

Brewer's yeast as a protein source in
the diet of tilapia (*Oreochromis
niloticus*) and freshwater prawns
(*Macrobrachium rosenbergii*) reared in
a clear water or biofloc environment

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Cover: Tilapia and giant freshwater prawns next to imhoff cone with biofloc inside
(photo: Nguyen Huu Yen Nhi, 2017)

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Brewer's yeast as a protein source in the diet of tilapia (*Oreochromis niloticus*) and freshwater prawns (*Macrobrachium rosenbergii*) reared in a clear water or biofloc environment

Abstract

This thesis investigated the effects of dietary protein replacement of fishmeal or soybean meal with spent brewer's yeast (SBY) in farmed tilapia and giant freshwater prawns. The effect of rearing tilapia and prawn in two different rearing systems, clear water recirculating aquaculture system (CW-RAS) and biofloc recirculating aquaculture system (Bio-RAS), were also investigated.

The fish reared in Bio-RAS displayed higher growth, a higher protein efficiency ratio and a lower feed conversion rate than fish reared in CW-RAS. This difference between systems was not as apparent in the growth and protein efficiency ratio of freshwater prawns.

In freshwater prawns, the survival rate was not affected by aquaculture water system nor by the replacement of fishmeal with SBY. The growth performance of prawns was not significantly different between any dietary treatments in Bio-RAS or CW-RAS. Significant differences were only found between brewer's yeast replaced fishmeal at 60% in a CW-RAS and at 40% in Bio-RAS.

Tilapia reared in CW-RAS with replacement of fishmeal with SBY showed a significantly ($p < 0.05$) lower weight gain, daily weight and specific growth rate than fish in Bio-RAS when all diet groups per treatment was combined. However, at diet group level this difference was significant only in the 100% replacement group of CW-RAS when compared with the control in Bio-RAS.

The protein requirement experiment showed that 27% and 31% crude protein can be used for tilapia in Bio-RAS and CW-RAS, respectively. The study demonstrated that tilapia reared in the Bio-RAS had a higher capacity to compensate for a reduction in dietary protein levels, as demonstrated by a higher growth rate than in fish reared in CW-RAS. The apparent digestibility of crude protein by tilapia was high in diets with high crude protein, but there was no difference between the two rearing systems.

SBY replace up to 100% of soybean meal without a significant reduction in tilapia growth. Tilapia exhibited the best performance when fed a diet where 30% soybean meal was replaced with SBY.

This thesis concludes that SBY represents a sustainable, high-volume protein substitute for fishmeal and soybean meal in tilapia and giant freshwater prawn production and that the protein requirement of tilapia can be reduced if reared in a high-density microbial environment, i.e. a so-called Bio-RAS.

Keywords: Nile tilapia, spent brewer's yeast, Bio-RAS, CW-RAS, biofloc, freshwater prawns, alternative protein, growth performance, recirculation system, soybean meal

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Dedication

To my parents with my respectful gratitude,
My husband Nguyen Thanh Tam,
My daughter Nguyen Phuong Quyen,

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List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Nhi NHY*, Da CT, Lundh T, Lan TT, Kiessling A (2018). Comparative evaluation of Brewer's yeast as a replacement for fishmeal in diets for tilapia (*Oreochromis niloticus*), reared in clear water or biofloc environments. *Aquaculture*, 495, 654-660.
- II Nguyen Huu Yen Nhi*, Trinh Thi Lan, Chau Thi Da, Kartik Baruah, Torbjörn Lundh and Anders Kiessling. Spent brewer's yeast as a replacement for fishmeal in diets for giant freshwater prawn (*Macrobrachium rosenbergii*), reared in either clear water or a biofloc environment. (Accepted, *Aquaculture nutrition*).
- III Nguyen Huu Yen Nhi, Trinh Thi Lan, Kartik Baruah, Torbjörn Lundh and Anders Kiessling. Nitrogen retention and protein requirement in Nile tilapia (*Oreochromis niloticus*) reared in clear water or biofloc water and fed different levels of crude protein. (Manuscript).
- IV Nguyen Huu Yen Nhi, Trinh Thi Lan, Kartik Baruah, Torbjörn Lundh and Anders Kiessling. Brewer's yeast as a protein source replacement for soybean meal in tilapia (*Oreochromis niloticus*) cultured in biofloc or clear water recirculation systems. (Manuscript).

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The contribution of Nguyen Huu Yen Nhi to the papers included in this thesis was as follows:

- I Participated in planning of the experiment and carried out the experiment, sampled fish, evaluated the results, performed the statistical analyses and wrote the manuscript.
- II Participated in planning of the experiment and conducted the experiment, sample collection, evaluated the results, performed the statistical analyses and wrote the manuscript.
- III Participated in planning and conducted the experiment, fed and collected waste from fish, sampled fish, evaluated the results, performed the statistical analyses and wrote the manuscript.
- IV Participated in planning of the experiment, carried out most feeding and collection of fish and biofloc samples, evaluated the results, performed the statistical analyses and wrote the manuscript.

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Abbreviations

AD	Apparent digestibility
ADiCP	Apparent digestibility of crude protein
ADiDM	Apparent digestibility of dry matter
ADiE	Apparent digestibility of energy
ADiOM	Apparent digestibility of organic matter
BF	Biofloc
BFT	Biofloc technology
Bio-RAS	Biofloc - Recirculating aquaculture system
BW	Body weight
CF	Crude fibre
C:N	Carbon: Nitrogen
COD	Chemical oxygen demand
CP	Crude protein
CSM	Cottonseed meal
CW	Clear water
CW-RAS	Clear water - Recirculating aquaculture system
DM	Dry matter
DO	Dissolved oxygen
DWG	Daily weight gain
EE	Ether extract
FCR	Feed conversion ratio
FI	Feed intake
GIFT	Genetically improved farmed tilapia
GLM	General Linear Model
GSI	Gastro-somatic index
HPLC	High performance liquid chromatography
HSI	Hepato-somatic index
iEns	Entero-somatic index
NFE	Nitrogen free extract
NO ₂ -N	Nitrite-nitrogen
NPU	Net protein utilization

PER	Protein efficiency ratio
PI	Protein intake
Qi	Intestinal quotient
RAS	Recirculating aquaculture system
SBM	Soybean meal
SBY	Spent brewer's yeast
SGR	Specific growth rate
SR	Survival rate
TAN	Total ammonia nitrogen
VSI	Viscero-somatic index
WG	Weight gain
WMB	Wheat milling by-product

1 Background

1.1 Current status of global aquaculture production

Global fish production peaked at about 171 million tons in 2016, with aquaculture representing 47 percent of the total. The total first sale value of fisheries and aquaculture production in 2016 was estimated at USD 362 billion, of which USD 232 billion was from aquaculture production (FAO, 2018).

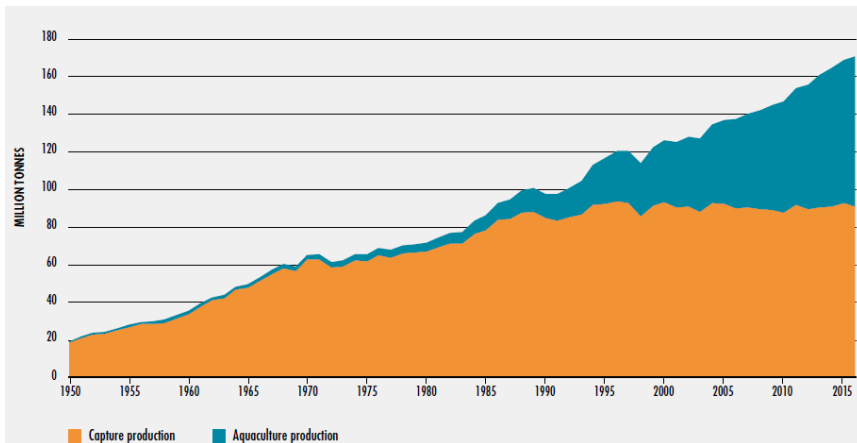


Figure 1. World capture fisheries and aquaculture production (FAO, 2018)

Aquaculture is growing very fast with an average annual growth of 5.8 percent during the years 2000 to 2016. The total global aquaculture production during 2016 consisted of 80.0 million tons of food fish and 30.1 million tons of aquatic plants, as well as 37 900 tons of non-food products. Farmed food fish production was comprised of 54.1 million tons of finfish, 17.1 million tons of molluscs, 7.9 million tons of crustaceans and 938 500 tons of other aquatic animals (FAO, 2018).

Aquaculture development varies greatly among and within geographical regions; a few major producers dominate the production of main groups of farmed species produced in inland aquaculture and in marine and coastal aquaculture. Developing countries dominate inland finfish farming, and Asia has accounted for about 89 percent of world aquaculture production for over two decades. China is a major producer of farmed food fish and has produced more than the rest of the world every year since 1991. India, Indonesia, Viet Nam, Bangladesh, Egypt and Norway were the other major producers (FAO, 2018).

Aquaculture is heterogeneous in terms of farmed species. Species are divided into omnivorous, herbivorous, and carnivorous fish. Carnivorous fish are meat eaters with a large mouth and sharp teeth used to catch and tear their prey. On the opposite end of the food chain are the herbivorous fish, which eat plants, algae, and fruits. Omnivorous fish eat everything in between the previous categories such as, detritus, meat and plants due to their having some of the traits of both the carnivore and the herbivore. Fed aquaculture, species including both carnivorous, herbivorous and omnivorous species, is produced in intensive and semi-intensive systems and fed with farm-made feeds or commercial compound feeds formulated to meet their nutritional requirements. Unfed aquaculture species include filter-feeding molluscan shellfish (e.g. oysters, clams, mussels) and aquatic plants (e.g. microalgae, seaweed). Farming of fed aquatic animal species has grown faster than that of unfed species. In 2016, the total unfed species production climbed to 24.4 million tons (30 percent of total farmed food fish), consisting of 8.8 million tons of filter feeding finfish raised in inland aquaculture (mostly silver carp and bighead carp) and 15.6 million tons of aquatic invertebrates, mostly marine bivalve molluscs raised in seas, lagoons and coastal ponds (FAO, 2018). About 88 percent of total fish production, over 151 million tonnes, was utilised for direct human consumption and this number has increased significantly in recent decades. Of the remaining 12 percent of total fish production, about 20 million tonnes, are used for non-food purposes such as fishmeal and fish oil. Fishmeal is one of the main feed components in aquaculture and livestock industries. It is also considered the most nutritious and digestible of ingredients in farmed fish feeds, but their inclusion rates in

compound feeds for aquaculture have shown a clear downward trend because of its increasing cost and restricted availability.

With steady and growing demand, long term fishmeal and soybean prices are consistently high (Figure 2) and will continue to increase in the near future (Asche *et al.*, 2013). Therefore, finding alternatives for fishmeal and soybean meal is of great importance (FAO, 2018).

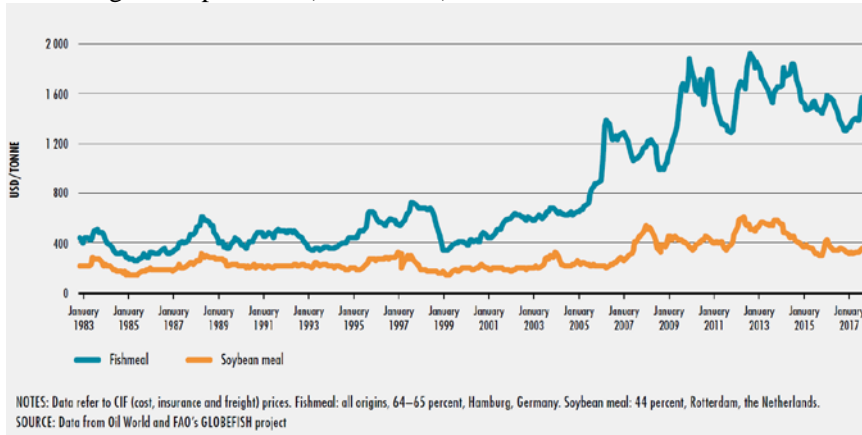


Figure 2. Fishmeal and soybean meal prices (FAO, 2018)

1.2 Vietnamese aquaculture

Viet Nam has a coastline of 3,260 kilometres and an Economic Exclusion Zone of 1 million square kilometres. The nation's aquaculture systems are diversified according to geographical and climatic conditions, which differ from North to South. The northern area is dominated by freshwater fish ponds, rice-cum-fish and marine cage culture. Central Viet Nam mostly consists of the intensive culture of giant tiger prawns and the marine cage culture of finfish or lobster. The southern area has the most diversified farming activities including pond, fence and cage culture of *Pangasius* catfish as well as several indigenous species such as tilapia, giant freshwater prawn, snakehead fish and climbing perch. Moreover, the southern area has various intensification levels and integrated culture such as rice-fish, rice-prawn and mangrove-aquaculture. Therefore, Viet Nam is an important producer of aquaculture products, and is one of the major producing countries accounting for 4.5% of total world aquaculture food fish production in 2016 (FAO, 2018). The total aquaculture production of Viet Nam in 2016 was 3,640.6 thousand metric tons including 2,576.2 and 663 thousand metric tons for fish and shrimp, respectively, an increase of 3.1% compared with the year 2015 (Vietnam, 2016). Shrimp and tilapia are two of Viet Nam's most

important aquaculture products. They share many ecological characteristics, and are cultured common worldwide.

