Evaluation of Nematodes and Artificial *Artemia* as Feed for Pacific White Shrimp in a Biofloc Nursery System

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

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Abstract

The global aquaculture production is growing immensely in all aspects and has already surpassed the output from wild caught fish and shellfish industries. The farming of *Penaeus vannamei* is one of the biggest contributors to this market. But many early stages of aquaculture depend on the finite and volatile resource *Artemia* as a live feed. This dependency has been identified as a bottleneck for future growth and sustainability progress. In this experiment, one artificial *Artemia* product and one nematode species were tested and evaluated in a feeding trial as potential replacements for live *Artemia* in a zero-water exchange biofloc nursery system. *P. vannamei* post larvae (PL) were stocked at a density of 60 PL/L in 60-L tanks. They were reared from PL12 – PL33 and fed 8 times per day with a dry feed (DF). Different treatments with four replicates each received a diet supplement of either live nematodes (N), live *Artemia* (LA), artificial *Artemia* (AA), or DF (control, C). The PL that received the live diets had nearly twice the survival rate compared to the ones only fed inert diets (N: 94 ± 6%, LA: 91 ± 7%, AA: 53 ± 11%, C: 51 ± 10%). Growth parameters were slightly better in the two inert diet groups (wet weight: N: 22.5 ± 5 mg, LA: 22.5 ± 5 mg, AA: 35 ± 5.7, C: 35 ± 10; Total length: N: 15.8 ± 3.9 mm, LA: 15.8 ± 3.2, AA: 17.1 ± 5.1 mm, C: 17.3 ± 5.0). No significant differences were detected in survival to salinity stress. In addition, beneficial effects on the biofloc and on the water quality were observed in the live diet groups and their causation should be further investigated. The results show that the nematode species *Panagrolaimus* sp. (NFS 24-5) can completely replace live Artemia in a co-feeding regime.

Keywords: *Penaeus vannamei*, nematodes, artificial *Artemia*, biofloc, nursery

Resumo

Nas últimas décadas a produção aquícola global experimentou um imenso crescimento em quantidade, variedade de espécies cultivadas e sistemas de produção. Desde 2014 que a aquicultura ultrapassa a produção mundial de peixes e mariscos capturados. O crescimento sem precedentes da população humana é o principal motor de uma procura crescente de proteína aquática de alta qualidade. O cultivo do crustáceo marinho *Penaeus vannamei* é um dos maiores contribuintes para este mercado. No entanto as fases iniciais das produções de muitos moluscos e peixes dependem de *Artemia* como alimento vivo, um recurso finito e volátil. Os cistos de *Artemia* só podem ser colhidos em algumas regiões do planeta e sua produção está sujeita a grandes flutuações naturais, o que limita sua exploração contínua. A dependência de *Artemia* por parte da indústria da aquicultura foi identificada como um estrangulamento ao crescimento e progresso do sector. A grande procura de proteína aquática levou a uma enorme pressão sobre muitos recursos naturais, em todos os tipos de ecossistemas aquáticos, desde os oceanos às águas interiores e passando pelos ambientes costeiros. A resposta da aquicultura tem de ser persuasiva no sentido de dar garantias de sustentabilidade, mantendo as suas atividades dentro de limites saudáveis. A obtenção de autonomia relativamente à *Artemia* poderá simplificar significativamente os processos de alimentação durante as fases larvares e, eventualmente, promover a sustentabilidade da aquicultura em geral.

Os produtos artificiais de *Artemia* já estão comercialmente disponíveis mas sua capacidade de alimentar eficientemente larvas de camarão ainda carece de testes científicos. Mais recentemente, uma espécie de nematode foi identificada como tendo propriedades semelhantes às dos cistos de *Artemia*: armazenamento a longo prazo em estado desidratado, processo de reidratação simples, tamanho pequeno para fácil ingestão e valor nutricional adequado para larvas de camarão. Além disso, estão disponíveis métodos de produção em massa, sustentáveis e economicamente viáveis, que facilitam sua aplicação comercial.

Nesta experiência, um produto artificial de *Artemia* e uma espécie de nematode foram testados e avaliados num ensaio de alimentação como substitutos potenciais de *Artemia*

viva num sistema de viveiro biofloco sem troca de água. Os sistemas Biofloc dependem do controle de nitrogénio através de processos microbianos que ocorrem dentro do tanque de cultivo, o que reduz substancialmente a renovação de água com o benefício adicional de fornecer proteína bacteriana. As pós-larvas (PL) de *P. vannamei* foram estabuladas com uma densidade de 60 PL por L em tanques semi-cónicos de 60 L. As larvas foram criadas a partir de PL12 - PL30 e alimentadas 8 vezes por dia com ração seca. Diferentes tratamentos com quatro replicados receberam um suplemento alimentar de nemátodos vivos (N), *Artemia* viva (LA), *Artemia* artificial (AA) ou ração seca (controle, C). As PL que receberam as dietas vivas tiveram quase o dobro da taxa de sobrevivência quando comparadas às alimentadas apenas com dietas inertes (N: 94 ± 6%, LA: 91 ± 7%, AA: 53 ± 11%, C: 51 ± 10%). No entanto, os parâmetros de crescimento foram ligeiramente melhores nos dois grupos de dieta inerte (peso húmido: N: 22,5 ± 5 mg, LA: 22,5 ± 5 mg, AA: 35 ± 5,7, C: 35 ± 10; Comprimento total: N: 15,8 ± 3,9 mm, LA: 15,8 ± 3,2, AA: 17,1 ± 5,1 mm, C: 17,3 ± 5,0). O efeito do stress salino não foi significativo. Nos grupos de dieta viva foram observados efeitos benéficos na formulação de bioflocos com reduzida acumulação de sólidos suspensos, totais e voláteis.

A dieta de nematode também teve níveis de nitrito significativamente menores, indicando melhor controlo do nitrogénio e parâmetros gerais de qualidade da água. O contexto e a causalidade dessas diferenças devem ser investigados, pois podem ser relevantes para operações de aquicultura usando sistemas de bioflocos. Em conclusão, a experiência verificou que a espécie nematode *Panagrolaimus sp*. (NFS 24-5) pode substituir completamente *Artemia* viva num regime de co-alimentação. Os resultados deste estudo de alimentação podem promover a sustentabilidade da criação de camarão por meio de alimentos alternativos, independentes dos recursos finitos de *Artemia*.

Palavras-chave: *Penaeus vannamei*, nematodes, *Artemi*a artificial, biofloco, viveiro

List of Abbreviations

AA	Artificial *Artemia*	**m**	Meter
[AA]	Artificial *Artemia* Group	**mg**	Milligram
AOB	Ammonia Oxidizing Bacteria	**mm**	Millimeter
		NH_3	Ammonia, unionized type
ANOVA	Analysis of Variance	**n**	Numbers, Sample Size
APHA	American Public Health Association	**[N]**	Nematode Group
		N	Nitrogen
ARA	Arachidonic Acid	**NH_4^+**	Ammonia, ionized type
BFT	Biofloc Technology	**NO_2^-**	Nitrite
°C	Degree Celsius	**NO_3^-**	Nitrate
C	Carbon	**NOB**	Nitrite Oxidizing Bacteria
[C]	Control Group	**NV**	Naamloze Vennootschap, Belgium Limited Company
CO_2	Carbon dioxide		
DHA	docosahexaenoic Acid	**O**	Oxygen
DO	Dissolved Oxygen	**p**	p-value, Probability Value
DF	Dry Feed	**PL**	Post Larvae
EPA	Eicosapentaenoic Acid	**PVC**	Polyvinyl-Chloride
EU	Experimental Unit	**RAS**	Recirculating Aquaculture System
FAO	Food and Agriculture Organization		
		RGR	Relative Growth Rate
g	Gram	**SPF**	Specific Pathogen Free
h	Hour	**SPR**	Specific Pathogen Resistant
H	Hydrogen	**t**	Time
HSD	Honest Significant Differences	**TAN**	Total Ammonia Nitrogen
		TSS	Total Suspended Solids
IPCC	Intergovernmental Panel on Climate Change	**UFSC**	Universidade Federal de Santa Catarina
		VSS	Volatile Suspended Solids
L	Liter	**W_0**	Initial Wet Weight
[LA]	Live *Artemia* group	**W_t**	Final Wet Weight
LCM	Laboratório de Camarões Marinhos	**WSSV**	White Spot Syndrome Virus
LTDA	Limited Company	**YSI**	Yellow Springs Instruments

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1. Introduction

1.1. Aquaculture Overview

In the last decades, the global aquaculture production experienced immense growth in total quantity, the variety of farmed species, and applied production systems (Bostock et al., 2010; Engle et al., 2017). In the year 2014, the total volume of farmed aquatic animals matched the total of wild caught fish and shellfish for the first time in history (FAO, 2016). This vast growth in output is largely driven by the urgent and imminent need to feed an ever-increasing human population of already 7.5 billion people to date, estimated to reach close to 9.5 billion in the year 2050 (United Nations, 2017). Along with this unprecedented growth in the total population emerges a soaring demand for high-quality protein, especially from aquatic sources. On a global average, mankind's growing appetite for fish and shellfish has already reached an annual consumption of more than 20 kg per capita (FAO, 2016). Estimates from the World Bank, the United Nations, and the Food and Agriculture Organization concluded that by 2030 over 60 percent of the consumed fish and shellfish will be provided by aquaculture (The World Bank, 2013). This shift towards more contribution from aquaculture to human nutrition will most likely continue hence after. Effects such as ocean warming and acidification, attributed to ongoing anthropogenic greenhouse gas emissions as most recently and prominently presented by the Intergovernmental Panel on Climate Change (IPCC, 2018), may lead to drastic redistributions of wild catch potentials in the global oceans with possible negative implications for many regions (Cheung et al., 2010). Particularly affected will most likely be the lower latitudes and the tropics, where also the fastest population increase and, hence, the highest requirement of fish as food is expected.

The high demand for aquatic protein, which has led to enormous pressure on many natural resources, from offshore oceans - through coastal environments - to inland freshwater systems, must be met with a persuasive movement towards more sustainability and nature conservation to keep exploitation of wild catches and

aquaculture activities in prospering and healthy limits. Therefore, in the face of global population growth and climate change, the world is required to find ways to produce enough food to feed mankind while preserving the planets natural habitats. In this scenario, especially the relatively new industry of aquaculture has been identified as a food producing sector with immense potential in growth and innovation. If aquaculture further continues towards more sustainability, it will play a significant role in the provision of nutritional and economic needs for future generations (Diana, 2009).

One important production segment of aquaculture is the group of marine crustaceans, with a contribution of almost 10% to the global market. And within this group, the species *Penaeus vannamei* (*P. vannamei*, Boone), commonly known as the Pacific white shrimp or whiteleg shrimp, accounts for over 80% of all farmed marine shrimp (Dugassa & Gaetan, 2018; FAO, 2016; Zhang et al., 2019). When considering all produced species in the world aquaculture industry, *P. vannamei* was ranked 6th in 2016 with a total volume of over 4 million tons (FAO, 2018).

1.2. *Penaeus vannamei*

In the late 1990s, a controversial taxonomic revision proposed by Pérez Farfante & Kensley (1997) split the former monophyletic *Penaeus* group into six genera, one of which was the genus of *Litopenaeus*, containing the species *vannamei*. Though, more recent genetic and molecular analysis of the Penaeid shrimp refutes the six-genus classification, resulting in a now widely accepted resumption of the former genus and species name: *Penaeus vannamei* (Ma et al., 2011).

Originally, *P. vannamei* is native to the tropical marine habitats of the Eastern Pacific, ranging from northern Mexico through Central America until North Peru. Water temperatures in this region are usually above 20-25 °C all year round (Dugassa & Gaetan, 2018). Adults live near or offshore in depths reaching up to 70 m and mate and spawn in the open ocean. The life-cycle of *P. vannamei* is rather complex and passes through

several morphologically differing stages. Maturity is attained with approximately 6-7 months of age when males have reached over 20 g and females over 28 g of body weight. A full-grown female can spawn up to a quarter million eggs at once, which are fertilized by male sperm in the external environment. About 16 hours after spawning and successful fertilization, the first larval stages, called nauplii, hatch from the eggs. The nauplii can swim intermittently and are independent of food but rely on internal yolk sack reserves as nutrition. After a few days, through metamorphic processes, the first larval stage is developed into the protozoa, which actively feeds on phytoplankton and unicellular algae. Further development phases lead through the mysis and early postlarvae (PL) stage, both of which mainly prey on zooplankton, such as rotifers, *Artemia*, and copepods. The late PL stage is already very similar in morphology to juvenile and adult stages. An adult will continue to grow and experience on average 50 molting periods during its lifetime (Dugassa & Gaetan, 2018; Zhang et al., 2019). After the final molting into a PL, the planktonic habitat is left behind, and migration inshore occurs passively through ocean currents and actively through impulsive swimming motions. In coastal waters, the PL find shelter and prey in mangroves, lagoons, and estuaries. During their benthonic adult life, the omnivorous *P. vannamei* feeds on detritus, worms, bivalves, and other crustaceans (Bailey-Brock & Moss, 1992). There is also evidence of sporadic cannibalistic behavior when they occur in high densities, which is usually only the case in captivity or aquaculture production (Romano & Zeng, 2017). This behavior has also been reported for several other penaeid shrimp species, such as *P. monodon* and *P. esculentus* (Abdussamad & Thampy, 1994; Arnold et al., 2005).

1.3. History of *Penaeus vannamei* Aquaculture

The relatively young aquaculture history of *P. vannamei* started in the 1970s when specimen caught from the wild off the coast of Panama were successfully spawned by French scientists in Tahiti and the species' life-cycle was closed for the first time (Michel et al., 2013). Further research achieved advances in the predictable maturation promotion through unilateral eyestalk ablation of females, which induces a hormonal

cascade affecting all aspects of the shrimp's physiology and eventually leads to spawning (Kannan et al., 2015). Also, the enhanced and specialized nutrition of the broodstock has improved the spawning of eggs in quantity and quality (Wouters et al., 2001). Intensive breeding techniques and genetic domestication programs eventually led to widespread shrimp aquaculture applications in the Hawaiian Islands, South United States of America, and many Central and South American countries. From the 1980s the commercial production in Latin America was growing rapidly but was periodically diminished by disease outbreaks, usually during the colder La Niña years. In the late 1980s, specific pathogen free (SPF) strains were developed at the Oceanic Institute in Hawaii, that reduced the impact of certain diseases and supported further shrimp farming activities (Wyban & Sweeney, 1991). In the late 1990s, *P. vannamei* was also introduced in the Asian aquaculture industry as an alternative to the major indigenous species *P. monodon* and *P. chinensis,* with which many farmers had disease susceptibility issues (Briggs et al., 2004). Since the implementation of *P. vannamei* in Asia, the species has experienced an immense increase of production until today, with China now being by far the biggest player worldwide and current producer of over 1 million tons annually (FAO, 2016).

1.4. Production Techniques of *Penaeus vannamei*

The aquaculture production of *P. vannamei* is fundamentally based on the grow-out of larvae obtained from a broodstock, which was originally caught in the wild or, as nowadays is the norm, has been domesticated and genetically improved over many generations in a specialized facility. Domestication has resulted in the production of broodstock shrimp strains with desirable traits, such as faster growth, lower salinity or temperature tolerance, or disease resistance. Significant success and a step forward was the development of specific pathogen free (SPF) and specific pathogen resistant (SPR) shrimp lines, which helped to overcome early issues with certain disease outbreaks (Browdy, 1998). The animals used as broodstock are reared, either individually or in groups, in so-called maturation tanks with optimal husbandry conditions comprised of

filtered seawater, fresh and specialized broodstock feeds, optimal photoperiod and temperature, and under continuous care by trained personnel. One eyestalk of the females is ablated, which will induce a repeated spawning event through a hormonal cascade. After successful spawning and fertilization by the males, the freshly hatched and positively phototactic nauplii are concentrated with a light source and transferred to a larval rearing tank or hatchery tank. In the ensuing hatchery phase, the nauplii are usually reared in "U" or "V"-shaped tanks with pure oxygen or air supply introduced from the bottom in order to keep the larvae afloat and emulate their planktonic life stage. They are predominantly fed with live feed, microalgae, and *Artemia*, often supplemented by liquid or dry formulated diets. The water is regularly exchanged at a rate of more than 100% per day to ensure optimal water conditions. When the larvae reach the PL10-12 stage, they are transferred directly to the grow-out tanks or an intermediate nursery tank system. In the shrimp aquaculture industry, the PL10 stage, for *e.g.*, is defined as an animal that went through all phases of larval development after hatching; from nauplii (1-2 days), zoea (3-5 days), mysis (3-5 days), until it reaches the PL stage, plus the 10 days it has already lived as a PL. This means that a PL10 is actually between 17 and 22 days old, depending on the development time in each pre-PL stage (Dugassa & Gaetan, 2018).

