Promoters:	Prof. dr. ir. Willy Verstraete Department of Biochemical and Microbial Technology Prof. dr. ir. Peter Bossier Department of Animal Production
Dean:	Prof. dr. ir. Guido Van Huylenbroeck
Rector:	Prof. dr. Paul Van Cauwenberge

UNIVERSITEIT
GENT

Faculty of Bioscience Engineering

Bioflocs technology: an integrated system for the removal of nutrients and simultaneous production of feed in aquaculture

Roselien Crab

Thesis submitted in fulfillment of the requirements for the degree of
Doctor (PhD) in Applied Biological Sciences:
Environmental technology

2010

Dutch translation:
Biovlok-technologie: een geïntegreerd systeem voor de verwijdering van nutriënten en de simultane productie van voeder in aquacultuur

Cover illustration: Eva Verschaeren & Ruben Accou
Reference: Crab, R. (2010). Bio-flocs technology: an integrated system for the removal of nutrients and simultaneous production of feed in aquaculture. PhD thesis, Ghent University, Belgium.
ISBN: 978-90-5989-395-5

This work was funded by the "Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen" (IWT grant no. 53256 – 2005-2009) and the "Fonds voor Wetenschappelijk Onderzoek" (Stay abroad – K.2.236.07.N.00).

Exam Committee

Prof. dr. Yoram Avnimelech
Department Civil and Environmental Engineering
Technion, Israel

Prof. dr. ir. Nico Boon
Department of Biochemical and Microbial Technology
Ghent University, Belgium

Prof. dr. ir. Peter Bossier
Department of Animal Production
Ghent University, Belgium

Prof. dr. ir. Peter Goethals
Department of Applied Ecology and Environmental Biology
Ghent University, Belgium

Prof. dr. Patrick Sorgeloos
Department of Animal Production
Ghent University, Belgium

Prof. dr. ir. Mieke Uyttendaele
Department of Food Safety and Food Quality
Ghent University, Belgium

Prof. dr. ir. Marc Verdegem
Aquaculture and Fisheries Department
Wageningen University, the Netherlands

Prof. dr. ir. Willy Verstraete
Department of Biochemical and Microbial Technology
Ghent University, Belgium

Chairman

Prof. dr. ir. Patrick Van Oostveldt
Department of Molecular Biotechnology
Ghent University, Belgium

Voorwoord

Nu alles stilaan vorm begint te krijgen, is het tijd om een gevreesd stukje te schrijven: 'hét' voorwoord. Alleen al de gedachte aan deze inleiding doet allerlei namen in me opwellen, vergezeld van warme gevoelens. Maar tegelijkertijd angst, angst iemand te vergeten die net op dat moment dat je het bos door de bomen niet meer zag, je met je beide voeten stevig op de grond trok. Tijdens deze rit kwam ik veel mensen tegen die me (bewust en onbewust) geholpen hebben bij het bereiken van mijn doel –ooit voor jullie te kunnen staan als 'Dr. Crab' (uitgesproken op zijn James Bonds)– en ik heb daarvoor alleen maar dankbaarheid te over die uitgaat naar jullie allemaal.

Maar zonder oorsprong, geen 'happy end' en dat brengt me bij de eerste warme dankbetuiging die uitgaat naar mijn promotoren, professor Verstraete en professor Bossier. Professor Verstraete, ik zal deze kans nooit vergeten. Uw energie, motivatie en begeleiding zijn de steunpilaren van dit werk en maakten het tot een stevige woonst en niet een bouwvallig tuinhuisje. Professor Bossier, ik dank u voor de geboden kansen en het delen van uw kennis.

Naast mijn Belgische promotoren had ik 'stiekem' nog een mentor in Israël. Professor Avnimelech, you gave my work an interesting turn and opportunities beyond what I dreamed of. Professor Verstraete was right about stating that going abroad can only expand your horizon and those horizons I saw in Israel were breathtaking. Besides the scientific enrichment that I took home with me from Israel, I will never forget how you, your family and friends made me feel like I found a second home.

Naast deze gevestigde waarden binnen de wetenschappelijke wereld, wil ik nog iemand bedanken die mij vanaf het begin alles heeft geleerd over deze waarden en de vestiging is van mijn wetenschappelijke avontuur. Dr. Tom, al van thesisstudente af heb ik je begeleiding erg op prijs gesteld en dat doe ik tot op de dag van vandaag nog steeds.

Dit brengt me naar LabMET en zijn labo's, bureau's en secretariaat, waar het thuisgevoel nooit ver weg was en waar alles begon tijdens het uitvoeren van mijn thesisonderzoek. Al mijn collega's van LabMET: dank u voor deze fijne tijd! Een extra regel voor mijn bureaugenoten die een fijne werksfeer vrijwaarden en af en toe met

mijn rare grillen overweg konden. Een klein gilletje voor jullie allemaal: Tom, Peter & Peter, Liesje, Ann, Pieter & Pieter. Nog een extra regel voor 'het secretariaat' (Kris, Regine en Annelies) waar het steeds fijn vertoeven was en die mijn stunteligheid in administratieve taken bijstuurden. Nog één regeltje extra voor Nico, Rita, 'dé Rotonde' en iedereen in, op, rond en naast LabMET. Ook op het labo voor aquacultuur was het fantastisch vertoeven. Dank u Geert van De Wiele, Jorg Desmyter, Mathieu Wille en alle medewerkers voor de hulp en ondersteuning en de plezierige tijd. Naast deze twee Gentse labo's bracht ik ook tijd door aan het Genosar Research Station en het labo van Prof. Avnimelech aan het Technion in Israël. Een ervaring om nooit te vergeten. Fantastische mensen om nooit te vergeten...

Tenslotte nog één groep mensen die ik vanuit professioneel oogpunt wil bedanken – mijn thesisstudenten. Ik hoop dat jullie het een even leerrijke ervaring vonden als ikzelf. Jullie begeleiden was een plezier en een voorrecht: Bram, Julie, Hendrik, An & Karen. Julie, I enjoyed working with you & wish you all the best in your future career.

En dan nu familie en vrienden. Mams, Paps, Mamie, Sassie, Kapitein Flo, Jana & Kurt, een kleine familie, maar ik had me er geen betere kunnen wensen. Mia (+ Boebitsa), Evatje & Ruben, een ode aan jullie om mijn 'eeuwigdurende' zorgenkindje mee te sussen. Last, but certainly not least, niet te vergeten (en waarschijnlijk sommigen vergeten, maar dit gaat ook uit naar jullie): Yannick, Julie, Alex, Marilyn, Medina, Steven, Cathy, CSI Charlotte, Isabelle aka Isa, Michael, Tinne, Pan, Youssef, kine Peter, mijn NICC collega's, ... en tenslotte mijn 2 katten Lucy & Gusar.

Iedereen, alles, overal... woorden komen te kort voor die kleine schouderklop, die grote reddingsactie, die veelzeggende stilte en dat spraakwater van motivatie. Dank u!

Roselien

Common – The light

There are times when you'll need someone
I will be by your side
There is a light, that shines, special for you, and me

I never knew a love like this
Got to be something for me to write this
Queen, I ain't seen you in a minute
Wrote this letter, and finally decide to send it
Signed sealed delivered for us to grow together
Love has no limit, let's spend it slow forever
...

Notation index

AA	Ascorbic acid
ABW	Average body weight
AHL	N-acyl-homoserine lactone
ALA	Linolenic acid
ANOVA	Analysis of variance
AOB	Ammonia oxidising bacteria
BFT	Bioflocs technology
C/N	Carbon to nitrogen ratio
CCD	Charge-coupled device
CF	(Fulton's) Condition factor
CFU	Colony forming units
Co	Community organisation
COD	Chemical oxygen demand
cSGR	Cumulative specific growth rate
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
DLVO	Derjaguin, Landau, Verwey and Overbeek
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DW	Dry weight
EDTA	Ethyleendiaminetetraacetic acid
EPA	Eicosapentaenoic acid
F	Feed
FAME	Fatty acid methyl ester
FAO	Food and agriculture organization
FCR	Food conversion ratio
FISH	Fluorescent in situ hybridisation
FSB	Fluidised sand biofilter

FVI	Floc volume index
G	Mean shear rate or Average velocity gradient
HPLC	High pressure liquid chromatography
HRT	Hydraulic residence time
HUFA	Highly unsaturated fatty acids
Kj-N	Kjehldal-nitrogen
LA	Linoleic acid
Lv	Viscous length
MBR	Membrane bioreactor
Meq	Molar equivalent
No	Initial number of shrimp
Nt	Number of dead shrimp
OD	Optical density
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PHB	Poly-β-hydroxybutyrate
PUFA	Polyunsaturated fatty acids
RAS	Closed or recirculating aquaculture system
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SBR	Sequence batch reactor
SC	Starvation control
SGR	Specific growth rate
SVI	Sludge volume index
T	Culture period
TAE	Mixture of tris base, acetic acid and EDTA
TAN	Total ammonia nitrogen
TCBS	Thisulfate citrate bile salt sucrose agar
TSS	Total suspended solids
TVC	Total *Vibrio* count
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultra violet
VSS	Volatile suspended solids
Wo	Initial weight
Wt	Final weight
γ	Relative uptake factor

Table of Contents

CHAPTER 1

NITROGEN REMOVAL TECHNIQUES IN AQUACULTURE FOR A SUSTAINABLE PRODUCTION

As the aquaculture industry intensively develops, its environmental impact increases. Discharges from aquaculture deteriorate the receiving environment and the need for fishmeal and fish oil for fish feed production increases. Rotating biological contactors, trickling filters, bead filters and fluidized sand biofilters are conventionally used in land-based intensive aquaculture systems to remove nitrogen from culture water. Besides these conventional water treatment systems, there are other possible modi operandi to recycle aquaculture water and simultaneously produce fish feed. These double-purpose techniques are the periphyton treatment technique, which is applicable to extensive systems, and the proteinaceous bioflocs technology, which can be used in extensive as well as in intensive systems. In addition to maintaining good water quality, the two techniques provide an inexpensive source of feed and a higher efficiency of nutrient conservation. The bioflocs technology has the advantage over all other techniques that it is relatively inexpensive; this makes it an economically viable approach for sustainable aquaculture.

Redrafted after:

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P. and Verstraete, W., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1-14.

De Schryver, P., Crab, R., Defoirdt, T., Boon, N. and Verstraete, W., 2008. The basics of bioflocs technology: The added value for aquaculture. Aquaculture 277, 125-137.

1.1 Overview of problem

Aquaculture is a rapidly growing food producing sector. The sector has grown at an average rate of 8.9% per year since 1970, compared to only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat-production systems over the same period (FAO, 2004). A closer look at the recent history of aquaculture growth shows that it has not been uniform. It has been faster in some regions of the world than in others. The same pattern appears when production is broken down by species. The annual change in average yearly growth in world aquaculture production was 11.8% during the period of 1985 – 1995 and 7.1% in 1995 – 2005 (FAO, 2009). In contrast to aquaculture, capture fisheries landings as a whole is stagnant. Although catch rates for some species did not declined during the 1990s, most ocean fisheries stocks are now recognized as fully exploited or over fished. The worldwide decline of ocean fisheries stocks and the further expansion of the human population is an incentive for the further growth of aquaculture. Despite the growth of the sector, aquaculture production still needs to increase 5-fold in the next 2 decades in order to satisfy the minimum aquatic food protein requirement for human nutrition (FAO, 2004).

The intensive development of the aquaculture industry has been accompanied by an increase in environmental impacts. The production process generates substantial amounts of polluted effluent, containing uneaten feed and feces (Read and Fernandes, 2003). Discharges from aquaculture into the aquatic environment contain nutrients, various organic and inorganic compounds such as ammonium, phosphorus, dissolved organic carbon and organic matter (Piedrahita, 2003; Sugiura et al., 2006). The high levels of nutrients cause environmental deterioration of the receiving water bodies. In addition, the drained water may increase the occurrence of pathogenic microorganisms and introduce invading pathogen species (Thompson et al., 2002).

To produce 1 kg live weight fish one needs 1-3 kg dry weight feed (assuming a food conversion ratio about 1-3) (Naylor et al., 2000). About 36% of the feed is excreted as a form of organic waste (Brune et al., 2003). Around 75% of the feed N and P are unutilized and remain as waste in the water (Piedrahita, 2003; Gutierrez-Wing and Malone, 2006). An intensive aquaculture system, which contains 3-ton tilapia, can be compared on a biomass basis to a human community with 50 inhabitants (Helfman et al., 1997). This intensive aquaculture system can also be compared in terms of waste generation to a community of around 240 inhabitants (Aziz and Tebbutt, 1980; Flemish government, 2005). It can thus be concluded that live fish biomass generates approximately 5 times

more waste than live human biomass. The reason is that the scope of digestion in fish is limited; a relatively large fraction of feed remains undigested and is excreted (Amirkolaie, 2005). The feeding habit of fish is reflected in the digestive anatomy. The gut length of fish is short and the ratio of gut length to body length is small (Hertrampf and Piedad-Pascual, 2000). For instance, the intestine of carp is 2.0-2.5 times longer than the body, while that of cattle and sheep is respectively 20 and 30 times longer. The human intestine is about 3 to 4 times longer than the body. Consequently, in fish, the chyme stays in the gut only for a short time. For this reason, fish feed must have a high digestibility. Typically, fish body contains 65 to 75% protein (Hertrampf and Piedad-Pascual, 2000). In addition, fish use proteins for energy production to a large extent, unlike terrestrial animals that use mostly carbohydrates and lipids (Hepher, 1988). Fish protein requirement, therefore, is about two to three times higher than that of mammals. Ammonium is one of the end products of protein metabolism (Walsh and Wright, 1995). All these factors contribute to the high nitrogen residues in aquaculture water (Figure 1.1). In water, NH_3 (ammonia) and NH_4^+ (ammonium) are in equilibrium depending on the pH and the temperature (Timmons et al., 2002). The sum of the two forms is called total ammonium nitrogen (TAN). Although both NH_3 and NH_4^+ may be toxic to fish, unionized ammonia is the more toxic form attributable to the fact that it is uncharged and lipid soluble and consequently traverses biological membranes more readily than the charged and hydrated NH_4^+ ions (Körner et al., 2001). Ammonia-N is toxic to commercially cultured fish at concentrations above 1.5 mg N/l. In most cases, the acceptable level of unionized ammonia in aquaculture systems is only 0.025 mg N/l (Neori et al., 2004; Chen et al., 2006). However, the toxicity threshold depends strongly on the species, size, fine solids, refractory organics, surface-active compounds, metals, and nitrate (Colt, 2006).

In addition to the generation of large amounts of waste, the use of fishmeal and fish oil as prime constituents of feed is another non-sustainable practice in aquaculture. Of note is the large share of fishmeal now consumed by the aquaculture industry, estimated at 60% of the world production (FAO, 2009). At the same time, the poultry industry has drastically reduced its fishmeal use (FAO, 2009). For fish oil, the role of aquaculture is even greater than for fishmeal, with close to 85% of production consumed by the sector (FAO, 2009). Hence, aquaculture is a possible panacea, but also a promoter of the collapse of fisheries stocks worldwide. The ratio of wild fish/fed farmed fish (both live weight base) is about 1.41/1 for tilapia and 5.16/1 for marine finfish (Naylor et al., 2000). Herbivorous, omnivorous and carnivorous finfish all necessitate about the same amount of dietary protein per unit weight, but herbivorous and omnivorous species utilize plant-based proteins and oils better and they require minimal quantities of fishmeal to supply essential amino acids (Naylor et al., 2000). Nevertheless, compound feeds for herbivorous and omnivorous fish often exceed required levels (Naylor et al., 2000).

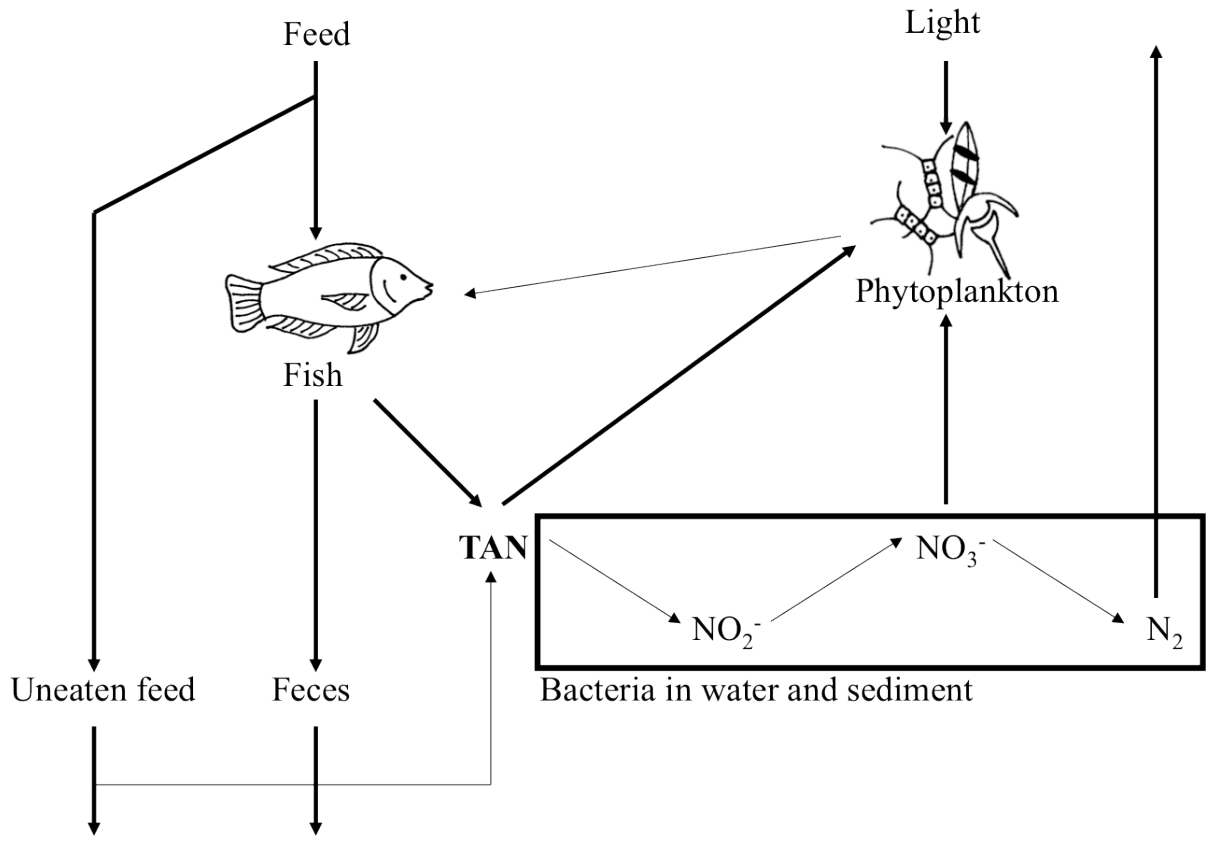


Figure 1.1 Nitrogen cycle in aquaculture ponds with a long hydraulic residence time. The N-input considered is formulated feed. A part of the feed remains unconsumed in the system (Franco-Nava et al., 2004b). The consumed feed is partially converted into fish biomass and partially excreted as ammonium or egested as feces (Jiménez-Montealegre et al., 2002). The uneaten feed and feces contribute to the organic matter load of the system. The microbial decomposition of organic matter in the system lead to increased levels of TAN and nitrite, both harmful to fish even at low concentrations (Meade, 1985; Jiménez-Montealegre et al., 2002; Torres-Beristain et al., 2006). The TAN present in the system may be transformed into nitrite, nitrate and gaseous nitrogen. The formation of nitrogen gas is considered negligible in aquaculture ponds (El Samra and Olàh, 1979). The bacteria present in the water and sediment carry out these nitrogen transformations by nitrification and denitrification. Both TAN and nitrate can be assimilated by the phytoplankton, present in the water column. The phytoplankton can be consumed by the cultured organism (Turker et al., 2003). The consumption of phytoplankton by fish is minimal in this network, although function of the selected species. In stagnant water ponds TAN tends to accumulate within the system due to insufficient nitrification activity (Grommen et al., 2002).

Purchase of commercially prepared feed for fish culture comprises 50% or more in the production costs; this is primarily due to the cost of the protein component (Bender et al., 2004). On average some 25% of the nutrient input of these feed sources is converted

into harvestable products (Avnimelech and Lacher, 1979; Boyd, 1985; Muthuwani and Lin, 1996; Avnimelech and Ritvo, 2003). To make further sustainable increase of aquaculture production possible, the search for inexpensive protein sources and a higher efficiency of nutrient conversion of feed is needed.

The co-occurring problems of growing aquaculture mentioned above, together with the increased interest in land-based intensive systems and the trend of producing seafood close to the captive market in temperate climates, asks for new technologies. The bioflocs technology, dealt with in this work, is a technology that can contribute to future sustainable aquaculture through the simple modus operandi of elevating the carbon to nitrogen ratio in the aquaculture system water. Through increasing the carbon level, the microbial community present in the water is stimulated and will control water quality while simultaneously recycling waste components into nutritious bioflocs, available for the culture organism to feed on (Avnimelech, 2009).

1.2 N removal outside the culture unit

For long, the most common method for dealing with pollution of the culture water by an excess of organic materials and nutrients has been the use of continuous replacement of the pond water with external fresh water (Gutierrez-Wing and Malone, 2006). However, the water volume needed for even small to medium aquaculture systems can reach up to several hundreds of cubic meters per day. For instance, penaeid shrimp require about 20 m^3 sea water per kg shrimp produced (Wang, 2003). For a medium-sized trout raceway system of 140 m^3, even a daily replacement of 100 times the water volume is applied (Maillard et al., 2005). A second approach is the removal of the major part of the pollutants in the water as is performed in recirculating aquaculture systems (RAS) with different kinds of biologically based water treatment systems (Guitierrez-Wang and Malone, 2006). The amount of water in RAS that needs to be replaced on a daily basis generally is decreased to about 10% of the total water volume (Twarowska et al., 1997).

The most common water purification treatments in aquaculture systems can be subdivided in different types of water treatments: 1) earthen treatment ponds or reservoirs, and 2) a combination of solids removal and nitrification tanks as also used in domestic wastewater treatment plants. It should be noted that the real nitrogen removal processes are those that involve the release of fixed nitrogen back to the atmosphere (van Rijn et al., 2006). However, these are not discussed here.

1.2.1 Earthen treatment ponds or reservoirs

This treatment procedure consists of the direct linkage of, and water recirculation between the intensive production ponds and treatment ponds. The effluent water of the production pond is retained in a basin for several hours to days to allow natural physical, chemical, and biological processes to improve its quality for reuse (Diab et al., 1992; Hargreaves, 2006). Important practical parameters in this system are the hydraulic retention times of the intensive fish culture unit and the treatment pond, homogeneous mixing of the treatment pond, and the periodic aeration of the pond sediment by drainage. The use of treatment ponds encounters problems due to algal collapse and anaerobiosis of the sediment (van Rijn, 1996). The main disadvantage is the unstable purification resulting from unpredictable fluctuations of phytoplankton biomass and speciation in the treatment pond (Hargreaves, 2006). An important advantage is that the microalgae grown in the treatment pond can be used to produce a second crop, such as bivalves, seaweed or *Artemia*, which can generate extra income (Wang, 2003).

A possible system configuration comprises a fish farm with nutrient assimilation by molluscs and seaweed (Figure 1.2).

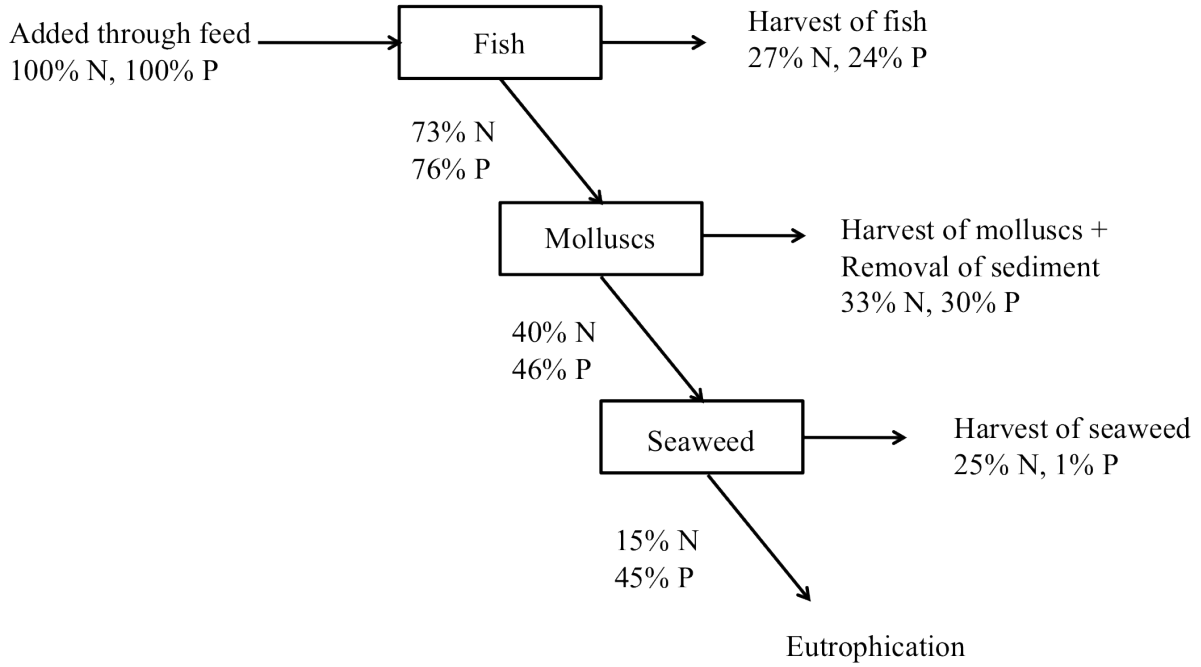


Figure 1.2 Integrated farming: nitrogen and phosphorus budget (after Kautsky, 2004).