1.3 Tilapia

Tilapia is the second major species produced in world aquaculture (Figure 3) after carp species (FAO, 2018). However, in Viet Nam, tilapia is the second white, farmed fish produced after *Pangasius*. There are two main species of tilapia including red tilapia (*Oreochromis sp.*), which is mainly raised for domestic-use, and Nile tilapia (*Oreochromis niloticus*), which is mainly raised for export. These two species have been cultured in two main farming models, monoculture and polyculture with shrimp. Cages in rivers, reservoirs, lakes and earth ponds are the main kinds of farming facilities. Tilapia farms are concentrated mainly on the Mekong Delta, Central and Northern part of Viet Nam with a total production in 2014 of 125,000 tons (an increase of 25% compared to 2013) and a total value exported of USD 35.8 million (an increase of 265.3% compared to 2004) (Dzung, 2015).

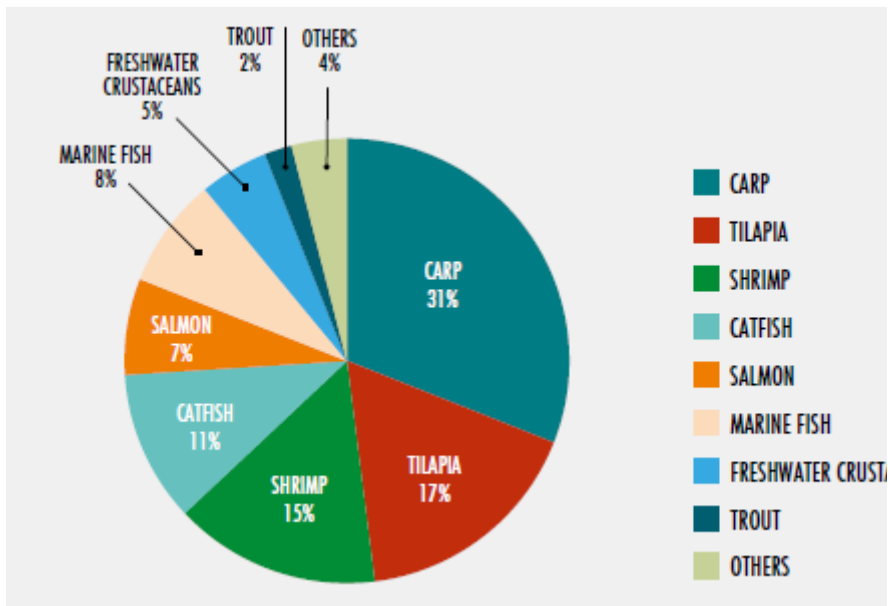


Figure 3. Share of consumption of total aquaculture feed by species group, 1995–2015 (%) (FAO, 2018)

1.3.1 Nutritional characteristics of tilapia

Tilapia is generally classified as an omnivorous species and has a low position in the aquatic food chain with little selectivity of food items. Tilapia feed mainly on phytoplankton, periphyton and detritus. Normally, they show increased preference for debris with increasing size. They can efficiently ingest the food sources mentioned through 'filter-feeding'. In nature, tilapia feed initially on zooplankton, especially crustaceans (copepods) during larval stages (El-Sayed, 2006).

However, for tilapia culture in hatcheries, newly hatched fry can utilise a complete diet of commercial feed which has a high protein content (around 50 percent) and energy to meet the demands of the fast growing fry. The size of tilapia feed is gradually increased in relation to growth and follows the rule, "small fish, small feed; large fish, large feed". Tilapia fingerlings of 5 to 40 grams use feed less than 2 mm in size. However, tilapia larger than 40 grams can be fed pellets, with the most common pellets 2 to 3 mm in size. Normally, tilapia are fed with floating pellets because this allows culturists to observe feeding responses. In addition, heat extruded floating feed pellets are more digestible for fish due to gelatinisation of starch which increases the amount of energy available to tilapia (Riche & Garling, 2003).

1.3.2 Protein requirements of tilapia

Nowadays, tilapia are consumed everywhere in the world. To support this demand, tilapia has been raised in intensive systems which must be supplied with feed in pelleted form. Similar to other species, Nile tilapia requires amino acids from protein for their growth, in addition to fat, minerals and vitamins. Protein requirements for optimum growth are dependent on, among other things, dietary protein quality/source, fish size or age and the energy contents of the diets. The protein requirements of tilapia can vary and sometimes recommendations are contradictory. Generally, the protein requirement of tilapia decreases with an increase in body size (El-Sayed, 2006).

For larval stages, the protein requirement of Nile tilapia is 45-50% (Riche & Garling, 2003; El-Sayed & Teshima, 1992), while fingerling tilapia require 27.5 – 40% dietary protein for maximum growth performance (FAO, 2017b; NRC, 1993; Siddiqui *et al.*, 1988; Wee & Tuan, 1988). In the adult stage, the protein requirement is around 30% (Al Hafedh, 1999). Moreover, 35–45% dietary protein is required for brood-stock to achieve optimum reproduction, spawning efficiency and larval growth and survival (El-Sayed *et al.*, 2003; Siddiqui *et al.*, 1998). The protein needed depends on the tilapias' physiological stage and the protein source supply in the diet. This can explain differences in the protein

requirement (Table 1) for optimum growth rate. However, the protein requirement of fish is also dependent on the energy content of the diet.

Table 1. *Protein requirement of Nile tilapia*

Life stage	Weight (g)	Protein source	Requirement (%)	Reference
Fry	0.012	Fishmeal	45	(El-Sayed & Teshima, 1992)
	0.02-1.0		40-50	(FAO, 2017b)
Fingerlings	1.0-10.0		35 - 40	(FAO, 2017b)
	24	Fishmeal/Soy bean meal/ Blood meal	27.5	Wee and Tuan (1988)
	40	Fishmeal	30	Siddiqui et al. (1988)
	50		30	NRC (1993)
Adults	45–264	Fishmeal	30	Al Hafedh (1999)
	>200		28-30	(FAO, 2017b)
Broodstock		Fishmeal / Soy bean meal	40	(El-Sayed <i>et al.</i> , 2003)
		Fishmeal	45	Siddiqui et al. (1998)

Protein requirements of Nile tilapia also differ with salinity. FAO (2017b) reported that tilapia of 0.024g body weight require 28% protein at a salinity of 10-15ppt, but the protein requirement increases up to 30.4% when salinity is reduced to 5 ppt.

In addition, temperature can affect the digestibility and protein requirements of tilapia. The highest protein digestibility occurs at 25°C (Stickney, 1997) and the optimum dietary protein to energy ratio was estimated in the region of 110 to 120 mg per kcal digestible energy respectively for fry and fingerlings.

In our experiments, the protein requirement of tilapia could be affected by the rearing system (Bio-RAS or CW-RAS), which will be shown in this thesis.

1.3.3 Amino acid requirements of tilapia

Amino acid requirements of tilapia at farm level have not been determined. However, a few studies have considered essential amino acid requirements of tilapia (Table 2).

Table 2. *Essential amino acid requirements of Nile tilapia as % of dietary protein*

Amino acids	Santiago and Lovell (1988)	Fagbenro (2000)
Lysine (Lys)	5.12	-
Arginine (Arg)	4.20	4.1
Histidine (His)	1.72	1.5
Threonine (Thr)	3.75	3.3
Valine (Val)	2.80	3.0
Leucine (Leu)	3.39	4.3
Isoleucine (Iso)	3.11	2.6
Methionine (Met)	2.68	1.3
Cysteine (Cys)	0.53	2.1
Phenylalanine (Phe)	3.75	3.2
Tyrosine (Tyr)	1.79	1.6
Tryptophan (Try)	1.00	0.6

1.3.4 Protein sources in tilapia diets

In tilapia, purified or semi-purified protein sources are not recommended under commercial farming conditions. However, there are many kinds of protein sources that have economic potential and are locally available, especially in developing countries. According to (El-Sayed, 2006), potential animal protein sources for tilapia include fishmeal, fish silage, shrimp meal, shrimp head waste, poultry by-product meal, blood meal and hydrolysed feather meal. In addition, potential plant protein sources for tilapia include soybean meal, soy protein concentrate, cotton seed meal, palm kernel cake, macadamia press cake, azolla, duckweed, cassava leaf meal, cowpea leaf protein concentrate, maize gluten feed and toasted lima bean (El-Sayed, 2006). In extensive and semi-intensive fish farming several other feed resources can be used for tilapia. These include bakery waste, brewers' waste, poultry manure, buffalo and cow manure, restaurant wastes and rejects, fruit and vegetable market wastes and rejects, rice polishing wastes, sugarcane bagasse, duckweed, starch and yeast industry wastes, aquatic weeds and water hyacinth (El-Sayed, 2006)

1.4 Giant freshwater prawns

In recent years, the growth and intensification of shrimp aquaculture in Asia has been explosive with strong demand and high world prices. Thus, it is becoming an increasingly critical source of income and employment, especially in Viet Nam. In fact, shrimp farming is now one of the most important aquaculture practices for production in the coastal regions of the Mekong Delta (Clayton & Brennan, 1999). Giant freshwater prawns are an essential species inland of the Mekong Delta of Viet Nam, which comprises a vast freshwater surface area

consisting of 320,000 ha of rice fields, 25,000 ha of ponds, and more than 5,000 km of rivers and canals (Wilder & Phuong, 2002). In Viet Nam, giant freshwater prawn (*M. rosenbergii*) is cultured in many ways, but rice–prawn farming and fence culture are the most important production models. While the freshwater prawn culture industry is small compared to Viet Nam’s saltwater-brackish water shrimp industry and the freshwater prawn culture industry in other countries, it is one of the means allowing impoverished farmers to raise their incomes (Phuong *et al.*, 2006).

1.4.1 Distribution of giant freshwater prawns

The giant freshwater prawn (*M. rosenbergii*) is the largest freshwater prawn species (Mather & De Bruyn, 2003). *Macrobrachium* belong to the crustacean group and is distributed throughout the tropical and subtropical zones of the world. They are found in most inland freshwater areas such as lakes, rivers, swamps, irrigation ditches, canals and ponds, as well as in estuarine areas. Brackish water is required in the larval stage of their life cycle, the larvae die within a few days in either freshwater or high salinities (Sandifer & Smith, 1985). Therefore, they are found in water bodies that are directly or indirectly connected with the sea (Holthuis, 1980).

1.4.2 Nutritional characteristics of freshwater prawns

During the larval stages, *M. rosenbergii* are non-active hunters and cannot swim long distances to search for food. They consume food using their thoracic appendages when they have a chance encounter with prey. Therefore, it is very important that live and moving food remains suspended in the water column during the larvae stage in order to give them a greater chance to feed (Lavens *et al.*, 2007). In addition, the size of the feed is important at the early prawn larval stages. In this stage, the larvae should be fed with the brine shrimp, *Artemia* nauplii, which have been found to be more suitable to their mouth than the cladoceran *Moina*. The free-swimming *Artemia* nauplius (first instar) are brownish orange in colour and have been a popular food for a range of organisms for culture and research. In hatcheries, *M. rosenbergii* larvae cannot digest artificial diets, they depend on live feed because they have a low digestive capacity (Lavens *et al.*, 2007).

After they metamorphose into post-larvae (PLs), *Artemia* can continue to be used in nurseries. However, cost is the one deterrent factor with using *Artemia* at a later post-larval stage. Therefore, the nursery stage uses a combination of other diets as the prawns grow into juveniles. After post-larvae stage, the prawn can be fed with pellets that sink in the water column due to their ability to walk, crawl and climb on vertical or horizontal surfaces (Ismael & New, 2007).

For *M. rosenbergii*, the nutrients required for growth and associated physiological functions are similar to those required by other crustaceans. Current data (FAO, 2017a) suggest that a digestible protein level of 28% or higher is required for optimum growth and protein efficiency of prawns (e.g. protein needs for juvenile stage, adult and brood-stock were 35-37%, 28-30% and 38-40%, respectively). Furthermore, Al-Hafedh (2007) suggested that the dietary protein requirement of freshwater prawns is 35% at the juvenile stage (1.96 ± 0.2 g).

However, according to Al-Hafedh (2007) giant freshwater prawns in semi-intensive culture where natural food is available show satisfactory growth when fed a diet with 14% protein. Moreover, for juvenile prawns, optimal dietary protein levels range between 13 and 25% with no significant effect on growth found at increasing dietary protein levels from 30 to 55%. The optimal level of dietary protein for prawns is between 27 and 35% when they can utilise available natural food in the water. In freshwater prawn ponds, there are many kinds of feed sources, such as macroinvertebrates (Oligochaeta, Chironomidae, gastropoda, bivalvia, ostracoda), macroalgae, microalgae, insect larvae and fragments of plant and animal origin used as natural feed for prawns (Correia *et al.*, 2002).