When a nursery system is applied before the grow-out phase, the PL are reared for one to five weeks with relatively high density in tanks that can be easily monitored and controlled for their water conditions. This ensures higher survival and faster growth in the early PL phases and leads to stronger PL that can then be transferred to the harsher conditions of grow-out ponds. This technique is especially applied in colder areas with shorter growing seasons, where the nursery tanks are set up in greenhouses or have heated water (Briggs, 2006).

The final grow-out systems can be classified as extensive, semi-intensive, intensive, and super-intensive, mainly depending on the densities of stocked PL. The simplest technique, largely applied in developing countries, is the extensive culture in irregularly shaped, earthen ponds that can be flooded and drained with the tidal flow. None or only minimal pumping and aeration is applied, the stocked PL are mainly fed on naturally

occurring prey within the pond, and only small and few shrimps can be harvested at the end of a grow-out season. In semi-intensive systems, larger densities are stocked, the water is exchanged regularly by pumping, the shrimp are fed several times daily with formulated diets, and the final yield, but also the investment and workload is considerably higher (Briggs, 2006).

More technically sophisticated intensive farms have ponds covered with a plastic lining, concrete raceways or circular tanks in various dimensions that can be completely drained, independent of the tides. Often times, these farms are located away from the coast on budget-priced land and use non-oceanic water sources with lower salinities. The densely stocked ponds need to have heavy aeration to achieve sufficient oxygenation and water movement. The shrimp are more intensely fed, and the water must be renewed frequently and channeled to settling ponds in order to cope with deteriorating water conditions. High renewal rates of water can be expensive, deteriorating for the surrounding environment, and may be a source of viral diseases. Alternatively, reduced water exchange systems, such as the biofloc technology (BFT), are now widely applied to confront these issues. Nevertheless, rigorous biosecurity measurements with careful monitoring and management of water, aeration, feed, and stocked PL are inevitable to achieve high yields in intensive farming systems.

In world regions without favorable all year-around temperatures of above 29 °C, the grow-out systems are located in greenhouses or even structured indoor facilities to help maintain high water temperatures during colder periods. Due to the higher cost of roofed space in more urbanized areas, PL are stocked in very high density in raceways or circular tanks, so-called super-intensive rearing systems. Often times, these enterprises rely on recirculating aquaculture systems (RAS) with a zero-water exchange, where only the evaporated water is replaced, and all accumulated biological waste is processed with biological filters. These systems are therefore considered to have high biosecurity and a low ecological footprint while allowing to produce shrimp of good quality in a sustainable manner. Recent technical advances in RAS have led to an increased interest in their application at locations completely independent of oceanic

water sources, temperate climate and with close proximity to consumers interested in a fresh and sustainably produced seafood.

1.5. Current Issues and Measures

One of the main issues that have arisen with the intensified large-scale shrimp aquaculture is disease outbreaks that threaten to reduce yields to a minimum or wipe out entire farms in affected regions. Vibriosis, a bacterial disease transmitted by various species of *Vibrio* spp., is one of the major problems and responsible for high mortalities in affected shrimp farms worldwide (Chandrakala & Priya, 2017). *Vibrio spp*. are naturally occurring opportunistic bacteria that can become pathogenic when the shrimp's immune system is suppressed, which can be the case at high densities with poor water and husbandry conditions. Many producers, especially in developing countries, have been counteracting the outspread of diseases with the broad administration of antibiotics during the production cycle (Bermudez-Almada & Espinoza-Plascencia, 2012). Indiscriminate usage of antibiotics in shrimp farms is associated with environmental issues, such as bacterial resistance and persistence of the disease and toxic residues in the water, sediment, and adjacent aquatic ecosystems. Farmers that are handling antibiotics regularly and local communities nearby that are extensively exposed to antibiotics in their environment and are in risk of suffering severe health issues. Also, antibiotic residues in the edible parts of the shrimp may also negatively affect the health of the consumers and have already led to concerns about food safety (Bermudez-Almada & Espinoza-Plascencia, 2012; Grigorakis, 2010; Holmström et al., 2003).

Another cause of widespread diseases in the shrimp aquaculture stems from the occurrence of several different types of pathogenic viruses. One extremely virulent species, among the 20 identified viruses affecting shrimp thus far, is the White Spot Syndrome Virus (WSSV). At affected shrimp farms, it can cause very rapidly emerging mortalities of up to 100% within 10 days. The financial losses in the 1990s alone, caused

by WSSV outbreaks worldwide, have been estimated to be worth more than 5 billion US dollars (Ganjoor, 2015). However, viruses are a common and naturally occurring biological agent in the marine environment, thus, their complete exclusion from aquaculture systems is practically impossible. The transmission may occur vertically from parent to the next generation, or horizontally from individual to individual. Applied methods to combat infestation and dispersion include strict biosecurity measures to avoid transmission, filtration and treatment of water sources, the usage of PL from certified SPF strains, the augmentation of the shrimp's natural immune responses through probiotics and specialized feed, and good overall husbandry conditions to reduce animal stress (Ganjoor, 2015; Walker & Mohan, 2009). A drastic reduction of water exchange, as is possible with BFT and RAS, indoor facilities, and strict feed controls can significantly reduce the risk of exposure to these disease agents, which may otherwise use transmission vectors such as water, feed, intruding animals, and lack of hygiene (Emerenciano et al., 2013).

A serious problem concerning the natural environment and the integrity of coastal regions in the tropics is the vast destruction of invaluable and endangered mangrove habitats through large constructions of shrimp farming ponds (Ashton, 2008; de Graaf & Xuan, 1998; FAO, 2016). Further, many farms, especially in developing countries with lax environmental protection laws and poor water management systems, have been reported to release large volumes of effluents to their surroundings that contaminate fresh and marine water habitats (Ahmad et al., 2017). The cumulative issues of diseases, mangrove destruction, and environmental pollution in the shrimp aquaculture industry preceded a decrease of consumer trust, especially in the sustainability and healthy food conscious group (Grigorakis, 2010; Shepherd & Little, 2015). Therefore, modern, biosecure, zero-water exchange systems located near urban areas may be able to reduce the pressure on the natural environment by producing healthy shrimp without antibiotics in the vicinity of the consumer. The absence of long transportation routes would also reduce carbon footprint and allow the marketing of a fresh and unfrozen high-value sustainable seafood product.

1.6. Biofloc Technology

The intensification of aquaculture brings with it an immense excess of organic pollutants, feed residues and biological wastes that accumulate in the rearing water. In addition to feces, aquatic organisms also excrete ammonia from their gills as the nitrogen end product of the protein catabolism. Depending on the temperature, alkalinity, and pH of the water, ammonia nitrogen can be present as the ionized type (NH_4^+) or the unionized type (NH_3). The sum of both types is usually referred to as the total ammonia nitrogen (TAN) and is used as a limiting water quality parameter (Chen et al., 2006). Ammonia is a small molecule, very soluble in water and permeates cell membranes relatively easily (Wright, 1995). In intensive culture systems with a high density of organisms and large biomass, the accumulation of ammonia in the water can quickly reach toxic levels. The most common method to attend this issue is the continuous replacement of the rearing water. This procedure requires very large volumes of clean incoming water and also produces equally large volumes of polluted effluents that deteriorate the adjacent environment. The RAS technology addresses this issue, and, with the use of biological filters hosting autotrophic nitrifying bacteria that oxidize ammonia to the lesser toxic nitrogen forms of nitrite and nitrate, only about 10% of the total water volume needs to be replaced (Ahmad et al., 2017). Though, the large financial investment and the high operational and maintenance costs of technology in RAS is inhibiting the broad application of this approach, especially in developing countries.

On the contrary, BFT has gained a lot of attention in recent years for its ability to control toxic nitrogen accumulation in a cost-effective, simple, and sustainable way. Its underlying principle of ammonia control is the promotion and maintenance of high levels of heterotrophic bacteria through the manipulation of the carbon:nitrogen (C:N) ratio in the water. By increasing the available carbon in the system, either by directly adding carbon-rich carbohydrates or the use of low protein feed with a high C:N ratio, heterotrophic bacterial growth is stimulated and nitrogen is assimilated through microbial protein metabolism (Ahmad et al., 2017; Avnimelech, 1999). The fast-growing

heterotrophic bacteria rapidly develop into dense communities of microorganisms and algae, which establish a flocculated suspension in the water, also called biofloc. This biofloc functions as a bioreactor controlling the water quality and can also serve as an additional source of protein for grazing shrimp (Avnimelech et al., 1994). Within a mature biofloc, toxic TAN and nitrite (NO_2^-) can be immobilized by heterotrophic assimilation into bacterial biomass and by autotrophic nitrification from TAN to nitrite and, in a further step, to nitrate (NO_3^-). When the C:N ratio is elevated to 15-20:1, the excreted nitrogen becomes the limiting growth factor and the heterotrophic bacteria readily incorporate it into their cells and thereby remove it from the water (Avnimelech, 1999). Compared to autotrophic nitrifying bacteria, which are primarily responsible for ammonia breakdown in RAS biofilters, heterotrophic bacteria have a 10 times higher growth rate and will, therefore, become the predominant bacteria in an environment with a high C:N ratio. Thus, toxic nitrogen species are immobilized much faster and more efficiently in a system with primarily heterotrophic bacteria (Hargreaves, 2006). The microbial processes, described by Avnimelech (1999) and Ebeling et al. (2006) in great detail, are taking place inside the rearing water, and are therefore independent of external biofilters and pumping systems, significantly reducing system space and cost.

Due to the extremely fast growth of heterotrophic bacteria in carbon-rich conditions, large amounts of bacterial biomass are produced and the accumulation of total suspended solids (TSS), which includes the volatile suspended solids (VSS) of organic origin, must be monitored and managed accordingly. It has been observed that TSS levels in the range of 400-600 mg/L are the most beneficial for shrimp rearing in BFT as they create favorable factors for the system stability and nitrogen control while providing a healthy environment for the shrimp. Extreme high TSS levels of >800 mg/L can lead to occlusions of the gills and lower the overall performance and survival of shrimp in such systems, while low levels of <200 mg/L are insufficient in controlling the TAN accumulation (Schveitzer et al., 2013). Another adverse effect of the fast increase of organic matter ignited through the addition of carbohydrates to the system is the corresponding increase of carbon dioxide (CO_2) in the water. High levels of CO_2 need to be avoided and must, therefore, be compensated through the elevation of dissolved oxygen (DO), which is essential to support the aerobic microbiological processes and

husbandry conditions for the shrimp. Hence, constant and sufficient aeration of the rearing water is extremely important in BFT, in order to maintain elevated DO levels and a well-mixed biofloc solution (Hargreaves, 2006).

The heterotrophic bacterial reaction also consumes a modest amount of alkalinity as an additional carbon source, therefore continuously reducing the alkalinity level in the water. Water sources with initial low alkalinity may require the addition of external carbon, such as sodium bicarbonate or calcium hydroxide. To support a sufficient and constant heterotrophic bacterial assimilation of nitrogen, the alkalinity levels should be kept between 100-150 mg/L (Ebeling et al., 2006).

To start and stimulate the initial biofloc formulation, a calculated amount of bottom soil containing beneficial microorganisms, ammonium sulfate, and carbon sources have to be added to the water. A preferable alternative, where possible, is the inoculation of a new biofloc with 30-50% water from an already existing and well performing mature biofloc solution (Ahmad et al., 2017; Schveitzer et al., 2017).

1.7. *Artemia*

The natural diet of shrimp larvae generally consists of diverse phytoplankton, zooplankton and biofloc compositions with various sizes and different nutritional values. But the feeding of *P. vannamei* larvae in commercial hatcheries, usually only consists of a few microalgae species and *Artemia spp.* nauplii as the main source of animal protein (de Lourdes Cobo et al., 2015). During the following production stages, the feeding exclusively with dry feed is common practice. Though, the quantity and quality of the nutritional diet offered to the shrimp larvae and juveniles have large implications for the further growth and development in the ensuing life stages. It has been reported that a supplement of live *Artemia* during the nursery phase is beneficial and produces larger PL that are more resilient and perform better in the grow-out phase (Zelaya et al., 2007).

The brine shrimp *Artemia* is an extremophile micro-crustacean that naturally occurs in hypersaline environments and has become a very popular live food for many aquaculture enterprises involved in the production of early stages of marine fish and shellfish (Gajardo & Beardmore, 2012). *Artemia* have a good nutritional value, are small and can, therefore, be readily ingested by the larvae Its dormant cysts can be stored for extended periods. The short incubation time of 24 hours to convert the desiccated cysts into free-swimming nauplii make them a convenient live food that requires relatively little labor and equipment for preparation (Sorgeloos et al., 1998). Starting with the emersion of hatchery operations in the 1970s, the overall positive attributes of *Artemia* have made it a highly sought-after essential in the aquaculture industry. But regrettably, the global supply of this natural resource is not sufficient to support the ongoing growth of this industry (Lavens & Sorgeloos, 2000). *Artemia* cysts can only be harvested in very few regions on the planet, mostly in hypersaline inland salt lakes or man-managed salt extraction ponds. Currently, the largest fishery of *Artemia franciscana*, the most abundant and most used species of brine shrimp, is located in the southern arm of the Great Salt Lake in the USA. This region alone contributes over 50% to the global *Artemia* cyst production, although precise data on production statistics are lacking (Calderon et al., 2004; Litvinenko et al., 2015; Mechaly et al., 2013). This condition has led to a dependency of the world aquaculture industry on a single location and exporting country (Sorgeloos et al., 1998). Additionally, the cyst production in the Great Salt Lake is subject to very dynamic natural fluctuations, limited by relatively strict environmental regulations, and prone to be affected by climatic changes. Recent research has also revealed that the long-term selective harvest of buoyant cysts may cause unforeseen evolutionary changes of *A. franciscana*, which could further challenge the sustainable management and future productivity of this fishery (Sura & Belovsky, 2016).

The accumulated inconsistencies have led to supply shortages and volatile prices of *Artemia* cysts in the past, affecting most of all marine aquaculture hatcheries and was therefore identified by researchers as one of the major bottlenecks for future growth (Lavens & Sorgeloos, 2000; Sorgeloos et al., 2001). To cope with this unreliability, other locations and different species of *Artemia*, such as the harvest of *A. persimilis* in Chile and Argentina or the aquaculture of the non-endemic *A. franciscana* in artificially

constructed salt ponds in the Mekong Delta of Vietnam, are being explored and investigated. Though, further research is needed to confirm their potential in commercial-scale applications that could contribute significantly to the ongoing global demand of *Artemia* cysts (Le, 2018; Mechaly et al., 2013).

To ensure the future sustainability of marine aquaculture production, the search for alternatives or complete substitutes of *Artemia* as live diets for the early larval stages is ongoing. Besides the adequate nutritional values, live diet alternatives must meet certain standards, which include the correct particle size, similar buoyancy in the water column, stability, minimal loss of nutrients through leaching, high digestibility, and the capability to be stored for long periods (Zelaya et al., 2007). Several products are already commercially available and some research has even pointed to potential nutritional and economic benefits when used in co-feeding regimes with live *Artemia*. But their broad application as a complete replacement for a live feed in all production phases has to be further investigated and published in peer-reviewed literature (Calderon et al., 2004; Gamboa-Delgado & Le Vay, 2009).

The main requirements by predatory shrimp and marine fish larvae to its prey are the attractiveness, the nutritional value, and especially the size. The successful uptake of diet particles is mechanically restricted by the size of the predators feeding apparatus, and thus, the prey must be small enough to be ingested (FAO, 1996). *Artemia* greatly fulfills this requirement, as well as certain roundworms of the phylum *Nematoda* that have caught the interest of scientists for various advantages.

1.8. Nematodes

Nematodes are an extremely diverse animal group that is found in all imaginable habitats including freshwater, marine, or terrestrial environments. Their ecological niches range from plant or animal parasites and predators to free-living non-parasitic forms that mainly feed on bacteria. Nematodes also occur naturally in biofloc and

Brüggemann (2012) has proposed several species as potential live diets for shrimp larvae based on their specific characteristics of being mass producible, storable, high in nutritional value, and capable to be enriched with specific fatty acids. Depending on the species of nematodes, individual sizes range between 150-2000 µm in length and 15-100 µm in diameter, which puts the smaller individuals in the size class of rotifers and the larger ones in the size class of *Artemia* (Sautter et al., 2007).

