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Biofloc Technology as an Integral Approach to Enhance Production and Ecological Performance of Aquaculture

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I dedicate this work to

My parents, Edi Widodo H.S. and Tintin Setiawati

My husband, Wiyoto,

My daughters, Azka Nadia K. and Nabila Aulia W.

My parents in law, Parman and Marpungah

My brothers, Indra Dwi A. (Rip) and Daffa Feri T.

All the family members

All my teachers

For their endless love and support

NOTATION INDEX

ABL	Average body length
ABW	Average body weigth
a.e.	Atom excess
AHPND	Acute hepatopancreatic necrosis disease
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
APHA	American Public Health Association
ATP	Adenosine triphosphate
BFT	Biofloc technology
BOD	Biochemical oxygen demand
CF	Condition factor
CFU	Colony forming unit
CP	Crude protein
DGGE	Denaturing gradient gel electrophoresis
DIN	Dissolved inorganic nitrogen
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DON	Dissolved organic nitrogen
DW	Dry weight
E/A	Essential amino acid ratio
EAA	Essential amino acid
EAAI	Essential amino acid index
EMS	Early mortality syndrome
EPS	Extracellular polymeric substances
FAO	Food and Agriculture Organization
FCR	Food conversion ratio
FVI	Floc volume index

GC	Gas chromatography
GHG	Green house gas
GSI	Gonadosomatic index
HAB	Harmful algal bloom
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
HSI	Hepatosomatic index
HSP	Heat shock protein
HUFA	Highly unsaturated fatty acid
IAEA	International atomic energy agency
IMNV	Infectious myo necrosis virus
IMT-culture	Integrated multitrophic culture
LD	Lethal dose
L-DOPA	L-dihydroxyphenylalanine
LGBP	Lipopolysaccharide glucan binding protein
LT	Lethal time
MAMP	Microbe associated molecular pattern
mas	Masquerade-like serine protease
NACA	Network of Aquaculture Centres in Asia-Pacific
NBT	Nitroblue tetrazolium
OD	Optical density
OM	Organic matter
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PO	Phenoloxidase
PPAE	Prophenoloxidase activating enzyme
ProPO	Prophenoloxidase
PRR	Pattern recognition receptor
RAS	Recirculating aquaculture system
RB	Respiratory burst
RNA	Ribonucleic acid

RSD	Relative standard deviation
SD	Standard Deviation
SE	Standard error
SGR	Specific growth rate
SP	Serine protease
SVI	Sludge volume index
TAN	Total ammoniacal nitrogen
TBC	Total bacteria count
TCBS	Thiosulfate citrate bile salts sucrose
THC	Total haemocyte count
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
T-SOD	Total superoxide dismutase
TSS	Total suspended solids
USD	US dollar
WSSV	White spot syndrome virus

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Background

The Food and Agriculture Organization (FAO) reported in 2014 that on average, aquaculture supplied 9.41 kg of fish per person for consumption in 2012 on a global scale, and that the contribution to the global total fish production is now comparable to that of capture fisheries reaching up to 42.2% (Figure 1.1). With the steady level of global capture fisheries, the contribution of aquaculture to the total fish production is required to continuously grow to meet the global demand of aquatic products. As aquaculture is a resource-based industry, the development of this sector is, however, constrained by the high competition in economic, social, physical and ecological resources with other industries (Ross et al., 2013). These limitations in resources, in particular in land and water, have led to the need for aquaculture intensification to increase the units of output for each unit of resource input.

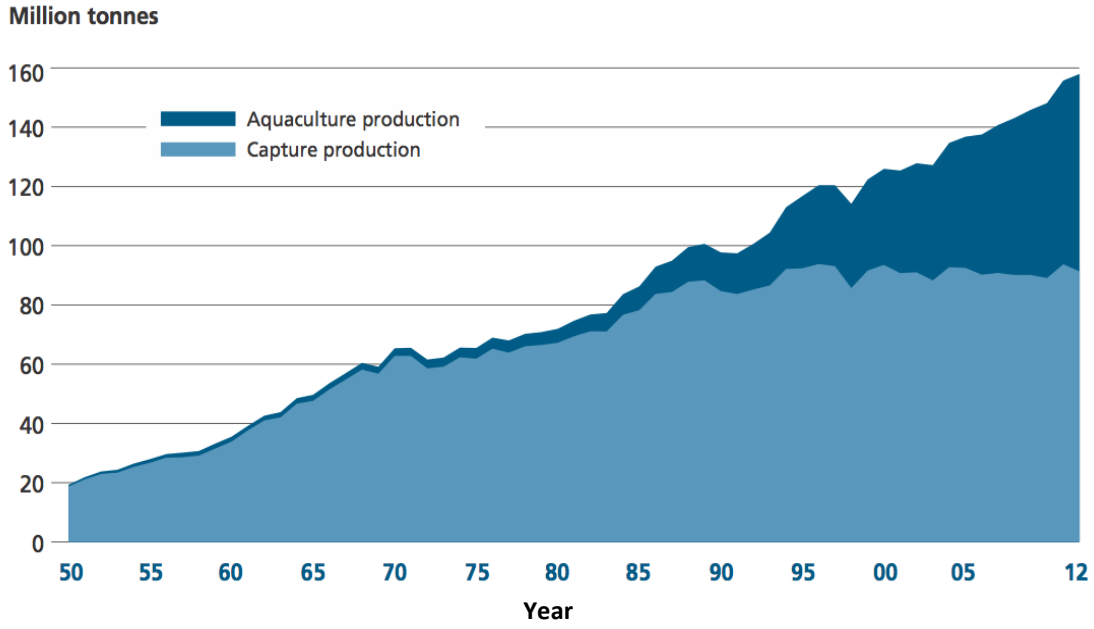

Figure 1.1. The contribution of aquaculture to total fish production (excluding aquatic plants and non-food products)(FAO, 2014).

To make efficient use of water and space, aquaculture intensification implies high stocking densities and consequently a high intensity of feed or nutrient input

per unit of aquaculture area. As the consequences of high input in seed and nutrients, not only a higher production is obtained, but also more waste is generated. Waste accumulation in an aquaculture system eventually deteriorates the water quality in the culture unit, and subsequently hampers growth and welfare of the cultured animals (Kautsky et al., 2000). Furthermore, when this waste is discharged without further treatment, it also contributes to the eutrophication of natural water bodies. This ultimately could threaten the sustainability of the aquaculture industry itself as well as bring about negative impacts to other human activities. Ross et al. (2013) highlighted the importance of the concept "carrying capacity" in ecosystem-based management that sets the upper limits of aquaculture production based on given environmental limits and social acceptability of aquaculture. Application of this concept can avoid unacceptable change to both the natural ecosystem and the social functions and structures. In this regard, the authors suggested that the development of aquaculture should consider not only improving the production carrying capacity (which estimates the maximum aquaculture production at farm scale), but also the ecological carrying capacity (which estimates the magnitude of aquaculture production that can be supported without leading to significant changes to ecological processes, services, species, populations or communities in the environment). According to this carrying capacity concept, the efforts to increase aquaculture productivity should therefore be approached by increasing the production through interventions in seed and feed quality, animal welfare and health, water quality maintenance, while at the same time minimizing the negative impacts on the environment.

Biofloc technology (BFT) is a relatively new aquaculture concept system that allows high density culture at limited or zero water exchange. Waste nutrients from the residual feed, faecal materials and metabolic products, with the presence of additional organic carbon source, are utilized by the heterotrophic bacteria to produce new biomass which can be further consumed by the cultured animals (Avnimelech, 1999 and De Schryver et al., 2008). Compared to other aquaculture systems, the biofloc system, a relatively straightforward, simple and robust technology, provides on one hand a more economical alternative in land and water

usage, and on the other hand an additional microbial protein source (Crab et al., 2012 and Hargreaves, 2006). The potential of this system in increasing land, water and nutrient utilization efficiency has raised attention for research and application during the past decade. As a consequence, more beneficial effects of the biofloc system have been discovered including the nutritional properties of bioflocs (Ju et al., 2008), exogenous digestive enzymes contribution (Xu and Pan, 2012 and Xu et al., 2013), potential control of pathogens (Crab et al., 2010a) and immunostimulatory effects (Kim et al., 2014 and Xu et al., 2013). On the other hand, more challenges in the application of this technology have also been identified such as the accumulation of microbial biomass (Ray et al., 2011), the requirement of efficient mixing and aeration system, as well as the high CO_2 emission from biofloc systems (Hu et al., 2014).

1.2 Objectives

The general objective of this study was to explore the possible contribution of biofloc technology application to aquaculture production, while maintaining sustainable practices. Aquaculture production can be optimized by increasing the production carrying capacity through sufficient supply of good quality seed, providing an optimum nutrition to ensure maximum growth as well as providing a healthy environment to support the health and welfare of the cultured biota. This study identify possible modifications to improve the ecological performance of a biofloc system and to explore possible beneficial effects of the system on the key factors in the enhancement of aquaculture production including seed supply, nutrition and health of the cultured animals. Figure 1.2 illustrates how the different chapters in this manuscript contribute to knowledge in those two fields.

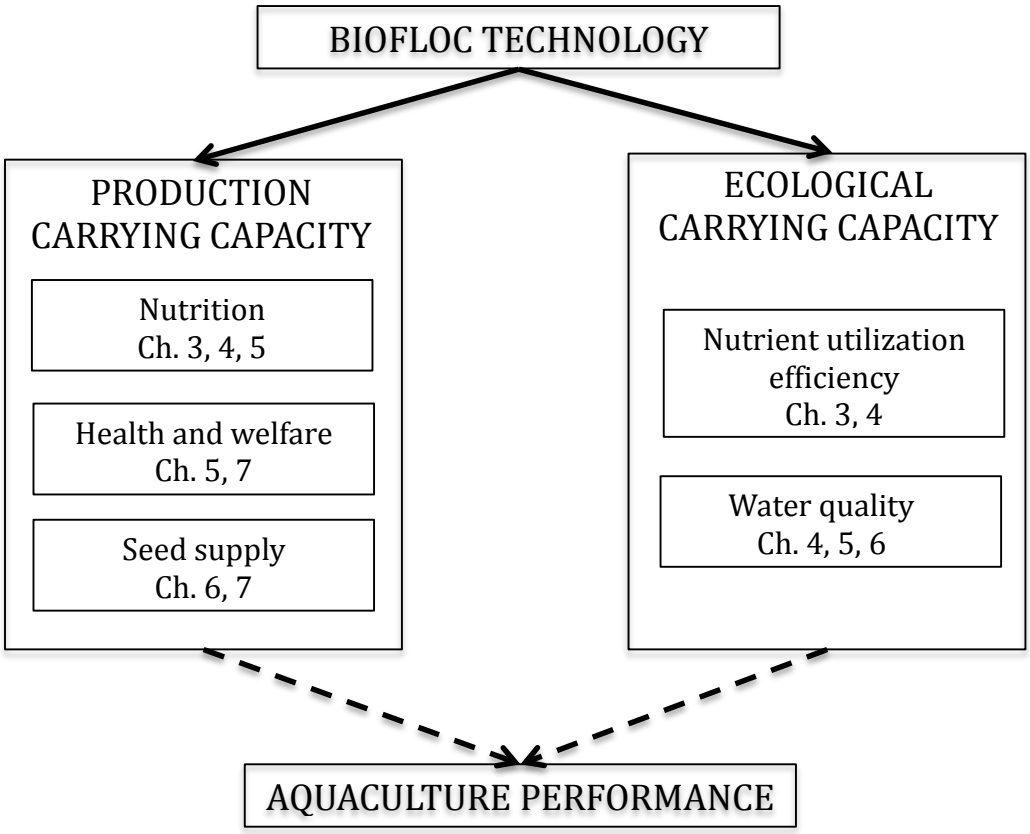

Figure 1.2. Overview of possible roles of biofloc technology in the enhancement of aquaculture performance and the coverage of these aspects in the chapters of the present manuscript .

1.3 Thesis outline

The first part of this manuscript (**Chapter 2**) reviews the development of culture systems including biofloc technology that facilitate the increase of nutrient utilization efficiency in aquaculture. The experimental results of **Chapter 3** show the effect of biofloc size on the nutritional composition and nitrogen recovery by some aquaculture animals. Using the stable isotope ^{15}N as a nutrient tracer, the feed-biofloc-animal nutrient utilization axis could be discussed more into detail. The consumption levels of bioflocs of different sizes by different aquaculture animals shown in Chapter 3 were used as basic information to develop an integrated biofloc-based aquaculture system described in **Chapter 4**. Possible integration of biofloc technology in shrimp-tilapia co-culture and shrimp integrated multitrophic culture

system was assessed through production performance, water quality and nutrient recovery of the system. **Chapter 5** provides information on the immunostimulatory feature of bioflocs for shrimp and how this can vary depending on the carbon source supplied. Additionally, the effects of different carbon sources on the nutritional property of bioflocs and water quality profile in a shrimp rearing unit are also discussed. Based on the information concerning the nutritional properties of bioflocs and water quality in the system gained from previous chapters, it was hypothesized that a biofloc system might also exert positive effects on the seed production. In this regard, **Chapter 6** describes the investigation of the reproductive performance of Nile tilapia in a biofloc system. Water quality, nutritional quality and biological composition of bioflocs were studied to explore their contribution to fish reproductive performance. The high larvae production in the biofloc system described in Chapter 5 became the main background of the next experiment (**Chapter 7)**. Here, Nile tilapia larval quality in larvae originating from a biofloc-based broodstock is further elucidated. The last chapter (**Chapter 8**) provides an overview and discussion of the research outcomes from previous chapters and formulates recommendations for future research.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction

Aquaculture as a food-producing sector is recognized for its ability to contribute to the global fish supply. Aquaculture production is projected to rise from 40 million tonnes by 2008 to 82 million tones in 2050 (FAO, 2010). The necessity to increase aquaculture production has been triggered by the increasing demand per capita in parallel to the increase of global population. However, the development of a sustainable aquaculture industry is particularly challenged by the limited availability of natural resources as well as the impact of the industry on the environment (Costa-Pierce et al. 2012 and Verdegem, 2013). With these limitations in mind, the development of the aquaculture industry should focus on the conceptualisation of systems that despite their high productivity and profitability, utilize fewer resources including water, space, energy and eventually capital, and at the same time have lower impact on the environment (Asche et al., 2008). One possible way to increase aquaculture productivity is by intensifying the production (Asche et al., 2008 and Avnimelech et al., 2008). Avnimelech et al (2008) described that intensifying fish production by adding more feed, working at high density, and supplying more aeration, could increase the productivity from less than 2000 kg/ha/year up to 100.000 kg/ha/year and at the same time reduce the water consumption per unit produced to half of the extensive system. However, the challenge of intensive aquaculture is to maintain environmental sustainability. As the system intensifies, more nutrients will be loaded into the system and subsequently discharged with the effluent water. As this may eventually contribute to the deterioration of the receiving water and the sustainability of the industry itself, interventions to minimize the impact of the discharged water should be an integral part of striving to increase aquaculture productivity.

It has been suggested that the nutrient (N, P and organic matter) utilization efficiency, the volume of waste water, the amount of nutrients discharged per kg production, and the on-farm conversion of waste products into secondary crops should be part of an aquaculture sustainability assessment (Verdegem, 2013). Table 2.1 presents N and P nutrient retention of some aquaculture species and the possible

quantity of nutrients discharged from their production unit of some aquaculture species at different trophic levels. The level of possible waste discharged from an aquaculture system is strongly dependent on the nutrient retention by the cultured animals and it appears to be irrespective of the trophic level. For example, with total N and P waste from aquaculture production system of about 20 kg/ton and 3 kg/ton, respectively, it can be estimated that a total world aquaculture production of 66.7 million metric tons in 2012 (excluding aquatic plants, FAO, 2014), generates about 1.3 million metric tons of N and 0.20 million metric tons of P wastes. Verdegem (2013) pointed out that in 2008 the nutrient waste generated from global aquaculture production of finfish and crustaceans represented 0.9% of the human input to the N-cycle and 0.4% of the global N-cycle.

Waste accumulation in an aquaculture system generates various toxic compounds such as ammonia (NH_3), nitrite (NO_2^-), and hydrogen sulfide (H_2S). These compounds deteriorate water quality, which directly affect the growth performance and welfare of the cultured animals, and increase the risk of disease outbreaks (Folke and Kautsky, 1989 and Kautsky et al., 2000). Kautsky et al. (2000) reported that viral and bacterial diseases together with poor soil and water quality are the main causes of shrimp mortality and that the risk of this mortality increases with the intensity of farming. The significant effect of disease outbreaks on shrimp production in several countries has been illustrated by Walker and Mohan (2009) (Figure 2.1). Episodes of new disease outbreaks, such as recently the shrimp early mortality syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) that causes large-scale mortality of cultivated shrimp in several countries in Asia (FAO, 2013), are no exceptions. As disease outbreaks pose a considerable threat to the sustainability of the aquaculture industry and it can be caused by water quality deterioration, it is clear that environmental management both within the culture system and the surrounding environment is an essential approach to minimize the risk of disease outbreak (Kautsky et al., 2000).

This literature review focuses on the development of aquaculture systems that facilitate the increase of nutrient recycling. The enhancement of nutrient utilization

in an aquaculture system should be performed using two approaches: firstly by increasing the feed quality and feeding strategy in a way that the nutrients can be efficiently delivered and finally utilized, and secondly by re-utilizing the nutrient waste through modifications in the culture system. The present review, however, will put more emphasis on culture systems that have so far been developed to re-utilize nutrient waste on the basis of nutrient recycling, and on integrated culture systems with species that directly or indirectly utilize the waste.

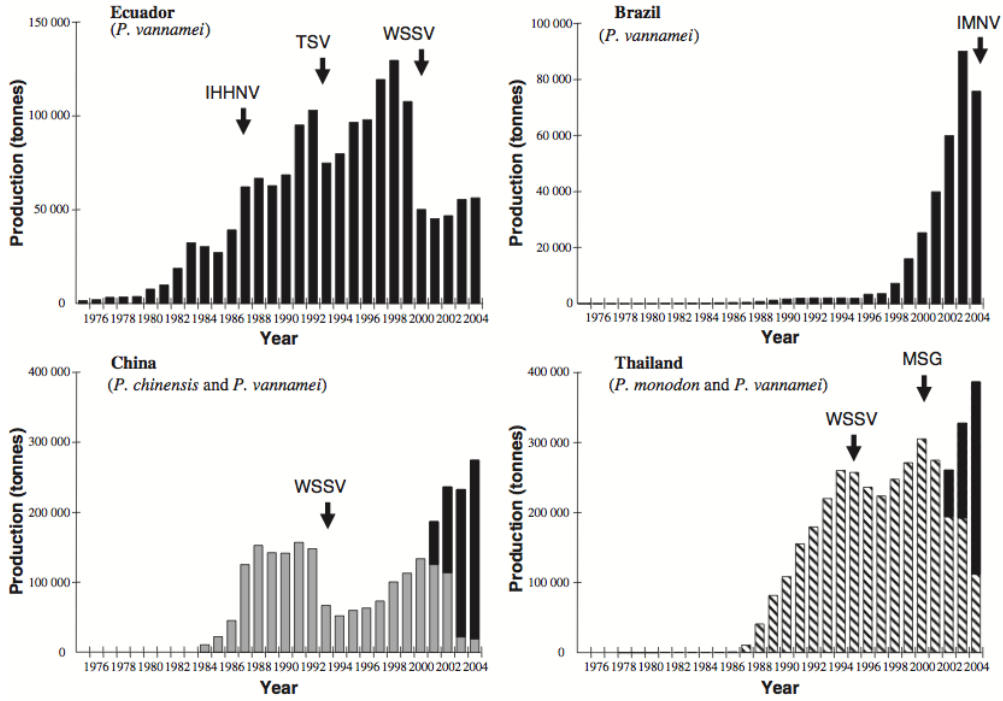

Figure 2.1. The effect of viral disease outbreaks on shrimp production (Walker and Mohan 2009). IHHNV= infectious hypodermal and haematopoietic necrosis virus, TSV= taura syndrome virus, WSSV= white spot syndrome virus, IMNV= infectious myonecrosis virus, MSG= monodon slow growth.

Table 2.1. Nitrogen and phosphorus waste from culture units of some species classified according to trophic level (www.fishbase.be). The data were calculated according to the production values and N retention of the feed. FCR=food conversion ratio, TN=total nitrogen, TP=total phosphorus.

Species	FCR	Nutrient input (% in compound diet)		Nutrient Retention (% nutrient input)		Nutrient discharged per tonne of production		Reference
		N	P	N	P	TN (kg)	TP (kg)	
Trophic level >4								
Atlantic Salmon	0.7	7.3	2.0	58	30	23	10	Storebakken et al., 1998
Grouper	2.3	7.2	1.2	39	64	101	10	Lupatsch, 2003
Trout	0.8	7.4	1.0	49	52	29	4	Dalsgaard and Pedersen, 2011
Tuna	15 – 25	2.9*	0.2*	4 – 7	8 – 11	99 – 254	16 – 41	Aguado-Giménez et al., 2006
Trophic level 2 - 4								
Red Sea bream	1.2	7.8	2.1	34	26	62	19	Biswas et al., 2007
Tiger shrimp	1.5	6.7	1.0	29	13	71	13	Thakur and Lin, 2003
Stripped catfish	2.3	6.0	0.7	66	-	46	14 - 18	De Silva et al., 2010
Carp	1.0	5.6	1.4	42	30	32	10	Jahan et al., 2000
White shrimp	1.8	5.6	0.9	28	13	72	14	Casillas-Hernández et al., 2006
Trophic level 2								
Grass carp	1.5	5.8	1.6	31	32	57	15	Cai et al., 2005 and Du et al., 2006
Milkfish	2.6	5.0	1.3	26	13	98	29	Holmer et al., 2002
Nile tilapia	1.1	4.8	0.4	43	35	31	3	Liebert and Portz, 2005

* fresh fish was used as the food source, calculation was therefore performed on wet weight basis

2.2 Nutrient waste in aquaculture

Waste in an aquaculture system mainly consists of solid and dissolved waste (Bureau and Hua, 2010). Solid waste is mostly in the form of faecal materials and uneaten feed, whereas dissolved waste is the excretion products of metabolic processes such as ammonia and orthophosphate. Once solid waste enters the water, part of the organic material will be demineralized by decomposers and eventually contributes to the dissolved nutrient waste accumulations (Crab et al., 2007).

2.2.1 Nitrogen

Nitrogen is one of the major nutrients required by most organisms. For most fish and shellfish species this nutrient is assimilated in the form of protein and nucleic acids (Olsen et al., 2008). Following ingestion, the indigestible protein and nucleic acids will be egested as faecal material, whereas the digestible protein will be broken down into amino acids which can further be used as building blocks of new proteins in the cells, converted into fatty acids or carbohydrates, or be used as a metabolic energy source (Olsen et al., 2008 and Wilson, 2002). The use of protein as an energy source or as a substrate for synthesis of other compounds, involves transdeamination, producing ammonia (NH_3). Due to its toxicity, ammonia should therefore be excreted rapidly from the body, and for most aquatic organism ammonia is excreted by direct diffusion from blood to water and/or functional Na^+/NH_4^+ exchange in the gill (Wilson, 2002).

The fraction of dietary protein utilized by the cultured organisms to build new proteins for growth or to generate energy determines the level of protein or nitrogen recovery and the level of nitrogenous waste. Most aquaculture organisms require food with a level of 15 to 50% protein on dry weight basis (2.4 - 8% N) and retain 28 – 66% of the dietary N (Table 2.1). This depends on the species, feeding habits, developmental stages, and dietary energy levels (Cho and Bureau, 2001). In a semi-intensive shrimp culture system, for instance, about 78% of the N entering the system originates from the feed, and only 18% of the total N input could be recovered and

harvested (Figure 2.2). Olsen et al. (2008) described that nitrogenous waste in fish culture consists of ammonia and particulate organic N, which accounts for 42% and 20% of the dietary N, respectively. Following decomposition, 15% of the particulate organic N may be released as ammonia and contribute to the total dissolved inorganic N. Ammonia represents 70 – 90% of the total nitrogenous waste in fish, with 5 – 15% excreted as urea, depending on the fish species (Folke and Kautsky, 1989 and Wilson, 2002).

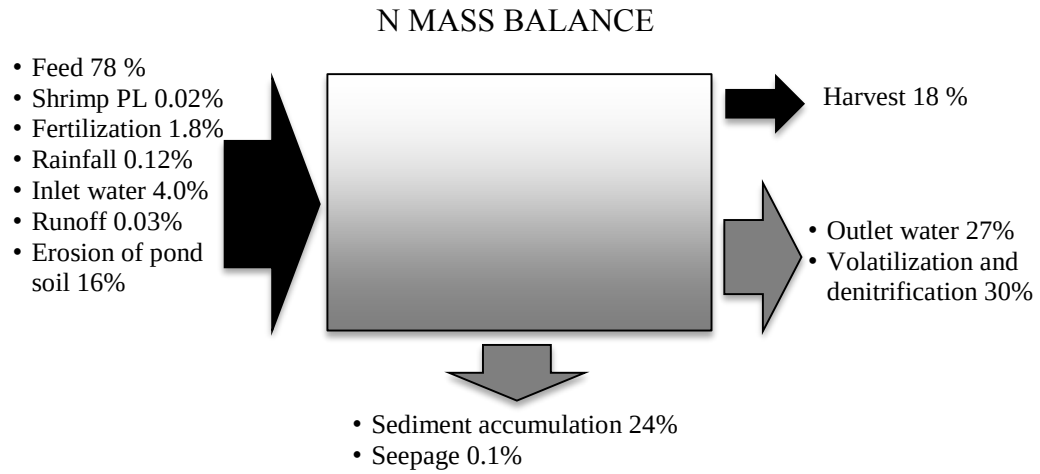

Figure 2.2. Nitrogen mass balance in a semi-intensive shrimp culture (redrafted from Funge-Smith and Briggs, 1998)

2.2.2 Phosphorus

Phosphorus is an essential component in the nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and in adenosine triphosphate (ATP), which is an important energy storage and transfer molecule in all organisms, as well as in phospholipids of the cell membranes, and in calcium phosphate forming teeth and bone. Furthermore, P in the form of phosphate is also important in carbohydrate, lipid, and amino acid metabolism, in muscle and nervous tissue metabolism and in various metabolic processes involving buffers in body fluids (Lall, 2002 and Olsen et al., 2008). The uptake of dietary P in the fish gastrointestinal tract involves the degradation of P compound into inorganic phosphate. The degree of degradation is determined by the plasma phosphate concentration (Bureau and Cho, 1999).

Excessive phosphate in the plasma will be excreted through the urine, while undigested phosphorus is egested with the faeces (Bureau and Cho, 1999, and Roy and Lall, 2004).

Lall (2002) noted that P exists naturally as inorganic phosphate (Pi) and as organic phosphate, bound in molecules such as sugars, proteins, and other components of the cell. Aquaculture feed mostly contains 0.4 – 2.1% P, whereas P assimilation efficiency by most aquaculture species is in the range of 13 – 64% (Table 2.1). The feed ingredients also determine the level of P assimilation efficiency. Animal ingredients such as fishmeal, contains bone-P, while plant ingredients contain phytate-P, which are almost indigestible, and most likely be egested in the faeces. Olsen et al. (2008) demonstrated that 38 – 50% of P waste in fish culture is egested with the faecal material, and 10 – 20% is excreted with urine or through the gills. Figure 2.3 presenting phosphorus mass balance in a semi-intensive shrimp culture system shows that most of P input was originating from feed (51%), and most of the P waste (84% of P input) is accumulated in the sediment (Funge-Smith and Briggs, 1998). Furthermore, as concentrations of soluble phosphorus in water are quite low, and the sediment in the pond strongly adsorbs phosphate, P input from fertilizer is required to stimulate phytoplankton bloom in a semi-intensive aquaculture system (Boyd and Tucker, 1998).

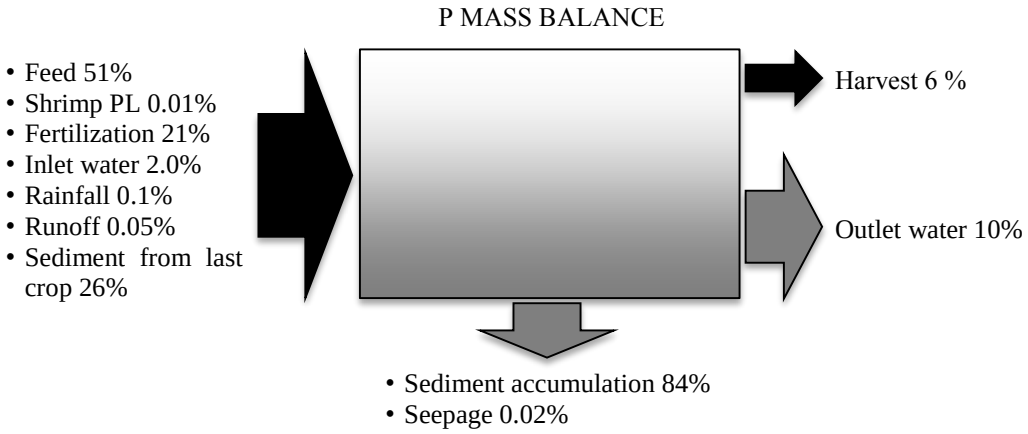

Figure 2.3. Phosphorus mass balance in a semi-intensive shrimp culture (redrafted from Funge-Smith and Briggs, 1998).

2.2.3 *The impacts of nutrient waste in aquaculture system*

As dietary nutrients provided in the feed are not totally recovered and each nutrient contributes to the total feed cost, improving nutrient utilization efficiency will enhance the economics of fish or shellfish production (Hargreaves, 1998). The accumulation of nutrient waste in an aquatic ecosystem may bring about adverse effects that hamper the production and deteriorate the environment. Ammonia as the main nitrogenous waste compound is toxic and should be maintained at a low concentration (<0.1 mg/L) (Boyd and Tucker, 1998 and Wedemeyer, 1996). Exposure to a high concentration of ammonia has been reported to cause hyperactivity, convulsions, loss of equilibrium, lethargy, coma, growth impairment, and suppression of immune competence in aquatic organisms (Liu and Chen, 2004). Nitrite is an intermediary product of nitrification and denitrification that may also accumulate in an aquaculture system. This compound is a potential toxic due to the competitive binding of nitrite to haemoglobin forming methemoglobin, which does not have the capacity to carry oxygen (Hargreaves, 1998).

When no further treatment is applied, the disposal of nutrient rich effluent water from an aquaculture unit would likely contribute to the eutrophication of the surrounding water bodies. Phosphorus has been recognized as a limiting nutrient in natural waters, hence introducing aquaculture discharge water (that is rich in P) may contribute significantly to the eutrophication of the receiving natural water bodies (Bureau and Cho, 1999). Negative impacts of eutrophication on natural water bodies have been well documented (Bonsdorff et al., 1997, Gowen, 1994, Herbeck et al., 2013, and Smith et al., 1999). The majority of them relates to the shift in phytoplankton abundance and composition including the risk of harmful algal bloom, alteration in natural macro biota including fish, macrophytes, and coral reefs, and the deterioration of water quality. Although the nutrient contribution of aquaculture activities has not yet been considered high, it is likely that the level of nutrient waste would follow the increasing production level of this sector. To anticipate the potential negative impacts of eutrophication as well as other environmental impact generated from aquaculture activities, some countries have

implemented restrictive legislation on fish production and farm effluents as regulatory approaches that may eventually compromise the economic viability of aquaculture (Bergheim and Brinker, 2003, Crawford, 2003, Read and Fernandes, 2003, and Tacon and Forster 2003).

It is well known that nutrients discharged from an aquaculture unit not only affect the production but may also generate economical losses. If the cost of N source (calculated as N in the protein source) is approximately 6 – 15 USD per kg, it can therefore be estimated that the total loss due to nitrogen discharged is within the range of 390 – 975 USD per ton of fish production. Furthermore, the implementation of restrictive legislation concerning farm effluents discharge requires the tax to each unit of nutrient discharged to be considered as one of the operational cost components. Chopin et al. (2001) for instance noted that the unit cost of N and P removal, which are in the range of 6.4 – 12.8 USD/kg and 2.6 – 3.8 USD/kg, respectively, should be taken into consideration in the economical appraisal of an aquaculture business unit. While, Bureau and Hua (2010) noted that the magnitude of environmental changes related to nutrient discharge from aquaculture activities might depend on biological, chemical, physical, as well as socio-economical characteristics of the receiving environment. It is clear that aquaculture operations should still be performed in environmental and socio-economically sustainable manners, and significant efforts should be invested to minimize the release of waste and/or the environmental impact of aquaculture operations.

2.3 Enhancement of nitrogen utilization efficiency in aquaculture system

2.3.1 Nutrient recycling by biofloc technology

In an aquatic system, nutrients can be removed by various natural biogeochemical processes involving mostly microorganisms with various functions in nutrient cycles. Ebeling et al. (2006) pointed out that the main N conversion pathways in an aquaculture system include photoautotrophic removal by algae, chemoautotrophic removal by nitrifiers, and heterotrophic conversion (Table 2.2).

Further, the microbial biomass (microalgae and/or bacterial biomass) resulting from these processes could be consumed by the cultured animals, eventually creating a nutrient recycle, enhancing the overall feed nutrient utilization efficiency. In general, there are three aquaculture systems that apply the principle of nutrient recycling for the enhancement of nutrient utilization, i.e. biofloc technology (Avnimelech, 2009), periphyton-based aquaculture systems (Asaduzzaman et al., 2008) and photosynthetic-based aquaculture systems (Brune et al., 2003).

Table 2.2. Stoichiometric equation of possible N conversion pathways in aquaculture system.

N conversion	Stoichiometric equation	Reference
Heterotroph	$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \rightarrow C_5H_7O_2N + 6.06\ H_2O + 3.07CO_2$	Ebeling et al., 2006
Photoautotroph	$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 106O_2$	Ebeling et al., 2006
	$16NO_3^- + 124CO_2 + 140H_2O + HPO_4^{2-} \rightarrow C_{106}H_{263}O_{110}N_{16}P + 138O_2 + 18HCO_3^-$	
Nitrification	$NH_4^+ + 1.83O_2 + 1.97HCO_3^- \rightarrow 0.024C_5H_7O_2N + 0.98NO_3^- + 2.90H_2O + 1.86CO_2$	Ebeling et al., 2006
Heterotrophic Denitrification	$C_6H_{12}O_6 + 2.8NO_3^- + 0.5NH_4^+ + 2.3H^+ \rightarrow 0.5C_5H_7O_2N + 1.4N_2 + 3.5CO_2 + 6.4H_2O$	Matějů et al., 1992

2.3.1.1 Nutrient conversion in biofloc system

The main principle of biofloc technology is to recycle waste nutrients, in particular nitrogen, into microbial biomass that can be used *in situ* by the cultured animals or be harvested and processed into feed ingredients (Avnimelech, 2009, De Schryver et al., 2008, Hari et al., 2004, and Kuhn et al., 2010). Heterotrophic microbiota as the major driving force are stimulated to grow by steering the C/N ratio in the water through the modification of the carbohydrate content in the feed or by the addition of an external carbon source in the water (Avnimelech, 1999), so that the bacteria can assimilate the waste ammonia for new biomass production (Figure 2.4). Hence, ammonia can be maintained at low concentration so that water

replacement is no longer required. Furthermore, the addition of an external carbon source in the water can be estimated according to the amount of feed dosed to the pond and the organic C requirement for heterotrophic microbial nitrogen conversion as described in Figure 2.5.

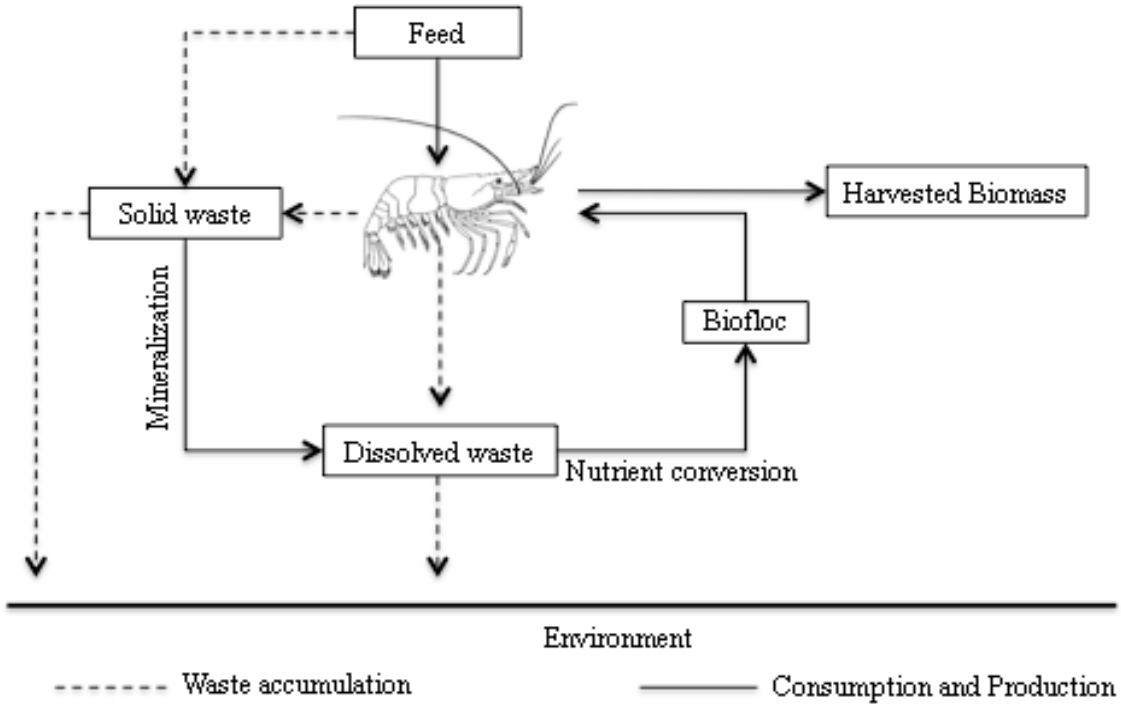

Figure 2.4. Schematic illustration of nutrient flow in a biofloc system (redrafted after Crab et al., 2007). Shrimp image is obtained from http://www.clipartpal.com.

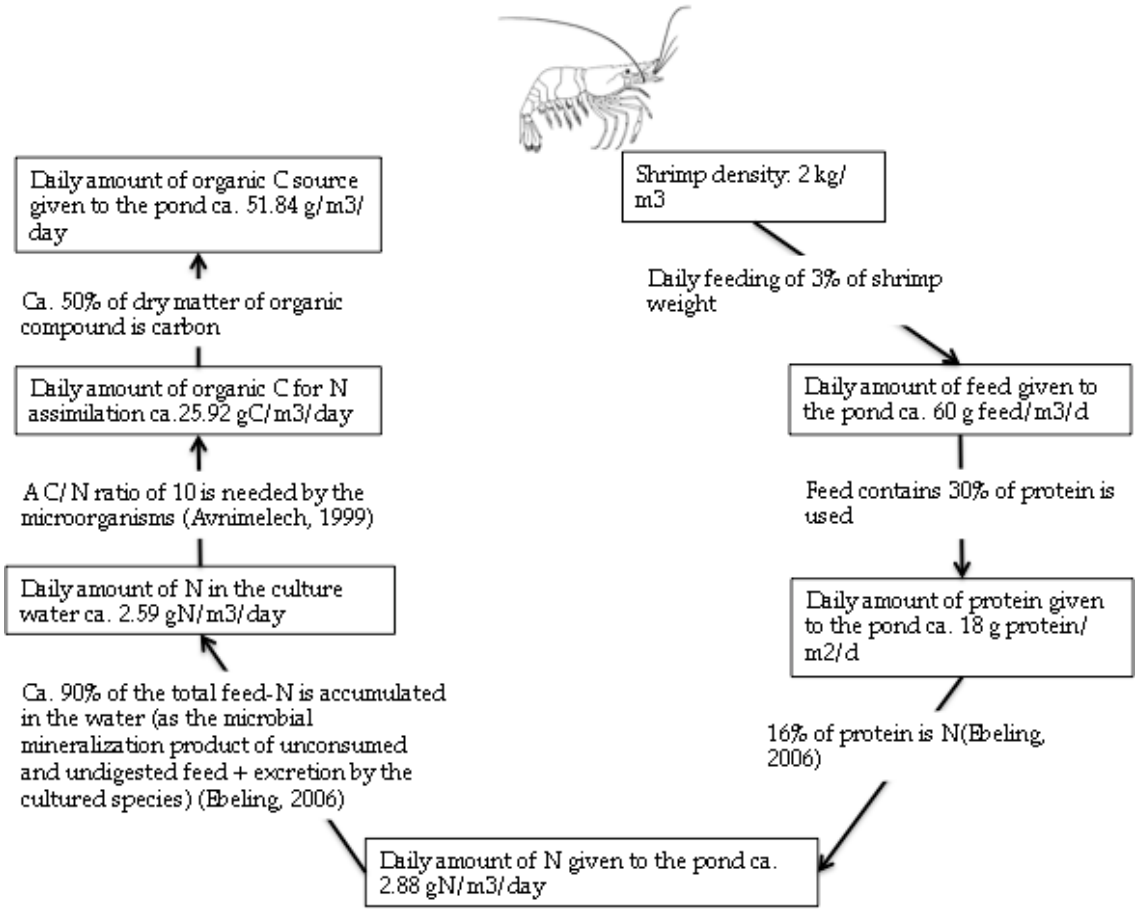

Figure 2.5. Schematic calculation of daily addition of organic carbon source in a biofloc-based shrimp culture system (redrawn with some modifications after De Schryver et al., 2008). Shrimp image is obtained from http://www.clipartpal.com

Although heterotrophic immobilisation is expected to be the main N conversion pathway in biofloc system, autotrophic and photoautotrophic nitrogen transformations evidently contribute to the nitrogen dynamics in the system (Azim and Little, 2008, Burford et al., 2003, Ebeling et al., 2006, and Hargreaves, 2006). It can be seen in the stoichiometric equations (Table 2.2) that N transformation processes, except denitrification, in a biofloc system mostly share similar requirements in oxygen (including phytoplankton during respiration) and alkalinity (Ebeling et al., 2006). The stoichiometric equations may also describe the variables in N transformation processes and their implication on the dynamics of some water quality parameters in biofloc systems. For instance, it can be seen in Table 2.2 that heterotrophic N immobilization resulted in higher dissolved oxygen (DO)

consumption, and higher production of microbial biomass and CO_2 than that of nitrification (Ebeling et al., 2006). On the other hand, nitrification and photoautotrophic N removal requires more alkalinity than that of heterotrophic immobilization.

