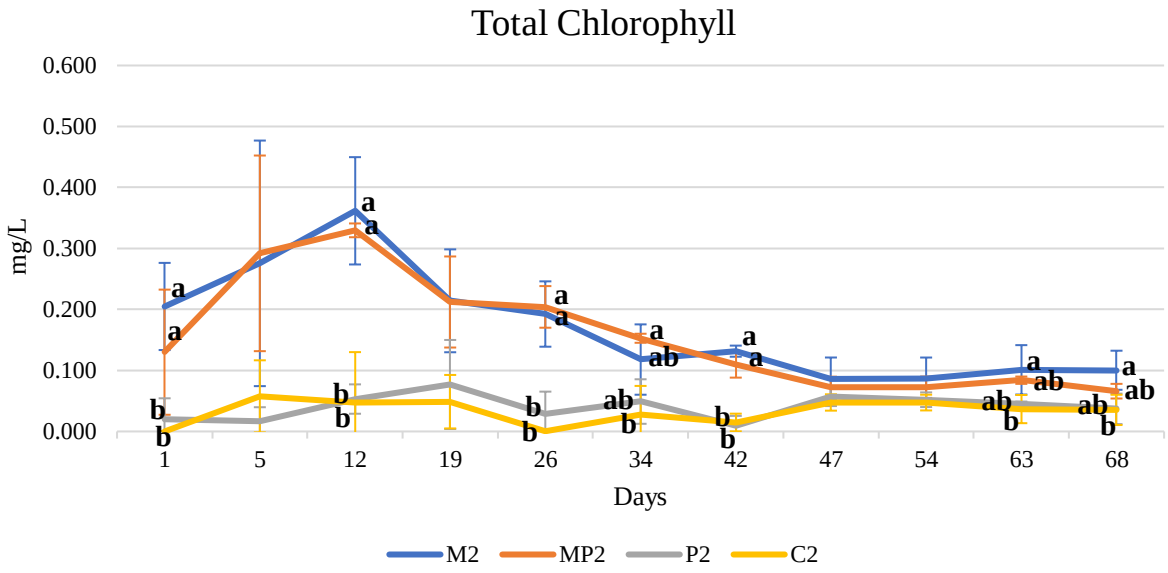

Figure 3.2. Total chlorophyll concentration (mg/L) throughout Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly ($p < 0.05$).

3.2.3. Shrimp growth performance and survival rate

The addition of probiotics and/or microalgae significantly reduced the survival of the animals. However, the weight gain presented no significant differences among treatments ($p > 0.05$). In terms of growth rate, C2 and P2 had similar results, which were significantly higher than M2 or MP2 ($p < 0.05$). Productivity was significantly higher in the C2 treatment when compared to M2 and MP2 ($p < 0.05$). Furthermore, regarding FCR results, P2 had significantly lower values than M2 treatment ($p < 0.05$) (Table 3.8).

Table 3.8. Shrimps' survival rate (%), Weight gain (g), Feed conversion ratio (FCR), Growth rate and Productivity at Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)).

Treatment	*Survival rate (%)*	*WG (g)*	*FCR (Feed Conversion Ratio)*	*Growth rate (g/day)*	*Productivity (g/m^3)*
C2	65.83 ± 6.27^{a}	6.34 ± 0.56	1.58 ± 0.16^{bc}	0.094 ± 0.008	919.8 ± 156.8^{a}
P2	42.26 ± 24.46^{b}	6.72 ± 0.74	1.32 ± 0.17^{c}	0.098 ± 0.010	417.0 ± 683.8^{ab}
M2	21.31 ± 4.06^{b}	4.97 ± 0.42	2.18 ± 0.35^{a}	0.073 ± 0.006	-242.7 ± 86.6^{b}
MP2	26.60 ± 12.37^{b}	5.42 ± 0.51	1.92 ± 0.34^{ab}	0.079 ± 0.007	-318.3 ± 375.9^{b}

Data correspond to the average values of three replicates ± SD. Different letters in the same column indicate the averages differ significantly ($p < 0.05$).

3.3. Shrimp survival under environmental challenges

Survival rates of shrimp subjected to stress challenges are presented in Table 3.9. For the stress test, survival rates were calculated based on the initial 30 shrimps, whereas in the 24h (post challenge), the survival rate was calculated based on the number of shrimps that survived the previous stress tests. Since the experiments were not replicated, statistical analysis could not be performed. Hence, data was discussed using the individual values.

Table 3.9. Shrimp survival rates under Hypoxia, Density, and combined Hypoxia+Density challenges. Assessed treatments C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion).

	Hypoxia		*Density*		*Hypoxia + Density*	
	Survival after 6h	Survival after 24h	Survival after 6h	Survival after 24h	Survival after 3h	Survival after 24h
C2	96.6 %	100 %	96.6 %	96.6 %	83.3 %	100 %
P2	100 %	100 %	100 %	93.3 %	53.3 %	100 %
M2	100 %	100 %	96.6 %	93.3 %	100 %	88.8 %
MP2	93.3 %	100 %	96.6 %	93.3 %	96.6 %	94.1 %

Firstly, regarding the hypoxia challenge, from 6h to 24h after hypoxia challenge, all shrimp in the four treatments survived.

Regardless of the treatment, shrimps submitted to the density stress challenge presented very similar survival rates (Table 3.9). In the 6h stress test, only the probiotic (P2) group achieved a 100% survival rate, while the remaining treatments had a survival rate of 96.6%. After 24h, the control (C2) treatment presented the highest survival rate of 96.6%.

In the combined hypoxia and density challenge, survival rates after 3h was the lowest in the probiotic (P2) treatment, comprising 53.3%. The higher values w in the microalgae (M2) and microalgae + probiotic (MP2), with 100% and 96.6%, respectively. After 24h, the lowest survival rates were found in the treatments with microalgae inclusion, with a survival of 88.8% and 94.1% for the microalgae (M2) and microalgae + probiotic (MP2) groups, respectively. On the other hand, during this period, all shrimps from the probiotic (P2) and control (C2) treatments survived.

4. Discussion

The results have demonstrated the capacity of *Chlorella vulgaris* to improve water quality, mainly in terms of nitrate and phosphate accumulation. In addition, a stable microalgae community was achieved in both phases, improving shrimp's health status. However, the initial high level of nitrates and phosphates, caused by the microalgae inoculation at the beginning of the trial, led to decreased survival and growth performance.