Here, nutrients released in the culture system can be converted into plant or other biomass, which can easily be removed and may often be a valuable by-product. The nutrient-assimilating photoautotrophic plants can be used to turn nutrient-rich

effluents into profitable resources (Neori et al., 2004). Biofiltration by plants generates in the culture system a mini-ecosystem, in which, if properly balanced, plant autotrophy counters fish (or shrimp) and microbial heterotrophy, not only regarding nutrients but also with respect to oxygen, pH and CO_2 (Neori et al., 2004). As a result, plant biofiltration diminishes the net environmental impact of aquaculture production systems. Today's integrated intensive aquaculture approaches, developed from traditional extensive polyculture, integrate the culture of fish or shrimp with vegetables, microalgae, shellfish and/or seaweed (Neori et al., 2004). By dividing the production process into stages, we can increase the constancy of the biomass in the system and improve the utilization efficiency of the physical facility (Wang, 2003).

1.2.2 Biofiltration

The treatment methods that are applied to treat aquaculture wastewater are broadly classifiable into physical, chemical and biological processes. Physical unit operations apply physical forces to remove contaminants. Solids removal is accomplished by sedimentation (settleable solids) or mechanical filtration (suspended and fine solids) (van Rijn, 1996). Two commonly used types of mechanical filtration in aquaculture include screen filtration and expendable granular media filtration (Twarowska et al., 1997; Franco-Nava et al., 2004a). For fine solids removal, foam fractionation - a process also referred to as air stripping or protein skimming - is often employed (Timmons, 1984; Hussenot, 2003). Chemical unit processes used for aquaculture wastewater treatment are customarily used in conjunction with physical unit operations and biological processes. The inherent disadvantage of most chemical unit processes is that they are additive processes; the chemicals tend to stay for a major part in the water. This is a significant factor if the wastewater is to be reused. The main chemical unit process used in aquaculture is disinfection by means of ozonation at a cost of 0.20-0.24 € m^{-3} water (Summerfelt, 2003). Disinfection by UV irradiation (0.045-0.11 € m^{-3} treated) is considered as a credible alternative to chemical disinfection, because of the absence of toxic by-products that are usually generated and identified during chemical disinfection (Hassen et al., 2000; http://martob.ncl.ac.uk/). These techniques avoid the addition of chemical substances that are hazardous to the cultured organism. Biological processes are the most important ones with respect to aquaculture wastewater treatment and the major biological process is nitrification. Nitrification is carried out in a variety of systems, which can be grouped into 2 general types: emerged (rotating biological contactors, trickling filters) and submerged (e.g. fluidized bed filters, bead filters) fixed film filters (van Rijn, 1996; Ling and Chen, 2005; Malone and Pfeiffer, 2006). Biological filters are used for freshwater and marine operations (Hovanec and DeLong, 1996;

Gutierrez-Wing and Malone, 2006; Malone and Pfeiffer, 2006). This part reviews recirculating systems on biofiltration technologies for freshwater systems.

Nitrification in the bacterial film of the biofilter are affected by a variety of parameters such as substrate and dissolved oxygen concentrations, organic matter, temperature, pH, alkalinity, salinity and turbulence level (Satoh et al., 2000; Chen et al., 2006). Nitrifying bacteria are sensitive organisms and are susceptible to high concentrations of ammonia and nitrous acid, low dissolved oxygen levels (< 1 mg/l) and pH outside the optimal range (7.5-8.6) (Masser et al., 1999; Villaverde et al., 2000; Ling and Chen, 2005). Nitrification, and especially the second step ($NO_2^- \rightarrow NO_3^-$), is quite sensitive to even traces of sulphides (Joye and Hollibaugh, 1995). Sulphides are present in sediments and in sludges accumulated in intensive aquaculture systems. For higher C/N ratios, the heterotrophic bacteria out-compete nitrifiers for available oxygen and space in the biofilters (Michaud et al., 2006). Hence, nitrification necessitates a low C/N ratio. Figure 1.3 illustrates the N cycle in aquaculture systems equipped with an external biofilter.

Rotating biological contactors have been used in the treatment of domestic wastewater. For decades they are now widely used as nitrifying filters in aquaculture applications. Rotating biological contactor technology is based on the rotation of a submerged substrate, which is made of high-density polystyrene or polyvinyl chloride, attached to a shaft (Tawfik et al., 2004; Park et al., 2005; Brazil, 2006). Nitrifying bacteria grow on the media and because of the rotation they are alternately contacting nitrogen rich water and air. As the rotating biological contactor rotates, it exchanges carbon dioxide, generated by the bacteria, with oxygen from the air. In general, rotating biological contactor systems are divided into a series of independent stages or compartments (Lavens and Sorgeloos, 1984; Brazil, 2006). Compartmentalization creates a plug-flow pattern, increasing overall removal efficiency. It also promotes a variety of conditions where different organisms can flourish to varying degrees. As the water flows through the compartments, each subsequent stage receives influent with a lower organic content than the previous stage; the system thus enhances organic removal (UN, 2003; Watten and Sibrell, 2006). Complimentary, the rotating biological contactor has low head requirements to move water through the vessel. This advantage implies passive aeration and carbon dioxide removal, and low chance of clogging (Brazil, 2006).

Miller and Libey (1985) demonstrated that a rotating biological contactor provided better TAN areal removal rates, in the range of 0.19-0.79 g TAN/m^2.day, than a packed tower or fluidized bed reactor (0.24 g TAN/m^2.day), when treating the same fish culture water at comparable hydraulic loadings. Brazil (2006) described the performance and operation of a rotating biological contactor in a tilapia recirculating aquaculture system. The system obtained an average TAN areal removal rate of about 0.42 g/m^2.day.

Increasing influent dissolved organic carbon levels decreased ammonia removal efficiency. However, there was no detectable relationship between the feed loading rate and ammonia oxidation performance. In addition to organic loading rate, mass and hydraulic loading rate, rotational speed and staging affected the ammonia oxidation performance.

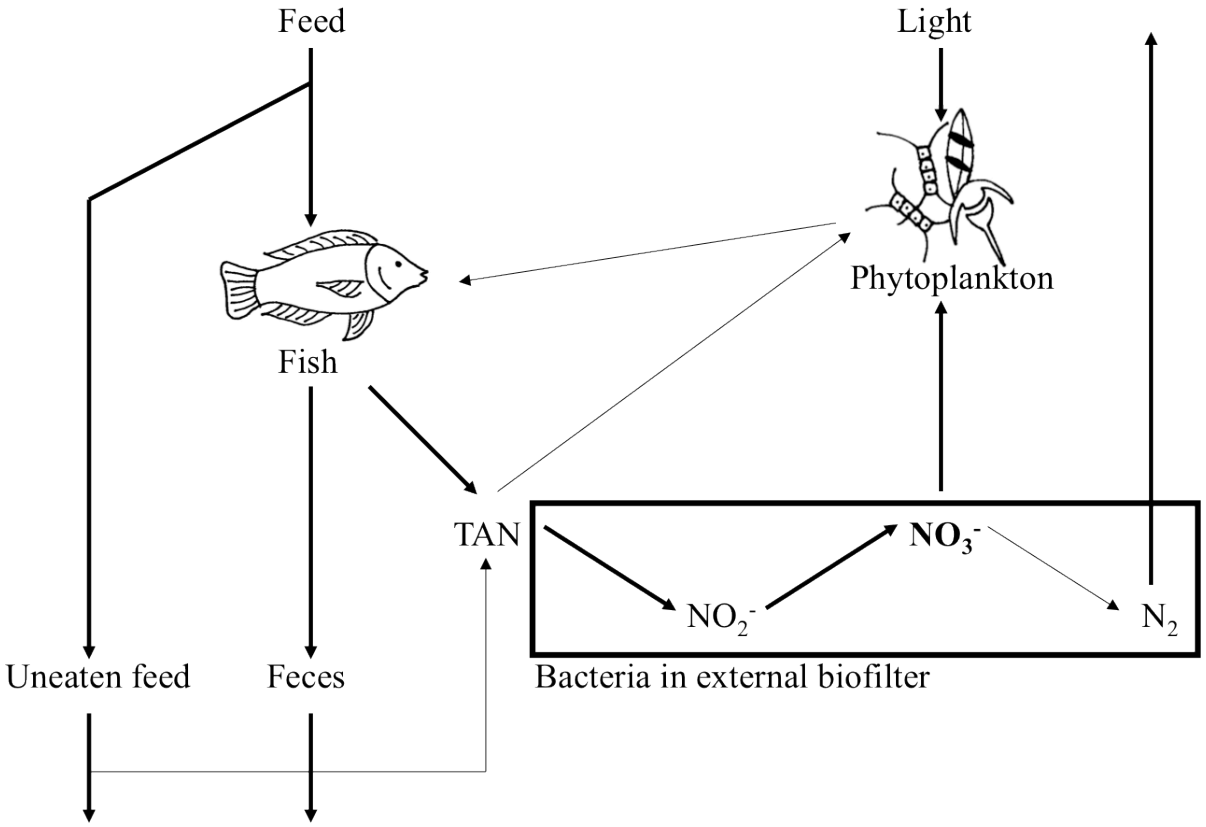


Figure 1.3 Nitrogen cycle in aquaculture systems equipped with an external biofilter. The nitrogen cycle is similar to that in water ponds with a long hydraulic residence time, but now the water rich in TAN is drained to an external biofilter. In this biofilter, nitrification is enhanced and through nitrite, nitrate is formed out of TAN. Nitrate is less toxic to fish than is TAN or nitrite (Meade, 1985; Lyssenko and Wheaton, 2006). Although such a system avoids TAN to accumulate, nitrate build up may take place. The biofilter creates space and optimal conditions for nitrifying bacteria to grow.

Trickling filters consist of a fixed medium bed through which aquaculture wastewater flows downwards over a thin aerobic biofilm (Eding et al., 2006). As it trickles down, the water is continuously oxygenated, while the carbon dioxide is degassed and removed by the ventilated air. Trickling medium has a specific surface area ranging from 100 to 1000 m^2/m^3. Finturf artificial grass (284 m^2/m^3), Kaldnes rings (500 m^2/m^3), Norton rings (220 m^2/m^3) and Leca or lightweight clay aggregate (500-1000 m^2/m^3) are some of the most frequently used media (Greiner and Timmons, 1998; Lekang and Kleppe, 2000; Timmons

et al., 2006a). The organic material present in the wastewater is adsorbed on the biological slime layer and degraded by aerobic microorganisms.

Kamstra et al. (1998) reported TAN areal removal rates between 0.24 and 0.55 g TAN/m^2.day for a commercial-scale trickling filter. For three different applied filter medium types in commercial farms and a range of hydraulic surface loading conditions, the highest observed TAN areal removal rate for a trickling filter was 1.1 g TAN/m^2.day, with an average TAN areal removal rate of about 0.16 g TAN/m^2.day (Schnel et al., 2002; Eding et al., 2006). Lyssenko and Wheaton (2006) reported TAN areal removal rates of 0.64 g TAN/m^2.day. In the same study they found similar TAN areal removal rates for a submerged expandable up flow sand filter.

Down flow microbead filters are combinations of trickling filters and granular type biological filters (Timmons et al., 2006a). The use of floating media in down flow configurations has the advantage of being capable of using smaller media and the associated higher specific surface areas. As the recirculating water passes through the packed bed, suspended solids are captured and biofiltration processes are active (Malone and Beecher, 2000). The configuration offers the added advantage of using high hydraulic loadings without the need for sophisticated mechanical structures in the reactor to retain the media within the reactor vessel (Greiner and Timmons, 1998). The medium consists of polystyrene beads that are 1-3 mm in diameter and have a porosity of 36-40% (Timmons et al., 2006a). Depending on these features the specific surface area ranges from 1150 to 3936 m^2/m^3 (Greiner and Timmons, 1998; Malone and Beecher, 2000; Timmons et al., 2006a).

Greiner and Timmons (1998) observed TAN specific surface removal rates of about 0.45-0.60 g/m^2.day. A study using a commercial microbead filter system reported an average TAN specific surface removal rate of 0.30 g/m^2.day (Timmons et al., 2006a).

Fluidized sand biofilters (FSB) have been widely adopted in recirculating systems that must reliably maintain excellent water quality (Summerfelt, 2006). Filter sand has a high specific surface area, i.e. 4000-20000 m^2/m^3 and has a moderate cost (Summerfelt, 2006). A disadvantage of the FSB is that they do not aerate, as do trickling filters (Summerfelt, 2006). Therefore, additional aeration is needed. These filters also must operate within a narrow water flow range in order to maintain proper bed expansion (Summerfelt, 2006). Miller and Libey (1985) demonstrated that the TAN removal rate of a fluidized bed reactor was around 0.24 g N/m^2.day. Timmons and Summerfelt (1998) found similar rates in their research.

Table 1.1 gives an overview of the average TAN areal removal rate and the cost per kg of fish produced per year for each biofilter type. Rotating biological contactors have the highest TAN areal removal rate, followed by bead biofilters and trickling filters, and fluidized sand biofilters. Although rotating biological contactors show good performance concerning TAN removal rate, they are together with trickling filters more expensive then the other biofilter types discussed. Fluidized sand biofilters and bead

biofilters are the least expensive options for water treatment when the cost per kg of fish produced per year is considered.

Table 1.1 General overview of the average TAN areal removal rate for frequently used biofilters in aquaculture systems. Also the costs for various biofilter choices based on their capitalization cost to support a 454-ton per year tilapia farm are mentioned (data from Timmons et al. (2006b)).

Biofilter type	**Average TAN areal removal rate** (g TAN/m^2.day)	**Cost** (€/kg N removed)	**References**
Rotating biological contactor	0.19-0.79	5.4	Miller and Libey, 1985; Brazil, 2006
Trickling filter	0.24-0.64	4.9	Kamstra et al., 1998; Schnel et al., 2002; Eding et al., 2006; Lyssenko and Wheaton, 2006
Bead filter	0.06-0.35	2.4	delos Reyes and Lawson, 1996; Sastry et al., 1999
Fluidized sand biofilter	0.24	0.9	Miller and Libey, 1985; Timmons and Summerfelt, 1998

The overall cost estimated for a full-scale wastewater treatment plant is 3.0 – 4.0 €/kg N removed for nitrification/denitrification application (Fux and Siegrist, 2004). They are, considering that the costs in Table 1.1 only represent the nitrification application, within the same range as for aquaculture water treatment cost estimates.

1.3 N removal within the culture unit

The three nitrogen conversion pathways naturally present for the removal of ammonia-nitrogen in aquaculture systems are photoautotrophic removal by algae, autotrophic bacterial conversion of ammonia-nitrogen to nitrate-nitrogen, and heterotrophic bacterial conversion of ammonia-nitrogen directly to microbial biomass (Ebeling et al., 2006).

Developing and controlling dense heterotrophic microbial flocs in the water column or attached microorganisms called periphyton can accelerate the biological removal of organic and inorganic wastes in ponds (Avnimelech, 2005; Azim et al., 2003a; Azim et al., 2003c). These processes are integral parts of the culture unit (Hargreaves, 2006). An important advantage is that microbial bioflocs and periphyton can be consumed and used as a source of feed by the cultivated or other aquatic organisms (Burford et al., 2003; Hari et al., 2004; Burford et al., 2004; Azim and Wahab, 2005; Keshavanath and Gangadhar, 2005). As explained in the following paragraphs, both approaches are possible solutions for water quality problems, and can decrease the use of fish oil and fishmeal utilization in aquaculture.

1.3.1 The periphyton treatment technique

The periphyton community consists of attached aquatic biota on submerged matrices. It harbours algae, bacteria, fungi, protozoa, zooplankton and other invertebrates (Azim et al., 2005). As with phytoplankton, periphyton can be found in almost every type of water body from small ponds to large oceans and in trophic conditions that range from the most oligotrophic to the most eutrophic (Azim and Asaeda, 2005). Given adequate light, up to about 0.5-meter depth in the water, high rates of photosynthesis and autotrophic production can be achieved (Craggs et al., 1996; Vermaat, 2005). Values for periphyton productivity are typically in the range of 1-3 g C/m^2 substrate.day or 2-6 g dry matter/m^2.day (Azim et al., 2005). Periphyton entraps organic detritus, removes nutrients from the water column and helps controlling the dissolved oxygen concentration and the pH of the surrounding water (Azim et al., 2002; Dodds, 2003; Bender et al., 2004).

Supplying surface to attach on, further called substrate, improves the nitrogen-related processes developing in the water column and the nitrogen flow is mainly linked to autotrophic and heterotrophic activity that takes place in the periphyton (Figure 1.4) (Milstein, 2005). The beneficial influence of periphyton on the water quality in different aquaculture systems has been investigated, as well as the impact of grazing by fish on periphyton communities (Huchette et al., 2000; Azim et al., 2001; Azim et al., 2002; Azim et al., 2003a; Azim et al., 2003b; Azim et al., 2003c; Azim et al., 2004). Not all fish are able to graze on periphyton; morphological and physiological adaptations to periphyton grazing are required (Azim et al., 2005). Although direct experimental evidence is scarce, the aquaculture fish species that can effectively utilize the periphyton assemblage are probably more numerous than those that are exclusively phytoplanktivorous (van Dam and Verdegem, 2005). Besides specialist

(macro)herbivores, more general detritus and benthos feeders can also thrive on periphyton (van Dam et al., 2002).

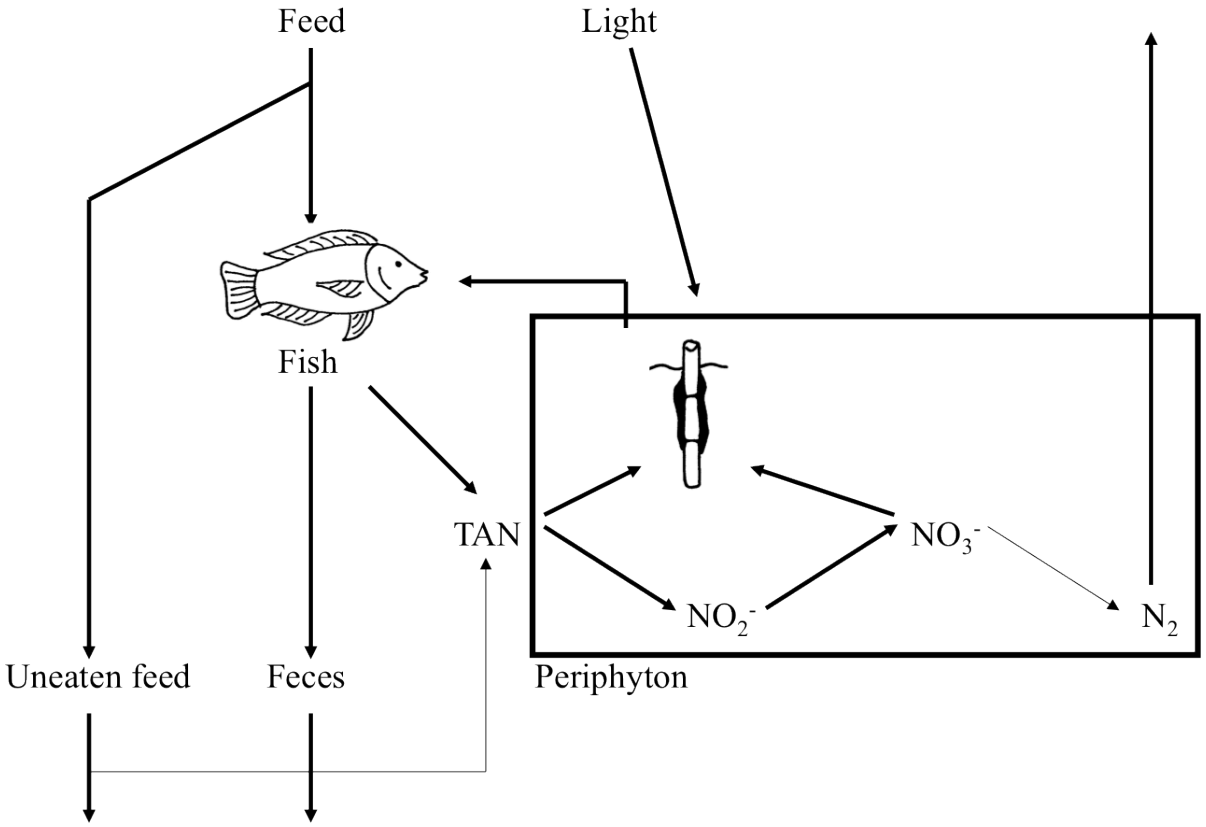


Figure 1.4. Nitrogen cycle in extensive aquaculture ponds with substrate for periphyton growth. The nitrogen cycle is similar to that in water ponds with a long hydraulic residence time, but now TAN concentration does not build up in the water column, neither is the nitrate concentration. The periphyton community takes up both TAN and nitrate and edible biomass is formed. The cultured fish can graze on the periphyton community and hence nitrogen, originating from wasted feed and excretion by fish, is redirected towards the cultured organism and therefore, this technique enhances the overall efficiency of nutrient conversion of feed.

Periphyton has an average C/N ratio of 10 (Azim and Asaeda, 2005). Its assimilation capacity is around 0.2 g N/m^2.day. From this it is clear that one needs a large surface, which allows periphyton growth, to treat intensive aquaculture wastewater without compromising the water quality. Besides N removal, biomass is formed. The yield is around 4 g dry matter/m^2.day and the protein content of periphyton is around 25% of the dry matter (Azim et al., 2002; Azim et al., 2005). This corresponds to a particular feed quantity that can diminish the overall feed cost.
Besides the large area needed, the problem with this system is that the process is completely dependent on the availability of sunlight (Azim and Asaeda, 2005). On cloudy days or on days with insufficient sunlight, the maximum nitrogen uptake rate will not

be reached. Another problem is the laborious task to harvest the periphyton. One can conclude that application of the periphyton treatment technique in the intensive aquaculture sector is not cost effective in many cases. Nevertheless, the technique of using this natural feed may be significant, particularly in smaller, extensive-level aquaculture systems in developing countries. The addition of the 'periphyton loop' in aquaculture ponds can be accomplished by adding static substratum to the pond (Azim et al., 2005), such as poles horizontally planted in the ponds. Substratums used are bamboo, hizol and kanchi (Azim et al., 2002; Azim et al., 2003c). Since periphyton can be easily cultured in modified fishponds and demands little management, the benefits may be substantial.

1.3.2 Bioflocs technology

If carbon and nitrogen are well balanced in the solution, ammonium in addition to organic nitrogenous waste will be converted into bacterial biomass (Schneider et al., 2005). By adding carbohydrates to the pond, heterotrophic bacterial growth is stimulated and nitrogen uptake through the production of microbial proteins takes place (Avnimelech, 1999). The technique of enhancing water quality through the addition of extra carbon to the pond, through an external carbon source or elevated carbon content of the feed, is called the bioflocs technology. This promoted nitrogen uptake by bacterial growth decreases the ammonium concentration more rapidly than nitrification (Hargreaves, 2006). Immobilization of ammonium by heterotrophic bacteria occurs much more rapidly because the growth rate and microbial biomass yield per unit substrate of heterotrophs is a factor 10 higher than that of nitrifying bacteria (Hargreaves, 2006). The microbial biomass yield per unit substrate of heterotrophic bacteria is about 0.5 g biomass C/g substrate C used (Eding et al., 2006).

Suspended growth in ponds consists of phytoplankton, bacteria, aggregates of living and dead particulate organic matter, and grazers of the bacteria (Figure 1.5) (Hargreaves, 2006). Typical flocs are irregular by shape, have a broad distribution of particle size, are fine, easily compressible, highly porous (up to more than 99% porosity) and are permeable to fluids (Chu and Lee, 2004).