The high concentration of organic C and the presence of nitrate may also facilitate heterotrophic denitrification to play a role in N transformation in biofloc systems. Some indications including high level of nitrate reduction rates (Hu et al., 2014) and nitrogen losses (Luo et al., 2014, Nootong et al., 2011, and Ray et al., 2011) have been reported. Furthermore, the presence of micro-niches in floc structure may also facilitate anoxic denitrification to take place in a biofloc system (Pochana and Keller, 1999 and Schramm et al., 1999). This is in agreement with the study by Gao et al. (2012) who showed a relatively high density of denitrifying bacteria (4×10^5 cells/mL) in biofloc-based shrimp culture system.

2.3.1.2 Biofloc morphology and composition

Under particular conditions, the microbial biomass growing in a biofloc system forms aggregates and lives in floc structures. This condition is mainly influenced by the substrate availability and the presence of biological stressors such as grazers (Bossier and Verstraete, 1996 and De Schryver et al., 2008). The irregular shape, variable particle size, high porosity and permeability to fluids are the general morphological properties of bioflocs (De Schryver et al., 2008) that could be influenced by various factors including microbial composition (Lee et al., 2002 and Wilen et al., 2008), mixing intensity, shear rate, dissolved oxygen and temperature (De Schryver et al., 2008). Furthermore, morphological structure of bioflocs could also be an important parameter for the assessment of biofloc quality and may also be used as an indicator of environmental conditions in the culture system. For instance, bioflocs with a higher floc volume index are usually dominated by filamentous bacteria, which have a higher affinity towards oxygen limitation (De Schryver et al., 2008).

In an aquaculture system, bioflocs consist of a heterogeneous mixture of bacteria, microalgae, fungi, particles, colloids, organic polymers, cations, dead cells, and microbial grazers including protozoa, ciliates, flagellates and nematodes (De Schryver et al., 2008, Ray et al., 2010b, and Zhao et al., 2012). Jang and Kim (2013) reported that there were totally 43 phyla, 105 classes, 263 orders, 606 families and 1265 genera of microbes found in biofloc systems. Furthermore, several factors have been identified to affect the biological composition of bioflocs e.g. salinity (Maica et al., 2012), biomass removal (Ray et al., 2010b), carbon source used to grow the flocs (Crab, 2010), and probiotic and microalgae addition (Ju et al., 2008 and Zhao et al., 2012). Zhao et al. (2012), for instance, found that the addition of sucrose together with probiotics resulted in *Bacillus* sp. predominance in the biofloc group rather than *Vibrio* predominance in the control group that also received probiotics but not carbon source (Table 2.3). Moreover, De Schryver et al. (2008) suggested that the microbial composition of bioflocs in aquaculture is under the influence of organic loading rate and dissolved oxygen.

Table 2.3. Predominant level of bacterial communities in a biofloc (with sucrose) and the control (without sucrose) systems (redrafted after Zhao et al., 2012).

Bacteria	Predominant level (%)	
	With Sucrose	Without sucrose
Cytophaga sp	0.97	3.90
Roseobacter sp.	1.53	-
Bacillus sp.	27.71	-
Vibrio sp.	-	22.65
Halomonas sp.	-	1.45
Photobacterium sp.	-	1.53
Paracoccus sp.	-	5.45
Pseudoalteromonas sp.	-	11.02
Other Proteobacteria*	43.89	29.16

* non-pathogenic proteobacteria associated to shrimp culture (Zhao et al., 2012)

The absorption of biofloc nutrients by the cultured animal is strongly related to its biochemical properties. Although the levels can vary among studies, it has been

reported that bioflocs contain considerable levels of protein, lipid, essential amino acids and fatty acids as well as carotenoids (Crab, 2010, Ju et al., 2008, and Kuhn et al., 2010). Table 2.4 presents the comparison between biofloc nutritional content and the dietary nutrient of shrimp and fish. It can be seen that the levels of most of the essential nutrients in bioflocs are comparable to those required by the shrimp or fish. The concentrations of lipid and essential fatty acids in bioflocs, however, were low relative to the animal's requirements. Some factors have been identified to affect biofloc biochemical composition including microbial composition, light exposure, nutrient loading, carbon source and salinity (Baloi et al., 2013, Crab et al., 2012, Maica et al., 2012, and Ju et al., 2008).

2.3.1.3 Biofloc as a food source for aquaculture animals

In situ utilization of microbial flocs generated in biofloc systems by some aquaculture organisms (Burford et al., 2004 and Hari et al., 2004) as well as the utilization of processed bioflocs as a feed ingredient (Anand et al., 2014, Kuhn et al., 2009, and Kuhn et al., 2010,) have been well documented. The mechanism by which a substance is recognized as food by most aquatic organisms mainly involves water borne chemical signs that direct the animals to identify and orient toward potential prey (Lee and Meyer, 1996). Ju et al. (2008) demonstrated that the concentrations of free amino acids such as alanine, glutamate, arginine and glycine, which have been known as potential attractants in shrimp diet (Nunes et al., 2006) and are present in bioflocs, were found to be comparable to that of the shrimp commercial diet. This suggests that bioflocs are likely to be recognized as food particles by some aquaculture organisms. Furthermore, the consumption of bioflocs by the cultured animals seems to depend on their capability to harvest these particles from the water column. Jang and Kim (2013) demonstrated that the difference in the 3rd maxilliped morphological structure, which functioned as a mesh that traps suspended particles, resulted in higher capability of *Litopenaeus vannamei* juvenile to utilize bioflocs than *Fenneropenaeus chinensis* and *Marsupenaeus japonicus* (Figure 2.6).

Table 2.4. Comparison of bioflocs nutritional composition (Azim and Little, 2008 and Kuhn et al., 2010) and dietary nutrient requirement of shrimp and fish (Tacon, 1987a). DW=dry weight

% DW	Biofloc (Azim and Little, 2008 and Kuhn et al., 2010)	Dietary requirement (Tacon, 1987a)	
		Shrimp	Fish
Crude protein	38 - 41	35 - 40	35 - 37
Crude lipid	0.1 – 3.2	10 - 11	6 - 7
Carbohydrate	31	30 - 35	< 40
Essential fatty acids			
Linoleic acid	0.54	1.0	1.0
Linolenic acid	0.02	1.0	1.0
Arachidonic acid	0.02	-	1.0
Eicosapentaenoic acid	0.01	1.0	1.0
Docosahexaenoic acid	0.02	1.0	1.0
Essential amino acids			
Arginine	2.0	1.9 - 2.7	1.5
Histidine	0.8	0.5 - 0.6	0.7
Isoleucine	1.1	0.8 - 1.0	1.0
Leucine	1.9	1.7 - 2.0	1.9
Lysine	1.3	1.8 - 2.1	2.1
Methionine	0.8	0.7 - 0.8	0.7
Phenylalanine	1.7	0.9 - 1.1	1.0
Threonine	1.7	1.2 - 1.3	1.2
Tryptophan	0.4	0.3 - 0.4	0.2
Valine	1.3	1.0 - 1.2	1.2
Mineral			
Ca	1.5	2.0 - 2.5	2.0
P	1.2	1.2	0.7
K	0.7	0.7 - 0.8	-
Mg	0.4	0.08 - 0.1	0.07

The nutrient conversion by bioflocs followed by its consumption by cultured animals clearly suggests the potential of the biofloc system in enhancing nutrient utilization efficiency in aquaculture systems. Da Silva et al. (2013) reported that the application of biofloc technology on Pacific white shrimp super intensive culture

considerably enhanced N and P utilization efficiency up to 70% and 66%, respectively, relative to conventional intensive culture systems with regular water exchange. Another report by Avnimelech (2007) noted that applying biofloc systems in tilapia intensive cultures increased nitrogen recovery from 23% to 43%. Furthermore, as there is no or limited water exchange applied in a biofloc system, it can be expected that this system uses water more efficiently than the conventional system with regular water exchange or flow through system.

Figure 2.6. Comparison of 3rd maxilliped structure of (A) *Litopenaues vannamei*, (B) *Fenneropenaeus chinensis*, and (C) *Marsupenaeus japonicus* showing the different capability of the shrimps in collecting bioflocs from the water column (Jang and Kim 2013).

2.3.2 Integrated aquaculture

Polyculture or integrated aquaculture systems has been applied by traditional extensive farmers for years (Chopin et al., 2001). This aquaculture system applies different species of fish or other aquatic organisms that have different food and feeding habits or different spatial distribution allowing more efficient use of resources within one aquaculture unit. Polycultures in general can be classified into two different groups, monotrophic polyculture which applies polyculture of different aquaculture species that are in the same trophic level but inhabit different

space in pond or consume different type of natural food, and multitrophic polyculture or integrated multitrophic culture system where organisms from different trophic levels are added to the system (Martínez-Porchas et al., 2010). Integrated multitrophic aquaculture systems mainly consist of a major fed aquaculture organism usually a high trophic level species, dissolved inorganic waste extractor such as seaweed as the primary producer, and low trophic level species that consume solid waste such as sea cucumber (Troell et al., 2009).

Figure 2.7 represents possible nutrient utilization in an integrated multispecies system with shrimp as the major fed organism. The deposit feeders such as sea cucumbers may consume part of the solid waste, whereas decomposers mineralize the organic material and contribute to the dissolved waste accumulation. Dissolved waste can be utilized by phytoplankton or bacteria, which are subsequently consumed by plankton feeder or filter feeder such as mollusc or fish, or can also be assimilated by seaweed. The combination of these species creates a synergistic relationship acting as bioremediators and simultaneously increases overall system productivity (Bostock et al., 2010).

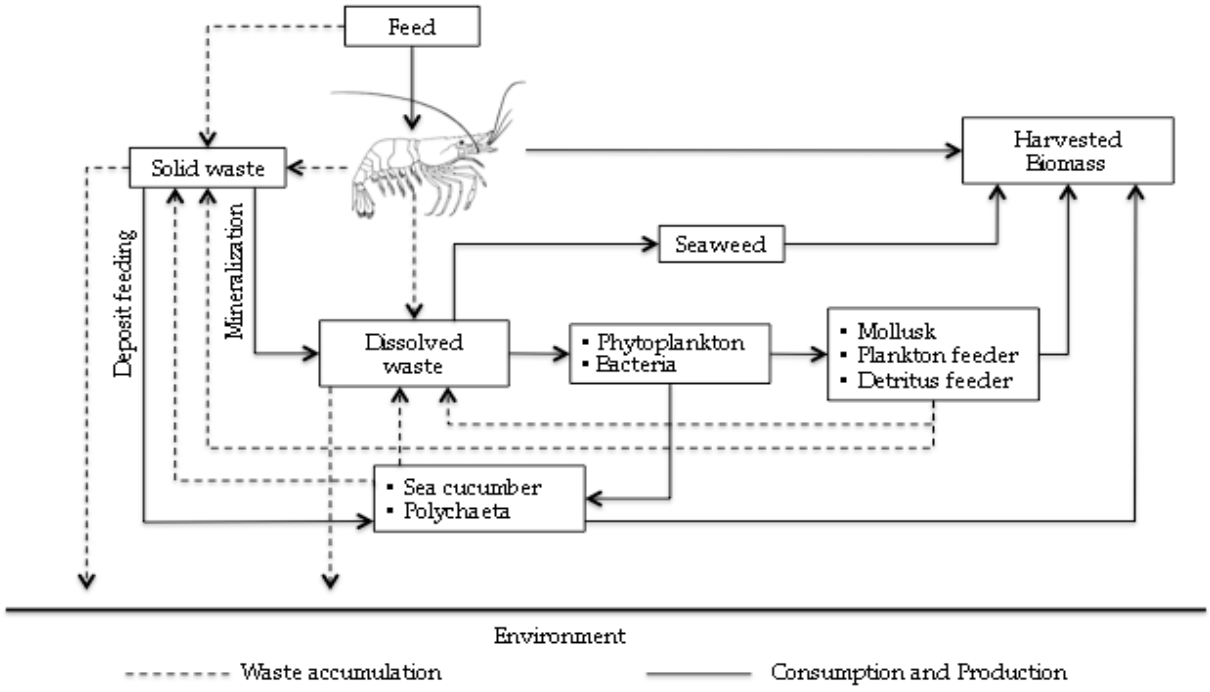

Figure 2.7. Schematic illustration of nutrient flow in integrated multitrophic culture system. Shrimp image is obtained from http://www.clipartpal.com

There are at least three general types of polyculture: direct polyculture which refers to two or more species mixed in the same culture unit without any partition (Figure 2.8a), cage-cum-pond polyculture which is a direct polyculture with partitioning (Figure 2.8b), and sequential polyculture which applies interconnected different culture units for each species (Figure 2.8c) (Martínez-Porchas et al., 2010). Each type of polyculture exerts different prerequisites and characteristics. Direct polyculture, which is the simplest type of polyculture, does not require extra investment for water circulation, cage installation or extra space, but most likely needs extra aeration and water exchange (Martínez-Porchas et al., 2010), as well as extra effort for product separation during harvest. This system also requires knowledge on the food and feeding habits, natural habitat, as well as the possibility of cannibalism or aggressive behaviour of each species to be combined. Similar to direct polyculture, cage-cum-pond polyculture uses different aquaculture species in the same culture unit; either the main organism or the subordinate species is, however, enclosed within cages (Martínez-Porchas et al., 2010). This arrangement could prevent direct interaction between the species, hence, reducing the risk of food competition, cannibalism or aggressive territorial behaviour. The application of this polyculture type, however, requires investment for cage installation and extra effort to regularly clean the cages. In comparison to other types, the production carrying capacity of direct polyculture and cage-cum-pond polyculture is limited by space and oxygen availability. In sequential polyculture system, the main species culture unit is separated from the subordinate species unit. The effluent water from the main culture compartment flows toward the secondary species compartment and is eventually discharged or recirculated back to the main culture compartment (Martínez-Porchas et al., 2010). Secondary species in the subsequent compartments likely act as bioremediator that utilizes uneaten feed, organic matter, dissolved waste or phytoplankton or bacteria generated from the dissolved waste. This process may improve the quality of the discharged water. The application of this type of polyculture, however, requires more investment for space and water installation.

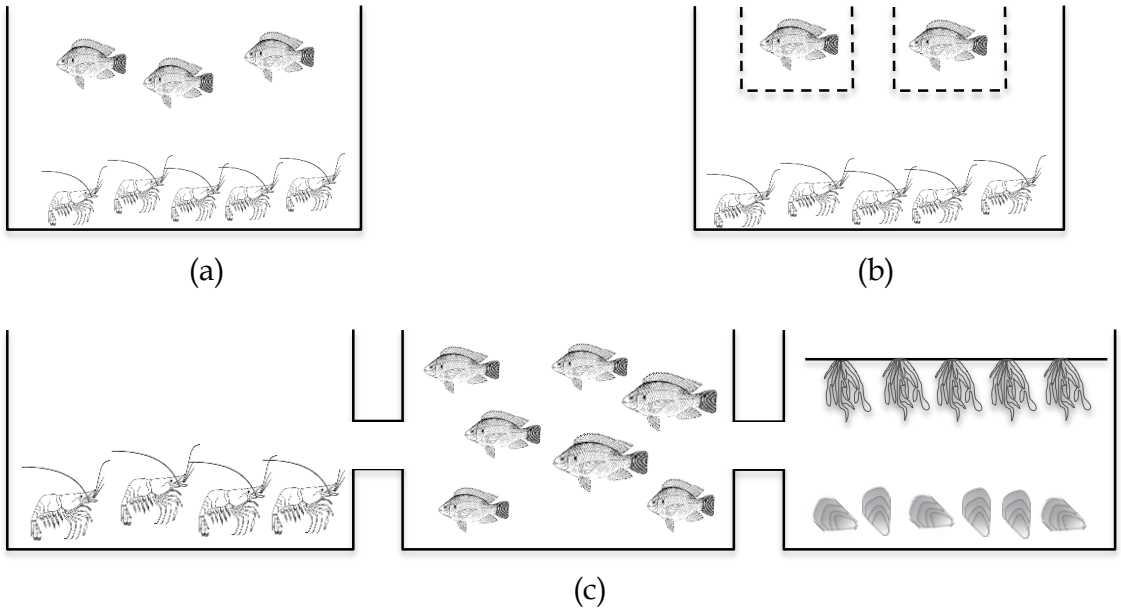

Figure 2.8. An illustration of shrimp polyculture types (a) direct polyculture, (b) cage-cum-pond polyculture, and (c) sequential polyculture (redrawn with some modification after Martínez-Porchas et al., 2010). Shrimp and tilapia image are obtained from http://www.clipartpal.com and http://www.fao.org.

The beneficial effects of polyculture application in aquaculture production have been well documented (Table 2.5). It appears that polyculture systems not only enhance total yield and productivity, but also reduce nutrient waste, improve water and sediment quality as well as control the presence of pathogenic microorganisms. Integrated culture systems have been shown to improve N and P utilization efficiency (Table 2.6). For example, integrated culture of Chinese shrimp with red tilapia and mollusc has been reported to increase N recovery to 75% higher than that of shrimp monoculture. Furthermore, the addition of dissolved inorganic waste extractor such as seaweed appears to significantly increase nutrient utilization efficiency. *Gracilaria*, for instance, may remove >90% of the dissolved inorganic N and 80% of the phosphate waste in the culture unit of *Sebastodes fuscescens* (Zhou et al., 2006). It is important to note that the beneficial effects of polyculture are determined by several factors including the species involved, the size of the organisms, the stocking density of each species, oxygen requirements and the carrying capacity of the system, as well as the food quantity and quality (Martínez-Porchas et al., 2010).

Several studies related to the economical evaluation of polyculture showed that the addition of some other species of commercial value could increase the economical profitability with little or no additional investment because most of the costs have already been met (Martinez-Porchas et al., 2010). Shi et al. (2013) demonstrated that applying polyculture of kelp and scallop resulted in almost double economic benefit. In term of environmental benefit, defined as the cost of nutrient treatment, polyculture of kelp and scallop resulted in similar benefit as kelp monoculture (50,000 USD/km^2), but four times higher than that of scallop monoculture (12,800 USD/km^2) (Shi et al., 2013).

In spite of the advantages, the application of polyculture particularly in intensive culture is still not common (Bostock et al., 2010 and Martinez-Porchas et al., 2010) mostly due to additional investment requirements (for sequential polyculture system), practical reasons such as harvesting techniques, and the farmer's limited knowledge. Furthermore, the addition of secondary species in a polyculture could lead to a growing complexity of biogeochemical processes, which accordingly influence the water quality. In addition, knowledge on the food and environmental requirements as well as potential social interactions between species is also an important consideration in designing a polyculture system. In this regard, more research is required to elucidate the variables that influence on the production of the target species as well as the environmental performance of polyculture system.

Table 2.5. Effect of polyculture in aquaculture production. DIN=dissolved inorganic nitrogen, TAN=total ammoniacal nitrogen, TP=total phosphorus, TN=total nitrogen, TSS=total suspended solids, OM=organic matter, TOC=total organic carbon.

Effects	Species	Reference
Shrimp growth and quality improvement (providing carotenoids for better pigmentation), decreased water turbidity	*L. vannamei, Ulva clathrata*	Cruz-Suárez et al., 2010
DIN removal >90%; PO_4^{3-}-P removal 80%	*Sebastodes fuscescens, Gracilaria lemaneiformis*	Zhou et al., 2006
DIN removal, 41 - 77% N uptake efficiency by different species of seaweed	*Chondrus crispus, Gracilaria bursa, Palmaria plamata, Scophthalmus maximus, Dicentrarchus labrax*	Matos et al., 2006
Maximum reduction of phosphate, TAN, NO_2^--N, and NO_3^--N of 58%, 48%, 61%, and 47%, respectively	*Pseudosciaena crocea, Gracilaria verrucosa*	Huo et al., 2012.
Maximum reduction of TAN, NO_2^--N , NO_3^--N and DIN of 30%, 100%, 72%, and 45%, respectively. But significantly increased PO_4^{3-} concentrations	Shrimp farm effluent, *Artemia fransiscana, Gracilaria caudata*	Marinho-Soriano et al., 2011.
N and P uptake efficiency by *Gracilaria* of 84% and 70%, respectively	*Gracilaria lemaneiformis, Chlamys ferreri*	Mao et al., 2009
Periphyton significantly reduced DIN, chl-a and total phosphate concentrations, and significantly increased water transparency. Tilapia significantly reduced TP and increased water transparency	*Macrobrachium rossenbergii, Oreochromis niloticus,* periphyton	Asaduzzaman et al., 2009
Reduction of TP and TN in culture water	*L. vannamei, Scatophagus argus, Ipomea aquatic*, biofloc	Liu et al., 2014
Reduction of TAN (22 - 37%) and TSS (10 - 20%)	*L. vannamei, Crassostrea gigas, Chione fluctifraga*	Martinez-Cordova and Martinez-Porchas, 2006

Table 2.5. Effect of polyculture in aquaculture production. DIN=dissolved inorganic nitrogen (continue).

Effects	Species	Reference
Co-culture of jellyfish and sea cucumber resulted in higher growth rate of sea cucumber and lower benthic nutrient loading (OM, TOC, TN and TP)	*Rhopilema esculenta, Apostichopus japonicus*	Ren et al 2014
Nutrient recycling and bioturbation	*L. stylirostris, Holothuria scabra*	Purcell et al., 2006
Utilization of biodeposit of mussel and reduction of waste output	*Perna canaliculus, Australostichopus mollis*	Slater and Carton, 2007 and Slater et al., 2009
Luminous bacteria control	*P. monodon,* oyster, green mussel, brown mussel	Tendencia, 2007
Luminous bacteria control, antibacterial effect of tilapia mucus on *Vibrio sp.*	*Epinephelus coioides, Tilapia hornorum, P. monodon, Oreochromis niloticus*	Tendencia et al., 2006
Bio-accumulation of *Vibrio anguillarum* by mussel and shedding virulent bacteria through their faeces	Cod, *Mytilus edulis*	Pietrak et al., 2012
Reduction of aggressive behaviour and cannibalism	*Diplodus puntazzo, Sparus aurata*	Karakatsouli et al., 2006
Control of recruit	Nile tilapia, *Tor putitura*	Shrestha et al., 2011

Table 2.6. Nitrogen utilization enhancement of culture systems that have been developed to re-utilize nutrient waste on the basis of nutrient recycling, and on integrated culture systems with species that directly or indirectly utilize the waste.

Species	N input (kg/ha)	% N harvested	N utilization enhancement (%) relative to monoculture	P input (kg/ha)	% P harvested	P utilization enhancement (%) relative to monoculture	Reference
White shrimp, biofloc	352	39	31 - 70	45	35	66	Da Silva et al., 2013
Nile tilapia, periphyton	1560	37	45	211	83	36	Hendriana et al., unpublished data
White shrimp, red tilapia	236	41	50	59	19	109	Yuan et al., 2010
Chinese shrimp, red tilapia, constricted tagelus	-	21	75	-	14	100	Tian et al., 2001
African catfish, common carp, Nile tilapia, microalgae	1882	57	12	423	77	105	Gál et al., 2007

2.3.3 *Introducing the nutrient recycle principle in integrated aquaculture systems*

A possible modification of the polyculture system to maximize nutrient utilization efficiency is by the applying nutrient recycle principle in an integrated aquaculture system. As an illustration, Figure 2.9 represents the possible combination of nutrient recycling in an integrated aquaculture system. The faster conversion of nutrient by the microbes associated in bioflocs or periphyton, may provide more digestible and nutritious additional food source for both main cultured organism and other species added into the system. In this way, utilization of the wasted nutrients is expected to be more efficient and less pollution is generated. However, there are not many studies focusing on such systems. Asaduzzaman et al. (2009) reported that the addition of a substrate for periphyton in prawn-tilapia polyculture resulted in significantly reduced dissolved inorganic nitrogen and total phosphate as compared to the control (no addition of substrate). The recent study by Liu et al. (2014) showed that the addition of maize to stimulate bioflocs grown in an integrated culture of shrimp, spotted scat and water spinach significantly increased shrimp total yield, reduced total food conversion ratio (FCR), and lowered total P and total N in the cultured water.

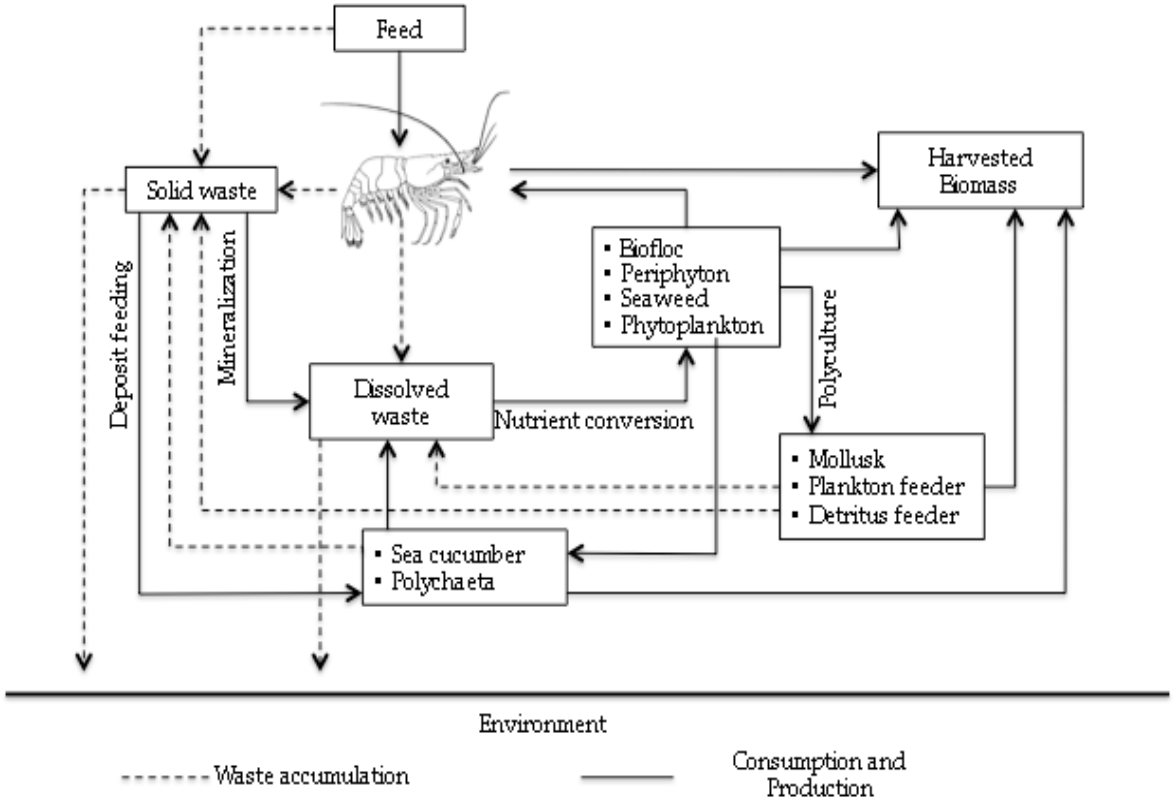

Figure 2.9. Schematic diagram of nutrient waste and possible enhancement of nutrient utilization efficiency in a shrimp culture unit by combining nutrient recycling through the stimulation of bioflocs, periphyton, seaweed or phytoplankton that convert dissolved nutrient into food biomass. This food biomass is expected to be more digestible and nutritious, thus allowing more nutrient recovering by both the shrimp as the main fed organism and subordinate species cultured in integration with the shrimp. Shrimp image is obtained from http://www.clipartpal.com

CHAPTER 3 THE SIZE OF BIOFLOCS DETERMINES THE NUTRITIONAL COMPOSITION AND THE NITROGEN RECOVERY BY AQUACULTURE ANIMALS

The effect of biofloc size on the nutritional composition of the flocs and the nitrogen utilization by white shrimp (*Litopenaeus vannamei*), red tilapia (*Oreochromis niloticus*) and mussels (*Perna viridis*) was investigated. Biofloc was collected from a shrimp culture unit and labelled with $(^{15}NH_4)_2SO_4$. The flocs were sieved grouping them into 4 different size classes (un-sieved, < 48 μm, 48 – 100 μm, and > 100 μm) and subsequently offered to shrimp, red tilapia and mussels. The biofloc class of > 100 μm contained the highest levels of protein (27.8%) and lipid (7.5%), whereas biofloc < 48 μm seemed to be richest in essential amino acids. Based on the Essential Amino Acid Index (EAAI), bioflocs produced in this study can be considered as a good quality protein source for shrimp (0.93 – 0.97) and a useful protein source for tilapia (0.83 – 0.90) and mussel (0.81 – 0.88). The total amount of nitrogen that could be derived from biofloc was highest when the bioflocs were larger than 100 μm, i.e. 4.06 g N/kg shrimp, 3.79 g N/kg tilapia, and 1.17 g N/kg mussel, respectively. Nitrogen recovery from the bioflocs, however, was highest when the flocs were < 48 μm. Overall, this study showed that biofloc consumption by shrimp, red tilapia and mussels occurs irrespectively of floc size but that floc size can play an important role in the quality of bioflocs in terms of nutritional composition and nitrogen retention by the animals.

Redrafted after:

Ekasari, J., Angela, D., Waluyo, S.H., Bachtiar, T., Surawidjaja, E.H., Bossier, P., De Schryver, P., 2014. The size of biofloc determines the nutritional composition and the nitrogen recovery by aquaculture animals. Aquaculture 426 – 427, 105 – 111.

3.1 Introduction

Biofloc technology has been widely studied and applied in aquaculture. This system applies the principle of assimilation of excreted dissolved nitrogen by heterotrophic bacteria by managing the C/N ratio in the water (Avnimelech, 1999). This heterotrophic bacterial biomass further forms aggregates (bioflocs) comprising not only the bacteria itself but also other microorganisms such as microalgae and zooplankton, as well as trapped (in)organic particles or solids (De Schryver et al., 2008). Biofloc technology (BFT) is not only an adequate approach in maintaining water quality in the aquaculture system but it also generates biomass that can contribute as a protein source for the cultured organisms *in situ* (Avnimelech, 2009 and Crab, 2010) or can be harvested for use as a feed ingredient (Kuhn et al., 2009 and Kuhn et al., 2010). Hence, the use of bioflocs as a food source may imply a decrease in the requirement of formulated feed protein (Xu et al., 2012) and also improve nitrogen utilization efficiency by the cultured animals (Avnimelech, 2006). In order to evaluate the use of bioflocs as a food source, general criteria of aquaculture feed can therefore be applied, i.e. the size of particles, attractiveness and palatability, digestibility, and nutritional content (Tacon, 1987b).

Specifically for *in situ* biofloc utilization, particle size distribution of the food may be of influence on the efficiency by which cultured animals with different feeding behaviour (filter feeders, scavengers, etc.) utilize the flocs as feed. Furthermore, in an aquaculture system with biofloc technology application, floc size may also relate to the uptake potential of the biofloc by the cultured organism, the digestibility of the flocs as well as the nutritional value of the flocs (De Schryver et al., 2008).

The protein, lipid, carbohydrate and vitamin C contained within the biofloc represent a considerable fraction of the nutritional requirements of several aquaculture species (Crab, 2010 and Tacon, 2002). This has been supported by previous studies which showed that biofloc supplementation into shrimp feed (Kuhn et al., 2010) or biofloc offering as a food source to pink shrimp resulted in comparable results in growth and survival to the control feeds (Emerenciano et al.,

2012a). Others have reported that the application of biofloc technology improved the feed conversion and protein retention indicating indirectly that the consumption of bioflocs contributes to the growth of the cultured organisms (Avnimelech, 2009, Gao et al., 2012, Hari et al., 2004, Wasieliesky et al., 2006, and Xu et al., 2012). Further investigations, however, are still needed on the amino acids, fatty acids and micronutrients such as vitamins and minerals contents in bioflocs.

This study investigated the effects of biofloc particle size on its consumption and nitrogen utilization by Pacific white shrimp (*Litopenaeus vannamei*), red tilapia (*Oreochromis niloticus*) and mussel (*Perna viridis*). For this purpose, the stable isotope ^{15}N was used as a tracer. In addition, we examined the relationship of the nitrogen retention from the bioflocs by the tested animals with the essential amino acids profiles for bioflocs of different particle size.

3.2 Materials and methods

3.2.1 Biofloc preparation, ^{15}N enrichment, and multilevel filtration

The schematic summary of the overall experiment is presented in Figure 3.1. A biofloc suspension was prepared in a 300 L fiber tank filled with seawater and stocked with 60 shrimp (5 g). The biofloc production unit as well as the experimental units (see below) were located indoors at a light/dark regime of 12h/12h and light intensity of 450 lux. Feed pellets containing 40 % protein (Chuen Sin, PT. Grobest Indomakmur, Indonesia) were administered three times daily at a total level of 8% of the shrimp biomass/day. To promote biofloc formation, 25 g of molasses containing 44% of carbon was added daily to obtain an estimated C/N ratio of 20. The biofloc preparation was performed for 3 weeks until the total suspended solids (TSS) reached a level of more than 500 mg/L. The shrimps and part of the water were subsequently removed, whereas the remaining 40 L of the shrimp culture medium containing the majority of the biofloc was enriched with ^{15}N. The addition of $(^{15}NH_4)_2SO_4$ (20% atom excess (a.e.) ^{15}N) was performed at a ^{15}N enrichment level of 12.5% on total N in biofloc biomass, coupled with the addition of molasses

(Avnimelech and Kochba, 2009) at an estimated C/N ratio of 20. The suspension was gently aerated for 2 days until the total ammoniacal nitrogen (TAN) of the floc suspension was 0 mg/L.

Part of the enriched biofloc suspension was diluted using fresh seawater to obtain an estimated TSS of 500 mg/L. This was considered as the un-sieved biofloc group. The remaining suspension was sieved over 2 stacked nylon filters of 100 μm and 48 μm mesh size, respectively, to obtain the > 100 μm, 48 – 100 μm, and < 48 μm floc size groups. A subsample of the enriched floc suspension was brought on the sieves and gently swirled to avoid inducing disintegration of the flocs until all suspension passed through. The flocs that did not pass the filters (> 100 μm group and 48 μm – 100 μm group) were gently transferred to fresh seawater before the next volume of biofloc suspension was sieved. Finally, all floc size classes were resuspended in seawater at an estimated TSS concentration of 500 mg/L and aerated for 1 h resulting in 4 different batches of flocs produced with different sizes: original un-sieved flocs, flocs which passed through the 48 μm sieve (< 48 μm), flocs which passed through the 100 μm but not the 48 μm sieve (48 μm – 100 μm), and flocs which did not pass the 100 μm sieve (> 100 μm). Five samples from each batch were collected for TSS measurement to determine initial TSS. In addition, duplicate samples of each biofloc suspension were analysed in terms of particle size distribution, nutritional composition, total nitrogen content and ^{15}N content. To determine the mass fractions of the < 48 μm, 48 – 100 μm, and > 100 μm floc size groups in the un-sieved biofloc group, 1 L of un-sieved biofloc suspension was fractioned into the respective floc size groups according to the previously described procedure, and the dry weight of each size group was determined after drying at 105 °C for 4h.

3.2.2 Feeding experiment

Following resuspension, each size class of ^{15}N labelled bioflocs was distributed into 20 plastic tanks of 2 L (80 tanks in total). From each experimental species

(shrimp, fish, and mussels), one individual was stocked per tank (5 replicates per floc size class). The remaining 5 replicated tanks per floc size class filled with only biofloc suspensions were used to observed possible biofloc degradation during the experiment. Five additional replicate tanks per species were also prepared as a control, filled with seawater without any biofloc addition and stocked with one individual. Aeration was provided in each tank to ensure a homogenous suspension of floc particles. The stocking size of the shrimp, red tilapia, and mussels were 10.6 ± 1.2 g, 9.6 ± 1.2 g, and 10.5 ± 1.2 g (6 - 7 cm in length), respectively. The animals were adapted to the environmental conditions of the experiment, i.e. the temperature, light condition and salinity in the laboratory for 1 week prior to the start of the feeding experiment. As for red tilapia, the adaptation to seawater salinity was performed three weeks before the experiment. Twenty-four hours before the biofloc feeding experiment, feeding with commercial pellet was stopped to ensure that the animals would feed on the biofloc suspension. The animals were kept in the biofloc suspensions for 4 days during which no additional feed was provided.

3.2.3 Sampling of animals

The total N and ^{15}N content in the test animals was determined after the feeding experiment. For this purpose, each animal was transferred to clean seawater and allowed for gut evacuation for 2 h, which in preliminary tests using ^{15}N labelled biofloc was shown to be adequate to obtain constant ^{15}N body values indicating complete emptying of the gut of the different species. Subsequently, the animals were sacrificed and oven dried (70 ^{o}C for 24 h) for further analyses.

3.2.4 Physical and chemical analyses

The size distribution of each sieved biofloc fraction was measured using a Coulter particle size distribution counter (Coulter LS 100Q Laser Diffraction Particle Size Analyzer, Beckman Coulter, USA) at a size range of 0.3 – 1000 μm. The distribution of particle sizes is expressed as the percentage of each size by volume

yielding a distribution curve where the total area under the curve represents the total biofloc volume, which is 100%. The volume fraction of each biofloc size class was calculated according to the summation of biofloc volume (% of total volume) for this range under the curve. Biofloc proximate composition was analysed following the procedure described in Takeuchi (1988). Total suspended solids and total ammoniacal nitrogen were analysed according to Standard Methods for Water Quality Analysis (APHA, 1998). ^{15}N stable isotope analyses were performed using mass spectrophotometry following the procedure of IAEA (2001).

The amino acid composition of the bioflocs was measured by a professional laboratory (Sarawanti Indo Genetech, Indonesia) using high performance liquid chromatography (HPLC). Briefly, a biofloc sample (100 mg) was hydrolysed with 5 mL 6N HCl for 22 h at 100 °C. The sample was subsequently diluted in 50 mL distilled water and filtered through a 0.45 μm filter. Into a 500 μL aliquot, 40 μL of internal standard (alpha amino butyric acid) and 460 μL of distilled water were subsequently added. Prior to HPLC injection (AccOtag column 3.9 x 150 mm, 37 C, AccQTag eluent A), pre-column derivatization was performed using a AccQ-Tag reagent kit followed by incubation for 1 h at 55 °C. Validation of the method was performed by using a standard amino acids mixture (Thermo Scientific Amino Acid Standard H catalog no. NCI0180) and a reference protein sample (bovine serum albumin, Sigma Aldrich catalog no. P3569), and amino acids recovery was obtained in a range of 91.3% – 106.9%).

A selection of amino acids that is considered as essential for aquatic animals in general was chosen as described by Tacon (1987a) and Babarro et al. (2011). The essential amino acid ratio (E/A) for each essential amino acid (EAA) was expressed as the percentage of this amino acid on the total amount of essential amino acids measured (Arai, 1981). The essential amino acids index (EAAI) was calculated according to Penaflorida (1989) using the formula:

$$EAAI = \sqrt[9]{aa1/AA1 \times aa2/AA2 \times \ldots \times aa9/AA9}$$

where aa being the essential amino acid ratio (E/A) in the biofloc, AA being the E/A ratio in the animal body, and 1, 2, 3,..., 9 being each of the essential amino acids.

Based on this index, Penaflorida (1989) classified dietary protein sources into good quality (EAAI > 0.9), useful (0.7 < EAAI < 0.9), and adequate (EAAI < 0.7).

3.2.5 Statistical analyses

Homoscedasticity and normality of biofloc uptake, total N uptake and N recovery were assessed using Levene's test and a Kolmogorov-Smirnov test, respectively. It was found that the variances of these variables were not equal therefore a non-parametric test (Kruskal-Wallis test) was applied, followed by a Mann-Whitney U test ($P < 0.05$).

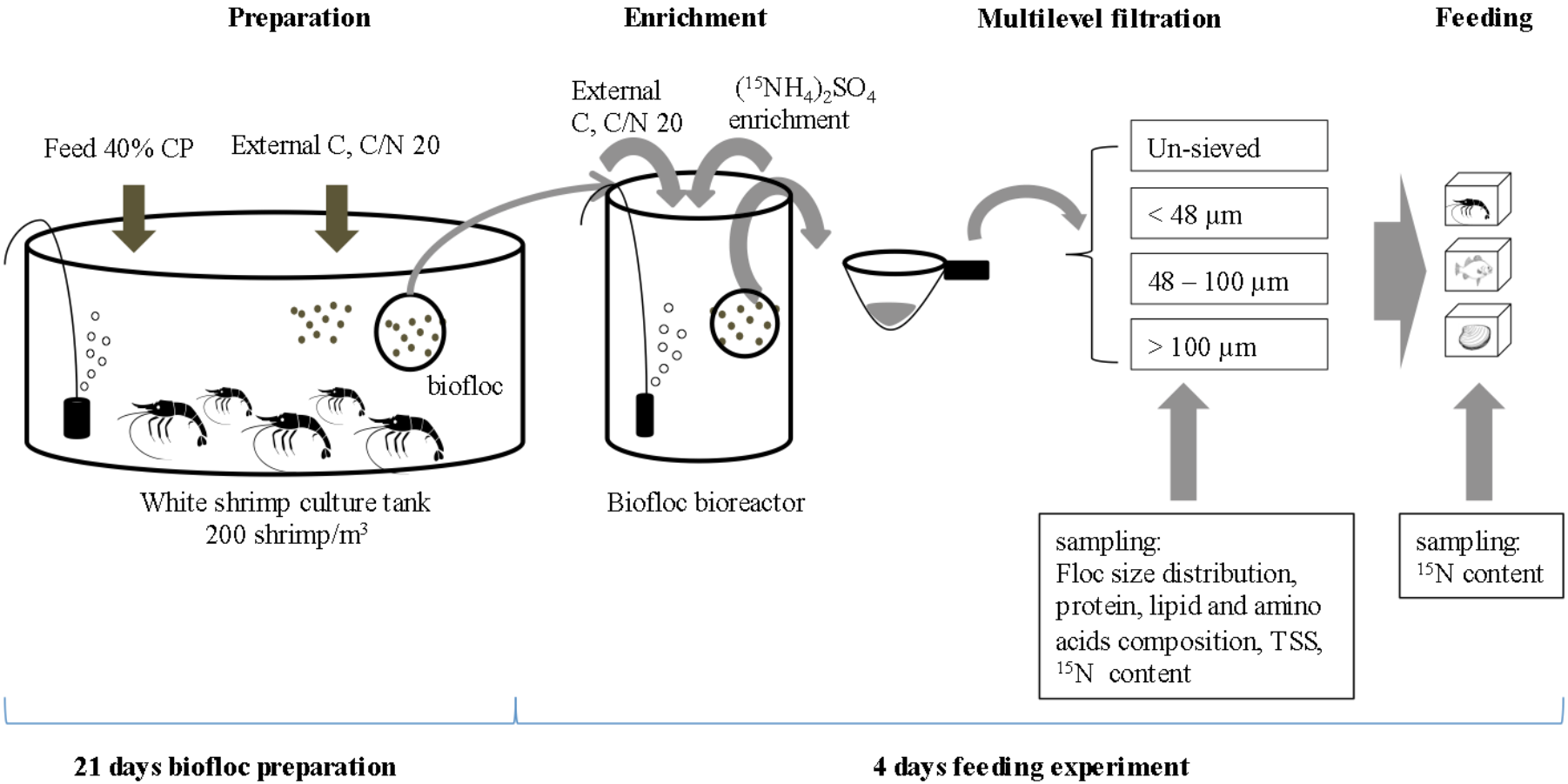

Figure 3.1. Schematic summary of the experimental procedure. CP = crude protein content, C = carbon, TSS = total suspended solids

3.3 Results

3.3.1 Biofloc size distribution

The particle size distribution in relation to the volume of bioflocs before and after sieving is presented in Figure 3.2. The smallest (< 48 µm) biofloc size was dominating as 44.8% of the total biofloc volume consisted of < 48 µm floc particle. The 48 – 100 µm and > 100 µm floc fractions represented 26.0% and 29.2%, respectively, of the total volume of un-sieved bioflocs.