In terms of water quality, during both phases, the addition of bacterial probiotics or microalgae did not result in higher levels of ammonia (NH_3) or nitrites (NO_2^-). This suggests that both populations remained stable throughout production, with no significant mortality events associated with these additions. In respect to the microalgae population, this hypothesis is supported by the chlorophyll results, which consistently showed higher concentrations in the groups where microalgae were inoculated. In previous studies such as those by Rios Da Silva et al., (2013) and Jiménez-Ordaz et al., (2021), an eventual increase in ammonia and nitrites levels was observed in biofloc systems during ongoing production of *Penaeus vannamei*. However, in the current study, no such increases in these nutrients were observed, implying that nitrifying processes were effectively taking place in the biofloc systems used.

Regarding total chlorophyll levels, Phase 1 showed stable growth of the microalgae population throughout the totality of the trial in M1 and MP1 groups. However, during Phase 2, a slight decrease in the microalgae population was observed about 40 days into the trial. Despite this, microalgae populations remained stable until 40 days into both phases, proving to be capable of evolving under biofloc conditions and improving water quality parameters. In line with the findings by Ekasari et al., (2021), where a stable microalgae population was maintained

through microalgae inoculation every 2 weeks, this approach could be explored further to enhance the stability of the microalgae community by a periodically reinforcing the population. The established microalgae community in both assays proved to be capable of controlling nitrates (NO_3^-) and phosphates (PO_4^{3-}). This hypothesis is supported by the observed increment values in these nutrients in the groups without microalgae compared to those with microalgae. In addition, these results are consistent with the results reported by Ekasari et al., (2021), where a decrease in these nutrients was observed in a culture of *Macrobrachium rosenbergii* inoculated with *Chlorella sp*. Additionally, regarding a study by Huang et al., (2022), in which it was observed a significantly decrease of NO_3^- and PO_4^{3-} in the rearing of *Penaeus vannamei* with *Thalassiosira pseudonana* addition. On the other hand, the high initial levels of nitrates and phosphates can be attributed to the fact that these nutrients were already present at high concentration in the microalgae inoculum used to initially inoculate the respective tanks, as they were added during the microalgae production process to induce a faster growth. As described by Nunes et al., (2009), a possible solution for the high concentration of nitrates and phosphates in the microalgae inoculum could involve centrifuging the microalgae culture water. This process would allow the nutrient- rich water to be removed, being left with microalgae paste that can be dissolved again in clean water with the required salinity. According to Antara et al., (2024), this method can still maintain high cell viability. However, after trying this method, it was concluded that it can be time-consuming and not sustainable to work with as the main used technique. In this sense, further exploration of improved techniques is necessary.

An increase in nitrates and phosphates was observed throughout the trial period in both phases, especially in P1/P2 and C1/C2 treatments. This problematic has been previously reported by Luo et al., (2020) and Rios Da Silva et al., (2013), who mentioned that this event is commonly associated with the growth and dominance of nitrifying bacteria in closed biofloc systems, even under conditions that should be more favorable for heterotrophic bacteria growth.

In respect to the first phase, the absence of differences in shrimps' growth performance may be due to the short duration of the trial. Even so, the results were consistent with data obtained in other studies. Suwoyo & Hendrajat, (2021), Xu et al., (2016) and Tinh et al., (2021) reported growth rate values ranging from 0.14 to 0.19 g/day under similar culture conditions. However, as a likely consequence of the elevated nitrate and phosphate levels, a lower growth performance and survival were observed in phase 2. Similar findings were obtained by Furtado et al., (2015), who reported that an exposure to high nitrate concentrations in a period of 42

days resulted in a high impact on zootechnical performance parameters (weight gain (WG), feed conversion ratio (FCR), and growth rate). Given the prolonged experimental period of 68 days in this study, this aligns with the lower growth performance observed in phase 2.

In comparison with the results of this study, lower FCR values were found in other studies which utilized similar *Penaeus vannamei* production conditions under BFT. Weldon et al., (2021) and Tinh et al., (2021), which both used similar densities and experimental periods to those used in this study, obtained FCR values ranging from 1.04 to 1.19 and 0.98 to 1.14, respectively. In the 2016 study by Xu et al., in which higher production densities (300 shrimps/m^3) and similar experimental period were tested, FCR values were obtained that were similar to the ones found in this study's first phase, ranging from 1.29 to 1.47. Moreover, given the lack of differences between treatments' FCR values in this first phase, no impacts associated with the high nutrient concentration could be proven. However, FCR values obtained in this study's phase 2 were still notably higher compared to the results from Xu et al. (2016), Weldon et al., (2021), and Tinh et al., (2021), especially M2 and MP2 treatments in which the greatest impacts in growth performance parameters were expected, due to the high nutrients concentrations throughout the prolonged trial period of this phase. Furthermore, in P2 treatment it was observed the lowest FCR value of this second phase, which goes in concordance with a study performed by Xie et al., (2019). This author, which tested producing *Penaeus vannamei* with different bacterial probiotic loads, observed an improvement of growth performance indicators (including FCR) in the treatments where probiotic was added compared to the treatment with no probiotic inclusion.

Regarding survival rates, the work performed by Dong et al. (2022) is in concordance with the results obtained in the present study, describing that the high concentration of nitrates and phosphates in the culture water may induce the appearance of bacteria such as *Vibrio*. Moreover, Furtado et al., (2015) found that rearing shrimps in high nitrate (NO_3^-) conditions (>300 mg/L) led to histopathological damage in the gills and hepatopancreas, as well as lower survival rates. On the other hand, the low survival rate may also be associated with the appearance of opportunistic harmful cyanobacteria. According to Lukwambe et al., (2019), the increase in N:P (nitrogen and phosphorus) concentration might influence the succession of dominant toxins of cyanobacteria. Additionally, this author demonstrated that adding bacterial probiotics to microalgae treatments could improve shrimp survival rates. This last observation is consistent with our findings, since a higher survival rate was obtained in the microalgae + probiotic (MP2) treatment, when compared to the microalgae (M2) group. In order to explore the possible pathogenic outbreaks throughout this study, a more detailed assessment of the

biofloc culture water in the treatments is needed, specifically through metagenomic analysis. This approach would provide a comprehensive understanding of the genomic composition of the bacterial community present in the biofloc.