Furthermore, large scale mass production methods of a specific nematode species, *Panagrellus redivivus,* have already been shown by Focken et al. (2006), which is paramount for a successful application as a commercial *Artemia* substitute and would allow implementation in aquaculture productions without supply shortages. Recently, research has focused on another mass producible nematode species, *Panagrolaimus spp.* (strain NFS- 24-5), for its ability to survive a specific dehydration level that makes it eligible for long term storage, similar to *Artemia* cysts (Honnens et al., 2013). To reactivate the nematodes from their desiccated state, simple rehydration for one hour is needed, after which they are also able to survive extended periods in saline water. This species also synthesizes two essential fatty acids, eicosapentaenoic acid (EPA) and arachidonic acid (ARA), and can be enriched with docosahexaenoic acid (DHA) (Honnens et al., 2014), making it a favorable nutritional diet for shrimp larvae culture. Recently, Seychelles et al. (2018) and Seychelles *et al.* (2017) have already successfully tested *Panagrolaimus sp.* as a live feed for *P. vannamei* during the hatchery phase from the Zoea 2 stage until PL6 with promising results. Several authors conclude that, when they are provided in sufficient amounts, nematodes are an effective and favorable live food for the rearing of many crustacean species, yielding excellent survival and growth performances that make them suitable as a replacement of *Artemia* (Brüggemann, 2012; Focken et al., 2006; Seychelles et al., 2018, 2017). But further feeding trials during different PL stages and under various rearing conditions are needed in order to thoroughly evaluate the capability of *Panagrolaimus sp.* (strain NFS-24-5) to be a complete substitute of live *Artemia.*

1.9. Objectives and Goals

The objective of this work is to evaluate an artificial *Artemia* diet and a desiccation-tolerant nematode species to replace live *Artemia* nauplii, supplemented during the nursery phase of *P. vannamei* from PL12 to PL 30 in a zero-water exchange biofloc rearing system. The results of this feeding trial may potentially lead to increased sustainability of shrimp farming through alternative feeds that are independent of finite *Artemia* resources. In addition, simplifying the feeding processes in shrimp hatcheries may eventually increase the availability of PL and benefit sustainable shrimp aquaculture operations.

2. Material and Methods

2.1. Preparation of the Experimental Facility

The experiment was carried out in March 2018 at the Laboratório de Camarões Marinhos (LCM) of the Universidade Federal de Santa Catarina (UFSC) in Barra da Lagoa, Florianopolis (Santa Catarina, Brazil). Prior to the arrival of the PL from the shrimp hatching company Aquatec LTDA (Rio Grande do Norte, Canguaratema, Brazil), the experimental room and all of the equipment was prepared. Sixteen experimental units (EUs) with a filling capacity of 60 L and consisting of semi-cylindrical blue plastic tanks, polyvinyl-chloride (PVC) pipes to supply constant aeration in the bottom of the tanks, and immersion heaters were set up in a wet laboratory of the LCM (Figure 1). Each tank was evenly equipped with six vertical PVC frames (0.16 m^2 each) spreading an artificial surface of Needlona®, a synthetic filter media fabric. The surface area of six Needlona® frames was equivalent to 100% of the inside surface area of the semi-cylindrical tanks (0.89 m^2), thus doubling the total surface area within each tank. Previous studies have shown that an artificial substrate of Needlona® is capable to reduce the accumulation of suspended solids while ensuring high survivals of PL reared in a biofloc nursery system (Costa Rezende et al., 2018). The air leading PVC pipes ran along the bottom of the tanks to provide a constant flow of air to assure efficient stirring of the biofloc solution and to increase the DO (Figure 1 A). The immersion heaters were connected to electrical contacts hanging from the ceiling above the tanks and were configured to maintain a constant water temperature of 28-30 °C. The room was also equipped with three additional radiators to help maintain high temperatures within the experimental room. One day before the start of the feeding trial, all equipment was thoroughly cleaned, disinfected with bleach, and again tested for orderly functioning. In addition, the water pipes of the facility were properly flushed and cleaned by purging a sponge through them.

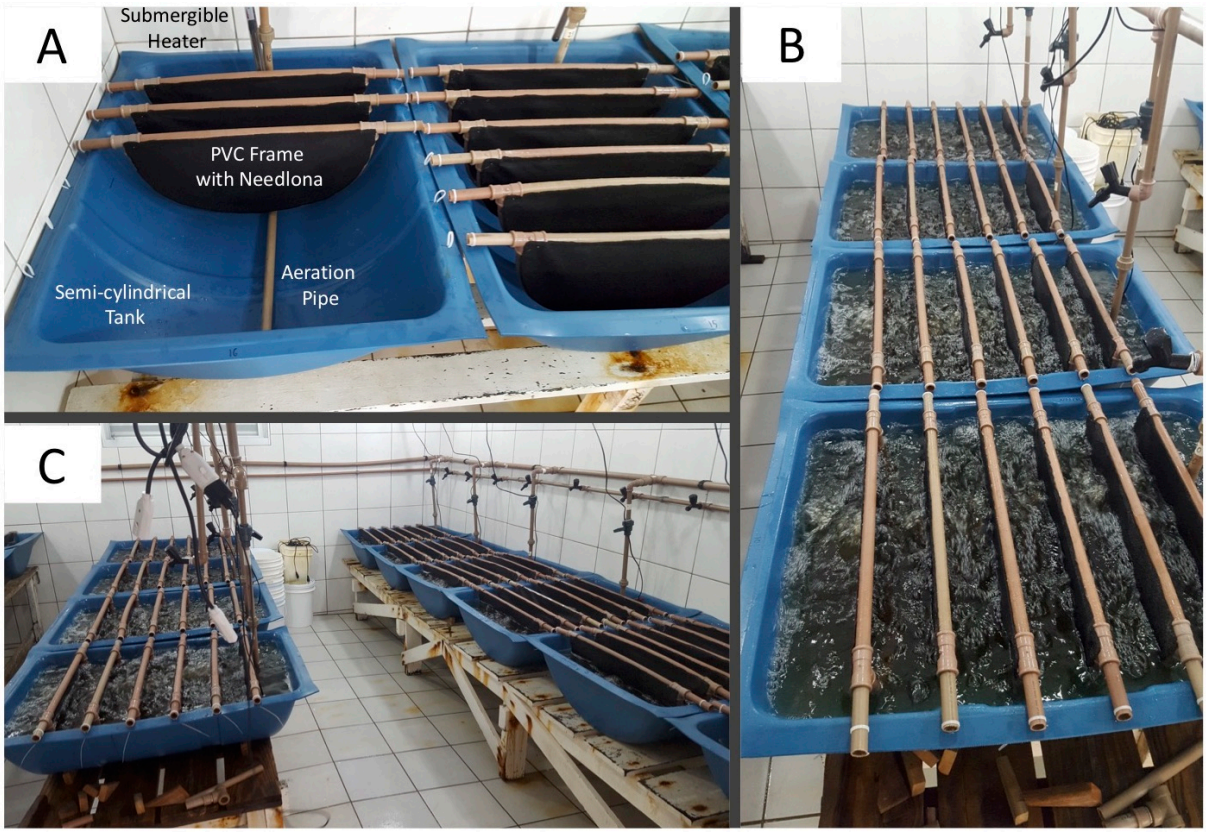

Figure 1: A: Experimental Unit (EU); B: Row of EUs filled with biofloc water; C: Overview of the wet laboratory room.

Two days before the arrival of the PL via air transportation, a 4000-L semi-cylindrical holding tank was set up with a bottom PVC tube for aeration and two flow-through heaters set at 28 °C (Figure 2 A). The tank was filled halfway with mature biofloc water from a grow-out pond at the LCM. The other half was filled up with filtered seawater at 33 ppt. The PL arrived late in the evening on the 9th of March 2018 at the Airport of Florianopolis. They were immediately picked up with a transporter and driven to the LCM. The total amount of approximately 165000 PL was packaged in 21 carton boxes containing plastic bags filled with seawater under a pure oxygen atmosphere. The plastic bags were immediately opened upon arrival to the lab and poured into a specialized bucket for concentration. The overflow of the bucket was covered by a 500 µm mesh to keep the PL from washing out (Figure 2 B). It was constantly flushed to exchange the transport water with clean and filtered seawater. After emptying 7 plastic bags and washing the PL with seawater, they were carefully transferred to the prepared holding tank. Another batch of 7 plastic bags was processed again until there were no bags left. The PL were kept in the holding tank for the next 4 days to allow for acclimation. During

this time they were hand-fed 8 times per day (8h00, 10h00, 12h00, 14h00, 16h00, 18h00, 21h00, and 24h00) with a dry feed (DF) (INVE). According to the feed producer's recommendations, ±1 g of DF was given at each feeding time.

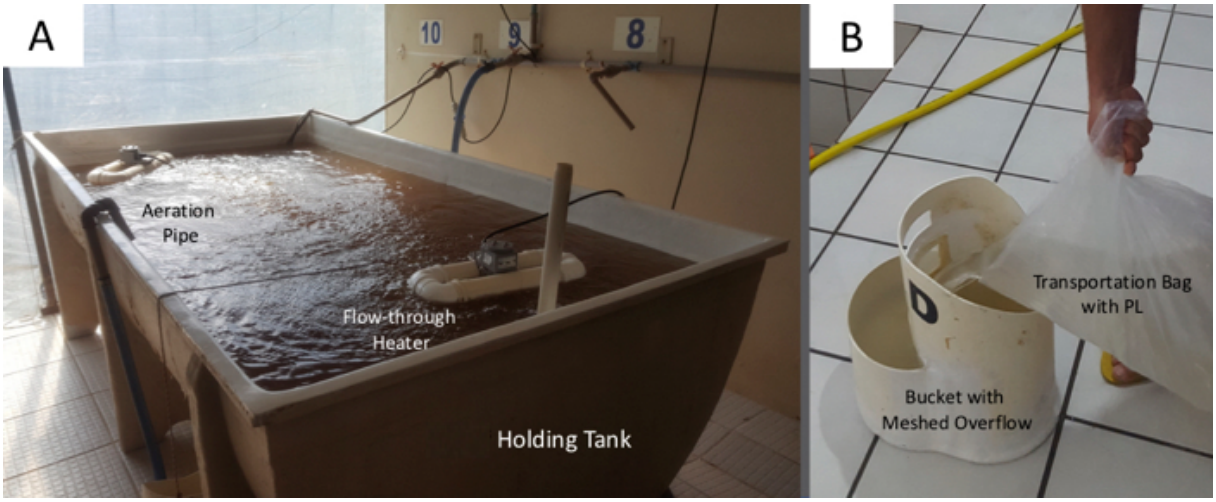

Figure 2: A: Holding Tank; B: Bucket with meshed overflow.

During acclimation, EUs were filled with 50% water coming from a mature biofloc grow-out tank and 50% filtered seawater at 33 ppt. In the morning of the 14th of March 2018, PL were flushed out through the bottom drain of the holding tank, concentrated and transferred to a 1000 L mixing tank, filled with fresh seawater. Very strong aeration was applied to achieve a homogenous mixing of the PL inside the tank. A 1-L container was used to quickly determine the density in PL/L. The container was emptied gradually over a sieve to count the number of PL retained. All counted PL were quickly returned to the tank by submerging the sieve. The total number of PL/L was calculated by the sum of all counted shrimp. This technique was repeated in three replicates to increase the accuracy of the PL density. Water from the 1000 L mixing tank was reduced to reach a density of 900 PL/L. While the mixing tank was under very strong aeration, 3600 PL contained in 4 scoops of 1-L were concentrated onto a sieve and transferred to each EU. Four EUs were randomly assigned to one of the four dietary treatment groups (Control [C], Artificial *Artemia* [AA], Nematodes [N], and Live *Artemia* [LA]).

2.2. Feeding Trial

The feeding trial started on the 15th of March 2018, one day after the PL have been transferred to the EUs. For the entire experiment, PL were hand-fed 8 times per day (8h00, 10h00, 12h00, 14h00, 16h00, 18h00, 21h00, and 24h00), whereas at 12h00 and at 18h00 the different treatment groups received their respective special diet. The control group [C] received the normal DF in the same volume as it was given at all other feeding times. The [AA] group received a special dry feed composed of extracted and processed *Artemia* cyst content. Both feed types, the DF by INVE and the artificial *Artemia* (AA) DF, were available in different pellet sizes, called "PL" and "XL" for the DF and "Standard" and "Large" for the AA. According to the producer's recommendations, depending on the PL age, the smaller, larger, or a mix of both pellet sizes, was given. The live diet groups received a freshly prepared live feed in a quantity based on a study by Suita et al. (2016), who proposed 70 *Artemia* individuals per PL per day. The [LA] group received this quantity of *Artemia* nauplii, whereas the [N] group received an equivalent amount in nematode dry weight of the species *Panagrolaimus sp.* (NFS 24-5). The precise amount of DF and special feed that was calculated and administered at each time during the experiment can be seen in Table 1, and the preparation and properties of each type of feed are described below in more detail. In order to maintain a stable biofloc formulation with good nitrogen control, dextrose was added to increase the C:N ratio and calcium hydroxide was added to increase alkalinity. The added amounts were determined depending on the results from the water analysis for TAN, nitrite, and alkalinity (Table 2).

Table 1: Feeding schedule.

Day 1- Day 5 (PL11-PL14) *								
	Feeding Times							
Treatment	8h00	10h00	**12h00**	14h00	16h00	**18h00**	21h00	24h00
Control [C]	0.28g DF	0.28g DF	**0.28g DF**	0.28g DF	0.28g DF	**0.28g DF**	0.28g DF	0.28g DF
Artificial *Artemia* [AA]	0.28g DF	0.28g DF	**0.57g AA**	0.28g DF	0.28g DF	**0.57g AA**	0.28g DF	0.28g DF
Nematodes [N]	0.28g DF	0.28g DF	**0.22g N**	0.28g DF	0.28g DF	**0.22g N**	0.28g DF	0.28g DF
Live *Artemia* [LA]	0.28g DF	0.28g DF	**126000 LA**	0.28g DF	0.28g DF	**126000 LA**	0.28g DF	0.28g DF
Day 6 – Day 9 (PL15 –PL18) **								
	Feeding Times							
Treatment	8h00	10h00	**12h00**	14h00	16h00	**18h00**	21h00	24h00
Control [C]	0.36g DF	0.36g DF	**0.36g DF**	0.36g DF	0.36g DF	**0.36g DF**	0.36g DF	0.36g DF
Artificial *Artemia* [AA]	0.36g DF	0.36g DF	**0.57g AA**	0.36g DF	0.36g DF	**0.57g AA**	0.36g DF	0.36g DF
Nematodes [N]	0.36g DF	0.36g DF	**0.22g N**	0.36g DF	0.36g DF	**0.22g N**	0.36g DF	0.36g DF
Live *Artemia* [LA]	0.36g DF	0.36g DF	**126000 LA**	0.36g DF	0.36g DF	**126000 LA**	0.36g DF	0.36g DF
Day 10 – Day 13 (PL19 - PL22) **								
	Feeding Times							
Treatment	8h00	10h00	**12h00**	14h00	16h00	**18h00**	21h00	24h00
Control [C]	0.45g DF	0.45g DF	**0.45g DF**	0.45g DF	0.45g DF	**0.45g DF**	0.45g DF	0.45g DF
Artificial *Artemia* [AA]	0.45g DF	0.45g DF	**0.72g AA**	0.45g DF	0.45g DF	**0.72g AA**	0.45g DF	0.45g DF
Nematodes [N]	0.45g DF	0.45g DF	**0.28g N**	0.45g DF	0.45g DF	**0.28g N**	0.45g DF	0.45g DF
Live *Artemia* [LA]	0.45g DF	0.45g DF	**157500 LA**	0.45g DF	0.45g DF	**157500 LA**	0.45g DF	0.45g DF
Day 14 – Day 21 (PL23 – PL30) **								
	Feeding Times							
Treatment	8h00	10h00	**12h00**	14h00	16h00	**18h00**	21h00	24h00
Control [C]	0.56g DF	0.56g DF	**0.56g DF**	0.56g DF	0.56g DF	**0.56g DF**	0.56g DF	0.56g DF
Artificial *Artemia* [AA]	0.56g DF	0.56g DF	**0.89g AA**	0.56g DF	0.56g DF	**0.89g AA**	0.56g DF	0.56g DF
Nematodes [N]	0.56g DF	0.56g DF	**0.35g N**	0.56g DF	0.56g DF	**0.35g N**	0.56g DF	0.56g DF
Live *Artemia* [LA]	0.56g DF	0.56g DF	**196875 LA**	0.56g DF	0.56g DF	**196875 LA**	0.56g DF	0.56g DF

* From Day 1 to Day 5, DF was given as a 1:1 mix of the sizes “PL” and “XL”; and AA was given as a 1:1 mix of the sizes “Standard” and “Large”.