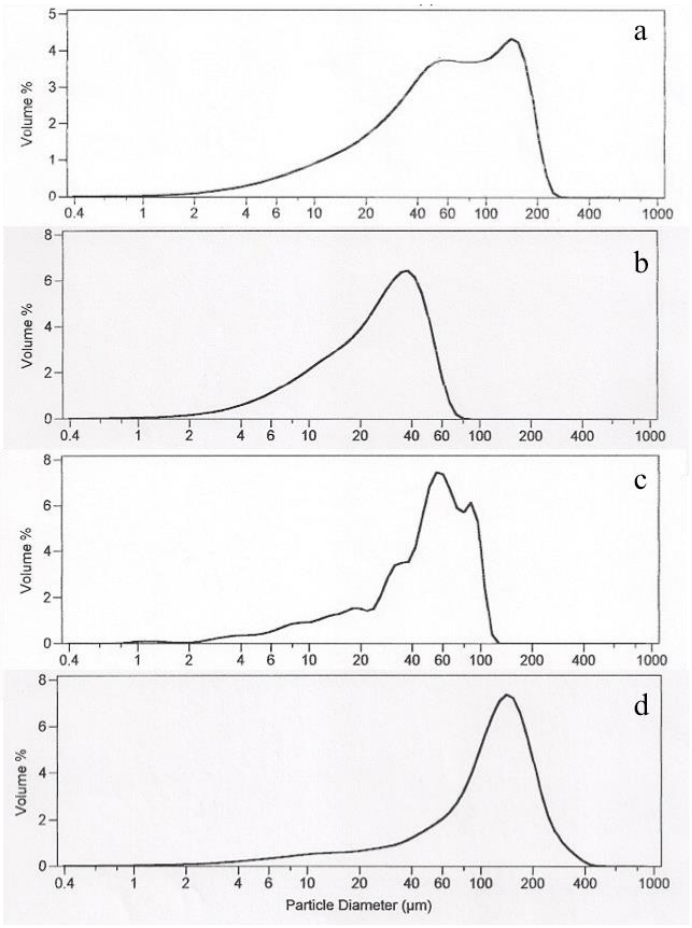

Figure 3.2. Volumetric floc size distribution of a) the un-sieved biofloc (mean particle diameter 73.83 ± 54.30 µm), b) the biofloc of the <48 µm fraction (mean particle diameter 26.84 ± 14.70 µm), c) the biofloc of the 48 – 100 µm fraction (mean particle diameter 50.75± 27.60 µm), and d) the biofloc of the >100 µm fraction (mean particle diameter 119.4 ± 71.80 µm). The area under the curve represents the total floc volume, which is 100%.

3.3.2 Biofloc protein, lipid and essential amino acids composition

The protein content of the bioflocs increased according to the particle size (Table 3.1). The > 100 µm floc size group also contained the highest lipid content, whereas the lowest lipid content was observed in the 48 – 100 µm biofloc.

Table 3.1. Nitrogen, protein and lipid content of biofloc of different size classes

Biofloc size class	Nitrogen content (% on dry weight)	Protein content (% on dry weight)	Lipid content (% on dry weight)
Un-sieved	4.0	25.0	7.2
< 48 µm	2.8	17.2	6.7
48 – 100 µm	3.7	23.4	6.0
>100 µm	4.5	27.8	7.5

The amino acid composition of the bioflocs differed amongst biofloc size groups (Figure 3.3). In general, bioflocs seem to be rich in valine, lysine, leucine, phenylalanine and threonine but deficient in methionine. Furthermore, though not essential, there was no cysteine found in any of the biofloc group. Nevertheless, the essential amino acid index values of bioflocs in the present experiment was in a high range of 0.81 – 0.97 (Figure 3.4).

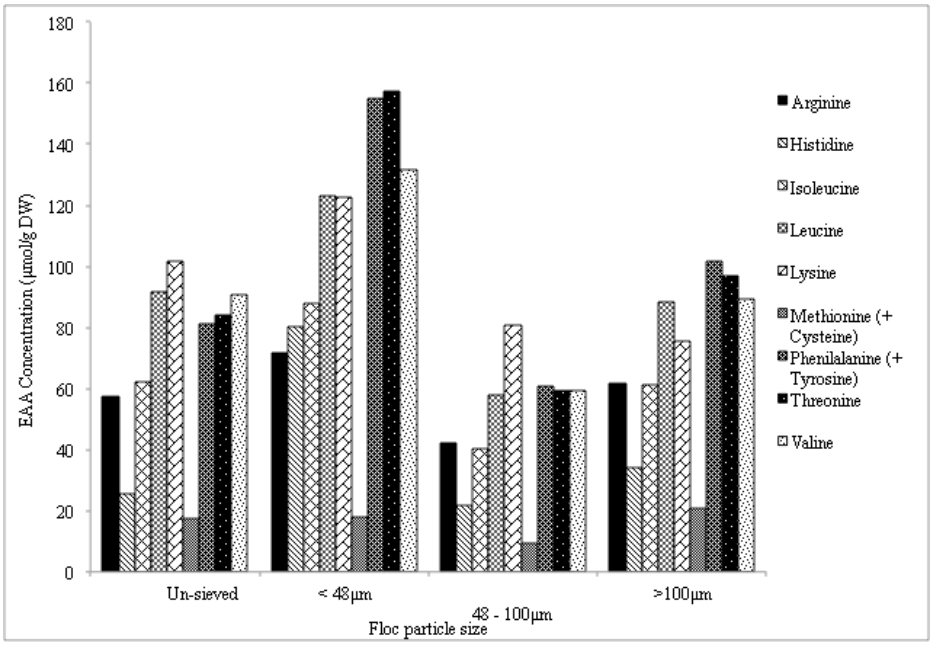

Figure 3.3. Essential amino acids pattern (µmol/g floc dry weight) of bioflocs of different size classes

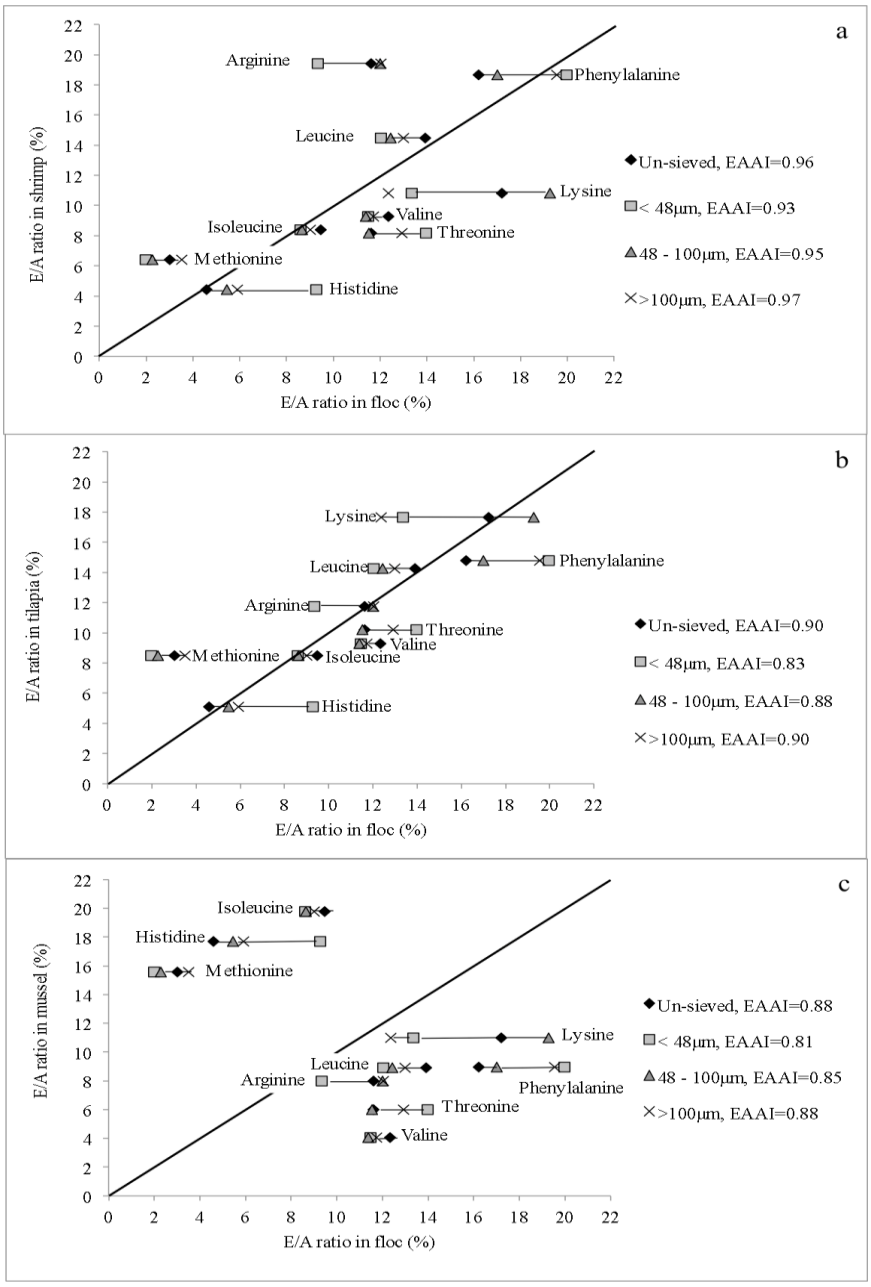

Figure 3.4. Relationship between the E/A ratio in flocs of different size classes with a) the E/A ratio in shrimp (shrimp whole body E/A ratio data from Tacon et al. (2002)); b) the E/A ratio in tilapia (tilapia whole body E/A ratio data from Clement and Lovell (1994)); and c) the E/A ratio in mussels (mussel meat E/A ratio data from Babarro et al. (2011)). The 45º line represents the situation where the E/A ratio in the flocs and in the animal are in perfect balance. Above the line, there is a shortage in the flocs in the essential amino acid under consideration whereas below the line there is an excess. The EAAI for each floc size is indicated in the figure.

3.3.3 Biofloc consumption

The calculated consumption of the flocs by the tested animals is given in Table 3.2. After 4 days, the < 48 µm floc was completely consumed by all animal species. Tilapia showed no preference regarding floc size as they consumed all the flocs irrespectively of the size. Though the differences were not significant ($P < 0.05$), the highest floc consumption by shrimp and mussels was observed for the flocs of 48 – 100 µm (64 and 57 g TSS/kg biomass, respectively).

Table 3.2. Calculation of the biofloc uptake by the animals during the experimental period. Values are means ± standard deviation. TSS=total suspended solids.

	Floc Size			
	Un-sieved	< 48 µm	48 – 100 µm	> 100 µm
Initial biofloc TSS (g/L)				
	0.41 ± 0.09	0.20 ± 0.08	0.48 ± 0.08	0.58 ± 0.03
Biofloc TSS after 4 days (g/L)				
Shrimp	0.25 ± 0.03	0.01 ± 0.01	0.15 ± 0.11	0.42 ± 0.03
Tilapia	0.0 ± 0.0	0.0 ± 0.0	0.04 ± 0.05	0.04 ± 0.06
Mussel	0.16 ± 0.04	0.01 ± 0.01	0.26 ± 0.07	0.35 ± 0.08
Biofloc TSS reduction (g/L)				
Shrimp	0.16 ± 0.03	0.19 ± 0.02	0.33 ± 0.12	0.17 ± 0.03
Tilapia	0.40 ± 0.00	0.20 ± 0.00	0.44 ± 0.06	0.54 ± 0.09
Mussel	0.24 ± 0.04	0.18 ± 0.02	0.22 ± 0.10	0.23 ± 0.08
Animal wet weight (g)				
Shrimp	11.5 ± 1.3	10.4 ± 1.5	10.6 ± 1.3	9.8 ± 0.6
Tilapia	9.0 ± 0.7	10.0 ± 1.0	9.4 ± 0.9	9.3 ± 0.9
Mussel	10.2 ± 1.1	10.2 ± 1.1	10.3 ± 1.2	10.4 ± 1.5
Biofloc uptake (g TSS/kg animal wet weight)**				
Shrimp	27.9 ± 6.5^{a}	≥ 36.5 ± 7.2^{a}***	64.2 ± 22.6^{a}	34.3 ± 7.6^{a}
Tilapia	≥ 88.9 ± 6.4^{ab}***	≥ 39.3 ± 3.9^{a}***	≥ 93.2 ± 11.2^{ab}***	≥ 117.3 ± 24.9^{b}***
Mussel	47.9 ± 4.3^{a}	≥ 36.0 ± 4.8^{a}***	57.2 ± 22.4^{a}	41.6 ± 5.5^{a}*

* n=3 (due to 40% of mortality in mussels fed with > 100 µm size floc)
** Different superscript letters in the same row indicate significant differences ($P < 0.05$).
Biofloc uptake was calculated by the formula:
Biofloc TSS reduction (g/L) x 2 L tank volume x 1000 / animal wet weight (g)
*** minimal value (≥) because biofloc suspension was completely consumed after 4 days of feeding experiment

3.3.4 Nitrogen recovery from the flocs

The nitrogen in the animals that could be derived from the bioflocs after the 4 days feeding experiment is given in Table 3.3. The nitrogen uptake from bioflocs with a particle size of > 100 µm was found to be the highest regardless of the animal species under consideration. Shrimp and mussel seemed to take up the least N from un-sieved flocs (7 mg N/shrimp and 5 mg N/mussel). Tilapia showed the least N uptake from un-sieved and the 48 – 100 µm flocs (10 mg N/tilapia).

Table 3.3. Nitrogen content of the flocs, and nitrogen recovery by shrimp, tilapia and mussel from flocs of different sizes. Values are means ± standard deviation. Calculation of the nitrogen uptake by the animals from the biofloc is performed by means of the stable isotopic ^{15}N tracer data $(^{15}NH_4)_2SO_4$

	Floc Size			
	Un-sieved	< 48 µm	48 – 100 µm	> 100 µm
^{15}N % a.e. in flocs	6.6	3.1	8.6	2.2
Total N available in biofloc (mg) *	32.8	11.0	35.7	51.6
^{15}N % a.e.				
Shrimp	0.18 ± 0.09	0.16 ± 0.17	0.72 ± 0.36	0.43 ± 0.09
Tilapia	0.39 ± 0.15	0.38 ± 0.15	0.49 ± 0.14	0.47 ± 0.17
Mussel	0.60 ± 0.14	0.28 ± 0.11	0.95 ± 0.63	0.31 ± 0.04**
Total N in animals (mg N/animal)				
Shrimp	242 ± 21	204 ± 11	229 ± 12	198±27
Tilapia	170 ± 3	187 ± 19	176 ± 17	159 ± 17
Mussel	57 ± 4	60 ± 7	59 ± 5	93 ± 28**
Total N uptake from biofloc (mg N/ animal)***				
Shrimp	7 ± 4^{a}	11 ± 1^{a}	19 ± 9^{ab}	40 ± 12^{a}
Tilapia	10 ± 4^{a}	27 ± 3^{b}	10 ± 4^{a}	34 ± 8^{b}
Mussel	5 ± 1^{a}	5 ± 2^{a}	6 ± 4^{a}	13 ± 2^{b}**
N recovery (%)****				
Shrimp	21 ± 12^{a}	95 ± 5^{a}	53 ± 26^{a}	78 ± 23^{a}
Tilapia	32 ± 12^{ab}	245 ± 29^{b}	29 ± 10^{a}	66 ± 15^{ab}
Mussel	16 ± 4^{a}	49 ± 18^{a}	18 ± 10^{a}	26 ± 4^{a}

* Calculated by the formula:
Initial biofloc TSS (g/L) x 2L tank volume x nitrogen content of biofloc (%)
**n=3 (due to 40% of mortality in mussel fed with > 100 µm size floc)
*** Calculated by the formula (IAEA, 2001):
^{15}N in animal (% a.e.) x Total N in animal (mg N)/
^{15}N in biofloc (% a.e.)
**** Different superscript letters in the same row indicate significant differences ($P < 0.05$). N recovery was calculated by the formula:
(Total N derived from biofloc/Total N available in biofloc) x 100%

3.4 Discussion

The dominance of flocs of < 48 μm in the un-sieved biofloc was also supported by the mass fractions of the biofloc suspension before sieving as this was 53% (1.86 g) for the < 48 μm floc fraction, 12% (0.42 g) for the 48 – 100 μm floc fraction, and 36% (1.25 g) for the > 100 μm floc fraction, respectively. Expressed as volume/weight (%/%) ratio, this shows that the flocs of 48 – 100 μm were less dense (2.20) as compared to the < 48 μm flocs (0.85) and the > 100 μm flocs (0.82). The low density of floc aggregates may be attributed to the density of filamentous bacteria in the floc (Barbusinki and Koscielniak, 1995).

Interestingly, bioflocs of different sizes were characterized by a different nutritional composition. The high protein and lipid content of the flocs of > 100 μm might be attributed to the concentration of extracellular polymeric substances (EPS), which can account 10 – 40% of the VSS (Frolund et al., 1996, Comte et al., 2006, D'Abzac et al., 2010). Protein content in the diet is an important factor for the nutrition of most aquatic organisms. Most of aquaculture species require protein at a range of 20 – 50% in their diet (Tacon, 1987a).

Protein quality of a diet for aquaculture species is determined by the amino acid composition of the protein and the bioavailability of the amino acids present. According to the nutritional requirement and the capability of amino acid synthesis by an organism, an amino acid is classified as nutritionally essential (indispensable) or non essential (dispensable) for fish (Li et al., 2009). EAAs are those that either cannot be synthesized or are inadequately synthesized *de novo* by animals relative to their needs and consequently must be provided by the diet. As amino acids are building blocks for protein, an optimal synthesis of protein is therefore determined by the dietary amino acid profile (Mente et al., 2002). It has been reported that the dietary amino acid requirement of a particular aquatic organism is strongly depending on its body amino acid composition (Tacon, 1987a).

Penaflorida (1989) suggested the calculation of the EAAI to evaluate the EAA profile in the diet relative to the EAA composition of the animal and classified a diet

to be of good quality with an EAAI of more than 0.9, to be useful when it is in the range of 0.7 – 0.9, and to be inadequate when it is less than 0.7. The EAAI values in Figure 3.4 show that the EAA composition of bioflocs of all sizes in general could meet the requirement of the aquatic species tested. Based on this index, bioflocs produced in this study can be considered as a good quality protein source for shrimp and a useful protein source for both tilapia and mussel. The figure also shows that flocs with a particle diameter of more than 100 µm consistently showed the highest EAAI for the three species tested, which suggests that the > 100 µm floc size group was higher in quality in terms of EAAs than the other floc size groups in this study. Figure 3.4 also shows the situation for each of the EAAs individually as it relates the E/A ratio of the biofloc (what is available) with the animal E/A (what is required according to literature data). The diagonal line represents the situation where the E/A ratio of the biofloc equals that of the animal and is the ideal case (Tacon, 1987a). Data points that are situated below the diagonal line indicate an excess of that EAA in amino acid profile of the biofloc, whereas data points situated above the diagonal line indicate a shortage in EAA. In this respect, it can be seen that there were some EAAs lacking in each of the floc size classes depending on the species under consideration. For shrimp, bioflocs lacked mainly arginine and to a lower degree leucine and methionine whereas for tilapia the flocs were relatively deficient in methionine, arginine and lysine (Figure 3.4a and 3.4b). The requirement of EAAs for mussels seems to be different from shrimp and tilapia (Figure 3.4c). It can be seen that for mussels, the biofloc E/A ratio was considerably different from the mussel flesh E/A ratio and it was found to be deficient in isoleucine, histidine and methionine.

All animal species completely consumed the < 48 µm flocs, and therefore the calculated TSS uptake values for the < 48 µm flocs should be considered as minimum values and would thus most probably have been higher in the case of more biofloc availability. The floc consumption of tilapia was in the range of 39 to 117 g TSS/kg tilapia, but since tilapia was observed to completely consume all the flocs on the second day of experiment, the actual floc consumption could be higher than these values. Furthermore, it is also important to note that the calculated TSS uptake may

have been a slight underestimation of the actual floc consumption as there was the possibility of minor floc formation during the experiment due to nutrient recycling. This is suggested by the observation in the biofloc control tanks where an average TSS increase of 12% occurred (data not shown).

The data in Table 3.2 show that tilapia can harvest all the flocs regardless of size, and this could be attributed to the nature of tilapia allowing it, as a filter feeder, to harvest also the small-sized biofloc particles. As shrimp have a nibbling feeding behavior, it was expected that the shrimp of 10 g size would prefer to consume bigger floc. The results, however, showed that shrimp of this size also consumed small floc particles. Surprisingly, mussels appear to also consume larger floc sizes. However, it is important to note that feeding with > 100 µm flocs resulted in mortality of part of the mussels (40%). This is in accordance with a previous report (Tantanasarit et al., 2013) in which it was noted that mussel gills would likely become clogged and filtration rate reduced, when exposed to a solution with too many high-sized particles. This evidently implies that the application of biofloc technology in mussel culture will be limited to smaller particle sizes of bioflocs.

Regardless of the N uptake values, consumption of the < 48 µm bioflocs led to the highest N recovery from the bioflocs by all the animals tested, which may suggest that this particular floc size was more digestible and better utilized. A more than 100% value was noticed for the < 48 µm flocs consumed by tilapia (245%) which was also significantly higher than those of other floc size groups. This value is not possible and is likely due to an inconsistency in one of the analyses. This extremely high value could only be caused by the overestimation of N uptake by the animal and/or the underestimation of initial total N available in biofloc. It can be seen in Table 3.3 that the concentration of ^{15}N in the animals and biofloc as well as the total N in the animals are within a close range, suggesting that these values are most likely to be correct. The total N uptake from biofloc values have been verified by calculating the range of contribution total N uptake from biofloc of the conventional feeding (Avnimelech and Kochva, 2008). It is found that the contributions of N uptake from bioflocs of the conventional feeding were in the range of 11 – 69% which

are in agreement to previous study by Avnimelech and Kochba (2009) and Burford et al. (2004). However, verification on the N content of biofloc showed that the C/N ratio of biofloc of the < 48 µm flocs was higher as compared to other floc size groups as well as that reported by previous study (Avnimelech, 2007). This suggests that there was an incosistenscy in the analyses of the N content of biofloc of this particular size. The trend for the N recovery does, however, not change as the result of this value. Both shrimp and tilapia showed a considerably higher N recovery from the bioflocs than mussel, irrespectively of the floc size.

Bureau (2004) noted that the main factors affecting nitrogen retention by fish are amino acid composition (the concentration and profile), and the balance between digestible protein and digestible energy. Therefore, N recovery results may indicate that from a nutritional point of view, bioflocs of < 48 µm and > 100 µm particle sizes could meet the requirement of the tested animals. This is supported by the amino acids profile mentioned earlier. Although the protein content of < 48 µm floc was the least amongst other floc size groups, the concentration of essential amino acids in this particular floc size was generally the highest. The > 100 µm floc class was also superior with regard to protein and lipid content as well as the essential amino acids balance represented by EAAI value.

Shrimp, tilapia and mussel were able to consume and retain N from bioflocs at all particle sizes tested. Caution, however, must be taken in providing > 100 µm flocs for mussel. In term of nutritional value, overall results also showed that flocs with particle size of > 100 µm and < 48 µm are more favourable for the aquaculture species tested in this study. Therefore particle size seems to play an important role in the quality of bioflocs and consequently affects the capacity of N retention by each animal. The capability of shrimp, tilapia and mussel in utilizing bioflocs in this study may also be used as a basic information to develop an integrated multi-trophic – biofloc system in which managing suspended solids in BFT systems and enhancing nutrient utilization efficiency for a more sustainable and environmentally friendly aquaculture practices will be important.

CHAPTER 4 BIOFLOC-BASED SHRIMP INTEGRATED MULTITROPHIC CULTURE SYSTEM

The present study assessed the production, water quality, and nutrient recovery of biofloc-based shrimp-tilapia co-culture and integrated multitrophic culture systems. The first experiment consisted of a shrimp monoculture system as the control and sequential shrimp-tilapia co-culture systems at three different tilapia densities. Tilapia initial densities tested in the experiment were determined relative to shrimp biomass, i.e. ST5 (5% of shrimp biomass), ST22 (22% of shrimp biomass), and ST45 (45% of shrimp biomass). The results of the first experiment showed that co-culture with tilapia seems to alter water quality profile and floc physical characteristics. TSS levels of ST5 were constantly higher than those of other treatments, while TSS levels of the ST45 treatment were comparable to those of the control. This may indicate that the fish biomass in this co-culture system considerably affected the balance of biofloc generation and utilization by the tilapia. Shrimp-tilapia co-culture significantly improved total feed phosphorus utilization efficiency. The production, water quality, and nutrient recovery of biofloc-based shrimp integrated multitrophic culture (IMT-culture) were evaluated in the second experiment. Tilapia, mussel and seaweed were added at a density of 45/tank, 10/tank and 400 g/tank, respectively. Shrimp IMT-culture significantly increased productivity, feed efficiency, and total nitrogen and phosphorus recovery. Total suspended solids level was also reduced in IMT-culture system, whereas dissolved inorganic nitrogen concentrations were not significantly affected.

4.1 Introduction

In biofloc systems, where the availability of carbon and nitrogen is steered to accommodate waste nutrients conversion by microbial biomass, the increase of nitrogen loading during the culture period would likely cause microbial biomass accumulation in the system (Avnimelech, 2012, De Schryver et al., 2008). As the microbial biomass production occurs at a faster rate than the consumption rate, high density of microbial biomass is inevitable (Ray et al., 2010a and 2010b). This biomass accumulation may hamper the growth of the cultured animals by directly causing gill occlusion (Schveitzer et al., 2013), or indirectly affecting the water quality and stability through the consumption of dissolved oxygen and the production of other metabolites. Furthermore, anaerobic zones may also be generated if the mixing capacity fails to keep this biomass in suspension (Avnimelech, 2012 and Crab et al., 2012). Considering the beneficial effects of biofloc technology application for a sustainable and ecologically friendly shrimp culture, finding solutions to overcome the accumulation of microbial biomass in the biofloc culture system is an important priority. Ray et al. (2011) demonstrated that it is possible through physical intervention to minimize floc accumulation in biofloc-based shrimp culture by regular floc removal through settling chamber. This, however, would need extra investment and most importantly in term of nutrient utilization, the sludge removal would also mean a loss of nutrients.

Although it is not yet a common practice among farmers, shrimp polyculture or integrated culture system has been perceived as one of the sustainable approaches to increase aquaculture productivity (Martínez-Porchas et al., 2010). There have been many studies on shrimp integrated culture (Cruz et al., 2008, Muangkeow et al., 2007, Tian et al., 2001, Wang et al., 1998, Yi and Fitzsimmons, 2004 and 2011, and Yuan et al., 2010). The application of this system has been shown not only to increase production and eventually economic return but also to increase the sustainability and ecological return by facilitating more efficient use of space, water, and food nutrients, as well as reducing waste production (Brune et al., 2003, Martínez-Porchas et al., 2010, Neori et al., 2004, Wang et al., 1998, and Yuan et al., 2010). Furthermore,

some studies reported that shrimp-tilapia co-culture and polyculture systems have played an important role in disease control in shrimp culture, including luminous bacterial disease (Cruz et al., 2008, Tendencia, 2007, and Tendencia et al., 2006), acute hepatopancreatic necrosis disease (AHPND) (Tran et al., 2013a), and viral diseases such as WSSV (Withyachumnarnkul et al., 2013, Verdegem and Tendencia, 2013). It has been suggested that this beneficial effect of polyculture in shrimp disease control is related to the alteration of microbial community composition in the water due to the selective feeding and foraging by the fish, bioturbation of pond sediment that improves sediment and water quality, as well as the release of antimicrobial substances from the skin and gut mucus of the fish (Cruz et al., 2008).

Combining the culture with other species, in particular those that show higher capacity in harvesting the flocs might be an alternative solution to control microbial floc density and to increase the production and nutrient utilization efficiency of a biofloc system. Waste nutrients from microbial solid waste mineralization and metabolic products of the shrimp as the major fed organism accumulate in the form of dissolved waste. Heterotrophic bacteria in bioflocs immobilize parts of the dissolve nutrient waste into new biomass that can be consumed by the shrimp or by tilapia and mussel. Biomass generation by seaweed, which uptakes parts of the dissolved nutrients, and by tilapia and mussel that consume bioflocs, is expected to contribute to the total harvested biomass. If nutrient consumption and waste generation is in equilibrium, it is expected that nutrient utilization efficiency will be higher and only limited waste is discharged.

The present study consisted of two consecutive experiments. The first experiment aimed to evaluate the effects of different density of tilapia on biofloc-based shrimp-tilapia co-culture, whereas the second experiment compared the production performance of biofloc-based shrimp monoculture and sequential integrated multitrophic culture systems. In a previous study, Ekasari et al. (2014) showed that tilapia and mussel could harvest bioflocs and are therefore potential species for biofloc-based shrimp integrated multitrophic culture system. The use of seaweed as a waste nutrient bioremediator in aquaculture system has been well

documented (Neori et al., 2004), and therefore the addition of this biota into the system can be expected to improve water quality and overall nutrient utilization efficiency in the integrated multitrophic culture (IMT-culture) system.

4.2 Materials and methods

4.2.1 Experimental Design

4.2.1.1 Experiment **1**

A completely randomized experimental design with four treatments (in triplicates) was conducted for a rearing period of 6 weeks. The treatments consisted of a shrimp monoculture system as the control, and three shrimp-tilapia co-culture systems with different tilapia initial biomass proportions relative to the shrimp biomass, namely ST5 (5% of shrimp biomass), ST22 (22% of shrimp biomass), and ST45 (45% of shrimp biomass). Shrimp monoculture system was conducted with the same system configuration, only the tilapia tank was left empty.

4.2.1.2 Experiment 2

The second experiment consisted of two treatments in quadruplicate, shrimp monoculture and shrimp IMT-culture, and conducted for a rearing period of 7 weeks. Similar to the first experiment, the shrimp monoculture system was configured at the same manner as the integrated culture system; only the tilapia and mussel tanks were left empty. Red tilapia density was set according to the results of the first experiment. The density of mussel was determined according to green mussel filtration rate (50L/ind) as described in Tantanasarit et al. (2013). Seaweed density was determined according to Lapointe and Ryther et al. (1978).

4.2.2 Experimental setup

The experiments were performed in the Department of Aquaculture, Bogor Agricultural University, Indonesia. The first experiment was performed indoor in

June – August 2013, whereas the second experiment was conducted in semi outdoor facility in November – December 2013. In the first experiment, each rearing unit consisted of two plastic tanks with a dimension of 54 cm x 36 cm x 45 cm and a working volume of 60L. These tanks were arranged in a recirculating system with a hydraulic retention time (HRT) of 5.6 h per tank (Figure 4.1). For the second experiment, each rearing unit consisted of 3 plastic tanks, with similar dimension as in the first experiment, arranged in a closed system with similar HRT as the first experiment (Figure 4.2). Tilapia compartment in experiment 2 was arranged with two settlement plates horizontally fixed on two sides of the tank. This modification allowed larger flocs to concentrate in the tilapia compartment while only small flocs went into mussel-seaweed compartment (Figure 4.3). An air blower supplied aeration to all treatment tanks. Water heaters were also installed in each tank to maintain the temperature. No water replacement was performed, whereas freshwater addition was only added to replace losses due to evaporation. Sodium bicarbonate was added following the calculation described in Furtado et al. (2011) on week 4 to maintain alkalinity level of more than 100 mg $CaCO_3$/L.

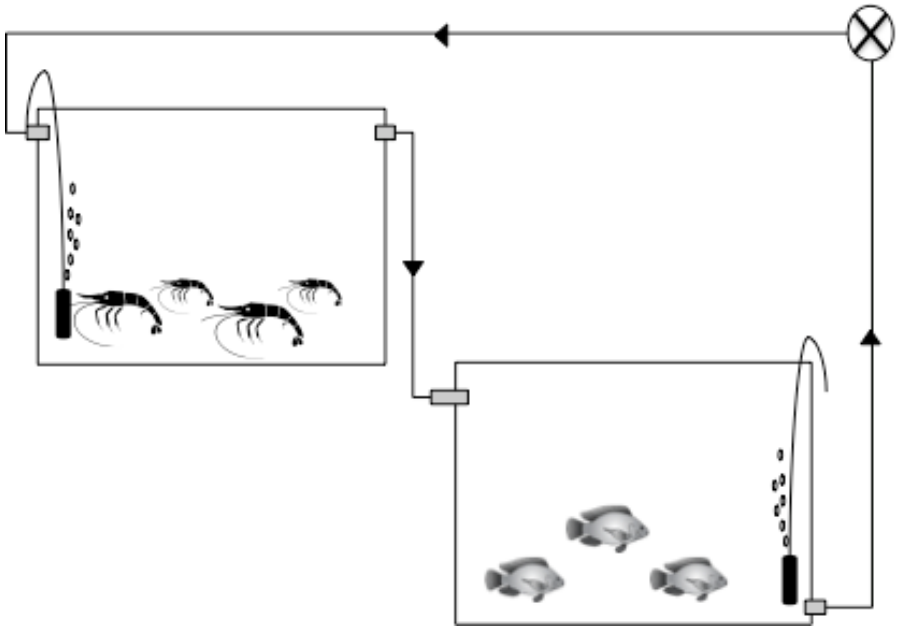

Figure 4.1. Schematic illustration of tanks configuration in each rearing unit in experiment 1

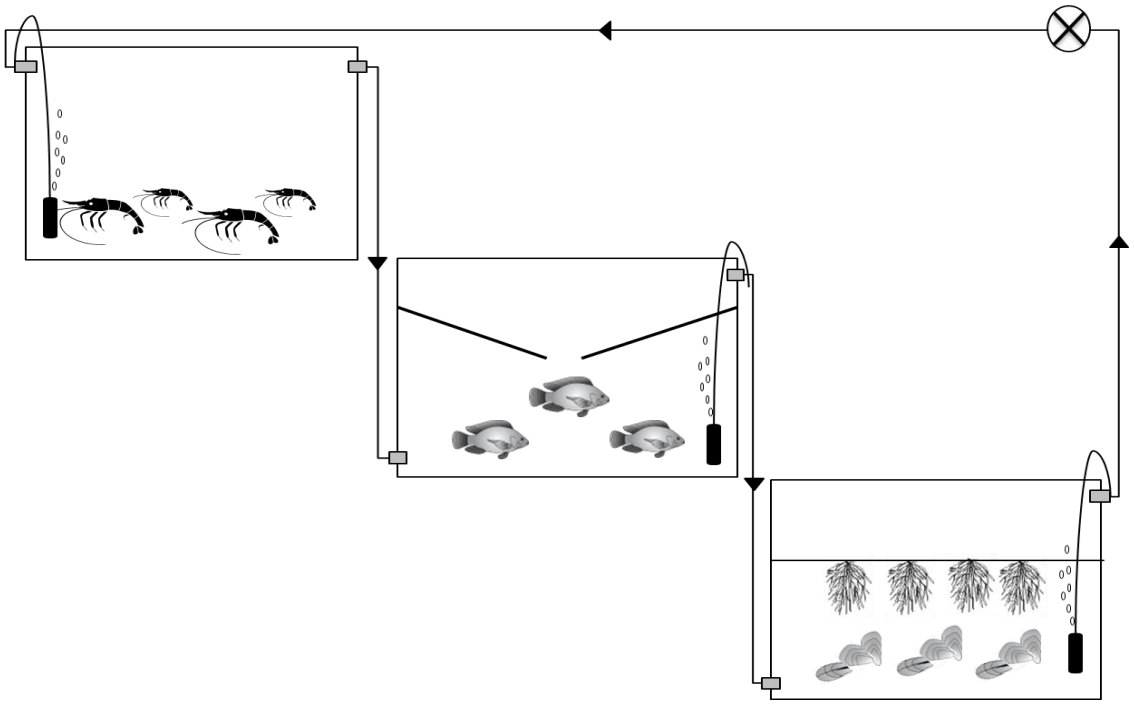

Figure 4.2. Schematic illustration of tanks configuration in each rearing unit in experiment 2

4.2.3 Experimental organisms

4.2.3.1 Experiment 1

Post larval of Pacific white shrimps (*Litopenaeus vannamei*) were obtained from a commercial hatchery (PT. Suri Tani Pemuka) in Anyer, Banten, Indonesia and were reared until they reached the experimental size. The shrimps at an initial average body weight of 5.80 g were randomly stocked at a density of 103/m^2 in one of the tanks. For the first 3 weeks the shrimps were maintained in the tank with regular organic C addition to allow floc accumulation to a TSS level of more than 100 mg/L. The shrimps were fed 4 times daily (07.00, 11.00, 15.00, and 19.00) at a level of 2.5 – 4.5% of biomass using a commercial pellet containing 36% protein (PT. Matahari Sakti). An organic carbon source, molasses (53% C), was applied daily to all shrimp tanks with an estimated C/N ratio of 15 calculated according to De Schryver et al., (2008) and Ebeling et al., (2006).

Red tilapia (*Oreochromis niloticus*) juveniles were obtained from a commercial hatchery in Bogor, Indonesia, and gradually acclimated to 35 g/L salinity for 3 weeks. On day 21, the tilapias that had reached an average body weight of 1.93 g were randomly assigned to the second tank of each rearing unit at the density according to the treatment. No external feed was given to the fish during the experiment.

4.2.3.2 Experiment 2

Shrimp post larvae and red tilapia juveniles were obtained from the same commercial hatcheries as in the first experiment. Seaweed (*Gracilaria verucossa*) and mussel (*Perna viridis*) were obtained from the seaweed pond farm in Muara Gembong, and mussel farm in Ancol, Indonesia. All the organisms were acclimated to laboratory conditions for 2 – 3 weeks prior to experimentation. Red tilapia juveniles were gradually acclimated to 30 g/L salinity for three weeks. The shrimps were reared until they reached a size of 5.23 g. The shrimps were then randomly assigned into the shrimp experimental tank at a density of 30 shrimps/tank (154/m^2). Similar to the first experiment, the shrimps were maintained in the tank for three weeks until TSS reached a level of more than 100 mg/L. Feeding and molasses addition were performed in the same manner as the first experiment.

At the onset of week 4, shrimp density was partially reduced to 20/tank and red tilapia was added at an initial biomass according to the best result of the first experiment, which was 45% of the shrimp biomass. Red tilapia, mussel and seaweed were added to the system at the density of 45/tank, 10/tank, and 400 g/tank, respectively. The initial average body weight of tilapia and mussel were 1.93 g and 13 g (5.4 cm in length), respectively.

4.2.4 Water quality analyses

Daily temperature, dissolved oxygen (DO), pH, and salinity were measured *in situ* using a portable DO meter (Lutron DO-5519, Taiwan), pH meter (Lutron

YK2001PH, Taiwan) and refractometer (ATAGO 2491-MASTER S, USA). Alkalinity, dissolved inorganic nitrogen (total ammoniacal nitrogen, NO_2^--N, and NO_3^--N), and total suspended solids (TSS) were determined weekly and subsequently analysed following the procedures in the Standard Methods for the Examination of the Water and Wastewater (APHA 1998). Floc volume was measured by recording the volume of floc in a 50 mL centrifuge tube after 30 min of sedimentation, whereas floc volume index (FVI) was measured by the determination of the volume of one mg dry weight of floc suspension (De Schryver et al., 2008).

4.2.5 Zootechnical performance

Survival was expressed as the percentage of live shrimps or tilapias on the final day of the experiment relative to their initially stocked number. Shrimp growth was monitored by weekly sampling and restocking of the measured animals. Specific growth rate was calculated according to Huisman (1987) with the following formula:

$$SGR\ (\%/day) = \left(\sqrt[t]{\frac{wt}{wo}} - 1 \right) x\ 100$$

SGR = specific growth rate (%/day)

wt = final average shrimp body weight (g)

wo = initial average shrimp body weight (g)

t = experimental period (day)

Feed efficiency was calculated according to Huisman (1987) with the formula:

$$Feed\ efficiency\ (\%) = \frac{Wt - Wo}{Total\ feed}\ x\ 100$$

Wt = final biomass (g)

Wo = initial biomass (g)

4.2.6 Nitrogen and phosphorus recovery

Nitrogen and phosphorus recovery was calculated according to the following formula (Storebakken et al 2000):

$$Nutrient\ recovery\ (\%) = \frac{(Final\ biomass\ x\ Nf) - (Initial\ biomass\ x\ Ni)}{(Feed\ intake\ x\ Ndiet)}\ x\ 100$$

N is the concentration of this compound in the biota or feed, the values of i and f represent initial and final sampling day. Nitrogen concentration in the organisms and suspended solids was determined according to the Kjeldahl method (Takeuchi 1988), whereas P content was determined according to the official methods of analysis (AOAC 1984).