Concerning the stress challenges, the lack of triplicates makes it difficult to draw definitive conclusions. The shrimp performance in Phase 2 was not as expected, and the high mortality rate among the animals prevented the production of triplicates for the subsequent stress challenge assay. In this sense, to better understand the impact of these microorganisms on the shrimp's immune system, these challenges should be repeated. Despite that, given the results, individually, hypoxia and density challenges did not show a relevant impact in the survival of reared shrimps. However, when both stressors were combined in the non-microalgae groups, survival dropped to nearly 50%. The obtained results in this study were concordant with findings from other studies. For instance, J. Chen et al., (2024) and Eissa et al., (2023) both found that the inclusion of *Chlorella vulgaris* in *Penaeus vannamei* production increased survival rates when the animals were subjected to a viral challenge. Similarly, the addition of other microalgae species as supplementation for *Penaeus vannamei* culture in biofloc systems has proven to be beneficial. According to Silva et al., (2023) and (Silva et al., 2022), the addition of *Scenedesmus obliquus* enhanced both shrimp immune status and survival rates. Furthermore, Medina Félix et al., (2019) demonstrated that the addition of *Dunaliella sp.* increased shrimp survival following exposure to a *Vibrio* viral challenge. Finally, the improved survival rates observed in the microalgae-groups may be also attributed to the role of carotenoids in shrimp immune resistance, which, as mentioned by Gui et al., (2022), can enhance stress tolerance and overall survival.

5. Conclusion

The establishment of a stable microalgae population into biofloc successfully reduced nitrate and phosphate accumulation. However, the selected methodology to inoculate the microalgae resulted in high levels of nitrate and phosphate from the start of the trials, which in trials longer than 40 days could lead to pathogenic outbreaks and reduced growth performance. On the other hand, despite these negative effects, the inclusion of microalgae in this system enhanced shrimp's immune response, as demonstrated by their survival rates after the environmental challenges.

Therefore, the main conclusions of this thesis are:

- A stable microalgae population can be achieved under biofloc conditions.

- The settlement of microalgae population into biofloc improved the water quality by reducing nitrate and phosphate accumulation.
- Alternative microalgae inoculation strategies are needed to prevent the initial high nitrate and phosphate concentrations in microalgae groups.
- The initial higher nitrate and phosphate levels likely contributed to lower growth and survival rates in the microalgae groups.
- Shrimps' immune system seems to be enhanced in microalgae experimental groups, however the environmental challenges showed be again performed in order to obtain statistical data that supports the results.

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7. Appendixes

Appendix I – Microalgae species selection study

The selection of the microalgae species was previously done in Alicante University, where two species were compared, *Chlorella* spp. and *Phaeodactylum tricornutum*. This study aimed to optimize the microalgae conditions for *Penaeus vannamei* production under biofloc technology, achieving an ecological balance between bacterial population and inoculated microalgae. More specifically, giving the fact that microalgae growth requires a light/dark cycle, three different photoperiods were tested (16:8, 12:12 and 8:16) for the purpose of determining the most suitable species (Cascales et al., 2023). Water parameters such as temperature, salinity, pH, dissolved oxygen, alkalinity, ammonium, nitrite, nitrate and TSS (Total Suspended Solids) were measured twice a week. The parameters were maintained at the optimal values, shown in Table 1.

Table 7.1. Optimal parameters' range values for Penaeus vannamei rearing.

Water Parameters	Values
Temperature	28 ºC
Salinity	19 ± 4 g/L
pH	8 – 8.8
Dissolved Oxygen	> 5 mg/L
Alkalinity	207 ± 40 mg/L
TSS	< 500 mg/L

The determining factors were Total Chlorophyll, analyzed by spectrophotometry (DLAB SP-UV1100), and the weight of the reared shrimps.

The results obtained from this study concluded that *Chlorella* spp., provided not only the least decline regarding chlorophyll concentration, but also, the highest weight gain of the reared animals. Hence, *Chlorella* spp. was determined as the best candidate for rearing *P. vannamei* under BFT. Furthermore, the best results were obtained with the photoperiod of 12:12 (L/D) (Cascales et al., 2023).

Appendix II – Microalgae production

In order to have a stable production of *Chlorella vulgaris*, a microalgae production facility was set up and initiated at the beginning of this project. A commercial inoculum (AqualGae, Spain) of 20L produced at 21 PSU, was used as the starter culture. The culture was developed by the inoculation in an Erlenmyer (1L), followed by a balloon (6L), and lastly a bag (40L).

Figure 7.1. Microalgae production facility.

The growth promotion was done through the addition of a commercial nutrients' solution (Easyphyt, EasyReef, Spain), containing phosphates and nitrates. Nutrients were added every 2-3 days, specifically 4 mL/L of nutrients solution per liter of culture water. The microalgae cells concentration was measured using a method provided by Alejos-Cabrera et al., (2023). The culture concentrations were measured every few days with a spectrophotometer (T60UV, PG Instruments, United Kingdom) at a wavelength of 680 nm, which presents a direct correlation with cells concentration.

Appendix III – Protocols for the analyses of water parameters

- **Total Suspended Solids (TSS)**

1) Weight and enumerate filtration filters.
2) Collect 50 mL of sample water.
3) Filtrate sample water using enumerated filters.
4) Dry filters using a laboratory oven (100 ºC) for at least 24 hours.
5) Weight dry filters.
6) Per each sample, make the calculation:

   Filter's final weight - Initial weight = Suspended solids weight (mg/50mL)

- **Ammonia (Koroleff method (1969) described in Grassof et al., 1983)**

1) In a test tube rack, prepare one test tube per sample, plus 5 for standard calibration curve.
2) Label the test tubes, these must be clean and dry.
3) Make standard curve (Table 7.2):

Table 7.2. Standard curve for ammonia.

Concentration in mg of $N\_NH_4^+$/L	0	0	0	0,07	0,28	0,49	0,70
Concentration in mL of $N\_NH_4^+$ (a 0,70 mg/L)	0	0	0	0,5	2	3,5	5
mL of distilled H_2O	5	5	5	4,5	3	1,5	0

4) Take 5mL of sample or standard and place it in a test tube.
5) Add 0.2 mL of phenol solution and shake.
6) Add 0.1 mL of citrate buffer solution and shake.
7) Add 0.2 mL of hypochlorite solution (DTT) and shake.
8) Leave the samples in the dark for a minimum of 6 hours (never more than 30 hours) or for half hour at 80 ºC.
9) In the spectrophotometer, calibrate to 0 with the blank and read the absorbance at a wavelength of 630nm.

- **Nitrites (Benschneider & Robinson)**

1) In a test tube rack, prepare one test tube per sample.
2) Previously label the test tubes, they must be clean and dry.
3) Make standard curve (Table 7.3):

Table 7.3. Standard curve for nitrites.