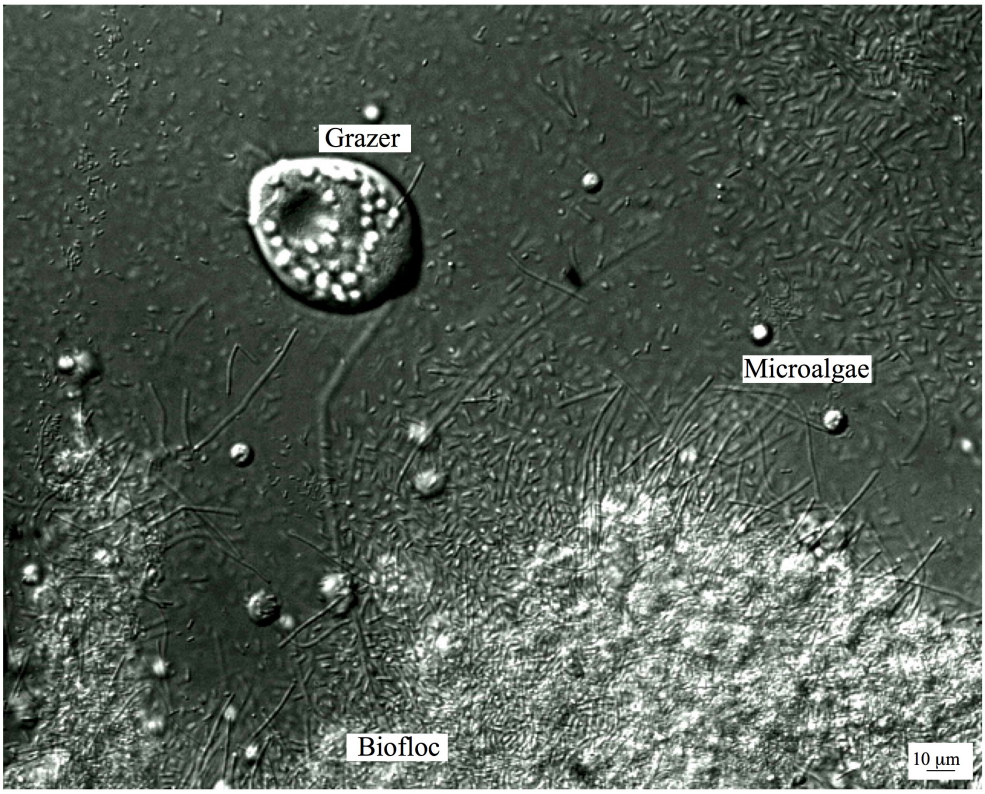


Figure 1.5. Amorphous aggregate, which consists of phytoplankton, bacteria, aggregates of living and dead particulate organic matter, and grazers. The flocs were examined with light microscopy, and digital images were captured with a 1-CCD camera (Crab, Unpublished data).

1.4 The basics of bioflocs technology

1.4.1 Selective forces for bacteria to live in floc structures

Individual bacterial cells are sized in the order of 1 μm (Madigan and Martinko, 2006). This implies that these organisms are in general surrounded by a layer of liquid that hampers the mass transfer of nutrients and waste products (Logan and Hunt, 1988). Calculation of the Reynolds number (Re = a dimensionless parameter that indicates whether a fluid flow in a particular situation will be laminar or turbulent) for bacterial cells, even for free swimming ones, will result in a value far below 2300 which is the upper limit for laminar flow. Indeed, a bacterium of 1 μm diameter (length or L) that is moving in a water column (20°C, viscosity $\mu = 1.002 \times 10^{-3}$ N/s.m^2, density $\rho = 999.86$ g/L) at speed of 1000 μm/s (Vs) (Mitchell and Kogure, 2006) results in a Reynolds number of

1.10^{-3}. Under such conditions, the viscosity of water dampens fluctuations smaller than the so called viscous length Lv, which is in the order of 1.0-6.0 mm. Below this dimension, the turbulence of the water is not important anymore for the substrate flux to a bacterial cell (Schulz and Jorgensen, 2001). In other words a laminar regime (also called diffusion sphere or Reynolds envelope), always present around bacteria or bacterial aggregate smaller than 100 μm, interferes with nutrient mass transfer as they move through the water column. This may result in mass transfer limitations when the rate of substrate consumption exceeds the rate of substrate supply (Simoni et al., 2001).

In natural environments, microorganisms tend to form amorphous aggregates. The settling velocity of these flocs appears not to relate to the square of the size, as expected from Stokes' law (Logan and Hunt, 1987; Logan and Hunt, 1988). If an aggregate is highly porous, fluid streamlines will penetrate the aggregate resulting in advective flow through it. This will improve the supply of nutrients to the cells present in the aggregate and will decrease the settling velocity of the flocs in the pond.
Using the relative uptake factor γ, defined as growth rate of aggregated cells/growth rate of free cells, one can make a comparison of the substrate uptake by aggregated versus dispersed cells. Figure 1.6 depicts the relative uptake predictions for microbial cells in permeable flocs (Logan and Hunt, 1988). The power input to the fluid originates from the aeration of the ponds. Different aeration techniques are available, such as diffuser aeration, mechanical aeration and packed column aeration. For turbulent fluids, the mean shear rate G is determined from the power input to the fluid per unit volume of the fluid. In intensive aquaculture systems the average power input to the fluid is around 1-10 W/m^3 = 10^1-10^2 cm^2/s^3 or 10/s < G < 100/s (Boyd, 1998; McGraw et al., 2001; Schuur, 2003). At these moderate mixing rates, cells growing in permeable aggregates can profit from advective flow and grow better than single dispersed cells (γ > 1). One can calculate that the relative growth rate of aggregated cells in this energy regime is greater than the growth rate of free cells (Logan and Hunt, 1987; Logan and Hunt, 1988). When more intense aeration is applied, the advantage of growing in flocs disappears and cells growing solely show higher growth.

Besides mass transfer, protection against protistan grazing as a biological stressor can drive bacteria to grow in flocs. It is postulated that grazing by protozoa (unicellular eukaryotic microorganisms sized 2.0-2000 μm) is one of the major causes of bacteria removal in soil, freshwater and marine ecosystems (Matz and Kjelleberg, 2005). In this respect, the aggregated way of life can be beneficial. By organizing themselves into aggregates, cells may become less susceptible to predation by protozoa (Young, 2006). This was shown in studies that revealed a shift towards smaller cells and a grouping of these into large multicellular flocs upon predation of a microbial community by mesobiota (Hahn and Hofle, 1999).

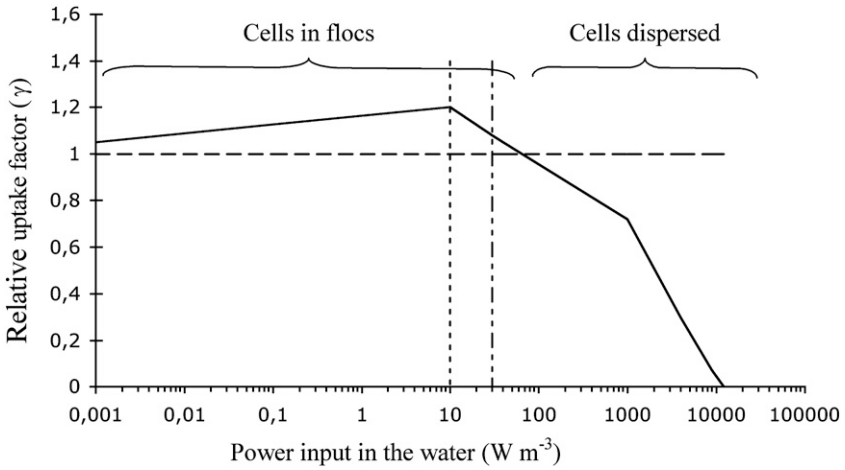


Figure 1.6. Predicted relative uptake for microbial cells within permeable flocs (after Logan and Hunt, 1988). A shear rate of 10 W/m^3 represents the mixing of the see; a shear rate of about 30 W/m^3 represents the mixing in aerated activated sludge and a shear rate of 0.1-10 W/m^3 corresponds to the mixing in most aquaculture systems.

The bacterial defence mechanisms against predation are diverse. Changes in bacterial size and shape to become over- and undersized, the exertion of high motility (swimming speeds of more than 30 μm/s that can considerably decrease capture (Matz and Jurgens, 2005)) or the attachment to surfaces that enhance survival have been reported (Young, 2006). A strategy evident for bacterioplankton communities against protozoan grazing is the grouping into large aggregates or flocs. Experimental field studies have shown that within 1-2 days after enhancing protistan grazing, the bacterial community shifted from small- and medium-sized single cells into communities dominated by filamentous and aggregated bacteria (Hahn and Hofle, 2001). By sticking together in such microcolonies, the group of bacteria reaches a size too large to be considered as a prey for the mesobiota. Only the organisms in the outer layers are susceptible to predation by grasping feeders (Matz and Kjelleberg, 2005).

1.4.2 Mechanisms of binding microbial cells into flocs

The flocculation of microbial communities is a complex process. Within the floc's matrix, a combination of physical, chemical and biological phenomena is operating. The exact mechanisms and the methods to engineer microbiological flocs remain largely unknown. The main constituents that can be found within the floc matrix are the extracellular polymeric substances. These structures form a matrix that encapsulates the microbial cells, and play a major role in binding the floc components together. The

presence of these structures in activated sludge systems can be substantial, up to 80% of the total mass (Hantula and Bamford, 1991; Liu and Fang, 2003). They are typically made up of polysaccharides, proteins, humic compounds, nucleic acids and lipids (Zita and Hermansson, 1994). They are produced as slime or capsule layers under various nutritional conditions but particularly in case of limitation by nutrients such as e.g. nitrogen (Steiner et al., 1976).

1.4.2.1 Surface interactions influenced by physicochemical parameters

The surface of a bacterium surrounded by polymeric compounds is in general negatively charged (Zita and Hermansson, 1994). The nature of these surface structures helps to determine the zeta-potential (Liu and Fang, 2003) that is an electrical potential generated by accumulation of ions from the surroundings at the bacterial surface (Sobeck and Higgins, 2002). The negative charge of flocs lies within the range of -0.2 to -0.6 meq/g volatile suspended solids (VSS) with a zeta-potential of -20 to -30 mV (Liu and Fang, 2003). The layer of oppositely charged counter-ions that is rather tightly fixed to the surface is the so-called Stern layer. Outside this layer a group of ions forms a cloud-like structure, the diffuse layer, which is electrically neutral (Hermansson, 1999) (Figure 1.7). Starting close at the surface and going to the outside, the potential of the particle gradually drops until it becomes the value of the surrounding bulk (in generally taken to be zero). When such a particle moves through a liquid medium, the fixed layer and part of the diffuse layer move along. However, some of the charges from the diffuse layer are lost resulting in a new edge of the particle, the shear plane, at zeta-potential. Due to equal surface charges, particles are repelled from each other and are kept in dispersion.

However, the latter is countered by Van der Waals forces. These are forces resulting from polarization of molecules into dipoles and inducing an attractive power between particles possibly resulting in aggregation. Whether or not bacteria will group themselves into flocs will thus depend on both the zeta-potential and the Van der Waals forces (Sobeck and Higgins, 2002). If the zeta-potential is substantial and thus the repelling surface charge of the particle is likely to be larger than the attractive Van der Waals forces, the bacteria will stay in dispersion and will not aggregate. In the opposite case of low zeta-potential or low surface charge, the Van der Waals forces will dominate and bacterial floc formation is likely to occur (Zita and Hermansson, 1994). The interaction of charged surfaces through a liquid is also known as the DLVO-theory, named after its developers Derjaguin, Landau, Verwey and Overbeek (Hermansson, 1999). An influencing factor regarding the DLVO theory can be deducted from the proton translocation-dehydration theory (Tay et al., 2000; Teo et al. 2000). During transport of electrons in the bacterial respiration chain, protons are actively pumped

out of the membranes. This ruptures the hydrogen bonds between the water molecules adhered to the cell and the negatively charged cell surface, and results in dehydration of the cell surface. In addition, the protonation of the cell surface neutralizes part of the cell negative charge. This results in an increased hydrophobicity of the cell surface, which has been shown to result in an increased adhesion strength (Van Loosdrecht et al., 1987). It seems reasonable to assume that the bacterial proton translocation activity plays a role in the initiation of microbial aggregation.

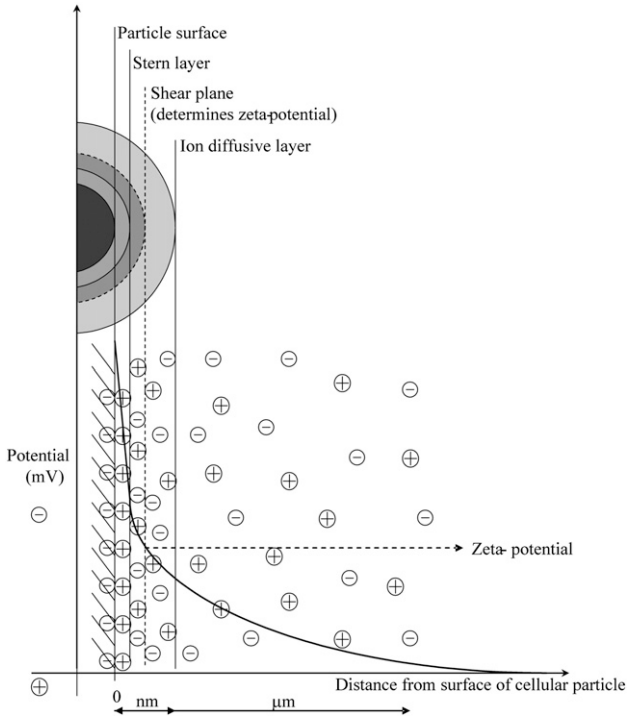


Figure 1.7. Schematic view of a charged cellular particle with its counter charges and the potential in the area of a particle surface (after Hermansson, 1999).

The divalent cation bridging theory states that divalent cations, mainly Ca^{2+}, bridge negatively charged functional groups within the bacterial surface structures (Higgins and Novak, 1997). Keiding and Nielsen (1997) stated for activated sludge that the "cloud" of surface structures comprises humic substances as major (adsorbed) compound. In BFT, the cells will be younger than those in activated sludge systems (where the residence time is ca. 20 days), thus comprising less adsorbed matter. Their extracellular polymeric composition is however also depending on sludge residence time (Sanin et al., 2006). Characterization of extracted extracellular polymeric substances from sludge flocs revealed that part of the polysaccharides are made up out of uronic acids having a carboxyl-group located at the fifth carbon. At neutral pH values, these carboxyl-groups are unprotonated. Also the protein is rich in carboxyl-group containing amino acids that will contribute to the negative charge as well (Sobeck and Higgins, 2002).

For cells which are not carrying any electrical charge or are living at high ionic strengths (≥ 0.1M), it can be assumed that the binding of microorganisms mainly is the result of steric interactions (comparable to the Velcro concept or the hydrogen bounding between two single DNA strands) as was observed by the interactions between microorganisms and substrata (Rijnaarts et al., 1999). At low ionic strengths (< 0.001M), the binding is hindered by the DLVO-theory of electrostatic repulsion. In case aggregation does occur, this is postulated to be due to extracellular polymers that make distance bonds between equally charged surfaces to counteract repulsion (Burdman et al., 2000). The latter interaction has been shown on an experimental basis. Blocking of extracellular polysaccharide synthesis resulted in a decrease of the microbial adhesion (Cammarota and Sant'Anna, 1998).

Since bioflocculation is based on the previously mentioned mechanisms, it may possibly be steered to a certain degree within the aquaculture ponds by means of the ionic strength in the environment. The balance between repulsive and attractive forces working within flocs depends on the electrolyte concentration (Zita and Hermansson, 1994). The influence of divalent cations on flocculation is positive. This can e.g. be ascribed to a decrease of the diffuse double layer (DLVO-theory). Even small changes in the ionic strength and ion composition of the water can have substantial influence on the structural properties of flocs. Particularly Ca^{2+} has been shown to be a significant factor in floc formation (Keiding and Nielsen, 1997). In addition, the presence of calcium ions also seems beneficial in the protection of fish species against heavy metal toxicity (Abdel-Tawwab et al., 2007; Wood et al., 2006). Bioflocculation may also be steered by the choice of organic compound used as food for the bioflocs. It is postulated that the addition of high-energy carbohydrates like sucrose or glucose sustains fast acidogenic growth (Tay et al., 2000). The sooner acidogens are able to take up and metabolize the substrate; the more rapidly the proton pumps will be activated (proton translocation-dehydration theory). This may result in a faster and facilitated process of floc formation. Finally, the production of extracellular biopolymeric flocculants by bacteria, fungi, yeasts and algae can also be engineered to some extent. To promote the overall aggregation efficiency of the microbial biomass, selecting for a start-up inoculum with co-aggregative species can be of interest. Culture conditions like C/N-ratio, pH, temperature and agitation speed within the pond are important factors for the activity of these organisms (Salehizadeh and Shojasadati, 2001). A selection of the most adequate floc forming species for pond practice can be performed and the resulting improvement in the starting-up period and flocculation efficiency should be assessed.

1.4.2.2 Quorum sensing as biological control

The grouping of microorganisms may be controlled by cell-to-cell interaction called quorum sensing. Quorum sensing is the regulation of gene expression programs (Spoering and Gilmore, 2006) and a way of cell-to-cell communication between bacteria thought to be depending on cell density (Lazazzera, 2000). It is known to regulate the expression of genes encoding for the production of lytic enzymes and toxins in biofilms (Cosson et al., 2002; Defoirdt et al., 2004). By secreting and detecting small, signalling molecules (*N*-acyl-homoserine lactones or AHL's in case of Gram-negative bacteria and peptides in case of Gram-positive bacteria) that accumulate in the surrounding environment, bacteria can induce a certain response when a signalling molecule threshold concentration level is reached (Miller and Bassler, 2001). It has been shown that a wild type *Pseudomonas aeruginosa* biofilm was not subject to flagellate grazing, whereas grazing of its quorum sensing deficient mutants *P. aeruginosa rhIR/lasR* could not be avoided (Matz et al., 2004). This indicates that a quorum sensing-dependent mechanism may be involved in the protection of bacterial biofilms and microcolonies (Queck et al., 2006). Quorum sensing has been shown to be active in biofilms (Kjelleberg and Molin, 2002) and because of the similar bacterial cell density in flocs, it can reasonably be expected to be also active in flocs. In addition, Valle et al. (2004) and Morgan-Sagastume et al. (2005) reported AHL production in different strains isolated from activated sludge flocs. Until now, the influence of quorum sensing on biofilms, and thus probably also on bioflocs, has mainly been shown to result in a differentiation of existing aggregated structures (Liu et al., 2006; Stanley and Lazazzera, 2004). It appears that the microcolony formation, as it occurs in biofilms, induces an activation of the quorum sensing mechanisms and finally results in a differentiated biofilm. This was shown in biofilm experiments with *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Lynch et al., 2002; Shrout et al., 2006). A clear relationship such as e.g. the excretion of signalling molecules by microorganisms under starvation circumstances resulting in flocculation has not yet been shown. Only one paper describes such a possible interaction (Johnson et al., 2005). Co-cultivation of *Thermotoga maritima* and *Methanococcus jannaschii* induced increased flocculation compared to a *Thermotoga maritima* monoculture. This could be related to an increased activity of the genes encoding for the production of polypeptide signaling molecules known to induce extracellular polymeric substance production. It seems that cellular communication in this case can be considered as a significant component in the microbial interaction for aggregation. Consistent with this, Eboigbodin et al. (2006) showed that quorum sensing affects bacterial cell surface electrokinetic properties. It was hypothesized that this was due to changes in the composition or presence of functional groups in the outer membrane macromolecules.

It is possible that quorum sensing mechanisms are at hand in flocs. The molecular and biochemical mechanisms involved in quorum sensing dependent biofilm production remain far from known and comprise an interesting line of exploration. It is certain that aggregation is the net result of many independent interactions in which the quorum sensing system can play a role (Kjelleberg and Molin, 2002). Since the understanding of the quorum sensing mechanisms for microorganisms is far from complete, it is difficult for use in the control of BFT. However, some interesting application prospective and related research certainly exists. E.g. the seeding of quorum sensing species within the ponds may allow them to integrate in the flocs and thus improve floc formation. Alternatively, the disruption of cell-to-cell communication in flocs e.g. may possibly be used as biocontrol effect. Many pathogens in aquaculture have been found to control virulence factor expression by quorum sensing. Inactivation or degradation of the signaling molecules or the use of antagonistic molecules can possibly be developed (Defoirdt et al., 2004). In both cases, considerable research efforts have to be performed to gain insight and understanding of the phenomena before practical applications come into perspective.

1.4.3 Factors influencing floc formation and floc structure in bioflocs technology

The knowledge on how to promote floc formation in activated sludge systems can be used for application in BFT. Yet, the parameters listed in Table 1.2 may need adjustment to obtain good aggregation and high quality of the bioflocs together with optimal growth conditions for the aquaculture organisms. In the next paragraph, the application of these parameters in BFT aquaculture is discussed. Since most of them are strongly interrelated, in many cases it is not easy to predict a certain outcome due to changing parameters. As far as known, no research has been performed on the relation between the operation parameters discussed below and the functioning of the BFT systems or bioflocs quality. Therefore, the following can be seen as an overview of possible research topics within the BFT aquaculture.

1.4.3.1 Mixing intensity

The mixing intensity imposed by a chosen aeration device at a certain power input will determine the steady-state floc size, this is the equilibrium between the rate of aggregation and the rate of breakage, and the floc size distribution (Chaignon et al., 2002; Spicer and Pratsinis, 1996). In aquaculture, energy dissipation in general is in the

range of 1 – 10 W/m^3 (Boyd, 1998). However, in highly intensive systems, more realistic values can reach up to 100 W/m^3. At higher mixing intensities and thus higher shear rates, the average floc size decreases due to increased floc breakage. Biggs and Lant (2000) showed in case of activated sludge that for a mean shear rate or G-value of 19.4/s (~ 0.4 W/m^3), the stable floc-size was ca. 130 μm whereas this was decreased to ca. 20 μm for a velocity gradient of 346/s (~ 120 W/m^3). The relationship between floc size and mixing intensity has been represented by Parker et al. (1972) with the power law relationship $d = C.G^{-x}$, where d is the maximum stable floc size, G is the average velocity gradient, C is the floc strength component and *x* is the stable floc size component.

Table 1.2 Overview of the main operational parameters for bioflocs technology based aquaculture, the floc parameters they influence and how these can be manipulated.

Parameter	Floc parameters influenced	Manipulation possibilities	Related to
Mixing intensity/shear rate	- Floc structure & final floc size	- Choice of power input (W/m^3) - Aeration device	- Dissolved oxygen
Organic carbon source (e.g. glucose, acetate, starch, glycerol)	- Chemical floc composition (fatty acids, lipids, protein, polyhydroxyalkanoates) - Microbial floc composition (filamentous vs. floc forming bacteria)	- Type of organic carbon source	- Organic loading rate - Dissolved oxygen
Organic loading rate	- Chemical floc composition (fatty acids, lipids, protein, polyhydroxyalkanoates) - Microbial floc composition (filamentous vs. floc forming bacteria)	- Feeding strategy (continuous feeding/regular interval feeding)	- Dissolved oxygen
Dissolved oxygen	- Microbial floc composition (filamentous vs floc forming bacteria) - Floc structure & floc volume index	- Choice of power input (W/m^3) - Aeration device - Floc production in the ponds vs in external unit	- Mixing intensity - Organic carbon source - Organic loading rate
Temperature	- Floc structure & activity	- Addition of heat	- Dissolved oxygen
pH/ionics	- Stability of the flocs	- Addition of acid/base, mono- or polyvalent ions	- Alkalinity - Conductivity

For BFT, the steady-state floc size is an important feature as it has already been shown that the quality of food for different aquaculture species is also dependent on the food size (Garatun-Tjeldsto et al., 2006; Knights, 1983). In order to represent a nutrition source, the food particle size in case of e.g. cod larvae and *Macrobrachium rosenbergii* larvae should be within the range of 250 – 1200 μm (de Barros and Valenti, 2003).

1.4.3.2 Dissolved oxygen

A change in mixing intensity, by alternative aeration device or power input, will directly influence the dissolved oxygen (DO) concentration in the water. The DO level is not only essential for the metabolic activity of cells within aerobic flocs but it is also thought to influence floc structure. A trend towards larger and more compact flocs at higher DO concentrations was noted by Wilen and Balmer (1999), although no clear relation could be found with average floc diameter. Poorer settling properties, a sludge volume index (SVI) of on average 250 mL/g, occurred at low DO values (0.5 – 2.0 mg/L) compared to settling at higher DO values (2.0 – 5.0 mg/L) where the SVI was ca. 100 mL/g. This can be ascribed to the presence of a higher amount of filamentous bacteria compared to the zoogloeal bacteria at DO-levels of less than or equal to 1.1 mg O_2/L as was observed by Martins et al. (2003). As filaments have a higher affinity towards oxygen, they are able to outcompete their zoogloeal counterparts at periods of oxygen limitation and thus dominate the microbial flocs (Martins et al., 2003). From the previous, it can be expected that bioflocs with a higher floc volume index or FVI (mL/g) are produced at lower DO-levels in the bioflocs ponds. We suggest, although experimental values are lacking, that the FVI should be higher than 200 mL/g to avoid the flocs from sedimenting too fast in regions of lower turbulence. This gives the aquaculture organisms enough opportunity to filter the flocs from suspension before they sediment to the bottom of the ponds and are lost as food. Negative impacts of a higher FVI however, like e.g. possible clogging of fish gills, have to be taken into account as well. In addition, the growth characteristics and stress resistance of aquaculture crop species largely depends on the amount of dissolved oxygen available in the water (Colt, 2006; Huntingford et al., 2006). For instance, exposing channel catfish to periodic oxygen levels of less than 1.5 mg/L results in a decrease of food consumption by the fish, a lower average body weight and a decreased net production (Torrans, 2005).