4.2.7 Statistical analyses

Homoscedasticity and normality of all data were assessed using Levene's test and a Kolmogorov-Smirnov test, respectively. All data in experiment 1 and 2 were shown to be normally distributed and homoscedastic. In experiment 1, the dissolved inorganic nitrogen concentrations, alkalinity, TSS, floc volume and FVI data were analysed using repeated analysis of variance (ANOVA) using the linear model with two factors (co-culture systems with different tilapia density and time), whereas the production performance, and N and P recoveries data were analysed using one-way ANOVA. In experiment 2, Student's t-test for independent samples statistical analyses was performed on the final TSS concentrations, production performance, and N and P mass balances. Repeated ANOVA using the linear model with two factors (culture systems and time) was used to analyse the alkalinity, pH, and dissolved inorganic N concentrations data. Due to the difference in sample size, comparison in production profile between shrimp-tilapia co-culture system in experiment 1 and shrimp IMT-culture in experiment 2 was performed using non-parametric statistics Mann-Whitney U test. Statistical analyses were conducted using SPSS statistics version 18 for windows (SPSS Inc.) at a significance level of 0.05. Significant differences between treatments were determined using a post-hoc Tukey test. Correlation coefficients were calculated using Pearson's Product-Moment Correlation.

4.3 Results

4.3.1 Experiment 1

4.3.1.1 Water quality

Between the monoculture and co-culture systems, no significant differences were observed in temperature and salinity, which were within the range of 27 – 32 ^{o}C and 28 – 38 g/L, respectively. Dissolved oxygen concentrations and pH tended to decrease as the rearing period progressed, ranging from 8.7 to 5.2 mg/L and from 7.6 to 7.1, respectively.

Despite the addition of sodium bicarbonate on day 28 to increase alkalinity to more than 100 mg $CaCO_3$/L, the alkalinity in treatment ST45 constantly decreased to a level of 65 mg $CaCO_3$/L (Figure 4.3). Other co-culture treatments (ST5 and ST22) showed a considerable increase of alkalinity after bicarbonate addition, but this only lasted for two weeks as on day 42, the alkalinity again dropped to less than 100 mg $CaCO_3$/L. Indeed, repeated ANOVA analyses showed that although no significant differences were observed amongst the treatments, there was a significant effect of sampling time on the alkalinity concentrations.

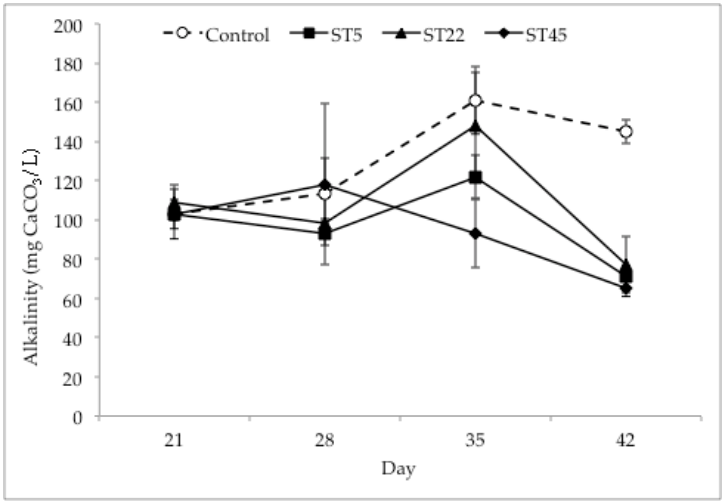

Figure 4.3. Mean values of alkalinity concentration in the rearing water of shrimp monoculture (control) and shrimp-tilapia co-culture with different tilapia density. Error bars indicate standard error of three replicates. Sodium bicarbonate was added to all tanks on day 28.

The addition of fish did not significantly affect total ammoniacal nitrogen (TAN) concentrations (Figure 4.4). Repeated measures ANOVA analyses showed that there were no significant differences observed in TAN and nitrate-N concentrations between treatments. A significant effect of time was, however, observed in these parameters. Indeed, nitrate concentrations of co-culture treatments were observed to increase significantly on day 35. There were no significant effects of treatments (tilapia co-culture density) and time observed on nitrite-N and total dissolved inorganic N concentrations in co-culture treatments.

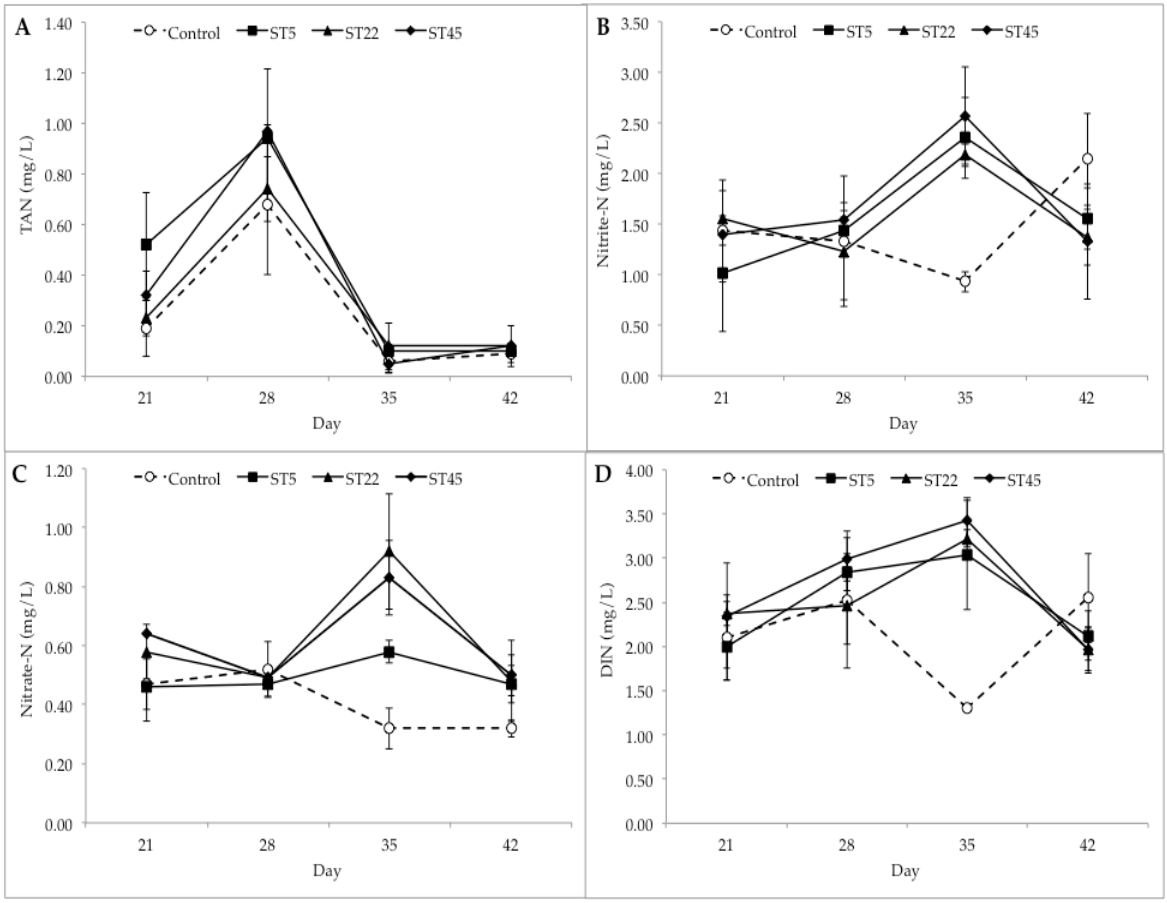

Figure 4.4. Mean values of dissolved inorganic nitrogen concentration in the rearing water in the rearing water of shrimp monoculture (control) and shrimp-tilapia co-culture with different tilapia density; (A) Total ammoniacal nitrogen, (B) Nitrite-N, (C) Nitrate-N, and (D) total dissolved inorganic N. Error bars indicate standard error of three replicates. Note: tilapia was added on day 21.

Following the addition of fish in day 21, TSS levels in ST5 and ST22 were significantly higher than those of the control and treatment ST45 in particular on day

25, 34 and 37 (Figure 4.5). On day 40, however, ST5 showed the highest TSS level, which was significantly different from those in other treatments. Furthermore, the repeated ANOVA analyses showed that TSS levels were significantly affected by time of sampling and the density of tilapia. Figure 4.6 shows that in spite of the relatively higher suspended solids concentrations in the co-culture treatments relative to the control, the volumes of the flocs in these treatments were significantly lower than those of the control. The significant effect of sampling time as well as significant interaction between factors (time and tilapia density) on floc volume showed the differences between sampling points and time-dependent effect of tilapia density. Similar trend was shown by the FVI levels of co-culture treatments that were significantly lower than those of the control. The differences were significant in particular from day 31 onwards. Compared to other co-culture treatments, it can also be seen that the TSS levels and floc volumes of ST45 were considerably lower than those of ST5 and ST22.

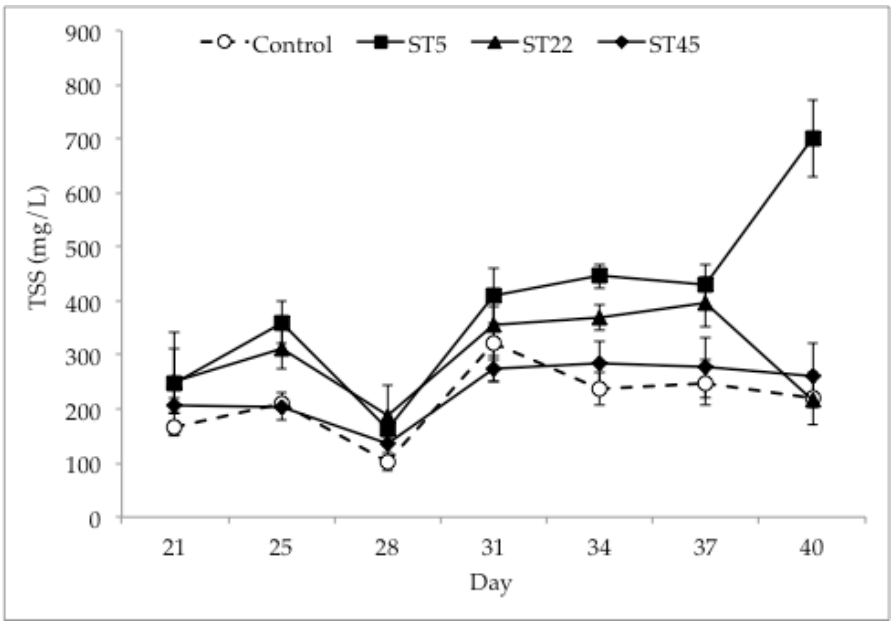

Figure 4.5. Mean values of total suspended solids levels in the rearing water of shrimp monoculture (control) and shrimp-tilapia co-culture with different tilapia density. Error bars indicate standard error of three replicates.

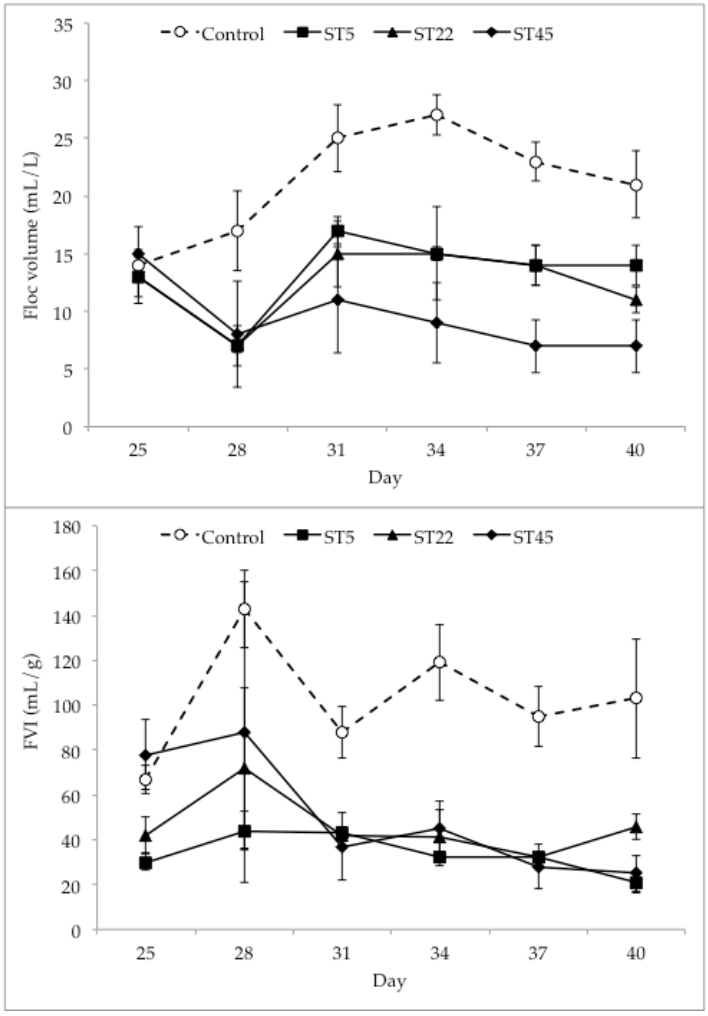

Figure 4.6. Mean values of floc volume (A) and floc volume index (B) in the rearing water of shrimp monoculture (control) and shrimp-tilapia co-culture with different tilapia density. Error bars indicate standard error of three replicates.

4.3.1.2 Production performance

There was no significant difference in the shrimp production between the treatments (Table 4.1). As it was expected, fish initial density clearly resulted in higher fish final biomass. The survival of fish in ST5 was significantly higher than that in ST22 and ST45. The tilapia biomass gain in ST22 and ST45 were significantly higher than that in ST5. However, there was no significant difference in biomass gain between ST22 and ST45. Furthermore, there were no significant differences in total biomass gain and total feed efficiency between the co-culture treatments and the shrimp monoculture.

Table 4.1. Production in biofloc-based shrimp monoculture (control) and shrimp-tilapia co-culture with different tilapia densities. Data represents means ± standard error of three replicates. Mean values (± SE) in the same row with different superscript letters were significantly different ($P < 0.05$). SGR=specific growth rate.

	Monoculture	ST5	ST22	ST45
Shrimp				
Initial biomass (g)	118 ± 4	120 ± 4	116 ± 9	116 ± 5
Biomass gain (g)	146 ± 2^{a}	130 ± 12 ^{a}	146 ± 4 ^{a}	140 ± 13 ^{a}
Survival (%)	97 ± 3^{a}	82 ± 2^{a}	83 ± 12^{a}	93 ± 5^{a}
SGR (%/day)	1.94 ± 0.03^{a}	1.77 ± 0.14^{a}	2.04 ± 0.08^{a}	1.92 ± 0.15^{a}
Tilapia				
Initial biomass (g)	-	9 ± 1	43 ± 1	89 ± 4
Biomass gain (g)	-	5 ± 1^{a}	24 ± 6^{b}	32 ± 3^{b}
Survival (%)	-	100 ± 0^{a}	81 ± 10^{b}	81 ± 2^{b}
SGR (%/day)	-	2.15 ± 0.25^{a}	1.82 ± 0.41^{a}	1.26 ± 0.14^{a}
Total biomass gain (g)	146 ± 2^{a}	135 ± 12^{a}	170 ± 8^{a}	172 ± 15^{a}
Feed efficiency (%)				
Shrimp	55 ± 2^{a}	42 ± 5^{a}	50 ± 6^{a}	52 ± 6^{a}
Total	55 ± 2^{a}	44 ± 5^{a}	56 ± 7^{a}	60 ± 7^{a}

4.3.1.3 Nitrogen and phosphorus recovery

There were no significant differences observed in total nitrogen recovery between treatments (Figure 4.7). With a significant correlation factor with the fish initial density of $r = 0.91$ ($P < 0.01$), the addition of tilapia tended to increase the P recovery. Phosphorus recovery levels of ST22 and ST 45 were significantly higher than those of the control and ST5 treatments.

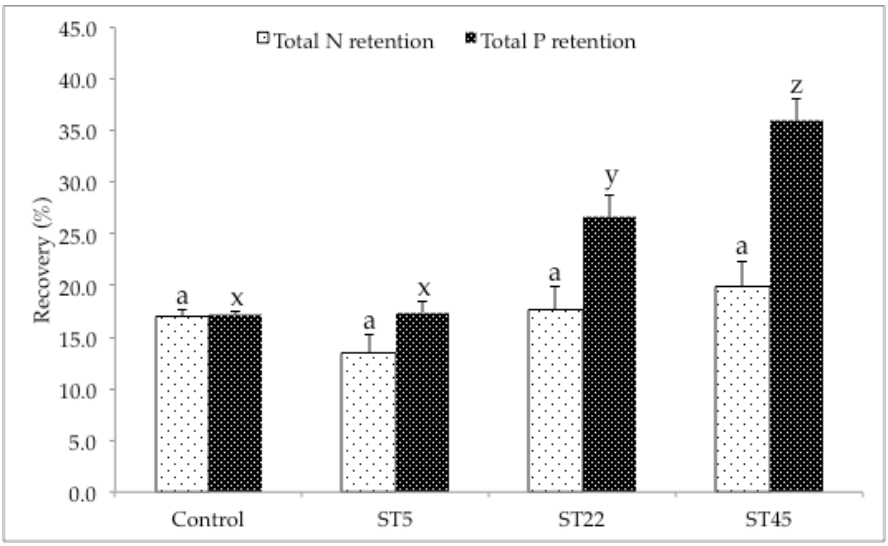

Figure 4.7. Mean values of total (feed and molasses) nitrogen and phosphorus recoveries by shrimp and fish in shrimp monoculture (control) and co-culture treatments with different density of tilapia. Error bars represent standard error of three replicates, and bars of the same series (Total N and Total P, respectively) with different superscript letters are significantly different.

4.3.2 Experiment 2

4.3.2.1 Water quality

There were no significant differences observed in temperature and salinity between treatments, with a range of 25 – 32 ^{o}C and 32 – 42 g/L, respectively. Dissolved oxygen concentrations could be maintained at a range of 5.2 – 7.8 mg/L. Despite of sodium bicarbonate addition on week 4, to obtain sufficient alkalinity level, a gradual decrease ($r = -0.96$, $P < 0.01$) of alkalinity was observed in IMT-culture system as the culture period progressed (Figure 4.8A). Similar pattern was also observed in pH, although the correlation was not significant ($r = -0.77$, $P > 0.05$) pH also tended to decrease during the experimental period (Figure 4.8B). Furthermore, repeated ANOVA analyses on both alkalinity and pH clearly suggested the significant effects of time of sampling and the culture systems ($P < 0.05$). It was observed that the mean of alkalinity and pH levels in IMT-culture system were indeed significantly lower than those in the monoculture system.

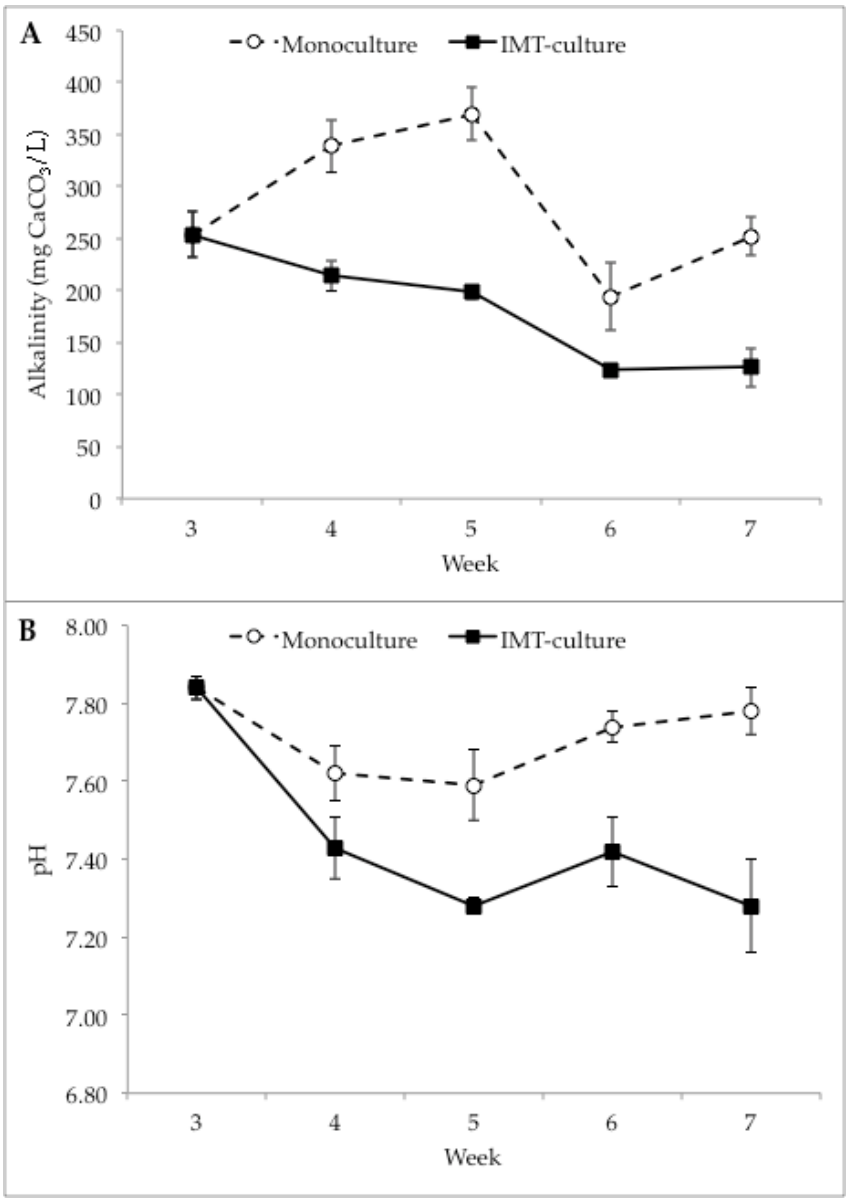

Figure 4.8. Mean values of alkalinity concentrations (A) and pH (B) in the rearing water of biofloc-based shrimp monoculture and shrimp IMT-culture. Error bars indicate standard error of four replicates.

There was no significant difference in TAN, nitrite-N and total dissolved inorganic N between culture systems (Figure 4.9). A significant effect of sampling time was observed in TAN and nitrate-N. Furthermore, nitrate-N concentrations in IMT-culture system were generally higher than those in the monoculture system ($P < 0.05$).

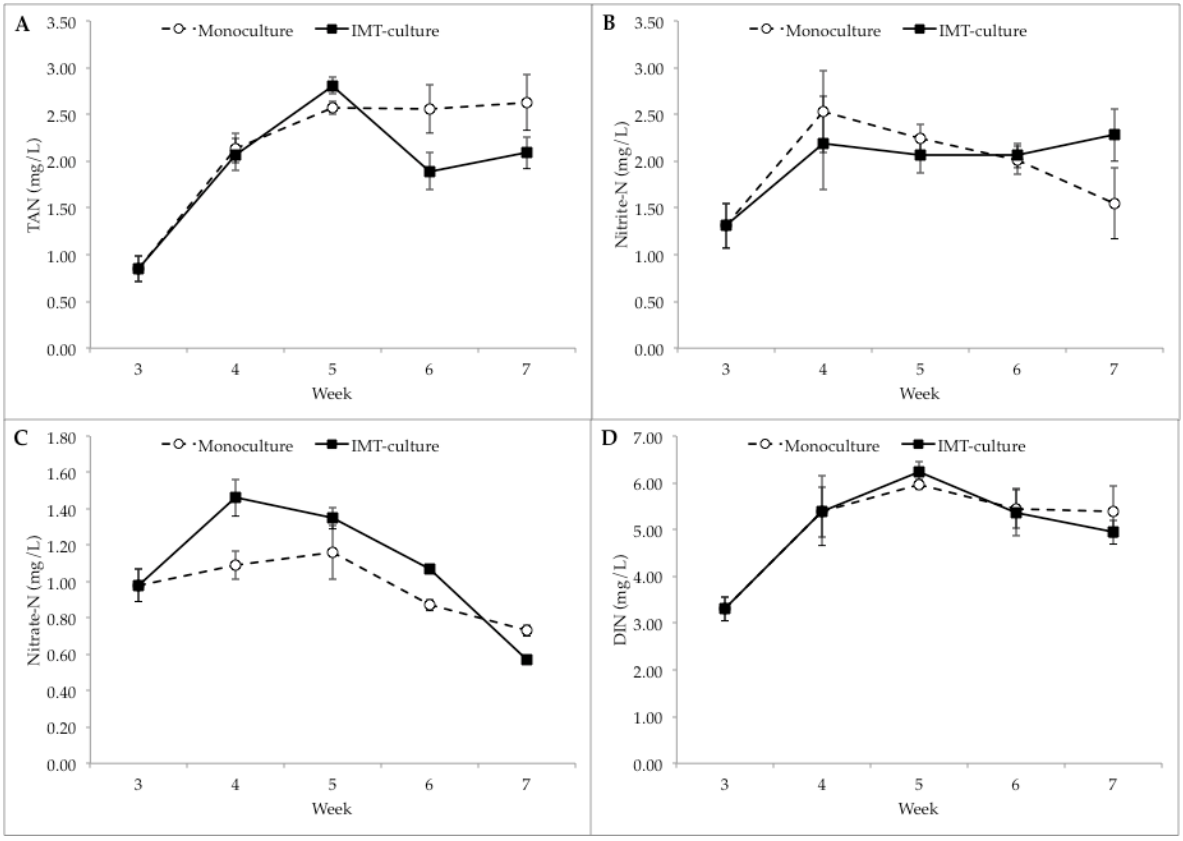

Figure 4.9. Mean values of dissolved inorganic nitrogen concentrations in the water of biofloc-based shrimp monoculture and shrimp IMT-culture; (A) Total ammoniacal nitrogen, (B) Nitrite-N, (C) Nitrate-N, and (D) total dissolved inorganic N. Error bars indicate standard error of four replicates.

Table 4.2 shows that the means of total suspended solids levels in both the shrimp and tilapia compartments in IMT-culture system on the final day of the experiment were significantly lower than those in monoculture, while that in the mussel & seaweed compartment was not significantly different. The mean TSS level of all compartment of IMT-culture was found to be 36% lower than that of monoculture system.

Table 4.2. Mean value (± SE, n = 4) of total suspended solids (mg/L) in the water of each compartment in a biofloc-based shrimp monoculture and shrimp IMT-culture on day 49. Mean values in the same row with different superscript letters were significantly different (P < 0.05). TSS=total suspended solids, IMT-culture=integrated multitrophic culture.

Compartment	TSS (mg/L)	
	Monoculture	IMT-culture
Shrimp	359 ± 26[a]	250 ± 25[b]
Tilapia	748 ± 72[a]	298 ± 59[b]
Mussel & seaweed	760 ± 59[a]	647 ± 101[a]
Mean of all compartment	623 ± 27[a]	398 ± 28[b]

4.3.2.2 Production

There was no significant effect of the culture system on shrimp survival (Table 4.3). In contrast, high mussel mortality was observed during the last week of culture period. Similar to survival, the growth rate of shrimp was also not significantly different. Compared to shrimp and tilapia, mussel seemed to grow much slower. Seaweed biomass on the final day of experiment was found to be similar to the initial biomass indicating zero growth of this biota. The contribution of tilapia and mussel productions in IMT-culture system significantly improved the system productivity as well as production per unit of water usage. Although the feed efficiency by shrimp was not significantly different amongst treatments, the total feed efficiency of IMT-culture system was significantly higher than that of monoculture system.

4.3.2.3 Nitrogen and phosphorus recovery

Nitrogen retained by the suspended solids was considerably higher in monoculture (22% of the total N input) than that in IMT-culture system (13% of the total N input) (Table 4.4). Total unaccounted N represents the dissolved organic N, and N loss through volatilization and denitrification, which were not measured in the present study. The percentage of unutilized N relative to the total N input, as

represented by the total unaccounted N and DIN, in IMT-culture system (61%) was significantly lower than that in the monoculture (74%) ($P < 0.01$). Nitrogen output in the form of shrimp was significantly higher in IMT-culture treatment, resulting in a 50% higher N recovered from feed and molasses than that of the monoculture ($P < 0.05$). Tilapia and mussels contributed 24% and 13% of the total N recovered from feed and molasses in IMT-culture system. Seaweed, however, showed negative contribution to feed and molasses N utilization. The total percentage of N recovered from feed and molasses in IMT-culture system was about two times higher than that of the monoculture system ($P < 0.01$).

Table 4.3. Mean value (± SE, n = 4) of survival, specific growth rateand feed efficiency of the biota in biofloc-based shrimp monoculture and shrimp IMT-culture. Mean values in the same row with different superscript letters were significantly different ($P < 0.05$). SGR=specific growth rate, IMT-culture=integrated multi trophic culture.

	Monoculture	IMT-culture
Survival (%)		
Shrimp	86 ± 1[a]	80 ± 5[a]
Tilapia	-	72 ± 7
Mussel	-	63 ± 9
SGR (%/day)		
Shrimp	1.47 ± 0.05[a]	1.49 ± 0.04[a]
Tilapia	-	2.17 ± 0.22
Mussel	-	0.43 ± 0.13
Seaweed	-	0.01 ± 0.11
Biomass gain (g)		
Shrimp	127 ± 6[a]	125 ± 2[a]
Tilapia	-	48 ± 5
Mussel	-	0.46 ± 0.11
Seaweed	-	1.4 ± 6.5
Total biomass gain (g)	127 ± 6[a]	175 ± 7[b]
Total feed (g)	347 ± 7[a]	336 ± 2[a]
Productivity (g/m^2)	219 ± 9[a]	299 ± 12[b]
Water utilization (m^3/kg)	1.43 ± 0.07[a]	1.04 ± 0.04[b]
Feed efficiency (%)		
Shrimp	36.7 ± 1.0[a]	37.2 ± 1.0[a]
Total	36.7 ± 1.0[a]	52.8 ± 4.7[b]

Table 4.4. Nitrogen mass balance in biofloc-based shrimp monoculture and shrimp IMT-culture. Mean values (± SE, n = 4) in the same row with different superscript letters were significantly different (P < 0.05). DIN=dissolved inorganic nitrogen, DON=dissolved organic nitrogen, IMT-culture=integrated multi trophic culture.

	Monoculture	IMT-culture
Input (g)		
Shrimp	3.24 ± 0.02	3.25 ± 0.01
Tilapia	-	1.44 ± 0.02
Mussel	-	0.35 ± 0.01
Seaweed	-	1.48 ± 0.00
Feed	18.47 ± 0.44	17.93 ± 0.17
Molasses	1.43 ± 0.03	1.38 ± 0.01
Total	23.14 ± 0.49	25.84 ± 0.18
Output (g)		
Shrimp	5.43 ± 0.11^{a}	6.45 ± 0.07^{b}
Tilapia	-	2.51 ± 0.10
Mussel	-	0.94 ± 0.08
Seaweed	-	1.05 ± 0.02
Suspended solids	5.08 ± 0.22^{a}	3.25 ± 0.23^{b}
DIN	0.97 ± 0.08^{a}	0.89 ± 0.05^{a}
Total	11.49 ± 0.27^{a}	14.83 ± 0.50^{b}
Total unaccounted N (DON, N volatilization, denitrification)	11.65 ± 0.62^{a}	10.74 ± 0.58^{a}
% N recovery from feed and molasses		
Shrimp	11.03 ± 0.57^{a}	16.58 ± 0.45^{b}
Tilapia	-	5.57 ± 0.54
Mussel	-	3.07 ± 0.44
Seaweed	-	(2.24 ± 0.09)
Total	11.03 ± 0.57^{a}	22.97 ± 1.29^{b}

The phosphorus retained in the suspended solids in monoculture system (52% of total P input) was significantly higher than that in IMT-culture system (29% of total P input) (Table 4.5). Total unaccounted P, consisting of total P in the water that was not measured in the present study, represents the unutilized P. About 35% of the total P input was not utilized in monoculture system, which was by far higher than that of the IMT-culture system (1% of the total P input). No significant difference in P recovery by shrimp between the culture systems (Table 4.5). Seaweed showed a remarkably high P retention, recovering 35% of the total feed and molasses P input. The contributions of tilapia, mussel and seaweed in shrimp IMT-culture system resulted in a 6 times higher total P utilization efficiency relative to that of monoculture system ($P < 0.01$).

4.3.3 Shrimp-tilapia co-culture vs. shrimp integrated culture systems

In general, a similar water quality profile was observed in both experiments. The reduction in alkalinity and pH in the polyculture system was, however, more evident in experiment 2. Furthermore, a higher dissolved inorganic N was also observed in IMT-culture system. Shrimp survival and growth in experiment 2 were slightly lower than those in the experiment 1. Tilapia growth on the other hand was 72% higher than that in the first experiment with the same stocking density. The percentage of enhancement (relative to the respective control in each experiment) in total biomass gain, total feed efficiency, as well as N and P recoveries in shrimp IMT-culture were higher than those in the co-culture, but only significant for P (Table 4.6).

Table 4.5. Phosphorus mass balance in biofloc-based shrimp monoculture and shrimp IMT-culture. Mean values (± SE, n = 4) in the same row with different superscript letters were significantly different (P < 0.05).

	Monoculture	IMT-culture
Input (g)		
Shrimp	0.11 ± 0.00	0.11 ± 0.00
Tilapia	-	0.25 ± 0.00
Mussel	-	0.06 ± 0.00
Seaweed	-	0.16 ± 0.00
Feed	2.56 ± 0.06	2.49 ± 0.02
Molasses	0.08 ± 0.00	0.07 ± 0.00
Total	2.75 ± 0.06	3.14 ± 0.02
Output (g)		
Shrimp	0.37 ± 0.01[a]	0.34 ± 0.01[a]
Tilapia	-	0.70 ± 0.04
Mussel	-	0.08 ± 0.00
Seaweed	-	1.06 ± 0.12
Suspended solids	1.43 ± 0.06[a]	0.92 ± 0.06[b]
Total	1.80 ± 0.07[a]	3.09 ± 0.09[b]
Total unaccounted P (Total P)	0.95 ± 0.10[a]	0.04 ± 0.11[b]
% P recovery from feed and molasses		
Shrimp	9.70 ± 0.26[a]	8.82 ± 0.33[a]
Tilapia	-	17.91 ± 1.49
Mussel	-	0.78 ± 0.13
Seaweed	-	35.03 ± 4.42
Total	9.70 ± 0.26[a]	62.55 ± 4.21[b]

Table 4.6. Comparison of the performance and nutrient recovery of shrimp-tilapia co-culture (n = 3, 42 days) and shrimp IMT-culture (n = 4, 49 days) systems. Mean values (± SE) in the same row with different superscript letters were significantly different ($P < 0.05$).

% Relative to the monoculture	Co-culture	IMT-culture
Total biomass gain	10 ± 10^{a}	38 ± 11^{a}
Total feed efficiency	10 ± 10^{a}	45 ± 13^{a}
N recovery	24 ± 13^{a}	126 ± 24^{a}
P recovery	84 ± 14^{a}	548 ± 55^{b}

4.4 Discussion

Two experiments were performed to elucidate the production, water quality and nutrients recovery of biofloc-based sequential integrated culture system. The first experiment evaluates the effects of different density of tilapia on biofloc-based shrimp-tilapia co-culture, whereas the second experiment compared the production, water quality and nutrient recovery of biofloc-based shrimp monoculture and shrimp integrated multitrophic culture with tilapia, mussel and seaweed.

4.4.1 Experiment 1

Dissolved oxygen concentration decreased during the rearing period, which might relate to the increase of biomass in the system. Although additional fish biomass might contribute to the consumption of dissolved oxygen, the differences amongst treatments were not significant. A reduction of alkalinity, by both heterotrophic bacteria and nitrifiers, seems to be a common water quality characteristic in biofloc systems (Ebeling et al., 2006 and Hargreaves, 2006,). In this regard, regular addition of alkalinity has been recommended to improve water stability (Furtado et al., 2013). In the present experiment, a constant reduction of alkalinity also occurred and it appears that the addition of fish in the co-culture system also contributed to the level of reduction. Furthermore, higher reduction

levels of alkalinity in co-culture treatments might also relate to the increase of nitrification activity in the respective treatments.

The addition of fish seems to alter the dynamics of dissolved inorganic nitrogen species in the culture water. The consumption of bioflocs by tilapia allows nitrogen assimilation but at the same time also results in the release of ammonia nitrogen as the product of protein catabolism, and therefore contributes to the accumulation of TAN. The sharp reduction in TAN concentration on day 35 might be explained by either immobilization by heterotrophic bacteria or conversion by nitrification. In control system, TAN reduction is likely to be caused by the heterotrophic bacteria assimilation rather than nitrification, as it was not followed by an increase in nitrite and/or nitrate concentrations. Carbon source addition in the present experiment was determined according to the presumptive of nitrogen utilization by the shrimp and did not include the possibility of nitrogen turnover by tilapia. Hence, it can be speculated that the added carbon source might not have been sufficient for the heterotrophic bacteria to assimilate this extra production of ammonia; instead it facilitated the nitrifiers to use the excess ammonia as a substrate. This was supported by the increase of nitrite and nitrate concentration subsequent to TAN reduction in the co-culture treatments on day 35.

One of the possible consequences of increased nitrogen turnover through the consumption of bioflocs by tilapia is the release of ammonia and accordingly the generation of more microbial biomass. It is interesting to note that TSS levels of the control were relatively lower than those of co-culture treatments except treatment ST45. This may indicate that the generation rate and consumption rate of microbial biomass in treatment ST45 were in equilibrium, thus resulting in comparable TSS levels to that of the control.

The floc volume and FVI levels (Figure 4.7) that represent floc physical characteristics were altered by the addition of fish into a biofloc-based shrimp culture system. The high FVI of the control indicated that the flocs in this system were more voluminous than that of co-culture treatments. Jin et al. (2003) noted that for the settleability of flocs in activated sludge, floc size, floc structure, and

domination of filaments could be the main influencing factors of the floc sludge volume index (SVI). The lower floc SVI in the co-culture treatments might be related to the secretion of mucus by tilapia that increases the settleability of the flocs. Shephard (1994) pointed out that the macromolecules in fish mucus are able to swell and interact with one another to give a three-dimensional structure to water. Hence, it can be expected that the mucus production by the tilapia also affected the structure of the floc, although it is not clear if that would influence FVI positively or negatively. It is also possible that the addition of tilapia to shrimp culture with biofloc system influenced the microbial community of the flocs, subsequently altering the structural characteristics of the floc. This was supported by Ray et al. (2010a) showing that suspended solids management by settling chamber resulted in different composition in the microbial communities in shrimp culture with biofloc system.

Although not significantly different from the control, it can be seen that shrimp productions in co-culture treatments were slightly lower than that of the control. Furthermore, the lower survival and growth of the shrimp in ST5 resulted in the lowest harvested biomass being 19% lower than the control. A possible explanation for this reduction in ST5 possibly relates to the higher TSS levels in this treatment that might cause gill occultation (Ray et al., 2010a and 2010b, and Schveitzer et al., 2013).

As the present study is the first studying tilapia density in biofloc-based shrimp-tilapia co-culture, the results could only be compared to co-culture systems in conventional stagnant water (Yi and Fitzsimmons, 2004 and Yuan et al., 2010) or non-biofloc sequential co-culture systems (Muangkeow et al., 2007 and 2011). As observed in the present study, those studies reported no significant effect on the shrimp growth, survival and biomass production, except Yi and Fitzsimmons (2004) who reported 19 to 29% increase of shrimp production in co-culture system relative to that of monoculture. The significant increase in total harvested biomass in co-culture system in the present study is in agreement with those studies in shrimp-tilapia co-culture. While Yuan et al. (2010) reported substantial enhancement, the

present study showed insignificant increase in total feed efficiency. High stocking density has been known to exert adverse effects on fish growth and survival. However, in this present study this was not quite evident as the growth and the biomass gain as well as the survival of the fish in ST45 were not significantly different from those in ST22.

The addition of fish biomass into shrimp culture system showed no effect on the overall nitrogen utilization efficiency, which is in contradiction to the studies by Yuan et al. (2010) and Muangkeow et al. (2011) demonstrating that tilapia addition significantly contributed to the feed nitrogen utilization efficiency in shrimp culture. Feed P recovery on the other hand was significantly improved at co-culture system with the highest tilapia density and was comparable to that of reported in sequential shrimp-tilapia co-culture (Muangkeow et al., 2011). This might be explained by the fact that tilapia retains P more efficiently relative to shrimp (0.18%/g tilapia wet weight vs. 0.06%/g shrimp wet weight), thus contributing significantly to the total P recovery in the system.

4.4.2 Experiment 2

Instead of freshwater addition to replace water loss due to evaporation, we observed that the salinity in all rearing units increased gradually to a level of 40 – 42 g/L on the last week of the experiment. The constant reduction of alkalinity in IMT-culture system might indicate that the activity of N conversion by both nitrifiers and heterotrophic bacteria was considerably higher in IMT-culture system than that in monoculture. Similar results in alkalinity were also reported by Brito et al. (2014a) in biofloc-based shrimp-seaweed co-culture. As a consequence of the decline in alkalinity, which represents the water buffering capacity, a decrease of pH was also observed in integrated culture system.

Most of the suspended solids were concentrated in tilapia and mussel & seaweed compartments. In IMT-culture system, TSS in tilapia compartment was significantly lower than that of the monoculture suggesting biofloc consumption by

tilapia. In the mussel & seaweed compartment this reduction was not significant, which might be explained by the lower consumption level of bioflocs by mussel and also the high level of bioflocs being trapped in seaweed thali. This is in agreement with Brito et al. (2014a and 2014b) who demonstrated a significant increase in settleable solids and a reduction in suspended solids in biofloc-based shrimp-seaweed co-culture system. Nonetheless, the mean of suspended solids in all compartments was significantly lower in IMT-culture than in monoculture systems, demonstrating the potential of this system to control microbial biomass in biofloc system.