Concentration in mg/L of $N\text{-}NO_2$	0	0,017	0,067	0,118	0,168
Standard curve solution concentration mL de $N\text{-}NO_2$ (a 0,168 mg/L)	0	0.5	2	3.5	5
mL of distilled H_2O	5	4.5	3	1.5	0

4) Take 100 mL flask.
5) Pour a nitrite stock solution (reagent III) into the stopper.
6) Add 0.24 mL of the solution to the volumetric flask.
7) Pour the remaining cap into the bottle.
8) Make up the volumetric volume with distilled water.
9) Shake by hand.

- **Nitrates**

1) Make standard curve (Table 7.4).
2) Label the tubes with the concentrations (0 0.25 0.625 1.25 and 2.5).
3) Add the corresponding volume of the prepared nitrate stock solution (0, 0.05, 0.1, 0.25, 0.5 and 1).
4) Add the corresponding volume of milliQ water (distilled water) to each tube.

Table 7.4. Standard curve for nitrates.

Concentration in mg NO_3/L	0	0,05	0,1	0,25	0,5	1
Concentration mg NO_3/L	0	0,250	0,5	1	2,5	5
mL of milliQ water	5	4,750	4,5	4	2,5	0

1) Add 5 mL of filtered sample to each test tube (samples are diluted 1:200) or 25µL filtered sample + 4975µL milliQ water.
2) Add 200 µL of N1 solution.
3) Add 200 µL of N2 solution.
4) Add 400 µL of N3 solution.
5) Add reactants also added to the standard curve.
6) Vortex (mix well).
7) Incubate in the dark for 24 hours at room temperature.
8) Measure the absorbance at 540nm in the spectrophotometer.

- **Phosphates**

Analysis performed with Phosphates kit containing Phosphates high range checker reagent (HI717-25, HANNA Instruments, Italy).

PROCEDURE:

1) Collect water samples.
2) Filter water samples through a filtration filter. Collect water after filtration.
3) In a glass cuvette add 10 mL of sample filtrated water.
4) Add 10 drops of phosphate reagent (HI717AS).
5) Add a prepared reactive (HI717B-0) and wait 5 min.
6) Read using HANNA in “Phosphate HR” reading option.

Appendix IV – Ammonia and Nitrites parameters' evolution graphs – Phase 1

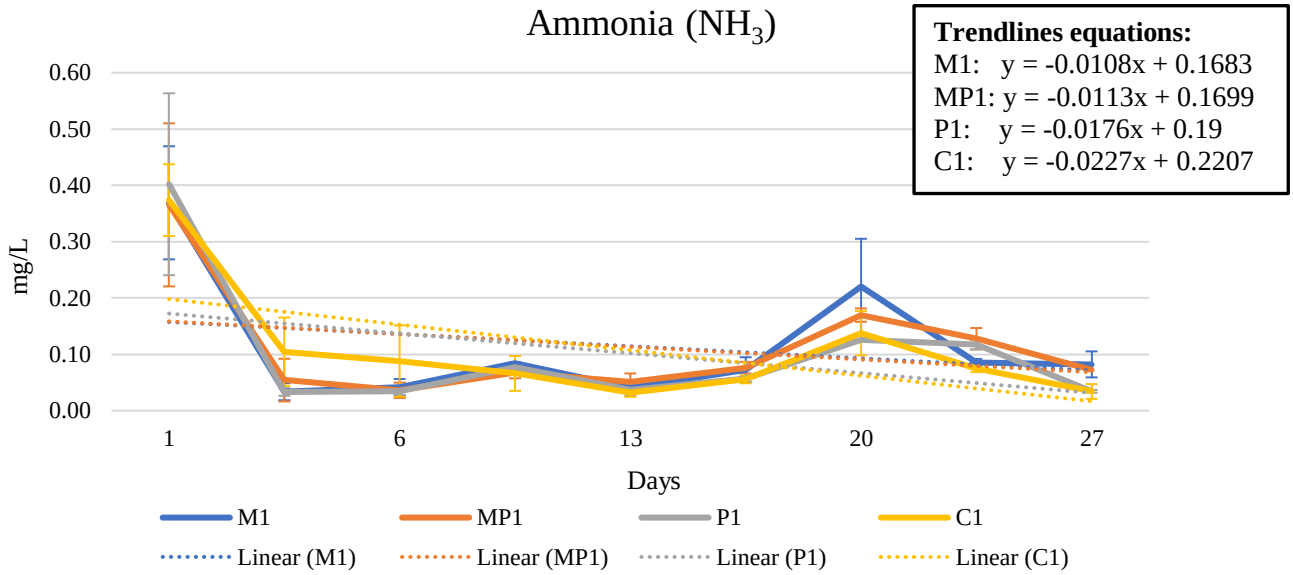

Figure 7.2. Ammonia concentration (mg/L) throughout Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