1.4.3 Amino acid requirements of freshwater prawns

The complete quantitative essential amino acid requirements for *M. rosenbergii* have not yet been published. However, the result of experiments with other aquatic species have indicated that amino acid requirements, shown as a per cent of dietary protein, are closely associated with the relative proportions found in their muscle tissue (Wilson, 2003). Actually, guidance on the potential quality of a dietary protein source or mixture of sources can be obtained from whole body essential amino acid profiles, which are in turn used to produce an evaluative index such as A/E. This index is based on the calculation of the relative proportion of each essential amino acid (A) to total essential amino acids (E). D'Abramo (1998) calculated A/E indices of tail muscle and whole body tissue of juvenile *M. rosenbergii* which were similar (Table 3). These proportions of essential amino acids in the tissue and body of prawns may be similar to the nutrient requirements of *M. rosenbergii*.

Table 3. Essential amino acids of prawn (*M. rosenbergii*) tail muscle tissue or whole body (A/E indices), %. (D'Abramo, 1998)

Amino acids	Tail muscle	Whole body tissue
Lysine (Lys)	16.6	16.4
Arginine (Arg)	19.0	14.6
Histidine (His)	4.6	6.8
Threonine (Thr)	8.1	8.8
Valine (Val)	8.2	7.3
Leucine (Leu)	14.7	14.8
Isoleucine (Iso)	8.2	7.3
Methionine (Met)	6.0	7.0
Phenylalanine (Phe)	14.8	17.2

1.4.4 Protein sources in freshwater prawn diets

In order to obtain maximum growth of *Macrobrachium*, protein from a single protein source or a mixture of protein sources must be provided at appropriate levels and proportions such that the requirements of all essential amino acids are satisfied. Most feedstuffs provided as sources of amino acids are limited in arginine, lysine and methionine. Thus, protein from ingredient sources which are substantially deficient in one or more essential amino acids will be required in greater amounts to achieve a maximum growth rate for prawns (D'Abramo & New, 2009). Most protein sources in farm-made diets for giant fresh water prawns consist of trash fish, soybean meal, corn meal or silkworm pupae, earthworms and golden apple snails (Jintasataporn *et al.*, 2004). These sources are hard to obtain due to the lack of the source supply and high cost. Therefore, research to find new alternative sources for fishmeal in the diets of freshwater prawn is needed.

1.5 Microbial protein as a new strategy for sustainable aquaculture

Aquaculture is more and more developed all over the world. New strategies and alternative protein sources are needed to achieve sustainable aquaculture development. The use of microorganisms such as micro-algae, bacteria, filamentous fungi and yeast in aquaculture has greatly increased during the last two decades (Nevejan *et al.*, 2016). These microorganisms could come from, for example, the beer industry in the form of SBY or from biofloc technology (BFT). These sources represent the most viable strategies to achieve sustainable aquaculture. Brewer's yeast is one of the microbial protein sources which has been used as an alternative protein source in animal feed. The biofloc systems

are based on the promotion of microbial growth and proliferation of either autotrophic or heterotrophic microorganisms; these microbes are expected to use, recycle and transform the excess nutrients from faeces, dead organisms, unconsumed food and diverse metabolites into the biomass, which would be further consumed by the cultured organisms. According to Avnimelech (2015), BFT is a consequence of the development of permanently mixed and aerated ponds, systems resembling bio-technological plants that maximize the potential of microbial processes. The introduction of the biofloc technology is a natural consequence of the water restriction exchange due to costs and environmental regulation and as a means to provide bio secure systems to minimize disease.

1.5.1 Biofloc

‘Biofloc’ is a generic term for farming fish and shrimp in a high-density microbial environment (for an extensive overview, see Avnimelech (2015)). In the past, biofloc has been dominated by naturally occurring phytoplankton, and is therefore also termed ‘green-water aquaculture’. In open tropical systems, phytoplankton are always present to some degree. Due to the facultative metabolism of phytoplankton, which can switch from phototropic metabolism during light conditions to aerobic metabolism during darkness, high concentrations of phytoplankton result in marked daily fluctuations in water gas content/composition. This creates unstable production conditions for fish or shrimp. However, the farmed fish or shrimp can feed on the phytoplankton, deriving additional nutrients, and providing some degree of water purification by reducing nitrogenous waste (Avnimelech, 2007). More recently, practices that change the carbon to nitrogen (C:N) ratio have been used to favour bacterial growth compared with phytoplankton growth (Avnimelech, 2015; Avnimelech, 1999). This reduces the risk of externally induced changes in the biofloc as most heterotrophic bacteria use aerobic metabolism independent of light conditions, conditional upon enough oxygen being present in the water. Provided the floc remains suspended in the water column, bacteria also offer a more stable water purification service than phytoplankton due to their much higher proliferation rates. This also results in a higher protein content of the biofloc (Avnimelech, 1999). It is even suggested that a high-intensity biofloc may function as a barrier to pathogen transmission between animals, significantly reducing the risk of severe disease outbreaks (Avnimelech, 1999).

The BFT was used in studies in the early 1980s (Serfling, 2006) and focused mainly on shrimp culture. Many studies have demonstrated that BFT can improve the water quality of shrimp culture systems (Xu *et al.*, 2012; Zhao *et al.*, 2012; Ray *et al.*, 2010; De Schryver *et al.*, 2008), enhance shrimp growth performance through additional natural food and stimulated digestive enzyme

activities (Xu & Pan, 2012; Xu *et al.*, 2012), improve the antioxidant status and immune defence of shrimp (Kim *et al.*, 2014; Souza *et al.*, 2014; Xu & Pan, 2013), and enhance shrimp biosecurity (Zhao *et al.*, 2012). However, few studies have been conducted on fish in BFT systems. In particular, the studies of tilapia in BFT systems have mainly documented water quality, growth and production performance (Crab *et al.*, 2009; Azim & Little, 2008; Avnimelech, 2007).

1.5.2 Brewer's yeast

Since the early fifties intense efforts have been made to explore new alternate protein sources as food supplements not only for humans, but also for animals and aquaculture. This occurred primarily in anticipation of a repeatedly predicted food security crisis. SBY is a new, microbial protein source. It originates from *Saccharomyces* yeast which is the second major by-product of the brewing industry (Figure 4). During fermentation, yeast biomass increases three to six fold depending on the fermentation conditions of each brewery (Ferreira *et al.*, 2010). The total amount of brewer's *Saccharomyces* yeast biomass produced in fermentation is about 1.7 kg/m³ - 2.3 kg/m³ of final product (Huige, 2006).

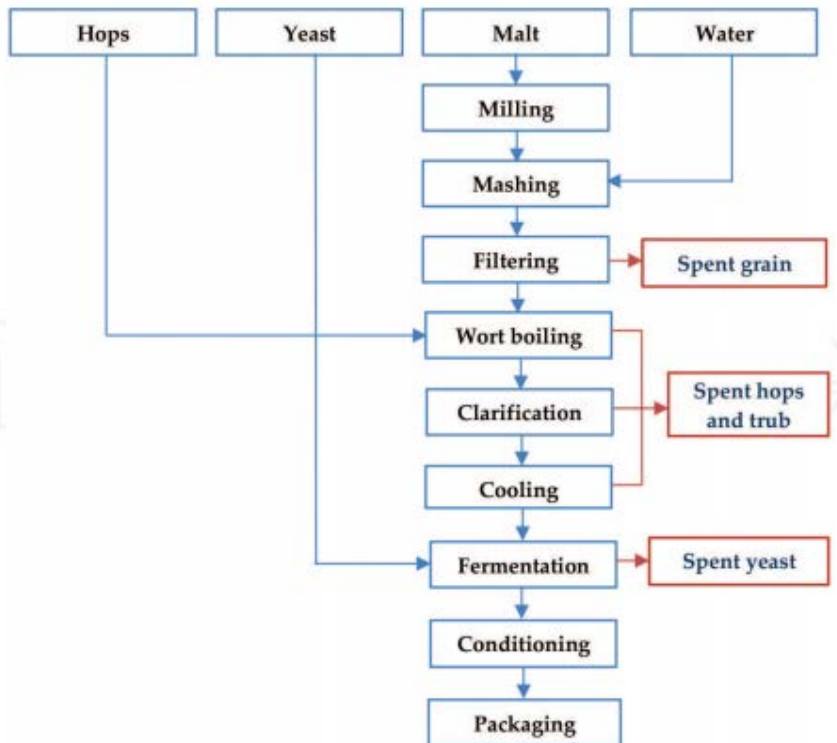


Figure 4. Schematic representation of the brewing process and points where the main by-products are generated (Fărcaș et al., 2017)

The creation of millions of tons of yeast residue will lead to ecological and economic problems. Therefore, research has identified several potential uses of SBY, such as a food source or an additive in feedstuff for livestock or aquaculture species (Vieira *et al.*, 2018; Zhang *et al.*, 2018; Ferreira *et al.*, 2010).

SBY collected from brewery plants and inactivated by heat is generally sold as inexpensive animal feed. Dried yeasts are an excellent source of protein for livestock and fish (Ferreira *et al.*, 2010; Huige, 2006). SBY have a protein content which is higher than many other ingredients, but a little bit lower than soybean meal and fishmeal (Table 4).

Table 4. Nutritional composition (% dry matter basis) of spent brewer's yeast, fishmeal, soybean meal, wheat flour and rice bran. Modified from NRC (2011)

Ingredients	Crude protein	Crude fat	Crude fiber	Ash
Brewer's yeast	42.6	1.0	3.2	6.6
Fishmeal	59-72	7.6-10.1	0.7-1.0	10.2-21.5
Soybean seed	35.2	18	5.0	4.5
Soybean meal	44.0-48.5	0.9-1.5	3.5-7.3	5.8-6.3
Wheat flour	11.7	1.2	1.3	0.4
Rice bran	13.0-15.7	1.7-7.4	13.7-17.9	16.7-24.7

Yeast has a balanced amino acid profile, and is abundant in lysine content, a source of many essential B vitamins. Yeast biomass can also be used as a source of production of functional foods, agents of detoxifying effluents containing heavy metals and a source of nutrients for human, microbial growth and animal nutrition, especially fish nutrition (Vieira *et al.*, 2018). The use of SBY as a dietary protein for farmed fish is not a new concept, as studies have investigated this possibility since the 1970s (Kohler & Pagan-Font, 1978). SBY was used in diets for rainbow trout (*Oncorhynchus mykiss*) in 1991 and showed that brewer's yeast could be a primary nitrogen source in fish feeds. Rumsey *et al.* (1991a;1991b) showed that the absorption of nitrogen increased by more than 20%, and metabolisable energy by 10%, after the removal of all wall material and separation of nitrogen into amino acids and nucleic acids. In addition, growth was faster and feed conversion more efficient in rainbow trout fed diets consisting of 25% yeast (Rumsey *et al.*, 1991b). Studies in the 2000s have shown that brewer's yeast can be used as a protein source to replace fishmeal in the diets of many fish species. Oliva-Teles and Gonçalves (2001) studied the effect of partial replacement of fishmeal protein by brewer's yeast at 0%, 10%, 20%, 30% or 50% in diets of seabass juveniles with an initial average weight of 12 g. Their findings indicated that brewer's yeast can replace 50% of fishmeal protein with no negative effects on fish performance. In tilapia, Ebrahim and Abou-Seif (2008) studied the effect of partial to total replacement of fishmeal protein in the diet with the yeast protein, *Saccharomyces cerevisiae*, at 25, 50, 75 and 100% supplemented with biogenic L-carnitine. They showed that brewer's yeast at 50 and 75% replacement recorded the best growth performance, feed and protein utilisation. Therefore, these data suggested that, yeast *S. cerevisiae* supplemented with biogenic L-carnitine can totally replace fishmeal in fingerling tilapia diet without any adverse effect on growth performance. In later work in the 2010s, OzÓRio *et al.* (2010) evaluated the efficacy of replacing fishmeal with brewer's yeast *S. cerevisiae* at 0, 30, 35, 50, 70 or 100% in diets of pacu, *Piaractus mesopotamicus*, juveniles. They showed that growth performance and feed utilisation increased with increasing dietary yeast levels up to 50% fishmeal replacement (superior growth), and that 100% fishmeal replacement by brewer's yeast was without negative effects on fish performance. Recent studies investigating the use of 0%, 15%, 25%, 35% and 45% fishmeal replacement in the practical diet of goldfish (*Carassius auratus*) showed an increased weight gain (Gumus *et al.*, 2016). Furthermore, (Langeland *et al.*, 2016) determined the apparent digestibility coefficients (ADCs) of Arctic char (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*) using intact and extracted yeast (*S. cerevisiae*) indicated no significant differences for ADCs between yeast with and

without cell walls in Eurasian perch. However, the absence of intact cell walls had a positive effect on digestibility of *S. cerevisiae* for Arctic char. Moreover, Vidakovic *et al.* (2016) have shown that intact and extracted yeast (*S. cerevisiae*) can replace up to 40% of crude protein of fishmeal which suggests that intact *S. cerevisiae* yeast are a promising protein source for Arctic char. In addition, Huyben *et al.* (2016) showed that yeast is a potential alternative to fishmeal in diets for farmed fish, yet replacing more than 50 % of fishmeal resulted in reduced fish growth and induced haemolytic anaemia in rainbow trout. This may limit yeast inclusion to not more than 50% in diets for farmed fish.