1.4.3.3 Organic carbon source

The dosing of an organic carbon source to the culture water in bioflocs ponds induces a decrease in dissolved oxygen levels due to aerobic microbial metabolism. This may

induce (sub)lethal effects on sensitive culture species (Landman et al., 2005). In such cases, it can be advised to grow the heterotrophic biomass in external biofloc reactors rather than within the culture unit itself. The externally grown flocs can be redirected to the pond as food but without inducing stress through varying DO levels. The organic carbon can be supplied either as additional organic carbon source (e.g. glucose, acetate, glycerol) or by changing the feed composition thus increasing its organic carbon content (Avnimelech, 1999). It is possible to theoretically calculate the amount of organic matter needed for an intensive pond, based on the amount of nitrogen excreted by the aquaculture species (Figure 1.8). The organic carbon source of choice will to a large degree determine the composition of the flocs produced, this mainly regarding the type and amount of storage polymers (Hollender et al., 2002; Oehmen et al., 2004). It was observed e.g. that the dosing of acetate in a sequence batch reactor (SBR) resulted mainly in poly-β-hydroxybutyrate (PHB) as storage polymer while these were 3-hydroxy-2-methylvalerate and polyhydroxyvalerate in case of propionate dosing (Yagci et al., 2007). Also, the costs of the different organic carbon sources will be a determining choice factor (Salehizadeh and Van Loosdrecht, 2004). The road to go for BFT is the use of organic carbon sources that are considered low-value products in other processing units as e.g. glycerol, which is a by-product from bio-diesel production (Dube et al., 2007).

1.4.3.4 Organic loading rate

The organic loading rate at which the organic carbon source is dosed in the water is a major process technical factor. Filamentous bacteria have an advantage over non-filamentous bacteria at low substrate levels due to their higher surface-to-volume ratio. Moreover, the filaments can penetrate outside the flocs and thus are exposed to higher substrate concentrations than the non-filamentous bacteria that mainly grow within the flocs (Martins et al., 2003). The organic carbon feeding strategy can also be important for BFT. The organic carbon can be added in small amounts and thus almost continuous mode or be added in larger doses but at regular time intervals (e.g. 1/d). The second type of application is also known as a feast and famine regime (Salehizadeh and Van Loosdrecht, 2004) and results in transient conditions of substrate availability. The microbial biomass stores cellular reserves like PHB under conditions of excess nutrient availability with which the microorganisms can bridge the periods of nutrient shortage. As described further, the storage products may be of high importance to the added value that bioflocs bring to aquaculture. As such, it may not be advisable to apply the organic carbon sources in continuous mode if the goal is to produce reserve materials.

The parameters described above can all be adjusted in the aquaculture systems. Two other parameters, more specifically temperature and pH, are also known to influence floc characteristics but are more difficult to change.

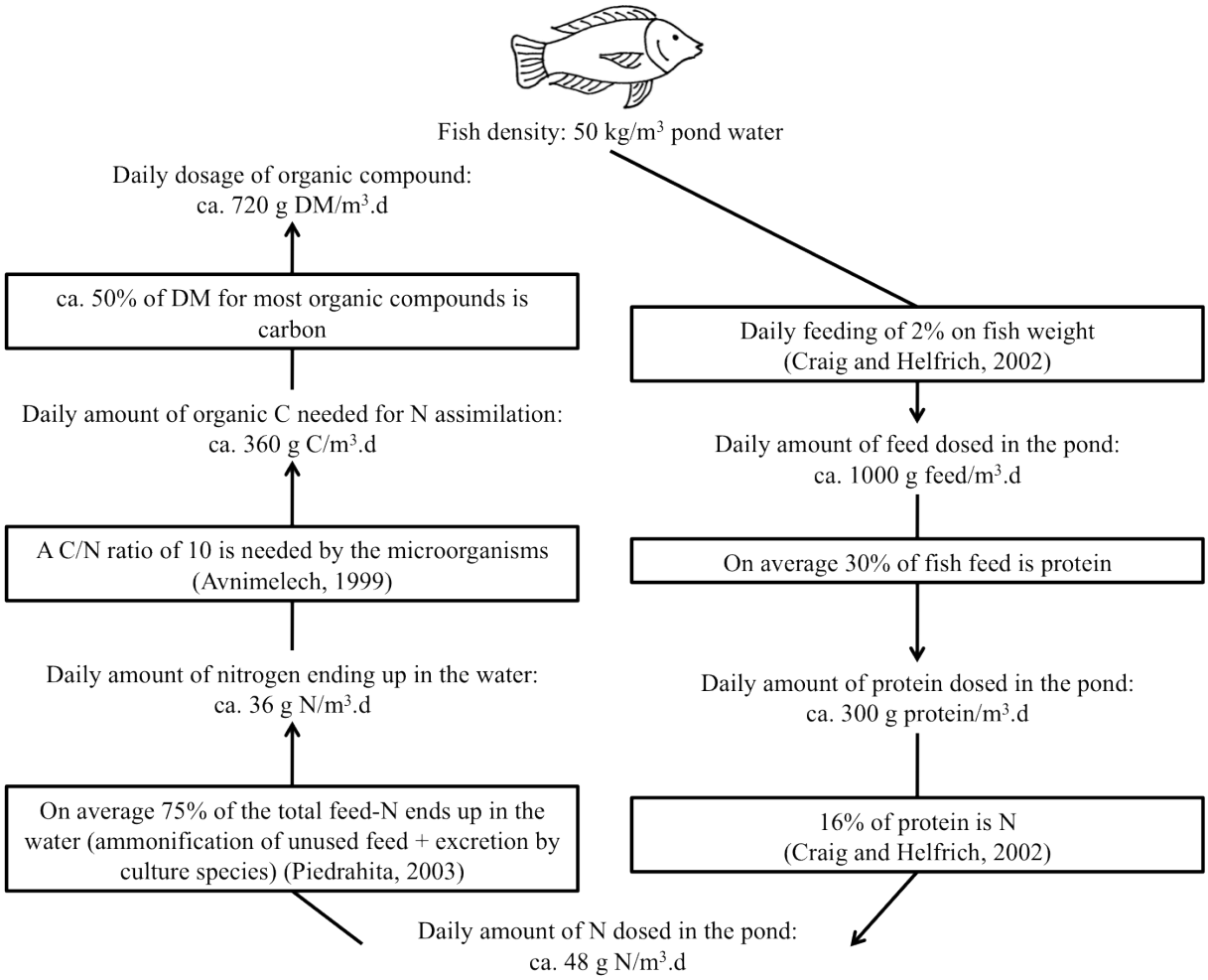


Figure 1.8 Schematic calculation of the daily amount of organic carbon needed by bioflocs to remove the nitrogen excreted in an intensive aquaculture pond of 50 kg fish/m^3.

1.4.3.5 Temperature

The influence of temperature is complex. Researches have been performed on activated sludge samples to find a relation between temperature and floc strength or floc morphology. Wilen et al. (2000) found that deflocculation of the flocs occurred at lower temperature (4°C) compared to higher temperatures (18-20°C), probably due to a decrease of the microbial activity within the flocs. Krishna and Van Loosdrecht (1999) observed that higher temperatures (30–35°C) resulted in bulking of the sludge (SVI ≥ 500 mL/g) due to the excessive production of extracellular polysaccharides. From the previous, it can be expected that an intermediate water temperature of 20-25°C would

be best to obtain stable flocs with an intermediate floc volume index of about 200 mL/g. The temperature is of major importance for the microbial metabolism, also concerning the previously mentioned storage polymers that may be important for aquaculture. It was shown that higher temperatures (35°C) can result in up to 75% less PHB formation compared to lower temperatures (15°C) (Krishna and Van Loosdrecht, 1999). The temperature is closely related to the amount of dissolved oxygen in the water (Boyd, 1998). The culture species will thus not only be influenced by the chosen temperature (changes in growth rates, food conversion efficiencies and even mortality), but also by the associated dissolved oxygen level. The water temperature in BFT ponds is not a factor that can be easily adjusted without imposing considerable additional operating costs, especially in outdoor ponds. In most cases, the climatic conditions determine the operation temperature and thus the species that can be cultured.

1.4.3.6 pH

Changes in pH determine the stability of the bioflocs present in the ponds (Mikkelsen et al., 1996). In several fish experiments, pH has been shown to be an environmental stressor resulting in aberrant physiological functioning, of course depending on the species. For various salmonids, near-lethal or sub-lethal pH levels are 4.2-5.0 causing decreased osmotic pressure, and increased hematocrit, plasma protein concentration, and blood viscosity (Portz et al., 2006). However, in case additional stressors like handling are absent, it seems that tilapia are able to acclimate to pH 4.0 without negative impacts on physiology (van Ginneken et al., 1997). Upper range levels also exist like a pH value of ca. 10 for the Klamath Largescale and Shortnose sucker (Portz et al., 2006). In general, next to the fact that it is not an easy parameter to control, possible changes in pH are limited to the optimal range for the cultured animals to avoid mortality and disfunctioning.

1.4.4 Biological biofloc monitoring techniques for aquaculture

The most obvious way to determine the presence and type of microorganisms in a sample is microscopy. However, since the method is based on visual morphology it is generally not possible to identify them. It can be used to gain a value of the proportion of filamentous and zoogloeal flocs within a water sample.

The fluorescent in situ hybridization or FISH procedure is based on the binding of fluorescently labelled DNA probes with the ribosomal RNA (= rRNA) of bacteria (Amann

et al., 1995). The DNA probes can be designed to exclusively bind to the rRNA of a chosen type of microorganism and thus allow detecting a certain species in a community. Since rRNA is only present in biologically active organisms, it only allows detecting the ones that are performing a specific task (non-active cells are not detected).

Real-time polymerase chain reaction (PCR) is a molecular technique that allows to simultaneously amplify and quantify the extracted DNA from a sample (Heid et al., 1996). This technique is very often used to determine the amount of a certain type of microorganisms in a sample or to determine relative proportion of different types of genes. A quantitative array allows for the simultaneous quantification of phylogenetic and functional genes involved in the activity of interest, e.g. nitrification and denitrification processes (Geets et al., 2007). As such, the evolvement of a complete system can be analyzed.

Denaturing gradient gel electrophoresis (DGGE) is a molecular approach that furnishes information concerning the genetic microbial diversity within any sample such as water, sludge and air (Muyzer et al., 1993). The technique is based on the separation of extracted and by PCR amplified genes (mostly 16S rRNA genes), unique for a group of microorganisms. The analysis of an environmental sample by means of DGGE results in a band pattern in which roughly each band represents a specific microorganism.
The information that can be obtained from a DGGE band pattern is limited, except for comparative purposes. For instance, only bacteria that are present at more than 1% of the total community are detected. The technique is mostly used as a research tool to visualize shifts in the microbial population composition in time. However, in this review some relatively new concepts that offer the possibility to make use of the DGGE band patterns in an alternative way are presented.
Moving Window Analysis is a technique based on DGGE to detect shifts in the microbial community in time (Wittebolle et al. 2005). The DGGE patterns from samples taken subsequently in time can be compared and thus reveal at what rate the microbial community is changing (Figure 1.9). By means of BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium), DGGE patterns can be analyzed and compared, thereby quantifying the differences. The percentage change between two subsequent samples (= % similarity) can be plotted in function of time.
Pareto curves can be made based on the DGGE pattern of one sample (~ one lane in the pattern). The cumulative proportion of band intensities (~ cumulative proportion of abundances) is plotted as function of the cumulative proportion of DGGE bands (~ cumulative proportion of species), the latter with the highest proportions first. This results in the Pareto curves (Figure 1.10) (Lorenz, 1905; Mertens et al., 2005). If every microorganism (~ DGGE band) is present in an equal amount (~ DGGE band intensity), the curve reveals a perfect evenness (the diagonal).

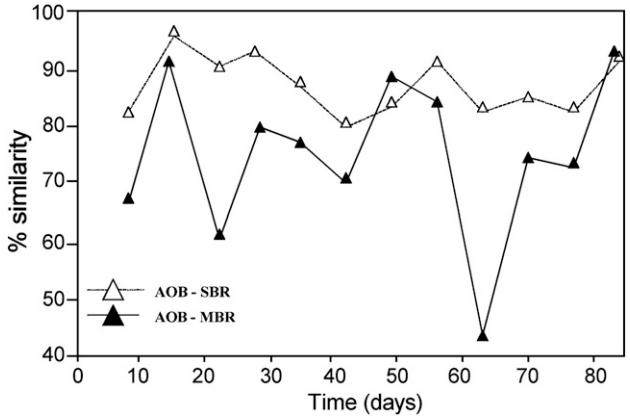


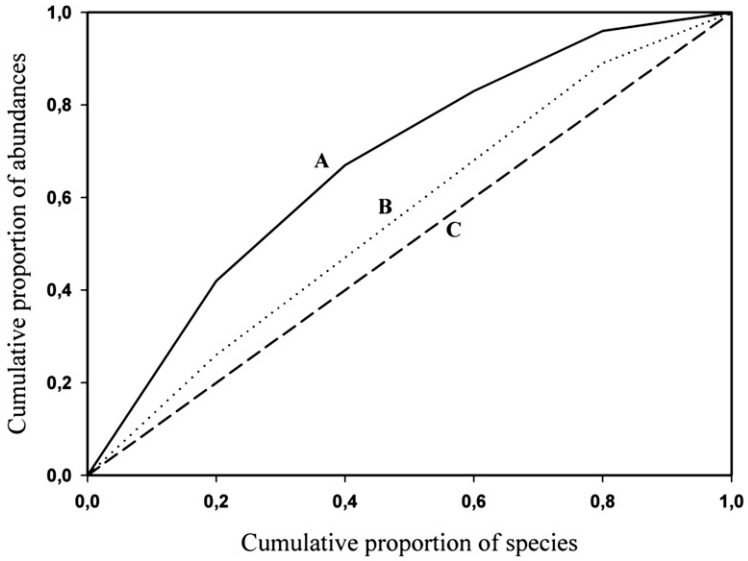
Figure 1.9 Example of moving window analysis: moving window correlation on the DNA level of ammonia oxidizing bacteria in a sequential batch reactor (AOB-SBR) and in a membrane bioreactor (AOB-MBR). The variability between two consecutive sampling dates ($\Delta_{t(week)}$) was calculated based on the denaturing gradient gel electrophoresis patterns. The sequential bioreactor reveals a stable performance ($\Delta_{t(week)}$=12.6 ± 5.2) while the membrane bioreactor shows a variable performance ($\Delta_{t(week)}$=24.6 ± 14.3) (Wittebolle et al., 2005).

Both techniques may be interesting for application in BFT. Relations between the shifts in microbial populations and changes in performance may be established. For BFT, particularly an incomplete nitrogen removal or a change in floc volume index (filamentous bacteria vs. floc forming bacteria) is of critical importance. It may even be possible to establish a maximum change value for the system and larger variations would suggest an immature system.

1.4.5 Nutritious compositions and protective effects of flocs for aquaculture

BFT offers the potential to use zero-exchange recirculation aquaculture systems. However, the added value that bioflocs bring to aquaculture is mainly determined by their potential to be used as additional fish food. Currently, most of the need for the essential compounds in fish food is fulfilled in the form of fishmeal and fish oil, due to their optimal nutritional quality (Watanabe, 2002). It is common practice that 1-5 kg of fish has to be caught in the oceans to be able to produce 1 kg of live aquaculture fish (Naylor et al., 2000). It represents a non-sustainable way of producing food that can be solved by the production of new biomass (microalgae and heterotrophic bacteria) grown on the nutrient waste streams of aquaculture systems. The new biomass is used as alternative food source (Avnimelech, 2006; Hari et al., 2006; Ponis et al., 2003;

Spolaore et al., 2006; Wang, 2003). In this view, the nutritional composition of the bioflocs is of uppermost importance to economically produce a healthy, high quality product (Watanabe, 2002). As discussed before, purchase of commercially prepared feed for fish culture comprises 50% or more in the production costs (Bender et al., 2004). Hence, the use of BFT might imply a considerable decrease in feeding and production costs. Most fish farmers use complete diets comprising protein (18-50%), lipid (10-25%), carbohydrate (15-20%), ash (< 8.5%), phosphorus (< 1.5%), water (< 10%), and trace amounts of vitamins, and minerals (Craig and Helfrich, 2002). The composition of the produced flocs should thus be compared with these values. High protein, polyunsaturated fatty acid (PUFA) and lipid content are the most important parameters determining the feasibility of the bioflocs as feed in aquaculture.



X-axis		Y-axis					
		Curve A		Curve B		Curve C	
n° of band = n° of species	Proportion of the species	Abundance of the species = intensity of the band	Proportion of the abundance	Abundance of the species = intensity of the band	Proportion of the abundance	Abundance of the species = intensity of the band	Proportion of the abundance
1	0,2	10	0,42	10	0,26	2	0,2
2	0,2	6	0,25	8	0,21	2	0,2
3	0,2	4	0,17	8	0,21	2	0,2
4	0,2	3	0,12	8	0,21	2	0,2
5	0,2	1	0,04	4	0,11	2	0,2
Total: 5	Total: 1,0	Total: 24	Total: 1,0	Total: 38	Total: 1,0	Total: 10	Total: 1,0

Figure 1.10 Example on how to calculate the Pareto curves made up out of three samples A, B and C based on DGGE analysis. This may be a tool to monitor the microbial community evolution in BFT.

Not only the nutritional value of the bioflocs is important. Other internal compounds may also be beneficial to the aquaculture species. Short chain fatty acids as biocontrol agents against pathogenic diseases are of particular interest. It was reported that the

application of 20 mM of butyric acid (as was the case with formic, acetic, propionic or valeric acid) to the culture water of *Artemia franciscana* resulted in the protection of these organisms against pathogenic *Vibrio campbellii* (Defoirdt et al., 2006). In this respect, research concerning certain special components in microbial cells is warranted. Emphasis can be put on the organic storage product poly-β-hydroxybutyrate (PHB). This is an intracellular biodegradable polymer produced by a wide variety of microorganisms and is involved in bacterial carbon and energy storage (Defoirdt et al., 2007). It is considered to be depolymerised in the gut of higher organisms and has also been shown to act as a preventive or curative protector of *Artemia franciscana* against *Vibrio* infections (Defoirdt et al., 2007). The accumulation of PHB by mixed cultures in BFT can occur under specific conditions determined by the presence of a growth-limiting factor such as nitrogen and the presence of an excess carbon source (Salehizadeh and Van Loosdrecht, 2004). Upon release from the bacterial cell, e.g. in the case of cell death and lyses, degradation of PHB is performed by the activity of extracellular PHB depolymerase enzymes which are widely distributed among bacteria and fungi (Jendrossek and Handrick, 2002). This results in the release of 3-hydroxybutyrate into the surrounding environment (Trainer and Charles, 2006). As such, PHB might offer a prebiotic advantage for aquaculture.

1.4.6 Overall added value of bioflocs technology for aquaculture

The added value that BFT brings to aquaculture is represented by the decreased costs for water treatment that can be reduced. Crab et al. (2007) gave an overview of the costs for different treatment techniques ranging from 1.1€/kg of fish production in case of rotating biological contactors to 0.2€/kg fish production in case of fluidized sand biofilters. Bioflocs do not allow for a complete replacement of the traditional food but still can bring about a substantial decrease of the processing cost since the food represents 40-50% of the total production costs (Craig and Helfrich, 2002). Current research should mainly focus on the composition and food value of these *in situ* feed products, maximizing their energy content and assess their digestibility for the aquaculture species.

The potential savings on food that can be obtained by BFT can be theoretically calculated. Tilapia can e.g. be produced with food at a 30% protein content and at an average food conversion ratio of 2.2 (Kang'ombe et al., 2007).
For a tilapia culture unit without application of bioflocs technology, one doses 2.2 kg feed per kg fish produced. About 30% of the feed is protein and 25% of the feed is actually taken up by the fish (Piedrahita, 2003). This results in a protein uptake of about

0.165 kg protein per kg fish produced, which is in accordance with the protein content of 14-17% on wet fish biomass for tilapia earlier reported (Hanley, 1991).
In a system with bioflocs, part of the feed will be recycled into flocs, which can also be used by the animals as feed source. Therefore, lower amounts of the conventional feed needs to be dosed to the water. Take *F* the amount of conventional feed added to the system if BFT is applied. With the same assumptions stated above, one can calculate that 0.3F kg protein is dosed per kg fish produced (30% protein feed) and of this, 0.075F kg protein is taken up per kg fish. This means that 75% of the added feed or 0.225F kg protein per kg fish is not taken up by the culture animals. This amount can be recycled into bioflocs. If we assume complete conversion into bioflocs and that the fish will consume only 25% of the bioflocs, the protein uptake through the bioflocs will be around 0.056F kg protein per kg fish. This indirect uptake together with the direct uptake of commercial feed, leads to a total uptake of the feed by the fish of about 0.131F kg protein per kg fish, which is a factor 1.75 higher than in a tilapia culture unit without application of bioflocs technology. Now we can calculate the amount of external feed F needed when BFT is applied when compared with the system without BFT. The total protein requirement by the fish is 0.165 kg protein per kg fish produced as calculated above. With a total uptake of about 0.131F kg protein per kg fish, this means that an amount of about 1.257 kg feed still needs to be applied.
To create a well-balanced carbon to nitrogen ratio, needed for establishing bioflocs in the culture water, one can add an external carbon source to the water. The amount of organic carbon needed to grow flocs in our case study needs to convert 75% of the 1.257 kg feed applied to the pond per kg fish that remains in the water as waste. Hence 0.943 kg feed per kg fish with a 30% protein content or 0.283 kg protein per kg fish remains unused. Proteins contain on average 16% nitrogen, which means that 0.045 kg nitrogen is unused per kg fish produced and is recycled into bioflocs, which have a C/N ratio of about 4 and a bacterial biomass yield of circa 0.5 (Avnimelech, 1999). Therefore 0.362 kg carbon needs to be added in the water per kg fish so that bioflocs can assimilate the excess of nitrogen in the water. In case acetate (40% C) is used as an organic carbon source, one needs to add about 0.905 kg acetate to the water per kg fish produced.
To evaluate the cost saving for tilapia breed by the application of BFT we calculate the feed costs for the production of 1 kg fish without and with bioflocs, considering a cost of 0.6 €/kg feed (in Belgium). Without BFT application feed costs are 1.32 € per kg fish produced and with BFT application 0.75 € per kg fish produced. With BFT one needs to consider the costs of the carbon source applied to the pond. With acetate (0.43 €/kg acetate) this means an extra cost of 0.39 € per kg fish produced. The overall costs for a tilapia BFT pond will be around 1.14 € per kg fish produced. The gain thus appears to be in the order of 14% in terms of feed costs per kg fish produced. For an intensive culture system producing at e.g. 500 ton fish/yr, this represents a gain of 90000€/yr. Clearly, these economics are only indicative and depend largely on the price of organic carbon

source added. Moreover, the potential gain on feed (here estimated to be in the order of 10-20%) must be compared to possible increase of costs (of the order of 30% as mentioned earlier) if one has to invest in water treatment in which nutrients are removed by processes such as e.g. nitrification/denitrification (Crab et al., 2007).

1.5 Studies regarding bioflocs technology in aquaculture systems

We can conclude that bioflocs can be considered as a kind of fast growing microbial mixed culture in which the 'waste'-nitrogen is recycled to young cells, which subsequently are grazed by the fish (Figure 1.11). Uptake of the bioflocs by fish depends most probably on the fish species and feeding traits, fish size, floc size and floc density (Avnimelech, 2007). With respect to feeding, this technique operates at "neutral cost", because it upgrades starch to protein. Moreover, one does not need to invest in an external water treatment system. This method is applicable to extensive as well as intensive aquaculture systems. In addition, the heterotrophic microbial biomass is suspected to have a controlling effect on pathogenic bacteria (Michaud et al., 2006). Preliminary results at our laboratories have shown the presence of poly-β-hydroxybutyrate in bioflocs. PHB-accumulating bacteria may abate pathogenic bacteria in aquaculture (Defoirdt et al., 2007; Halet et al., 2007).

In what follows, some examples are discussed concerning performance of the bioflocs technology in practice in freshwater systems.
Avnimelech (1999) pointed out the use of the C/N ratio as a control element in aquaculture systems. Nitrogen control was induced by adding carbohydrates to the water, and through the subsequent uptake of nitrogen by heterotrophic bacteria. This resulted in the synthesis of microbial proteins that can be eaten by the cultured fish species. Experiments with sediment suspension amended with about 10 mg N/L ammonium and glucose at a concentration 20 times higher than that of the TAN showed that almost all the added ammonium disappeared over a period of about 2 hours. Avnimelech et al. (1994) found that protein utilization by fish in intensive biofloc systems is almost twice as high as the protein utilization in conventionally fed intensive aquaculture ponds, due to a recycling of the excreted nitrogen into utilizable microbial protein. Protein recovery by tilapia rose from 23% in the control to 43% in the floc treatment. It was concluded from this study that the price of feed for fish production using sorghum supplemented granules (pellets containing only 20% protein and

sorghum as a carbonaceous substrate) is just about 50% of the conventional cost when 30% protein pellets were used.