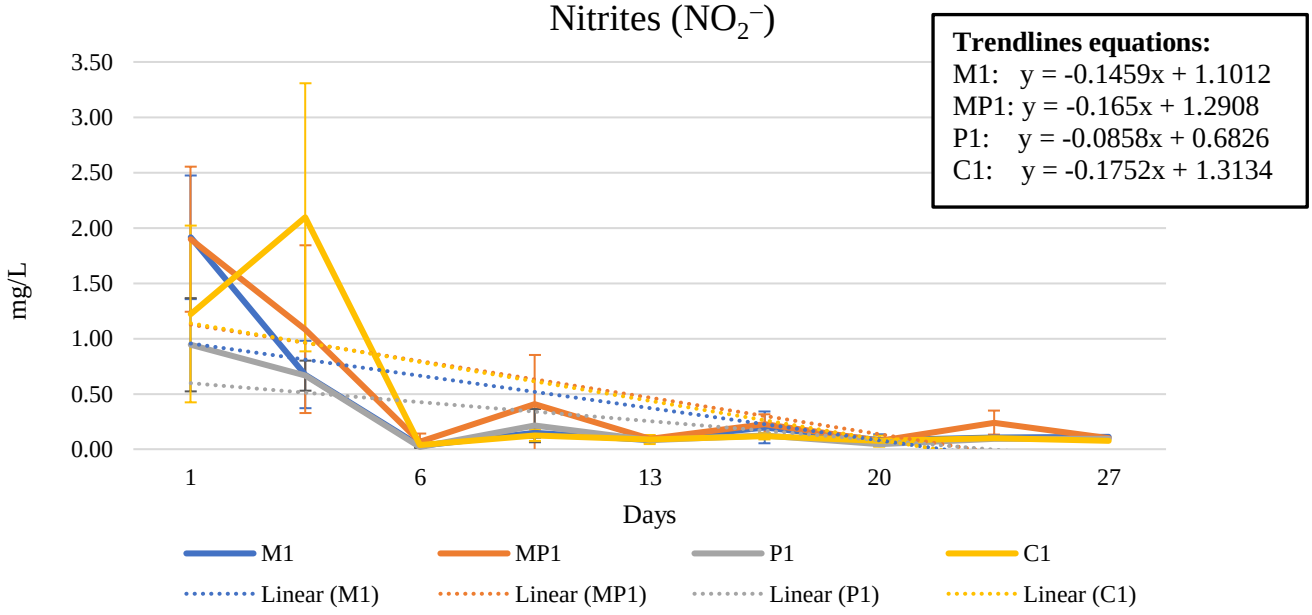

Figure 7.3. Nitrites concentration (mg/L) throughout Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

Appendix V – Nitrates and Phosphates parameters' evolution graphs – Phase 1

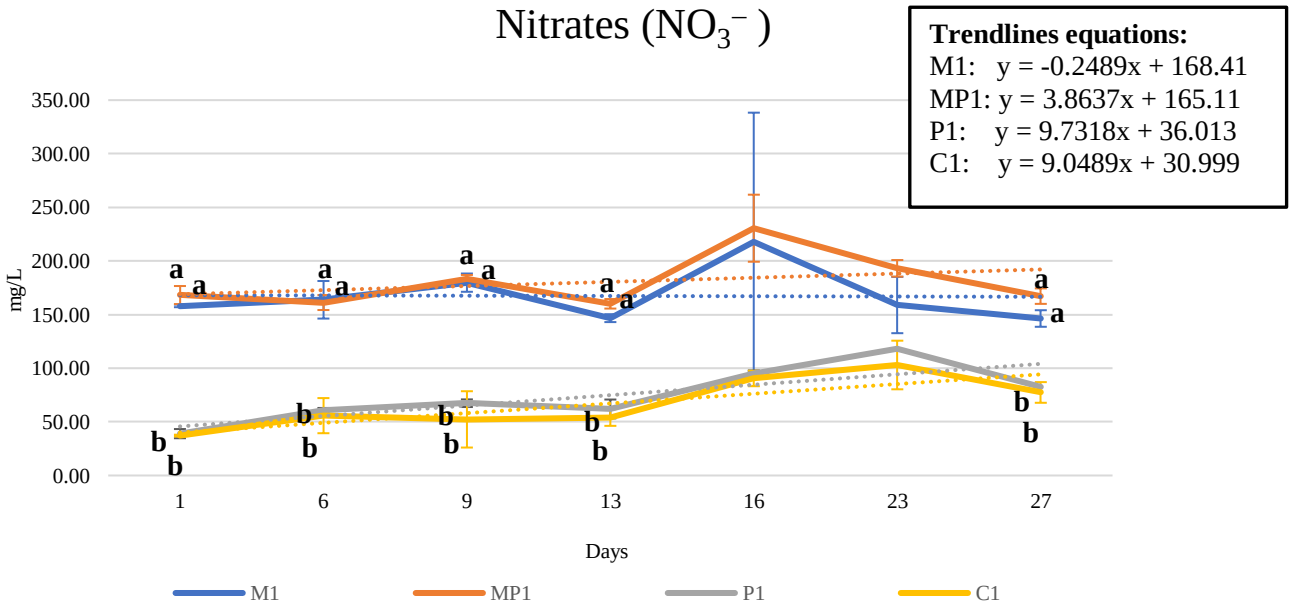

Figure 7.4. Nitrates concentration (mg/L) throughout Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly among treatments ($p < 0.05$).

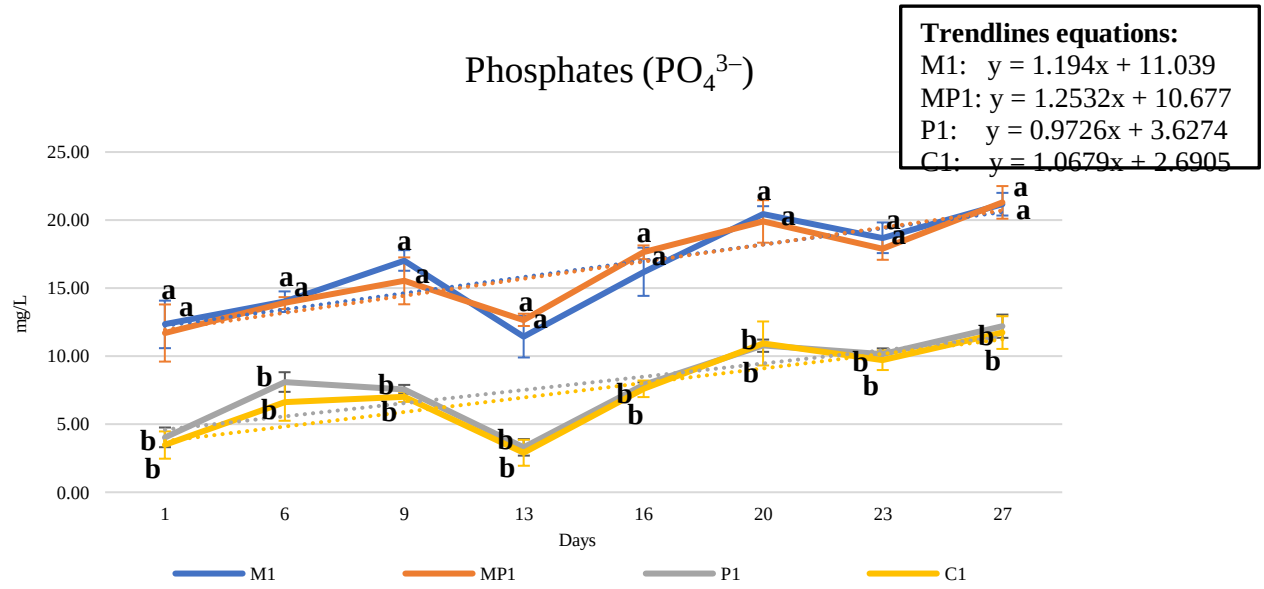

Figure 7.5. Phosphates concentration (mg/L) throughout Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly among treatments ($p < 0.05$).