The profile of TAN, nitrite-N and nitrate-N concentrations as well as the reduction of alkalinity in IMT-culture system might indicate that nitrification activities were higher than that of the monoculture. But in term of total dissolved inorganic nitrogen in Figure 4.9D, it is clear that culture systems exerted no significant effect. The consumption of bioflocs by tilapia and mussel was likely to contribute to nitrogen turnover and ultimately affected the dynamics of dissolved inorganic N. As the difference in DIN was insignificant it could be assumed that the generation rate of ammonia by tilapia and mussel were in balance with the uptake rate of this compound by either the microbes (heterotrophic and/or nitrifiers) or by the seaweed. The use of seaweed in an integrated aquaculture system has been reported to significantly reduce the concentration of DIN (Al-Hafedh et al., 2012 and Huo et al., 2012). Furthermore, Brito et al (2014a and 2014b) demonstrated that the addition of *Ulva lactuca* and *Gracilaria* in biofloc-based shrimp culture resulted in a significant reduction in TAN, nitrite and phosphate concentrations. However, an absence of growth and insignificant nitrogen recovery of seaweed were observed (see later), which may signify a suboptimal role of seaweed in DIN uptake in IMT-culture system in the present experiment.

The mortality of mussel was more pronounced during the last week of culture, which might be due to the increase of biofloc size. Exposing mussel to biofloc suspension with a size range of more than 100 μm has been reported to cause mortality most likely due to gill clogging (Ekasari et al., 2014a). As the tilapia

compartment had been modified to accommodate for the collection of large-sized bioflocs in anticipation to this possibility, it is likely that the small size flocs formed larger aggregates in the mussel & seaweed compartment.

Since the success of seaweed cultivation in shrimp pond has been well documented in some studies (Brito et al., 2014a and 2014b, Cruz-Suárez et al., 2010, Lombardi et al., 2006, and Nelson et al., 2001), the absence of growth in seaweed in the present experiment was unexpected. It has been reported, however, that salinity of more than 40 g/L may reduce the growth of some species of seaweed as a result of energy mobilization for osmotic adjustment (Choi et al., 2010, Hayashi et al., 2011, and Karsten, 2012). In this regard, the exposure of seaweed to a salinity of more than 40 g/L especially during the last week of culture period might explain the zero growth of seaweed. This is also supported by the profile of nitrogen and phosphorus content in seaweed (the later part of the discussion).

As it was expected, the contribution of additional biota in IMT-culture resulted in significantly higher productivity and total feed efficiency. Some polyculture studies showed that although no or minor effects on the yield of main fed organism is observed, the addition of other species, which utilize either the solid waste or the dissolved waste, significantly improved the total feed efficiency (Lombardi et al., 2006, Liu et al., 2014, Purcell et al., 2006, Ren et al., 2014, and Troell et al., 1999). Liu et al. (2014) recently demonstrated that combining biofloc system in shrimp-spotted scat-water spinach integrated culture resulted in a 43% higher production and 2.7 factors lower food conversion ratio (FCR) as compared to that of the same system but without bioflocs. These results suggest that bioflocs contributed significantly to the feed utilization by additional biota in the integrated system and hence to the increase of production.

The higher level of TSS in monoculture system resulted in higher percentage of N retained in the suspended solids, most likely in the form of biofloc biomass. The lower proportion of total unutilized nitrogen in IMT-culture system indicated the higher nitrogen utilization efficiency in this system. Considerably higher nitrogen recovery by shrimp in IMT-culture system, which was mostly attributed to the

higher content of N in the shrimp body (2.42 ± 0.28 vs. 1.83 ± 0.01 % wet body weight), suggests an influence of the culture system on nitrogen assimilation by the cultured animals. The level of dietary N assimilation, which represents protein retention by the shrimp, is strongly affected by the digestibility of protein and amino acids composition (Ekasari et al., 2014a). Therefore, the higher N concentration in shrimp in an IMT-culture system compared to the monoculture system might indicate a difference in the protein quality (i.e. the protein digestibility and amino acids composition) between bioflocs in monoculture and IMT-culture systems. The results of the first experiment clearly suggested that the addition of tilapia significantly affected the settling characteristics of the floc likely caused by the alteration in the floc microbial composition. It is possible that the changes in the floc microbial community composition have also influenced the biochemical composition of the respective flocs. Our previous study (Ekasari et al., 2014a) demonstrated that the different amino acids profile and essential amino acids indexes in different floc particle sizes might influence the N assimilation levels by the cultured animals. With the increase of N utilization by shrimp and the contribution from tilapia and mussel, the total feed N recovery in the IMT-culture system was significantly higher than that in the monoculture. This is supported by significantly lower level of unutilized N in the IMT-culture system. The lack of contribution of seaweed on feed N utilization was mainly due to the absence of biomass gain in seaweed as well as the reduction of N content of seaweed after the experimental period from 0.37% of wet weight at the initial to 0.26% of wet weight at the final.

Considerably high proportion of P retained in the suspended solids may indicate the potential of bioflocs in assimilating this particular nutrient. This confirms previous study (Da Silva et al., 2013) that demonstrated the level of P retention by bioflocs at about 26% of the total P input. Despite its zero growth, a significant contribution of feed P utilization by seaweed was unexpectedly observed. This mostly attributed to the increase of seaweed P content from 0.04% of wet weight in the beginning to 0.33% of wet weight at the final day of the experiment. The reduction of N and the increase of P contents in seaweed in the present study might

be related to the adverse effect of hyper salinity (> 40 g/L) exposure on seaweed (Choi et al., 2010, and Marinho-Soriano et al., 2006). Choi et al. (2010) pointed out that N uptake rate by *Ulva pertusa* was significantly reduced at a salinity of 40 g/L, whereas P uptake rate was relatively unaffected. The limited cell division and mobilization of enzymes to overcome the increased turgor pressure were considered as the main reason for a reduced N uptake rate, and consequently affecting N content of the seaweed (Kirst, 1990, and Parida and Das, 2005). Phosphorus content in seaweed on the other hand is not only determined by the P availability in the water as well as the uptake rate (Choi et al., 2010), but also by the capability of seaweed in accumulating P in anticipation to nutrient deficiency (Fogg, 1973, and Pedersen et al., 2010). Interestingly, uncoupling of growth and nutrient uptake as observed in the present experiment appears to be a characteristic of some species of macroalgae as physiological adjustment to seasonal and diurnal nutrient and environmental changes (Choi et al., 2010).

4.4.3 Shrimp-tilapia co-culture vs. shrimp integrated multitrophic culture systems

The higher reduction in alkalinity and pH seems to be related to the high dissolved inorganic N concentrations in experiment 2 as compared to experiment 1, can be explained by the higher total feed input in experiment 2 (336 g) relative to experiment 1 (255 g). Generally, both systems showed only little effect on the dissolved inorganic N concentrations. This is in agreement with Tian et al. (2001) who reported no significant difference in the dissolved inorganic N species (TAN, nitrite-N and nitrate-N) concentrations between shrimp monoculture and integrated culture systems. Liu et al. (2014) on the other hand demonstrated that the concentrations of DIN species of the rearing water of shrimp integrated culture with bioflocs were considerably lower than those of shrimp monoculture system and shrimp integrated culture without bioflocs.

Despite the zero growth of seaweed, the application of IMT-culture system appears to have more significant effects in productivity enhancement and feed nutrient utilization efficiency in biofloc-based shrimp culture than in co-culture

system. The nitrogen recoveries in both experiments in the present study were comparable to that of shrimp-tilapia-constricted tagelus, which was about 24% (Tian et al., 2001). Phosphorus utilization efficiency in biofloc-based shrimp-tilapia co-culture (31%) and shrimp integrated culture (63%) systems in the present study on the other hand were remarkably higher than that reported in non-biofloc shrimp integrated culture system that was ranging from 10 to 15% (Tian et al., 2001).

The results of the present study showed that co-culture with tilapia altered water quality profile and floc physical characteristics as well as improved the total harvested biomass and feed phosphorus utilization efficiency in the biofloc-based shrimp culture system. In addition, the stocking density of tilapia in this co-culture system significantly influenced the degree of alterations in water quality, production performance as well as the nutrient recovery of the system. The application of integrated multitrophic culture system in biofloc-based shrimp culture appears to be a promising technique to control the accumulation of microbial biomass and at the same time to increase nutrient utilization efficiency. Nonetheless the zero growth of seaweed in this system warrants further confirmation. With the high capacity of DIN uptake by seaweed and assuming that seaweed growth would be possible, a further decline in the dissolved inorganic nitrogen concentration in the rearing water in IMT-culture system could be obtained.

CHAPTER 5 IMMUNE RESPONSE AND DISEASE RESISTANCE OF SHRIMP IN BIOFLOC SYSTEM

The objective of this study was to document the immunological effects of growing shrimp in biofloc systems. The experiment consisted of four types of biofloc systems in which bioflocs were produced by daily supplementation of four different carbon sources, i.e. molasses, tapioca, tapioca-by-product, and rice bran, at an estimated C/N ratio of 15 and a control system without any organic carbon addition. Each biofloc system was stocked with shrimp juveniles that were reared for 49 days. The use of tapioca-by-product resulted in a higher survival (93%) of the shrimp as compared to the other carbon sources and the control. The highest yield and protein assimilation was observed when tapioca was used as the carbon source. After 49 days, phenoloxidase (PO) activity of the shrimp grown in all biofloc systems was higher than that of the shrimp from the control system. Following a challenge test by injection with infectious myo necrosis virus (IMNV), the levels of PO and respiratory burst (RB) activity in the shrimp of all biofloc treatments were higher than that of the challenged shrimp from the control treatment. An increased immunity was also suggested by the survival of the challenged shrimp from the experimental biofloc groups that was significantly higher as compared to the challenged shrimp from the control treatment, regardless of the organic carbon source used to grow the bioflocs. Overall, this study demonstrated that the application of biofloc technology may contribute to the robustness of cultured shrimp by immunostimulation and that this effect is independent of the type of carbon source used to grow the flocs.

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5.1 Introduction

Diseases remain a limiting factor for the aquaculture industry (FAO 2012). With respect to the shrimp culture industry, disease outbreaks have been the primary cause of production loss during the last two decades (FAO 2012). Disease outbreaks not only result from the mere presence of a pathogen in the system, a compromised health status of the cultured animals in combination with suboptimal environmental conditions are also factors facilitating disease outbreaks (De Schryver et al., 2012 and Liu and Chen 2004). Therefore, disease prevention and control should not only focus on implementing biosecurity measures, but must be performed in an integral approach involving, among others, adequate nutrition, enhancing the immunity of the cultured animals and maintaining a good water quality.

Biofloc technology has been studied in several occasions and contributes to the maintenance of good water quality in the system and to the nutrition of the cultured animals (Avnimelech, 1999). The basic principle of the biofloc system is to recycle waste nutrients, in particular nitrogen, into microbial biomass that can be used *in situ* by the cultured animals or be harvested and processed into feed ingredients (Avnimelech, 2012, Crab et al., 2007, De Schryver et al., 2008, Hari et al., 2004, and Kuhn et al., 2009). Heterotrophic microbial aggregates are stimulated to grow by steering the C/N ratio in the water through the modification of the carbohydrate content in the feed or by the addition of an external carbon source (Avnimelech, 1999), so that the bacteria can assimilate the waste ammonia for new biomass production. Biofloc systems have been shown not only to maintain ammonia below toxic levels and to improve the feed nutrient utilization efficiency of the cultured animals (Avnimelech, 1999, Hari et al., 2004, and Zhao et al., 2012), but also to provide extra nutrients (Xu et al., 2012) and exogenous digestive enzymes (Xu and Pan, 2012). Biofloc application can also lead to increased growth, survival and reproductive performance of the cultured animals (Ekasari et al., 2013 and Emerenciano et al., 2012b, 2013b).

So far, very few studies (de Jesús Becerra-Dorame et al., 2014, Kim et al., 2014, Xu and Pan, 2013 and 2014) investigated the immunological potential of the biofloc

technology although it is widely known that microorganisms, their cell components or their metabolites can act as immunostimulants that enhance the shrimp innate immune system and provide improved protection against pathogens (Smith et al., 2003 and Vazquez et al., 2009). Xu and Pan (2013) reported that the total haemocyte count and phagocytic activity of the haemocyte of the shrimp from biofloc containing culture units were significantly higher than those of the shrimp in the non-biofloc control group. Furthermore, the authors also noted that shrimp grown in a biofloc environment harbored a higher total antioxidant capacity both in the plasma and hepatopancreas. A recent study reported that the expression of six selected genes (prophenoloxidase [ProPO1 and ProPO2], serine protease [SP1], prophenoloxidase activating enzyme [PPAE1], masquerade-like serine protease [mas] and Rat-sarcoma-related nuclear protein), directly and indirectly related to the shrimp immune response, were significantly upregulated in biofloc-grown shrimp (Kim et al., 2014). Immune stimulation may thus be a very important feature in biofloc-grown shrimp contributing to disease control. It could for example (partly) explain the lower prevalence of AHPND observed in farms that apply biofloc system (NACA 2012). AHPND is currently causing very large problems in the culture of shrimp post larvae in Asia (Tran et al., 2013b).

Carbon source plays an important role in the application of biofloc technology. The sources of organic carbon in this system have been reported not only to alter the nutritional value of biofloc (Crab et al 2010b; Ekasari 2010), but also to affect the inorganic nitrogen removal rate (Avnimelech, 2009). Furthermore, as organic carbon addition in a biofloc system contributes to the operational cost, selection of functional carbon source is therefore an important consideration. The objective of this study was to perform a study on biofloc-grown shrimp. The water quality was monitored over a 49-day period in biofloc systems supplied with four locally purchased organic carbon sources; tapioca, and three agricultural by products, i.e. molasses, tapioca, tapioca by products, and rice bran. The shrimp growth performance, immune responses and resistance to the IMNV were also verified.

5.2 Materials and methods

5.2.1 Experimental design

Twenty glass tanks (90 cm x 40 cm x 35 cm) each filled with 100 L seawater were used as the experimental culture units. Temperature in all tanks was maintained in the range of 27.3 – 28.3 °C during the entire experiment, aeration was provided in each aquarium using an air blower and the light regime was set at 12h light/12h dark. Inter-molt phase Pacific white shrimp juveniles, previously acclimatized collectively to the experimental room and conditions for 1 week, at an initial average body weight of 2.02 ± 0.05 g were randomly distributed in the tanks at a density of 30 shrimp/tank (83 shrimp/m^2). Four times daily, a commercial pellet containing 30% of crude protein (Feng Li, PT Matahari Sakti, Indonesia) was provided for 49 days to all tanks. The feeding level was determined at 7% on wet body weight per day and the daily feed amount was adjusted to the biomass in the tanks.

The experiment consisted of five treatments (four replicate tanks per treatment): one control treatment without organic carbon addition and with a weekly water exchange of 50%, and four treatments with different organic carbon sources added for biofloc development (molasses, tapioca, tapioca by-products, and rice bran). Freshwater was regularly added only to make up for water loss due to evaporation. All organic carbon sources were locally purchased. Organic carbon was added daily two hours after feeding at an estimated C/N ratio of 15 (De Schryver et al., 2008). Proximate composition and organic carbon content in the different types of organic carbon source were determined according to Takeuchi (1988) and Walkley and Black (1934) (Table 5.1).

Table 5.1. Proximate composition and total organic carbon content of molasses, tapioca, tapioca- by-product, and rice bran (all values, except moisture, are expressed as percentage on dry weight). nd: not detected.

	Molasses	Tapioca	Tapioca-by-product	Rice bran
Moisture (%)	31.9	10.0	13.8	9.6
Ash (%)	5.9	0.3	0.6	7.4
Protein (%)	3.8	1.6	nd	6.6
Lipid (%)	0.4	nd	nd	9.9
Fiber (%)	nd	nd	7.9	13.3
Nitrogen free extract (%)*	58.1	88.1	77.7	53.4
Organic carbon (%)	38.0	50.3	48.8	43.5

*Nitrogen free extract represents soluble carbohydrate content (minus fiber)

5.2.2 Water quality

Temperature, dissolved oxygen (DO), pH, and salinity were measured *in situ* using a portable DO meter (Lutron DO-5519, Taiwan), pH meter (Lutron YK2001PH, Taiwan) and refractometer (ATAGO 2491-MASTER S, USA). Biochemical oxygen demand (BOD), alkalinity, dissolved inorganic nitrogen (total ammoniacal nitrogen, NO_2^--N, and NO_3^--N), and total suspended solids (TSS) were determined following the procedures in the Standard methods for the examination of the water and wastewater (APHA, 1998).

5.2.3 Zootechnical performance of the shrimp

Survival was expressed as the percentage of live shrimp on the final day of the experiment relative to the total initially stocked shrimp. Shrimp growth was monitored by weekly sampling and restocking of the measured animals. Specific growth rate was calculated according to Huisman (1987) with the following formula:

$$SGR\ (\%/day) = \left(\sqrt[t]{\frac{wt}{wo}} - 1 \right) x\ 100$$

SGR = specific growth rate (%/day)

wt = final average shrimp body weight (g)

wo = initial average shrimp body weight (g)

t = experimental period (day)

The food conversion ratio (FCR) was expressed as the ratio of the total feed given relative to the shrimp biomass gain, whereas the input/output ratio was measured as the summed weight of feed and carbon source given per unit of biomass gain. These parameters were calculated for each tank at the end of the culture period.

5.2.4 Protein and lipid assimilation

Shrimp protein and lipid content were determined according to the Folch and Kjehdahl method as described in Takeuchi (1988). The assimilation of protein and lipid originating from the feed by the shrimp (%) were subsequently calculated according to the following formula (Takeuchi, 1988):

$$Protein\ assimilation\ (\%) = \frac{Final\ protein\ content - Initial\ protein\ content}{Protein\ input}\ x\ 100$$

$$Lipid\ assimilation\ (\%) = \frac{Final\ lipid\ content - Initial\ lipid\ content}{Lipid\ input}\ x\ 100$$

5.2.5 IMNV challenge test

An IMNV challenge test was performed at the end of the experimental period. The IMNV was obtained from IMNV infected Pacific white shrimp (as determined by a IQ2000 IMNV detection kit, Genereach, Taiwan) from the Brackish Water Aquaculture Development Institute (BBAP Situbondo, East Java Indonesia). This stock was free of taura syndrome virus (TSV), white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV) as verified by polymerase chain reaction using IQ2000 detection kits for TSV, WSSV and IHHNV, respectively. The preparation of the IMNV stock and the determination of virus titer

were conducted according to Escobedo-Bonilla et al. (2005). Briefly, muscle tissue of the naturally infected shrimp was suspended and grinded in phosphate buffer saline (PBS, 10x) and centrifuged at 3000 x *g* for 20 min at 4°C. The supernatant was transferred into a new tube and centrifuged again at 13 000 x *g* (4°C for 20 min). The supernatant was then filtered over a 0.45 µm syringe filter and stored at -80°C until further use. Prior to the actual challenge test, a preliminary experiment was performed to determine the LD_{50} and the effective period of infection (LT_{50}).

Ten healthy appearing inter-molt stage shrimp were kept in each carbon source treatment replicate tank while the remaining shrimp were removed. Sixty healthy inter-molt stage shrimp from the control treatment tanks were randomly selected and redistributed over 6 tanks containing new seawater to make up the negative (non-challenged) and positive (challenged) control for the challenge test (n = 3, each). The challenge test was performed by injecting 100 µL of virus suspension (100 µL of PBS for negative control) in between the third and fourth abdominal segment. The challenge test was run for 6 days during which feed was given four times a day to visual satiation according to feeding tray observation. Shrimp mortality was determined daily; most of the dead shrimp showed clinical signs of IMNV infection and this was further confirmed by PCR using a IQ2000 IMNV detection kit. PCR conditions were applied according to the manufacturers protocol.

5.2.6 Immune parameters

Immune parameters were measured at prior to and after IMNV injection. The measurement of total haemocyte count (THC), phenoloxidase activity and respiratory burst were performed for two inter-molt stage shrimp from each replicate tank according to Liu and Chen (2004). Briefly, 200 µL of haemolymph sample was taken with a 1 mL syringe containing 200 µL of precooled anticoagulant solution (30mM trisodium citrate, 0.34 M sodium chloride, 10mM EDTA, 0.12 M glucose, pH 7.55). For total haemocyte counting, duplicates of 50 µL of diluted haemolymph were counted for the number of haemocytes using a haemocytometer under a light microscope.

Phenoloxidase activity measurement was performed by adding 200 µL of the diluted haemolymph into 1 mL with anticoagulant solution followed by centrifuging at 700 x *g* for 20 min at 4°C. The supernatant was discarded and the pellet was rinsed and resuspended in cacodylate-citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0) and centrifuged again. The pellet was resuspended in 200 µL cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.26 M magnesium chloride, pH 7.0), and a 100 µL aliquot was incubated with 50 µL trypsin (1 mg/mL) as an activator for 10 min at 25 °C, followed by adding 50 µL of L-dihydroxyphenylalanine (L-DOPA) and 800 µL of cacodylate buffer 5 min later. A no-activation control measurement was prepared at the same time consisting of 100 µL cell suspension in cacodylate buffer, 850 µL cacodylate buffer, and 50 µL L-DOPA. The optical density of the shrimp's phenoloxidase activity was expressed as dopachrome formation in 100 µL of haemolymph at 490 nm.

Respiratory burst activity (production of superoxide anion O_2^-) was determined by reduction of nitroblue tetrazolium (NBT) to formazan according to Song and Hsieh (1994) with some modifications. Fifty µL of the diluted haemolymph was incubated for 30 min at room temperature, followed by centrifugation at 1000 x *g* for 20 min at 4 °C. The pellet was then incubated with 100 µL nitroblue tetrazolium (0.3% in Hank's balanced salt solution) for 2 h at room temperature. The suspension was subsequently centrifuged at 1000 x *g* for 10 min, and fixed with 100 µL of absolute methanol. The formazan pellet was then rinsed three times with 70% methanol, air-dried, and redissolved in120 µL KOH (2M) and 140 µL dimethylsulfoxide (DMSO). The optical density was measured at 630 nm using a microplate reader, and respiratory burst was expressed as NBT-reduction in 10 µL of haemolymph.

5.2.7 Bacterial quantification

After 49 days of rearing, a total viable bacterial count and estimated *Vibrio* count from tank water and shrimp intestine was determined by the spread-plate

technique on seawater complete agar (Atlas, 1946) and thiosulfate citrate bile salts sucrose (TCBS) agar, respectively. Three shrimp from each replicate tank were collected and aseptically dissected. The intestine was removed, pooled and homogenized in PBS.

5.2.8 Statistical Analyses

Correlation coefficient between the protein and lipid content of the carbon sources and the protein and lipid assimilation by the shrimp was calculated using Pearson's Product-Moment Correlation. All survival data was arcsine transformed. Homoscedasticity and normality of all data were assessed using Levene's test and a Kolmogorov-Smirnov test, respectively. As all data were normally distributed and the variances of the variables were equal, the data were analysed using one-way analysis of variane (ANOVA). Repeated measures ANOVA using the linear model of two factors (C source and time) was used to analyse the post challenge survival and dissolved inorganic N concentrations data (Rodríguez et al., 2007). Statistical analyses were conducted using SPSS statistics version 18 for windows (SPSS Inc.) at a significance level of 0.05. Significant differences between treatments were determined using a post-hoc Duncan test.

5.3 Results

5.3.1 Water quality

The water quality parameters DO, BOD, pH, salinity, and alkalinity were found to be highly similar among treatments (Table 5.2). While there was no significant difference observed amongst carbon source treatments, the TSS levels in these treatments were significantly higher than in the control, in particular from day 28 onward. Repeated ANOVA analyses on the concentrations of TAN, nitrite-N, nitrate-N and total dissolved inorganic nitrogen showed significant effects of carbon sources and sampling time, as well as significant interaction between these factors ($P < 0.01$). Total ammoniacal nitrogen concentrations in the control were generally

higher than those of the carbon source treatments (Figure 5.1). In most sampling weeks, the dissolved inorganic N concentrations in the control appeared to be higher than in the treatments (Figures 5.1 and 5.2), and the difference was significant ($P < 0.05$) in week 2, 5, and 6. In comparison to the other organic carbon sources used in this study, dosing rice bran resulted in higher concentrations of total ammoniacal nitrogen (TAN), nitrite-N and nitrate N.

Table 5.2. The range and mean value (between brackets, n = 4) of water quality parameters in Pacific white shrimp culture water in biofloc technology systems supplied with different carbon sources. DO=dissolved oxygen, BOD=biochemical oxygen demand, TSS=total suspended solids.

	Control	Molasses	Tapioca	Tapioca-by-product	Rice bran
DO (mg/L)	5.9 - 7.2 (6.3)	5.8 - 7.3 (6.0)	5.6 - 7.3 (6.1)	5.8 - 7.2 (6.1)	5.9 - 7.0 (6.1)
BOD (mg/L)	1.05 - 3.88 (2.60)	0.93 - 4.38 (2.82)	1.08 - 4.20 (2.84)	0.85 - 4.60 (2.91)	1.13 - 4.20 (2.83)
pH	7.2 - 8.1 (7.4)	7.0- 8.1 (7.4)	6.9 - 8.1 (7.4)	6.9 - 8.1 (7.4)	6.9 - 8.1 (7.4)
Salinity (g/L)	29 – 30 (30)	29 – 30 (30)	29 – 30 (30)	29 – 30 (30)	29 – 30 (30)
Alkalinity (mg/L)	77 - 160 (121)	55 - 167 (107)	58 - 161 (118)	52 - 148 (107)	69 – 154 (114)
TSS (mg/L)	48 – 97 (93)	82 – 241 (180)	67 – 196 (155)	66 – 204 (160)	68 – 230 (184)

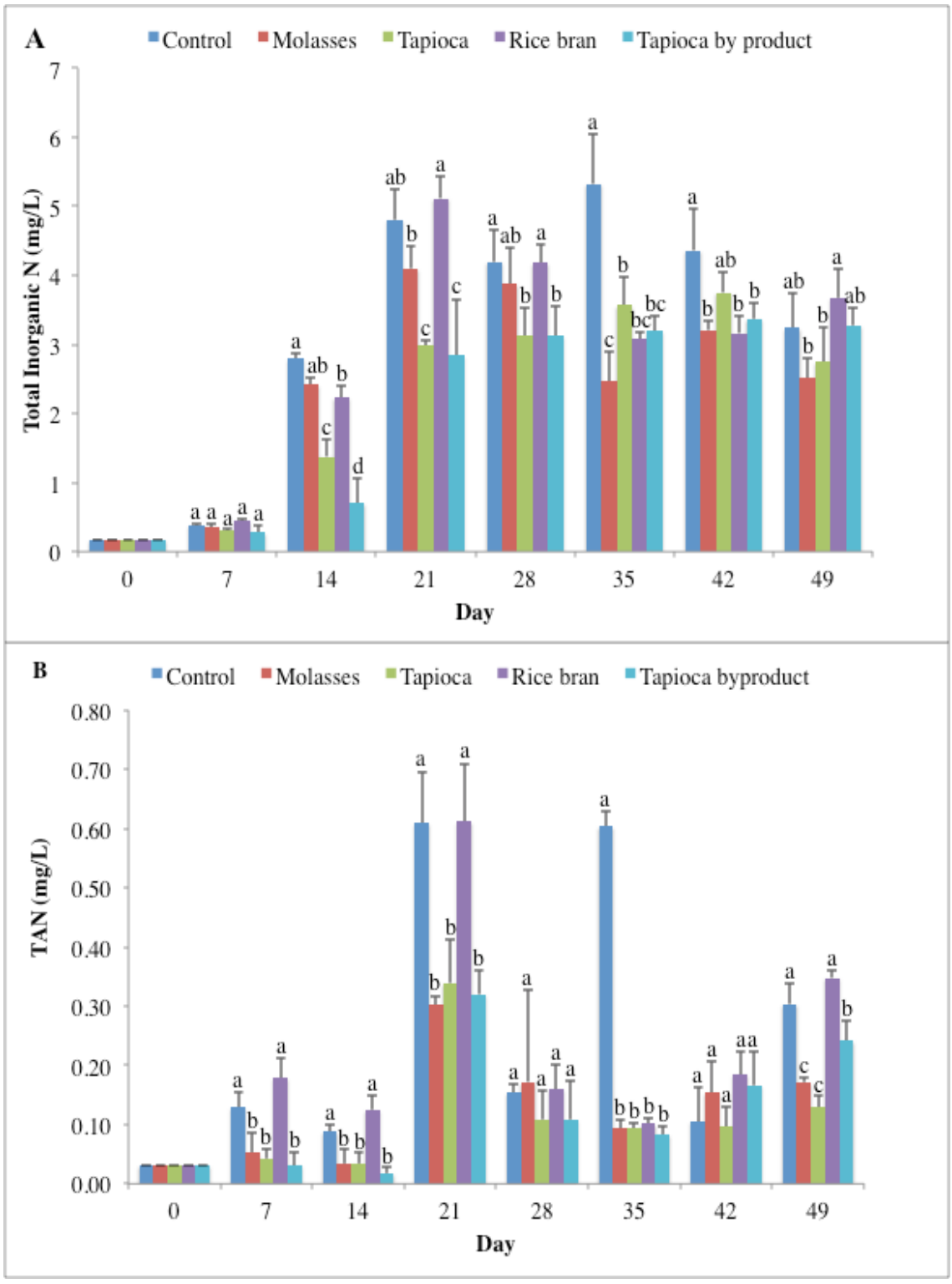

Figure 5.1. Dissolved inorganic nitrogen concentrations in Pacific white shrimp culture water in biofloc technology systems supplied with different carbon sources, A) total dissolved inorganic N; and B) total ammoniacal nitrogen (TAN). Values are means with error bars indicating standard deviations (n = 4). For each day, values marked with a different letter are significantly different ($P < 0.05$).

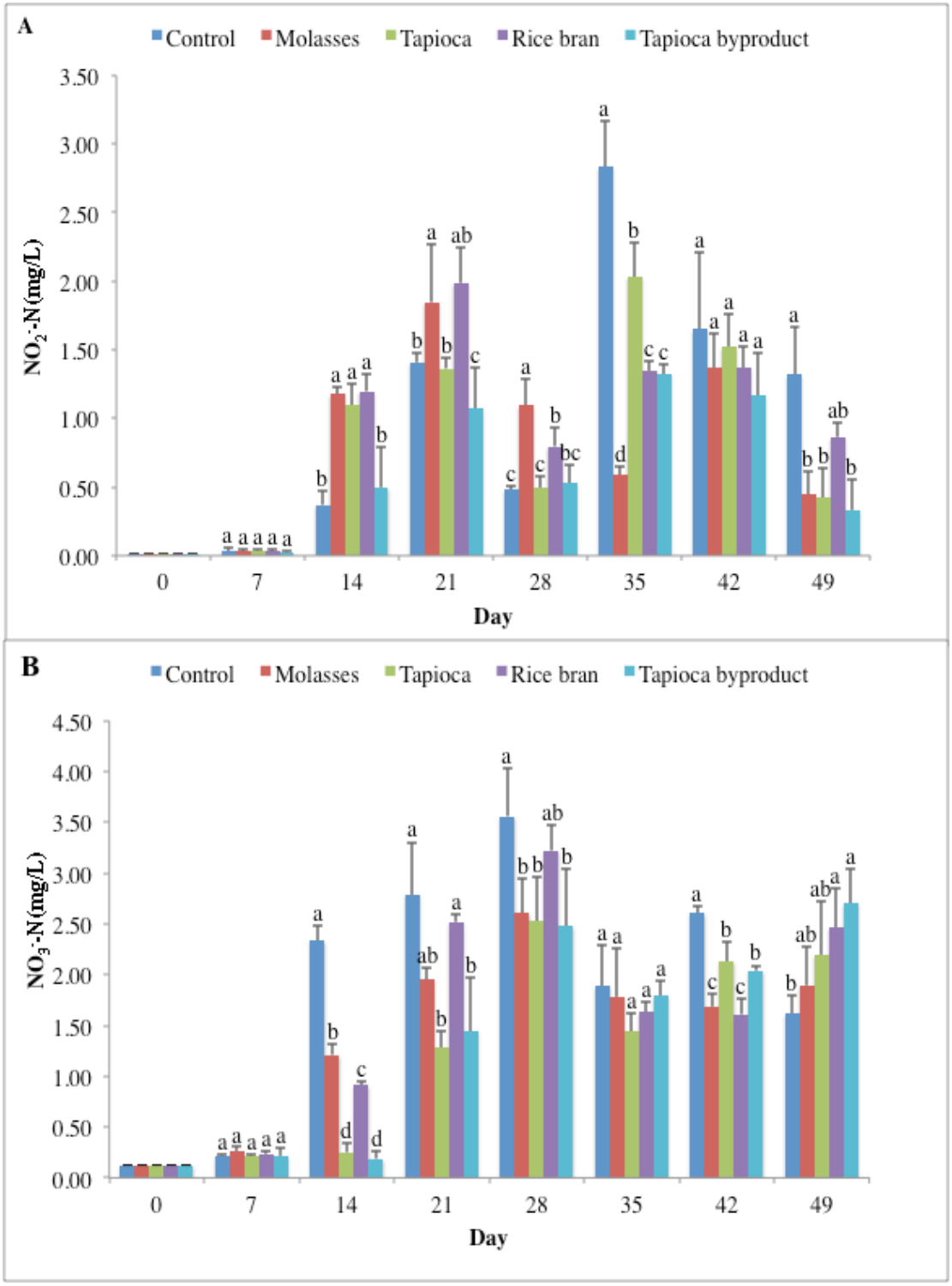

Figure 5.2. Dissolved inorganic nitrogen concentrations in Pacific white shrimp culture water in biofloc technology systems supplied with different carbon sources, A) nitrite nitrogen (NO_2^--N); and B) nitrate nitrogen (NO_3^--N). Values are means with error bars indicating standard deviations (n = 4). For each day, values marked with a different letter are significantly different ($P < 0.05$).

5.3.2 Survival and growth performance

There was a trend towards a higher survival in the biofloc treatments as compared to the control, although the difference was only significant for the tapioca-by-product treatment (Table 5.3). No significant differences were observed in the final body weight and specific growth rate among treatments. The protein assimilation was significantly higher for the tapioca and rice-bran treatments relative to the control while the lipid assimilation was significantly higher for the molasses and tapioca treatments. The addition of organic carbon source resulted in a significantly higher shrimp yield and significantly lower food conversion ratio for the tapioca and tapioca-by-product treatments as compared to the control. In term of input/output ratio, the rice bran treatment resulted in the highest value as compared to the other C source treatments.

Table 5.3. Mean values ± standard deviation of the growth parameters of Pacific white shrimp cultured in biofloc technology systems supplied with different carbon sources (n = 4). Values for the same parameter marked with a different superscript letter are significantly different (P < 0.05).

	Control	Molasses	Tapioca	Tapioca by-product	Rice bran	P value
Survival (%)	84±3^{a}	87±3^{ab}	88±3^{ab}	93±5^{b}	85±3^{ab}	0.030
Final body weight (g)	7.14±0.53^{a}	7.26±0.34 ^{a}	7.75±0.54 ^{a}	7.22±0.30 ^{a}	7.36±0.30 ^{a}	0.295
SGR* (% day^{-1})	3.06±0.19 ^{a}	3.08±0.15 ^{a}	3.24±0.13 ^{a}	3.05±0.10 ^{a}	3.15±0.17 ^{a}	0.391
Yield (kg m^{-2})	0.50 ± 0.02^{a}	0.52 ± 0.02^{ab}	0.57 ± 0.04^{b}	0.56 ± 0.04^{b}	0.52 ± 0.01^{ab}	0.030
Protein assimilation (%)	30.63±1.85^{a}	32.90±1.98^{ab}	37.61±3.38^{b}	32.37±3.03^{ab}	35.74±1.05^{b}	0.007
Lipid assimilation (%)	12.93±1.08^{a}	20.22±1.50^{c}	16.18±1.84^{b}	11.66±1.61^{a}	13.08±0.69^{a}	0.000
FCR**	1.67±0.10^{a}	1.56±0.09^{ab}	1.41±0.13^{b}	1.44±0.13^{b}	1.56±0.05^{ab}	0.021
Input/output ratio	1.67±0.10^{a}	2.77±0.15^{b}	2.81±0.24^{b}	3.01±0.22^{b}	3.46±0.12^{c}	0.000

5.3.3 Immune parameters

After 49 days of the experimental period, PO activity of the shrimp from the biofloc treatments was higher than that of the control shrimp and the differences were significant for the molasses and tapioca treatments (Table 5.4). There was no significant difference observed in THC. Respiratory burst activity of the carbon source treatments was not significantly different from the control. However, it can be observed that the RB activity was influenced by the carbon source used for biofloc culture as indicated by the significantly higher activity of RB in shrimp of the molasses treatment relative to the tapioca-by-product treatment. Following the IMNV challenge, a significant lower level of THC was observed for all treatments as compared to the negative control (the latter being non-challenged shrimp). Among these treatments, THC did not show significant differences. The IMNV challenge induced a decrease in the PO activity of the shrimp of all treatments. Nonetheless, the activity of PO in the challenged shrimp cultured in the tapioca treatment was significantly higher than the shrimp from the positive control. A similar pattern was observed for the RB activity. The RB activity in the challenged shrimp of all treatments with organic carbon addition was significantly higher than in the shrimp from the positive control.

During the first 3 days of challenge, a significant effect of time was observed, however, the survival of shrimp from the carbon treatments did not show significant differences relative to the shrimp from the positive and the negative control (Figure 5.3). In contrast, a significant effect of time and a significant interaction between time and C source were observed on day 4 - 6. A sharp decrease in survival was observed in all treatments in the period of day 3 - 5. On day 5, the survival in the positive control (23%) was significantly lower than in all other treatments. Although there was no significant difference amongst carbon treatments, the survival of the challenged shrimp in these treatments were significantly higher than the positive control. Similar pattern was observed on day 6, only now the survival of the shrimp from the molasses and tapioca treatments were not significantly different from that of the shrimp from the positive control. No significant differences in shrimp survival between the carbon treatments were observed after the IMNV challenge.

Table 5.4. Mean values ± standard deviation of immune parameters of Pacific white shrimp in biofloc technology systems supplied with different carbon sources prior to IMNV challenge (n = 4) and post IMNV challenge (n = 4 for biofloc treatments; n = 3 for negative and positive control). Values within the same column marked with a different superscript are significantly different ($P < 0.05$).

Treatment	Pre-challenge			Post-challenge		
	THC (x 10^6 cells m/L)	PO (OD_{490} 100 µ/L)	RB (OD_{630} 10 µ/L)	THC (x 10^6 cells m/L)	PO (OD_{490} 100 µ/L)	RB (OD_{630} 10 µ/L)
Negative control*	11.90±0.69^{a}	0.160±0.026^{a}	0.223±0.115^{ab}	11.68±0.64^{a}	0.144±0.039^{a}	0.257±0.155^{ab}
Positive control*				6.81±2.40^{b}	0.071±0.019^{b}	0.084±0.007^{c}
Molasses	12.03±3.06^{a}	0.491±0.224^{b}	0.470±0.147^{a}	6.80±2.54^{b}	0.090±0.020^{ab}	0.317±0.020^{a}
Tapioca	16.61±4.21^{a}	0.603±0.224^{b}	0.214±0.055^{ab}	5.10±0.81^{b}	0.192±0.090^{a}	0.215±0.052^{ab}
Tapioca by-product	16.21±2.50^{a}	0.277±0.084^{ab}	0.189±0.114^{b}	7.15±1.23^{b}	0.132±0.008^{ab}	0.194±0.055^{b}
Rice bran	15.08±4.48^{a}	0.435±0.094^{ab}	0.459±0.183^{ab}	6.49±1.26^{b}	0.127±0.003^{ab}	0.160±0.016^{b}
P value	0.255	0.036	0.048	0.007	0.009	0.003

* The negative and positive controls prior to challenge are the same non-biofloc control treatment and therefore have the same value

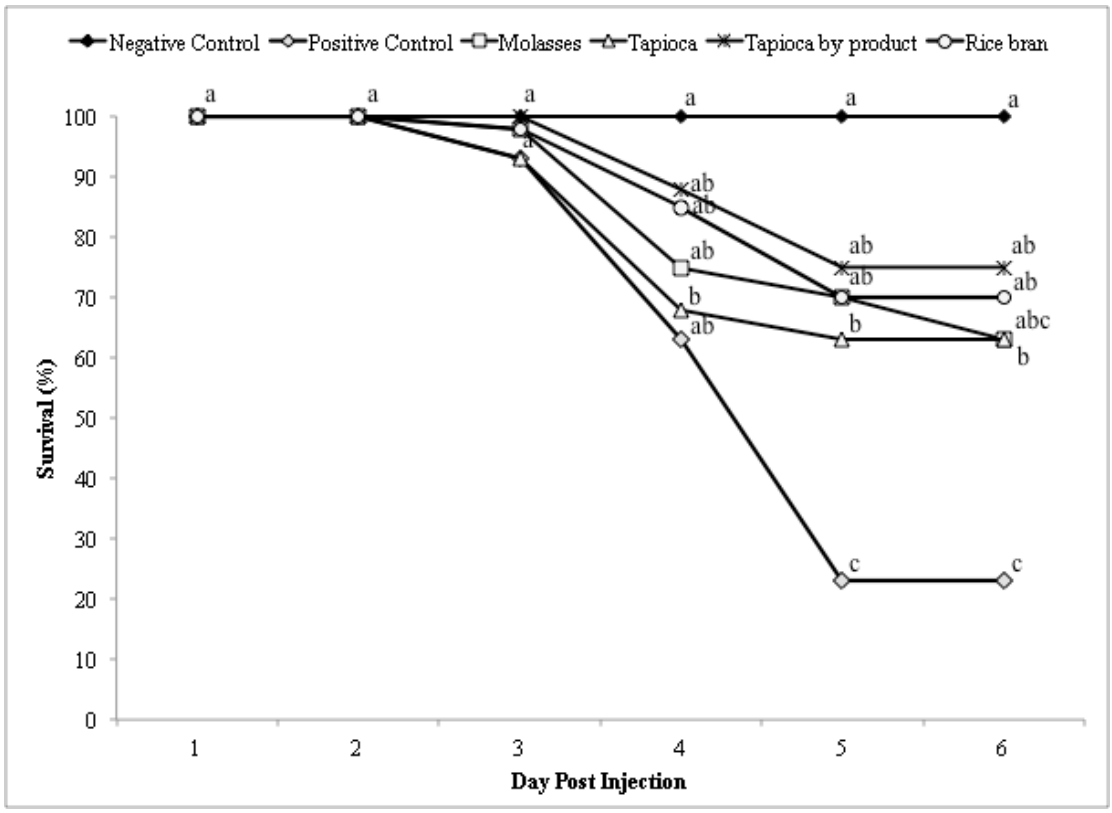

Figure 5.3. Mean values ± standard deviation of survival (%) of Pacific white shrimp cultured in biofloc systems supplied with different carbon sources following a challenge test with IMNV (n = 4 for biofloc treatments; n = 3 for negative and positive control). Standard deviations are not presented for clarity of the figure. At each time point, values marked with a different letter are significantly different ($P < 0.05$).