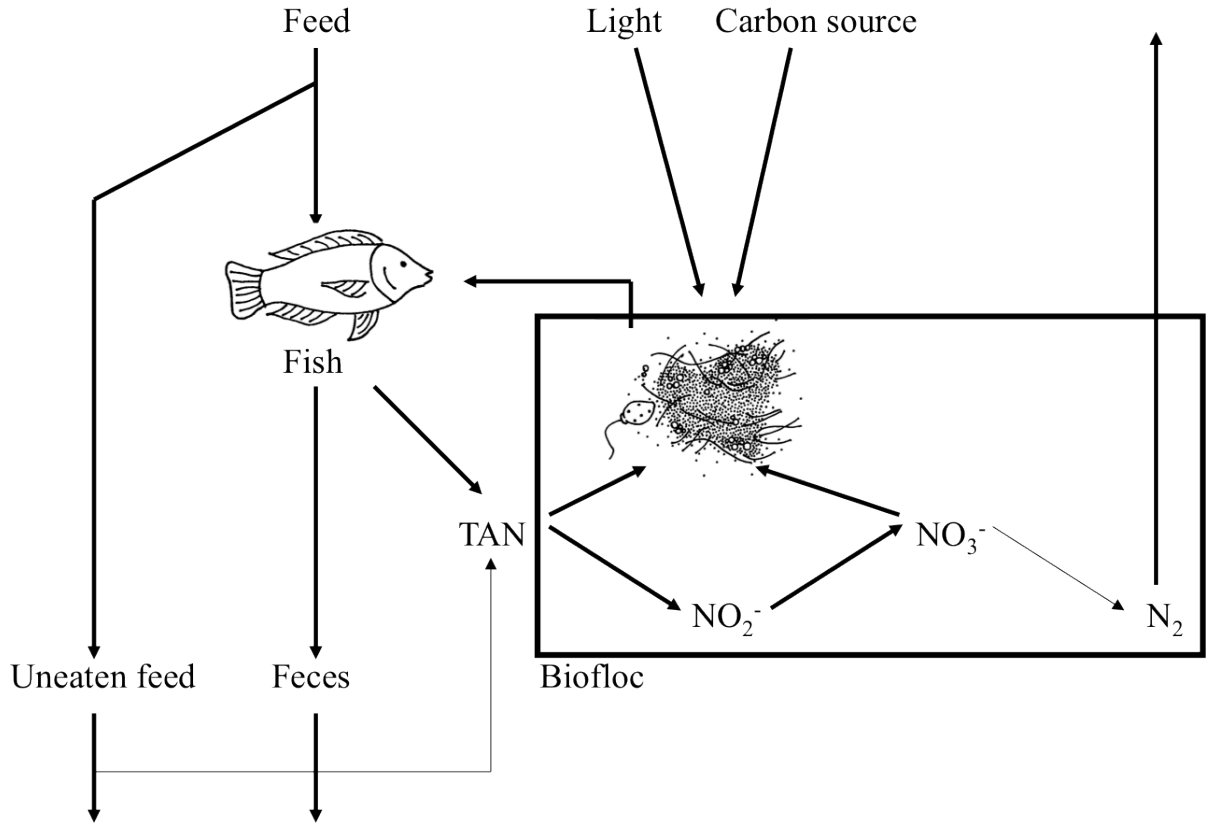


Figure 1.11 Nitrogen cycle in biofloc ponds. The nitrogen cycle is similar to that of in water ponds with periphyton. In contrast to the periphyton system, this system is also applicable to intensive systems. The added carbon source, together with the waste nitrogen, is converted into microbial bioflocs, which in turn can be eaten by the cultured organism. This technique provides an inexpensive protein source with a higher efficiency of nutrient conversion of feed.

The bioflocs technology is also applicable to saline systems, as discussed below.
Hari et al. (2004) facilitated the development of heterotrophic bacteria and the related *in situ* protein synthesis by increasing the C/N ratio of the feed and by further increasing the C/N ratio through carbohydrate addition to the ponds. The added carbohydrate facilitated increased heterotrophic growth thereby augmenting shrimp production. The levels of inorganic nitrogen species in the water column were lower due to uptake by heterotrophic bacteria, making farming more sustainable. The TAN levels in the water column in the study were 0.01 mg/L, which is low compared to levels reported in other studies (0.5-3.0 mg/L) (Hopkins et al., 1993). Consumption of microbial flocs increased nitrogen retention from added feed by 13% (Hari et al., 2004).
Burford et al. (2004) promoted the growth of the natural microbiota in ponds by routine addition of grain feed (18-22% protein) and molasses as carbon sources. Fishmeal-based

feeds and ammonia from shrimp excretion were used as nitrogen sources. The study supports the theory that natural biota can provide a nitrogen source for shrimp, and that flocculated particles are likely to be a significant proportion of this.
Hari et al. (2006) reported that carbohydrate addition in combination with a decreased dietary protein level improved the sustainability of shrimp farming in extensive shrimp culture systems through 1) increased nitrogen retention in harvested shrimp biomass, 2) reduced demand for feed protein, 3) reduced concentrations of potentially toxic TAN and NO_2-N in the system, and 4) reduced water based nitrogen discharge to the environment. If carbohydrate was added to the water column to enhance heterotrophic bacterial protein production, the protein level in the diet could be reduced from 40% to 25%, without compromising shrimp production.

1.6 Conclusions and future perspectives

Possible effluent treatment technologies in aquaculture are diverse. The challenge to the designers of aquaculture systems is to develop systems that maximize production capacity per cost unit of capital invested. To do so, components used in recirculating systems need to be designed and developed to reduce the cost of the unit while maintaining reliability. The bioflocs technology, the periphyton treatment technique, integrated treatment ponds, fluidized sand biofilters, bead filters, trickling filters and the rotating biological contactors can be considered as good wastewater treatment technologies. The bioflocs technology provides a sustainable method to maintain water quality in aquaculture systems and moreover concurrently feed is produced. Since the purchase of commercially prepared feed in fish culture has a share of 50% or more in the production costs, a wastewater treatment technique that maintains water quality and simultaneously produces *in situ* fish feed has a large asset over other techniques. Additional research in this field concerning management of the biofloc production, the biofloc dynamics in intensive aquaculture systems, the nutritional value of bioflocs and the health effects of bioflocs is needed, more specifically the effect on growth and survival of the cultured organisms. Also microbiological aspects need further investigation, particularly the microbiological characterization of the bioflocs, possible manipulation of the microbial community and presence of pre/probiotic organisms in the microbial community of the bioflocs are challenging fields of interest.

Intensive aquaculture must deal with its impacts on the environment in the form of water pollution and the use of fish oil respectively fishmeal. BFT offers the possibility to simultaneously maintain a good water quality within aquaculture systems and produce

additional food for aquaculture organisms. A good understanding of the microscopic mechanisms that are involved in bioflocculation, e.g. advective flow and quorum sensing, will be important for future BFT practice. These will argument our capability to steer the microbial aggregation to obtain optimal morphological characteristics (floc size and floc size distribution) to serve as food for the culture species. Currently, research is mainly focusing on the nutrient removal from the water and not so much on the compositional aspects (for example protein, polyunsaturated fatty acids, lipids, poly-β-hydroxybutyrate, vitamins and micronutrients) of the bioflocs, although the latter can represent a major added value for aquaculture. The nutritional value of the bioflocs, as well as their morphological characteristics, are dependent on a large set of operational parameters in BFT aquaculture systems. Mixing intensity, dissolved oxygen, organic carbon source, organic loading rate, temperature and pH are all influencing factors that are interrelated. The effects they exert on the bioflocs are largely unknown and thus warrant in depth investigation. Research should focus on the optimal way to manage the BFT aquaculture ponds with respect to optimal biofloc morphology and compositional and nutritional value of the bioflocs so that indeed it can replace to a large extent both water treatment and protein supply based on fishery products.

1.7 Thesis outline

In the second half of 2007 and early in 2008, energy costs and the prices of basic foodstuffs rose rapidly worldwide. This also affected fish prices, which rose in real terms for the first time in years. Nevertheless, by 2010, global demand for fish and fish products will probably continue to increase following the pattern of recent decades (FAO, 2009). To support aquacultural growth, taking into account sustainability, researchers stand for challenges regarding water quality control tools and fish feed technology. This work aimed at evaluating the possibility of using bioflocs technology as a sustainable tool to support the necessary future increase in aquaculture production in the field of water quality control as well as *in situ* feed production. Various parameters influence the bioflocs technology as discussed. In Figure 1.12 an overview is depicted of possible steering parameters of bioflocs technology and probable effects that are discussed in this work. Due to the novelty of the technique, many research fields remain unexplored and further development of those domains is needed. Since so many study directions show a great potential, the objective here was to look at bioflocs technology from various research angles to enlighten some aspects of the basics of bioflocs technology. This work chose to enlighten different paths to go for bioflocs technology,

rather than an in depth research in a specific field. One aspect that returns throughout the work is the influence of the type of carbon source used.

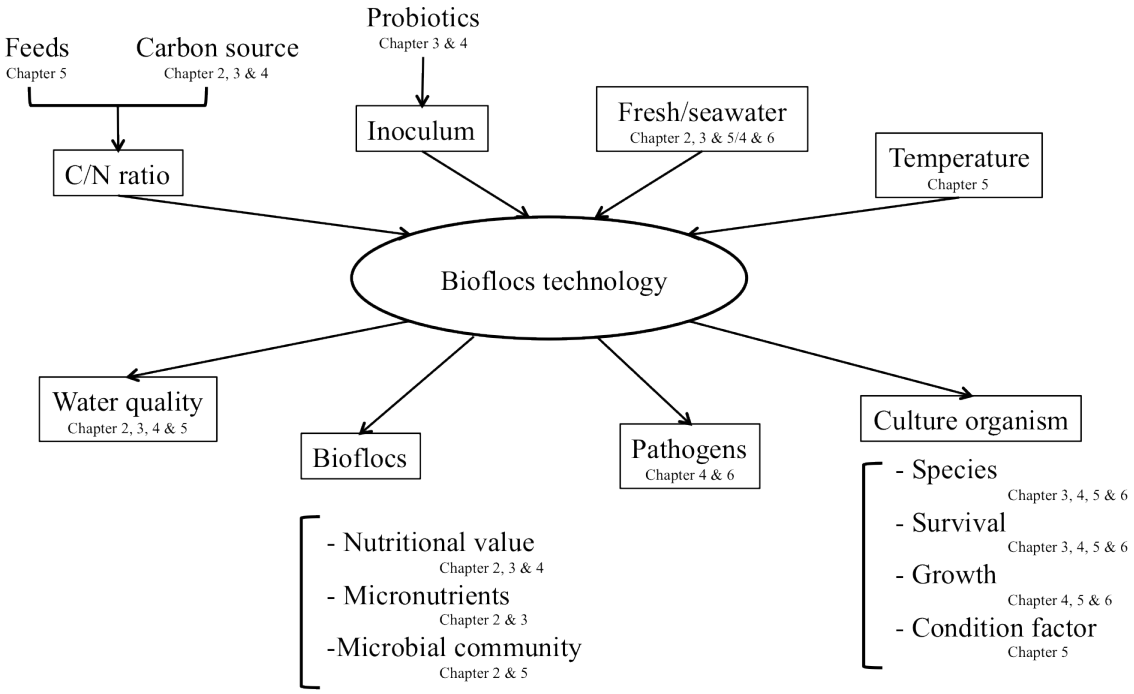


Figure 1.12 Overview of possible steering parameters of bioflocs technology and their probable effects as discussed in this work.

In the first part of this work, the investigation focuses on the effect of different carbon sources on the water cleansing properties of the freshwater bioflocs and the nutritional qualities of the bioflocs, particularly in relation to two micronutrients; vitamin C and PHB (**Chapter 2**). For a better understanding of the microbial community of the bioflocs, a microbial analysis was also performed and discussed. **Chapter 3** describes the use of these nutritional freshwater bioflocs as a feed for *Macrobrachium rosenbergii* postlarvae through assessment of its survival. The bioflocs were grown on different types of carbon source and the influence of a probiotics inoculum was investigated. The nutritional properties of the bioflocs were evaluated and the micronutrient vitamin C measured. Besides the research regarding the use of bioflocs as a probable feed for freshwater species, the possibility of using bioflocs as a feed for a saline culture species was investigated in **Chapter 4**. The use of bioflocs as a feed for *Litopenaeus vannamei* was studied through follow up of the survival and growth of the animals. Different carbon sources were used to stimulate bioflocs formation and the addition of a probiotics to the biofloc reactors was investigated, measuring the bioflocs' nutritional value, effect on water quality and impact on *Vibrio* counts. The work described in **Chapter 5** handles the specific case of the use of bioflocs technology as a means in overwintering of tilapia hybrid fingerlings (*Oreochromis niloticus* x *Oreochromis aureus*). The establishment of proper C/N ratios in the tilapia overwintering ponds was achieved through feed with

elevated carbon content and/or through the addition of an external carbon source. Fish survival, growth and condition factors were examined. The microbial community of the bioflocs was also analyzed to better understand the effect of elevated C/N ratios. Besides water quality management and feed properties, the possible protective action of bioflocs regarding Vibrios was researched in **Chapter 6**. The survival and growth of gnotobiotic *Artemia franciscana* nauplii, challenged with *Vibrio harveyi* and *Vibrio campbellii* was investigated, looking at its survival and growth. The last part (**Chapter 7**) provides a brief overview and discussion of all the research outcomes and formulates recommendations for future research on bioflocs technology.

CHAPTER 2

DIFFERENT ORGANIC CARBON SOURCES SHAPE THE NUTRITIONAL PROPERTIES AND THE MICROBIAL COMMUNITY COMPOSITION OF BIOFLOCS

During a 20-day experiment the possible use of different carbons sources were examined as a water quality control tool. Glucose, acetate and starch controlled the water quality within acceptable range for aquaculture species when a C/N ratio of at least 10 is maintained. The added carbon source influenced the nutritional quality of the bioflocs. All bioflocs had good nutritional properties, but were deficient in vitamin C. Those based on sugar and starch supplementation had high energy contents (27 and 21 kJ/g DW, respectively); those grown on acetate had a high level of PHB (18% DW). In the biofloc reactors, a distinct microbial and algal community developed depending on the organic carbon source used. All microbial communities showed an organization that allowed them to deal with changing environmental conditions while preserving their functionality. These results emphasize the importance of the choice of organic carbon source used in bioflocs technology applications and particularly highlight that plain starch is well suited for this purpose.

Redrafted after:

Crab, R., Boon, N., Defoirdt, T., Bossier, P., Verstraete, W., 2010. Different organic carbon sources shape the nutritional properties and the microbial community composition of bioflocs. Aquaculture, Submitted.

2.1 Introduction

The bioflocs technology (BFT) is a sustainable technique used in aquaculture to maintain good *in situ* water quality through the development and control of dense heterotrophic microbial bioflocs by adding carbohydrate to the water (Avnimelech et al., 1989; Avnimelech, 1999; Crab et al., 2007; Crab et al., 2009; Crab et al., 2010a). The intensive development of the aquaculture industry has been accompanied by an increase in environmental impacts. The production process gives rise to substantial amounts of wastewater, comprising wasted feed and fecal matter (Read and Fernandes, 2003). Only about 25% of the feed nutrients are converted into harvestable products (Avnimelech and Lacher, 1979; Muthuwani and Lin, 1996, Avnimelech and Ritvo, 2003). This contributes to high nitrogen residues in aquaculture water, especially total ammonia nitrogen (TAN), which is the sum of both ammonia and ammonium. Even a low concentration of ammonia, which is in equilibrium with ammonium predominantly depending on the pH, is very toxic to most aquaculture species (Timmons et al., 2002). Maintaining ammonia below toxic levels is vital for the success of cultures of fish and shrimp. If organic carbon and nitrogen are well balanced in a culture system, nitrogenous waste will be converted into bacterial proteinaceous biomass (Avnimelech, 1999; Schneider et al., 2005). Hence, developing and controlling dense heterotrophic microbial bioflocs in the water column by adding carbohydrates improves the water quality in the pond and in addition to this, the bioflocs can subsequently be consumed and used as a source of feed by the cultivated aquatic organisms (Burford et al., 2003; Burford et al., 2004; Hari et al., 2004; Avnimelech, 2005).

The BFT approach is a possible solution for water quality problems, and can decrease fish oil and fishmeal utilization in aquaculture by providing an alternative protein source to the cultured organism. In addition to their potential as a water quality control tool and as a protein source, bioflocs could also contain extra added value components. Vitamins are necessary compounds in the diet of fish and shrimp needed for normal growth and health. Of these, vitamin C is the most important one because it is a powerful antioxidant, enhances tolerance to environmental stressors and increases immunoresistance (Merchie et al., 1997). Besides vitamins, poly-ß-hydroxybutyrate (PHB) is an interesting compound to consider. A wide variety of microorganisms is known to accumulate PHB as an intracellular energy and carbon storage compound, usually when an essential nutrient such as nitrogen is limited in the presence of excess carbon (Lee, 1996). Defoirdt et al. (2007) and Halet et al. (2007) showed that the addition of PHB particles or PHB-containing bacteria to the culture water of *Artemia* nauplii,

infected with the pathogen *Vibrio campbellii*, significantly increased the survival of the shrimp. In BFT systems, organic carbon is added to the pond and as a consequence, nitrogen becomes limited and such conditions are known to select for PHB producers (Salehizadeh and Van Loosdrecht, 2004).

The objectives of this study were to investigate the effects of three organic carbon sources (glucose, starch and acetate) on BFT, particularly with respect to water quality and nutritional value of the bioflocs. Apart from water quality and biofloc quality we also investigated the vitamin C and PHB content of the bioflocs to screen for possible added value features. Finally, since molecular analysis of bioflocs has only recently been described by Crab et al. (2009), the bacterial and algal communities developed after addition of the different carbon sources were examined using recently developed analysis methods based on PCR-DGGE (Marzorati et al., 2008).

2.2 Materials and methods

2.2.1 Experimental design and conditions

The bioflocs were grown in nine identical rectangular reactors with each a water volume of 5 L (360 x 210 x 145 mm). An artificial aquaculture system effluent entered the tank continuously at a flow rate of 0.21 L/hr and the excess of reactor water was removed by means of an overflow resulting in a hydraulic residence time of 1 day. The reactors were continuously aerated. The influent contained about 25.0 mg TAN/L and 3.6 mg PO_4^{3-}-P/L to simulate an aquaculture effluent from an intensive system. Three carbon sources were investigated: starch, glucose and acetate. The carbon source was added once a day in an amount corresponding with an organic C/N ratio of 10 (1250 mg organic C per reactor per day). Each treatment was performed in triplicate, resulting in a reactor A, B and C for every carbon source treatment. Sludge, obtained from the drumfilter of an intensive tilapia aquaculture farm (VitaFish, Dottignies – Mouscron, Belgium) was used as an inoculum and 0.25 L of drum filter slurry was supplied to each 5 L reactor at the start-up. After a start-up period of 4 days for initial establishment of the bioflocs, collection of reactor water and biofloc samples was initiated. Samples were taken every 4 days over a 20-day period.

2.2.2 Water quality

The reactor water samples were analyzed for pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), chemical oxygen demand (COD), total suspended solids (TSS) and volatile suspended solids (VSS). The physicochemical properties of the water samples were determined according to the standard methods for the examination of water and wastewater (APHA, 1998). The temperature of the reactors was measured at each sampling.

2.2.3 Nutritional and extra added value of the bioflocs

The biofloc samples were analyzed for Kjeldahl nitrogen (Kj-N), total ammonia nitrogen (TAN), total suspended solids (TSS) and volatile suspended solids (VSS). These properties were determined following APHA (1998). The difference between Kjeldahl-N and TAN was used to calculate the protein content of the bioflocs by multiplying the organic nitrogen content by 6.25 (Jauncey, 1982). The ash content was determined using TSS and VSS values. Lipids were extracted according to Folch et al. (1957), using the modification of Ways and Hanahan (1964). Protein, lipid and ash content were expressed as percentage of the dry weight (% DW) of the bioflocs. The total carbohydrate was calculated according to the following formula: carbohydrate (% DW) = 100 – (crude protein (% DW) + lipid (% DW) + ash (% DW)) (Manush et al., 2005). The gross energy content of the diets was calculated using kilo joule (kJ/g DW) values of 23.0, 38.1 and 17.2 for protein, lipid and carbohydrate respectively (Tacon, 1990).

Fatty acid methyl esters (FAME) were prepared by transesterification for gas chromatography according to Coutteau and Sorgeloos (1995) and identified by a gas chromatograph equipped with temperature programmable on-column injector.

A paired-ion, reversed phase, high-performance liquid chromatography (HPLC) procedure coupled with electrochemical detection and internal standard quantization based on isoascorbic acid was used for the determination of the vitamin C or ascorbic acid in biofloc samples. Extraction was done following a modification of the method of Kutnink et al. (1987).

Poly-β-hydroxybutyrate (PHB) concentrations were measured with a gas chromatograph following the procedure described by Oehmen et al. (2005). PHB content is given as the percentage of the total dry weight of the bioflocs.

2.2.4 Statistical analysis

The results were subjected to statistical analysis using the software SPSS 16.0. For measurements over a time interval, a paired Student's test was used for analyzing significant changes over time (when $p < 0.05$). If the parameter was stable over time, a time average and standard deviation were calculated (Table 2.1, 2.2 and 2.3). Data represent the average and standard deviation of triplicate reactors.

2.2.5 Microbial analysis

Biofloc samples for DNA extraction were taken 14 days after the first day of sampling from reactor A, B and C of every organic carbon source treatment. Total DNA was extracted from bioflocs in duplicate (resulting in six samples per carbohydrate treatment) by a protocol reported previously by Boon et al. (2000). PCR was performed using a Taq polymerase kit (Fermentas GmbH, St-Leon Rot, Germany). Primers used in this study were as described by Muyzer et al. (1993), amplifying the V3 region of the 16S rRNA gene (338f-GC and 518r), as well as those described by Díez et al. (2001), amplifying the eukaryotic 18S rRNA gene (Euk1A and Euk 516r-GC). DGGE based on the protocol of Muyzer et al. (1993) was performed using the Bio-Rad D gene system (Bio-Rad, Hercules, CA). PCR fragments were loaded onto 8% (w/v, prokaryotic) and 6% (w/v, eukaryotic) polyacrylamide gels in 1 x TAE (20 mMTris, 10 mM acetate, 0,5 mM EDTA, pH 7,4). To separate the amplified DNA fragments, the polyacrylamide gels were made with denaturing gradients ranging from 45% to 60% (prokaryotic) and from 30% to 45% (eukaryotic). On each gel, a marker containing different PCR fragments covering the whole denaturing gradient was loaded. The electrophoresis was run for 16 hr at 60°C and 38 V (prokaryotic) and 100 V (eukaryotic). Staining and analysis of the gels was performed as described previously (Boon et al., 2002).

The DGGE patterns were normalized and analyzed using the BioNumerics software version 5.1 (Applied-Maths, St-Martens-Latem, Belgium). Clustering was carried out using Pearson correlation.

The organization of the communities was visualized through Pareto curves (Lorenz, 1905). A Pareto curve is obtained as follows: abundances of the species are ranked from high to low. The cumulative proportion of species is used as x-axis and the cumulative proportion of abundances presents the y-axis. This yields a concave curve. Based on the Pareto curves, the Gini-coefficients were calculated as described by Mertens et al. (2005). The Gini coefficient is the numerical value of equality and is calculated as twice the area above the Pareto curve. The community organization (Co) can then be

calculated and is a value between 0 and 100, based on the Gini value (Gini x 100). A value of 0 is a complete uneven community; a value of 100 is a complete even community. For Pareto curves applied to DGGE data, the abscissa gives the cumulative proportions of bands or operation taxonomic units (OTUs) and the ordinate the cumulative proportions of the peak heights of the densiometric curves.

2.3 Results

2.3.1 Water quality

The water quality results for pH, dissolved oxygen (DO), total ammonia nitrogen (TAN), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N) were measured and are represented in Table 2.1. The pH was between 6.5 and 8.4 for all treatments, the dissolved oxygen concentration between 4 and 9 mg O_2/L. TAN concentrations were low for the glucose and acetate reactors (1.3 ± 0.6 and 1.0 ± 0.7 mg N/L, respectively), but high in the starch reactors (13.0 ± 1.0 mg N/L). Nitrite concentrations were around zero for the glucose and acetate reactors, and around 0.12 ± 0.01 mg N/L in the starch reactors. Nitrate concentrations ranged from zero to 0.62 mg N/L in all reactors. The temperature of the biofloc reactor water was controlled and maintained around 25°C in all treatments.

Table 2.1 Water quality measured in the biofloc reactors after addition of three different carbon sources. Samples were taken over a 20 days period on regular time intervals (4 days) and the reactors were stable in time. Every organic carbon treatment was performed in triplicate.