Appendix VI – Ammonia and Nitrites parameters' evolution graphs – Phase 2

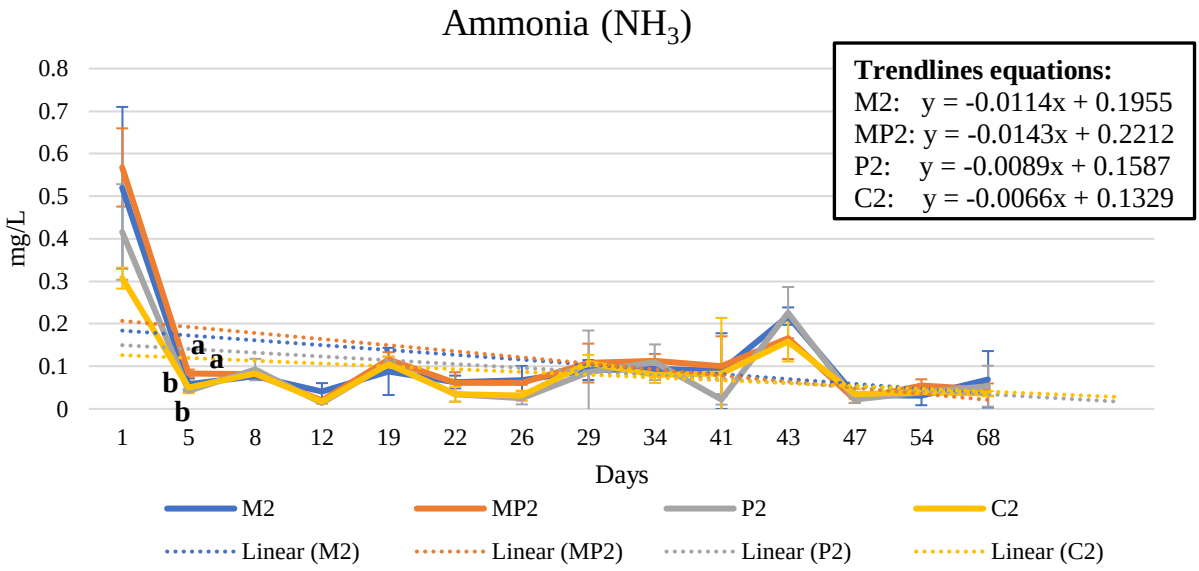

Figure 7.6. Ammonia concentration (mg/L) throughout Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly among treatments ($p < 0.05$).

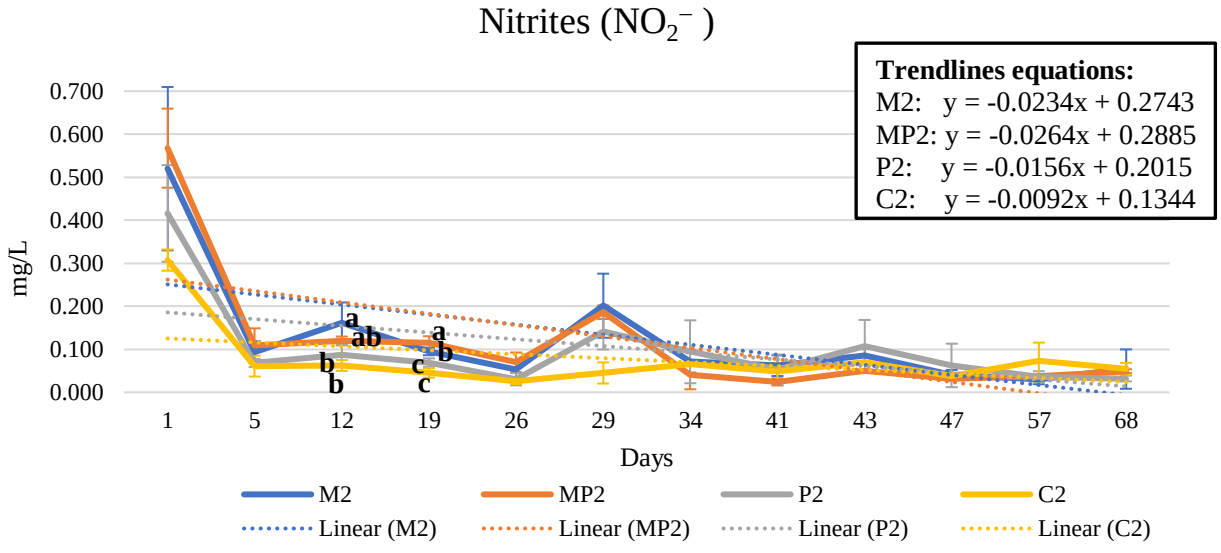

Figure 7.7. Nitrites concentration (mg/L) throughout Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly among treatments ($p < 0.05$).

Appendix VII – Nitrates and Phosphates parameters' evolution graphs– Phase 2

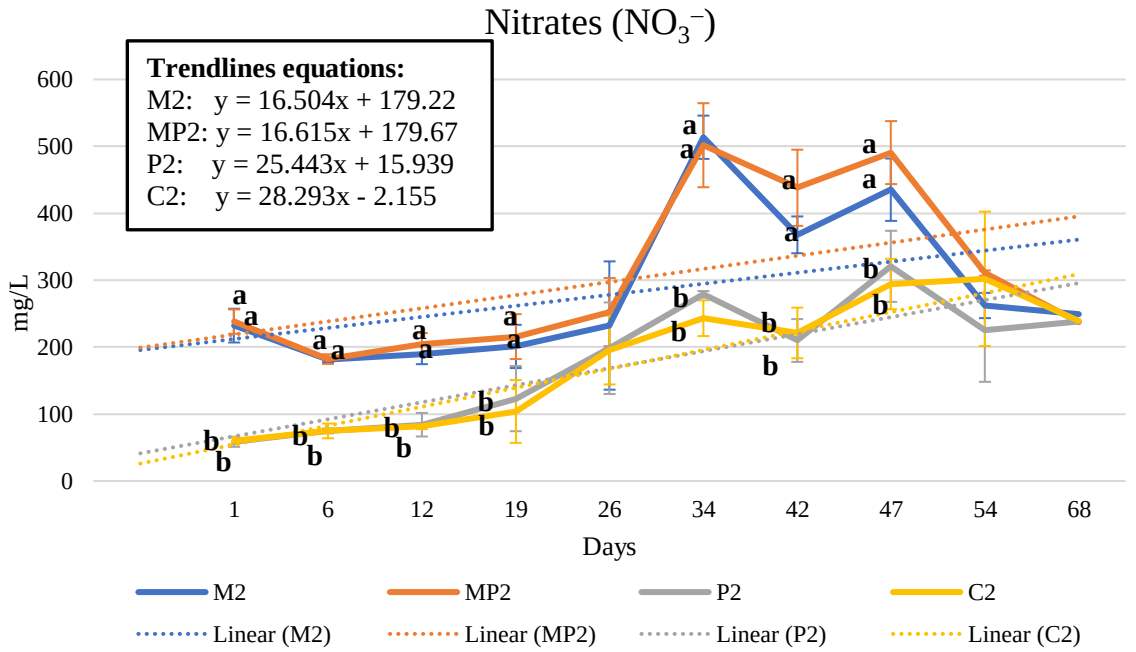

Figure 7.8. Nitrates concentration (mg/L) throughout Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly among treatments ($p < 0.05$).