5.3.4 Bacterial counts in water and shrimp intestines

The total viable bacterial count in the water of the control treatment was significantly lower than that in the water of the biofloc treatments ($P < 0.05$; Figure 5.4A). The number of presumptive vibrios in the shrimp intestines of the control treatment was significantly higher than that in the shrimp intestines of the biofloc treatments, irrespectively of the organic carbon sources used ($P < 0.05$; Figure 5.4B). There was no significant difference observed in the total viable bacterial count and the presumptive *Vibrio* count in the shrimp intestines and in the water, respectively, between the different organic carbon source treatments.

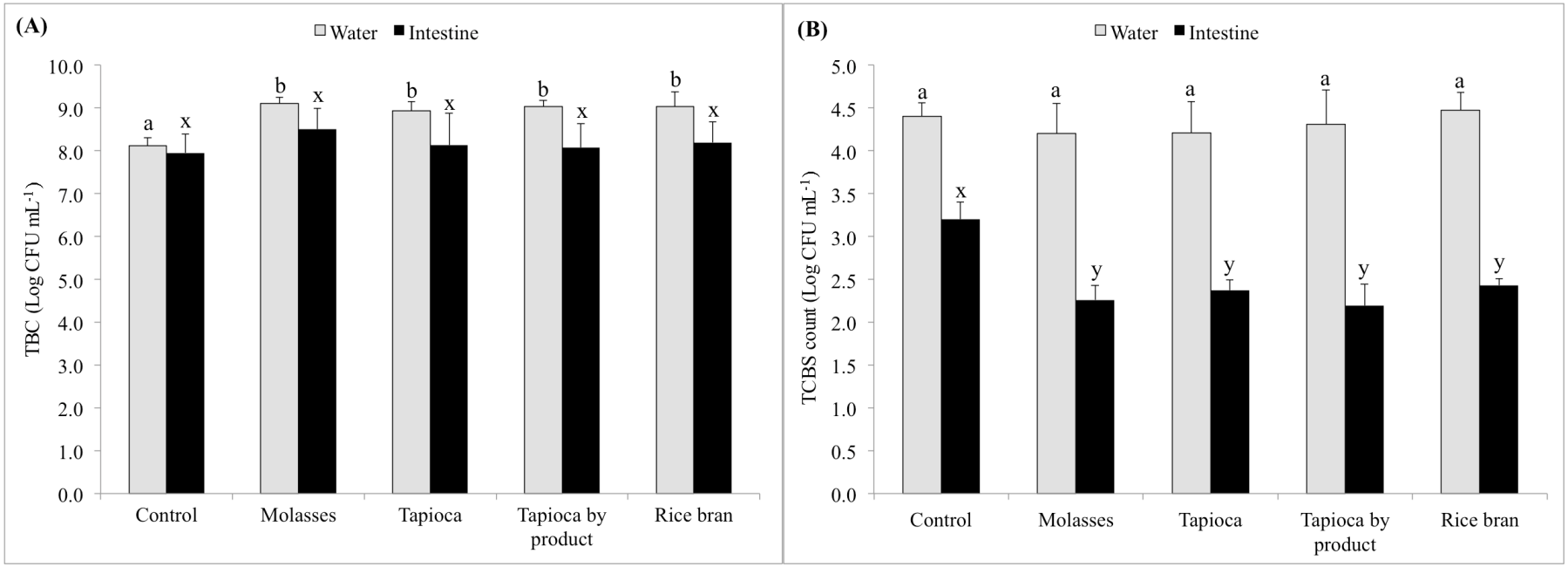

Figure 5.4. Mean values ± standard deviation of (A) total viable bacterial counts (TBC), and (B) presumptive *Vibrio* counts (TCBS) in the water and intestines of Pacific white shrimp cultured in biofloc systems supplied with different carbon source (n = 4). Bars of the same series (water and intestine, respectively) with different superscript letters are significantly different ($P < 0.05$).

5.4 Discussion

In this research, we illustrate that the application of biofloc technology for Pacific white shrimp culture significantly affects the shrimp immune response, a currently underexplored feature of bioflocs, and may increase the robustness of shrimp to resist infection. These effects seem to be independent of the type of carbon source used to grow bioflocs.

Each type of bioflocs grown on a different carbon source (molasses, tapioca, tapioca by-product, rice bran) was adequate in maintaining the overall water quality parameters in a normal range for shrimp growth. The lower level of dissolved inorganic nitrogen generally observed in the tanks with biofloc treatment as compared to the control with regular water replacement confirmed previous reports regarding the effect of biofloc technology (BFT) application on water quality in shrimp culture. Avnimelech (2012) pointed out different effects of simple versus more complex carbohydrates applied as the carbon source in biofloc-based ponds. Simple sugars, such as sucrose, result in a faster ammonia removal, while more complex carbohydrates require more time for decomposition into simple sugars, thereby resulting in slower ammonia removal. This may explain the higher TAN levels at several time points during the experimental period for the rice bran treatment, the carbon source that contained the highest fiber level, as compared to the other carbon source treatments.

In earlier studies, it was shown that the application of BFT in general results in an increased growth performance and survival of the cultured shrimp (Hari et al., 2004, Krummenauer et al., 2011, Ray et al., 2011, and Zhao et al., 2012). In our study, there also seemed to be a trend towards a slightly increased growth rate and survival for the BFT treated shrimp, although no significant differences were observed in comparison to the shrimp from the control treatment. Also, no differences were observed between the different carbon source treatments. The different organic carbon sources, however, appeared to have an effect on the assimilation of protein and lipid by the shrimp. The protein assimilation by the shrimp in the biofloc treatments was higher than that of the control shrimp but only significantly in case

of the tapioca and rice bran treatments. A similar observation was obtained for the lipid assimilation that was significantly higher for the molasses and tapioca treatment. As the shrimp in all treatments received the same dietary supplementation of proteins and lipids contained in the commercial diet, and only little correlations ($r = 0.17$ and $r = -0.33$, respectively) was observed between the shrimp protein and lipid assimilation and the protein and lipid content in the carbon sources, the altered assimilation values can only result from protein and lipid uptake in the form of biofloc biomass. One of our recent studies (Ekasari et al., 2014) showed that according to its essential amino acid composition, bioflocs could be considered a good quality protein source. Xu et al. (2012) suggested that the improvement of protein assimilation by animals reared in BFT systems is related to the increase in digestive proteinase activity in the intestinal tract as a result of the contribution of both exogenous digestive enzymes by the microbes in the biofloc and the endogenous digestive enzymes production as stimulated by the biofloc. The enhancement of protein and lipid assimilation in biofloc treatments clearly showed positive contribution of bioflocs biomass generated from nutrient waste as a food source for the cultivated animal. This in turn could result in lower feed conversion ratio in biofloc system (Xu et al., 2012, Gao et al., 2012, Zhao et al., 2012, and Megahed, 2010), which was also observed in the present study. Furthermore, the input/output ratio that represents the gain in biomass relative to the combined input of feed and C source indicates the effectiveness of the source of organic C in relation to biomass gain. In this regard, it can be observed that the use of rice bran as an organic C source was the least effective as compared to the other C sources in this study.

The bioflocs clearly affected the shrimp innate immune response. For shrimp reared in a biofloc system, the total haemocyte count and phenoloxidase activity prior to challenge showed higher values as compared to the control. This stimulation effect seems to be a general feature of bioflocs, although the extent of the stimulation seemed to be carbon source dependent. The circulating haemocytes of crustaceans and other invertebrates are essential in immunity, performing functions such as phagocytosis, encapsulation, and storage and release of the prophenoloxidase

system (Johansson et al., 2000). Phenoloxidase is an enzyme of the crustacean defense mechanisms that leads to melanisation of foreign cells to inactivate them and prevent their spread throughout the body. This enzyme is highly stimulated by microbial cell wall components such as lipopolysaccharides (LPS) and ß-1,3-glucans (Cerenius and Söderhäll, 2012, Perazzolo and Barracco, 1997, and Sritunyalucksana and Söderhäll, 2000). As the shrimps were cultured in BFT-based systems they evidently consumed the microbial floc *in situ* (Crab et al., 2012) so that the increases in total haemocyte number and PO activity point in the direction of a stimulatory effect of the (digested) biofloc on shrimp immunity. When considering biofloc-based stimulation of PO activity, Kim et al. (2014) clearly showed that the expression levels of proPO1, proPO2 and PPAE1 genes, which regulate the phenoloxidase activation systems, were significantly higher in biofloc-cultured shrimp than those of the shrimp from a control treatment. The absence of significant effects in terms of respiratory burst activity, a measure to determine the generation of reactive oxygen species associated with phagocytosis by shrimp haemocytes (Song and Hsieh, 1994), indicates that actual phagocytosis might not occur more in comparison to the control. It can thus be that the immune system is stimulated by a yet uncharacterized variety of immunostimulatory microbial cell wall material resulting in a higher immune response capability (Amparyup et al., 2013, Van de Braak et al., 2002). Similar observations were obtained by Xu and Pan (2013). These authors described that bioflocs stimulated the release of haemocytes into the circulation but that antibacterial and bacteriolytic responses were not significantly affected.

Following IMNV challenge, a decrease in the levels of THC, PO and RB activity was observed for the positive control, which is a normal physiological response in case of infection (Chang et al., 2003, Costa et al., 2009, Le Moullac and Haffner, 2000, Li et al., 2010, and Liu and Chen, 2004). The recovery of the shrimp immune system from viral infections, if not mortal, can take rather long (9-12 days) (Chang et al., 2003). Although the level of THC in all challenged treatments was similar 6 days after infection, the higher levels of PO and RB activity for the biofloc treatments as compared to the positive control pointed towards a faster recovery, or a more constant activity of the immune system in these treatments. The increased activity or

efficiency of the immune system of the shrimp from the biofloc treatments was also illustrated by the shrimp survival after infection that was significantly higher than in the positive challenge control.

It is also interesting to note that the application of bioflocs in shrimp culture results in similar effects in terms of growth, feeding efficiency, pathogenic bacteria inhibition and immune responses as the application of probiotics (Castex et al., 2009, Li and Mai, 2009, Xia et al., 2013, Zhang et al., 2011, Zokaeifar et al., 2012, and Zokaeifar et al., 2013). For instance, Zokaeifar et al. (2012 and 2013) reported that adding *Bacillus subtilis* into water or the feed of white shrimp resulted in better growth and survival, inhibition of *Vibrio* growth in the intestine, enhanced protease and amylase activities, as well as up-regulation of immune related genes such as LGBP, proPO, peroxinectin, and serine protease. The mechanisms by which probiotic bacteria affect shrimp performance have been reviewed by several authors (De Schryver et al., 2012, Farzanfar, 2006, and Ninawe and Selvin, 2009). They include immunomodulation, competitive exclusion, bioremediation, providing a source of nutrients and enzymatic contribution to digestion and quorum sensing blocking. The effects that bioflocs can have for shrimp culture as shown in the present study, as well as in recently reported studies (de Jesús Becerra-Dorame et al., 2014, Kim et al., 2014, Xu and Pan, 2012, Xu and Pan, 2013, and Xu et al., 2012,) strongly suggest that the beneficial effects associated with biofloc run at least partly parallel to those observed by addition of probiotics.

In conclusion, the present study showed that bioflocs have positive effects on the immune response of white shrimp leading to a higher resistance against IMNV challenge. Only slight differences were observed amongst the different organic carbon treatments.

CHAPTER 6 BIOFLOC-BASED REPRODUCTIVE PERFORMANCE OF NILE TILAPIA *Oreochromis niloticus* L. BROODSTOCK

The experiment evaluated the effect of biofloc technology application on reproductive performance of Nile tilapia broodstock. In this 84 days of trial, two treatments with 4 replicates were applied, control and BFT. Eight units of concrete tanks (3mx2mx0.6m) were each stocked with 60 tilapia broodstocks with an average body weight of 84.56 ± 4.81g at a female : male ratio of 4 : 1. In BFT treatment, molasses was added daily to obtain an estimated C/N ratio of 15. Total fry production of broodstocks in BFT tanks was significantly higher than that in the control tanks (8491 vs. 5154). Overall results of this experiment indicated that reproductive performance of tilapia broodstocks in floc-based system was better than in the control.

Redrafted after:

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6.1 Introduction

Intensive aquaculture, applying high density of animals per unit of area, needs to be supported by adequate production inputs including seeds and feed. Intensive aquaculture at the same time also needs good management, in particular water quality, to ensure that environmental conditions remain conducive for optimal growth. Various water quality management strategies for intensive aquaculture system have been proposed and applied, including biofloc technology (Crab et al., 2007). In biofloc technology, the growth of heterotrophic bacterial biomass is stimulated aiming at the assimilation of the excreted ammonia waste into microbial aggregates by providing an external organic carbon source. This biomass can be further harvested as a food source for or by the cultured animals, therefore generating a nutrient recycle and an efficient feed nutrient utilization. It appears that the application of biofloc technology (BFT) not only maintains the water quality, but it may in addition be enhancing the reproductive performance as observed in blue shrimp (Emerenciano et al., 2012b), pink shrimp (Emerenciano et al., 2013a), and Pacific white shrimp (Emerenciano et al., 2013b and Braga et al., 2013).

Tilapia is a promising aquaculture species that has been produced intensively all over the world. In the period of 2002 - 2012 global tilapia production showed a remarkable annual growth rate of about 10.4 %, with a production value of 7.7 billion USD in 2012 (FAO Global Aquaculture Production Statistics 1950 - 2012). One of the keys to a successful tilapia production as well as to other aquaculture species is the continuous supply of high quality seed. Tilapia seed production, however, is limited especially by its asynchronous spawning and low number of eggs (Bhujel, 2011). Various techniques have been introduced and developed ever since to boost tilapia seed production, most of them involving genetic improvement including hybridization and all male population seed production (Fitzsimmons, 2013). In this particular study, an experiment was set up to see the effect of BFT application on the reproductive performance of Nile tilapia *Oreochromis niloticus* L., an effect that has not been reported so far.

6.2 Materials and methods

6.2.1 Broodstock rearing

The experiment was carried out in the Field Laboratory of the Department of Aquaculture, Bogor Agricultural University, Indonesia. Eight units of outdoor concrete tanks with a dimension of 3 m x 2 m x 0.7 m were filled with 3 m^3 of water and randomly assigned for control and BFT treatment (4 replicates). Nile tilapia at respective average body length (ABL) and weight (ABW) of 16.7 ± 0.5 cm and 85 ± 5 g, were acclimatized for 7 days, and stocked at a density of 20 fish/m^3 at a male:female ratio of 1:4. Molasses (44% C) was added in BFT treatment tanks as an external carbon source at an estimated C/N ratio of 15 (Avnimelech, 2007). Commercial feed (30% crude protein, Central Proteina Prima, Jakarta, Indonesia) was provided twice a day to satiation for 13 weeks of culture period. There was no water replacement performed during the experimental period.

6.2.2 Biological and chemical analyses

Four females (16 fish per treatment) were randomly collected from each tank every two weeks to measure gonadosomatic index (GSI), hepatosomatic index (HSI), absolute fecundity, egg diameter, total cholesterol, and blood glucose. Gonadosomatic index represents the ratio between the weight of gonad and the fish body weight, whereas HSI represents the proportion of liver of the fish body weight (Rocha, 2008). Absolute fecundity was measured by counting the total number of eggs per female. A hundred fish eggs were randomly collected from the dissected fish gonad and the diameter was measured under microscope. Fish fry was collected daily and manually counted.

Proximate analyses were performed according to Takeuchi (1988), whereas fatty acids composition was measured by a professional laboratory (Sarawanti Indo Genetech, Indonesia) using gas chromatography (GC). Serum glucose and total cholesterol were measured using enzymatic colorimetric test kits respectively, HUMAN Glucose liquicolor complete kit (catalog no. 10260, Germany) and

HUMAN Cholesterol liquicolor complete kit (catalog no. 10017, Germany). Water quality analyses were regularly performed. Temperature, pH, and dissolved oxygen (DO) were measured *in situ* using a pH meter (Lutron YK2001PH, Taiwan), and portable DO meter (Lutron DO-5519, Taiwan). Alkalinity and dissolved inorganic nitrogen (total ammoniacal nitrogen, NO_2^--N, and NO_3^--N) and plankton enumeration were performed according to Standard Methods for the Examination of Water & Wastewater (APHA, 1998).

6.2.3 Statistical analyses

Plankton abundance was log transformed. Homoscedasticity and normality of the data were assessed using Levene's test and a Kolmogorov-Smirnov test, respectively. Accordingly, Student's t-test for independent samples statistical analyses was performed. Repeated measures ANOVA using the linear model of two factors (culture systems and time) was used to analyse the inorganic nitrogen concentrations (Rodríguez et al., 2007). Statistical analyses were conducted using SPSS statistics version 18 for windows (SPSS Inc.) at a significance level of 0.05.

6.3 Results

6.3.1 Reproductive performance of Nile tilapia

Fish of both treatments seems to grow relatively well over the culture period as it is shown by the increasing ABW (Table 6.1). Moreover, the final body weight of female brood fish in BFT tanks in the final day of the experiment was significantly higher than the fish in the control. The total feed intake in biofloc group (8.3 ± 0.4 kg) was significantly lower than that of the control (9.3 ± 0.3 kg). There was no significant difference observed in HSI of the female fish during the experimental period. Until day 56, gonadosomatic index of female brood fish in BFT seemed to increase, reached its peak at a level of 4.01%, and remained relatively constant afterwards at around 3% (Table 6.1). Gonadosomatic index of female broodfish in the control however seems to be relatively constant at a range of 2.43 to 2.92%. Egg diameter in BFT

treatment was found to be insignificantly different over the experimental period. Fish fecundity was constantly higher in the BFT treatments except on day 70. This is also reflected in the total fry produced during the 84 experimental days, which was 65% higher than that of the control (Figure 6.1). Blood glucose levels in the fish in BFT treatment were constantly higher than those of the control, and significantly different on days 42 and 56 (Table 6.2). Though not statistically significant, blood cholesterol levels were also found to be consistently higher in BFT treatment (Table 6.2).

Table 6.1. Reproductive parameters of female Nile tilapia *Oreochromis niloticus* L. in control and BFT tanks (mean ± SD, n per treatment =4, 4 fish per replicate). Different superscripts following values in the same row indicate significant difference for the respective parameters ($P < 0.05$). ABW=average body weight; HSI= hepatosomatic index; GSI= gonadosomatic index.

Day	ABW (g)		HSI (%)		GSI (%)		Fecundity (egg/ind)		Egg diameter (mm)	
	Control	BFT	Control	BFT	Control	BFT	Control	BFT	Control	BFT
0	85±5		1.03±0.35		1.31±0.61		-		-	
14	81±14^{a}	90±7^{a}	2.46±0.44^{a}	2.18±0.31^{a}	2.43±0.17^{a}	1.90±0.40^{a}	755±188^{a}	948±92^{a}	0.99±0.05^{a}	1.23±0.12^{a}
28	91±7^{a}	91±3^{a}	1.95±0.27^{a}	2.15±0.40^{a}	2.78±0.24^{a}	2.26±0.23^{b}	874±153^{a}	875±375^{a}	1.40±0.18^{a}	1.25±0.15^{a}
42	92±17^{a}	99±2^{a}	1.68±0.44^{a}	1.90±0.44^{a}	2.55±0.73^{a}	2.94±0.58^{a}	768±130^{a}	1159±83^{b}	1.45±0.17^{a}	1.39±0.13^{a}
56	96±8^{a}	110±5^{b}	1.86±0.43^{a}	2.02±0.34^{a}	2.92±0.59^{a}	4.01±1.21^{a}	744±121^{a}	1078±145^{b}	1.58±0.31^{a}	1.58±0.31^{a}
70	112±20^{a}	121±26^{a}	1.90±0.40^{a}	1.90±0.48^{a}	2.72±1.00^{a}	3.20±0.69^{a}	849±174^{a}	828±158^{a}	1.56±0.21^{a}	1.67±0.19^{a}
84	107±16^{a}	129±5^{b}	1.90±0.39^{a}	1.84±0.39^{a}	2.64±0.94^{a}	3.08±0.47^{a}	890±73^{a}	1243±217^{b}	1.45±0.25^{a}	1.55±0.09^{a}

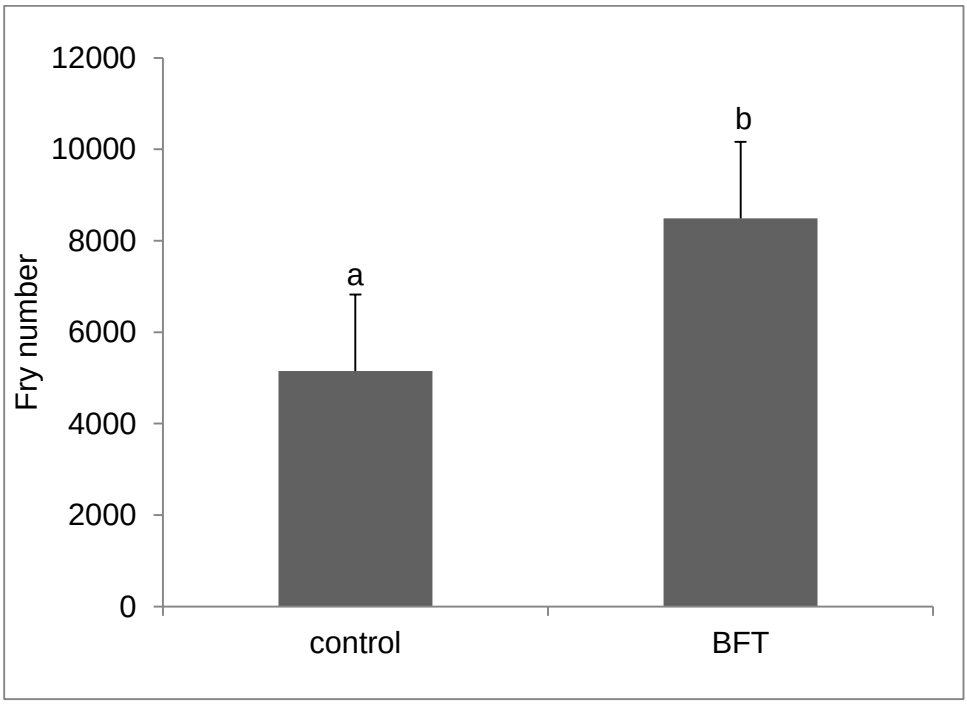

Figure 6.1. Mean ± SD of total Nile tilapia *Oreochromis niloticus* L. fry production in control and BFT tanks for 84 days of culture (n per treatment = 4). Values annotated with different superscript are significantly different ($P < 0.05$).

Table 6.2. Blood glucose and total cholesterol concentration of female Nile tilapia *Oreochromis niloticus* L. in control and BFT tanks (mean ± SD, n per treatment =4, 4 fish per replicate). Different superscripts following values in the same row indicate significant difference for the respective parameters ($P < 0.05$).

Day	Glucose (mg/dL)		Total Cholesterol (mg/dL)	
	Control	BFT	Control	BFT
0	103±27		143±25	
14	69±3 [a]	85±15 [a]	148±33 [a]	172±17 [a]
28	73±11 [a]	83±22 [a]	136±9 [a]	145±15 [a]
42	71±13[a]	93±8[b]	129±23 [a]	146±11 [a]
56	85±16[a]	105±19[b]	142±32 [a]	168±24 [a]
70	70±16 [a]	74±12 [a]	153±18 [a]	171±41 [a]
84	51±13 [a]	55±7 [a]	157±33 [a]	168±16 [a]

6.3.2 *Biofloc composition*

Table 6.3 presents the proximate composition of bioflocs collected on the final day of experiment. Crude protein content of bioflocs was higher than that of the commercial diet used in this study, and was comparable to that of the tilapia broodstock diet recommended by El-Sayed (2006). Similarly, biofloc lipid content was also higher than those of the commercial diet in this study and the reference broodstock diet.

Table 6.3. Proximate and essential fatty acid composition of bioflocs collected from broodstock tanks and a reference of broodstock diet (El-Sayed, 2006). DW=dry weight.

Composition	Biofloc (this study)	Commercial diet used in this study	Broodstock diet (El-Sayed, 2006)
Protein (%DW)	37.4	29.9	38.4
Lipid (%DW)	11.9	6.4	9.8
Fiber (%DW)	16.0	6.5	5.9
Ash (%DW)	17.7	14.6	13.7
Essential fatty acids (%total lipid)			
Linoleic acid	4.94	-*	7.45
Linolenic acid	0.13	-	0.63
Arachidonic acid	0.49	-	3.4
Eicosapentaenoic acid	0.20	-	0.24
Docosahexaenoic acid	0.11	-	2.37

* not measured

6.3.3 *Water quality*

Water temperature, pH and dissolved oxygen concentrations were relatively similar between the control and biofloc tanks in the range of 27.8 – 32.0 ^{o}C, 7.28 – 8.29, and 3.6 – 6.5 mg/L, respectively. Total ammoniacal nitrogen concentrations in both experimental groups were fluctuating during the experimental period (Figure 6.2). However, there were no significant differences observed in TAN concentrations between treatments. Nitrite-N concentrations in both treatments increased for the

first 3 weeks and followed by a constant reduction. After week 8, nitrite-N concentrations in BFT tanks seemed to be stable at less than 0.1 mg/L. Nitrate-N concentration in BFT tanks was fairly constant throughout the rearing period. Nitrite-N and nitrate-N concentrations were significantly higher in the control tanks than in the BFT tanks ($P < 0.05$). However, the effects of the treatments on these parameters were significantly influenced by the time of sampling ($P < 0.05$). Nonetheless, the total dissolved inorganic N concentrations were comparatively lower ($P < 0.05$) and more stable in BFT tanks than those in the control. Interestingly, in the control tank *Microcystis* dominated the microalgae composition accounting 68% of the total phytoplankton abundance. The abundance of *Microcystis* sp. in the control tanks was significantly higher than in BFT tanks (Table 6.4). In BFT tanks, on the other hand, phytoplankton composition was dominated by *Scenedesmus* (44%).

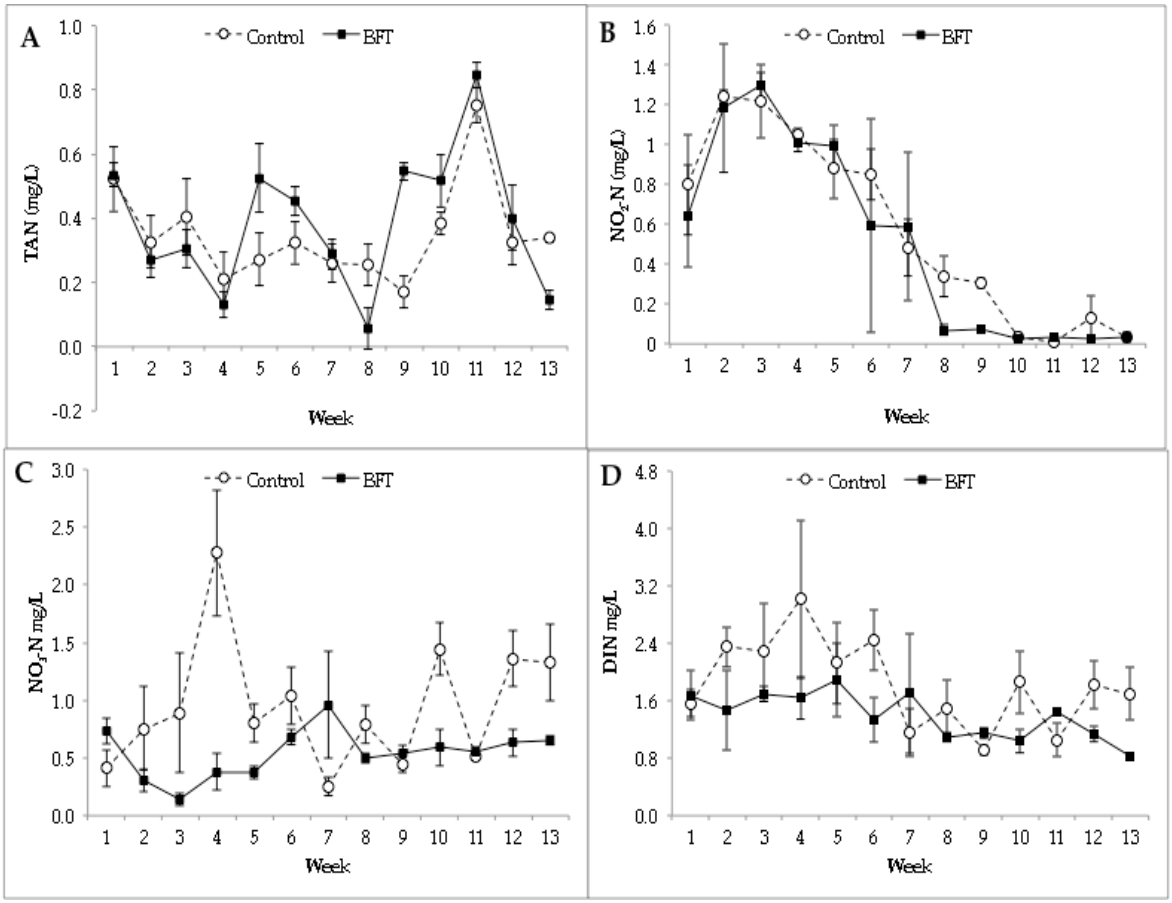

Figure 6.2. Dissolved inorganic N concentrations in the broodstock culture water for 13 weeks of rearing period; (a) total ammoniacal nitrogen, (b) nitrite-N, (c) nitrate-N, and (d) total dissolved inorganic N. Values are means with error bars indicating

standard deviations (n = 4).

Table 6.4. Mean (± SD) of dominant phytoplankton and zooplankton abundance in the water of Nile tilapia broodstock rearing tanks. Different superscripts following values in the same row indicate significant difference for the respective species ($P < 0.05$).

Organisms	Biofloc	Control
Phytoplankton (x 10^3 cells/L)		
CYANOPHYCEAE		
Microcystis sp.	343 ± 249[a]	7342 ± 614[b]
CHLOROPHYCEAE		
Scenedesmus sp.	1194 ± 182 [a]	1596 ± 318 [a]
Pediastrum sp.	648 ± 193 [a]	1009 ± 129 [a]
Dictyosphaerium sp.	186 ± 108 [a]	86 ± 37 [a]
Zooplankton (x 10^2ind/L)		
PROTOZOA		
Arcella sp.	3.3 ± 0 [a]	0.4 ± 0.2 [a]
Diflugia sp.	4.9 ± 2.3 [a]	0.3 ± 0.4 [a]

6.4 Discussion

A higher ABW gain was demonstrated by BFT treatment suggesting that, although more energy was allocated for reproduction (see later), the fish grew better in this environment. Despite the higher growth, it is interesting to point out that the total amount of feed given to the broodfish in BFT tanks was significantly lower than that of the control. This might indicates that the fish gained other food source which reduced their requirement for external feed, yet this alternative food source was sufficient to meet their nutritional requirement for a good growth and reproduction performance.

There was no significant difference in fish HSI level amongst treatments, whereas the low level of this parameter in both treatments may indicate that energy reserve in the liver was mobilized for gonadal maturation (Rocha, 2008). A high

variability in GSI at any time point in both treatments was also observed, which might be related to the asynchronous spawning behaviour of tilapia (Coward and Bromage, 2000). Blood glucose values observed in this study may designate a higher availability and mobilisation of energy for the fish in BFT. Cholesterol is known as a precursor for the biosynthesis of steroid hormones, including those that function in reproduction such as testosterone, estrogens, and progestogens (Lubzens et al., 2010). Higher levels of blood cholesterol in BFT treatment can therefore be supportive to improved reproductive performance of broodfish in this treatment. Lower total cholesterol level in the blood (less than 180 mg/dL) compared to that in the study by Chen et al. (2003), could be associated with the fact that the fish had been undergoing spawning activities (McDonald and Milligan, 1992).

Rocha (2008) discussed the factors triggering fish gonad maturation and spawning which include: (1) the nutrition of female (food availability, amino acids, fatty acids, ascorbic acids, vitamin E); (2) physiological factors (hormones, morphological changes, mobilization of energy reserves); and (3) ecological factors (food availability for the larva, water quality, and exposure and the presence of toxins). Some factors, listed above, might have contributed to a higher reproductive performance of tilapia reared in the BFT treatment in comparison to the control. From a nutritional point of view, bioflocs in the BFT treatment may contribute as a potential readily available food source which support not only the broodfish reproduction but also the survival and development of the produced larva. As can be seen in Table 6.3, the nutritional composition of biofloc collected from the BFT broodstock tank was comparable to that of the requirement of tilapia broodstock (El-Sayed, 2005) and fry (El-Sayed, 2006). Our previous experiment (Ekasari et al., 2014) showed that according to the essential amino acids index, biofloc could be considered as a good protein source for tilapia. Several studies show that bioflocs contain not only considerable level of protein but also highly unsaturated fatty acids (Ekasari et al., 2010) and vitamin C (Crab et al., 2012) which are required to support gonadal maturation and high egg quantity and quality (Dabrowski and Ciereszko, 2001). Equally important, recent reports showed that biofloc might also contribute

exogenous digestive enzymes (Xu and Pan, 2013) which are essential for the early stage larvae when their digestive system have not fully developed.

As it was observed in this experiment, previous studies have reported that the application of BFT could improve the stability of water quality in an aquaculture system (Avnimelech, 2007). Water quality fluctuation and deterioration on has been reported to cause stress that eventually affects the fish growth and reproductive performance (Bhujel, 2000). Furthermore the presence of blue green algae, *Microcystis* sp. that dominated the control tanks is also an important ecological factor. It was observed that as the culture period progressed, blue green algae tended to dominate the control media and on day 84 the density of *Microcystis* sp. had reached 7.34×10^6 cell/mL (Table 6.4). This blue green algae is known for the toxin, microcystin, which has been reported for its adverse effects for fish including internal haemorrhage, lowered growth rate and reduced reproduction success (Semyalo et al., 2011). In BFT treatment on the other hand, the growth of *Microcystis* is supressed, and the microalgae community was dominated by the Chlorophyceae, *Scenedesmus* sp. (1.19×10^6 cell/mL). Because of these different microbial communities in the tanks, it is anticipated that the light quantity and quality in the control and BFT tanks must have been very different (although measurements were not performed). Also these differences might have contributed to the observed recruitment difference (El-Sayed, 2006).

Finally, the results of the present study pointed out that the application of BFT effectively enhanced tilapia reproductive performance and therefore in situ biofloc production can be suggested as a way to increase tilapia seed production. The high production of fish larvae in BFT tanks in the present experiment may not only stem from higher production of eggs, but it is also possible that the biofloc system also support the survival and growth of the larvae.

CHAPTER 7 BIOFLOC TECHNOLOGY POSITIVELY AFFECTS NILE TILAPIA LARVAL PERFORMANCE

The effect of producing and culturing Nile tilapia (*Oreochromis niloticus*) larvae in biofloc technology (BFT) based systems was investigated in terms of larval growth performance and robustness. Broodstock fish were maintained in a system with and a system without the application of biofloc technology in order to produce larvae. Molasses (53% C) was added daily into the biofloc system at an estimated C/N ratio of 10. A growth test was performed with larvae that were harvested from the biofloc system and the control system and that were subsequently housed in a biofloc environment or a control environment according to a 2 x 2 factorial design. The survival of the larvae from BFT origin (90-98%) was higher than the survival of the larvae from control origin (67-75%). The growth performance of the larvae did not seem to be affected by the origin or the housing of the larvae, although the growth seemed to be more uniform when the larvae were housed in BFT water. Upon infection with the pathogenic bacterium *Streptococcus agalactiae*, the larvae from BFT origin showed a significantly higher survival (75-80%) than the control larvae housed in control water (ca. 55%). Housing of the control larvae in BFT water resulted in an increase in infection resistance (about 70% survival). In a salinity stress test, the tolerance of the larvae to osmotic stress at 35 g/L for 1 h was assessed. The larvae of BFT origin showed a survival of 72% and 42% at 1h and 24h post salinity stress, respectively. This was significantly higher than for the larvae of the control origin that showed a survival of 33% and 5% at these respective time points. Overall, the data show that the application of biofloc technology for tilapia broodfish maintenance and larval production can improve Nile tilapia fry quality and production performance.

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7.1 Introduction

Tilapia is an aquaculture species that is being produced intensively all over the world. In 2012, the global tilapia production reached 4.5 million metric tons (FAO, 2014) and is expected to increase further exponentially in the future. A key to the success of tilapia production, as well as other aquaculture species, is the continuous supply of high quality seed. With the assumption of 80% survival and an average harvest size of 500 g, it can be calculated that about 11.3 billion tilapia larvae are needed annually on a global scale. This is, however, not straight forward. Although tilapia breeds naturally and does not require any hormonal stimulation for their reproduction in captivity, this fish spawns asynchronously and only produces low numbers of eggs (about 1000 per spawn) (Bhujel, 2011). Bhujel (2011), for instance, reported shortage of premium quality tilapia fry in Thailand in the 1980s. Various techniques have been introduced and developed ever since to boost tilapia seed production, most of them involving genetic improvement through hybridization and all male population seed production with varying degrees of success (Fitzsimmons, 2013 and Little, 2004).

Biofloc technology is an approach in aquaculture that has gained much interest and is currently widely being studied and applied. The basic principle of this technology is the generation of a nitrogen cycle in a stagnant water aquaculture system by the stimulation of heterotrophic microbial growth that assimilates nitrogenous waste and that can be utilized as a feed source by the cultured species (Avnimelech, 1999 and De Schryver et al., 2008). Biofloc systems have not only been shown to maintain nitrogenous waste below toxic levels and to improve the feed nutrient utilization efficiency of the cultured animals (Avnimelech, 1999, Crab et al., 2009 and Hari et al., 2004), but also to provide extra essential nutrients (Ekasari et al., 2009, Ju et al., 2008 and Xu et al., 2012) and exogenous digestive enzymes (Xu and Pan, 2013). Biofloc application can also lead to improved reproductive performance of the cultured animals as has been shown for Pacific white shrimp (Braga et al., 2013), pink shrimp (Emerenciano et al., 2013) and Nile tilapia (Ekasari et al., 2013). The immunological effects associated with the microbes that make up the bioflocs,

comprising enhancement of the non-specific defense and providing protection against disease infection, has been explored as another beneficial feature of bioflocs (Kim et al., 2014 and Xu and Pan, 2013).

Our previous study on Nile tilapia illustrated that the number of offspring that could be collected from biofloc-based broodstock tanks was significantly higher than that of broodstock tanks without BFT (Ekasari et al., 2013). It is not clear, however, if the application of BFT only increases the fecundity of the parental generation or if the biofloc system also supports the survival of the offspring. Only few studies have dealt with the effect of biofloc-based systems on fish larvae or seed rearing (Crab et al., 2010 and Emerenciano et al., 2011). Emerenciano et al. (2011) attributed the significantly higher survival and growth of pink shrimp post larvae reared in biofloc systems in comparison to those reared in clear water system to the extra food and nutrients provided in biofloc systems.

In the present study, it was investigated how biofloc systems affect the performance of Nile tilapia larvae. Our assessment for the larval quality included survival and growth, uniformity in size, and tolerance to stress and disease (MacNiven and Little, 2001 and Mohan, 2007).

7.2 Materials and methods

7.2.1 Broodstock rearing

The experiment was performed in the field research facility of the Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Indonesia. Male and female tilapia broodstock were obtained from the Wanayasa Nila Nirwana Development Center, the Department of Fisheries of West Java Province (BPBIAT Wanayasa, West Java, Indonesia). The fish were acclimatized to the local rearing conditions for two weeks before the start of the experiment. During this period, female and male fish were kept separately in three outdoor tanks (3 m x 2 m x 0.7 m) previously filled with 3000 L chlorinated and aerated water at a

density of 40 fish per tank for female broodstock and 20 fish per tank for male broodstock.

As a start of the experiment, 2 concrete rectangular outdoor tanks with a dimension of 3 m x 2 m x 0.7 m and a working water volume of 3 m^3 were stocked with 25 brood fish at a female:male ratio of 4:1. The two tanks were maintained in a different way, one without organic C addition (control) and one with organic C addition (BFT). Aeration was provided using an air blower. Commercial floating feed (Sinta Feed, Indonesia) was provided twice daily in each tank at a feeding level of 2.5%/day on fish biomass. Molasses (53% C) was used as the organic C source in the BFT treatment and was added daily at an estimated C/N ratio of 10 as described in De Schryver et al. (2008).