	Glucose	Starch	Acetate
pH	7.4 ± 0.3	7.0 ± 0.5	8.0 ± 0.4
DO (mg O_2/L)	7 ± 2	6 ± 2	7 ± 1
TAN (mg N/L)	1.3 ± 0.6	13.0 ± 1.0	1.0 ± 0.7
NH_3-N (mg N/L)*	0.017 ± 0.008	0.069 ± 0.005	0.05 ± 0.04
NH_4^+-N (mg N/L)*	1.3 ± 0.6	13.0 ± 1.0	0.9 ± 0.7
NO_2^--N (mg N/L)	0 ± 0	0.12 ± 0.01	0 ± 0
NO_3^--N (mg N/L)	0.05 ± 0.01	0.58 ± 0.04	0 ± 0

* Values were calculated based on TAN concentrations, pH and temperature.

2.3.2 Nutritional quality of the bioflocs

The biochemical composition and the energy contents of the bioflocs are shown in Table 2.2. Protein content was high in the glucose bioflocs, being 40 ± 6% DW, while about half that value in the other treatments. Crude lipid was also high in the glucose bioflocs (41 ± 9% DW). High ash content was noted in the acetate bioflocs, up to 30% DW. The carbohydrate content of the bioflocs was high in the starch treatment (59 ± 6% DW). The gross energy content of the bioflocs was the highest in the glucose fed reactor (27 ± 2 kJ/g DW). Starch and acetate bioflocs showed considerably lower energy contents.
A total of forty long chain fatty acids were detected (Table 2.3), although concentrations were low in the three treatments. Glucose and starch bioflocs contained the highest concentration of n-6 fatty acids, while glucose bioflocs showed the highest sum of n-3 fatty acids.

Table 2.2 Nutritional qualities of the bioflocs. Samples were taken over a 20 days period on regular time intervals (4 days) and the reactors were stable in time. Every carbon treatment was performed in triplicate.

	Glucose	Starch	Acetate
Crude protein (% DW)	40 ± 6	21 ± 3	19 ± 8
Crude lipid (% DW)	41 ± 9	17 ± 1	21 ± 11
Ash (% DW)	5 ± 2	3 ± 2	20 ± 10
Carbohydrate (% DW)*	14 ± 17	59 ± 6	40 ± 13
Gross energy (kJ/g DW)**	27 ± 2	21 ± 1	19 ± 1

*Values were calculated based on crude protein, crude lipid and ash content.
** Values were calculated based crude protein, crude lipid, ash and carbohydrate content.

Table 2.3 Long chain fatty acid composition, determined by FAME analysis, of the bioflocs. The flocs in all three treatments were analyzed at day 10 and every treatment was performed in triplicate.

	Glucose	Starch	Acetate
18:2(n-6) (mg/g DW)	0.5 ± 0.3	0.7 ± 0.2	0.4 ± 0.2
18:3(n-3) (mg/g DW)	0.05± 0.01	0.04 ± 0.03	0.06 ± 0.03
20:5(n-3) (mg/g DW)	0.50± 0.10	0.15 ± 0.02	0.08 ± 0.03
22:6(n-3) (mg/g DW)	0.04 ± 0.01	/	/
sum n-6	1.0 ± 0.3	1.0 ± 0.1	0.6 ± 0.1
sum n-3	0.80 ± 0.03	0.30 ± 0.07	0.19 ± 0.08

The PHB content of the different bioflocs was determined. It was high in the acetate bioflocs (18.0± 8.0% DW), lower in the glucose bioflocs (6.0± 3.0% DW) and PHB was almost absent in the starch bioflocs (0.5 ± 0.5% DW). No vitamin C was detected in the bioflocs, the concentration was below the detection limit of 5 μg vitamin C/g DW in all samples.

2.3.3 Microbial analysis

The cluster analysis of the DGGE patterns of the bacterial community (based on amplification of the 16S rRNA gene) is given in Figure 2.1. The samples from the reactors fed with the same carbon source tend to cluster together. The dendograms indicate that there is a substantial difference in prokaryotic community structure between the different treatments. The result of the cluster analysis of the DGGE of the eukaryotic community, including algae and protozoa, is given in Figure 2.2. The samples from the reactors fed with the same carbon source tend to cluster together and the dendograms created using Ward linkage indicate that in between treatments there is a substantial difference in eukaryotic community structure. The prokaryotic community organization was evaluated by establishing Pareto curves of the obtained DGGE patterns as depicted in Figure 2.3.

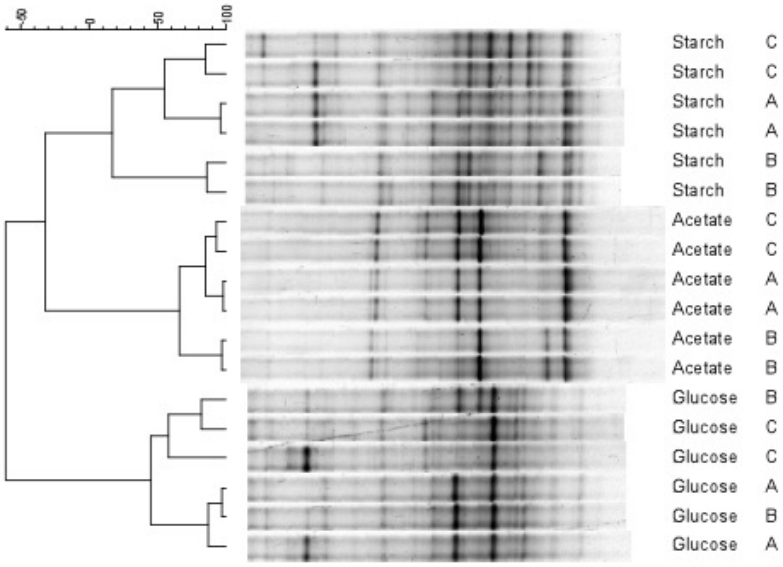


Figure 2.1 Bacterial analysis of the bioflocs. Pearson correlation of microbial communities in the biofloc reactors based on the obtained 16S rRNA DGGE patterns and dendograms were created by using Ward linkage. Each carbon source treatment was performed in triplicate resulting in reactor A, B and C per treatment. Total biofloc DNA was extracted in duplicate for every reactor.

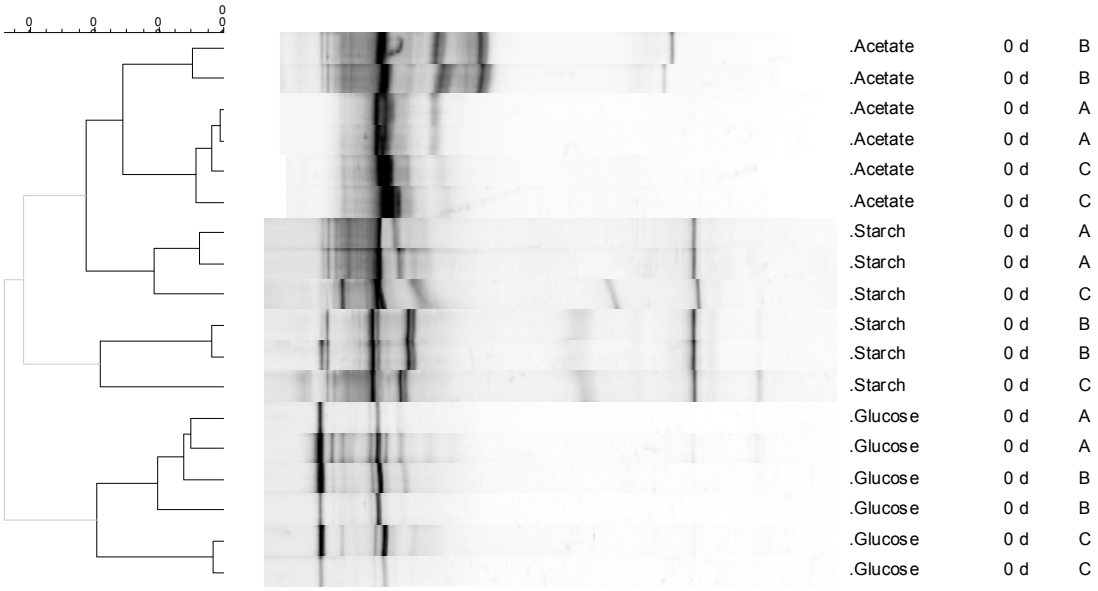


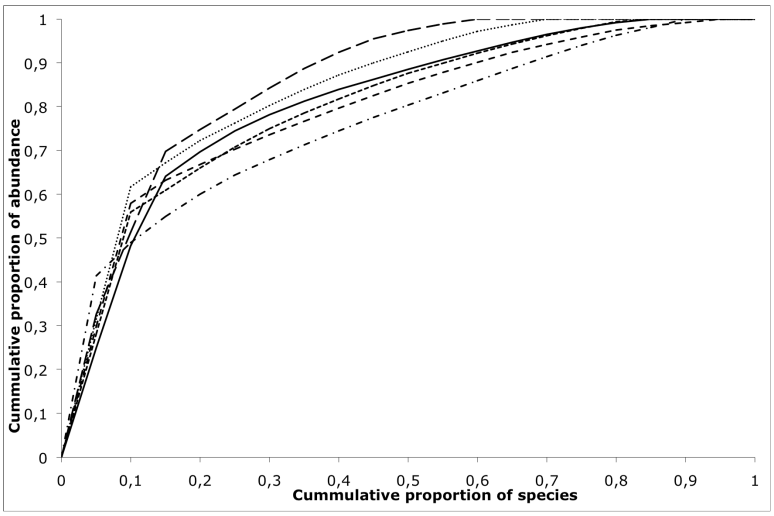
Figure 2.2 Eukaryotic analysis of the bioflocs. Pearson correlation of microbial communities in the biofloc reactors based on the obtained 18S DGGE patterns and dendograms were created using Ward linkage. Each carbon source treatment was performed in triplicate resulting in reactor A, B and C per treatment. Total biofloc DNA was extracted in duplicate for every reactor.

2.4 Discussion

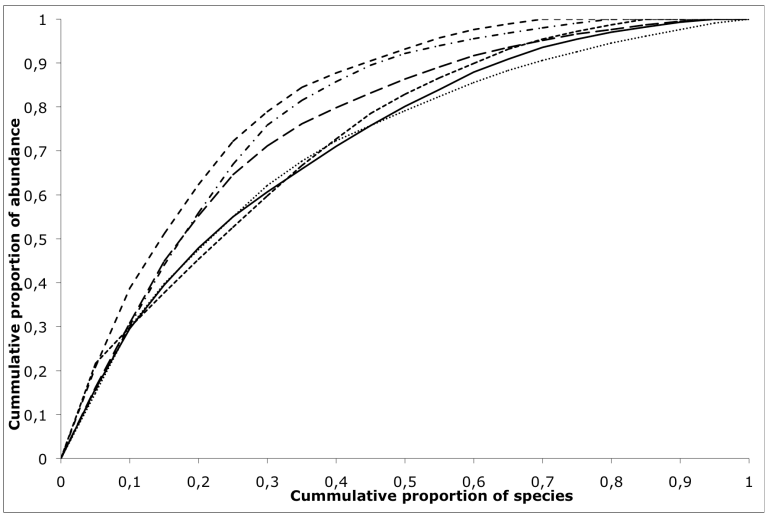
The water quality parameters (Table 2.1) were within acceptable range over the whole testing period of 20 days for all three treatments. The water quality parameters did not significantly fluctuate over time; hence average values are discussed here. The addition of glucose and acetate decreased TAN to 1.3 mg N/L and 1.0 mg N/L, respectively, while nitrite-N and nitrate-N were both around 0 mg N/L. The calculated ammonia (NH_3) levels (based on TAN and pH measurements) indicate that for the glucose and the acetate treatment toxic levels are not exceeded. Ammonia is toxic to most commercial fish at concentrations above 1.5 mg N/L (Neori et al., 2004). The addition of starch had a minor effect on the TAN and measured concentrations were about 13 mg N/L. Also some nitrite and nitrate could be found in the water (respectively 0.12 and 0.58 mg N/L). We observed that starch did not completely dissolve after addition to the reactors. As a consequence, we can assume that not all starch carbon was available to the biofloc microorganisms, thereby hampering the development of the bioflocs. Due to incomplete solubility of this organic carbon source, the ratio of bio-available carbon to nitrogen will

have been below the optimal level for microbial biomass production, resulting in suboptimal uptake of inorganic nitrogen (Table 2.1) and poorer water quality.

a)



b)



c)

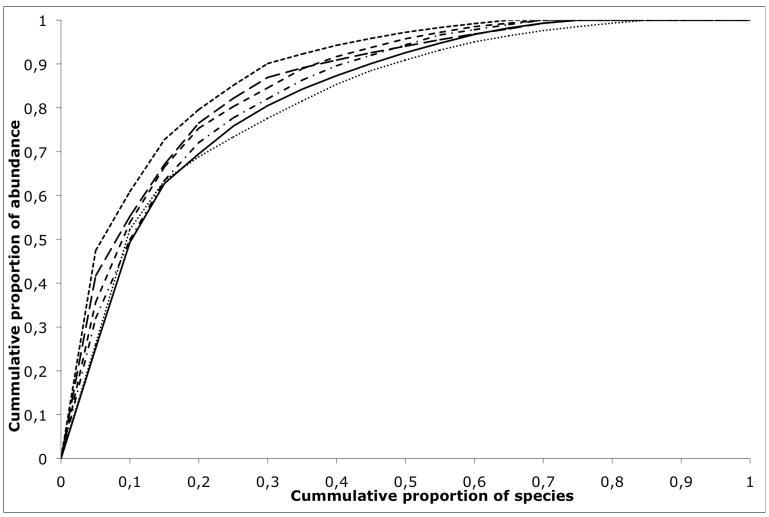


Figure 2.3 Pareto analysis of the 16S DGGE pattern for a) glucose, b) starch and c) acetate bacterial communities. The cumulative normalized number of bands is represented on the x-axis and their respective cumulative normalized intensity is represented on the y-axis.

The composition of the bioflocs grown on different substrates (Table 2.2) did not differ significantly between sampling dates and therefore, average values are discussed here. Bioflocs grown on glucose had a high protein content (up to 40% of the DW). Acetate and starch yielded bioflocs with an average protein content of about 20% of the DW. This microbial protein can serve as an additional high value feed for fish or shrimp, recycling the non-utilized fraction of the added conventional feed (Avnimelech et al., 1989; McIntosh, 2000; Velasco et al., 1998). Protein levels in conventional feeds generally average 20-40% of the DW (Craig and Helfrich, 2002). Proteins are required in the diet to provide essential amino acids (Ghaly et al., 2005). Fats are high-energy nutrients that can be utilized to partially substitute for protein in aquaculture feeds (Craig and Helfrich, 2002). Fats supply about twice the energy of proteins and carbohydrates. Lipids typically comprise about 15% of the DW of fish diets, and have an important function in supplying essential fatty acids. Diets deficient in fats lead to lower growth and poor food conversion efficiency in fish (Tacon, 1990). In this bioflocs experiment about 41% fat of the DW was measured in the glucose grown bioflocs, 17% of the DW in the starch grown bioflocs and 21% of the DW in the acetate grown bioflocs. Hence, the lipid content of all three types of bioflocs was high when compared to conventional feeds. High energy diets are widely used in salmon and trout farming, given the significant benefits of high levels of non-protein energy on improved protein retention and lower nitrogen excretion (Lee and Putnam, 1973; Kaushik and Oliva-Teles, 1985; Cho and Kaushik, 1990; Cho et al., 1994). High dietary fat can lead to an increase of the whole body fat content. This increase in body lipid is mainly due to an increase in lipid content of liver and digestive tract, and not of the muscle tissue (Jobling, 2001; Dias et al., 1998; Peres and Oliva-Teles, 1999; Boujard et al., 2004). The ash content of the flocs in the glucose and starch treatment was low, respectively 5 and 3% on the DW. Complete diets are advised to contain less then 8.5% of the DW ash (Craig and Helfrich, 2002). High ash content lowers the digestibility of other ingredients in the diet resulting in poor growth of the fish. In the acetate bioflocs, we measured a higher ash content was observed, i.e. 20% of the DW. This can decrease the digestibility of the acetate bioflocs. Carbohydrates are the most economical and inexpensive sources of energy for fish diets (Craig and Helfrich, 2002). Yet most of the fish species have a poor ability to utilize carbohydrates and they only represent a minor source of energy for fish (Ghaly et al., 2005). The carbohydrate content of glucose and starch bioflocs was within the limits for typical carbohydrate content in fish feed (Ghaly et al., 2005). The flocs grown on glucose showed a low carbohydrate content; yet the starch bioflocs showed a high carbohydrate content. The latter can be a result of the poor solubilisation because starch particles may still be present in the flocs. Although bioflocs are overall a possible good additional nutritious aquaculture feed, it is of crucial importance that the cultured species will accept this type of feed and digest it. The beneficial influence of bioflocs on the water quality in aquaculture systems has been investigated, but not all fish will be able to utilize bioflocs.

Morphological and physiological adaptations to take up the bioflocs can be required. Experimental evidence however, showed that certain aquaculture species could effectively utilize bioflocs (Avnimelech et al., 1994; Hari et al., 2004; Burford et al., 2004; Hari et al., 2006; Crab et al., 2009; Crab et al., 2010b; Crab et al., 2010c). Besides herbivores, more general detritus and benthos feeders can thrive on bioflocs.
Although bacteria and algae are also rich in vitamins and minerals (Anupama, 2000), the vitamin C content in all bioflocs was below detection limit and supplementation of vitamin C will be needed.

The results from the clustering analysis of the bioflocs indicates that both the prokaryotic and the eukaryotic community are influenced by the choice of carbon source. The impact of the carbon source is manifested through distinct clustering of the band patterns obtained from the reactors per used carbon (Figure 2.1 and 2.2). This makes the bioflocs technology a controlling tool by which one can steer the developing microbial communities in the biofloc ponds. Through the choice of the carbon source, one can influence and optimize the capacity of the technique to control the water quality in the culture system. Moreover, the nutritional properties of the flocs, especially since both microbial and algal communities are influenced (Figure 2.1 and 2.2). Also the overall cost can be manipulated by the choice of the organic carbon source.
Pareto curves illustrating the species evenness of the biofloc communities were calculated based on the data obtained from the PCR-DGGE analysis. These curves (Figure 2.3) showed that the profiles of the bioflocs for glucose and acetate positioned similarly at a community organization value (Co) of about 66. The starch bioflocs had a Co value of 51, indicating a more evenly distributed community, as the Pareto curves were more flat. This evenness can make the community better adapted to changes in the overall conditions (Wittebolle et al., 2009). Future research in large scale systems is needed to see to what extend indeed the growth of better quality flocs by using specific organic carbon sources correlates with more stability in the microbial community patterns.

In conclusion, the results confirm that bioflocs technology is a method to control water quality within the culture component by developing and controlling dense concentrations of heterotrophic bacteria. The accumulation of toxic inorganic nitrogen species, ammonia and nitrite, is prevented in biofloc systems when nitrogen and carbon are well balanced. Glucose and acetate can manage water quality so that zero or minimal water exchange is needed when applied at an organic C/N ratio of 10. While maintaining good water quality, nutritious feed is produced *in situ*. All tested carbon sources generate a potential feed source and hence can increase overall nutrient conversion efficiency by providing an inexpensive protein source produced on nitrogenous waste products. Differences in nutritional quality were noted depending on

the organic carbon source used. These differences were also reflected in the prokaryotic and eukaryotic community composition. For a better understanding of the community structure, clustering analysis was performed and the community organization was calculated. Each organic carbon source stimulated specific bacteria, protozoa and algae, and hence influenced the composition and community organization of the bioflocs, both the prokaryotic and eukaryotic component, and thus indirectly the water quality control function and feed qualities. Future research should focus on the use of low-cost non-conventional agro-industrial residues and hence upgrade waste to nutritious feed. Cycling and recycling of these residues will not only decrease pollution, but will also serve as a source of carbon and energy for very inexpensive production of bioflocs rich in nutrient components.

In this study the obtained bioflocs had different nutritional properties and community composition, depending on the carbon source used. Therefore, the question was raised whether the use of different carbon sources would also affect the taste and acceptance of the bioflocs by cultured animals. For that reason the research in the following chapter describes the use of these nutritional bioflocs grown on different carbon sources as a feed for postlarva of the freshwater prawn *Macrobrachium rosenbergii*.

CHAPTER 3

THE EFFECT OF DIFFERENT ORGANIC CARBON SOURCES ON THE NUTRITIONAL VALUE OF BIOFLOCS, A FEED FOR *MACROBRACHIUM ROSENBERGII* POSTLARVAE

A 15-day lab-scale experiment was designed to determine the possible use of bioflocs as a feed for *Macrobrachium rosenbergii* postlarvae. The bioflocs were grown on acetate, glycerol and glucose. A mixture of *Bacillus* spores was added to a glycerol fed reactor during start up. Nutritional analysis showed that the highest protein content was obtained in the [glycerol + *Bacillus*] bioflocs, i.e. 58 ± 9% on the dry weight (DW) of the bioflocs. The glycerol and acetate bioflocs showed a lower, but similar protein content (42 – 43% on DW) and glucose bioflocs contained 28 ± 3% on DW. The total n-6 fatty acid content in the glycerol and [glycerol + *Bacillus*] bioflocs was a factor 2 – 4 higher than in all other bioflocs. The vitamin C content of the bioflocs was variable, but values up to 54 µg/g DW were measured in the glycerol bioflocs. Daily harvested bioflocs were fed to *Macrobrachium rosenbergii* postlarvae as the sole feed. Survival was significantly higher than in a starvation control, i.e. 0% survival after 15 days, which indicated that the prawns were able to use the bioflocs as a feed. High survival levels were obtained in the [glycerol + *Bacillus*] and glucose groups, i.e. 75 ± 7% and 70 ± 0%, respectively. These results on survival are in accordance with the measured nutritional parameters of the

bioflocs and suggest that the choice of the carbon source used for growing bioflocs is of prime importance.

Redrafted after:

Crab, R., Chielens, B., Wille, M., Bossier, P. and Verstraete, W., 2010. The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. Aquaculture Research 41, 559-567.

3.1 Introduction

There has been a considerable expansion of the global freshwater prawn production during the last decades. The total global production of farmed *Macrobrachium rosenbergii* was only about 5000 tonnes in 1984 and 17000 tonnes in 1993 (FAO, 2005-2008). By 2002, official FAO data indicate that global production exceeded 200000 tonnes. Even at these numbers, the current production of freshwater prawns is relatively small compared to that of marine penaeid shrimp farming. However, the latter has been levelling of in recent years (New, 2005). In looking at global trends, markets for freshwater prawns will continue to expand in both industrialized and developing countries (New, 2005).

Research concerning *Macrobrachium* spp. has focused mainly on nutrition and feeding. Coyle and Tidwell (2002) suggested that more research should be carried out on management practices that maximize the availability of natural food, like for example in earthen ponds. In pond systems, microbial and micro-invertebrate populations develop in the first period of operation and they are a highly nutritional diet to the prawns (Allan et al., 1995). The prawn growth is thus depending on the consumption of these endogenously available live food organisms in addition to the externally supplied feed (Correia et al., 2002). The addition of fertilizers or carbon sources directly to the pond water is a way to augment the natural production of these food organisms (Uddin et al., 2007; Crab et al., 2007). Correia et al. (2003) reported that increasing the natural productivity of ponds significantly improved the feed conversion ratio, with concomitant savings in farm operation costs.

The bioflocs technology (BFT) promotes suspended growth in ponds, consisting of phytoplankton, bacteria, aggregates of living and dead particulate organic matter, and grazers of the bacteria (Hargreaves, 2006). If carbon and nitrogen are well balanced in the solution, the nitrogenous waste generated by the cultivated organisms and especially ammonium will be converted into bacterial biomass (Schneider et al., 2005). By adding carbohydrates to the pond, bacterial growth is stimulated and nitrogen uptake through the production of microbial proteins takes place (Avnimelech, 1999). This promoted nitrogen uptake by bacterial growth can decrease in a matter of hours the putatively toxic ammonium while nitrification in conventionally used biofilters is slower (Hargreaves, 2006). With respect to feeding, this technique can operate at "neutral cost", because it upgrades carbohydrate to protein. Moreover, one does not need to invest in an external water treatment system (Crab et al., 2007). The BFT is achievable by the use of different types of organic carbon. The road to go for the BFT is the use of carbohydrates that are considered low value products in other processing

units like for example glycerol, which is a by-product from biodiesel production (Dube et al., 2007; De Schryver et al., 2008).

The goal of the present research was to assess the nutritional value of bioflocs grown on different carbon sources and their ability to serve as a feed for *Macrobrachium rosenbergii* postlarvae. Bioflocs were grown on three different carbonaceous substrates: acetate, glycerol and glucose. For the glycerol treatment, the influence of the addition of a mixture of *Bacillus* spores was also investigated. The general nutritional composition of the bioflocs was investigated, as was the survival rate of *Macrobrachium rosenbergii* postlarvae fed with bioflocs.