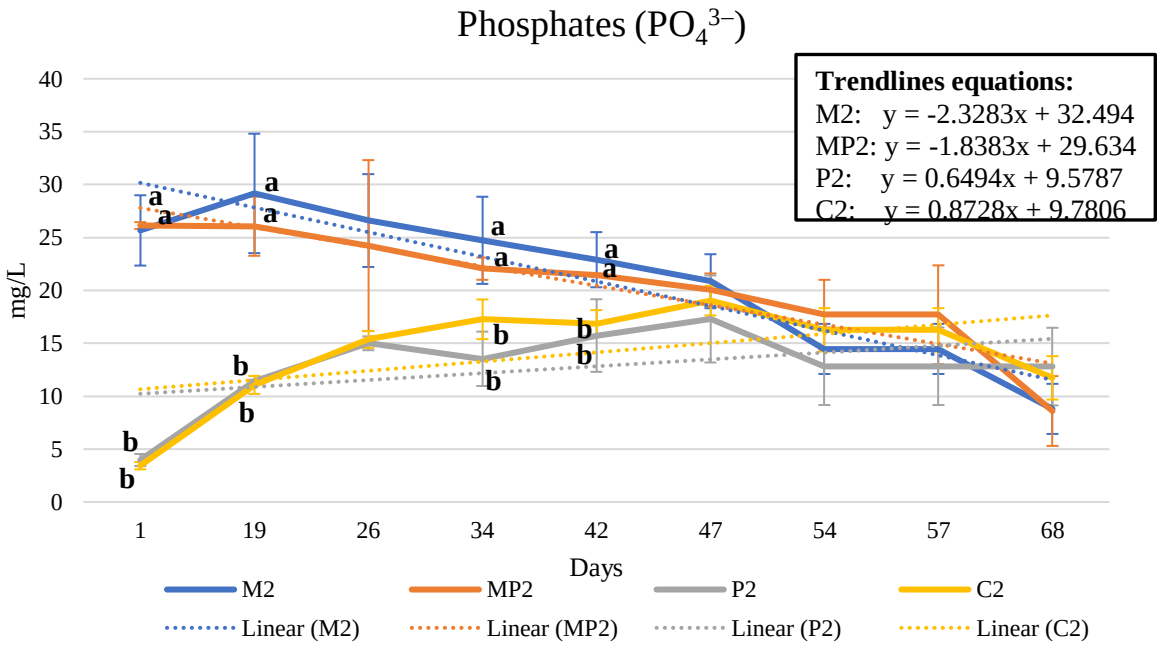

Figure 7.9. Phosphates concentration (mg/L) throughout Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)). Different letters in the same sampling moment between groups indicate the averages differ significantly among treatments ($p < 0.05$).

Appendix VIII – Nitrates and Phosphates' increment graphs – Phase 1

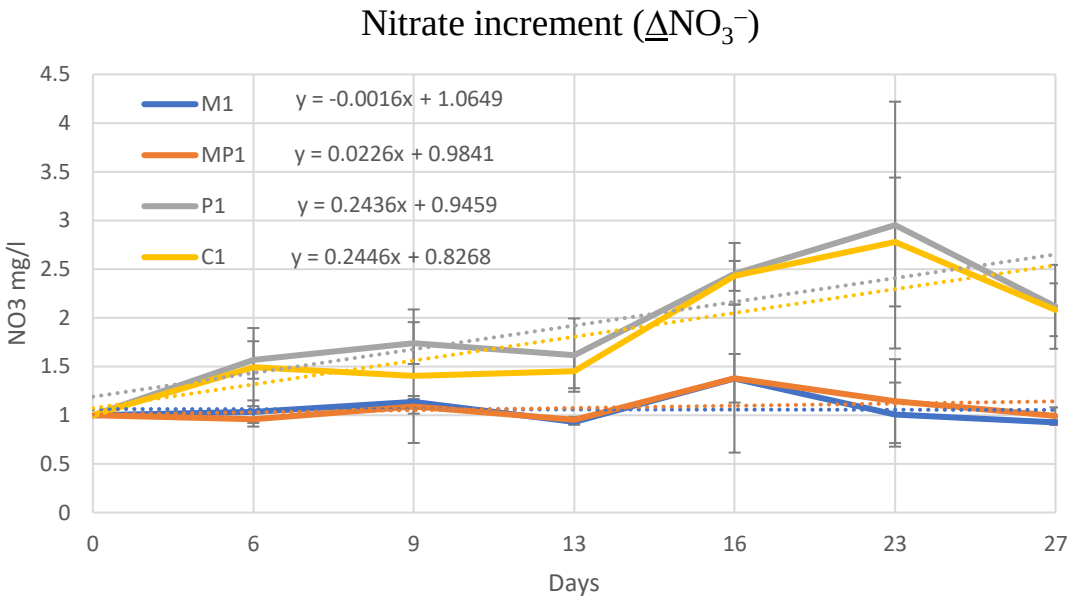

Figure 7.10. Nitrates increment throughout time - Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