7.2.2 Larval growth experiment

A growth experiment was performed using tilapia at late larval stage. Eight weeks after placing the parental generation in the broodstock tanks, about 1000 larvae were collected from each tank and graded according to size. Out of each batch, 120 larvae of similar size were selected for use. The average body weight (ABW) and average body length (ABL) were 23 ± 4 mg and 10.3 ± 0.8 mm, and 20 ± 4 mg and 10.0 ± 0.8 mm for the larvae from the control tank and the BFT tank, respectively (further called "origin of the larvae"). The larvae were subsequently randomly distributed in 2 L plastic tank, previously filled with 1.5 L of water from either the BFT tank or the control tank, (further called "culture water") at a density of 15 fish/tank (10 fish/L). Feeding was performed using a commercial feed (40% protein) (PT. Matahari Sakti, Indonesia) at a fixed level of 40% on the initial fish biomass per day, spread over 4 times a day. For the tanks containing BFT water, molasses (53% C) was added daily at an estimated C/N ratio of 10. Artificial light was provided at a photoperiod of 24 h light. According to the previous, the growth experiment consisted of 4 treatments, each performed in quadruplicate:

1. BFT/BFT: fish larvae collected from the BFT broodstock tank and transferred to BFT water (with molasses addition)

2. BFT/C: fish larvae collected from the BFT broodstock tank and transferred to control water (without molasses addition)
3. C/BFT: fish larvae collected from the control broodstock tank and transferred to BFT water (with molasses addition)
4. C/C: fish larvae collected from the control broodstock tank and transferred to control water (without molasses addition)

No water exchange was applied during the experiment. Freshwater was, however, regularly added to replace loss due to evaporation.

Growth parameters were determined after 14 days of larval rearing in the 2L tanks. The survival was expressed as the percentage of surviving larvae over initially stocked larvae. The specific growth rate (SGR) was calculated according to Huisman (1987) using the equation:

$$SGR = \sqrt[t]{\frac{wt}{wo}} - 1\ x\ 100$$

with SGR = specific growth rate (%/day), wt = final average fish body weigh (g) or length (mm), wo = initial average fish body weight (g) or length (mm), t = experimental period (day)

The condition factor (CF) was calculated according to Ali et al. (2008) using the equation:

$$CF = \frac{W}{L^3}\ x\ 100$$

with k = condition factor, W = average body weight (g), L = average body length (cm)

The relative standard deviation (RSD) of the fish total length per treatment was calculated as the percentage of the standard deviation over the mean of the total length.

7.2.3 Water quality

The temperature in the tanks during the growth test was monitored on a daily basis. Other water quality parameters including pH, dissolved oxygen (DO), alkalinity, total ammoniacal nitrogen (TAN), nitrite-N, and nitrate-N were measured on the final day of the growth experiment. Temperature was measured using a thermometer, whereas pH and DO were measured using portable pH meter (Lutron YK2001PH, Taiwan) and DO meter (Lutron DO-5519, Taiwan), respectively. Alkalinity, TAN, nitrite-N and nitrate-N were measured according to the Standard Method for the Examination of Water and Wastewater (APHA, 1998).

7.2.4 Challenge test

A challenge test was performed by immersing the surviving larvae from the growth experiment in a *Streptococcus agalactiae* suspension. Pathogenic *Streptococcus agalactiae* (isolate no. NK1) was kindly provided by the Agency for Marine Affairs and Fisheries Research and Development of the Ministry of Fisheries and Marine Affairs, Indonesia. About 10 µL of stock culture was inoculated into Brain Heart Infusion broth (Criterion C5141, Hardy Diagnostics, California, USA) and incubated for 24 h at 30°C under constant agitation (140 rpm). After culture, the cells were washed twice in sterile saline. Twelve units of 2 L plastic tanks were filled with 1.5 L of chlorinated and aerated freshwater and added with *Streptococcus agalactiae* culture at a density of 10^7 CFU/mL. From three out of four growth test tanks per treatment, ten healthy fish were selected and transferred to an immersion tank. Following 6 h of immersion, the fish were transferred to a tank containing new freshwater and reared for 5 more days. Culture condition such as temperature, light, and aeration was arranged similar to the growth test experiment. During this period, a commercial feed (PT. Matahari Sakti, Indonesia) was offered 4 times a day at a daily level of 20% on fish biomass and no carbon source was added to the BFT treatment tanks. After 5 days, the survival of the larvae was determined.

7.2.5 Salinity stress test

A salinity stress test was performed using tilapia at late larval stage. Twelve weeks after placing the parental generation in the broodstock tanks, about 1000 tilapia larvae were collected from each tank. The larvae were graded according to size and sixty larvae with a similar size were selected from each batch for use in the experiment. The average body weight and body length was 18 ± 3 mg and 9.9 ± 0.5 mm, and 15 ± 2 mg and 9.8 ± 0.8 mm for the larvae from the control tank and the BFT tank, respectively. The salinity stress test was performed at 35 g/L NaCl, a concentration that during a preliminary experiment was determined as the LT_{50} for the tilapia larvae after 50 min. Six units of 2 L plastic tanks were filled with 1.5 L of 35 g/L saline water. The sixty larvae from the BFT broodstock tank and the 60 larvae from the control tank were distributed into the tanks containing saline water at a density of 20 fish/tank. After immersion in the saline water for 60 minutes, the larvae were transferred to another tank containing freshwater with aeration and 1h later the number of survivors was determined. The survival of the larvae was also determined 24h after transfer into the freshwater tanks.

7.2.6 Statistical analyses

Fish survivals and relative standard deviation of total length were arcsin and square root transformed, respectively. Homoscedasticity and normality of the data were assessed using Levene's test and a Kolmogorov-Smirnov test, respectively. A two-way analysis of variance (ANOVA) followed by a post hoc Tukey's test (P level of 0.05) was performed for all parameters in the growth experiment except for water quality parameters, which was analysed using one-way ANOVA. The results of the salinity stress test were compared using a Student's t-test at a significance level of 0.05.

7.3 Results

7.3.1 Larval growth performance

The survival of the larvae originating from the BFT broodstock tank was higher than for the larvae originating from the control tank, regardless of the type of water in which they were housed (Table 7.1). No significant differences between the treatments were observed in terms of specific growth rate (determined based on body weight or body length) and the condition factor. The relative standard deviation that represents the variability of the fish total length was lower in case the larvae were reared in BFT water. The difference was significant with larvae originating from the BFT broodstock tank. No significant interaction between the origin of the larvae and the larvae culture water was observed for any of the growth parameters measured in this study.

The post challenge survival of tilapia larvae originating from the BFT broodstock tank was significantly higher than those originating from the control broodstock tank ($P = 0.044$) (Figure 7.1). The culture water in which the larvae were housed did, however, not significantly affect the survival post challenge ($P = 0.245$). Interestingly, the post challenge survival of larvae from the C/BFT treatment was also 36% higher than that of larvae from the C/C treatment. No significant interaction was observed between the origin of the larvae and the larvae culture water ($P = 0.472$).

7.3.2 Water quality parameters

The water temperature and DO levels was similar in all tanks during the growth test (Table 7.2). Significantly lower alkalinity concentrations were observed in the tanks with control water. Total ammoniacal nitrogen concentrations were significantly lower in the tanks with BFT water than in those with control water. Nitrite-N concentrations seemed to vary randomly according to type of water and origin of the larvae. No significant differences were observed for the nitrate-N concentrations.

7.3.3 Salinity stress test

One hour after the salinity stress, a significantly higher survival was observed for the larvae originating from the BFT broodstock tank than for the larvae originating from the control broodstock tank (Figure 7.2). After 24 h in freshwater, an additional 30% mortality was observed for both treatments. However, the number of survivors among the larvae originating from the BFT tank was still significantly higher than for the larvae of control origin.

Table 7.1. Mean (± standard deviation) of the performance parameters of Nile tilapia larvae (n=4). Values within the same row marked with different superscript letters are significantly different ($P < 0.05$). The P values resulting from the two-way ANOVA analysis are presented in the last three columns. ABW=average body weight, ABL=average body length, SGR=specific growth rate, CF=condition factor, RSD=relative standard deviation.

	BFT/BFT	BFT/C	C/BFT	C/C	P-value larval origin	P-value rearing system	P-value interaction larval origin and rearing system
Final ABW (mg)	108 ± 15^{a}	112 ± 44^{a}	119 ± 24^{a}	105 ± 18^{a}	0.891	0.712	0.515
Final ABL (mm)	16.6 ± 1.2^{a}	16.2 ± 1.3^{a}	17.3 ± 0.7^{a}	16.4 ± 1.3^{a}	0.394	0.293	0.689
SGR ABW (%/day)	12.0 ± 1.0^{a}	10.9 ± 2.2^{a}	11.7 ± 1.5^{a}	10.8 ± 1.2^{a}	0.736	0.308	0.200
SGR ABL (%/day)	3.7 ± 0.5^{a}	3.6 ± 0.6^{a}	3.7 ± 0.3^{a}	3.3 ± 0.6^{a}	0.264	1.207	0.163
CF (*k*)	2.4 ± 0.4^{a}	2.3 ± 0.4^{a}	2.3 ± 0.4^{a}	2.4 ± 0.4^{a}	0.507	0.537	0.855
RSD total length (%)	10.6 ± 1.6^{a}	14.7 ± 1.4^{b}	12.2 ± 4.8^{ab}	14.2 ± 1.1^{b}	0.744	0.032	0.486
Survival (%)	98 ± 3^{a}	90 ± 9^{ab}	67 ± 9^{c}	75 ± 15^{bc}	0.000	0.455	0.066

Table 7.2. Mean (± standard deviation) of water quality parameters at day 14 of the growth experiment and the optimal ranges for tilapia culture (Boyd and Tucker, 1998 and El-Sayed, 2006). Values within the same row marked with a different superscript letter are significantly different ($P < 0.05$). DO=dissolve oxygen, TAN=total ammoniacal nitrogen.

	BFT/BFT	BFT/C	C/BFT	C/C	Reference (Boyd and Tucker 1998 and El-Sayed 2006)
Temperature (^oC)	27.0 ± 0.1 ^{a}	26.8 ± 0.5 ^{a}	27.3± 0.3 ^{a}	27.0 ± 0.1 ^{a}	26 – 32
pH	7.7 ± 0.3^a	7.0 ± 0.1^c	7.8 ± 0.1^a	7.3 ± 0.1^b	6.5 – 9.0
DO (mg/L)	6.9 ± 0.4 ^{a}	7.3 ± 0.4 ^{a}	6.9 ± 0.3 ^{a}	7.0 ± 0.1 ^{a}	> 4.0
Alkalinity (mg $CaCO_3$/L)	62 ± 10^b	28 ± 5^c	94 ± 15^a	30 ± 8^c	> 20
TAN (mg/L)	0.54 ± 0.10^a	1.30 ± 0.20^b	0.56 ± 0.09^a	1.05 ± 0.13^b	
NO_2^--N (mg/L)	2.04 ± 0.35^a	0.23 ± 0.13^c	0.56 ± 0.10^b	0.09 ± 0.04^c	< 8
NO_3^--N (mg/L)	1.15 ± 0.41 ^{a}	0.82 ± 0.10 ^{a}	0.56 ± 0.11 ^{a}	0.87 ± 0.27 ^{a}	> 1000

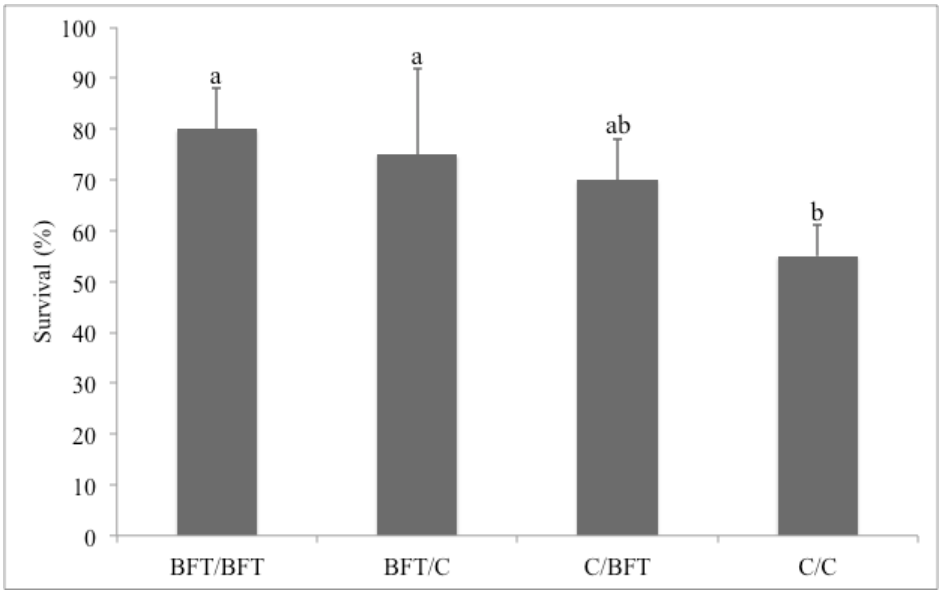

Figure 7.1. Survival (± standard deviation) of Nile tilapia larvae originating from the BFT broodstock tank or the control broodstock tank, and further reared in either BFT water (BFT/BFT or C/BFT treatments) or control water (BFT/C or C/C treatments) and subsequently challenged with *Streptococcus agalactiae* (n=3). Bars with different superscript letters are significantly different ($P < 0.05$).

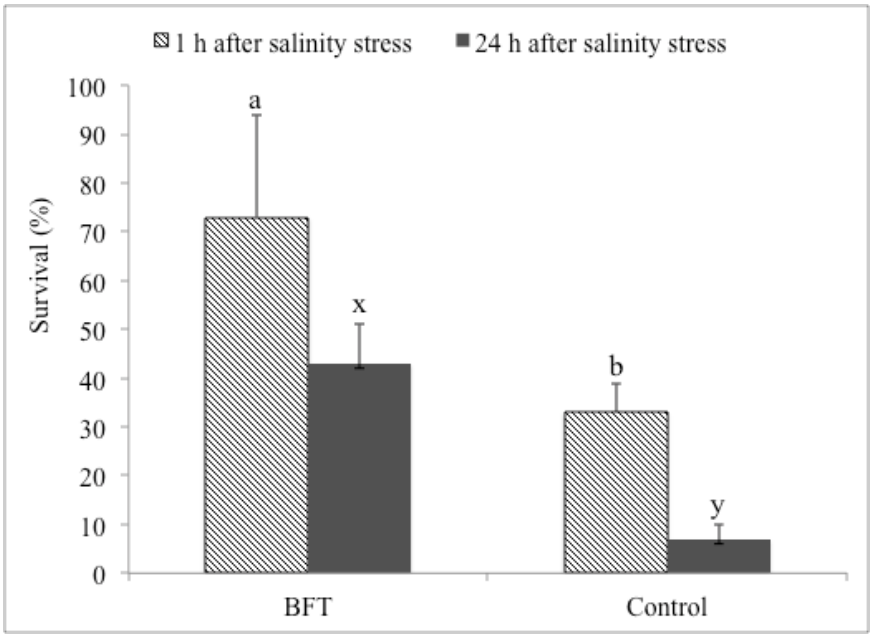

Figure 7.2. Survival (± standard deviation) of Nile tilapia larvae originating from the BFT broodstock tank or the control broodstock tank, and subsequently immersed for 1h in 35 g/L salinity water (n=3). Bars with different superscript letters at the same time (1 h and 24 h after salinity stress, respectively) are significantly different ($P < 0.05$).

7.4 Discussion

Most studies on biofloc systems report a significant increase in the growth performance of the cultivated animals (for example Azim and Little, 2008, Luo et al., 2014 and Xu and Pan 2012). In the present study, there was also a slight increase in the body weight and body length of the larvae reared in biofloc water, although these were not significantly different from the larvae reared in control water. Similar trends for the growth rate of animals reared in biofloc systems (and their controls) have also been reported in white shrimp (Xu et al., 2012) and rohu (Mahanand et al., 2013). El-Sayed (2002) reported that the maximum specific growth rate of Nile tilapia larvae of similar initial fish size (15 mg) as in the present study attained about 10%/day. This is comparable to the specific growth rate of the fish in the present study, even though the fish density was three times higher than that of the previous studies (El-Sayed, 2002 and El-Sayed and Kawanna, 2004). The insignificant differences in the growth of the larvae from the different treatments in this study might thus indicate that the nutritional requirements for larval growth were equally satisfied in both systems and that the additional consumption of bioflocs in this regard did not contribute significantly to the growth of the fish.

The relative standard deviation of the total length can be used to assess the size uniformity of the larvae. As the marketable size of fish is frequently determined according to the length, length variation in a fish larval population can also be considered an important parameter during production (Bondad-Reantaso, 2007). In contrast to larval survival and feed efficiency, the size variation of the fish was significantly affected by the culture system but not by the origin of the larvae. Growing tilapia larvae in BFT water resulted in more similar size of the fish than when these reared in control water. The variation in fish size within a population can be influenced by the accessibility to feed (Martin et al., 2005). One of the benefits of the biofloc system might have been the continuous availability of an easily accessible food source for the larvae. Therefore, it appears that the larvae in BFT system had access to feed outside the regular feeding moments, alleviating possible negative consequences of social interaction during feeding.

Despite of significant differences observed in some parameters between treatments, the water quality parameters in the present experiment were within the optimal range for normal growth of tilapia (Boyd and Tucker, 1998 and El-Sayed, 2006). Moreover, differences in pH, alkalinity, and dissolved inorganic nitrogen concentrations seem to be typical features of biofloc systems (Azim and Little, 2008 and Luo et al., 2014).

The conditions under which broodfish are reared have been reported to significantly influence the larval quality and quantity (Gunasekera et al., 1996). Biofloc technology application has been shown to improve the reproductive performance of tilapia broodstock fish by increasing fecundity and the quantity of the produced larvae (Ekasari et al., 2013). In the present study, it was indicated that the application of biofloc technology in tilapia broodstock tanks also improves larval quality and performance. However, due to the fact that there was no biological replicate for each broodstock culture system in this study, a generalization of these results on the tilapia broodstock population cannot be made, and therefore further research is required to confirm these effects of biofloc system. The results indicate that indeed there was a substantial difference in the survival of larvae collected from control and biofloc tanks. The higher survival of larvae originating from the BFT tank might suggest that the robustness of larvae produced in this particular system was significantly improved. These observations could be confirmed by the stress tests. A slight influence of the culture water was illustrated by an 8% reduction in survival of larvae from the BFT/C treatment as compared to larvae from the BFT/BFT treatment.

Stress tests are commonly used methods to assess the robustness of larvae of aquaculture species (Gunasekera et al., 1996 and MacNiven and Little, 2001). The basic principle of these tests is to increase the intensity of stress to more than the acclimation capacity of the organism so that it will affect their performance in growth, survival and reproduction (Wedemyer et al., 1990). A commonly used type of stress is infection with pathogenic bacteria (Dierckens et al., 2010 and Forberg et al., 2011). Saline challenges on the other hand have also been used to assess the osmotic capability as an indicator of the general robustness of the larvae

(Gunasekera et al., 1996, Kjørsvik et al., 2003, MacNiven and Little, 2001 and Salze et al., 2008). The current experiments pointed out that the origin of the larvae significantly affected their resistance to *Streptococcus agalactiae* infection, with BFT larvae being more resistant than control larvae. Furthermore, the higher robustness of BFT larvae coincided with a higher tolerance to osmotic stress during a saline challenge.

The increased resistance of the larvae to *Streptococcus agalactiae* infection may be explained by maternally derived immune protection or broodstock nutritional status. It is known that the female brood fish confer some form of protection or immunity to the embryo by loading unfertilized eggs with both innate and adaptive immune factors including serine protease like molecules, various types of lectins, Ig/antibody, complement factors, serine protease like molecules and macroglobulins (Schreck et al., 2001, Seppola et al., 2009, Swain and Nayak, 2009, Yousif et al., 1995, and Zhang et al 2013). Interestingly, in the review of Zhang et al. (2013) it is described that broodstock fish that are exposed to particular pathogens will synthesize more immune factors that can be transferred to their offspring, providing them with rapid and efficient immune responses when challenged with a pathogen. The nutritional status of the broodstock fish has been reported to affect the robustness of offspring in the first weeks after hatching (Izquierdo et al., 2001). Several studies have shown that a well balanced diet for the broodstock fish ensures sufficient nutrient supply to the eggs and larvae and therefore improves the quality of the eggs and larvae produced (Cecchini et al., 2000, Hamre, 2011, Izquierdo et al., 2001, and Zakeri et al., 2011). Therefore, the results of the present study may also indicate that the nutritional condition of the broodstock fish kept in the BFT tank was generally better than that of the broodstock fish kept in the control tank.

In addition to parental factors, it has been suggested that the application of biofloc technology increases the immunity of the cultured animals (Kim et al., 2014, Xu and Pan, 2013, and Xu and Pan, 2014). The consumption of microbial flocs exposes the animals to microbe associated molecular patterns (MAMPs) such as ß-1,3-glucan, lipopolysaccharides and peptidoglycan that may lead to the activation of the non-specific immune system (Xu and Pan, 2013, and Xu and Pan, 2014). This has

been supported by the observed upregulation of lipopolysaccharide glucan binding protein (LGBP) in white shrimp (Kim et al., 2013) and the observation of the activation of phenoloxidase (Ekasari et al., unpublished data, and Kim et al., 2014), antimicrobial activity (Kim et al., 2014), phagocytic activity (Xu and Pan, 2013) and antioxidation (de Jesús Becerra-Dorame et al., 2012, and Xu and Pan, 2013) in shrimp grown in biofloc systems as compared to those grown in control systems. The effect of the culture environment on the degree of infection was observed in the present study where the larvae of C/BFT treatment showed less mortality than larvae of C/C treatment. This further confirms the previously described effects of biofloc consumption on the stimulation of the fish immune system.

It is also interesting to note that the larval tolerance to environmental stress might also be enhanced by the consumption of live microbial material (Liu et al., 2010, and Rollo et al., 2006). Rollo et al. (2006) reported that the uptake of live microorganisms via rotifers or *Artemia* resulted in a better tolerance of sea bream larvae to pH stress. The authors attributed this increased tolerance to the upregulated expression of the genes encoding for HSP70, a heat shock protein that is involved in protecting cells by binding and refolding damaged proteins. Although further confirmation is required, it can thus be suggested that the increased stress tolerance of the larvae produced in the BFT tank may not only find its cause in the nutritional status of the broodstock but also in the consumption of microbial flocs by the larvae.

CHAPTER 8 GENERAL DISCUSSION AND CONCLUSIONS

8.1 Discussion

Biofloc technology is a relatively new aquaculture system that allows high density culture at limited or zero water exchange. Waste nutrients from the residual feed, faecal materials and metabolic products, in the presence of added organic carbon source, are utilized by the heterotrophic bacteria to produce new biomass which can be further consumed by the cultured animals (Avnimelech et al., 1999 and De Schryver et al., 2008). The application of this technology allows a lower use of water and more efficient food utilization in an intensive aquaculture system, making it a potential aquaculture technology for the development of a more sustainable aquaculture industry (Crab 2012 and Hargreaves 2006,). Figure 8.1 presents an overview of three major aspects involved in the application of biofloc technology in aquaculture, i.e. 1) operational parameters, 2) biofloc characteristics and functionality, and 3) the production and environmental performance. Some studies focusing on biofloc technology application have indicated that biofloc characteristics and functionality in aquaculture system are under the influence of the operational parameters (e.g. De Schryver et al., 2008, Avnimelech, 2012). As the stimulation of microbial growth in a biofloc-based aquaculture system aims at both an increase of production and improved environmental performance, it is expected that the steering of biofloc characteristics by modifying the operational parameters could also bring about beneficial effects to the target species as well as to the environmental performance.

8.1.1 Biofloc characteristics

The basic principle of biofloc technology in an aquaculture system is the stimulation of microbial flocs that convert the waste nutrients into microbial biomass. To achieve this some adjustment and interventions in the operation of aquaculture system are required. As there is only limited scientific information, De Schryver et al. (2008) suggested that the operational parameters of biofloc technology (BFT) application in aquaculture system could be adapted from those applied in activated sludge systems. These operational parameters can be classified into *abiotic parameters* that include the choice of organic carbon source, mixing

intensity, organic loading rate, dissolved oxygen supply, temperature and pH, and *biotic parameters* that include floc concentration, and the addition of secondary species in an integrated system. Subsequently, these operational parameters could interrelatedly steer the biofloc characteristics such as microbial composition, nutritional composition, and physical characteristics as well as the functioning of the BFT system.

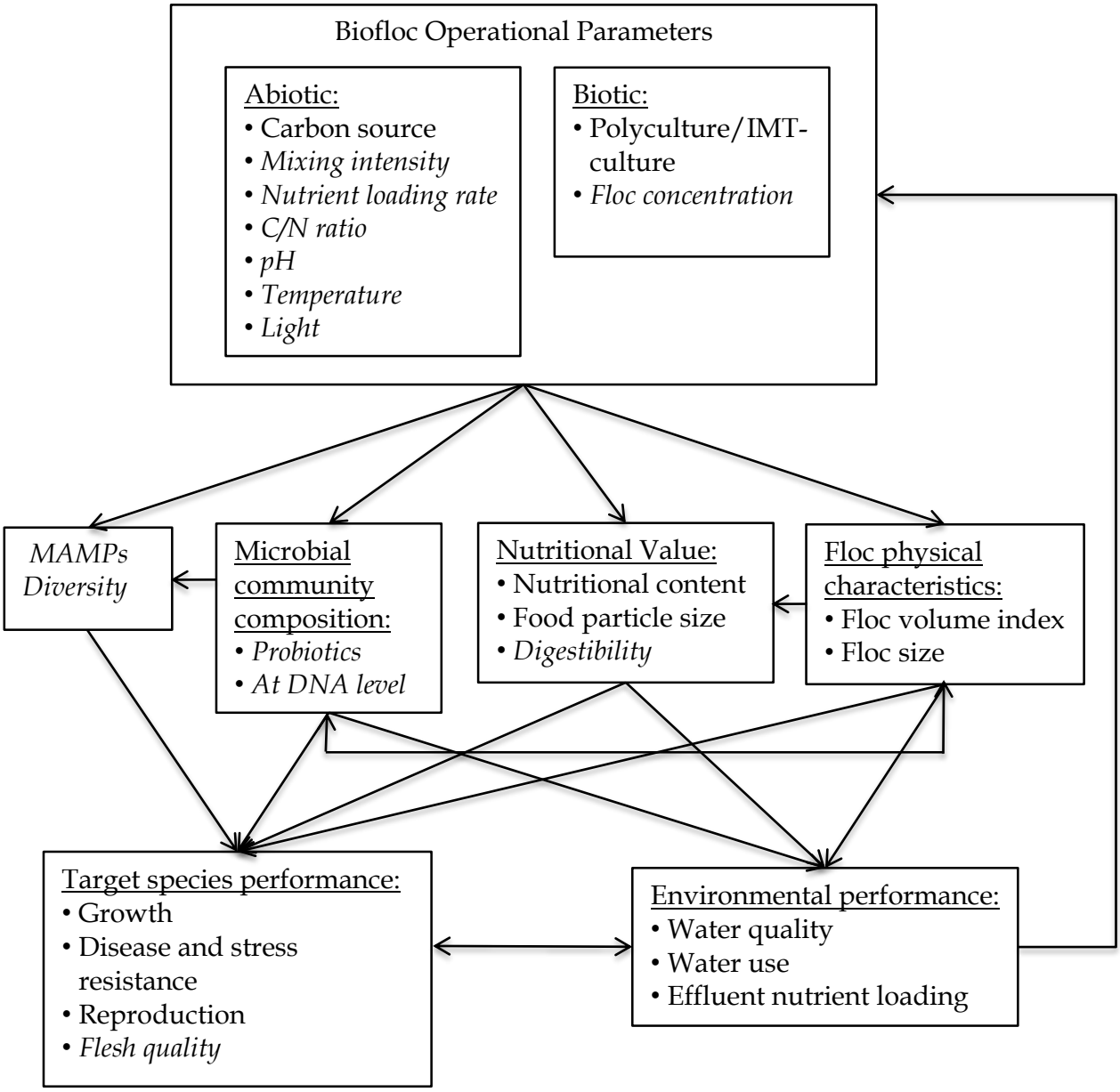

Figure 8.1. Overview of the effects of the operational parameters on the key parameters of biofloc technology and their relation to the production and the environmental performance of the culture system. The italic letters indicate possible topics, not covered in this thesis, that could be subjects for future research. IMT-culture: integrated multitrophic culture; MAMPs: microbe associated molecular patterns; DNA: deoxyribonucleic acid.

8.1.1.1 Microbial community composition

Some operational parameters have been identified that influence the microbial community composition of bioflocs in aquaculture system including salinity, solids removal, carbon source, probiotic, and microalgae addition. Maica et al. (2012) demonstrated that at a salinity level of 25 g/L the plankton community in biofloc-based shrimp culture system was dominated by flagellates and diatoms, whereas at lower salinity (0, 2 and 4 g/L) it was dominated by ciliates and chlorophytes. In addition, the relative abundance of plankton groups such as cyanobacteria and rotifer in biofloc-based aquaculture system seems to be affected by solids removal through a settling chamber (Ray et al., 2010b). Crab et al. (2012) showed that the prokaryotic and eukaryotic community, assessed by denaturing gradient gel electrophoresis (DGGE) pattern cluster analysis, was influenced by the choice of carbon source. Furthermore, using the same analysis, the authors also suggested that starch-grown bioflocs have a more evenly distributed community, which could result in a better adaptation to changes.

The addition of specific microorganisms has been suggested as a possible approach to stimulate floc formation (De Schryver et al., 2008), to increase biofloc nutritional quality (Crab 2012, De Schryver et al., 2008, and Ju et al., 2008), and to steer the microbial community (Zhao et al., 2012). Crab et al. (2009) suggested that although the microbial communities in bioflocs are considerably rich, the distribution of dominating microorganisms and resilient ones assure the competence of counteracting the effect of a sudden exposure to disturbance. In this regard, it is interesting to investigate the effect of operational parameters in biofloc-based aquaculture system on the microbial composition at DNA level. Furthermore, the possibility to introduce known species of microorganisms that are able to produce bioflocs with high functionality on the target species nutrition or water quality control is of particular interest for further research. Zhao et al., (2012) for example have indicated that the addition of *Bacillus* sp. as a probiotic could result in the predominance of this species in a biofloc system.

The microbial community composition could influence the physical structure and functions of bioflocs in an aquaculture system. De Schryver et al. (2008) suggested that biofloc formation could also be stimulated by the addition of microbial species that produce microbial cellular biopolymeric flocculants. It is also interesting to note that the microalgae-dominated bioflocs (which could be related to floc concentration, determining the amount of light penetration) possess relatively higher protein, essential amino acids and carotenoids levels than bacteria-dominated flocs (Ju et al., 2008), indicating the potential effect of microbial composition on the nutritional value of bioflocs grown in the culture systems. The presence of nitrifiers and denitrifiers associated with the bioflocs could also relate to the dissolved inorganic N profile, as well as dissolved oxygen and alkalinity concentrations in the culture water. Furthermore, we observed that the presence of cyanobacteria *Microcystis* sp., which may produce toxins such as microcystins, in biofloc system was significantly reduced (4.96×10^6 cells/mL in control and 0.23×10^6 cells/mL in biofloc) (Ekasari et al., 2013). This was in agreement with previous studies (Emerenciano et al., 2013b, Ray et al., 2010b, and Schrader et al., 2011) that demonstrated possible effect of bioflocs on the growth of cyanobacteria known to produce geosmin. Although further research is needed, this might indicate a possible role for the microbial community in bioflocs in controlling the growth of harmful algal blooms (HABs) and the production of off-flavours.

The microbial community composition in bioflocs might influence microbe-associated molecular patterns (MAMPs) that play an important role in the stimulation of innate immunity of aquaculture animals. The presence of pattern recognition receptors (PRRs) in the innate immunity system of plants and animals provide them a mechanism to identify classes of microbial compounds (such as chitin for fungi, peptidoglycan for bacteria, or glucan for yeasts). The PRRs allow for nonself recognition of compounds absent in the host (Boller and Felix, 2009). Furthermore, the molecular patterns recognized by the PRRs are typically associated with microbes regardless of pathogenicity. The contribution of MAMPs from bioflocs on stimulation of the innate immunity of aquaculture target species has been confirmed by the present study (Chapter 5 and Chapter 7), which demonstrated

enhanced immune parameters in shrimp grown in a biofloc system as well as a higher resistance of tilapia larvae to pathogenic bacterial infection. With the considerably rich microbial community in biofloc, it can be expected that the diversity of MAMPs is also high. In this regard, further research to elucidate the effect of biofloc operational parameters on the microbial community composition and the diversity of MAMPs in bioflocs is of particular interest.

8.1.1.2 Nutritional composition

Only a few studies have been performed to evaluate the effect of biofloc operational parameters on the nutritional composition of bioflocs in an aquaculture system. The operational parameters that have so far been reported to affect the nutritional composition of bioflocs include the choice of organic C source (Crab et al., 2012, De Schryver and Verstraete, 2009, and Ekasari et al., 2014b), C/N ratio (De Schryver and Verstraete, 2009), and microbial composition (Ju et al., 2008). A summary of proximate composition of bioflocs from this study and previous research is presented in Table 8.1. It can be seen in this table that the nutritional composition of bioflocs observed in the present work (Chapter 5) confirms the studies pointing out carbon source as one of the variables determining biofloc nutritional compositions. De Schryver and Verstraete (2009) noted that the protein content of bioflocs collected from a sequencing batch reactor with C/N ratio of 10 and 15 were considerably higher than those with C/N ratio of 2.5 and 5. Likewise, Xu and Pan (2012) reported that a C/N ratio of 20 resulted in higher protein content and extracellular protease activity than a C/N ratio of 15.

Although there is no study has been conducted to elucidate this effect in aquaculture systems, studies in activated sludge showed that sludge age could affect the extracellular polymeric substances (EPS) production, protein and carbohydrate content of the microbial floc. Biofloc culture age may be related to the food/microorganism ratio (the sludge loading rate) and the endogenous respiration of the microbes in the biofloc (Massé et al., 2006, Sheng et al., 2010) which may subsequently affect the biochemical composition and EPS content of the bioflocs. At

high culture age (because of shortage of nutrients: low food/microorganism ratio), the density of microbial flocs may increase substantially. That might be the consequence of an increase in degradation of EPS which is being used as a source of carbon. Furthermore, Massé et al. (2006) suggested that reduction of sludge protein/carbohydrate ratio at high solid retention time could be related to the reduction of protein production or the increase of polysaccharide production during low food/microorganism ratio, or the slower rate of carbohydrate hydrolyses as compared to protein. In addition to possible alteration on the bioflocs nutritional properties, high culture age might also increase the ash content in bioflocs (Ju et al., 2008). The high ash content in bioflocs may reduce its digestibility and therefore could be a drawback in bioflocs utilization as a food source (De Silva and Anderson, 1995). As typical biofloc-based aquaculture systems apply long retention time (Hargreaves, 2006, Crab et al., 2007), the manipulation of floc retention time is therefore necessary. This can be done by involving processes that control floc concentration by floc harvest, either mechanically or by filter feeders.

Table 8.1. Proximate composition of bioflocs grown with different carbon source. DW=dry weight .

Carbon source	Bioflocs proximate composition					Species	Reference
	Crude Protein (% DW)	Crude Lipid (%DW)	Ash (% DW)	Carbohydrate (%DW)	Gross Energy* (kJ/g DW)		
Molasses	19	3.9	48	30	10	*L. vannamei*	Ekasari et al., 2014a
Tapioca	17	2.4	48	33	9		
Tapioca by product	14	2.1	43	40	10		
Rice bran	15	3.2	45	38	10		
Molasses	37	12	18	33	19	Tilapia	Ekasari et al., 2013
Acetate	42	2.3	27	29	16	Reactor	Crab et al., 2010
Glucose	28	5.4	17	50	17	Reactor	Crab et al., 2010
	28 - 33	6.0 - 9.0	8 - 12	50 -54	16 - 17	Reactor	Ekasari et al., 2010
Glycerol	43	2.9	20	34	17	Reactor	Crab et al., 2010
	30 - 31	8 - 9	7 - 13	49 - 53	16 - 18	Reactor	Ekasari et al., 2010
Glycerol + Bacillus	58	3.5	25	14	17	Reactor	Crab et al., 2010
Molasses (90%) + wheat (10%)	18 - 26	0.3 - 0.7	35 - 42	20 - 36	10 - 11	*L. vannamei*	Emerenciano et al., 2012
	30	0.5	39	29	12	*F. paulensis*	Emerenciano et al., 2011
	25	0.6	47	26	10	*F. duorarum*	Emerenciano et al., 2013
Wheat	35	1.1	15.4	44	19	*Labeo rohita*	Mahanand et al., 2013
Molasses	15.7	1.6	-	-	3.3	*L. vannamei*	Kuhn et al., 2010
Sucrose	41	<0.1	12	31	18	Tilapia	Kuhn et al., 2009
	49	1.1	13	36	15	Tilapia	Xu and Pan, 2012
	27 - 32	3.7 - 4.2	44 - 49	20 - 21	9 - 10	*L. vannamei*	Azim et al., 2008
Wheat	38	3.2	12-13	46 - 47	19	Tilapia	Azim and Little, 2008
	51 - 54	1.9 - 2.6	7 - 8	37 - 39	21 - 22	Tilapia	Xu et al., 2012
Brown sugar	26 - 31	2.2 - 2.7	27 - 39	32 - 40	10 - 13	*L. vannamei*	Crab et al., 2010

* Estimated according to Bureau et al. (2002)

8.1.1.3 Physical characteristics

Because of the nature of ionic profile on the surface of a bacterium, floc formation may be influenced by the ionic strength in the water of a biofloc-based culture system (De Schryver et al. 2008). Furthermore, it has been suggested that floc formation is favored in the water with low zeta potential, an electrical potential of ions in the surroundings of the bacterial surface, and at high Van der Waals forces that induce an attractive power between particles (De Schryver et al., 2008, and Ritvo et al., 2003). Therefore, manipulating the ionic strength and ion composition in the water such as by the addition of Ca^{2+} ion could influence biofloc structural properties. Ritvo et al. (2003) noted that the point zero charge of salt effect, defined as the pH-values at which the net surface charge is zero, of biofloc collected from tilapia culture is in the pH range of 2.8 – 4.2. This suggests that decreasing the water pH would result in more flocculation of the suspension in water column. Furthermore, Ritvo et al. (2003) also demonstrated that increasing water salinity could stimulate floc formation.

The stability, size and structure of bioflocs formed in an aquaculture system may be influenced by some parameters in aquaculture technical operation. Mixing intensity will determine the shear rate and dissolved oxygen concentration, which directly affect the floc structure. Shear rate defines the equilibrium between the rate of aggregation and the rate of breakage, and the floc size distribution (De Schryver et al., 2008). Moreover, the dissolved oxygen concentration in the water has been reported to have influence on microbial balance between filamentous bacteria and floc forming bacteria, which in turn affects the floc size and settling property. An interesting result by Furtado et al. (2011) demonstrated that the addition of CaOH, carbonate or sodium bicarbonate, to anticipate the reduction of water pH and alkalinity in a biofloc-based shrimp culture, resulted in a higher floc volume and floc volume index. This suggests that the addition of these compounds into the biofloc water could also influence the floc characteristics. The choice of aquaculture species might also influence the biofloc physical characteristics. The present study (Chapter 4) showed that co-culturing shrimp with tilapia resulted in a lower floc volume

index. It is suggested that the mucus secretion by tilapia might be one of many factors that have influence on the floc setting ability.

Floc size has been suggested to affect the biofloc functioning in an aquaculture system (Ekasari et al., 2014a and Ritvo et al., 2003). Ritvo et al. (2003) suggested that a smaller floc size might provide a higher surface area for nitrifiers, thus facilitating N removal through nitrification. On the other hand, flocs with bigger and compact structure may facilitate anaerobic environment for the denitrifiers. The present study demonstrated that biofloc physical structure, represented by the size distribution, could influence the nutritional quality of biofloc as a food source for aquaculture animals (Ekasari et al., 2014a). The flocs with a size range of > 100 µm seem to be higher in protein and lipid content than smaller sized flocs. Additionally, the study also showed that flocs with a particle diameter of more than 100 µm contain essential amino acids of higher quality than the other floc size groups.Furthermore, the higher nutritional quality of this particular floc size seems to relate to the higher N uptake from biofloc by the tested animals.

8.1.2 Target species performance

A summary presented in Table 8.2 shows that the application of biofloc technology could improve net productivity in the order of 8 to 43% relative to the non-biofloc control (traditional with water exchange, clear water system or recirculating aquaculture system). A few studies reported negative effects of biofloc application (Emerenciano et al., 2013b, and Samocha et al., 2007). The reduced performance of biofloc-grown *L. vannamei* as reported by Samocha et al. (2007) appears to be a single observation, as several studies with the same species and the same carbon source showed positive results (Gao et al., 2012 and Xu and Pan 2012). Adverse effect of biofloc technology on the pink shrimp *Farfantepenaeus duorarum*, on the other hand, could be explained by the lower tolerance to high stocking density and water turbidity (Emerenciano et al., 2013b). Furthermore, the absence of morphological structures to capture the suspended particles in this species seems to be affecting its capability to ingest-digest biofloc particles (Emerenciano et al.,

2013b). This is confirmed by Jang and Kim (2013) who demonstrated that the capability of different species of shrimp in utilizing bioflocs was attributed to the structure of the third maxilliped that traps the suspended particles.

Table 8.2. The effect of biofloc technology application on the productivity of various aquaculture species.

Species	Net productivity (ton/ha)	ΔNet productivity relative to the control (%)	Reference
Penaeus monodon	0.5	> 100	Kumar et al., 2014
Oreochromis niloticus	370	29	Luo et al., 2014
Oreochromis niloticus	48	43	Azim and Little, 2008
Marsupenaeus japonicus	13	31	Zhao et al., 2012
Litopenaeus vannamei	8	< 0	Samocha et al., 2007
Litopenaeus vannamei	7	34	Xu and Pan, 2012
Litopenaeus vannamei	9	17	Gao et al., 2012
Litopenaeus vannamei	5	8	This study (Chapter 5)
Macrobrachium rosenbergii	5	17	Pérez-Fuentes et al., 2013
Farfantepenaeus duorarum	0.4	< 0	Emerenciano et al., 2013b
Penaeus semisulcatus	5	33	Megahed, 2010

8.1.2.1 Nutritional contribution of bioflocs

One of the primary characteristics of biofloc technology is the contribution of microbial biomass generated from nutrient waste as a food source for the animal cultured. In this regard, it can be expected that the feed utilization efficiency in a biofloc system is higher than that of clear water system with regular water exchange or recirculating aquaculture system (Table 8.3). Furthermore, our work that showed considerably higher protein and lipid assimilation by shrimp in biofloc group confirmed the more efficient dietary nutrient assimilation in this system (Ekasari et al., 2014b). Essentially, biofloc studies with Pacific white shrimp (Xu et al., 2012),

tilapia (Azim and Little, 2008), and green tiger shrimp (Megahed, 2010) clearly showed the possibility to reduce protein content in the feed. Moreover, Ray et al. (2010a) pointed out that the use of plant-based diet (96% protein obtained from plant-based ingredients) is favourable in a biofloc system. The reduction of protein content of the feed and the use of plant-based protein sources in the feed are considered to be more sustainable and eco-friendly because of the less nitrogenous and phosphorous waste produced. It also reduces the dependency on overexploited marine resources.