Besides being a potential alternative to fishmeal protein, SBY has been used as a source of nutrients and bioactive compounds for many species of fish in aquaculture (Ferreira *et al.*, 2010). Brewer's yeast contains various immunostimulating compounds, such as β -glucans, nucleic acids, as well as mannan oligosaccharides, (White *et al.*, 2002) with effects on immunity as demonstrated in giant freshwater prawns (*M. rosenbergii*) (Parmar *et al.*, 2012) and hybrid striped bass (Li & Gatlin Iii, 2004). Additionally, commercial formulations of brewer's yeast are suitable as a food source for the mass production of the nematode *Panagrellus redivivus* used to feed farm fish and crustacean larvae (Ricci *et al.*, 2003) during their early stages of development. Abass *et al.* (2018) evaluated the effects of varying dietary inclusions of *S. cerevisiae*, finding that 0%, 3%, 5% and 7% have beneficial impacts on growth, stress tolerance, and disease resistance in juvenile Nile tilapia (*O. niloticus*).

2 Objectives of the thesis

The overall aims of this thesis were to determine the effect of feeding SBY as a protein alternative for fishmeal and soybean meal in the tilapia (*O. niloticus*) and giant fresh water prawn (*M. rosenbergii*) reared in either clear water or biofloc environment. An additional aim was to determine if the protein requirement of tilapia reared in these two environments, clear water recirculating aquaculture system (CW-RAS) or biofloc recirculating aquaculture system (Bio-RAS), differed. A series of experiments were conducted in an attempt to identify possible replacement levels of SBY and to determine the protein requirement of tilapia.

2.1 The specific aims

- Determine the ability of using SBY to replace fishmeal protein in CW-RAS and Bio-RAS of tilapia (Paper I) and giant freshwater prawn (Paper II).
- Evaluate nitrogen retention and protein requirements of tilapia reared in either CW-RAS or Bio-RAS by feeding graded levels of a fixed mixture of three protein sources of animal, plant and microbial origin at a set gross energy level (Paper III).
- Evaluate changes in apparent digestibility of gross protein and gross energy in relation to diet protein levels and the rearing environment of CW-RAS or Bio-RAS (Paper III).
- Determine the effect of SBY on replacement soybean meal protein in tilapia diets on growth performance, survival rate, feed utilization and body indices of tilapia in CW-RAS and Bio-RAS (Paper IV).

2.2 Hypotheses examined in the thesis

- Biofloc water supports higher protein retention by recycling nitrogen in the farming environment directly from faeces to feed.
- Biofloc environment supports full growth with sub optimal amino acid profile.
- Brewer's yeast functions as a good protein alternative for fishmeal and soybean meal.

3 Materials and methods

3.1 Experimental design

This thesis is based on the different studies described in Papers I-IV. Four experiments were set up with four diets fed (Table 5) in triplicate for each experiment reared in two parallel systems including biofloc recirculating aquaculture system (Bio-RAS) and clear water recirculation aquaculture system (CW-RAS). The term, Bio-RAS, describes the combined system of BFT and RAS designed to provide the optimal environment for both the reared species and microbes, but in separate units, before circulating the microbes into the rearing compartment. Twenty homogeneous fish were distributed into each tank for each treatment in each experiment. At the beginning and end of the experiment, each acclimatised fish was individually weighed. The experiments reported in Papers I, III, IV were all performed with tilapia and Paper II was performed with giant freshwater prawns. The average initial body weight (BW) was 29 ± 3.2 g/tilapia, 6.7 ± 0.03 g/prawn, 39.1 ± 2.5 g/tilapia and 50.26 ± 0.74 g/tilapia for Papers I, II, III & IV experiments, respectively (Tables 6 and 7).

The experiments in all papers were arranged in a completely randomised design in regard to the four diet treatments, while the two parallel recirculation systems (CW-RAS and Bio-RAS) were organised as separate entities. In Paper I, diets containing different proportions (0, 30, 60, 100%) of brewer's yeast to replace fishmeal (three replicates per diet and water environment) were denoted B0% and C0% (control) and B30% and C30%, B60% and C60%, and B100% and C100%, where B signifies Bio-RAS and C signifies CW-RAS. In Paper II, four diet treatments with SBY with three replicates per diet and water environment were denoted B0% and C0% (control); B20% and C20%; B40% and C40%; B60% and C60% replacement of fishmeal and water environment,

respectively. In Paper III, four diet treatments with different crude protein levels with three replicates per diet and water environment were denoted B35% and C35% (control); B31% and C31%; B27% and C27%; B23% and C23% crude protein and water environment, respectively. Paper IV, diets containing different proportions (0, 30, 60, 100%) of brewer's yeast replace soybean meal (three replicates per diet and water environment) were denoted B0% and C0% (control); B30% and C30%; B60% and C60%; B100% and C100%.

Table 5. *Ingredient composition of diets with different levels of spent brewer's yeast (Y) as a replacement for fishmeal (Papers I, II) or soybean meal (Paper IV) and different crude protein levels (Paper III) (g kg⁻¹ DM)*

	Paper I- Tilapia				Paper II- Prawn				Paper III- Tilapia				Paper IV- Tilapia			
	Y0	Y30	Y60	Y100	Y0	Y20	Y40	Y60	35%	31%	27%	23%	Y0	Y30	Y60	Y100
Fish meal	200	140	80	0	260	208	157	105	80	80	80	80	10	10	10	10
Soybean meal	356	366	376	388	204	210	216	223	380	277	173	70	400	280	160	0
Blood meal	-	-	-	-	-	-	-	-	-	-	-	-	13	17	21	28
Spent brewer's yeast	0	90	180	300	0	78	155	232	180	180	180	180	0	152	304	505
Rice bran	202	185	168	148	120	100	80	60	155	210	253	303	250	238	227	210
Wheat flour	200	174	148	112	160	140	120	99	142	190	251	304	230	210	186	160
Maize meal	-	-	-	-	200	205	209	215	-	-	-	-	-	-	-	-
Squid oil	2	5	8	12	6	9	13	16	8	8	8	8	-	-	-	-
Fish oil	-	-	-	-	-	-	-	-	-	-	-	-	57	53	48	43
Premix (Vitamin-mineral)	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Lecithin	-	-	-	-	10	10	10	10	-	-	-	-	-	-	-	-
Methionine	-	-	-	-	-	-	-	-	-	-	-	-	3.6	3.6	3.6	3.6
CMC	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Cr ₂ O ₃	-	-	-	-	-	-	-	-	15	15	15	15	-	-	-	-

3.2 Fish and facilities

All the experiments in Papers I, II, III and IV were carried out on the experimental farm at An Giang University, Long Xuyen city, An Giang province, Viet Nam. The experimental tilapia (*O. niloticus*), of the GIFT strain (Dey *et al.*, 2000), used in Paper I and in Papers II and IV were bought at a hatchery in Cai Be, Tien Giang province and Cu Chi, Hoc Mon, Ho Chi Minh city, Viet Nam, respectively. In Paper II, all monosex, males of *M. rosenbergii* were obtained from the freshwater prawn nursery farm in Phu Thuan commune, Thoai Son district, An Giang province. The nursery farm obtained the prawn seeds from a local government hatchery and raised them to the desired weight. All fish and prawns were transported by car in sealed 0.5 m³ plastic bags filled with oxygen-saturated water and 1 kg fish per bag. On arrival at the research station, all fish were dipped in a solution of 3% NaCl for 5 min to eliminate ectoparasite infection. The fish were then reared and quarantined in two tanks (3 m³) for 2 weeks to acclimate to indoor conditions. The acclimatised fish were selected randomly, weighed and then transferred to each experimental tank (500-L round holding tanks) for one week before the experiment commenced for adaptation to experimental conditions.

3.3 Experimental diets

The recipes of diets in all experiments are in Table 5. In Papers I and II, four iso-nitrogenous (35% protein) and iso-energetic (19 MJ Kg⁻¹) diets were produced with fishmeal replaced by SBY at a level of 30%, 60%, 100% and 20%, 40%, 60% for tilapia and giant fresh water prawn, respectively. In Paper III, the recipes of the diet were based on the results of Paper I. The tilapia diets in Paper III had 23%, 27%, 31%, 35% crude protein and iso-energetic (19 MJ Kg⁻¹) for tilapia. In Paper IV, four iso-nitrogenous (28% protein) and iso-energetic (19 MJ Kg⁻¹) diets were produced with soybean meal replaced by SBY at a level of 30%, 60% and 100%. The level of fishmeal was kept constant and methionine crystal was added to the diets to obtain a proper balance of amino acids.

3.4 Feeding and feed preparation

In all four papers, the SBY was a by-product obtained from a brewery company in Hoc Mon district, Ho Chi Minh City, Viet Nam. Other ingredients were obtained from local markets in Long Xuyen City, Viet Nam. All ingredients were thoroughly mixed and a cold-pelleted feed was made with an electric meat grinder (Quoc Hung Company, Long Xuyen city, Viet Nam). Feed diameter and length was 1-2 mm and 2-3mm for giant fresh water prawn in Paper II and tilapia in Papers I, III, IV, respectively. All diets were sun-dried for one to two days, then weighed and stored in sealed plastic bags in small portions at 5 °C until use.

The tilapia (Papers I, III and IV) were fed by hand twice a day (between 7:00 and 9:00 h and between 16:00 and 18:00 h) until satiation. However, prawns in Paper II were fed to satiation (approximately 10% of BW) manually twice daily using a plastic tube that put feed into the square nets at the bottom of the tanks (Figure 5). Feeding was closely monitored in each tank, the feed residue was collected, and the feeding rate was adjusted depending upon the previous day's feed consumption. The amount of feed used was recorded during the experiment and used to calculate true feed intake.

3.5 Experimental system and management

All experiments was conducted in a closed recirculation system, so called RAS. In this study we employ a novel approach by separating the main rearing units of fish/shrimp and the microbial community, i.e. the biofloc. We denote this system Bio-RAS in order to separate it from more traditional clear water RAS, here denoted CW-RAS.

The experimental clear water recirculation system (CW-RAS) was comprised of 12 parallel-connected composite settlement tanks with a volume of about 500 L per tank, connected to a sedimentation tank containing sand and stones (1-2 mm Ø) that functioned both as a biological and mechanical filter. The water source was municipal tap water, de-chlorinated by aeration for 24 h before use. Water was circulated in the system at a rate of 3 L min⁻¹ into each tank. About 30% of the water was replaced every two days.

The experimental Bio-RAS consisted of 12 identical composite settlement tanks with a volume of about 500 L per tank, connected in parallel to four 10 m³ composite tanks, replacing the biological and mechanical sand filter of the CW-RAS. The four bioreactor tanks were connected in series and housed next to the clear water tanks in the same open building. The four 10 m³ tanks functioned as bio-generators for microbial growth and settlement of sludge and allowed the microbial and fish/shrimps to be handled separately. In all papers, two weeks before the start of the experiment, the biofloc was initiated as described by Pérez-

Fuentes *et al.* (2016). In brief, *Bacillus subtilis*, molasses (carbon source) and blood meal (nitrogen source and product of Huong Hoang Nam company, Ho Chi Minh city, Viet Nam) were added to each of the bioreactor tanks, at a C:N ratio of 10:1 and 20:1 for tilapia experiments and the giant fresh water prawn experiment, respectively. Each of the 24 experimental tanks were aerated via one air stone connected to a low-pressure electrical blower (Resun GF-370, produced in China). Each of the bioreactor tanks was equipped with four air stones and an internal circulation water pump (DAB FEKA VS 750M-A, Mestrino, Italy). In Paper II, each of the 24 experimental tanks was also equipped with a round net at the bottom, functioning as a feeding tray, and a net and plastic cluster located in the middle of the tank, functioning as an enrichment substrate (Figure 5).



Figure 5. Round nets and small square nets at the tank bottom functioning as a feeding tray, and a black net and plastic cluster hung in the middle of the tank as enrichment substrate

3.6 Sample collection and calculations

All tilapia in Papers I, IV and Paper III were weighed and length measured monthly and thrice weekly, respectively. All fish were starved the day before measurement, however, fish in the Bio-RAS system could still ingest biofloc. At the end of the experiment, five fish from each tank were randomly selected and

sacrificed by lethal anesthesia with ethylene glycol monophenyl ether at 0.5 mg L⁻¹. Weight of fillet, liver, stomach, intestine and length of intestine were recorded, in order to calculate percentage of fillet, hepato-somatic index (HSI, %), viscero-somatic index (VSI, %), gastro-somatic index (GSI, %), entero-somatic index (iEns, %) and intestinal quotient (Qi) index in accordance with Moreira *et al.* (2012) and Da *et al.* (2012) as follows:

$$\text{Percentage of fillet} = (\text{Fillet weight}/\text{Total weight of the individual}) \times 100$$

$$\text{HSI (\%)} = [100 \times (\text{Liver weight (g)}/\text{Body weight (g)})]$$

$$\text{VSI (\%)} = [100 \times (\text{Viscera-somatic weight (g)}/\text{Body weight (g)})]$$

$$\text{GSI (\%)} = (\text{Stomach weight}/\text{Total weight of the individual}) \times 100$$

$$\text{iEns (\%)} = (\text{Intestine weight}/\text{Total weight of the individual}) \times 100$$

$$\text{Qi} = \text{Intestine length}/\text{Total length of the individual.}$$

In Paper II, at the beginning and end of the experiment, prawns were starved for one day prior to measurement of weight and length (end of telson to posterior margin of orbit) (Balazs & Ross, 1976) in order to empty the digestive tract. Ethylene glycol monophenyl ether 0.3 mg L⁻¹ was used to anesthetize the prawns before all measurements.