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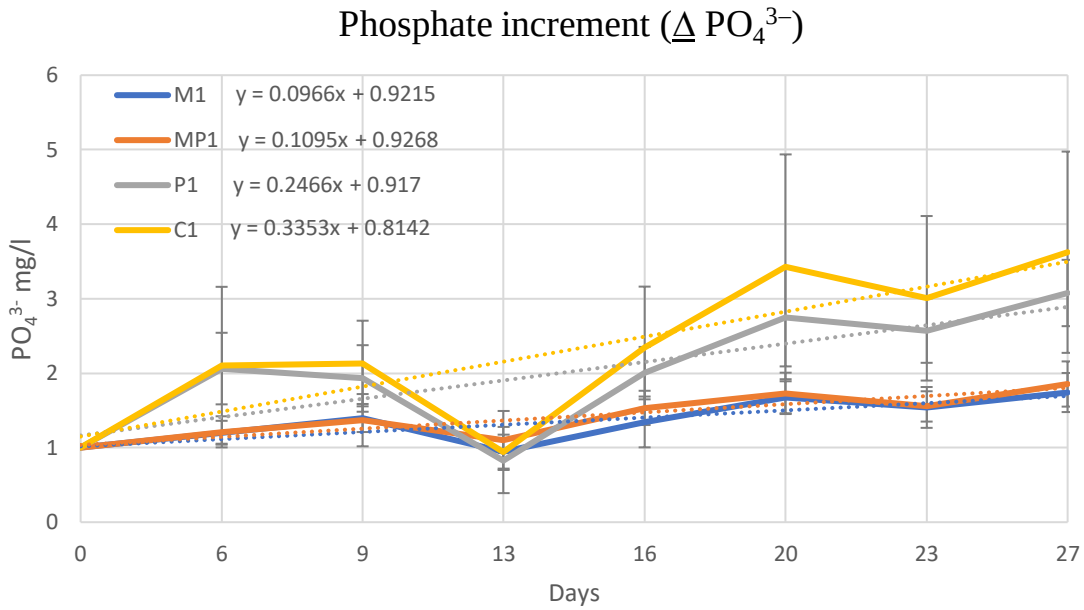

Figure 7.11. Phosphates increment throughout time - Phase 1 (C1 (control), P1 (Bacterial probiotic inclusion), M1 (Microalgae inclusion) and MP1 (Microalgae and bacterial probiotic inclusion)).

Appendix IX – Nitrates and Phosphates' increment graphs – Phase 2

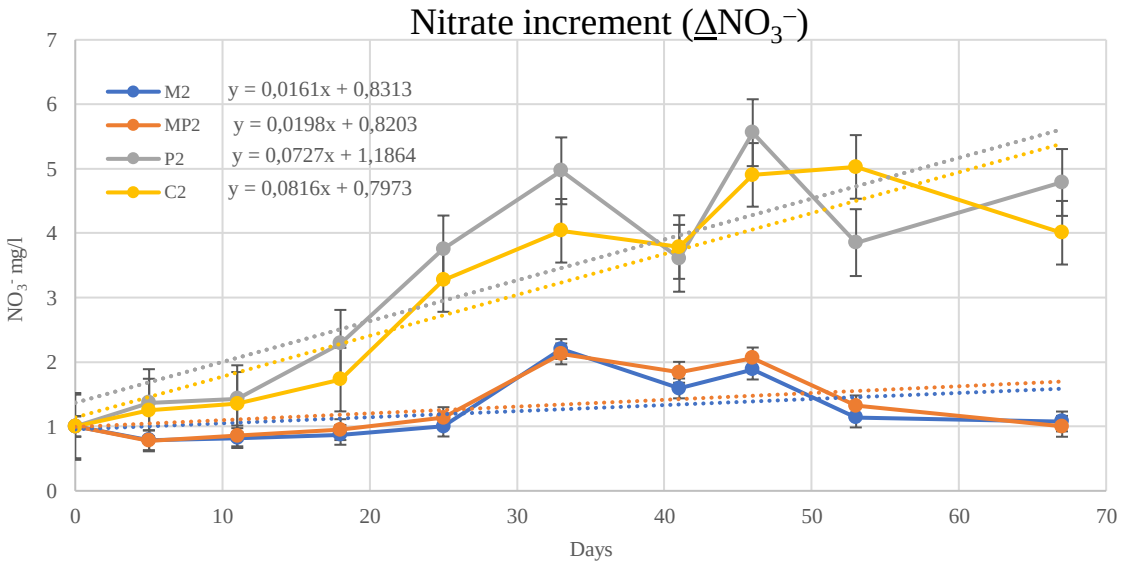

Figure 7.12. Nitrates increment throughout time - Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)).

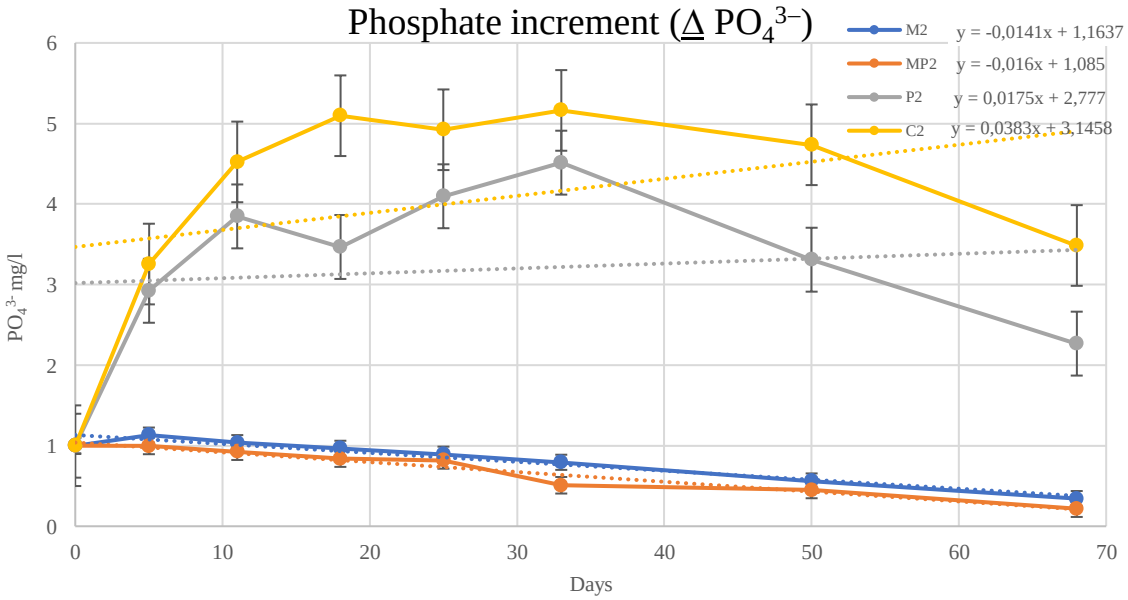

Figure 7.13. Phosphates increment throughout time - Phase 2 (C2 (control), P2 (Bacterial probiotic inclusion), M2 (Microalgae inclusion) and MP2 (Microalgae and bacterial probiotic inclusion)).