3.2 Materials and methods

3.2.1 Biofloc reactors

The bioflocs were grown in four rectangular reactors with a water volume of 5 L (360 x 210 x 145 mm). The synthetic aquaculture water continuously flowed in the tank at a flow rate of 0.21 L/hr and the excess of reactor water was removed by means of an overflow resulting in a hydraulic residence time (HRT) of 1 day. The reactors were continuously aerated. The influent contained about 25.0 mg TAN/L and 3.6 mg PO_4^{3-}P/L to simulate an aquaculture effluent from an intensive system. Three simple directly soluble carbon sources were investigated: glucose, glycerol and acetate. The carbon source was added once a day in an amount corresponding with an organic C/N ratio of 10. At start up, 0.25 L of drum filter slurry originating from an intensive tilapia aquaculture farm, VitaFish (Dottignies - Mouscron, Belgium), was supplied to each reactor. An additional treatment was inserted, where the bioflocs were grown on glycerol and inoculated with a mixture of *Bacillus* spores at an initial level of 5.10^6 CFU/mL reactor water (INVE technologies NV, A08/TT/0449). Although there was one reactor per carbon source, these results are reproducible and have been repeated. After a start up period of 4 days for initial establishment of the bioflocs, collection of water and biofloc samples was initiated. Samples were taken every 3 days and that over a period of 15 days in total. The bioflocs fed to the *Macrobrachium rosenbergii* postlarvae were freshly harvested every day by filtration.
The water of the biofloc reactors was analyzed for ammonium nitrogen, NO_2^--N and NO_3^--N using a colorimetric ammonium test kit (Aquamerck), nitrite and nitrate test kits (Merckoquant), respectively.

3.2.2 Nutritional value of the bioflocs

Biofloc samples were immediately analyzed after sampling. The Kjeldahl nitrogen (Kj-N), total ammonia nitrogen (TAN), total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (APHA, 1998). The difference between Kjeldahl-N and TAN was used to calculate the protein content of the bioflocs by multiplying the organic nitrogen content by 6.25. The ash content was determined using TSS and VSS values. Lipids were extracted according to Folch et al. (1957), using the modification of Ways and Hanahan (1964). Protein, lipid and ash content were expressed as percentage of the dry weight (% DW) of the bioflocs. The total carbohydrate was calculated according to the following formula: carbohydrate (% DW) = 100 – (crude protein (% DW) + lipid (% DW) + ash (% DW)) (Manush et al., 2005). The gross energy content of the diets was calculated using kilo joule (kJ/g DW) values of 23.0, 38.1 and 17.2 for protein, lipid and carbohydrate respectively (Tacon, 1990).
Fatty acid methyl esters (FAME) were prepared by transesterification for gas chromatography according to Coutteau and Sorgeloos (1995) and identified by a gas chromatograph.
A paired ion, reversed phase, high performance liquid chromatography procedure coupled to electrochemical detection and internal standard quantization based on isoascorbic acid was used for the determination of the vitamin C content or ascorbic acid (AA) in the biofloc samples on day 0 and day 15 of the experiment. Extraction was done following a modification of the method of Kutnink et al. (1987). AA levels were expressed per mg DW.

3.2.3 Experimental design

The animals used for experimental purpose were 14-day-old postlarvae of *Macrobrachium rosenbergii*. *Macrobrachium rosenbergii* breeders were maintained under standardized conditions at the Laboratory of Aquaculture and Artemia Reference Centre (Ghent University, Belgium) in two separate freshwater recirculation units (Cavalli et al., 1999). Larvae from a single female breeder were reared following the procedure described by Cavalli et al. (2000). Hundred postlarvae of *Macrobrachium rosenbergii* were distributed in 100 plastic cups. By placing one postlarvae in each cup, cannibalistic behavior could be eliminated. The experiment consisted of five separate treatment groups, performed in duplicate, resulting in 10 distinctive experimental groups comprising 10 postlarvae following a complete randomized design. The treatment groups were made out of a control treatment in which food was deprived to serve as a

control (starvation control) and four that were fed with the different bioflocs only. The different bioflocs were given to the test organisms as diets equal in nitrogen. The nitrogen content of the bioflocs was determined daily and on wet weight basis through analysis of the Kjeldahl nitrogen, TAN and wet weight of the biomass. The results of a control treatment with commercial feed are not included here since this work was primarily aimed for as a first screening of carbon sources for the growth of bioflocs.
The experiment was conducted for a period of 15 days in the wet laboratory of the Laboratory of Aquaculture and Artemia Reference Centre (Ghent University, Belgium). Animals were held in 170 mL plastic cups. The cups were filled with 100 mL brackish water of salinity about 3 ppt. On a daily basis, 50% of the water was replaced to maintain good water quality and to remove redundant bioflocs. The cups were placed in a warm water bath to maintain optimal water temperature in the cups (28°C). Aerators installed at the ceiling promoted ventilation of the air above the cups, and thus oxygen transfer between water and air to keep adequate dissolved oxygen concentrations in the cup water (> 5 mg O_2 L^{-1}).

3.2.4 Calculation and statistical methods

The results were submitted to statistical analysis using the software SPSS 16.0. For measurements over a time interval a paired student test was used for analyzing significant changes over time (when $p < 0.05$). If the parameter was stable over time, a time average and standard deviation was calculated. The ratings were analyzed for mean differences (95% of significance, ANOVA) and treatments and survival were considered significantly different after performing post hoc analyses using Tukey's test when $p < 0.05$.

3.3 Results

3.3.1 Performance of the biofloc reactors

The removal efficiency of TAN was > 98% in all biofloc reactors, which implicates a practically complete removal within 1 day (HRT). The bioflocs converted the waste TAN into microbial biomass. Nitrite and nitrate formation was minimal (< 0.5 mg N/L), which is consistent with heterotrophic growth and minimal nitrification. These parameters

were measured to monitor that the water quality in the bioreactors remained within the limits for prawn farming.

3.3.2 Nutritional value of the bioflocs

The biochemical composition and the energy contents of the experimental diets, being the different types of bioflocs is shown in Table 3.1. Protein content was similar in the acetate and glycerol treatment, being 42 ± 8 and 43 ± 1 % on DW, respectively. Bioflocs grown on glycerol that were initially inoculated with a mixture of *Bacillus* spores showed an increased protein content by 15% DW on average as compared to the bioflocs grown on glycerol without initial inoculation. Bioflocs grown on glucose showed a protein content that was significantly lower as compared to the other treatments. The lipid content was low in all treatments when compared to the reference diet and ranged from 2.3% on DW for the acetate treatment to 5.4 % on DW for the glucose treatment. The ash content had an average value ranging from 17% DW for the glucose treatment up to 27 % DW for the acetate treatment. The carbohydrate content showed a high variation in between treatments. In the glucose treatment the highest calculated carbohydrate levels (50% on DW) were noted. In the treatment [glycerol + *Bacillus*] the lowest values were registered (14% on DW). Since the addition was regulated so that all animals received similar amounts of protein, the energy addition varied. The acetate, glycerol and glucose biofloc fed groups received respectively 1.2, 1.3 and 2.1 times more energy compared with the [glycerol + *Bacillus*] biofloc group.

A total of forty long chain fatty acids were detected and the fatty acid profiles of the different bioflocs are shown in Table 3.2. The carbon source had a significant effect on the fatty acids profile of the bioflocs. Fatty acid concentrations in the bioflocs were low, except for three fatty acids, more specifically palmitic acid 16:0, palmitoleic acid 16:1(n-7) and linoleic acid 18:2 (n-6) (LA). The total n-6 concentration is mainly determined by the concentration in LA, other n-6 fatty acids are only present in low concentrations. The bioflocs fed with glycerol with/without inoculation with the mixture of *Bacillus* spores showed the highest total n-6 concentration that differed significantly from the other treatments. The total n-3 content of all bioflocs was low and ranged on average from 0.4 to 0.7 mg g^{-1} DW. The linolenic acid (18:3(n-3) or ALA), eicosapentaenoic acid (20:5(n-3) or EPA) and docosahexaenoic acid (22:6(n-3) or DHA) content of the bioflocs was low in all treatments. The n-6/n-3 ratio was 39 in the glycerol and [glycerol + *Bacillus*] bioflocs and about 18 in the acetate and glucose bioflocs.

Table 3.1. Nutritional quality of the bioflocs fed with acetate, glycerol and glucose and an additional glycerol fed treatment with initial inoculation by a mixture of *Bacillus* spores. Samples were taken over a 15 days period at a regular time interval of 3 days and the reactors were stable in that time (no significant changes over time per reactor, $p > 0.05$). Time averaged values are presented for the measured parameters ($n = 6$). Carbohydrate and gross energy content are calculated according to Manush et al. (2005) and Tacon (1990). A reference commercial prawn feed analyzed by Hossain and Paul (2007) is inserted in the table to serve as a comparison basis. Values on the same line followed by different uppercase letters are significantly different ($p < 0.05$).

Substrate	Acetate	Glycerol	[Glycerol + *Bacillus*]	Glucose	Reference diet*
Crude protein (% DW)	42 ± 8[a]	43 ± 1[a]	58 ± 9	28 ± 3	34
Crude lipid (% DW)	2.3 ± 0.4[a]	2.9 ± 0.9[ab]	3.5 ± 0.7[b]	5.4 ± 0.6	9
Ash (% DW)	27 ± 7[a]	20 ± 3	25 ± 5[a]	17 ± 1	25
Carbohydrate (% DW)	29 ± 14[ab]	34 ± 3[a]	14 ± 11[b]	50 ± 4	32
Gross energy (kJ/g DW)	15.5 ± 0.8	16.9 ± 0.5[a]	17.0 ± 0.6[a]	17.0 ± 0.2[a]	15.4

*Data after Hossain and Paul (2007)

The vitamin C content of the bioflocs was different on day 0 and day 15 of the experiment. In the acetate treatment the vitamin C content measured on day 0 and 15 was 6 and 0 µg vitamin C/g DW, respectively. In the glucose treatment concentrations were 0 and 39 µg vitamin C/g DW, respectively. In the glycerol treatments, the vitamin C content of the bioflocs went from 54 to 6 µg vitamin C/g DW and in the [glycerol + *Bacillus*] it went from 54 to 8 µg vitamin C/g DW. One can conclude that the glycerol treatments showed the highest vitamin C concentration values and that, except for the glucose treatment, the concentration decreased over time in all treatments. None to very small amounts were found in the other type of bioflocs.

3.3.3 Survival of *Macrobrachium rosenbergii* postlarvae

In all the biofloc trials, the prawns accepted the diets and none of the prawns showed any pathological signs since no abnormalities were observed. Data on survival (%) of *Macrobrachium rosenbergii* postlarvae are presented in Figure 3.1. After 15 days, the starvation control (SC) had no survival anymore. From day 10 onwards, no significant changes in survival were noted in all other experimental groups. Survival in the [glycerol + *Bacillus*] and the glucose treatments was the highest, 75 ± 7% and 70 ± 0%, respectively. Significantly lower survival was noted in the glycerol and acetate fed

animals, i.e. 60 ± 0% and 25 ± 7%, respectively. All treatments showed significantly higher survival than the starvation control.

Table 3.2 Long chain fatty acid composition of the bioflocs fed with different carbonaceous substrates determined by FAME analysis and expressed in mg/g DW. Samples were taken over a 15-day period on regular time intervals (every 3 days) and the reactors were stable in that time (no significant changes over time per reactor, $p > 0.05$). Hence, average values are presented for the measured parameters ($n = 6$). Values on the same line followed by different uppercase letters are significantly different ($p < 0.05$). Results below the detection limit are indicated by the symbol -.

	Acetate	Glycerol	[Glycerol + *Bacillus*]	Glucose
14:0	0.10 ± 0.01	0.6 ± 0.3^{ab}	0.80 ± 0.01^{a}	0.45 ± 0.07^{b}
14:1(n-5)	0.4 ± 0.1^{a}	0.4 ± 0.2^{ab}	0.20 ± 0.01^{b}	0.55 ± 0.06
15:0	0.15 ± 0.07	0.25 ± 0.05^{a}	0.25 ± 0.07^{a}	0.3 ± 0.1^{a}
15:1(n-5)	0.06 ± 0.03^{a}	0.10 ± 0.05^{ab}	0.10 ± 0.01^{b}	0.3 ± 0.1
16:0	2.2 ± 0.3	17 ± 9^{a}	26 ± 3	15 ± 7^{a}
16:1(n-7)	4 ± 2^{ab}	3.7 ± 0.4^{a}	3 ± 1^{a}	5 ± 1^{b}
17:0	0.05 ± 0.07	0.4 ± 0.2^{ab}	0.5 ± 0.1^{a}	0.2 ± 0.2^{b}
17:1(n-7)	0.10 ± 0.03^{a}	0.13 ± 0.07^{a}	0.10 ± 0.01^{a}	0.12 ± 0.06^{a}
18:0	0.5 ± 0.1	4 ± 1^{a}	7.1 ± 0.4^{b}	6 ± 2^{ab}
18:1(n-9)	1.8 ± 0.8	19 ± 8^{a}	30 ± 3	18 ± 8^{a}
18:1(n-7)	1.5 ± 0.7^{a}	3 ± 1^{b}	1.9 ± 0.8^{ac}	2.7 ± 0.4^{bc}
18:2(n-6) (LA)	5 ± 2	19 ± 5	28.2 ± 0.9	11 ± 4
19:0	0.05 ± 0.03^{a}	0.4 ± 0.2	0.05 ± 0.04^{a}	0.10 ± 0.05^{a}
18:3(n-6)	0.2 ± 0.1^{ab}	0.4 ± 0.2^{b}	0.15 ± 0.07^{a}	0.15 ± 0.06^{a}
19:1(n-9)	-	-	-	0.05 ± 0.03
18:3(n-3) (ALA)	0.04 ± 0.03	0.5 ± 0.2^{a}	0.45 ± 0.07^{a}	2.0 ± 0.4
18:4(n-3)	0.16 ± 0.06^{a}	0.15 ± 0.07^{a}	0.20 ± 0.01^{ab}	0.25 ± 0.07^{b}
20:0	-	0.10 ± 0.05^{a}	0.20 ± 0.01^{b}	0.2 ± 0.1^{ab}
20:1(n-9)	0.05 ± 0.03^{a}	0.10 ± 0.05^{ab}	0.15 ± 0.08^{b}	0.10 ± 0.02^{b}
20:1(n-7)	-	-	-	0.03 ± 0.03
21:0	-	-	-	0.05 ± 0.03
20:3(n-6)	0.15 ± 0.07^{a}	0.10 ± 0.08^{ab}	0.06 ± 0.04^{b}	0.07 ± 0.04^{b}
20:4(n-6)	0.7 ± 0.4	0.3 ± 0.2^{a}	0.15 ± 0.07^{a}	0.2 ± 0.1^{a}
20:3(n-3)	-	-	-	-
20:4(n-3)	-	0.10 ± 0.05	-	0.10 ± 0.02
22:0	-	0.14 ± 0.07^{a}	0.35 ± 0.07	0.15 ± 0.07^{a}
20:5(n-3) (EPA)	0.10 ± 0.03^{a}	0.11 ± 0.06^{a}	0.05 ± 0.07^{a}	0.25 ± 0.07
22:1(n-9)	0.1 ± 0.1	-	-	-
22:1(n-7)	0.1 ± 0.1	-	-	-
23:0	-	0.10 ± 0.06^{a}	0.10 ± 0.04^{a}	-

	Acetate	Glycerol	[Glycerol + *Bacillus*]	Glucose
21:5(n-3)	-	-	-	0.05 ± 0.03
23:1(n-9)	0.05 ± 0.03	-	-	-
22:4(n-6)	0.1 ± 0.1[a]	-	-	0.05 ± 0.05[a]
22:3(n-3)	-	-	-	-
22:5(n-6)	0.2 ± 0.2[b]	0.06 ± 0.06[a]	-	-
22:4(n-3)	-	-	-	-
24:0	-	-	-	-
22:5(n-3)	0.05 ± 0.04	0.4 ± 0.2	0.65 ± 0.08	0.15 ± 0.06
24:1(n-9)	-	0.09 ± 0.04	-	0.3 ± 0.2
22:6(n-3) (DHA)	0.05 ± 0.07[a]	-	0.07 ± 0.07[a]	0.05 ± 0.03[a]
Total n-6	7 ± 2	20 ± 5	27 ± 3	12 ± 4
Total n-3	0.4 ± 0.2[a]	0.6 ± 0.2[ab]	0.7 ± 0.1[b]	0.65 ± 0.07[b]
n-6/n-3	18 ± 8[a]	39 ± 25[ab]	39 ± 10[b]	18 ± 5[a]

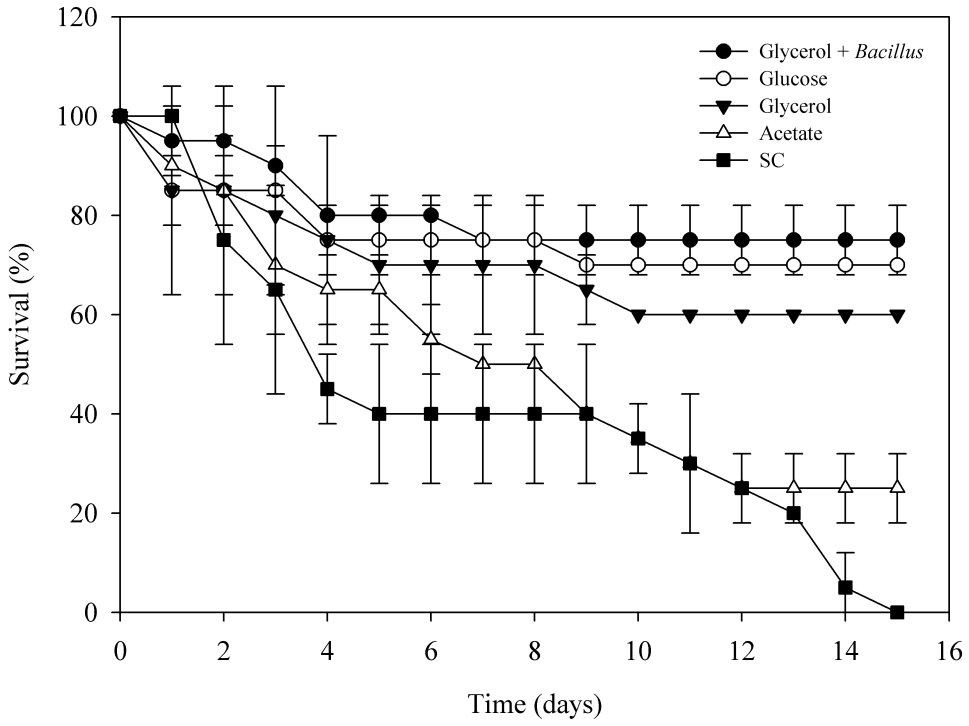


Figure 3.1 Effect of different bioflocs on the survival of *Macrobrachium rosenbergii* postlarvae. A starvation control (SC) treatment was included in the set-up, where the animals received no feed. Bioflocs were grown in 5 L reactors on the following carbonaceous substrates: acetate, glycerol and glucose. A biofloc reactor fed with glycerol was initially inoculated with a mixture of *Bacillus* spores (INVE technologies NV, A08/TT/0449). The treatments were performed in duplicate and are indicated as acetate, glycerol, glucose and [glycerol + *Bacillus*], respectively.

3.4 Discussion

The BFT may provide a sustainable method to maintain water quality within acceptable range for prawn species (Boyd and Zimmerman, 2000). In the biofloc reactors, high total ammonia removal was measured with minimal formation of nitrite and nitrate. This phenomenon was also noted in other researches, including experiments in larger test systems with and without cultured animals (Avnimelech et al., 1994; Burford et al., 2004; Hari et al., 2006). Low survival rates or decreased growth due to toxic ammonia and nitrite can be prohibited and therefore, the BFT may make it possible to increase growth yields and survival levels at low water replacement rates, while a potential additional natural food resource is provided, more specifically in the form of bioflocs (Naqvi et al., 2007; Mallasen and Valenti, 2006; Asaduzzaman et al., 2008).

The common sources of microbial energy in the BFT are flour, molasses and starch (Avnimelech, 1999; Hari et al., 2004; Burford et al., 2004). This study addresses the influence of three simple directly soluble carbon sources, i.e. acetate, glycerol and glucose. Although all have about the same energy content upon aerobic metabolism (i.e. 110 kJ/mol acetate-C, 181 kJ/mol glycerol-C and 165 kJ /mol glucose-C) there may be major differences by which the bacteria, taking part in the phenomenon of floc formation, profit from these potentialities (Rittmann and McCarty, 2001). Indeed, the impact of various carbon sources on the outgrowth of bioflocs in activated sludge systems has been documented (Bodík et al., 2009). It is remarkable that for acetate and glycerol the protein content of the bioflocs is much higher. The latter can be due to the fact that these substrates do not promote bacteria that produce large amounts of exopolysaccharides, but rather are used by species mainly investing in cellular growth. Moreover, growing within the structure of the flocs rather than living alone in a planktonic form selectively enhances these species. The latter points at organisms that merit from the positive effects of advective flow occurring within flocs (Logan and Hunt, 1987; Logan and Hunt, 1988; Hondzo and Wüest, 2009). This aspect certainly merits further investigation.

Since feed is the largest cost item in the production process (as it constitutes 40 – 60% of the operational cost), alternative low cost diets can lower production costs (Mitra et al., 2005). Here we investigated the bioflocs as a feed for *Macrobrachium rosenbergii* postlarvae. The bioflocs possess good prime nutritional values when compared to a reference commercial prawn feed analyzed by Hossain and Paul (2007) (Table 3.1). D'Abramo (1998) reported the use of commercial freshwater prawn feeds with protein levels ranging from 22% to 38.5% DW and stated that when natural food was also available, diets with about 13% protein on DW could successfully be used. The crude

protein content in the [glycerol + *Bacillus*] was the highest, followed by glycerol and acetate bioflocs. The lipid content was low in all bioflocs. Roustaian et al. (2001) demonstrated the importance of lipid as the major metabolic energy source for growing *Macrobrachium rosenbergii* postlarvae, which implicates a commercial feed will still be necessary to provide adequate lipid levels. Also the highly unsaturated fatty acid levels, more specifically that of EPA and DHA, are low. EPA and DHA have been recognized as important nutrients for the growth of various crustaceans and *Macrobrachium rosenbergii* in particular (Das et al., 2007). Besides EPA and DHA, *Macrobrachium rosenbergii* require both 18:2(n-6) (LA) and 18:3(n-3) (ALA) in their feed (Kamarudin and Roustaian, 2002; Mitra et al., 2005). The first component, also known as linoleic acid, is present in high concentrations in the bioflocs, especially in the glycerol fed bioflocs particularly with *Bacillus* as a probioticum. The second component, linolenic acid, is only present in low concentrations. The gross energy content of the bioflocs and the reference diet is in the same order of magnitude. The presence of vitamin C in the bioflocs, which has an influence on the survival, was lower than the advised level (60 to 150 µg/g of vitamin C), yet values up to half of this level were measured in the glycerol bioflocs (Hari and Kurup, 2002; D'Abramo, 1998; Mitra et al., 2005). Overall, the results show that the biofloc quality is significantly influenced by the added carbonaceous substrate.

Besides its composition, the nutritional value of the bioflocs is also dependent on the ability to both ingest and digest the bioflocs. Survival was measured in the postlarval stage, where feed digestibility is generally less than in the larval stages (Das et al., 2007). The glycerol fed bioflocs with and without *Bacillus* showed the highest nutritional value when looking at the biochemical composition and a high survival was noted (Figure 3.1). The acetate and glucose bioflocs showed inferior properties. The acetate fed animals showed a low survival, but the glucose treatment had a good survival. Gross energy of all type of bioflocs was similar, but since the bioflocs were administered isonitrogenously, the dosed energy differed in between treatments. This might explain the high survival in the glucose fed animals, since through the feed a higher amount of energy (up to twice that of in the other diets) was given to the postlarvae. Since the nutritional composition of the bioflocs was significantly influenced by the carbon source, the same can be observed when looking at survival. The diet probably also had an effect on growth and feed utilization, but this was not investigated in this set-up. There are possibly other means to influence the overall feed quality of the bioflocs. For instance the use of chemo-attractants that have been used to enhance consumption of an inferior feed or alter feeding behavior, thus achieving better food acceptance (Harpaz, 1997). The addition of chemo-attractants to the pond or incorporation in the bioflocs can be an interesting field of study that further aims at increasing the nutrient intake by the cultured animal through the BFT.

Correia et al. (2002) found that pond aging resulted in superior growth of *Macrobrachium rosenbergii* prawns, as compared with newly dug earthen ponds. Shortening the pond aging process and enhancing natural feed production would be beneficial to the farmer. Inoculation using water and/or sediment from older or BFT ponds can accelerate the biofloc development process (Correia et al., 2002). This is a biological jump-start that is commonly used in certain bioreactors, such as in wastewater treatment systems (Correia et al., 2002). One can also inoculate with probiotics. In this study there was a significant difference between the *Bacillus* amended glycerol treatment and the glycerol treatment. The nutritional value of the bioflocs, especially the protein content, increased, but the mechanism behind this increase is not known. This increase is also reflected in the survival measured. Possibly, the probiotic strains altered directly or indirectly the composition of the microbial community in the bioflocs, and so the rearing environment and the shrimp intestinal tract through biofloc uptake. Another potential advantage of bioflocs, but not tested in this experiment, is the inhibitory effect on pathogens. *Bacillus* spp. can influence the composition of the water borne microbial populations in such a way that they will decrease the number of pathogens in the vicinity of the farm species and hence have an antagonizing effect on potential pathogens (Farzanfar, 2006).