** Starting from Day 6, DF was given only in the feed size “XL”, and AA was given only in the size “Large”

Table 2: Amount of diet, dextrose, and calcium hydroxide added to all groups at each day.

Input	Day *																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Diet (g)	2.2	2.2	2.2	2.2	2.2	2.9	2.9	2.9	2.9	3.6	3.6	3.6	3.6	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Dextrose (g)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	2.0	1.0	1.0	1.0	1.0
Calcium Hydroxide (g)	-	-	-	-	0.6	0.6	0.6	1.1	1.1	1.1	1.1	0.9	0.9	0.9	0.5	0.5	0.5	0.5	0.5	0.5

* Starting at day 16, the feed was decreased by 50% due to deteriorating water quality.

2.3. Feed Preparation

2.3.1. Preparation of live *Artemia*

On every day, live *Artemia* nauplii were prepared for hatching 24 hours before they were used as feed. Based on the recommendations in a study by Suita et al. (2016), it was determined that during the nursery phase from PL1 to PL30, each PL should receive approximately 70 individuals of *Artemia* nauplii as feed per day. Thus, each tank of 3600 PL should receive 252000 nauplii per day, so half that amount per special feeding. Since four tanks received the LA treatment, approximately 1.0 - 1.5 million nauplii, depending on the experimental phase, were needed each day (Table 1). Prior conducted tests of hatching *Artemia* cysts (*Artemia* High 5, INVE) in this facility and with the available equipment have shown results with high fluctuations regarding the quantity of produced nauplii. On average, only between 75000 and 100000 nauplii were successfully hatched per gram of *Artemia* cysts. Therefore, to be on the safe side, 20 g of cysts were prepared for hatching each day, expected to be yielding an excess of 1 million *Artemia* nauplii per day. This volume of cysts was raised to 30 g during the later stages of the feeding trial when more nauplii were needed. Starting at 11h00 in the morning before the first day of the experiment, 20 g of *Artemia* cysts were put in 10 L of water at a salinity of 15-25 ppt. The solution was filled into an *Artemia* hatching container with an air supply attached at the bottom (Figure 3). The aeration was set up strong enough to sufficiently mix the solution without spilling it out of the container. A strong light source

emitting approximately 2000 lux was placed just above the water surface and an immersion heater was inserted in the tank and set to 29 °C. Twenty-four hours later, the light and aeration were switched off and the heater was removed. A lid was placed above the container so that the hatched *Artemia* would swim to the transparent bottom of the tank, now the only source of light. After half an hour, the water and the hatched *Artemia* nauplii were carefully drained from the bottom of the tank into a bucket through the disconnected aeration pipe, until only the empty shells that were floating atop were left in the hatching tank. This process of shell and nauplii separation was followed by removing any remaining floating *Artemia* shells with a plastic pipette. By filtration through a 100 µm sieve, the *Artemia* nauplii were concentrated and then washed into a beaker with 1000 mL of seawater at 33 ppt to have a known volume for the following quantity calculations. A small aeration stone, connected to an air delivering system, was added to ensure sufficient oxygen supply. An aliquot of 1 mL of the *Artemia* solution was diluted in 1000 mL of seawater. The total quantity of *Artemia* was estimated by counting nauplii contained in 10 samples of 1 mL, using a glass pipette. The calculation was extrapolated to the concentrated solution of *Artemia* nauplii to deliver the quantity required in each EU of LA dietary treatment. The remainder of the hatched *Artemia* were stored in a beaker with sufficient aeration to be used as feed for the second special feeding at 18h00. Any excess of produced *Artemia* nauplii was frozen inside small plastic cups and marked with the estimated quantity of individuals inside. These units of frozen *Artemia* nauplii were used as backup feed in case the hatching process on a given day would fail or yield too little live nauplii.

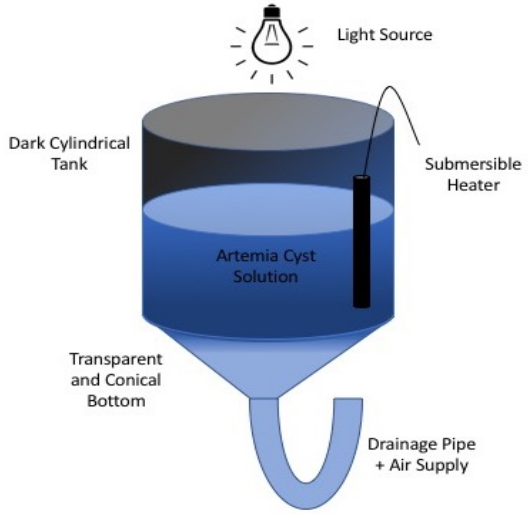

Figure 3: Diagram of *Artemia* hatching tank.

2.3.2. Preparation of Nematodes

The nematode diet was composed of pure dehydrated *Panagrolaimus sp.* (NFS 24-5) provided by the company E-nema GmbH (Schwentinental, Germany). Since the nematodes are not uniform in size but consist of small individuals (150-800 µm length) and large individuals (800-1300 µm length) it is more practical to estimate the needed amount equivalent to the LA in weight rather than density. One *Artemia* nauplius is estimated to have a dry weight of 1.6 ± 0.1 µg (Farhadian et al., 2009). Thus, the amount of dehydrated nematodes equivalent to 70 *Artemia* nauplii is 112 µg. To compensate the 10% of water still existent in the dehydrated state, 123 µg dry weight of nematodes was used per PL per day. The required quantity of dehydrated nematodes needed for each day (Table 1) was weighed using a digital scale and put in a glass beaker. They were rehydrated in 24 mL of freshwater every day 1-hour prior to feeding. The solution was then thoroughly mixed until it became completely homogeneous. After 30 to 60 minutes the nematodes were sufficiently hydrated to awaken from their desiccated state and began to show their typical sinuous movements, which could be observed under a binocular microscope. At each special feeding time, 3 mL of the nematode solution was administered to each tank of the [N] group with a plastic pipette. After the 12h00 feeding, the remaining half of the daily prepared nematode solution was kept in a fridge at 7 °C until the evening feeding at 18h00.

2.3.3. Preparation of dry feed and artificial *Artemia*

The DF used for this feeding trial was manufactured by INVE (Thailand) Ltd. (Nong Lum, Wachirabarami, Thailand) and consisted of the products INVE EPAC Black PL and INVE EPAC Black XL, differing only in their particle size. The smaller particle size product, INVE EPAC Black PL, was only used during the first 5 days, from PL12 – PL17, mixed in a 1:1 ratio with the larger product XL. Both products had the same inclusion levels of marine protein, plant protein, pigments, marine oils, yeast extract, minerals, and antioxidants. The composition of both feeds was also identical, with 45% protein,

7% lipid, 3% fiber, and 10% moisture content (INVE Technologies, 2018). The artificial *Artemia* (AA) supplement product contained an extract of *Artemia* cysts and other ingredients, such as fish gelatin, squid meal, hydrogenated vegetable fat, soy lecithin, and microalgae. The diet composition was 51% protein, 13.6% lipid, 8% ash, 3.5% fiber and 8% moisture. This product also came in two particle sizes, called Standard and Large, where the smaller particle size (Standard) was delivered during the first 5 days in the same way as indicated for DF. All specifications regarding ingredients were taken directly from publications provided by the producers. The required quantities of DF and AA feed (Table 1) needed for each day were weighed in small 50 mL plastic cups. When all cups were prepared, they were administered simultaneously to the different treatment groups at the designated time.

2.4. Water Quality Analysis

The temperature and the DO of each EU were measured daily with a YSI 55 device (YSI Incorporated, Yellow Springs, OH, USA) and recorded in the morning at 8:00 and in the evening at 18:00. Twice per week 200 mL water samples of each EU were taken in the morning at 8h00 and analyzed for alkalinity (APHA 2005-2320 B), total ammonia nitrogen (TAN) a nitrite (APHA, 2005), salinity (YSI 30, YSI Incorporated, Yellow Springs, OH, USA), and pH (YSI 100, YSI Incorporated, Yellow Springs, OH, USA). Total suspended solids (TSS) and volatile suspended solids (VSS) (APHA 2005-2040 D and 2005-2540 E) were measured using 0.6 µm glass fiber micro-filters (GF-6, Macherey-Nagel, Düren, Germany). TAN and nitrite analysis were carried out following a protocol from Strickland and Parsons (1972) and the guidelines of APHA (2005). All water analysis procedures were carried out by professional technical staff in a specialized laboratory of the LCM.

2.5. Salinity Stress Test

One day before the end of the experiment, a salinity stress test was performed to determine the PL's ability to survive an osmotic shock. Thirty PL from each EU were randomly selected with a 500 µm sieve and placed in a 500 mL plastic cup containing freshwater at the same temperature as the EU (~29 °C). To minimize handling stress, the whole sieve containing the PL was placed inside the freshwater cup. After 45 minutes, the sieve was removed and placed in a cup of seawater at 33 ppt and ~29 °C. Another 45 minutes later, all surviving and dead PL were counted. A PL was determined to be dead when it did not show any movement after a stimulus with a plastic probe.

2.6. Final Sampling

On the final day of the experiment (Day 20, PL31), the larvae from each tank were collected by siphoning the water through a 500 µm sieve. Each individual sieve and the PL within were carefully blotted with a piece of paper to remove excess water and were then weighed together. Thereafter, the sieves, freed of the PL, were dried in an oven at 60 °C for 2 hours and then weighed again. The individual weight of each sieve was subtracted from the total (sieve and PL) to obtain the total wet weight of all PL per tank. A smaller quantity of PL from each tank was weighed separately and subsequently counted in order to estimate the wet weight per larvae. The counted PL were stored and preserved in formaldehyde. All other remaining PL were stored in plastic bags and frozen at -20 ºC for further body composition analysis. At a later date, the formaldehyde-preserved PL were placed on a white paper and measured from rostrum to tail to obtain the mean total length of each treatment group. The relative growth rate (RGR) was calculated to analyze potential differences in growth between the groups with:

$$\text{RGR (\%/day)} = (e^{g}-1) \times 100, \qquad (1)$$

where $g = (\ln W_t - \ln W_o) \times t^{-1}$, W_t and W_o are the final and initial wet weights, respectively, and t is the duration of the trial in days (Aragao et al., 2007). After the finalization of the experiment, the frozen PL were transferred to a commercial laboratory where a body composition analysis for ash, lipid and protein contents was performed.

2.7. Statistical Analysis

All data was analyzed using the statistical software program RStudio (Version 1.1.453 – © 2009-2018 RStudio, Inc.). The Shapiro-Wilk test was used to test the obtained data for normal distribution (Royston, 1995) and the Levene's test determined the homogeneity of variance (Fox, 2016). When the data was found to be normally distributed and with homogeneous variance, a one-way analysis of variance (ANOVA) (Chambers & Hastie, 1992), followed by Tukey's honest significant differences (HSD) post-hoc test (Yandell, 1997) was performed. The water parameters measured over time were analyzed using a repeated measures ANOVA with the treatment group as the main factor and the sampling day as the additional factor. Any data set that was found to be not normally distributed and/or without homogenous variance, was analyzed using the non-parametric Kruskal-Wallis Rank Sum test (Hollander et al., 2015), followed by Dunn's test (Dunn, 1964). All significant differences were determined at a confidence level of $\alpha = 0.05$.

3. Results

3.1. Water Quality Parameters

3.1.1. Temperature and Salinity

Temperature and salinity did not show any significant differences among treatments from day 1 until day 20, the end of the experiment (Figure 4 andFigure 5). Utilizing electric submergible heaters, the mean temperature was kept between 28 °C and 29.5 °C at all times. The maximum recorded temperature was 29.2 ± 0.3 °C for the [N] group at day 16, while the lowest temperature of 28.3 ± 0.3 °C was recorded in the [AA] group at day 1. There were some minor fluctuations in salinity throughout the feeding trial, but the rearing water never reached more than 36 ppt in any tank. The highest salinity of 35 ± 0.4 ppt was measured on day 5 in the [C] group and the lowest salinity of 33.1 ± 0.2 ppt occurred at the start of the experiment and was equal in all treatments. Whenever an increase in salinity was observed in a specific tank, the adequate amount of disinfected freshwater was added to counter a rising salinity.

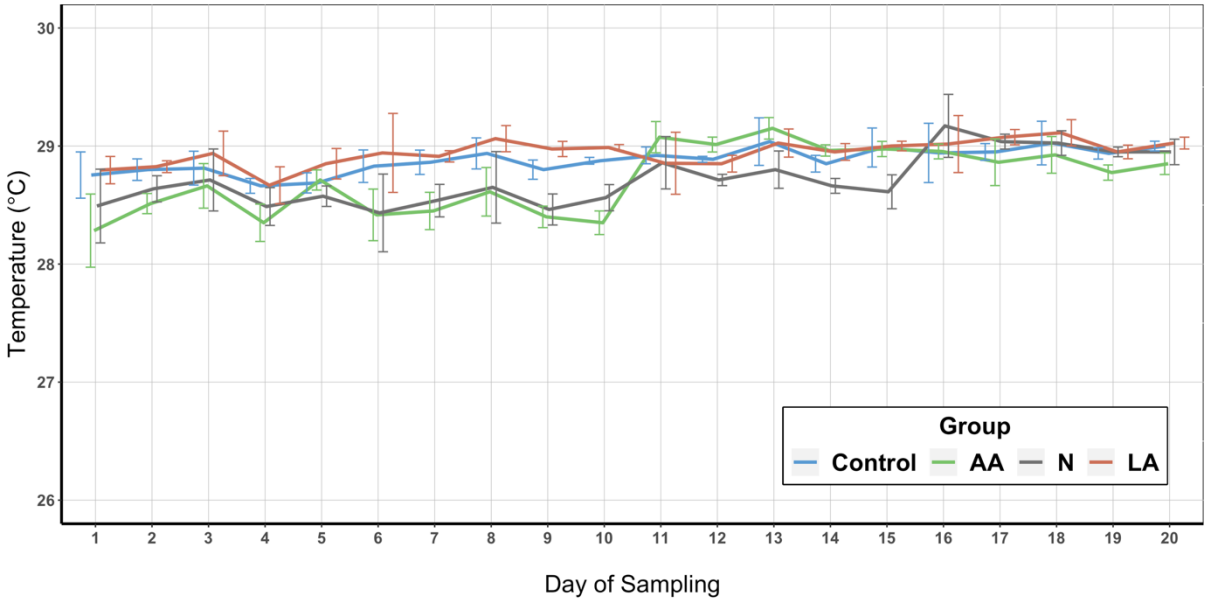

Figure 4: Temperature in each treatment along the experiment. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences on the given day.

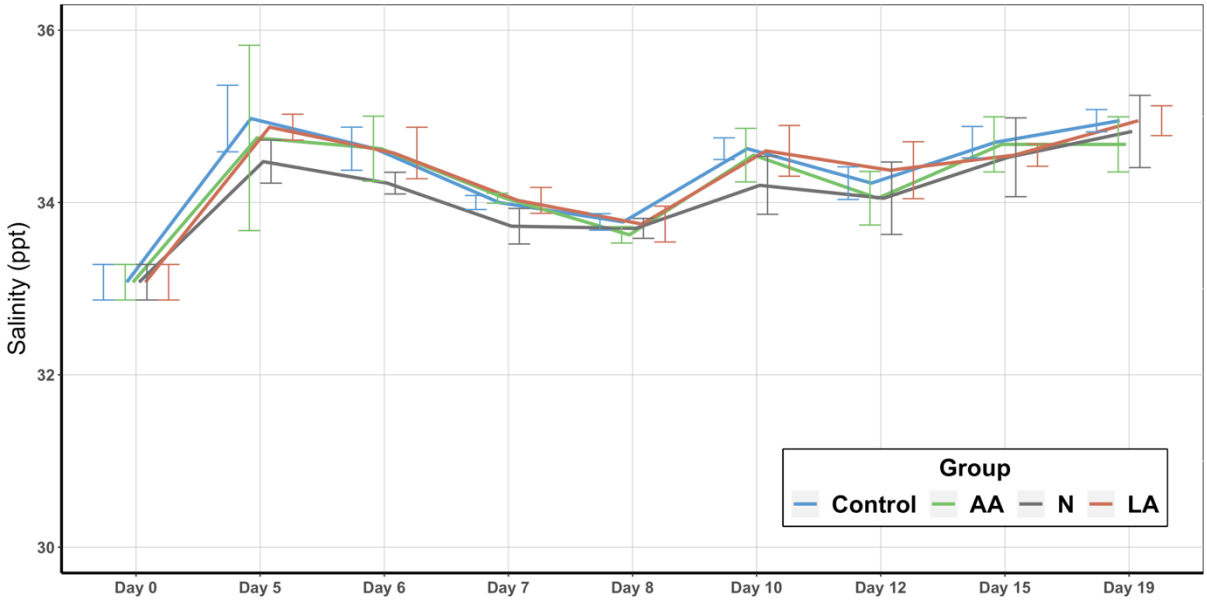

Figure 5: Salinity of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences on the given day.