The feed conversion ratio (FCR), protein efficiency ratio (PER), total feed intake per prawn (FI), protein intake (PI), specific growth rate (SGR), daily weight gain (DWG), weight gain (WG) and survival rate (SR) were calculated according to Ghosh *et al.* (2016) and Bhavani *et al.* (2013) as follows:

$$\text{FCR} = \text{total feed intake (g)}/\text{total wet weight gain (g)}$$

$$\text{PER} = \text{total wet weight gain (g)}/\text{protein intake (g)}$$

$$\text{FI (g)} = \text{total feed intake (g)}/\text{number of prawn}$$

$$\text{PI (g)} = \text{feed intake (g)} \times \text{protein in the diet (\%)}$$

$$\text{SGR (\%/day)} = [(\ln \text{ final Wt} - \ln \text{ initial Wt})/\text{days}] \times 100$$

$$\text{DWG (g/day)} = (\text{Final Wt} - \text{initial Wt})/\text{days}$$

$$\text{WG (g)} = \text{Final weight} - \text{initial weight}$$

$$\text{SR (\%)} = (\text{Total number of fish or prawn harvested}/\text{Total number of fish or prawn cultured}) \times 100.$$

The apparent digestibility of different diets in Paper III was determined using 1.5% chromic oxide as an indicator. Two weeks before the end of the feeding trial, faecal matter was collected using fine mesh nets. One hour after feeding, uneaten feed was removed from the tanks by opening the valves at the tank bottom. The faeces samples were collected on the water surface of the tanks as tilapia faeces are expelled in long strands encased within a clear gelatinous material and float. Only intact strands of faecal matter were collected at the surface of the tilapia tanks. As long as the faecal strands were intact, the effect of leaching on nutrient digestibility estimation was believed to be minimal

(Bahurmiz & Ng, 2007). After collection, the samples were pooled for fish fed each diet in each tank, freeze-dried and stored at -20 °C for subsequent analysis.

The apparent digestibility (AD) of dry matter (ADiDM), organic matter (ADiOM), crude protein (ADiCP) and energy (ADiE) of the diets were calculated as described by Cho *et al.* (1982):

$$AD_{diet} (\%) = 100 - 100 \times (\%M_{diet} / \%M_{faeces}) \times (\%N_{faeces} / \%N_{diet})$$

Where

%M = marker concentration (% in DM)

%N = nutrient content (% in DM).

The crude protein of whole fish in Paper III was determined before and after the experiment by randomly sampling 10 fish before starting the experiment and 5 fish per tank after finishing the experiment to calculate net protein utilization (NPU).

$$NPU (\%) = 100 \times (\text{Protein in final fish} - \text{Protein in initial fish}) / \text{protein intake}$$

3.7 Water quality monitoring

Water temperature (°C) in the tanks was recorded daily at 7.00-8.00 h and 13.00-14.00 h. Dissolved oxygen content (DO, mg L⁻¹) and pH were measured with a Hach HQ30d Multi-Parameter Meter (Hach, USA) every third day. Nitrite-nitrogen (NO₂-N, mg L⁻¹) and total ammonia nitrogen (TAN, mg L⁻¹) were measured twice a month and weekly for tilapia in Papers I, III, IV and prawn in Paper II, respectively, according to standard methods (APHA, 1998). In Paper II, chemical oxygen demand (COD, mg L⁻¹) were measured weekly according to standard methods (APHA, 1998) and water transparency was measured weekly by using a Secchi disk. Water quality parameters were measured at the water intake and outlet of the experimental bioreactors and filter tank.

In Papers II and III, biweekly phytoplankton and zooplankton at the water inlet and outlet of first and last of the paired experimental bioreactor tanks, were counted and identified at the level of genus as previously described by Bellinger and Sigeo (2015). The density of plankton was calculated by the following formula (Coutteau, 1996).

$$N = (P \times C \times 100) / V$$

Where

N is the number of plankton / liters of water in the tank

P is the number of planktonic organisms counted

C is the volume of the plastic bottle holding the sample (100 mL)

V is the volume of water sample from each tank.

In Papers III and IV, the biomass of micro components in the bioreactor tank was measured by using water filter bag (1 μ m) at the water outlet from the bioreactor tank to the rearing tanks. The bio floc samples were collected at the beginning, middle and the end of the experiment. During each sample period (start, middle and end) was three samples was taken on 3 consecutive days and analysed for total nitrogen, fat and amino acids.

3.8 Chemical analysis

In all papers, feed samples were analysed in triplicate as follows: dry matter was determined by drying in an oven at 105 °C until constant weight. Ash content was determined by incineration of the sample at 550 °C for 4 h. Crude protein was calculated as $6.25 \times \% \text{ N}$ analysed by the Kjeldahl method, ether extract (EE) was measured using the Soxhlet method and crude fibre (CF) content was analysed using standard methods (AOAC, 2000). Amino acid content was determined by high performance liquid chromatography (HPLC) according to Vázquez-Ortiz *et al.* (1995). Nitrogen free extract (NFE) was calculated according to methods described in (AOAC, 2000) ($\text{NFE} = 100 - (\% \text{ protein} + \% \text{ lipid} + \% \text{ fibre} + \% \text{ ash})$). Gross energy content (kcal kg^{-1}) was calculated according to NRC (1993) using the values 5.64, 9.44 and 4.11 kcal g^{-1} whole body for protein, lipid and carbohydrate, respectively.

Chromic oxide in feed and faeces in Paper III were determined by digestion in a mixture of perchloric acid, concentrated sulphuric acid and Na-molybdate at a temperature of 250 °C. The resulting dichromate was determined at 360 nm against Cr_2O_7 standard solutions (Furukawa & Tsukahara, 1966).

3.9 Statistical analyses

All the data on feed used, growth performance, digestibility and carcass index were analysed using a General Linear Model (GLM) ANOVA, with the Tukey pair-wise comparison, using a significance level of $p < 0.05$ (Minitab software 16.2.0; Minitab (2010)). Fixed factors were feed, system, feed \times system interaction and error.

4 Main results

4.1 Growth performance, feed utilisation and survival rate

In all studies, the fish kept in Bio-RAS were higher in growth, protein efficiency ratio and lower in FCR than fish kept in CW-RAS, except for the growth and protein efficiency ratio of freshwater prawns in Paper II (p_w value in Tables 6 and 7).

When water environments (Bio-RAS and CW-RAS) were compared (p_w values), prawns in Paper II did not result in significant differences in growth variables; even so, one could discern a trend of higher growth and an improved protein efficiency ($p < 0.1$), with 6%, 9%, 6% and 15% higher final body weight, WG, SGR and PER, respectively, in Bio-RAS compared to CW-RAS. However, there was a highly significant ($p < 0.01$) (21%) lower FCR observed in Bio-RAS kept prawns as compared to CW-RAS prawns, in combination with a trend towards lower feed intake and higher growth ($p < 0.05$). Moreover, feed and protein intake of tilapia were identical and independent of the water environment (Paper I and IV).

In Papers I and IV, when all treatment groups were compared, there were significant differences between treatments (Table 6). For example, tilapia given 0% yeast in Bio-RAS (B0) differed significantly from tilapia given 0% fishmeal in CW-RAS (C100) (Paper I). Additionally, only fish given 30% yeast in Bio-RAS (B30) differed significantly from fish given 0% and 100% yeast in CW-RAS (C0 and C100) (Paper IV), in regard to growth variables. However, Paper III showed that the growth variables and protein intake of tilapia were highest in the CW-RAS and Bio-RAS groups that were fed a 35% crude protein diet, and all of these indices decreased when the dietary protein level decreased (Table 7). Paper II showed the lowest growth performance in the treatment group with 60% protein replacement with SBY kept in CW-RAS (C60) and the highest growth

performance in prawns given 40% protein replacement kept in Bio-RAS (B40). FCR also displayed a significant effect by treatment, with all Bio-RAS kept groups displaying significantly lower FCR as compared to the group given 60% replacement in CW-RAS (C60). This group (C60) also displayed significantly higher FCR when compared to the 0% replacement group kept in the same water environment (C0). This difference between replacement levels was absent in prawns kept in the Bio-RAS (Table 7). The effect observed in FCR was also observed in PER, while feed intake and PI did not display any significant differences with treatment.

When yeast inclusion levels were pooled independent of water environment (p_y values) before analysis, no significant effect was noted between fish given the different levels of yeast on any growth related parameter, feed intake, protein intake, protein efficiency and survival rate (Papers I, II and IV) (Tables 6 and 7). However, there was a significant difference in growth performance of fish fed 30% SBY and 100% SBY replacement (Paper IV). When protein levels were pooled independent of water environment, the fish given 23% crude protein always displayed the lowest growth, a significant difference from fish given 35% crude protein. No significant difference in PER and NPU was found (Paper III) (p_p values in Table 7).

The survival rate (Tables 6 and 7) of fish was generally very high, exceeding 96% (Papers III and IV), and no difference between the two rearing systems (CW-RAS or Bio-RAS) was observed (Papers II, III and IV). However, in Paper I, the survival rate of tilapia kept in Bio-RAS environment was slightly higher than for fish reared in CW- RAS.

Table 6. Growth performance, feed utilisation and survival rate of tilapia in Bio-RAS (B) and CW- RAS (C) fed four levels of spent brewer's yeast where 0, 30, 60, 100 indicates 0% 30%, 60% and 100% replacement of fishmeal protein (Paper I) or soybean meal protein (Paper IV)

Paper I	Dietary treatments								SEM	P value	P _W value	P _Y value
	B0	B30	B60	B100	C0	C30	C60	C100				
Initial weight, g	29.4	29.44	29.3	29.4	29.3	29.4	29.4	29.3	0.10	0.776	0.797	0.73
Final weight, g	220.5 ^a	189.7 ^{ab}	206.4 ^{ab}	182.6 ^{ab}	184.3 ^{ab}	175.1 ^{ab}	186.9 ^{ab}	145.1 ^b	13.3	0.044	0.017	0.08
Daily weight gain, g/d	2.12 ^a	1.78 ^{ab}	1.97 ^{ab}	1.70 ^{ab}	1.72 ^{ab}	1.62 ^{ab}	1.75 ^{ab}	1.29 ^b	0.15	0.043	0.017	0.08
Specific growth rate, %/d	2.24 ^a	2.07 ^{ab}	2.16 ^a	2.03 ^{ab}	2.04 ^{ab}	1.97 ^{ab}	2.05 ^{ab}	1.77 ^b	0.08	0.028	0.016	0.06
Feed conversion rate	1.28	1.47	1.35	1.44	1.58	1.65	1.45	1.99	0.15	0.092	0.019	0.26
Protein efficiency ratio	2.26	1.96	2.12	2.01	1.81	1.85	1.97	1.46	0.20	0.252	0.032	0.431
Survival rate, %	95.0	100.0	96.7	95.0	88.3	93.3	88.3	88.3	4.04	0.363	0.012	0.603

Paper IV	Dietary treatments								SEM	P value	P _W value	P _Y value
	B0	B30	B60	B100	C0	C30	C60	C100				
Initial weight, g	50.4	50.4	49.8	50.6	50.3	50.4	50.4	50.0	0.48	0.939	0.93	0.93
Final weight, g	175.9 ^{ab}	199.2 ^a	181.4 ^{ab}	172.0 ^{ab}	158.4 ^b	166.4 ^{ab}	163.8 ^{ab}	148.4 ^b	8.24	0.018	<0.00	0.06
Daily weight gain, g/d	2.09 ^{ab}	2.48 ^a	2.19 ^{ab}	2.02 ^{ab}	1.80 ^b	1.93 ^{ab}	1.89 ^{ab}	1.64 ^b	0.14	0.018	<0.00	0.05
Specific growth rate, %/d	2.08 ^{ab}	2.29 ^a	2.15 ^{ab}	2.03 ^{ab}	1.91 ^{ab}	1.99 ^{ab}	1.96 ^{ab}	1.81 ^b	0.08	0.021	<0.00	0.05
Feed conversion rate	1.46 ^{ab}	1.28 ^b	1.37 ^b	1.60 ^{ab}	1.65 ^{ab}	1.64 ^{ab}	1.59 ^{ab}	1.86 ^a	0.09	0.007	<0.00	0.11
Protein efficiency ratio	2.41 ^{ab}	2.74 ^a	2.54 ^a	2.21 ^a	2.13 ^{ab}	2.12 ^{ab}	2.18 ^{ab}	1.88 ^b	0.13	0.005	<0.00	0.16
Survival rate, %	98.3	100.0	100.0	100.0	96.7	98.3	100.0	100.0	1.44	0.626	0.39	0.24

SEM = Standard error of the mean; Means with different superscript letters within rows are significantly different ($p < 0.05$).