Table 8.3. Food conversion ratio (FCR) of some aquaculture animals cultured in biofloc system

Species	FCR	Δ FCR relative to non biofloc control (%)	Reference
Penaeus monodon	1.47	34	Kumar et al., 2014
Oreochromis niloticus	1.20	18	Luo et al., 2014
Oreochromis niloticus	3.45	30	Azim et al., 2008
Marsupenaeus japonicus	1.67	7	Zhao et al., 2012
Litopenaeus vannamei	1.41 - 1.56	7 - 16	Ekasari et al., 2014b
Litopenaeus vannamei	1.79	< 0*	Samocha et al., 2007
Litopenaeus vannamei	1.47	25	Xu and Pan, 2012
Litopenaeus vannamei	1.75	18	Gao et al., 2012
Macrobrachium rosenbergii	2.27	21	Pérez-Fuentes et al., 2013.
Penaeus semisulcatus	1.16	63	Megahed, 2010

* < 0 : higher feed efficiency in the control

The improvement of feed efficiency in a biofloc system may be attributed to the contribution of bioflocs as a food source, more specifically the contribution of essential nutrients and digestive enzymes either through the stimulation of endogenous production or microbial secretion, and the enhancement of nutrient bioavailability that facilitates higher nutrient assimilation. This study (Ekasari et al., 2014a) showed that although the uptake level was affected by the particle size of bioflocs, shrimp, tilapia and mussel consumed bioflocs at a range of 2.8 % to about

12% of the animal body weight. This was confirmed by using ^{15}N stable isotope as an indicator for biofloc consumption and microbial protein uptake in a biofloc system (Ekasari et al., 2014a). It has been reported that daily nitrogen uptake by tilapia (Avnimelech and Kochba, 2009) or shrimp (Burford et al., 2004) contributed ca. 22 - 28% of the daily protein from conventional feeding using a formulated diet. Fatty acid is also a well-recognized biomarker for the study of trophic interactions in aquatic, especially marine, habitats (Dalsgaard et al., 2003, Kelly and Scheibling, 2012, and Troch et al., 2012). Odd and branched chain fatty acids such as 15:0, 17:0, 15:1, 17:1, 16:1n-7, 18:1n-7, and iso- and anteiso- branched saturated fatty acids have been known as putative biomarkers for bacteria (Dalsgaard et al., 2003, Kelly and Scheibling, 2012, and Ray et al., 2010b). The data of fatty acids profile of shrimp cultured with flocs (Izquierdo et al., 2006), showed that the concentrations of 15:0, 16:1n-7, 17:0, and 17:1 fatty acids, as the indicators of assimilation of heterotrophic bacteria in shrimp, were considerably higher in the shrimp cultured in floc system than those in the shrimp cultured in clear water system (Table 8.4). This again confirms the role of microbial flocs as a food source for the shrimp.

Table 8.4. The concentrations of fatty acids (heterotrophic bacteria biomarkers) in shrimp cultured in floc and clear water systems (data obtained from Izquierdo et al., 2006). DW=dry weight, nd=not detected.

Biomarkers	Diet (%DW)	Floc (%DW)	Shrimp (%DW)	
			Floc	Clear Water
15:0	0.011	0.025	0.006	0.003
15:1	nd	0.021	nd	nd
16:0iso	nd	0.014	0.018	0.016
16:0aniso	0.002	0.072	nd	nd
16:1n-7	0.119	0.875	0.03	0.011
17:0	0.013	0.011	0.013	nd
17:1	0.01	0.182	0.005	nd
18:1n-7	0.264	0.197	nd	nd

One of the factors influencing an efficient use of biofloc protein as a food source for the cultured animals is the amino acids composition. According to the essential amino acids index which is an index representing dietary amino acids compliance relative to the animal's requirement, bioflocs could be considered as a good protein source for shrimp and a useful protein source for tilapia and mussel (Ekasari et al., 2014a). Furthermore, it is also important to note that bioflocs also contains various bioactive compounds including carotenoids, free amino acids, and chlorophylls (Ju et al., 2008), trace minerals (Tacon et al., 2002) and vitamin C (Crab et al., 2012) which have been known to have positive effects on aquaculture animals including the enhancement of antioxidant status, growth, and immune response.

New findings that have been reported recently are the contribution of exogenous digestive enzymes of the microbes in bioflocs in improving the digestibility of the feed as well as the bioflocs (Anand et al., 2014, Luo et al., 2014, Xu and Pan, 2012, Xu et al., 2013, and Xu et al., 2013). It has been demonstrated that extracellular protease and amylase activities of bioflocs collected from a biofloc shrimp culture unit were about 11 – 14 and 294 – 335 μmol/min/g total suspended solids (TSS), respectively (Xu and Pan, 2012). These extracellular enzymes, most possibly excreted by the microbes in bioflocs, facilitate the breakdown of complex nutrients including protein and carbohydrate into smaller units thus facilitating higher digestibility and nutrient absorption of the ingested bioflocs. Furthermore, it is possible that these exogenous enzymes also work effectively in the digestive tract of the shrimp upon liberation from the lysed ingested microbial cells. This was shown by the significantly higher activity of protease, amylase, cellulose and lipase in the shrimp stomach that contributed to the increase of feed utilization efficiency (Xu and Pan, 2012 and Xu et al., 2013).

It is also important to point out that the consumption of bioflocs seems to stimulate the production or the activity of endogenous digestive enzymes. Xu and Pan (2012, 2014) demonstrated the considerably higher activity of total protease, trypsin, and cellulase in the digestive gland of shrimp cultured in biofloc system. The authors suggested that this stimulation effect was a response to changes in diet composition in the presence of biofloc. Interestingly, the stimulation of endogenous

digestive enzyme activity is also exerted by bioflocs incorporated in the feed. Anand et al. (2014) demonstrated that the activity of amylase, cellulase, protease and lipase in the hepatopancreas were significantly stimulated in the shrimp fed with a diet containing 4% of biofloc powder. Thus resulting in better utilization (digestion) of the formulated feed.

8.1.2.2 The effect of biofloc technology on the health condition of the cultured animals

The health condition and welfare of the cultured animal will certainly affect their growth and survival, and eventually the production. Hence, this aspect is an important indicator of the success of any innovations or modifications in an aquaculture system. Only few studies elucidating the possible effects of biofloc system on the health and welfare of the cultured animals have been reported so far. Gill occlusion is the most common adverse effect on the health of animals cultured in biofloc systems (Azim et al., 2008, Ray et al., 2011, and Schveitzer et al., 2013). Schveitzer et al. (2013), for instance, observed more than two times higher incidence of gill obstruction in shrimp cultured in biofloc systems with TSS levels of more than 400 mg/L. It has been suggested that gill obstruction by microorganisms can affect the shrimp's breathing and osmoregulatory function and in the case of severe condition it may lead to asphyxiation (Bauer, 1979, and Clifford and Cook, 2002). In the present study (Chapters 3), we observed an adverse effect of biofloc particle size of > 100 µm on the survival of mussel which may also relate to gill occlusion.

Antioxidant and immune responses are relatively common parameters in the assessment of aquaculture animal's health condition. Antioxidant activity is frequently used as an indicator to oxidative stress resulting from pathogen pressure and environmental perturbations, and therefore can reflect the animal health status (Liu and Chen, 2004 and Xu and Pan, 2013). Several studies showed that the application of biofloc system also improved antioxidant activity of the cultured animals (de Jesus Becerra-Dorame et al., 2012, Luo et al., 2014, Xu and Pan, 2013). Xu and Pan (2013) for instance reported that the total antioxidant activities of shrimp in biofloc groups were more than two times higher than that of the control (clear water).

It has been suggested that this antioxidant activity enhancement effect of bioflocs might relate to the contribution of some bioactive compounds known to have antioxidant effect such as carotenoids, vitamin C, and essential fatty acids (Xu and Pan, 2012 and Xu and Pan, 2013).

The effects of bioflocs on the immune response have been investigated in this study (Ekasari et al., 2014b). Although it is widely known that the microbial cell components or metabolites can act as immunostimulants that enhance the innate immune system and provide protection against pathogen, only few studies have been done to elucidate the immune-modulating effect of bioflocs. Our study showed that the shrimp immune response, as represented by the total haemocyte count and phenoloxidase activity, was significantly enhanced in the biofloc group relative to the control group in clear water (Ekasari et al., 2014b). This was in agreement with previous reports showing that maintaining shrimp in the bioflocs system increased the quantity of circulating haemocytes and increased phagocytic activity, but not antibacterial and bacteriolytic activities (Xu and Pan, 2013). As it is clear that bioflocs are consumed and digested by the shrimp, it is possible that microbial cell components or metabolites released during the digestion eventually stimulated the shrimp defence mechanisms. The immunomodulating effect of bioflocs was also confirmed by Kim et al. (2014) who reported the upregulation of six immune related genes (prophenoloxidase [ProPO1 and ProPO2], serine protease [SP1], prophenoloxidase activating enzyme [PPAE1], masquerade-like serine protease [mas] and Rat-sarcoma-related nuclear protein) in shrimp grown in biofloc systems. The increased activity of the immune system of the shrimp grown in the presence of bioflocs was confirmed by the higher survival of the shrimp following viral challenge (Ekasari et al., 2014b).

The health condition of aquaculture animals also strongly relates to pathogen pressure. Our study (Ekasari et al., 2014b) indicates that the presence of *Vibrio*, known to be potentially pathogenic, in the intestines of shrimp in biofloc system was significantly reduced. This is in agreement with the study by Crab et al. (2010) who reported a reduction in luminescent and viable cells of pathogenic *Vibrio harveyi* in *Artemia* cultured with biofloc as well as their higher survival following *V. harveyi*

challenge relative to control. The authors suggested that the reduction of *V. harveyi* population in biofloc environment might be attributed to the disruption of *V. harveyi* cell-to-cell communication also known as an important factor in determining the pathogenicity of this particular bacterium. Likewise, Zhao et al. (2012), by DGGE analyses on the Kuruma shrimp culture water, demonstrated a high predominance of *Vibrio* (23%) in the control, while there was no evidence of *Vibrio* presence in biofloc system.

It is interesting to point out that the mechanisms by which probiotic bacteria seems to affect shrimp performance such as immunomodulation, competitive exclusion, bioremediation, provision of nutrients sources and contribution to digestion and quorum sensing blocking have also been reported in some biofloc studies including the present study (De Schyrver et al., 2012, Farzanfar, 2006, and Ninawe and Selvin, 2009). This signifies that the beneficial effects of bioflocs are, to some extent, comparable to the effects of probiotic application in aquaculture.

The welfare of the cultured animals is most frequently represented by the animal's physical health. Ashley (2007) noted that stress and immune function could be used as the indicators to assess the welfare of aquaculture animals. There are only a few studies that measured the effect of biofloc technology application on the cultured animal welfare. Azim and Little (2008) for example reported that the welfare of tilapia, as represented by plasma cortisol following a stressor, was lower in biofloc-grown tilapia than in the control. On the contrary, different results recently reported by Luo et al. (2014) showed that the immune activity of tilapia in biofloc system was significantly higher than that of the control (recirculating aquaculture system). The result of the first study seems to be implausible because the growth performance, which is also a good indication for animal welfare, of the fish in biofloc system was significantly higher than that of the control and therefore did not support the conclusion that the fish welfare in biofloc system was poorer than that of the control. Interestingly, Becerra-Dorame et al. (2014) showed that the concentration of lactate, an indicator of anaerobic metabolism that might be related to stressful

situation (Ashley, 2007), in shrimp blood of biofloc group was significantly lower than that of the non-biofloc group.

8.1.2.3 The effect of biofloc technology on offspring production

The effect of biofloc technology on the reproductive performance of aquaculture animals has been reported recently. Ekasari et al. (2013) demonstrated that the total eggs and recruits number produced by Nile tilapia broodstock maintained in a biofloc system were significantly higher than that in the green water system. This was parallel to Emerenciano et al. (2013a) who demonstrated that maintaining pink shrimp (*Farfantepenaeus duorarum*) broodstock in biofloc system resulted in higher female fecundity and spawning rate, and an increased eggs size. This improvement in shrimp reproductive performance has been suggested to be a response to the nutritional contribution of bioflocs. Furthermore, bioflocs have also been reported to contain some essential nutrients, important for fish reproduction including essential fatty acids, carotenoids, essential amino acids, vitamin C as well as some minerals (Ju et al., 2008). This was confirmed by Ekasari et al. (2013) showing that blood glucose and total cholesterol in biofloc broodstock group were considerably higher than in those in the green water broodstock group, pointing towards a higher availability of energy and an increased availability of precursors for the biosynthesis of steroid hormones important for reproductive processes. Another study showed that maintaining white shrimp male broodstock in a biofloc system, with commercial artificial feed, resulted in a comparable spermatophore and sperm quality to those maintained in clear water system with a high daily water exchange with a fresh food (trash fish, squid and crab) diet (Braga et al., 2013). In this regard, applying biofloc technology in the pre-maturation stage of male broodstock brings about more advantages than the clear water technology approach as it requires less water and reduces fresh food utilization that may increase the risk of disease transmission.

Our study in Chapter 7 shows how bioflocs in broodstock fish rearing and larval culture affects the growth performance and robustness of Nile tilapia larvae.

We indicated that the higher larval production in biofloc system observed in the previous experiment (Ekasari et al., 2013) was not only caused by the increased broodstock fish productivity but also by the robustness of the larvae produced. It was demonstrated in a larval growth experiment in the present study (Chapter 7) that the survival of the larvae from broodstock fish in a biofloc system was significantly higher than that of larvae from the control system. Furthermore, the experiment also showed that the offspring originating from broodstock fish maintained in a biofloc system were significantly more resistant to bacterial disease infection and to salinity stress than those obtained from broodstock fish in the control system. Although this still needs to be confirmed in a follow up study which should include broodstock tank replicates, the results of the experiment in Chapter 7 strongly suggested that the microbial environment and nutritional contribution of biofloc in the biofloc-based broodstock culture system may positively contribute to the enhancement of maternally derived immunity as well as the resistance of the larvae to environmental disturbances. The more uniform fish size in biofloc-based Nile tilapia larvae rearing suggests the contribution of bioflocs as an easily accessible food source for the larvae. The continuous availability of bioflocs in the culture system provides additional food source for the larvae outside the regular feeding moments, thus minimizing possible negative social interaction during feeding (Chapter 7). Furthermore, the digestion of bioflocs by the larvae allows the release of MAMPs, which have been recognized as immunostimulants, resulting in higher resistance to bacterial disease of the larval cultivated in a biofloc system than in the control system (Kim et al., 2014, Xu and Pan, 2013, and Xu and Pan, 2014).

8.1.3 Environmental performance

Equally important as target species production enhancement, the application of biofloc technology may significantly reduce the quantity of water used, a main resource in aquaculture. To illustrate, an intensive zero exchange lined shrimp pond only required 1 – 2.26 m^3/kg shrimp, whereas a conventional system with regular water exchange may require water up to 80 m^3/kg (Hargreaves, 2006). In addition,

Luo et al. (2014) noted that water consumption of biofloc-based tilapia culture system was 40% lower than that of recirculating aquaculture system (RAS).

One of the potential major negative impacts of aquaculture activities is the high nutrient loading in the effluent water. The magnitude of the nutrient waste produced by an aquaculture unit is determined by the level of utilization efficiency of the system. The evidence of bioflocs consumption and the assimilation of its nutrient in an aquaculture system clearly confirmed the role of the microbial loop in returning the nutrients from the waste back into the food chain and eventually the cultivated animal. The microbial loop describes the interconnected dissolved organic matter utilization and excretion processes by various microorganisms including bacteria, microalgae and zooplankton that ultimately return the organic matter in the form of biomass into the food chain fuelling the higher trophic level (Fenchel, 2008).

8.1.3.1 Nutrient utilization and waste production

Our study, which used ^{15}N stable isotope to trace nitrogen, demonstrated a considerably high nitrogen recovery from bioflocs by shrimp, tilapia and mussel (Ekasari et al., 2014a). Moreover, as it was observed in the present study (Ekasari et al., 2013, 2014b, 2014d) most of the studies using bioflocs confirmed that external organic carbon addition reduced the dissolved inorganic N in the water of aquaculture system (Crab et al. 2009, Kumar et al., 2014, Luo et al., 2014, Megahed, 2010, Pérez-Fuentes et al., 2013, and Zhao et al., 2012,). Our results showed that the dissolved inorganic N (dynamic) in a biofloc system could be dependent on the type of carbon source added, with carbon source containing higher fibre resulting in slower N removal (Ekasari et al., 2014b).

Although heterotrophic bacteria are expected to outcompete the nitrifiers through the addition of organic C source to attain high C/N ratio, substantial levels of nitrite-N and nitrate-N clearly are evidence of ongoing nitrification. It is possible that the nitrifiers take advantage of living in biofloc environment by attaching to heterotrophic flocs (Hargreaves, 2006). The presence of nitrifiers on one hand may contribute to overall NH_3-N removal potential of biofloc system along with

heterotrophic immobilization, but on the other hand nitrification will also add more complexity and dynamic into the system influencing oxygen, CO_2 production, and the reduction of alkalinity (Azim and Little, 2008). The addition of secondary species in biofloc-based integrated culture may also affect the nitrification in the system (Chapter 4). The addition of secondary species may contribute to the N turnover in the system that may eventually affect the requirement of organic C by heterotrophic bacteria to completely immobilize ammonia-N. It has been demonstrated in the present study that the addition of organic C source, which was determined according to the presumptive nitrogen utilization only by the main organism (shrimp), might not have been sufficient for the heterotrophic bacteria to assimilate extra production of ammonia. Hence, it facilitated the nitrifiers to use this extra ammonia as a substrate.

The presence of phototrophic N uptake in biofloc system, as shown by the concentration of chlorophyll a or the density of phytoplankton, may also contribute to the overall water quality in a biofloc system (Baloi et al., 2013, Emerenciano et al., 2013b, Hargreaves, 2006, and Magondu et al., 2013). It seems that phytoplankton is also benefited by the high nutrient availability particularly ammonium, nitrate, as well as phosphate (Hargreaves, 2006). Moreover, the high mixing rate in biofloc system may provide continuous exposure to light and carbon dioxide (Burford et al., 2003). Differences in phototrophic N removal may be explained by the difference in light exposure (Baloi et al., 2013) (which in turn might be influenced by floc concentration).

Although more research is needed to confirm the level of denitrification in various biofloc systems, recently the results of Hu et al. (2014) indicated that denitrification is occurring. Denitrification may contribute to the reduction of nitrate accumulation from nitrification, by converting nitrate into N_2 gas that will be emitted from the water. It is interesting to point out that the addition of organic C into biofloc system facilitates a fast and complete conversion of nitrate into N_2, lowering N_2O, a greenhouse gas (GHG) that is an intermediary product of denitrification (Hu et al., 2012, Hu et al., 2013, Hu et al., 2014). However, the high heterotrophic microbial

respiration in biofloc system results in the release of CO_2. Hence, in terms of nutrient utilization efficiency, N_2 and CO_2 emission through microbial activities in a biofloc system may be accounted as a nutrient loss negatively contributing to the overall feed nutrient utilization efficiency. Therefore, further studies to control denitrification and to capture the CO_2 in biofloc-based systems aiming at increasing C and N retention in the aquaculture unit are required.

The nutrient recycling by the microbial loop involves the uptake of inorganic phosphorus by heterotrophic bacteria (Kirchman, 1994). In this regard, it is possible that the microorganisms in bioflocs could also enhance the bioavailability of this nutrient for the cultivated animals, converting indigestible P into more digestible P. The level of P assimilation efficiency of fishmeal and plant based ingredients by fish has been perceived to be limited by the high level of indigestible bone-P and phytate-P (Lall, 2002), therefore it is likely that this nutrient will be egested in the faeces rather than utilized by the cultivated animals. The consumption of the microbial biomass in the biofloc might therefore facilitate P assimilation, in particular the indigestible one, from the feed to the cultivated organisms thus reducing the nutrient waste. While more research is still needed to confirm this possibility, Luo et al. (2014) has recently reported that higher P recovery could be achieved in biofloc-based tilapia culture as compared to non-biofloc system.

While the potential of phosphorus conversion by heterotrophic bacteria has been reported by previous studies (Schneider et al., 2006, Kirchman, 1994), there are only few studies on biofloc technology in aquaculture that showed the effect of biofloc system on phosphorus concentration in the effluent water. Luo et al. (2014) reported that orthophosphate concentration in biofloc-based tilapia culture system was one log unit lower than that in RAS. Likewise, it has been noted that phosphorus output from a super intensive *L. vannamei* culture was three factors lower than that from semi-intensive culture and one log unit lower than that from intensive culture (Da Silva et al., 2013). These results showed that biofloc technology application may not only improve N, but also P utilization efficiency, corroborating the role of this system on the improvement of aquaculture productivity and the reduction of environmental impact from aquaculture unit.

Interestingly, combining biofloc system with integrated multi trophic culture system may also enhance nutrient utilization efficiency. This study demonstrated that combining biofloc system with tilapia resulted not only in higher production but also in higher feed P recovery (Chapter 4). With the higher efficiency in retaining P by tilapia (0.18%/g tilapia vs. 0.06%/g shrimp), tilapia addition on a biofloc-based shrimp culture resulted in substantially higher P utilization, accounting 31% of the feed and molasses P input. Furthermore, the level of P recovery in this study was indeed higher than that in the shrimp-tilapia co-culture without biofloc, which was about 19% (Yuan et al., 2010). This could point towards a potential contribution of bioflocs in P utilization in both monoculture and integrated culture systems. Moreover, the addition of secondary species such as mussel and seaweed to this system also improved N recovery by the shrimp itself as well as the overall feed N and P recovery of the system. The addition of tilapia and mussel in this regard increased the consumption of excess microbial biomass that was not utilized by the primary cultivated animal (shrimp). This study showed that tilapia and mussel recovered 8.58% of N and 19.25% of P inputs from the shrimp feed. Seaweed on the other hand, contributed significantly to the P utilization, showing a nutrient recovery level of 36 % of the feed P input. Furthermore, the addition of seaweed or macrophytes (Liu et al., 2014, Xu et al., 2008) in a biofloc-based integrated aquaculture system may also bring about the possibility to capture the excess CO_2, which may result in an increase in C utilization efficiency and a reduction in the emission of GHG. This additional benefit in nutrient utilization efficiency should stimulate further research on the possibility of incorporating biofloc system into an integrated multitrophic culture system to mitigate negative environmental impact of aquaculture nutrient wastes.

8.1.3.2 Water quality parameters

Apart from its potential role in the reduction of waste nutrient, another important water quality feature in a biofloc system observed in this study is the constant reduction of alkalinity during culture period (Chapter 4, 5 and 7). This can

be explained by the requirement of alkalinity in ammonia/ammonium transformation processes by heterotrophic bacteria, nitrification, and phytoplankton uptake (when ammonium is used as the N source). Most importantly, losing the buffering capacity of the water may eventually result in a high fluctuation or significant reduction of pH, particularly in biofloc system where CO_2 production from the cultured species and the microbial biomass is usually high (Furtado et al., 2011 and Furtado et al., 2013). This clearly confirms the importance of regular addition of alkalinity to maintain the buffering capacity of the system. Furtado et al. (2011) demonstrated that the mean of alkalinity concentrations, CO_2 and pH of a biofloc-based white shrimp culture without any addition of alkalinity were about 78 mg $CaCO_3$/L, 12.8 mg/L and 6.9, respectively. On the other hand, regular addition of sodium bicarbonate, calcium hydroxide or sodium carbonate could maintain alkalinity of more than 100 mg $CaCO_3$/L, pH of more than 7.5, and CO_2 concentrations of a range of 1 – 5 mg/L, which are acceptable levels for supporting a normal shrimp growth (Furtado et al., 2011). In addition, theoretical alkalinity requirement in a biofloc system can be calculated according to the stoichiometric equation of heterotrophic N conversion pathways (Ebeling et al., 2006). According to this equation, for every gram of ammonium nitrogen converted, ca. 3.57 g of alkalinity is required. In this regard, for a shrimp culture with a 30%protein content feed, it can be calculated that for every kg of feed added into a biofloc system, the quantity of alkalinity that needs to be added to support heterotrophic N conversion is at least 14%. This is in agreement with the study by Furtado et al. (2014) who recommended a daily addition of calcium hydroxide between 10 – 20% of the amount of daily feeding to maintain alkalinity and pH in a biofloc-based shrimp culture.

The most prominent limitation of regular carbon source addition in biofloc system is the microbial biomass built up (Avnimelech, 2012, Hargreaves, 2006, and Ray et al., 2011). Despite continuous consumption by the cultured animals, the high nutrient accumulation in the system boosts the microbial biomass production at a rate far exceeding the consumption rate by the cultured animals. As an illustration, an intensive shrimp farm with a production of 10 tonne/ha/season with an FCR of

about 1.5, feed protein content of 35%, and N retention about 30%, will generate 588 kg N waste. According to the stoichiometric equation of heterotrophic N conversion, the yield of bacterial biosynthesis is 10 mg dry weight/mg N (Ebeling et al., 2006). Hence, the microbial biomass yield generated in a shrimp culture system can reach about 5.9 ton which is about half of the harvested shrimp biomass.

Another consequence of microbial biomass built up in biofloc system, besides higher water turbidity, is a more dynamic and possibly unstable system. Furthermore, a high microbial biomass accumulation will also increase the risk of gill occlusion by suspended solids, O_2 consumption, and accumulation of CO_2 and other metabolites products (Ebeling et al., 2006, Hargreaves, 2006, Ray et al., 2010a; 2010b; 2011, and Vinatea et al., 2010). Hence, a regular removal of suspended solids in biofloc system is important (Ray et al., 2011 and Luo et al., 2014). Ray et al. (2011) for instance demonstrated that the use of settling chambers improved the water quality and production performance of *L. vannamei* in super intensive culture with biofloc technology. Additionally, Schveitzer et al. (2013) recommended maintaining TSS level between 400 – 600 mg/L for a stable system. Solid removal from a biofloc system, however, might reduce the nutrient utilization efficiency, therefore, alternative methods to harvest this excess microbial biomass for other purposes such as feed ingredient (Kuhn et al., 2010) or as food source for other species in an integrated aquaculture system (Emerenciano et al., 2012 and Liu et al., 2014) are of future research interest.

In the present work (Chapter 4), the possibility to utilize excess microbial biomass in a biofloc system by applying an integrated multitrophic culture system has been initiated. Integrating a biofloc-based shrimp culture system with tilapia at a density of 45% of shrimp biomass, mussel and seaweed resulted in 36% TSS reduction, 37% higher productivity, and 45% higher feed efficiency relative to that of monoculture system. However, it is also important to note that the addition of secondary species could also alter other water quality parameters. Our study showed that the consumption of bioflocs by secondary species in an integrated culture system could increase the N turn over in the system, thus contribute to the

production of additional total ammoniacal nitrogen (TAN) as the excreted product from bioflocs protein utilization. This might explain the absence of differences in the dissolved inorganic nitrogen concentrations between the biofloc-based monoculture and integrated culture. These results was indeed different from those reported by Liu et al. (2014) who demonstrated that a biofloc-based integrated culture of shrimp, spotted scat, and water spinach could reduce the total N in the cultured water. Nonetheless, these preliminary experiments demonstrated that combining biofloc system with an integrated multitrophic culture system might be a promising technique to control the accumulation of microbial biomass in aquaculture system and also to increase productivity and warrant further research at a larger scale.

8.2 Conclusion

The present study demonstrated new possible roles of biofloc technology in aquaculture production. We confirm that the application of biofloc technology could indeed improve the water quality of the culture system. However, it is also noticed that without the effort to control and to utilize the microbial biomass accumulation in the system, the application of biofloc technology indeed only converts the dissolved waste into solids waste. Therefore, in the attempt to increase biofloc and overall nutrient utilization, the potential of biofloc utilization by some aquaculture species has been elucidated. This study showed that particle size plays an important role in the nutritional quality of bioflocs and in situ utilization of bioflocs by some aquaculture species. With this knowledge in mind and the aim to increase nutrient utilization efficiency in an aquaculture system, a biofloc-based integrated culture has been constructed. Indeed, we demonstrated that combining biofloc system with an integrated culture system resulted in lower suspended solids, higher biomass production, total feed efficiency, as well as higher N and P recovery. Furthermore, it is shown in the present study that biofloc technology application not only plays essential roles in the water quality maintenance and the nutrient utilization efficiency, it also brings about other beneficial effects for the cultured species including enhancing their immunity as well as their reproductive performance. We

demonstrated that the consumption of bioflocs by shrimp resulted in an increase in itsimmune response leading to a higher resistance against infectious myo necrosis virus (IMNV) challenge. Similarly, a higher resistance against *Streptococcus agalactiae* was also observed in tilapia larvae cultured in a biofloc system. Moreover, a notable beneficial effect of biofloc system on tilapia reproduction was also confirmed.

It is important to note that the magnitude of the expected beneficial effects of biofloc system can be determined by the operational parameters applied in the aquaculture system. We confirmed that carbon source could affect the dissolved inorganic nitrogen profile in the cultured water, and that alkalinity should be closely monitored during the culture period in addition to dissolved oxygen concentration. It is also observed that the addition of secondary species in a biofloc-based integrated system might influence the water quality dynamic in the culture system and did not necessarily reduce the dissolved nutrient waste.

In conclusion, overall results of the present study suggest that biofloc technology application could indeed positively contribute to the enhancement of aquaculture production in a sustainable and ecological manner. However, it is also noticed that more research is needed to elucidate the factors determining the stability and functionality of a biofloc system. Furthermore, the question on whether these beneficial effects on the production and the environment can outbalance the extra cost needed for BFT application remains to be verified. Thus, a detailed business and life-cycle analysis is needed to quantify the profitability and sustainability of this system as well as its possible reductions in environmental impact.

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APPENDIX A. SUMMARY/SAMENVATTING

Biofloc technology offers beneficial effects on the production and environmental performances of intensive aquaculture system. Compared to other aquaculture systems, biofloc system provides more economical alternative in land and water usage, relatively straightforward, simple and robust technology, and additional microbial protein source. The potential of this system in increasing land, water and nutrient utilization efficiency has drawn more attention for research and application during the past decade.

The general objective of this study was to explore the contribution of biofloc technology application to aquaculture productivity enhancement, while maintaining sustainable practices.

An overview on the development of culture systems, including biofloc technology, that facilitate the increase of nutrient utilization efficiency in aquaculture is provided in **Chapter 2**. **Chapter 3** investigated the effect of biofloc size on the nutritional composition of the flocs and the nitrogen utilization by white shrimp (*Litopenaeus vannamei*), red tilapia (*Oreochromis niloticus*) and mussels (*Perna viridis*). This study showed that biofloc consumption by shrimp, red tilapia and mussels occurs irrespectively of floc size but that floc size can play an important role in the quality of bioflocs in terms of nutritional composition and nitrogen retention by the animals. The consumption levels of bioflocs with different size by different aquaculture animals shown in Chapter 3 were used as the basic information to develop an integrated biofloc- based aquaculture system assessed in **Chapter 4.** The results described this particular chapter showed that co-culture with tilapia seems to alter water quality profile and floc physical characteristics. Shrimp integrated multitrophic culture (IMT-culture) significantly increased productivity, feed efficiency, and total nitrogen and phosphorus recovery. Total suspended solids level was also reduced in IMT-culture systems, whereas dissolved inorganic nitrogen concentrations were not significantly affected.

The immunological effects of growing shrimp in biofloc systems with different carbon sources were documented in **Chapter 5**. An increased immunity was suggested by the immune parameters as well as the survival of the challenged shrimp from the experimental biofloc groups that was significantly higher as compared to the challenged shrimp from the control treatment, regardless of the organic carbon source used to grow the bioflocs. Overall, this study demonstrated that the application of biofloc technology may contribute to the robustness of cultured shrimp by immunostimulation and that this effect is independent of the type of carbon source used to grow the flocs.

Based on the information of nutritional property of bioflocs and water quality in the system gained from previous chapters, it was hypothesized that biofloc system might also exert positive effects on the seed production. In this regard, **Chapter 6 and 7** elaborate the investigation on the reproductive performance and offspring quality of Nile tilapia in biofloc system. Overall results of the experiments indicated that maintaining tilapia broodstocks in floc-based system resulted in better reproductive performance and higher quality of larvae.

Finally, the last chapter (**Chapter 8**) provides a general discussion of the research outcomes and some subjects of future research interest for the development of biofloc technology in aquaculture.

In conclusion, the present study demonstrated new possible roles of biofloc technology in the attempts to increase aquaculture productivity that include improving water quality, providing essential nutrients, improving resistance and tolerance to diseases and environmental disturbances, as well as increasing the production of high quality seed, in a sustainable and ecological manner.

SAMENVATTING

De biovlokken technologie heeft positieve effecten op productie en milieu performatie van intensieve aquacultuur systemen. Vergeleken met andere aquacultuur systemen heeft de biovlokken technologie voordelen op het gebied van land en watergebruik, is het een relatief simpele en robuste technologie en kan het een bijkomende bron van eiwitten betekenen. Het potentieel van dit systeem met betrekking tot land en watergebruiksefficientie heeft heel wat aandacht getrokken in zowel onderzoek als in de sector in de laatste tien jaar.

De objectieven van die PhD werk was na te gaan op welke manier de biovlokken technologie kan bijdragen tot verhoging van de efficientie in de aquacultuur, zonder negatieve effecten voor het milieu.

Een overzicht van de ontwikking van kultuursystemen, inclusief de biovlokken technologie, die een verhoogde retentie van nutrienten faciliteren wordt voorgesteld in **Hoofdstuk 2**. **Hoofdstuk 3** onderzoekt de effecten van biovlokken grootte op de nutritionale samenstelling van biovlokken en het stikstof gebruik door garnalen (*Litopenaeus vannamei*), tilapia (*Oreochromis niloticus*) en mosselen (*Perna viridis*). Deze studie toonde aan dat biovlokken geconsumeerd worden door garnalen, tilapia en mosselen onafhankelijk van de vlok grootte, maar dat de vlok grootte een belangrijke rol kan spelen in de kwaliteit van de vlokken in termen van nutritionele samenstelling en stikstof retentie door de dieren. Het consumptie niveau van biovlokken met verschillende grootte door verschillende dieren was de basis voor het onderzoek naar een geintegreerde biovlokken-gebaseerd aquacultuur systeem zoals onderzocht in **Hoofdstuk 4**. De resultaten toonden aan dat co-kultuur van tilapia met garnalen het profiel van de waterkwaliteitsparameters veranderde alsook de fysische karakteristieken van de vlokken. In de geintegreerde multitrofische cultuur verhoogde de productiviteit van garnalen, de voeder efficientie, en de totale stikstof en fosfor retentie. Het niveau van totale gesuspendeerde partikels was

verlaagd in geintegreerde kultuursystemen, terwijl de anorganische stikstof concentraties niet significant beinvloed werden.

Immunologische effecten op garnalen in het biovlokken systeem met verschillende koolstof bronnen zijn beschreven in **Hoofdstuk 5**. Blootstelling aan biovlokken schijnt een verhoogde immuniteit teweeg te brengen. Dat bleek uit verhoogde immuun parameters alsook uit verhoogde overleving bij blootstelling aan pathogenen in de groep van dieren die biovlokken vervoederd werden. Dit fenomeen was onafhankelijk van de gebruikte koolstofbron.

Gebaseerd op de informatie over de nutritionele waarde van biovlokken en de waterkwaliteit in het systeem, bekomen in de vorige hoofstukken werd er verondersteld dat biovlokken systemen ook een positieve invloed zouden kunnen hebben op de productie van larven. In verband hiermee wordt in **Hoofdstuk 6** en **7** de reproductieve performantie van tilapia bepaald alsook de kwaliteit van de larven. Uit dit onderzoek bleek dat inderdaad tilapia meer nakomelingen produceren in een biovlokken systeem en dat de larven in een dergelijk systeem van een hogere kwaliteit zijn.

Het laatste hoofdstuk bevat een algemene discussie van de onderzoeksresultaten en een omschrijving van het biovlokken onderzoek dat nuttig zou zijn in de toekomst.

In conclusie, het huidige onderzoek toonde aan dat biovlokken een positieve rol kunnen spelen in verhoogde aquacultuur productiviteit, inclusief een verhoogde productie van larven, terwijl het een positief effect kan hebben op waterkwaliteit, nutrientenretentie en ziekteresistentie. Ze kunnen dus bijdragen tot een verhoogde duurzaamheid van aquacultuur.

APPENDIX B. CURRICULUM VITAE

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EDUCATION

Ghent University, Belgium
Ph.D in Applied Biological Sciences **2010 – 2014**
Ph.D thesis: "Biofloc technology as an integral approach to enhance production and ecological performance of aquaculture"

Ghent University, Belgium
Master of Science in Aquaculture **2006 – 2008**
Thesis: "Bio-flocs technology: the effect of different carbon source, salinity and the addition of probiotics on the primary nutritional value of the bio-flocs"

Bogor Agricultural University, Indonesia
Bachelor in Aquaculture **1995 - 1999**

AWARDS

Aquaculture Engineering Society travel grant	**2013**
SEAMEO-SEARCA travel grant	**2011**
VLIR-UOS ICP PhD Scholarship award	**2010 – 2014**
VLIR-UOS Master Course Scholarship award	**2006 - 2008**

PROFESSIONAL EXPERIENCE

Bogor Agricultural University
Lecturer **2005 – present**

P.T. Charoen Pokphand Indonesia
Researcher **2000 – 2005**

Bogor Agricultural University
Teaching assistant **1999 – 2000**

PROFESSIONAL TRAINING

Bogor Agricultural University
Training in Laboratory Management **2013**

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Short Course in Stable isotopes in ecohydrology and plant ecology **2012**

Ministry of Fisheries and Marine Affairs
Training in Biofloc Technology **2007**

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Training in Human Relation **2002**

PUBLICATIONS AND PAPERS

Ekasari, J., Crab, R., Verstraete, W., 2010. Primary nutritional content of bio-flocs cultured with different organic carbon sources and salinity. Hayati Journal of Biosciences 17, 125–130.

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Rohmana, D., Surawidjaja, E.H., Sukenda, S., Ekasari, J., 2014. Water quality and production performance of catfish prawn co-culture with organic carbon source addition. Aquacult. Int., DOI 10.1007/s10499-014-9814-2

PARTICIPATIONS TO NATIONAL AND INTERNATIONAL MEETINGS AND WORKSHOPS

Ekasari, J., Azhar, M.H., Surawidjaja, E.H., De Schryver, P., Bossier, P. The effects of bioflocs grown on different carbon sources on shrimp immune response and disease resistance. Biofloc Technology and Shrimp Disease Workshop. Ho Chi Minh City, Vietnam December 9 – 10, 2013. Aquacultural Engineering Society. Oral Presentation

Ekasari, J., Angela, D., Waluyo, S.H., Bachtiar, T., Surawidjaja, E.H., Bossier, P., De Schryver, P. Utilization of biofloc with different particle size by Pacific white shrimp,

red tilapia and mussel. World Aquaculture Society, Asia-Pacific Chapter. Ho Chi Minh City, Vietnam December 9 – 10, 2013. Oral Presentation.

Suprayudi, M.A., Ekasari, J., Fauzi, I.A., Aritama, P., Cahaya, K.D. Apparent digestibility of rubber seed meal, cocoa pod meal, and corn cob meal in red tilapia *Oreochromis niloticus* and giant gourami Osphronemus gouramy diet. World Aquaculture Society, Asia-Pacific Chapter. Ho Chi Minh City, Vietnam December 9 – 10, 2013. Oral Presentation.

Ekasari, J. The benefits and challenge of biofloc-based shrimp culture system. International Symposium on Aquatic Product Processing. Bogor, 13 – 15 November 2013, Indonesia. Oral Presentation.

Ekasari, J., Napitupulu, I.D., Supriyono, E. Ionic strength manipulation to stimulate microbial aggregate formation in biofloc-based shrimp culture system. Indonesian Aquaculture Conference, Solo 3 – 4 September 2013, Indonesia. Oral presentations

Ekasari, J., Zairin, Jr. M., Putri, D.U., Sari, N.P., Widanarni, Surawidjaja, E.H., Bossier, P., Biofloc-based reproductive performance of Nile tilapia broodstock. World Aquaculture Society. Nashville, USA, 21 – 25 February 2013. Oral Presentation.

Ekasari, J., Chayati, T.N., Widanarni, Wiyoto, Surawidjaja, E.H., Bossier, P., Do bioflocs technology and Skt-B probiotics application contribute to the resistance of whiteleg shrimp *Litopenaeus vannamei* to IMNV and *Vibrio harveyii* coinfection? Fourth National Symposium of Biotechnology in Aquaculture, 18 October 2012. Bogor, Indonesia. Oral presentation

Ekasari, J. Grouper Nutrition. 7th Regional Grouper Hatchery Production Training Course. The Network in Aquaculture Centres in Asia-Pacific (NACA) and the Brackish water Aquaculture Development Center of the Ministry of Fisheries and Marine Affairs of the Republic Indonesia. Situbondo, Indonesia. 25 September – 15 October 2011. Oral Presentation

Ekasari, J., Widanarni, Maryam, S. Water quality and production performance of red tilapia intensive culture with biofloc technology. World Aquaculture Society. Kochi, India, 17 – 20 January 2011. Oral Presentation.

MEMBERSHIPS

World Aquaculture Society

Aquacultural Engineering Society

Indonesian Society for Scientific Aquaculture

Indonesian Microbiology Society

APPENDIX C. ACKNOWLEDGEMENT

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