3.1.2. Dissolved Oxygen

For the most part of the experiment, the DO levels were similar among the four treatments. But during the final stages, after day 15, some statistically significant differences were observed. The initial DO levels in all treatments was 7.14 ± 0.02mg/L and DO levels slowly dropped until they reached their lowest point of 6.8 ± 0.22 mg/L on day 15 in the [C] group, after which they slowly started to rise again. On day 18, the DO level of the [LA] group was significantly lower (6.78 ± 0.09 mg/L) than that of the [AA] group (6.99 ± 0.07 mg/L) and the [N] group (6.96 ± 0.05 mg/L). There was no significant difference to the [C] group (6.91 ± 0.07 mg/L). On day 19, the following day, no statistically significant differences were observed among groups, while on the final day, day 20, again the [LA] group (7.02 ± 0.06 mg/L) had significantly lower DO than the [AA] group (7.18 ± 0.09 mg/L). But neither group, [LA] nor [AA], had a significantly different DO level compared to the [N] group (7.13 ± 0.07 mg/L) or the [C] group (7.06 ± 0.04 mg/L), as shown in Figure 6.

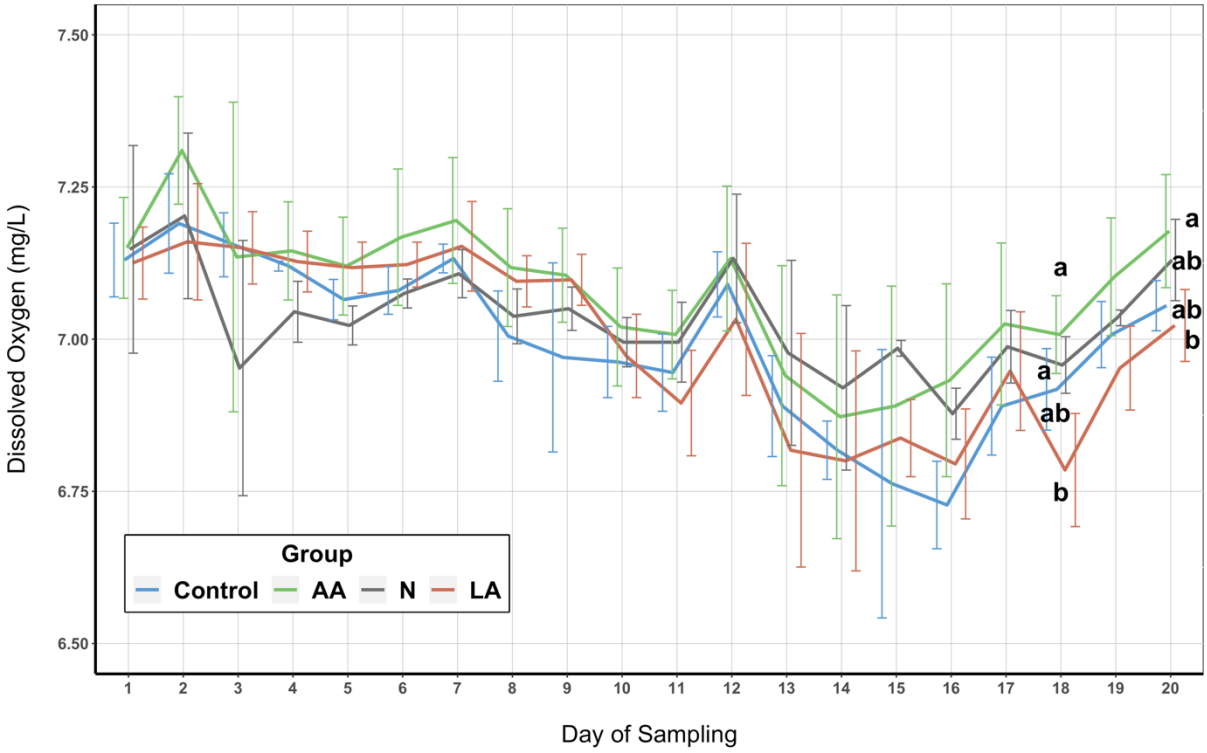

Figure 6: Dissolved oxygen in each treatment along the experiment. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences on the given day. Different letters represent statistically significant differences on the given day ($p < 0.05$).

3.1.3. Alkalinity

The water alkalinity analysis at the start of the experiment returned a starting value of 145.0 ± 0.0 mg/L in all treatments. At the first two sampling points, on day 5 and day 8, the alkalinity dropped to 120.0 ± 4.0 mg/L and then to 100.0 ± 3.6 mg/L, respectively, in all treatments. In order to increase the alkalinity in the water, calcium hydroxide was added to each tank at a rate of 20% of total feed given per day. The daily ratio was given in two servings at 11h00 and 15h00 starting on the afternoon of day 5. Since the alkalinity still decreased until day 8, the calcium hydroxide dosage was doubled to 40% of the total feed given per day. An overview of the exact quantities of calcium hydroxide added each day can be found in Table 2. At day 12, the alkalinity started rising slowly and was now again above 100.0 ± 5.5 mg/L in all treatments. Until this point in the experiment, there were no statistically significant differences among the groups. On the next sampling point on day 15, the alkalinity had risen to 130.0 ± 4.2 mg/L in all treatments except for

the [N] group, which showed a significantly higher value of 149.0 ± 6.0 mg/L. The alkalinity on the final day of the experiment, day 19, had decreased slightly in all groups to 123.7 ± 2.5 mg/L, whereas again, the [N] group had the highest and significantly different value of 142.0 ± 5.2 mg/L (Figure 7).

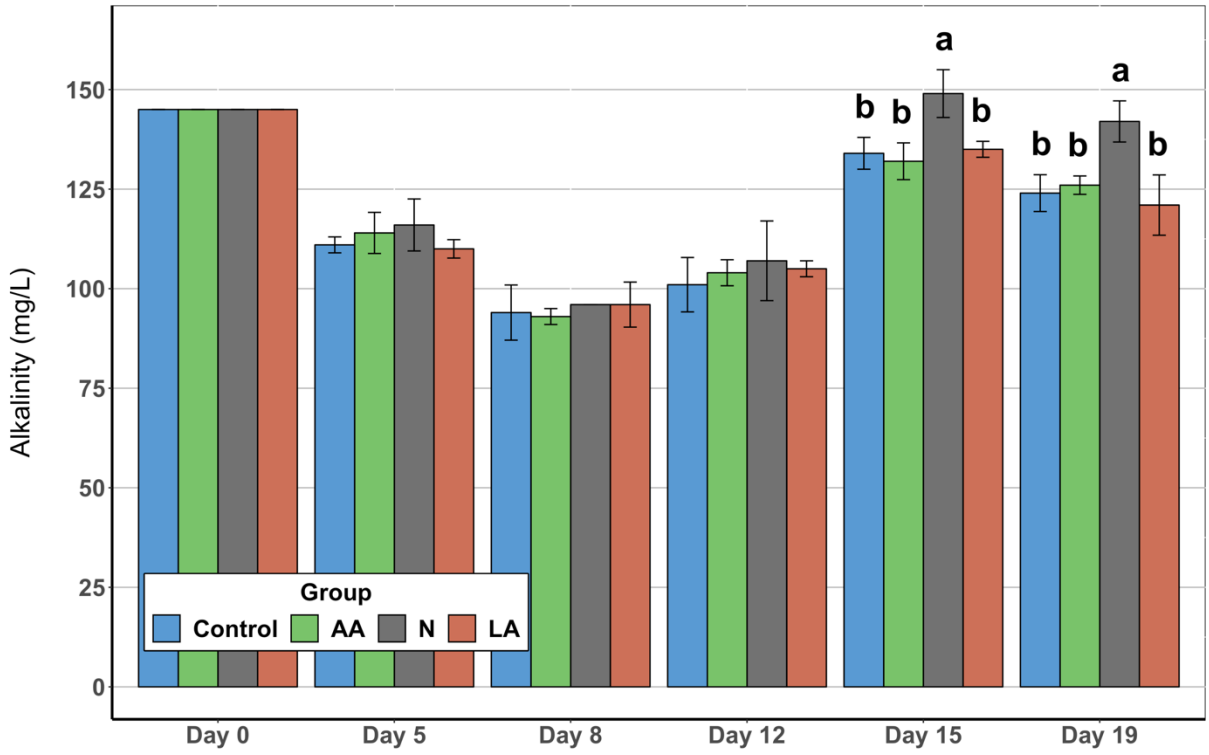

Figure 7: Alkalinity of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.4. pH

The pH of the biofloc water started out slightly basic in all treatments (8.21 ± 0.04) and decreased slowly until day 8 to 7.74 ± 0.07. Towards the end of the experiment, the pH in the [N] group was significantly higher on day 15 (8.00 ± 0.02) and day 19 (7.98 ± 0.03) than in the other three groups, where the pH was below 7.92 ± 0.03 and 7.91 ± 0.02, respectively (Figure 8). To determine a potential correlation between alkalinity and pH, a Spearman rank test was performed using the complete data sets of these two parameters. A positive correlation with a correlation coefficient of $r = 0.56$ was determined ($p < 0.001$; Figure 9).

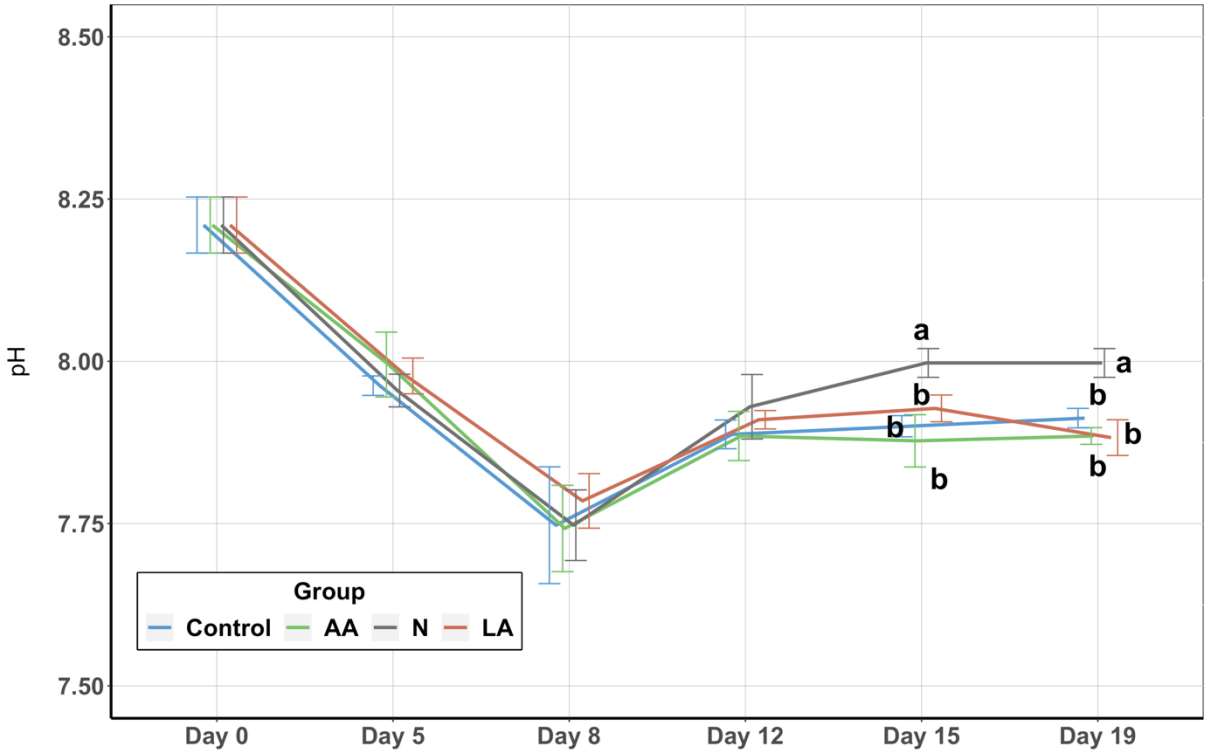

Figure 8: The pH level of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day on the given day ($p < 0.05$).

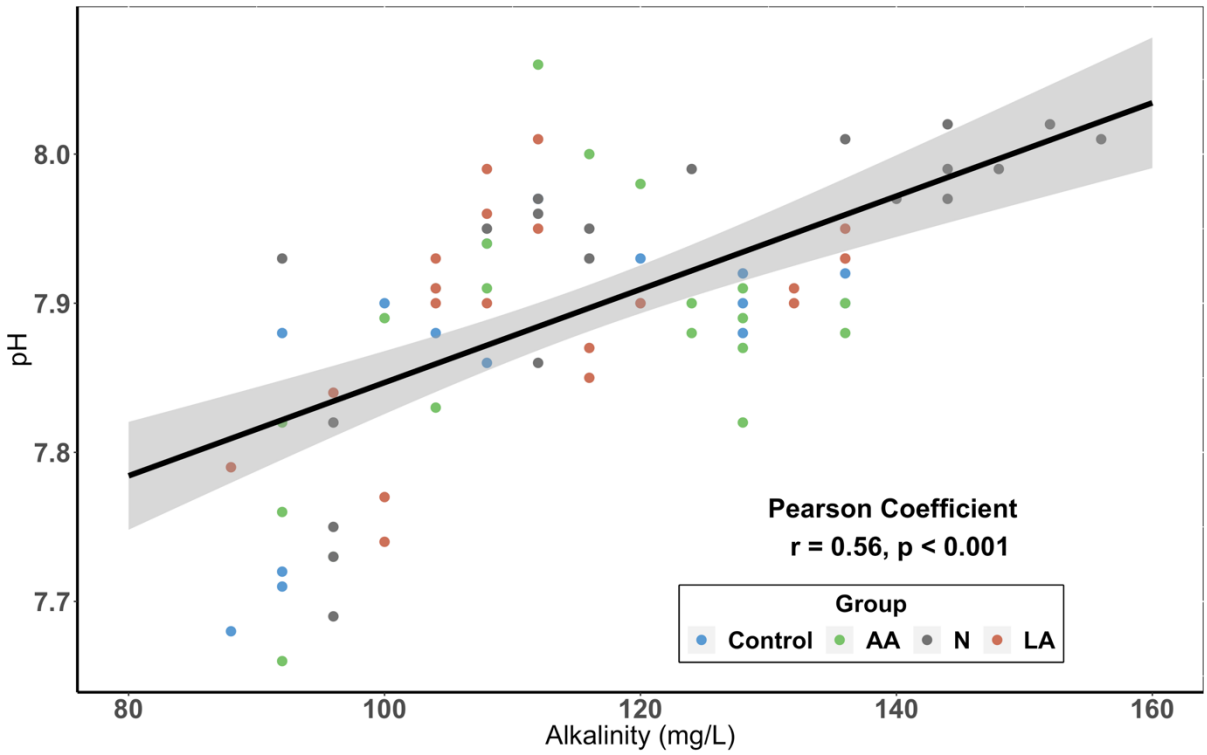

Figure 9: Pearson correlation of alkalinity and pH with data from all groups on all sampling days.