P_W value: between different water environments; P_Y value: between different levels of spent brewer's yeast.

Table 7. Growth performance, feed utilisation and survival rate of freshwater prawns fed four different levels of spent brewer's yeast (Paper II) or tilapia fed four different crude protein levels (Paper III) and kept in Bio-RAS (B) or CW-RAS (C) environments; 0, 20, 40, 60 indicates 0% 20%, 40% and 60% replacement of fishmeal; 23, 27, 31, 35 indicates 23% 27%, 31% and 35% crude protein.

Paper I	Paper II-Prawns								SEM	P value	P _w value	P _y value
	B0	B20	B40	B60	C0	C20	C40	C60				
Initial weight, g	6.6	6.6	6.6	6.7	6.7	6.7	6.7	6.7	0.02	0.99	0.08	0.87
Final weight, g	22.0 ^{ab}	20.9 ^{ab}	22.86 ^a	22.3 ^{ab}	22.3 ^{ab}	22.0 ^{ab}	21.0 ^{ab}	17.8 ^b	0.95	0.04	0.08	0.16
Daily weight gain, g/d	0.18 ^{ab}	0.17 ^{ab}	0.19 ^a	0.19 ^{ab}	0.19 ^{ab}	0.18 ^{ab}	0.17 ^{ab}	0.13 ^b	0.01	0.04	0.08	0.16
Specific growth rate, %/d	1.43 ^{ab}	1.36 ^{ab}	1.46 ^a	1.44 ^a	1.44 ^a	1.42 ^{ab}	1.36 ^{ab}	1.16 ^b	0.06	0.03	0.06	0.12
Feed conversion rate	4.49 ^b	5.13 ^b	4.32 ^b	4.46 ^b	5.07 ^b	5.32 ^{ab}	5.53 ^{ab}	7.33 ^a	0.41	0.02	<0.00	0.07
Protein efficiency ratio	0.39 ^a	0.41 ^a	0.41 ^a	0.43 ^a	0.43 ^a	0.41 ^a	0.34 ^{ab}	0.25 ^b	0.04	0.05	0.10	0.37
Survival rate, %	60.0	71.7	63.3	68.3	75.0	73.3	66.7	65.0	4.97	0.33	0.25	0.49
Paper IV	Paper III -Tilapia								SEM	P-value	P _w value	P _p value
	B23	B27	B31	B35	C23	C27	C31	C35				
Initial weight, g	39.13	39.03	38.99	39.03	39.05	39.09	39.08	39.08	0.069	0.881	0.534	0.862
Final weight, g	158.1 ^b	165.8 ^{ab}	170.9 ^{ab}	179.1 ^a	122.8 ^d	123.6 ^d	133.1 ^{cd}	141.4 ^c	3.64	<0.001	<0.001	0.025
Daily weight gain, g/d	1.89 ^b	2.01 ^{ab}	2.09 ^{ab}	2.22 ^a	1.33 ^d	1.34 ^d	1.49 ^{cd}	1.63 ^c	0.058	<0.001	<0.001	0.025
Specific growth rate, %/d	2.18 ^b	2.28 ^{ab}	2.32 ^{ab}	2.39 ^a	1.80 ^d	1.81 ^d	1.90 ^{cd}	2.02 ^c	0.038	<0.001	<0.001	0.035
Protein efficiency ratio	2.49 ^a	2.37 ^{ab}	2.31 ^{abc}	2.37 ^{ab}	1.94 ^{bcd}	1.84 ^{cd}	1.79 ^d	1.89 ^{bcd}	0.10	<0.001	<0.001	0.456
Feed conversion rate	1.66 ^{bcd}	1.6 ^{bcd}	1.39 ^{de}	1.22 ^e	2.24 ^a	1.99 ^{ab}	1.83 ^{bc}	1.55 ^{cde}	0.08	0.001	<0.001	<0.001
Net protein utilisation, %	33.5 ^{ab}	34.0 ^{ab}	35.6 ^a	35.3 ^a	25.8 ^c	25.1 ^c	25.9 ^c	27.7 ^{bc}	1.44	<0.001	<0.001	0.498
Survival rate, %	100	95	98.3	100	95	98.3	96.7	98.3	2.36	0.67	0.464	0.758

SEM = Standard error of the mean; Means with different superscript letters within rows are significantly different ($p < 0.05$).

P_wvalue: between different water environments; P_yvalue: between different levels of spent brewer's yeast; P_p-value: between different protein levels.

4.2 Protein requirement of tilapia (Paper III)

Protein intake and the growth variables of tilapia, such as WG, DWG, and SGR were highest in the treatments in each system (CW-RAS and Bio-RAS) that were fed with 35% crude protein diet, and all these indices decreased when the dietary protein level decreased. No significant differences in FCR, protein and growth variables were found between treatments within the Bio-RAS group, except for those fed with the 23% crude protein level diet. However, in CW-RAS group significant differences were found in FCR and growth variables between treatments fed 23%, 27% and 35% protein, respectively (Table 7). This indicated that the protein requirement of tilapia in Bio-RAS was lower than in CW-RAS, or that tilapia in Bio-RAS could tolerate a 27% protein level while those in CW-RAS could tolerate a 31% level.

4.3 Digestibility of diets (Paper III)

The apparent digestibility (%) of dry matter (ADiDM), organic matter (ADiOM), crude protein (ADiCP) and energy (ADiE) of tilapia fed different protein levels and reared in either Bio-RAS or CW-RAS were determined (Table 8) by adding the inert marker (Cr_2O_3) as an indicator in the diets. Significant differences in ADiDM and ADiE could only be detected between treatment groups fed 27% protein (B27) in Bio-RAS and 23% protein (C23) in CW-RAS ($p < 0.05$). However, the ADiCP of tilapia was high in groups fed with diets containing high levels of protein (B35, C35 and C31) and was significantly different from B23, C23 and C27. Fish kept in Bio-RAS had a significantly higher ADiDM and ADiE than those in CW-RAS (p_w values < 0.001). However, the significant difference in ADiCP was only due to differences between the two highest and lowest dietary protein levels and was not influenced by the different rearing systems, which was the opposite situation than ADiOM.

Table 8. Apparent digestibility (%) of dry matter (ADiDM), organic matter (ADiOM), crude protein (ADiCP) and energy (ADiE) in tilapia fed different crude protein levels and kept in different water environments: B: Biofloc-bioreactor (Bio-RAS) system; C: clear water recirculation system, 23, 27, 31, 35 indicates 23% 27%, 31% and 35% crude protein, respectively, in the diet.

	Dietary treatment								SEM	P-value	P _w value	P _p value
	B23	B27	B31	B35	C23	C27	C31	C35				
ADiDM	77.3 ^{ab}	79.4 ^a	77.7 ^{ab}	78.6 ^{ab}	73.4 ^b	74.8 ^{ab}	76.0 ^{ab}	75.4 ^{ab}	1.16	0.029	<0.001	0.387
ADiOM	82.5	83.4	82.0	82.2	80.3	80.0	81.1	79.8	0.83	0.06	0.001	0.845
ADiCP	83.7 ^c	86.0 ^{abc}	87.1 ^{ab}	87.8 ^a	84.3 ^{bc}	84.8 ^{bc}	87.8 ^a	88.1 ^a	0.59	<0.001	0.759	<0.001
ADiE	84.5 ^{ab}	86.0 ^a	85.4 ^{ab}	86.0 ^a	82.2 ^b	83.4 ^{ab}	84.0 ^{ab}	82.6 ^{ab}	0.76	0.013	<0.001	0.246

SEM = Standard error of the mean.

Means with different superscript letters within rows are significantly different ($P < 0.05$).

p_w value: between different water environments.

P_p value: between different protein levels.

4.4 Carcass and body indices (Papers I, III & IV)

Carcass and body indices were analysed in all tilapia experiments (Papers I, III and IV) (Table 9). Percentage of fillet, hepato-somatic index (HSI), viscero-somatic index (VSI), gastro-somatic index (GSI), entero-somatic index (iEns) and intestinal quotient (Qi) index were evaluated.

In all tilapia papers, fillet yield index at the end of the experiments ranged from 30.39% to 35.3%, and did not differ significantly between any of the treatments ($p>0.05$), regardless of whether data were analysed per treatment group, by water environment or by brewer's yeast inclusion (Paper I and IV)/protein level (Paper III) in the diets.

GSI did display significant and marked systematic differences when all treatments were compared. This indicated a slightly larger stomach in the fish kept in CW-RAS and fed a high fishmeal diet (Paper I) / fed a high protein level (Paper III). However, if the brewer's yeast inclusion levels were pooled independent of water environment, no significant effect on body indices was noted between fish given the different levels of yeast. An exception was GSI, where 30% brewer's yeast inclusion were higher and significantly different than 100% of SBM (Paper IV).

HSI and VSI were not significantly different among treatments in Papers I and IV. However, HSI and VSI of tilapia fingerlings reared in different environments and fed different protein levels were significantly different among treatments. They did not differ among treatments when each crude protein level in different environments was pooled ($p_p>0.05$) (Paper III).

Qi of tilapia was significantly higher in Bio-RAS than in CW-RAS (Paper III). However, this index was independent of the water environment in Papers I and IV (p_w values).

Table 9. Hepato-somatic index (HSI%), viscero-somatic index (VSI%), gastro-somatic index (GSI), entero-somatic index (iEns) and intestinal quotient (Qi) in tilapia fingerlings fed different levels of spent brewer's yeast to replace fishmeal (Paper I) or soybean meal (Paper II) or fed different protein levels (Paper III) in the diet. B: Bio-RAS; C: CW- RAS. 0, 30, 60, 100 indicates 0% 30%, 60% and 100% brewer's yeast replacement and 23, 27, 31, 35 indicates 23% 27%, 31% and 35% crude protein, respectively, in the diet.

Paper I	B0	B30	B60	B100	C0	C30	C60	C100	SEM	P value	P _w value	P _y value
HSI, %	1.6	1.3	1.6	1.6	1.7	1.3	1.3	1.5	0.10	0.09	0.408	0.026
VSI, %	7.0	6.9	6.9	6.7	7.5	6.9	6.1	6.5	0.40	0.43	0.650	0.267
GSI, %	0.4 ^b	0.4 ^b	0.5 ^{ab}	0.4 ^b	0.6 ^a	0.5 ^{ab}	0.4 ^{ab}	0.4 ^b	0.046	0.002	0.279	0.081
iEns, %	3.0 ^a	2.7 ^{ab}	3.2 ^a	2.5 ^{ab}	3.3 ^a	2.8 ^{ab}	2.4 ^{ab}	2.0 ^b	0.22	0.001	0.166	0.002
Qi,	4.6 ^{ab}	4.4 ^{ab}	4.6 ^{ab}	4.5 ^{ab}	5.1 ^a	5.0 ^a	4.7 ^{ab}	4.0 ^b	0.24	0.02	0.218	0.039
Paper III	B23	B27	B31	B35	C23	C27	C31	C35	SEM	P value	P _w value	P _p value
HSI, %	1.86 ^a	1.67 ^{ab}	1.18 ^b	1.38 ^{ab}	1.55 ^{ab}	1.40 ^{ab}	1.54 ^{ab}	1.64 ^{ab}	0.13	0.01	0.89	0.06
VSI, %	8.38 ^a	7.40 ^{ab}	7.07 ^{ab}	6.69 ^b	6.46 ^b	6.91 ^{ab}	6.94 ^{ab}	7.99 ^{ab}	0.36	0.003	0.23	0.66
GSI, %	0.40 ^{bc}	0.39 ^c	0.51 ^{abc}	0.34 ^c	0.31 ^c	0.64 ^{ab}	0.47 ^{bc}	0.75 ^a	0.06	<0.001	<0.001	<0.01
iEns, %	3.06 ^a	2.48 ^{ab}	2.65 ^{ab}	2.49 ^{ab}	2.02 ^b	2.58 ^{ab}	2.22 ^b	2.83 ^{ab}	0.19	0.005	0.05	0.69
Qi	5.92 ^a	4.75 ^{bcd}	5.29 ^{abc}	5.64 ^{ab}	3.85 ^d	4.54 ^{bcd}	4.49 ^{cd}	4.71 ^{bcd}	0.26	<0.001	<0.001	0.26
Paper IV	B0	B30	B60	B100	C0	C30	C60	C100	SEM	P value	P _w value	P _y value
HSI, %	1.66	1.90	1.86	1.85	1.44	1.63	1.58	1.49	0.14	0.17	0.01	0.47
VSI, %	8.92	9.35	9.22	8.05	7.99	7.93	7.75	7.82	0.37	0.31	<0.00	0.25
GSI, %	0.60 ^{abc}	0.77 ^a	0.71 ^{ab}	0.63 ^{abc}	0.36 ^c	0.63 ^{abc}	0.49 ^{abc}	0.44 ^{bc}	0.07	<0.00	<0.00	0.01
iEns, %	3.14 ^{ab}	3.35 ^{ab}	3.98 ^a	3.22 ^{ab}	2.59 ^b	2.91 ^b	2.51 ^b	2.82 ^b	0.24	<0.00	<0.00	0.37
Qi	6.04	6.04	6.37	5.80	5.83	6.08	5.53	5.59	0.25	0.31	0.10	0.49

SEM = Standard error of the mean. Means with different superscript letters within rows are significantly different ($p < 0.05$).