3.1.5. Total Suspended Solids

The water analysis at the start of the experiment accounted for 303.0 ± 36.5 mg/L of total suspended solids (TSS) in the initial biofloc water. During the following sampling days, several statistically significant differences were observed (Figure 10). Most strikingly, on the final two sampling days, day 15 and day 19, a significant difference between the [AA] and [C] group and the [N] and [LA] group were noticed. On day 15, the [AA] group (437.8 ± 34.6 mg/L) and the [C] group (431.5 ± 23.9 mg/L) had significantly higher TSS values than the [N] group (264.5 ± 38.6 mg/L) and the [LA] group (260.0 ± 62.8 mg/L). Similarly, although with a smaller difference in magnitude, the same pattern was observed on the measurements on day 19, where the [AA] (423.0 ± 12.3 mg/L) and [C] (416.3 ± 16.7 mg/L) groups had significantly higher values of TSS than the [N] (344.0 ± 17.8 mg/L) and [LA] (340.0 ± 40.5 mg/L) groups.

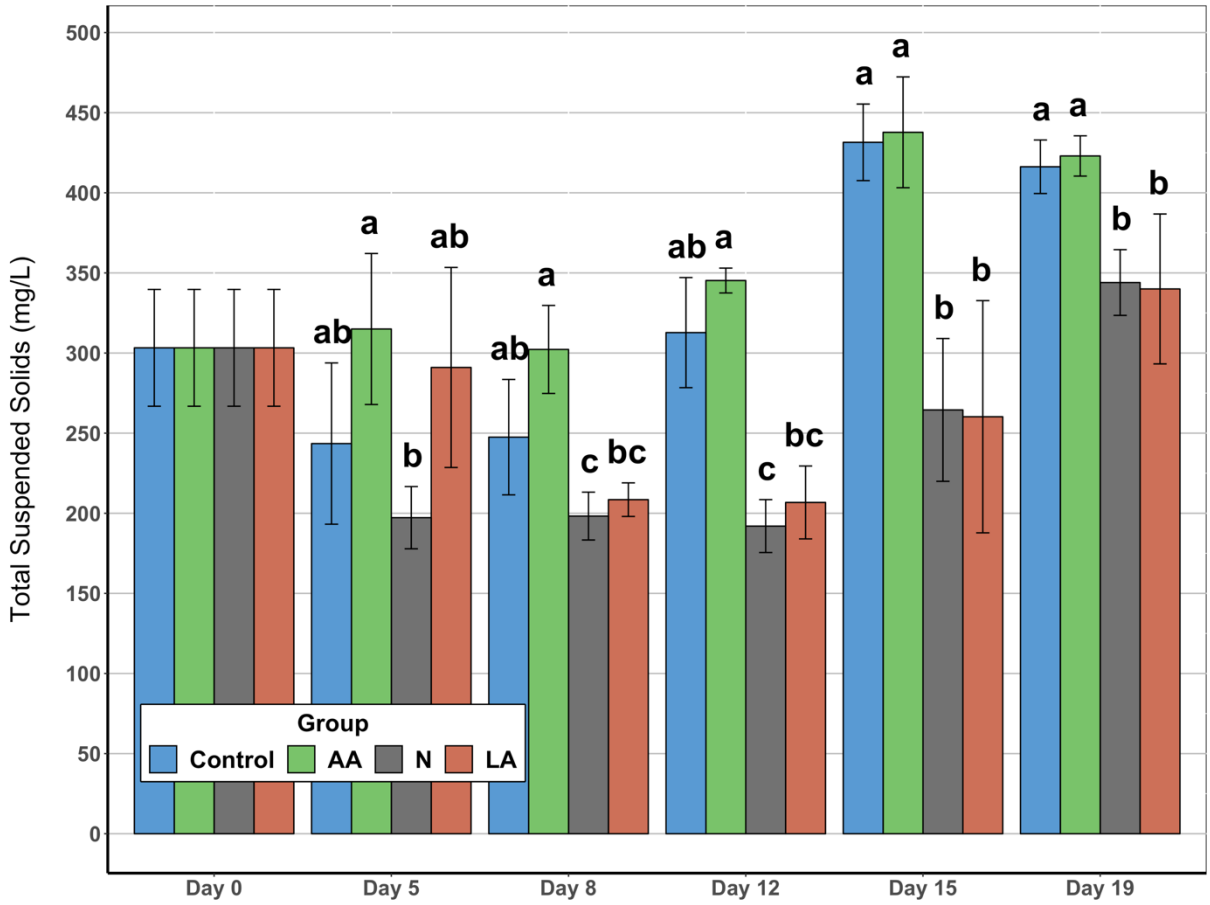

Figure 10: Total suspended solids of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.6. Volatile Suspended Solids

The volatile suspended solids (VSS) analysis showed a very similar pattern compared to the TSS. All treatments had the same initial value of 108.8 ± 16.0 mg/L VSS at the start of the experiment. During the course of the feeding trial, several statistically significant differences were determined among the groups, and again, during the final stage of the experiment, on day 15, a clearly significant difference between the control and the [AA] groups and the two life feed groups, [N] and [LA], was observed (Figure 11). On the last sampling, day 15, the [AA] (171.8 ± 26.6 mg/L) and the control (165.8 ± 10.7 mg/L) groups had significantly higher VSS values than the [N] (97.3 ± 8.5 mg/L) and the [LA] (89.0 ± 13.2 mg/L) groups. Looking at both parameters of suspended solids, TSS and VSS, during the latter phase of the experiment, and especially on the final day of sampling (day 19 and day 15, respectively), the inert diet groups (control and [AA]) always had statistically significant higher values than the groups fed with live diets ([N] and [LA]).

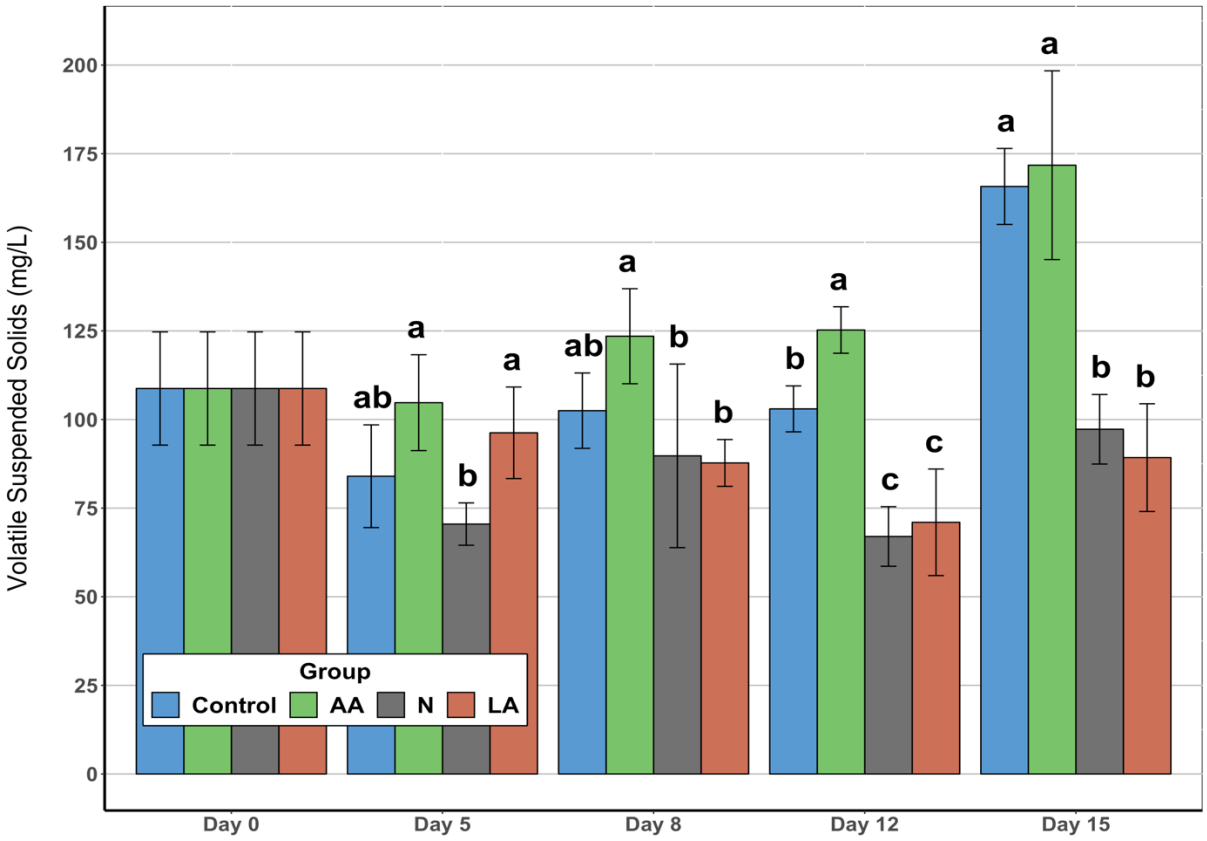

Figure 11: Volatile suspended solids of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.7. Total Ammonia Nitrogen

The total ammonia nitrogen (TAN) concentrations at the beginning of the experiment started relatively low with 0.25 ± 0.03 mg/L in all treatments but began fluctuating during the course of the trial (Figure 12). On sampling day 5, the [AA] group (0.35 ± 0.07 mg/L) had a slightly significant higher TAN concentration than the [N] (0.25 ± 0.02 mg/L) and [LA] (0.23 ± 0.01 mg/L) groups. No statistically significant differences were found between either group and the control group (0.27 ± 0.02 mg/L) on that day. On all other sampling days, no significant differences among the treatments could be determined. Overall, a decrease in the mean TAN levels towards the end of the experiment was observed. The highest measured peak of TAN during the feeding trial occurred on day 8 in the [LA] group with 0.44 ± 0.25 mg/L.

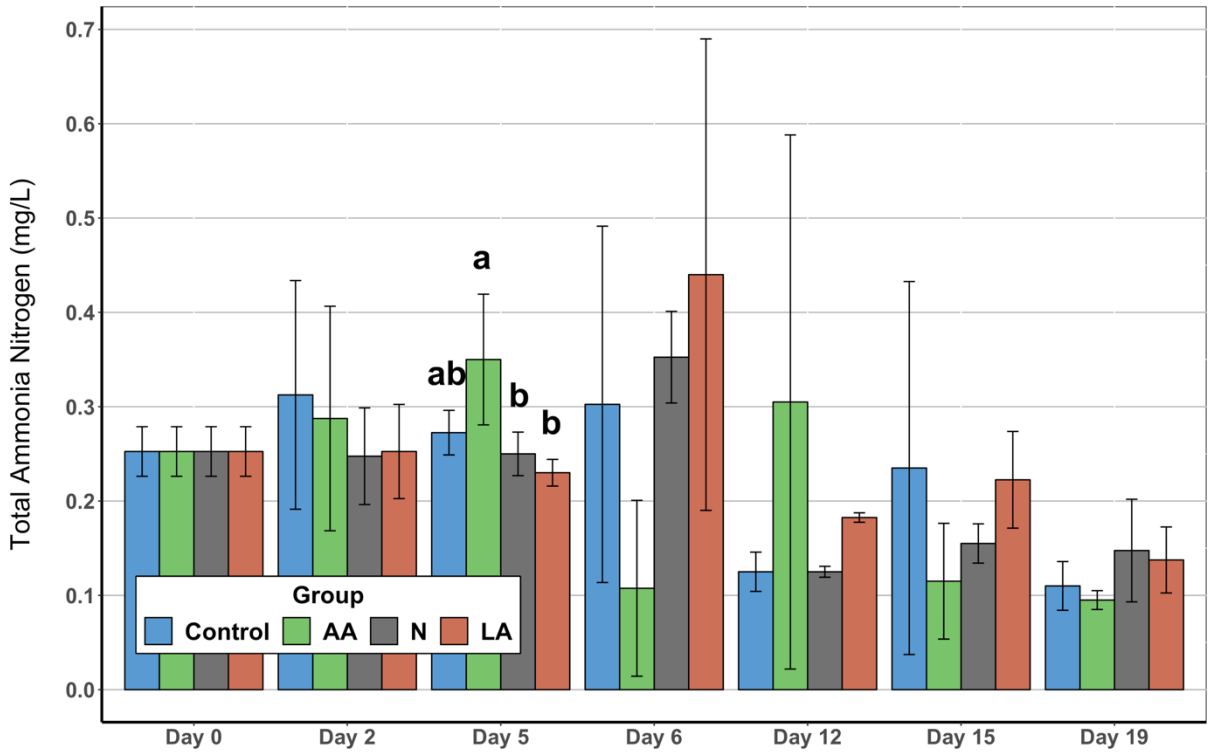

Figure 12: Total ammonia nitrogen values of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.1.8. Nitrite

From the start of the experiment, until past day 8, almost no nitrite was detected in the biofloc system of all treatments (< 1.4 ± 0.2 mg/L). Starting from day 12, nitrite levels started to rise relatively quick, until they reached critical levels on the final sampling, day 19. On day 12, 15, and 19, statistically significant differences among the groups were observed, whereas, on all of these days, the [N] group had significantly lower nitrite than all other groups (Figure 13). Nitrite peaked on the last day, with the highest value in the [LA] group (30.0 ± 1.7 mg/L) followed by [AA] (29.2 ± 1.5 mg/L) and the control group (26.1 ± 2.5 mg/L). Compared to the [LA] and [AA] group, the [N] group had a significantly lower nitrite level (23.6 ± 1.6 mg/L) on that day, while the [C] group was not significantly different to any other group.

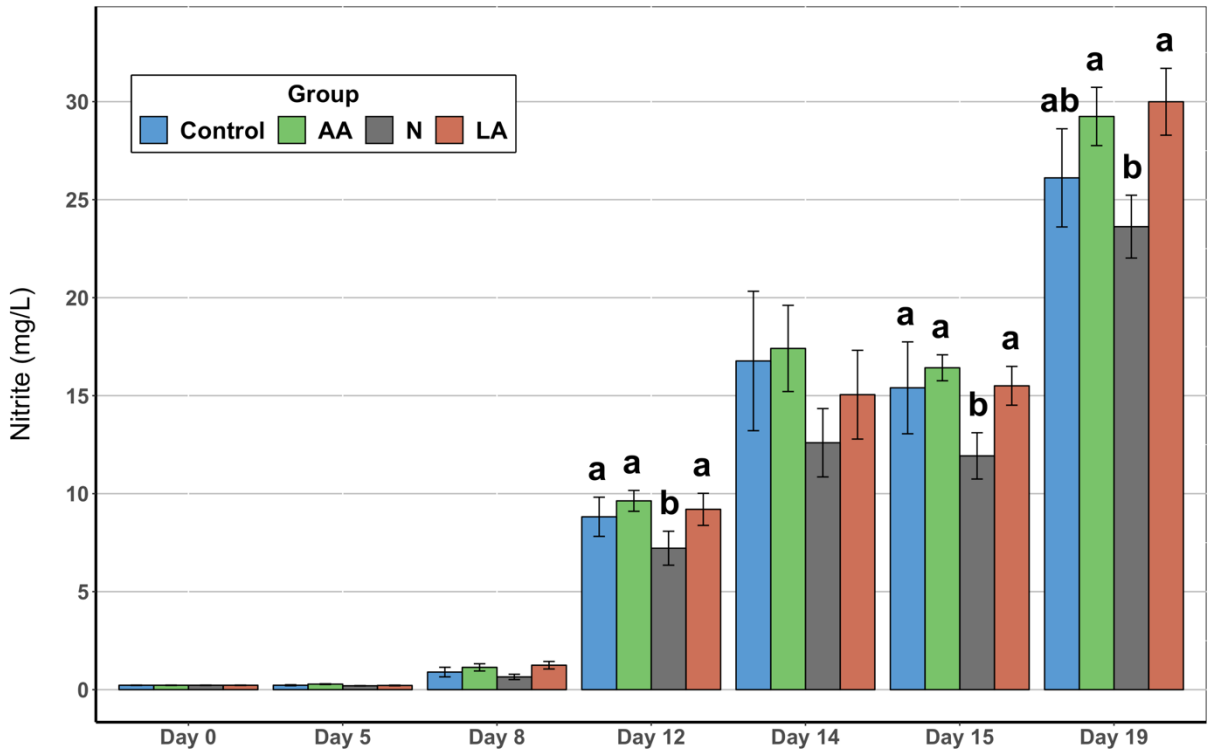

Figure 13: Nitrite values of the four treatments on each sampling day. Values are means ± SD (n=4). The absence of letters indicates no statistically significant differences. Different letters represent statistically significant differences among the treatments on the given day ($p < 0.05$).

3.2. Growth Performance

3.2.1. Survival

The results of the final survival showed that PL fed with live diet supplements, the groups [N] and [LA], had a statistically significant higher survival at the end of the experiment compared to the two groups fed only with an inert diet, [C] and [AA]. The [N] group had a final survival of 94 ± 6% and the [LA] group of 91 ± 7%, whereas the [C] and [AA] group only had a final survival of 51 ± 10% and 53 ± 11%, respectively (Figure 14).