P_w value: between different water environments. P_y value: between different levels of yeast (Papers I and IV) or protein (Paper III).

4.5 Water quality

Water quality parameters in Bio-RAS and CW-RAS were in the range suitable for culturing tilapia and freshwater prawns. Almost all water quality variables such as temperature, DO, pH and NO₂-N, TAN were similar between two systems. Water temperature was 27-29 °C, pH was 6.2-8.4 and dissolved oxygen (DO) content was 4.7-5.8 mg L⁻¹. TAN was 0.05-0.81 mg L⁻¹, NO₂ was 0.004-0.015 mg L⁻¹ (Papers I-IV). In Paper II, the chemical oxygen demand (COD) was 10.7-25.0 mg L⁻¹ and the transparency of water in Bio-RAS was 29.8 cm.

4.6 Plankton assessment, chemical composition and biomass of biofloc

The plankton communities in freshwater prawn (Paper II) and tilapia (Paper III) experiments were collected in bioreactor tanks consisting of six groups of phytoplankton and four groups of zooplankton.

4.6.1 Phytoplankton

Bio-RAS water environment culture of freshwater prawns contained forty-seven genera of phytoplankton belonging to Chlorophyta (23 genera), Bacillariophyta (12 genera), Cyanophyta (6 genera) and Euglenophyta (3 genera), Chrysophyta (2 genera) and Xanthophyta (1 genera). However, in tilapia Bio-RAS consisted of twenty-six genera of phytoplankton belonging to Chlorophyta (13 genera), Bacillariophyta (7 genera), Cyanophyta (4 genera) and Euglenophyta (2 genera).

4.6.2 Zooplankton

In Paper II, four genera of zooplankton were identified, including three genera of Crustaceans and one genus of Rotifera. Rotifers were the most abundant group, accounting for 99.04% of the zooplankton. 0.96% were Crustaceans. The most common genera were *Brachionus* (Rotifera), *Moina*, *Nauplius* larvae and *Daphnia* (Crustaceans). However, Bio-RAS water in the tilapia experiment (Paper III) recorded nineteen genera of zooplankton. This was higher than in the prawn culture, which included eight genera of Rotifera, four genera of Protozoa, four genera of Cladocera and three genera of Nauplius.

4.6.3 Chemical composition and biomass of biofloc

The biochemical compositions and amino acid contents of biofloc biomass in Paper IV are given in Table 10. There were significant differences in dry matter, protein and lipid contents of biofloc between the beginning and the end of the experiment. The protein and biomass biofloc increased with increasing culture time. Conversely, when protein content increased over time, the lipid content in biofloc was reduced with culture time. The essential amino acid contents of biofloc were not affected by the culture time except histidine, lysine, methionine and phenylalanine (Table 10). Histidine and phenylalanine gradually increased and lysine slowly decreased during the experimental periods (Table 10). However, in Paper III, the protein content of biofloc was 33.38% lower than in Paper IV, while lipid content was 5.37 higher.

Table 10. *Chemical composition and amino acid contents of biofloc biomass at different periods of the experiment (%) (Paper IV)*

	Periods			SEM	P- value
	Start (Week 0)	Middle (Week 4)	Final (Week 8)		
Dry matter (g.m ⁻³)	8.20 ^c	25.78 ^b	71.41 ^a	3.92	<0.00
Protein	39.58 ^b	43.80 ^{ab}	44.62 ^a	0.98	0.02
Lipid	2.79 ^a	1.50 ^{ab}	0.61 ^b	0.41	0.03
Essential amino acids					
Arginine	1.44	1.60	1.52	0.05	0.19
Histidine	0.60 ^b	0.71 ^{ab}	0.77 ^a	0.03	0.02
Isoleucine	0.77	0.82	0.82	0.04	0.52
Leucine	2.06	2.00	2.10	0.04	0.20
Lysine	1.34 ^a	1.20 ^b	1.21 ^b	0.02	<0.00
Methionine	0.56 ^a	0.44 ^b	0.49 ^{ab}	0.02	<0.00
Phenylalanine	1.53 ^b	1.59 ^{ab}	1.68 ^a	0.03	0.05
Threonine	1.21	1.23	1.23	0.04	0.89
Valine	1.24	1.28	1.35	0.04	0.15
Total	10.72	10.86	11.18	0.22	0.37

5 General discussion

5.1 The ability to replace fishmeal and soybean meal with spent brewer's yeast

The results obtained in Papers I and II indicated that SBY (*S. cerevisiae*) can be used to replace 100% of fishmeal in tilapia and 60% of fishmeal in giant freshwater prawn diets without negative effects on growth performance and feed conversion. Moreover, protein from SBM could be replaced by 100% protein from SBY in tilapia diets (Paper IV). Several studies have indicated that brewer's yeast has the potential to be used as a feed additive to improve growth performance in different fish species including hybrid catfish (Essa *et al.*, 2011), hybrid striped bass (Li & Gatlin Iii, 2005; Li & Gatlin Iii, 2004; Li & Gatlin Iii, 2003), rainbow trout (*Oncorhynchus mykiss*) (Rumsey *et al.*, 1991b) or tilapia (*O. niloticus*) (Lara-Flores *et al.*, 2003). Brewer's yeast has also been demonstrated to be a good fishmeal replacement candidate in the diets of various fish species such as, Pongpet *et al.* (2016) Thai Panga (*Pangasianodon hypophthalmus* × *Pangasius bocourti*); Gumus *et al.* (2016) gold fish (*Carassius auratus*); Rumsey *et al.* (1990) lake trout (*Salvelinus namaycush*); Oliva-Teles and Gonçalves (2001) and seabass (*Dicentrarchus labrax*). Additionally, no significant reduction in growth was observed in these replacement studies provided the inclusion level remained below 50-75% replacement of fishmeal. This is in line with findings in our experiments. Our study results for tilapia in CW-RAS tallies well with that of Ebrahim and Abou-Seif (2008), who reported that yeast protein (*S. cerevisiae*), supplemented with 100 mg biogenic L-carnitine per 100g, yielded good growth up to 50-75% replacement and that fishmeal replacement up to 100% had no significant ($p>0.05$) adverse effect on growth in tilapia fingerlings. In contrast, OzÓRio *et al.* (2010) reported that growth of pacu (*Piaractus mesopotamicus*) was reduced when fed diets with

both 70% and 100% replacement of fishmeal by brewer's yeast. Rumsey *et al.* (1991b) observed a similar decline in weight gain of rainbow trout fed with 50% and 75% fishmeal replacement by brewer's yeast. While differences tolerance of yeast as a diet substitute for fishmeal may be explained by species differences, divergent results have also been reported in similar species. Furthermore, pellet production, including ingredient drying, could have an effect on digestibility. Our studies differed from others in the method of pellet production. Our pellets were cold-pelleted. It has previously been suggested that extrusion increases the digestibility of intact *S. cerevisiae* through cell wall disruption (Langeland *et al.*, 2016; Vidakovic *et al.*, 2016; Hauptman *et al.*, 2014) in comparison to a cold-pelleted diet (Øverland *et al.*, 2013). It has also been shown that spray-drying of *S. cerevisiae* may reduce digestibility when compared to pellets dried on a fluidized bed (Øverland *et al.*, 2013). Consequently, variation in source quality could be an explanation for the variation in results amongst previous studies. The quality of brewer's yeast is known to vary not only between different production recipes, but also between batches from the same brewery (Gallone *et al.*, 2018), also yielding a possible source of variation. Similarly to fish meal, SBY could also replace 100% of SBM in tilapia diets. However, the research for alternative SBM in diets of fish is scarce. Barros *et al.* (2002) showed that cottonseed meal (CSM), can replace up to 50% of SBM in the diet of juvenile channel catfish (*Ictalurus punctatus*) and displayed the best performance compared to diets with no SBM or no CSM. In tilapia (*O. niloticus* × *aureus*), rubber seed meal can be included at a level up to 30%, as a replacement of SBM, in the diet without obvious adverse effects on growth. However, other effects, such as increased antioxidant capacity, non-specific immune response and resistance to *A. hydrophila* infection, have been reported by (Deng *et al.*, 2015). In juvenile hybrid tilapia (*O. niloticus* × *aureus*), Yue and Zhou (2008) showed that ~60% of SBM in diets could be replaced by cottonseed meal, including additional lysine. However, another study in juvenile channel catfish found that 100% of solvent-extracted SBM could be replaced by cottonseed meal supplemented with iron from ferrous sulfate heptahydrate (Barros *et al.*, 2002). Our present results indicate that when using SBY as a protein source for tilapia, 100% of SBM could be replaced without any significant reduction in growth.

5.2 Bio-RAS and CW-RAS

Biofloc technology (BFT) and clear-water (CW) recirculating aquaculture systems (RAS) are the two main categories of intensive closed aquaculture systems. BFT is dominated by closed pond culture, while clear water RAS by tank rearing.

Clear water (CW) systems use more filtration components, including an external biological filter for nitrifying bacteria and mechanical filters for removal of solids from the water. The costs of buying and operating additional filtration components for a CW system may make this a more expensive and technically vulnerable approach. However, the nitrification cycle in CW systems may be more stable than in BFT systems due to the controlled environment provided by the external mechanical and biofilters (Ray & Lotz, 2017; Ebeling & Timmons, 2012). BFT tank technology is the most recent RAS to have been developed for use in closed, intensive and zero-water exchange culture systems (Crab *et al.*, 2009; Azim & Little, 2008; De Schryver *et al.*, 2008; Avnimelech, 2007). The major drawback of a BFT system is the mix of farmed species and the microbial community sharing the same water and competing for the same resources, such as oxygen and nutrients. Housing a mixed community in the same tank also increases the risk of sludge accumulation, with low-oxygen zones and formation of toxic substances such as H₂S. However, in a system where the farmed animal and microbes live in a mixed environment, obvious advantages are gained. One advantage of the BFT system is the possibility to regain lost nutrients in the form of prey organisms. Another positive is the possibility of disease prevention through design. A possible way of maintaining the advantage of the high intensive indoor BFT system, is the separation of main rearing units for fish/shrimp and the microbial community. By exchanging the external bio- and mechanical filters of a CW- RAS with a bioreactor system one obtains a separate unit for microbial growth/sedimentation. By such separation, minerals, nutrients and gases favourable to microbes is added more efficient. Also, such separation facilitate effective ventilation of CO₂ and nitrogenous gases produced by the microbes. Furthermore, an external bioreactor will function as sediment trap and sludge easily isolated and removed, hereby significantly reducing the risk of H₂S formation.

In this study we employ a novel approach by separating the main rearing units of fish/shrimp and the microbial community, i.e. the biofloc. We denote this system Bio-RAS in order to separate it from more traditional clear water RAS, here denoted CW-RAS. This combined system of BFT and RAS was designed to provide the optimal environment for both the reared species and microbes, but in separate units, before circulating the microbes into the rearing compartment. Conical rearing tanks are utilised reducing the accumulation of sludge to zero, while providing nutrients specific to enhance microbial growth in external open bioreactors in the form of large tanks that function as sediment traps.

Our studies indicated that heterotrophic microbes circulating in the total water system seem to remove toxic ammonia more efficiently than a fixed bio-bed in which the water is exposed to nitrifying bacteria for a limited time. The Bio-RAS approach may have the potential to support equally high rearing densities as conventional RAS, while providing the advantages of BFT, with zero water exchange and reduced disease risk.

5.3 Growth performance and feed utilisation of fish in Bio-RAS versus CW-RAS environments

The most telling results in our present studies (Papers I-IV) showed that the growth performance and feed utilisation of tilapia in Bio-RAS were higher than in CW-RAS even though we used a very simple Bio-RAS as a complement to the pelleted feed. However, there was no significant difference between Bio-RAS and CW-RAS ($p < 0.1$) when freshwater prawns were cultured (Paper II), even though shrimp cultured in the Bio-RAS did display higher numbers (Table 7) as the level of SBY increased. The findings in our tilapia studies were supported by the results from previous studies such as Long *et al.* (2015), Azim and Little (2008), Luo *et al.* (2014), reporting a 9%, 10% and 22% higher body weight of fish grown in biofloc system compared to clear water system. This was similar to our findings.