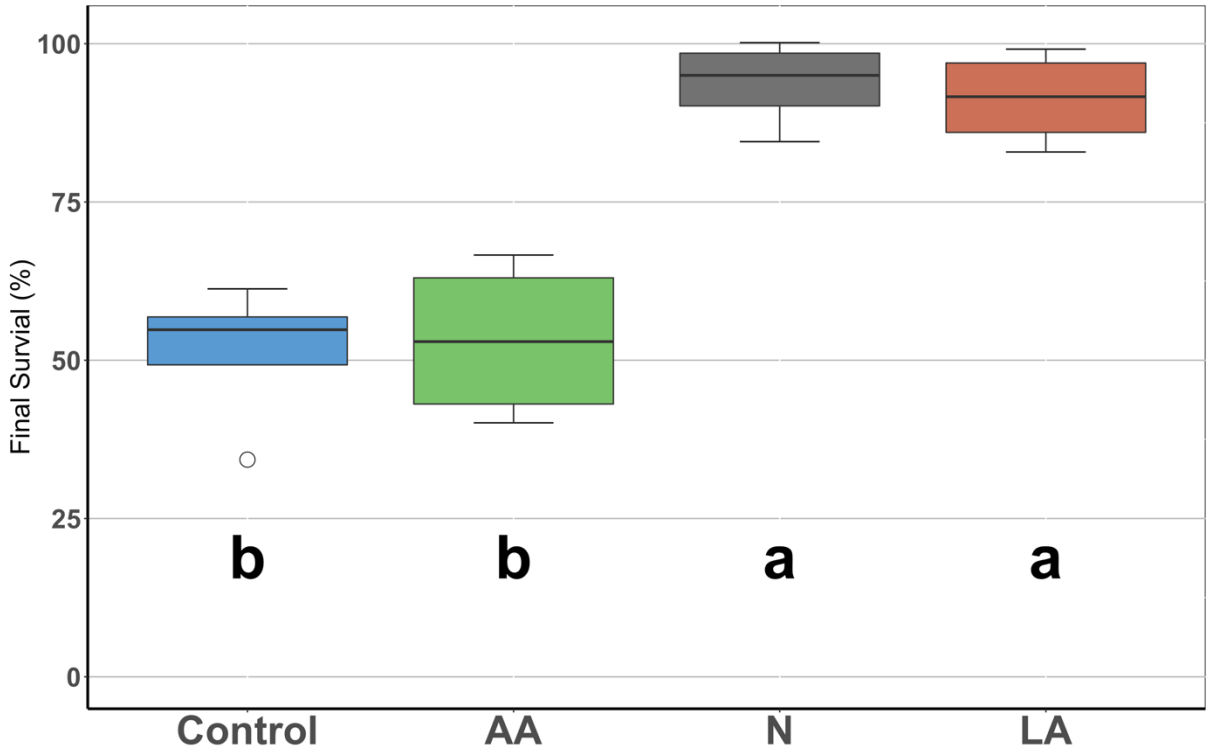

Figure 14: Final survival of *P.vannamei* post larvae from each treatment at the end of the experiment (n=4). Box = 25th and 75th percentile, Bars = min and max values, thick line = median value. Different letters indicate statistically significant differences, which were determined by a one-way ANOVA ($p < 0.0001$), followed by Tukey's HSD ($p < 0.05$).

3.2.2. Growth

The mean wet weight per larvae, determined right after the completion of the experiment, returned a statistically significant difference between the two inert diet groups and the two live diet groups (Table 3). The mean wet weight of the larvae from the [C] (35 ± 10 mg) and [AA] (35 ± 6 mg) groups was slightly, but significantly higher than the weight of the larvae from the [N] (23 ± 5 mg) and LA (23 ± 5 mg) groups. Similar to the wet weight results, the mean individual length results returned significant differences between the inert diet and the live diet groups (Table 3). The [C] (17.3 ± 5.0 mm) and [AA] (17.1 ± 5.2 mm) groups had significantly longer PL mean length than the [N] (15.8 ± 3.9 mm) and [LA] (15.8 ± 3.2 mm) groups.

Table 3: Growth of P. vannamei larvae from different treatments at the end of the experiment. RGR: relative growth rate.

Groups	Wet Weight (mg)	Total Length (mm)	RGR (% day $^{-1}$)
Control [C]	35 ± 10 ^{a}	17.3 ± 5.0 ^{a}	15.2 ± 1.5 ^{a}
Artificial *Artemia* [AA]	35 ± 5.7 ^{a}	17.1 ± 5.1 ^{a}	15.3 ± 1.0 ^{a}
Nematodes [N]	22.5 ± 5 ^{b}	15.8 ± 3.9 ^{b}	12.8 ± 1.2 ^{b}
Live *Artemia* [LA]	22.5 ± 5 ^{b}	15.8 ± 3.2 ^{b}	12.8 ± 1.2 ^{b}

* Values are means ± SD (n=4 for all except total length: C: n = 175; AA: n = 189; N: n = 222; LA: n = 246). Different superscript letters within the column indicate significant differences among groups ($p < 0.05$). Initial wet weight was 2 ± 0 mg.

The calculation and analysis of the relative growth rate returned statistically significant differences between the inert diet and the live diet groups. [C] and [AA] treatments had an RGR of 15.2 ± 1.5 and 15.3 ± 1.0%/day, respectively, whereas both treatments [N] and [LA] only had an RGR of 12.8 ± 1.2%/day (Table 3).

3.2.3. Body Composition

The analysis of the body composition for lipids, ash, and protein content was performed by a commercial laboratory (LABCAL) and the results are presented in Figure 15. Unfortunately, there was not enough sample tissue available to analyze replicates per group, thus, no standard deviation nor statistical significance could be calculated. PL from all treatments had around 10-12% protein, 4-5% ash, and 1% lipid content.

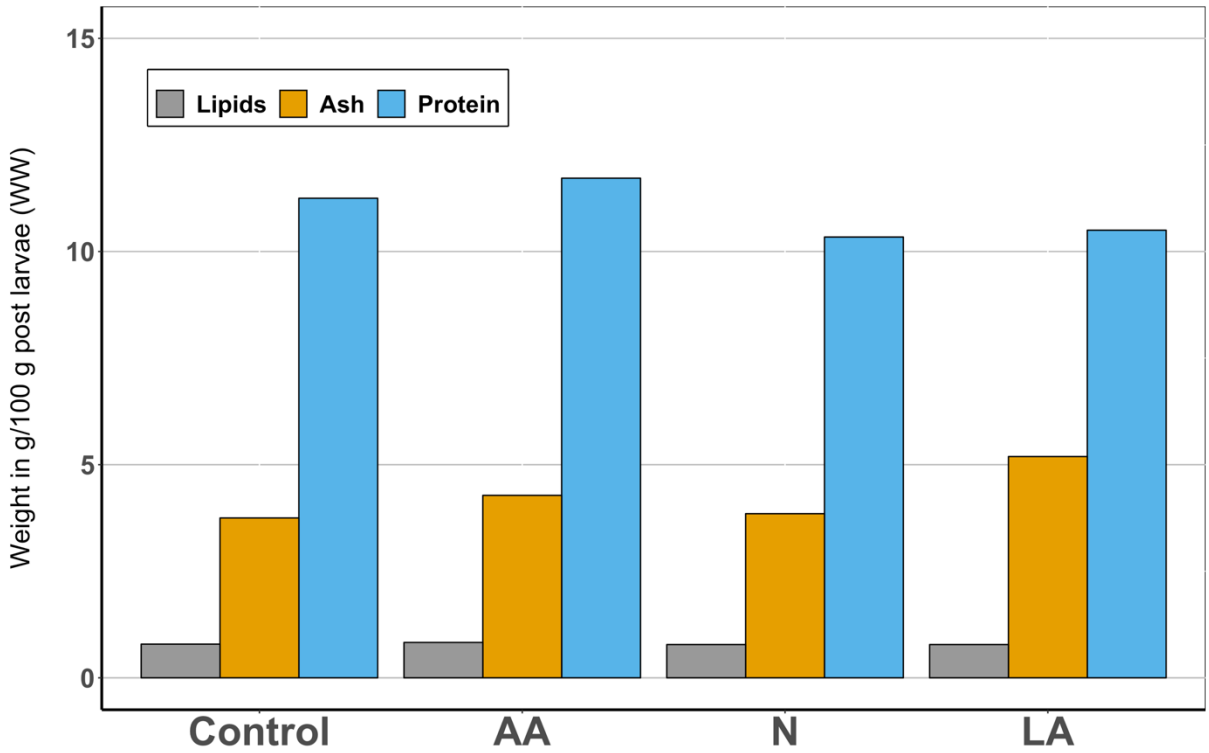

Figure 15: Post larvae body composition at the end of the experiment. Due to the lack of replicates, no statistical analysis could be performed.

3.2.4. Salinity Stress Test

The salinity stress test did not return any significant differences among the treatments. All groups had a high survival between 88 ± 6% for the [N] group and 94 ± 6% for the [LA] group (Figure 16).

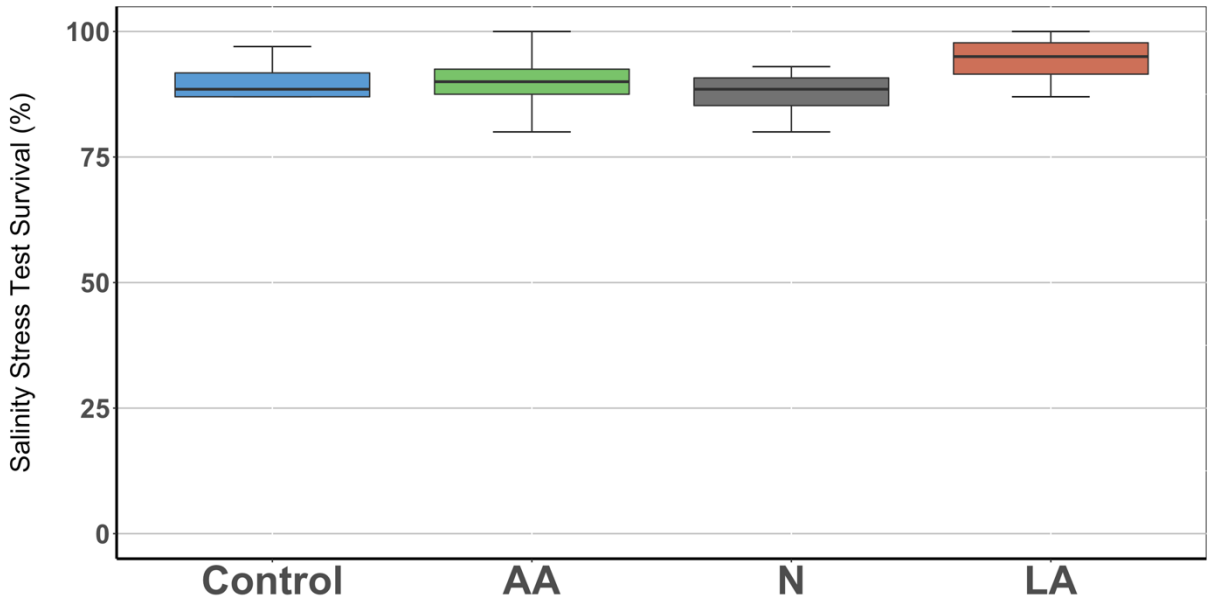

Figure 16: Survival of *P.vannamei* post larvae from each treatment (n=30) after the salinity stress test. Box = 25th and 75th percentile, Bars = min and max values, thick line = median value. The absence of letters indicates no statistically significant differences among the treatments.

4. Discussion

4.1. Water Quality Parameters

The temperature and salinity levels were kept relatively constant around 29 °C and 33-35 ppt for the entire experiment and no significant differences were observed among the groups. The species *P. vannamei* naturally occurs in oceanic habitats with all year temperatures above 25 °C (Dugassa & Gaetan, 2018), hence, the temperature and salinity of this experiment can be considered optimal. During all times DO concentrations were maintained well above the limits considered adequate for aquaculture (> 5 mg/L) (Boyd & Gautier, 2000). Thus, although statistically significant differences were observed among the groups at days 18 and 20, no effect of DO on the larval growth is expected.

The alkalinity decreased in all treatments despite the addition of calcium hydroxide before it slowly increased again towards the end of the experiment. The pH level in the water followed a very similar course to the alkalinity and this parallel behaviour was expected and confirmed with a positive correlation by the Spearman rank test. The alkalinity of water is very closely related to pH, and generally, pH tends to increase as alkalinity increases and vice versa (Boyd, 2000). Both parameters can have an impact on the bacterial composition of the biofloc and subsequently on the nitrogen cycle in the water. The nitrification of ammonia to nitrate is a two-step conversion performed by different genera of chemoautotrophic bacteria. In a first step ammonia oxidizing bacteria (AOB) of the genus *Nitrosomonas* metabolize ammonia to nitrite and in a second reaction, nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB) of the genus *Nitrobacter* (Chen et al., 2006). The optimum pH for the growth of nitrifying bacteria varies widely depending on the species, but the ideal pH for the nitrification process is between 7.2 to 9.0 for *Nitrosomonas* and 7.2 to 8.8 for *Nitrobacter* (Chen et al., 2006; Villaverde et al., 1997). At day 8 of the experiment, the mean pH in all treatments dropped to 7.8 ± 0.1, which was closer to the lower pH limit proposed by Villaverde et al. (1997). The authors also argue that AOB have faster growth than NOB and the latter also show a higher sensitivity to pH. It is therefore likely that selective

inhibition of NOB occurred, which subsequently resulted in the observed accumulation of nitrite starting after day 8 of the experiment. Both steps of the nitrification process also consume considerable amounts of alkalinity, as carbonate and bicarbonate are essential nutrients for nitrifying bacteria (Chen et al., 2006). A reduction in alkalinity leads to a decreased buffering capability of the rearing water resulting in acidification and a reduced pH. This negative feedback loop could have inhibited the nitrification process further and should have been avoided by timely addition of calcium hydroxide or other carbonate sources.

The authors Ebeling et al. (2006) and Hargreaves (2006) suggested that different pathways of nitrogen removal are to some degree present at the same time in biofloc systems. The three general ways to recycle nitrogen are photoautotrophic uptake by algae, autotrophic bacterial conversion from ammonia to nitrite and nitrate, and heterotrophic bacterial assimilation of nitrogen into microbial biomass. Vinatea et al. (2010) argue that the latter two bacterial processes play the dominant role in high density and indoor nursery systems, as photosynthesis by phytoplankton is minimal due to high turbidity and limited natural light. According to Avnimelech (2006), heterotrophic assimilation and autotrophic nitrification can take place at the same time on the surface of a mature biofloc. In order to support both bacterial processes of nitrogen reduction, the alkalinity levels should be kept between 100-150 mg/L at all times (Ebeling et al., 2006), which was only achieved after day 12 of the experiment. Chen et al. (2006) recommend even higher alkalinity concentrations of above 200 mg/L in zero water exchange systems, since a stratification of alkalinity and pH in the biofilm on the substrate surface may occur. Thus, the temporary drop in alkalinity and pH may have hampered the growth of the NOB and manipulated the microbial community in the biofloc towards an unfavourable composition regarding nitrite control. This assumption is in accordance with findings by Furtado et al. (2015) who studied the effects of different alkalinity levels for the rearing of *P. vannamei* in BFT and concluded that low alkalinity negatively effects NOB. In this experiment the nitrite levels started to rise in all groups beginning on day 12, suggesting an overall reduced alkalinity supply. In contrast, the TAN levels were continuously decreasing in all treatments toward the end of the experiment. This observation further indicates the presence of a chemoautotrophic

bacterial nitrogen conversion from TAN to nitrite, without the subsequent step from nitrite to nitrate. In a pure heterotrophic system, there should have been no nitrite build up at all, since the ammonia nitrogen would have been assimilated directly into bacterial biomass (Ebeling et al., 2006).