In our studies, fish kept in the Bio-RAS environment always surpassed fish kept in CW-RAS given the same inclusion level of brewer's yeast in the diet in terms of growth registered as body weight or as SGR, and feed efficiency. The Bio-RAS not only resulted in better growth and feed utilisation by fish compared to fish reared in CW-RAS, Bio-RAS also had a higher tolerance for fish with a high level of spent brewer yeast alternative for fishmeal in the diet. For example, yeast could totally replace fishmeal in Bio-RAS but only 60% SBY could be used in CW-RAS without significantly affecting growth performance (Paper I). Therefore, based on our data we conclude that not only will a Bio-RAS environment improve performance if the fish are given a fishmeal-based diet, but they will also be better able to tolerate a higher substitution level of brewer's yeast. Bio-RAS represents not only a more economical and environmentally friendly protein source, but also a nutrient source with lower biological availability (Langeland *et al.*, 2016).

BFT has been successfully applied to the farming of shrimp and prawns (Emerenciano *et al.*, 2013). It has been reported that intermediate levels of biofloc create factors propitious for maintaining the productivity and stability of super-intensive *Litopenaeus vannamei* aquaculture systems (Schveitzer *et al.* (2013). Additionally, bioflocs have also been shown to improve the growth performance and feed utilisation of cultured shrimp (Xu & Pan, 2012). In another study, *Marsupenaeus japonicus* under high-intensive production conditions with zero water exchange farming systems in biofloc system was associated with a 41.3% improvement in the production yield (Zhao *et al.*, 2012). BFT has also been applied to freshwater prawn co-culturing together with catfish (*Clarias batracus*) in a concrete pond system and was shown to increase economic viability (Rohmana *et al.*, 2015). Haque *et al.* (2015) showed that freshwater prawns cultured in an environment with the addition of both maize flour and periphyton substrates showed a higher net yield than prawns with only maize flour added and having a C:N ratio of 20:1, or prawns cultured in normal practice without the addition of periphyton substrates and carbohydrate.

Data from our studies have shown a stronger effect of biofloc in our tilapia experiments (Paper I, III and IV) than in freshwater prawn experiments (Paper II). Based on several practical observations (Crab *et al.*, 2010) we suggest that the underlying rationale for the observed difference between tilapia and freshwater prawn is based on feeding behaviour. The two species exhibit different feeding strategies, where tilapia is an excellent filter feeder, using the whole water column for foraging, and growing *M. rosenbergii* tend to mainly graze from solid objects and use their periopods (chelate legs) to bring food particles to the mouth. We, therefore, conclude that *M. rosenbergii* most likely have a more specific need of either highly flocculating bacteria, increased grazing surface or microbial assimilating zooplankton in order to efficiently utilise the floc compared to tilapia, an area presently being addressed in our laboratories.

5.4 Protein requirement of tilapia in Bio-RAS versus CW-RAS environments

Protein is the most expensive as well as the most crucial nutrient in fish feed. Previous studies have reported that the protein requirement of fish could be markedly influenced by the environmental conditions of the culture water/system (Mansour & Esteban, 2017; Megahed, 2010; Azim & Little, 2008). It is therefore important to define the appropriate requirement of protein for tilapia production in different culture systems with varying environments. The culture of filter-feeder tilapia in Bio-RAS could reduce the level of protein required in feed when compared to tilapia kept in conventional systems. Our results showed that the crude protein requirement of tilapia was 27% in Bio-RAS, and 31% in CW-RAS. These results are in close accordance with the findings of Avnimelech *et al.* (1989), Azim and Little (2008) and Mansour and Esteban (2017) who showed the possibility of using diets with less crude protein content for tilapia culture in a biofloc environment. Azim and Little (2008) have shown that dietary CP levels can be reduced from 35% to 24% for tilapia juveniles with a body weight of 100 g reared in BFT without any loss of performance, unlike fish reared in CW system. Furthermore, tilapia fed 20% crude protein and reared in (wheat milling product) biofloc significantly outperformed the fish fed 30% crude protein and reared in CW. Using wheat milling product as a carbon source could make up for a reduction in dietary protein levels of 10% and improve growth performance of *O. niloticus*. The growth of tilapia reared in biofloc tanks and fed 20% protein still surpassed that of the fish cultured in clear water, and fed the 20 or 30% protein diet (Mansour & Esteban, 2017). In addition, optimistic results have been found for shrimp culture on reduced-protein level diets in BFT. CP levels could be decreased from 35% to 25% for *Penaeus semisulcatus* (green tiger prawn) (Megahed, 2010) and from 31.15% to 16.25% for *L. vannamei* (Xu & Pan, 2014) without loss of growth performance. Our results are also in agreement with the findings of da

Silva *et al.* (2018) who evaluated CP levels (17, 21, 25, 29 and 33%) in tested diets for Nile tilapia in biofloc system. They showed that tilapia of 10–60 g and 60–230 g reared in biofloc system can be fed on diets with 28% CP (26% of digestible protein) and 22% CP (20% of digestible protein), respectively. The decreased nutrient requirement of tilapia cultured in Bio-RAS in our experiments could be explained by compensation, when tilapia consume high nutritional quality biofloc biomass in the water column, especially in terms of protein 33.38% (in Paper III) and 39.6–44.6% (in Paper IV). This has been demonstrated in several aquaculture species. According to Wang *et al.* (2015), the protein value of biofloc in crucian carp (*Carassius auratus*) systems was 29.39%, in environmental culture of red tilapia (*Oreochromis sp.*) was from 39.71% to 48.13% (Ekasari & Maryam, 2012) and in system-reared genetically improved farmed tilapia (*O. niloticus*) was 41.13% (Long *et al.*, 2015) and 34.06–38.53% (Zhang *et al.*, 2016). Many studies demonstrated that protein from biofloc could be used as a protein source for fish (Himaja & Rajagopalasamy, 2016; Crab *et al.*, 2012; Crab *et al.*, 2010; Avnimelech & Kochba, 2009; Avnimelech, 2007). Tilapia, a filtering species, are well-suited to biofloc feeding and graze on biofloc directly (Azim & Little, 2008; El-Sayed, 2006). This probably explains the lower CP levels estimated in this study in comparison with those normally recommended for tilapia (Kpundeh *et al.*, 2015; NRC, 2011; Al Hafedh, 1999).

5.5 Water quality and biofloc biomass

The water quality in our studies (Papers I-IV) was very good and within the limits for tilapia and freshwater prawn rearing (Boyd & Zimmermann, 2009). It should be noted that no water was exchanged in the Bio-RAS during the experimental period, but that the bioreactor tanks were cleared of non-digestible material, i.e. sludge, by siphoning the bottom every two weeks. The low TAN and NO₂-N values in the biofloc environment of our study is in agreement with earlier work. (Asaduzzaman *et al.*, 2008; Hari *et al.*, 2004; Avnimelech, 1999) have reported that the addition of carbohydrate to the production systems reduces the total ammonia nitrogen (TAN) concentration through converting inorganic nitrogen into the bacteria which have high quality of protein. Thus, the reduction in nitrite nitrogen and total ammonia nitrogen could be attributed to the addition of carbonaceous substrates that lead to an increased microbial activity. This immobilised TAN, allowing the synthesis of new bacterial cells (Hari *et al.*, 2004) and uptake of the nitrogenous compounds by bacteria and phytoplankton. These biomass increased during the experiment with high nutrient values, containing high amounts of amino acids, 33.4–44.6% protein and 0.61–5.37% lipid. Thus it was well in line with a fully nutritional diet except for a low crude lipid level (0.61–2.79%) in Paper IV. The nutrient content of the biofloc was similar to that analysed by Long *et al.* (2015), which had 41.13% crude protein and 1.03% crude fat. On the other hand, biofloc collected by Azim and Little (2008) from a zero-exchange indoor system rearing tilapia fed different protein

levels from 24% or 35% crude protein was found to contain 37.9-38.4% crude protein, 3.16-3.23% crude lipid, 11.8-13.4% ash and 18.6-19 kJ g⁻¹ energy, i.e. nearly twice the level of lipids found in our final biofloc sample. The floc density (g dry matter m⁻³) increased from 8.20 g m⁻³ at the start to 71.41 g m⁻³ at the end of our experiment (Table 5). This is higher than the density reported by Avnimelech (2007), and was possibly the underlying cause of the significant difference in growth of fish between the two systems.

6 General conclusions

Based on the results of the studies presented in this thesis, it can be concluded:

1. Separation of the BFT system into a major farming part and a bioreactor part has the potential to support an intensification of the BFT system, here denoted Bio-RAS.
2. Bio-RAS supports full growth and lower FCR by recycling nitrogen in the farming environment directly from faeces to feed.
3. The Bio-RAS environment of the present study seemed suboptimal for freshwater prawns (*M. rosenbergii*) and increased flocculation, grazing area and/or zooplankton can possibly lead to improved production performance.
4. Tilapia (*O. niloticus*) in Bio-RAS and CW-RAS can be fed on diets with 27% and 31% of CP, respectively.
5. Fishmeal can be replaced with spent brewer's yeast at a ratio of 100% in tilapia diets and 60% for giant freshwater prawns, if kept in a biofloc environment.
6. Soybean meal protein can be replaced with spent brewer's yeast at a ratio of 100% in tilapia diets with no negative effects in tilapia growth and feed efficiency.
7. Soybean meal protein replaced with spent brewer's yeast at a ratio of 30% in tilapia diets improved feed efficiency and growth performance in comparison to the control (0% yeast) or 100% yeast. i.e. it is possible that SBY inclusion can be favourable, especially if the fish is reared in a biofloc environment.
8. All growth indices, PER of tilapia in all experiment in Bio-RAS were higher than in CW-RAS.
9. FCR of tilapia and prawns in Bio-RAS were lower than in CW-RAS.
10. Tilapia kept in Bio-RAS environment have lower mortality rates than in CW-RAS when using diets with different levels of SBY replaced for fishmeal.

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Popular science summary

Aquaculture is one of the fastest growing food production industries in the world, playing an important role in increasing aquatic production, improving national income and improving living standards for people through job-production. The products of the aquaculture sector are very rich and diverse, a source of high quality food that can meet the nutritional demands of the population. Most aquatic products are protein rich, easily digestible and suitable for all ages. In addition, fishmeal, waste products and processed aquatic products provide feed for livestock, poultry and fish through industrial animal feed processing. However, the rapid development of the aquaculture sector is accompanied by the consequence of environmental pollution. Therefore, environmentally friendly aquaculture practices and management play an important role in the future of sustainable aquaculture development. Biofloc biotechnology (BFT) is considered a promising, new, sustainable production system that minimises the environmental impact of large-scale aquaculture.

BFT in aquaculture functions based on the principle of adding a carbon source in a proportion consistent with the amount of nitrogen available in pond water. Certain bacteria then convert nitrogen-containing compounds in the pond water into biomass, thereby reusing nitrogen from waste water. In addition, the natural biomass formed in a BFT system provides feed for production fish and prawns in an efficient manner. In this way, the application of BFT reduces the cost of fish feed in addition to providing an environmentally-conscience, sustainable solution for large-scale aquaculture. In this study we employ a novel approach by separating the main rearing units of fish/shrimp and the microbial community, i.e. the biofloc. We denote this system Bio-RAS in order to separate it from more traditional clear water RAS, here denoted CW-RAS.

Industrial brewery plants produce a large amount of by-product in the form of spent brewer's yeast (SBY). This SBY needs to be treated and utilised in order to minimise its effect on the environment. Interestingly, SBY has a high nutritional value, giving it the potential to be used as a protein-replacement

source for fishmeal and soybean meal in farmed tilapia and other aquaculture species. These factors provided the basis for the studies in this thesis.

The goal of this thesis was to determine the effect of feeding SBY as an alternative protein-source to fishmeal and soybean meal in the fish species, tilapia (*Oreochromis niloticus*), and giant fresh water prawns (*Macrobrachium rosenbergii*) in two different aquaculture environments, clear water or biofloc.

The results of our studies showed that SBY can 100% replace fishmeal in the diet of tilapia and also 60% replace fishmeal in the diet of giant freshwater prawns in recirculation systems, if kept in a biofloc environment.

Additional findings were:

- Feed was more efficiently used by tilapia and prawns in Bio-RAS compared to CW-RAS thanks to nutrients being recirculated back to the fish/shrimp as feed.
- Protein efficiency was better in tilapia in Bio-RAS than in CW-RAS.
- Protein content can be reduced to 27% and 31% in feed to tilapia if reared in biofloc or clear water systems, respectively.
- Soybean meal protein can be replaced with spent brewer's yeast at a ratio of 100% in tilapia diets with no negative effects in tilapia growth and feed efficiency.
- Inclusion of 30% spent brewer's yeast seems to be beneficial to tilapia, especially if reared in biofloc environment.
- Growth of tilapia was always higher in biofloc than in clear water environments.
- Our studies show that using the by-product, SBY, and the biofloc system, can reduce feed costs and minimise the environmental impact of large-scale aquaculture systems. In this way, aquaculture can continue to improve the lives of people worldwide by providing income and meeting growing demands for sustainable, high-quality fish, crustacean and shellfish.

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