On the final two sampling days (15 and 19 days of experiment) a clear statistically significant difference was observed, where the [N] group had higher alkalinity and pH then all other groups. Consequently, the [N] group had also significantly lower nitrite levels on these days, suggesting a different level of bacterial nitrogen control present in the nematode treatment. After day 12, the nitrite continued to increase in all groups until it reached dangerously high levels between 24-30 mg/L at day 19. In order to not further deteriorate the water quality by increasing the organic matter, the daily feed input in all tanks was decreased by 50% from day 16. But nevertheless, a continuous increase in nitrite was observed. Lin & Chen (2003) estimated that a concentration of 25.7 mg/L of nitrite nitrogen is the upper safe level limit for rearing *P. vannamei* PL of approximately 4 g at 35 ppt salinity. In the final days of the experiment, this limit was exceeded, so the nitrite accumulation may have had a negative effect on the shrimp's survival and growth. Especially, since the PL in this experiment were much smaller at the final sampling with an average of 28.8 ± 8.6 mg and previous studies suggested that penaeid shrimp tolerance to nitrite increases with age (Lin & Chen, 2003; Ramírez-Rochín et al., 2017). According to a study by Vinatea et al. (2010), nitrite concentrations close to 9 mg/L may already cause a significant reduction in the growth rate of shrimp. But since this level was exceeded only after day 14 in all treatments, differences in survival and growth of PL among the groups due to elevated nitrite concentrations may not be evident at the final sampling. The significant lower nitrite levels of the [N] group did not result in better growth compared to the other groups, as the highest RGR was observed in the [C] and [AA] groups. Since the highest values of nitrite and the excess of the safe level only occurred on the last days of the experiment, mortalities that would occur after long-term exposure should not have affected the final survival of the PL.

TAN in none of the treatments ever exceeded 0.5 mg/L and was constantly well below the safe levels for *P. vannamei* in biofloc systems, which are considered to be around 4.0

mg/L of TAN at 35 ppt salinity (Boyd & Gautier, 2000; Lin & Chen, 2001). Nitrate, the final product of the nitrification process, was not monitored during the experiment because the autotrophic conversion of nitrogen was not expected to have an important role. But since nitrate is the least toxic form of nitrogen (Romano & Zeng, 2013), it is assumed that a potential accumulation did not have adverse effects on the PL performance.

Microbial communities in biofloc are very complex systems, but they can be shifted towards predominantly heterotrophic bacterial growth by increasing the carbon:nitrogen ratio. It is possible that this shift did not occur at a sufficiently fast rate and more rigorous addition of dextrose earlier in the course of this experiment could have promoted a stronger growth of heterotrophic bacteria (Ebeling et al., 2006). The specific growth of the microbial community has implications on the formation of the biofloc, its ability to control nitrogen build-up in the system, and the accumulation of suspended solids. According to Schveitzer et al. (2013) and Samocha et al. (2007), TSS levels between 300-600 mg/L are optimal for rearing *P. vannamei* larvae. Within this range, the biofloc nitrogen control is most effective while the respiration of the shrimp is not handicapped. TSS levels in all groups fluctuated between 200-450 mg/L and were within the desired limits. Hence, a general adverse impact on the shrimp's growth performance through TSS is not expected. However, a clear distinction between the groups fed with inert diets, [C] and [AA], and live diets, [N] and [LA], started to develop over the course of the experiment. On the last two sampling days, day 15 and day 19, the live diet groups had statistically significant less TSS than the inert diet groups, indicating a possible effect of the live diet on the biofloc formation itself. At the last sampling at day 15, the groups that received a supplement of live *Artemia* or live nematodes also had statistically significant differences and approximately 50% lower VSS levels than the groups that received only DF.

Over the course of the experiment, the live diet groups received approximately 25% less DF than the inert diet groups. This difference in inert organic material could have caused divergent developments in the biofloc formulation, leading to greater TSS and VSS accumulations in the [C] and [AA] treatments. The final sampling revealed significant

lower survivals in the inert diet groups, but it remains unclear at what time during the experiment these mortalities occurred. Hence, the feed input was not adjusted to the reduced quantity of PL and it is, therefore, possible that overfeeding led to the increase in TSS and VSS. The exact cause of the mortalities is also unknown, but the most plausible hypothesis in this scenario is the nitrite increase, which reached levels above 10 mg/L at day 14. Although Lin & Chen (2003) did not report this nitrite level as lethal toxic to juvenile shrimp, the possibility that smaller shrimp may suffer mortalities cannot be excluded. But since a clear divergence in the TSS levels between inert and live diet groups occurred as early as day 12, two days before the nitrite peak, it may be assumed that the presence of live diets in the biofloc solution somehow reduced the accumulation of TSS and VSS in the [N] and [LA] groups as well.

Many free-living nematode species are bacterivores and predominantly feed on bacteria, while some species even occur naturally in biofloc environments (Emerenciano et al., 2013). If the *Panagrolaimus sp.* (strain NFS-24-5) survives extended periods of time in salt water, as other nematode species do (Brüggemann, 2012), they may have been ingesting bacteria from the biofloc and thereby reducing the extensive accumulation of VSS and TSS in the [N] group.

Artemia only start feeding once they reach the metanauplii stage, which happens after 6 – 8 h in temperatures above 25°C (Sorgeloos et al., 1998). Metanauplii are passive filter feeders that will ingest anything small enough, such as microalgae or bacteria (Fernández, 2001). Since the *Artemia* nauplii were stored for 6 h until the 18hoo feeding at ambient temperature (~25 °C), it is possible that they had developed into metanauplii by the second special feeding. Luo et al. (2017) and Yao et al. (2018) have recently shown that *Artemia* can feed very efficiently on the microorganisms in biofloc and thereby encapsulate valuable nutrients and probiotics. If nematodes and *Artemi*a metanauplii were able to survive predation by the PL, or if they were administered in excess so that a population of live diets remained in the rearing water, they may have consumed some part of the biofloc. This could explain why the TSS and VSS levels in the [LA] and [N] groups were statistically significant lower than the levels in the inert diet groups. Sorgeloos et al. (1998) also state that metanauplii are almost transparent, less visible and

fast swimming, therefore less acceptable as prey. Nevertheless, *P. vannamei* PL are very efficient hunters that feed frequently (Peixoto et al., 2017; Sanudin et al., 2014), and at least the nematodes have no means to actively avoid predation. Besides, the live diets in the [N] and [LA] groups are very different, coming from completely separated animal groups with varying feeding mechanisms, but the reduced TSS levels of both live diet groups were very similar. The assumption that nematodes and *Artemia* reduced the accumulation of suspended solids to the same degree by feeding on the biofloc remains questionable. But it could be further investigated with an experimental design, in which the tanks are periodically checked for survivors at certain times after feeding. Also, a gut analysis of nematodes and *Artemia* could be performed to estimate the rate of their biofloc ingestion and ability to reduce TSS and VSS.

According to the feeding regime, the [N] and [LA] group received about 25% less DF over the course of the experiment, which was supplemented by their specific live diet. If the provided feed was insufficient, it may be possible that the shrimp in these groups, in order to avoid starvation, were grazing more intensely on the biofloc and therefore partially removed it from the system. The final growth results showed that PL from the live diet groups were slightly but still significantly lighter and shorter than the PL from the inert diet groups, which would support the assumption of a suboptimal feeding regime compared to the inert diet groups.

Several authors have investigated the nutritional contribution of biofloc and it was reported that up to 29% of daily food uptake by *P. vannamei* could stem from biofloc material with concurrent improvements of growth rate (Burford et al., 2004; Emerenciano et al., 2013). But since all groups in this experiment were reared in biofloc and no quantitative analysis of the biofloc ingestion by the shrimp was done, this observation cannot be affirmed with this experiment. The interpretation of the water quality parameters rather points to the assumption that the supplement of live diets to the feeding regime led to a change of the biofloc composition, which in turn had an effect on the PL performance. Since both live diets had an impact on the TSS and VSS, but only the [N] group had significantly different results in nitrite, alkalinity, and pH, it can also be assumed that there is a disparity between nematodes and live *Artemia* and

their effect on the biofloc system with ensuing implications for the water quality. Further experiments investigating the impacts of live diets, especially nematodes, on biofloc and water quality should be designed in order to better understand the complexity of the processes. A first presumption from this experiment is that the nematode species *Panagrolaimus sp.* (strain NFS-24-5) may be beneficial for the water quality in a biofloc system.

4.2. Larval Performance

The analysis of the final survival of the PL revealed a clear distinction between inert and live diets with statistically significant differences. The [N] and [LA] groups had nearly twice the survival rate than the [C] and [AA] groups at the end of the experiment. As mentioned before, the exact cause of the mortalities and at what moment they occurred remains unclear. It is also plausible that the PL in the inert diet groups died gradually over the time of the experiment, rather than at one incident. No dead individuals were observed during the daily routines or at the final harvest of the PL. This occurrence is expected, as *P. vannamei* are omnivorous scavengers with occasional cannibalistic behaviour, especially during the nursery phase with high metabolic rates (Romano & Zeng, 2017). Any deceased PL would have been readily consumed by the survivors, potentially increasing their feed input and growth.

Compared to the low survival rates of the [C] and [AA] groups (51 ± 10% and 53 ± 11%, respectively), Costa Rezende et al. (2018) reported high survival (91 ± 12%) in a similar experimental setup with *P. vannamei* reared from PL5-PL20, fed with inert diet alone and no observed nitrite peak. The water quality of this experiment, in regard to elevated nitrite levels, was comparable among all treatments. Only the [N] group had significantly lower nitrite levels during the final days of the experiment. But since the [LA] group had a very high survival despite high nitrite levels, it can be suspected that elevated nitrite concentrations were not the sole reason for low survival in the inert diet

groups. It may be possible that the shrimp fed with an additional live diet were better capable to survive in water with lower quality.

The [N] and [LA] groups had a much higher survival (91 ± 7% and 94 ± 6%, respectively) than Seychelles et al. (2018) reported for *P. vannamei* reared until PL6 and fed with enriched nematodes *Panagrolaimus* sp. (NFS 24-5) and live *Artemia* (44 ± 24% and 33 ± 1%, respectively). Higher survival rates in later PL stages are expected, as larvae become more resistant to stress after the critical PL6 stage when gills and excretory organs become fully functional (McGraw et al., 2002).

As mentioned above, nematodes and *Artemia* can feed on bacteria from the biofloc (Brüggemann, 2012; Luo et al., 2017; Yao et al., 2018) and could thereby bioencapsulate valuable probiotics. Lakshmi et al. (2013) suggest probiotics in the diet of shrimp to increase their health and resistance to stress and diseases. It is plausible that the live diet groups had increased ingestion of probiotics from the biofloc, which could have improved their survival in elevated nitrite levels compared to the inert diet groups.

Live diets play an important role in the feeding of early shrimp larvae, and complete substitution of live *Artemia* has led to a reduction in survival and overall growth performance in several studies (Hoseinifar & Zare, 2013; Sorgeloos et al., 1998). The underlying issues and required nutrients of penaeid shrimp that undergo complicated life cycles are complex and not yet fully understood (Robinson et al., 2005). However, one identified problem that inert diets often pose, is the leaching of nutrients to the water that can impair growth and survival but also degrades water quality (Kolkovski et al., 2009; Villamar & Langdon, 1993). Gamboa-Delgado & Le Vay (2009) achieved good results with a co-feeding regime of inert diets and live *Artemia*, in which nutritional deficiencies of unenriched *Artemia* nauplii were compensated by specially formulated diets rich in essential nutrients. *Artemia* are known to be very digestible and it has been observed that penaeid larvae modulate their enzyme content in the gut in response to dietary quality (Le Vay et al., 2001). Therefore, a live diet supplement may be beneficial for the digestion of inert diets, resulting in better nutrient uptake and increased health of the shrimp. The results of this experiment certainly support this hypothesis in regard

to health, as final survival in the live diets groups were near twice as high as in strictly inert diet groups.

However, growth parameter results of wet weight, length, and RGR were statistically significant higher in the inert diet groups. The PL from the [C] and [AA] groups were on average 55.5% heavier at the end of the experiment. Although it must be noted, that the PL were weighed in their wet state directly after the experiment, assuming that the remaining water content was not perfectly identical among the groups. Therefore, wet weight results should be interpreted with reservation. The length measurements on the other hand stem from a very large sample size ([C]: n = 175; [AA]: n = 189; [N]: n = 222; [LA]: n = 246) and are much more robust. The data shows the same tendency, that PL from the inert diet groups are significantly different and on average 9.1% longer at the end of the experiment. The RGR also revealed a significant difference between live and inert diet groups, with the latter growing on average 2.5% more per day. But since the RGR is also based on the wet weight measurements, this result must also be interpreted with reserve. But the accumulated findings of the growth parameters point to the conclusion that PL from the two inert diet groups [C] and [AA] were significantly larger at the end of the feeding trial than PL that received a supplement of live diets.

The differences in size could be explained with the decreased PL density, due to the high mortalities that occurred in the inert diet groups. In addition, the survivors received an excessive amount of feed since the mortalities were unnoticed and the DF input was not adjusted. Results by de Lorenzo et al. (2016) indicate longer larvae length at lower stocking densities during the hatchery phase from mysis to PL5. Other studies with older PL also verified that the growth of *P. vannamei* increases with decreasing densities (Krummenauer et al., 2011; Moss & Moss, 2004).

There is also evidence that larger PL are more capable to survive adverse husbandry conditions such as elevated nitrite levels (Jayasankar et al., 2009; Laramore et al., 2001; Ramírez-Rochín et al., 2017). It is therefore plausible that the groups with the lowest final survival had a larger proportion of heavier and longer individuals at the end of the

experiment. An involuntary selection of such animals would thereby skew the results of the growth parameters towards the inert diet groups, which had the lowest survival.

Sorgeloos et al. (1998) reviewed the use of *Artemia* in the larval culture of several crustaceans and concluded that they provide an adequate feed with high nutritional value, especially when they are enriched with certain essential nutrients. More recent studies emphasize the benefits of co-feeding regimes with inert diets and live *Artemia* (Gamboa-Delgado & Le Vay, 2009; Zelaya et al., 2007). The results of this experiment verify these early findings, as the survival of the PL fed with live *Artemia* were very high compared with the inert diets and only the growth parameters were slightly reduced.

Seychelles et al. (2018, 2017) have already shown that the nematode species *Panagrolaimus sp*. (NFS 24-5) is capable to replace *Artemia* nauplii during the early larval stages of *P. vannamei* rearing. Nölting et al. (2017) successfully and completely replaced Artemia for PL12-PL33 in a zero water and partial biofloc nursery. The results considering the final survival in this study from PL10 - PL30 in complete biofloc system confirm the recent findings. In a co-feeding regime of inert and live diets in a biofloc system, nematodes can be used as a supplement replacing live *Artemia* nauplii. In addition to their good performance to support shrimp survival, beneficial effects on the water quality were also observed. The underlying mechanisms of this observation should be further investigated, as they may prove to be another advantage of this nematode species.

The simple preparation and application of desiccated nematodes outperforms the use of *Artemia* cysts in terms of time and effort needed. Although it was not quantified in this experiment, the preparation of live *Artemia* nauplii was the most time-consuming task and the one that needed most labour. The procedure included the preparation of the hatching tank, the hydration of cysts for 24 h, the harvest of nauplii, the separation of nauplii from shells and the quantification of live nauplii. In comparison, the desired quantity of nematodes only needed weighing and hydration in water for 30-60 min.

The results of the stress test revealed no differences among the groups in survival after exposure to zero salinity. Since there was high mortality in the groups fed with inert diets at the end of the feeding trial, it can be suspected that only the strongest survived and were hence subjected to the salinity stress test. This involuntary pre-selection of strong individuals would explain the lack of differences between the inert and live diet groups. It is also possible that the duration of exposure (45 min) was not exerting sufficient stress on the PL and that longer exposure times would have revealed differences in survival. To evaluate the larval quality, salinity stress survival is an important parameter that can ensure that PL will be resistant to transportation and grow-out in a farm. Values of above 75% are required by most aquaculture enterprises (FAO, 2003), and all treatments in this experiment had high survival values in the salinity stress test that fulfil this criterion.

The body composition analysis returned similar results for lipids, ash, and protein in all groups. The [LA] group had slightly higher ash levels. Unfortunately, not enough material was available to analyze replicates and thus, no conclusion can be taken. In further experiments, more emphasis should be given on such analysis approaches in order to better understand the effects of live diets on body composition.

4.3. Final Conclusions

PL fed with a supplement of live *Artemia* or nematodes had significantly higher survival but slightly lower growth than PL from control and artificial *Artemia* groups. The nematode species *Panagrolaimus* sp. (NFS 24-5) can completely replace *Artemia* nauplii in a co-feeding regime during the nursery phase in a zero-water exchange biofloc system. In addition, beneficial effects on the biofloc and on the water quality were also observed in the live diet groups. Further experiments investigating the complex interactions of live diets, biofloc, water quality, and shrimp health should be designed to better understand the underlying processes.

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