It may be concluded that bioflocs can be used as an additional feed source for *Macrobrachium rosenbergii* postlarvae. Yet, the effect is dependent on the carbon source used. Bioflocs fed with glucose and glycerol constitute a cost effective, sustainable and environment friendly diet, all prerequisites to further improve the production of prawn. In this study, the bioflocs grown on glycerol supplemented with an initial inoculation of a mixture of *Bacillus* spores and the ones grown on glucose, showed the best nutritional values based on biochemical analyses and survival measurements of *Macrobrachium rosenbergii* postlarvae. The BFT can be a key to sustainable environmental aquaculture prawn rearing with minimal need for investment in external water treatment systems and decreasing feed costs.

The potential importance of the results obtained here calls for further study of the use of BFT in aquaculture. The composition of the bioflocs and the compliance with the nutritional needs of the cultured animals need more in to depth research. Aspects that have a large potential and importance are the survival and growth of the cultured animal, the role of the different components of the bioflocs in terms of nutrition and the animal health.

Thus far only freshwater systems were investigated in this work. It was interesting to look at whether the bioflocs technology would work as well in seawater systems. Therefore, the biofloc reactor set up was used in the following chapter to investigate the

effects of bioflocs on the water quality in seawater. Yet again, the use of different carbon sources and their effect on water quality, nutritional parameters of the bioflocs and the use of bioflocs as a feed for white shrimp *Litopenaeus vannamei* was investigated.

CHAPTER 4

THE EFFECT OF BIOFLOCS ON THE SURVIVAL AND GROWTH OF WHITE SHRIMP (*LITOPENAEUS VANNAMEI*)

In order to determine the capacity of whiteleg shrimp (*Litopenaeus vannamei*) to use bioflocs as a replacement for artificial feed containing animal protein sources, two 28-day growth trials were conducted in an indoor tanks system. Experimental treatments included a control treatment in which the animals were fed an artificial feed containing 35% DW crude protein and different treatments in which the animals were fed either bioflocs alone or a combination of bioflocs and artificial feed. Bioflocs were grown on two different carbon sources, glucose or glycerol. Complete substitution of the artificial feed by bioflocs led to poor survival of animals fed glucose-grown bioflocs. Moreover, growth performance and highly unsaturated fatty acid content of biofloc-fed shrimp was lower than that of shrimp fed the artificial diet. When replacing 10 and 30% of the artificial feed by glucose and glycerol bioflocs, respectively, good survival was obtained. Moreover, shrimp fed glycerol-grown bioflocs showed the same growth performance as animals fed solely artificial feed. Finally, the inoculation of the biofloc reactors with a probiotic mixture of *Bacillus* strains resulted in lower levels of *Vibrio* spp. in the shrimp culture water. These results indicated that artificial feed can be partially replaced by bioflocs without affecting shrimp survival and growth performance. The performance is strongly dependent on the carbon source used to grow the bioflocs, possibly by affecting the accessibility, palatability or attractiveness of the bioflocs towards the animals.

4.1 Introduction

With increasing production of shrimp culture and intensification by applying higher stocking densities, the need to decrease nutrient concentrations in production units and their effluents has become of critical interest (Lezama-Cervantes and Paniagua-Michel, 2010). The accumulated uneaten feed and excreta often cause the water quality in the shrimp cultures to deteriorate, with especially ammonium accumulation being problematic. Deterioration of the quality of the culture water can lead to poor growth, low survival rates and disappointing production yields (Venero et al., 2007; Lezama-Cervantes and Paniagua-Michel, 2010). Together with the growth of crustacean culturing in aquaculture industry, there has been a shift towards an increase in feed inputs. The latter represents one of the primary variable costs associated with the production of shrimp (Venero et al., 2007). Future use of animal protein sources in shrimp feeds is expected to be considerably lower as a consequence of increasing economical, environmental and safety issues (Amaya et al., 2007). Shrimp research has recently focused on the development of feed replacement strategies with a minimal supply of fishmeal, which is replaced by alternative and cheaper sources of protein.

The problems associated with shrimp culturing have raised the call to rear shrimp in systems with minimal water exchange and careful monitoring of the water quality. Recirculating aquaculture systems use different technologies (such as biofilters) to clean outlet water for reuse within the culture system. An alternative, relatively new technique emerging in aquaculture is the bioflocs technology (BFT), which has numerous benefits over recirculating aquaculture systems. The basic principle of bioflocs technology is that through carefully adjusting the C/N ratio in the culture system, suspended growth is stimulated (Crab et al., 2007). When combined with constant aeration and agitation of the water, this practice allows maintaining levels up to 200 – 500 mg/L of microbial flocs in suspension (Azim and Little, 2008; Avnimelech, 2009). If carbon and nitrogen are well balanced in the culture water, the nitrogenous waste generated by the cultivated organisms will be converted into bacterial biomass (containing protein) that is available for feeding (Schneider et al., 2005). In addition to maintaining good water quality, the BFT technique thus generates a source of feed, which results in about 1.2 fold lower production costs and a 2 times more efficient nutrient conversion (Crab et al. 2007; Avnimelech, 2009). The bioflocs technology therefore offers the advantages of lower impact on the environment due to lower external water requirements, removal of toxic inorganic nitrogen species and *in situ* production of additional feed for the cultured animals (De Schryver and Verstraete,

2009). It is estimated that the decrease in feed costs per kg annually produced live weight using bioflocs technology can be in the order of 14% (De Schryver and Verstraete, 2009).

In addition to the problems of water quality deterioration, the marine shrimp industry is also challenged by shrimp diseases caused by (opportunistic) pathogens such as *Vibrio* spp. (Defoirdt et al., 2007). Because of the risk of antibiotic resistance development, there is a growing awareness that antibiotics should be used with more care in animal production. As a consequence, alternative biocontrol techniques are being explored. One of these alternatives is the use of probiotics, live microbial feed supplements that beneficially affect the host (Verschuere et al., 2000). Beneficial effects, ascribed to probiotic bacteria include improvement of the intestinal balance through improved feed value, enzymatic contribution to digestion and inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic activities, production of growth-promoting factors, inducing increased immune response and improving water quality (Liu et al., 2010). Several studies reported that *Bacillus* spp. improved shrimp growth performance, survival, immunity and disease resistance in aquaculture (Tseng et al., 2009). This has raised the interest of steering the microbial populations in bioflocs by adding a probiotic *Bacillus* mixture during the development of bioflocs (Crab et al., 2010b).

The goal of the present research was to assess the palatability and digestibility of bioflocs for white shrimp (*Litopenaeus vannamei*). To this end, bioflocs were grown on two different carbon sources, glucose and glycerol. Furthermore, the effect of inoculating a probiotic *Bacillus* mixture into the biofloc reactors was also evaluated. The capacity of bioflocs to serve as a feed for white shrimp was studied by evaluating the survival, average body weight and specific growth rate of the shrimp in two separate experiments with complete and partial artificial feed replacement by bioflocs.

4.2 Materials and methods

4.2.1 Biofloc reactors

The bioflocs were grown in reactors with a water volume of 40 L. The influent continuously flowed in the tank at a flow rate of 0.83 L hr^{-1} and the excess of reactor water was removed by means of an overflow resulting in a hydraulic residence time

(HRT) of 2 days. The reactors were continuously aerated. The influent contained 30 g L^{-1} artificial sea salt, 25.0 mg TAN L^{-1} and 3.6 mg PO_4^{3-}-P L^{-1} (simulating the effluent from an intensive aquaculture system). The carbon sources (glucose or glycerol) were added once a day at 250 mg L^{-1} (corresponding to an organic C/N ratio of 10). At start up, the reactors were inoculated with 2 L of drum filter slurry originating from an intensive tilapia aquaculture farm, VitaFish (Dottignies - Mouscron, Belgium). The reactors with probiotics were inoculated once at start up with a commercial mixture of *Bacillus* spores (INVE technologies NV, A08/TT/0449) at a level of 2.10^6 spores mL^{-1} reactor water. After a start up period of 4 days for initial establishment of the bioflocs, collection of water and biofloc samples was initiated. The bioflocs fed to the *Litopenaeus vannamei* test organism were freshly harvested every day by filtration.

4.2.2 Nutritional value of the bioflocs

Biofloc samples were immediately analyzed after sampling. The Kjeldahl nitrogen (Kj-N), total ammonia nitrogen (TAN), total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (APHA, 1998). The difference between Kjeldahl-N and TAN was used to calculate the protein content of the bioflocs by multiplying the organic nitrogen content by 6.25 (Jauncey, 1982). Protein content was expressed as percentage of the dry weight (% DW) of the bioflocs. Fatty acid methyl esters (FAME) were analysed by gas chromatography according to Coutteau and Sorgeloos (1995). The FAME profile was determined at day 28 of feeding trial 1.

4.2.3 Experimental design

The animals used for experimental purpose were *Litopenaeus vannamei*. For this work, approximately 250 Pacific white shrimp (12.6 ± 0.4 g) were received from the Laboratory of Aquaculture and Artemia Reference Centre (Ghent University, Belgium). The rearing units consisted of 40 L rectangular tanks. The tanks were set up as closed systems without biofilter unit and heaters were provided to control water temperature around 27-28°C together with an aeration device maintaining dissolved oxygen concentrations above 5 mg O_2/L. The tanks were filled with 40 L of seawater and on a daily basis 8 L of the seawater from the experimental tanks was replaced by fresh seawater. With this, uneaten food particles and bioflocs were largely removed one a day, but not measured. The photoperiod was 12/12 hours. The water of the rearing tanks was analyzed for total ammonium nitrogen (TAN), NO_2^--N and NO_2^--N using a colorimetric

ammonium test kit (Aquamerck) and nitrite and nitrate test kits (Merckoquant), respectively.

4.2.3.1 Feeding trial 1 with complete replacement of the artificial feed

At the start of the experiment, the shrimp had an average weight of 12.6 ± 0.3 g resulting in an initial density of 3.8 kg m^{-3}. The experiment consisted of five separate treatment groups, performed in duplicate. Each group comprised 12 shrimp that were divided in groups following a completely randomized design. The treatment groups (defined by the feed given to the white shrimp) were as follows:

- Control: Artificial feed with 35% DW crude protein;
- Glucose: Bioflocs grown on glucose as a carbon source;
- Glucose + Probiotics: Bioflocs grown on glucose as a carbon source were a probiotic *Bacillus* mixture was added to the biofloc reactor;
- Glycerol: Bioflocs grown on glycerol as a carbon source;
- Glycerol + Probiotics: Bioflocs grown on glycerol as a carbon source were a probiotic *Bacillus* mixture was added to the biofloc reactor.

In the control treatment, an artificial feed was given at a rate of 2.5% of the wet shrimp biomass. The shrimps were fed two times daily. The different bioflocs were given to the test organisms as diets equal in nitrogen. The nitrogen content of the bioflocs was determined daily and on wet weight basis through analysis of the Kjeldahl nitrogen, TAN and wet weight of the biomass. The experiment was carried out for 28 days at the Laboratory of Aquaculture and *Artemia* Reference Centre (Ghent University, Belgium). For each treatment, two individuals were taken on day 28 for whole body FAME analysis. The fatty acid composition of the shrimp was determined by gas chromatography following a modified procedure of Folch et al. (1957) as described by Ways and Hanahan (1964). The shrimp samples were first dried and pulverized to obtain a homogenous sample. Survival and growth were calculated based on the following formulae:

$$\text{Survival (\%)} = \{(No - Nt) / No\} \cdot 100\%$$

With No: initial number of shrimp; Nt: number of dead shrimp

$$\text{Specific growth rate (\% body weight day}^{-1}\text{)} = \{(\ln(Wt) - \ln(Wo)) / t\} \cdot 100\%$$

With Wo: initial weight (g); Wt: weight at day t (g); t: culture period (days)

4.2.3.2 Feeding trial 2 with partial replacement of the artificial feed

The second experiment again consisted of five separate treatment groups performed in duplicate, with each group comprising 12 shrimp following a completely randomized design. The initial weight of the shrimp averaged 16.3 ± 0.3 g, resulting in an initial density of about 4.9 kg m^{-3}. The treatment groups consisted of a control treatment where artificial feed with a 35% DW crude protein content was added and four groups that were fed with the different bioflocs in combination with the artificial feed. The different bioflocs and the artificial feed were given twice daily to the test organisms as diets equal in nitrogen. The experiment was conducted for a period of 28 days.
The treatments were as follows:

- Glucose: Artificial feed and bioflocs grown on glucose as a carbon source;
- Glucose + Probiotics: Artificial feed and bioflocs grown on glucose as a carbon source were a probiotic *Bacillus* mixture was added to the biofloc reactor;
- Glycerol: Artificial feed and bioflocs grown on glycerol as a carbon source;
- Glycerol + Probiotics: Artificial feed and bioflocs grown on glycerol as a carbon source were a probiotic *Bacillus* mixture was added to the biofloc reactor.

A substitution level of the artificial feed of 50% as compared to the control treatment was carried out during the first 14 days of the experiment. Due to low survival and visual observations regarding shrimp fitness (the shrimp were not eating from the glucose bioflocs from day 14 of the experiment onwards), the substitution level was decreased to 10% and 30% for the glucose and glycerol treatments, respectively. Survival and growth were calculated based on the same formulae as in trial 1. On day 20 of the experiment, the average total *Vibrio* count (TVC) of the shrimp rearing water was determined by plate counting on thisulfate-citrate-bile-salt-sucrose (TCBS) agar.

4.2.4 Statistical analyses

The results were submitted to statistical analysis using the software SPSS 16.0. A two-way ANOVA followed by a Tukey's test was employed to test the significant differences of the crude protein content of the bioflocs and the water quality parameters of the rearing tank water. A one-way ANOVA followed by a Tukey's test was employed to test the significant differences of shrimp survival and growth. The ratings were analyzed for mean differences at a 5% level of significance.

4.3 Results

4.3.1 Feeding trial 1 with complete replacement of the artificial feed

4.3.1.1 Nutritional properties of the bioflocs

The crude protein content of the bioflocs used in trial 1 did not change significantly throughout the experimental period. The average protein content was between 15 and 22 %, with no significant differences between the different treatments (Table 4.1). Total n-3 PUFA content ranged between 0.3 and 0.9 mg/g DW, with glucose flocs having lower n-3 PUFA content than the other treatments, although the difference was not significant ($p > 0.05$). Total n-6 PUFA content varied between 9 and 28%, with relatively high variability.

4.3.1.2 Water quality in the shrimp rearing tanks

The TAN and nitrite levels and temperature in the white shrimp rearing units were measured throughout the experiment right before the daily water replacement. TAN levels were relatively low in all treatments, with no significant differences between the different treatments (Table 4.2). Nitrite levels were significantly higher in the control treatment than in the other treatments, although the average concentration was still relatively low (Table 4.2).

Table 4.1 Primary nutritional value of the bioflocs used as a feed for *Litopenaeus vannamei* in feed trial 1, the bioflocs were grown on different carbon sources with or without addition of a probiotic *Bacillus* mixture.

	Glucose	Glucose + *Bacillus*	Glycerol	Glycerol + *Bacillus*
Crude protein (% DW)	19 ± 7	20 ± 4	15 ± 3	22 ± 10
Total n-3 PUFA (mg/g DW)	0.3 ± 0.3	0.7 ± 0.3	0.7 ± 0.1	0.9 ± 0.1
Total n-6 PUFA (mg/g DW)	9 ± 11	28 ± 32	12 ± 16	17 ± 16

Table 4.2 Average TAN and NO_2^--N concentrations and temperature of the shrimp culture water in feed trial 1. Bioflocs used as feed were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

	Artificial feed	Glucose	Glucose + *Bacillus*	Glycerol	Glycerol + *Bacillus*
TAN (mg/L)	0.5 ± 0.7[a]	0.7 ± 0.8[a]	0.9 ± 1.2[a]	0.5 ± 0.3[a]	0.8 ± 1.0[a]
NO_2^--N (mg/L)	0.7 ± 0.3[a]	0.2 ± 0.2[b]	0.4 ± 0.4[b]	0.3 ± 0.2[b]	0.3 ± 0.3[b]
Temperature (°C)	27 ± 2[a]	28 ± 1[a]	27 ± 1[a]	27 ± 2[a]	28 ± 2[a]

Mean values in the same row with a different superscript differ significantly ($p < 0.05$).

4.3.1.3 Shrimp performance

In the first trial, the feed of the shrimp in the floc treatments was completely replaced by bioflocs. The bioflocs collected from the reactors (Figure 4.1 A) for shrimp feeding were ingested by the shrimp as indicated by the color of their digestive tract (Figure 4.1 B and C).

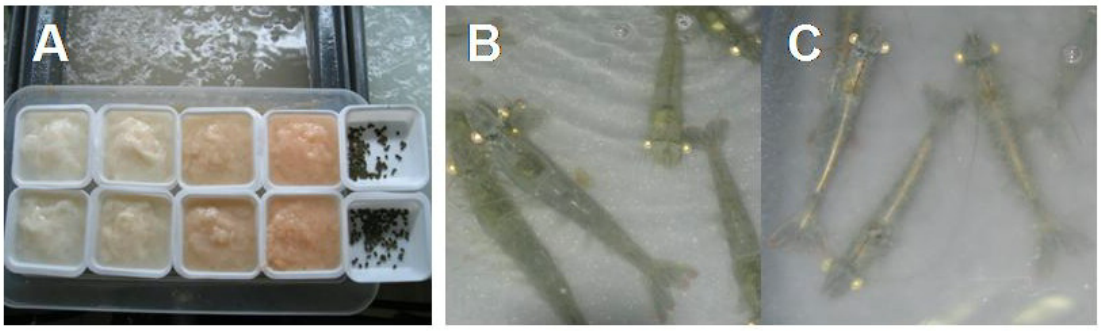


Figure 4.1 A) Bioflocs collected from the different reactors and the artificial feed used in the feeding trials. From left to right: glucose-grown flocs, glucose-grown flocs inoculated with *Bacillus*, glycerol-grown flocs, glycerol-grown flocs inoculated with *Bacillus*, artificial feed. B) Shrimp fed with artificial feed. Note the green digestive tract. C) Shrimp fed with bioflocs. Note the white digestive tract.

The survival of shrimp fed glucose flocs (with and without *Bacillus*) was lower than that of shrimp fed artificial feed or glycerol flocs (both with and without *Bacillus*), although the difference was only significant for the glucose + *Bacillus* treatment (Table 4.3). The shrimp fed an artificial diet showed better growth characteristics than shrimp fed bioflocs, as manifested by higher body weight and SGR (Table 4.3). The highly unsaturated fatty acid (HUFA) profile of the shrimp and the bioflocs measured on day 28

are presented in Table 4.4. In general, bioflocs from the reactors that were inoculated with the *Bacillus* mixture showed higher fatty acid content than flocs without *Bacillus*. However, this was not reflected in the fatty acid composition of the shrimp. Shrimp fed the artificial diet had the highest fatty acid content, followed by shrimp fed glucose-grown bioflocs (with and without *Bacillus*) and shrimp fed glycerol-grown bioflocs (with and without *Bacillus*) showed the lowest fatty acid content. During the experiment, some abnormalities were observed at the uropods of shrimp fed with bioflocs originating from the glucose reactors (Figure 4.2).

Figure 4.2 A) *Litopenaeus vannamei* with normal tail; B) Shrimp with abnormality at the uropods observed in animals from the glucose bioflocs-fed treatment.

Table 4.3 Survival, average body weight (ABW), specific growth rate (SGR) and cumulative specific growth rate (SGRc) of shrimp in feed trial 1. The shrimp were fed either an artificial feed or bioflocs. Bioflocs used as feed were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

	Artificial feed			Glucose			Glucose + *Bacillus*			Glycerol			Glycerol + *Bacillus*		
DOC (day)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGR (% BW
7	100 ± 0	13.5 ± 0.3	0.7 ± 0.4	100 ± 0	12.5 ± 0.3		100 ± 0	12.6 ± 0.6		100 ± 0	12.6 ± 0.1		100 ± 0	12.5 ± 0.5	
14	100 ± 0	14.6 ± 0.9	0.9 ± 0.5	100 ± 0	12.5 ± 0.0		100 ± 0	12.4 ± 0.6		100 ± 0	12.8 ± 0.1		100 ± 0	13.0 ± 0.2	
21	100 ± 0	15.4 ± 0.8	0.9 ± 0.3	88 ± 6	12.3 ± 0.3		88 ± 6	11.5 ± 0.3		100 ± 0	12.8 ± 0.7		100 ± 0	14.7 ± 2.6	
28	96 ± 6^{ab}	16.2 ± 1.6^{A}	0.8 ± 0.4^{x}	71 ± 6^{bc}	11.9 ± 0.8^{B}	-0.2 ± 0.2^{y}	54 ± 18^{c}	13.8 ± 1.0^{AB}	0.3 ± 0.1^{xy}	100 ± 0^{a}	12.7 ± 0.9^{B}	0.0 ± 0.0^{y}	92 ± 12^{ab}	12.6 ± 0.1^{B}	0.1 ± (

Mean values on day 28 with a different superscript differ significantly ($p < 0.05$).

Table 4.4 Fatty acids profile of shrimp and bioflocs in feed trial 1 on day 28. Bioflocs used as feed were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

	Shrimp					Bioflocs			
	Control (d 28)	Glucose	Glucose + Probiotics	Glycerol	Glycerol + Probiotics	Glucose	Glucose + Probiotics	Glycerol	Glycerol + Probiotics
16:0	7.8	5.0	5.1	3.1	2.7	4.6	14.9	9.9	6.8
18:0	3.3	3.0	3.1	2.7	2.2	1.9	6.5	6.8	4.1
18:1(n-9)	5.8	3.9	3.9	2.6	2.1	5.1	18.4	10.7	5.3
18:1(n-7)	1.2	1.0	0.9	0.6	0.7	3.4	5.0	1.3	3.5
18:2(n-6)	4.6	3.4	3.3	3.0	2.1	8.5	27.2	11.8	16.2
18:3(n-3)	0.5	0.3	0.3	0.1	0.1	1.4	1.6	0.4	0.2
20:1(n-9)	1.5	0.7	0.8	0.2	0.2	0.0	0.1	0.0	0.0
20:4(n-6)	0.7	1.1	1.0	0.9	0.9	0.1	0.4	0.1	0.2
20:5(n-3)	5.1	4.0	4.7	2.9	2.9	0.0	0.2	0.0	0.2
22:6(n-3)	5.1	2.9	3.1	1.7	1.7	0.0	0.1	0.2	0.3
Total n-6	5.3	4.6	4.4	4.0	3.0	8.6	27.6	12.0	16.6
Total n-3	10.9	7.2	8.2	4.7	4.8	0.2	0.7	0.7	0.9

4.3.2 Feeding trial 2 with partial replacement of the artificial feed

4.3.2.1 Nutritional properties of the bioflocs

The average protein content of the bioflocs used for trial 2 varied between 19 and 34 %. The crude protein contents of the glycerol bioflocs initially inoculated with a probiotic *Bacillus* mixture showed the highest levels and were significantly different from the non-inoculated glycerol bioflocs (Table 4.5). Total n-3 PUFA content ranged between 0.4 and 0.9 mg/g DW, with glucose flocs having lower n-3 PUFA content than glycerol flocs. Total n-6 PUFA content varied between 4 and 20%, with non-inoculated bioflocs showing higher values than *Bacillus* flocs (both for glycerol and glucose grown flocs), although the differences were not significant ($p > 0.05$).

Table 4.5 Primary nutritional value of the bioflocs used as a feed for *Litopenaeus vannamei* in feed trial 2. Bioflocs were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

	Glucose	Glucose + *Bacillus*	Glycerol	Glycerol + *Bacillus*
Crude protein (% DW)	29 ± 10^{ab}	27 ± 5^{ab}	19 ± 4^{a}	34 ± 2^{b}
Total n-3 PUFA (mg/g DW)	0.5 ± 0.1^{a}	0.4 ± 0.1^{a}	0.6 ± 0.1^{ab}	0.9 ± 0.1^{b}
Total n-6 PUFA (mg/g DW)	7 ± 9^{a}	1.0 ± 0.0^{a}	20 ± 10^{a}	4 ± 1^{a}

Values in the same row with a different superscript differ significantly ($p < 0.05$).

4.3.2.2 Water quality shrimp rearing units

The TAN, nitrite and nitrate levels and temperature in the white shrimp rearing units were measured throughout the experiment. TAN levels were relatively low in all treatments, with only the biofloc- fed treatments [glucose + *Bacillus*] and [glycerol + *Bacillus*] showing a significant difference between each other (Table 4.6). Nitrite levels were significantly higher in the control treatment than in the other treatments, although the average concentration was still relatively low (Table 4.6).

Table 4.6 Average TAN and NO_2^--N concentrations and temperature of the shrimp culture in feed trial 2. Bioflocs used as feed were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

	Artificial feed	Glucose	Glucose + *Bacillus*	Glycerol	Glycerol + *Bacillus*
TAN (mg/L)	0.4 ± 0.2^{AB}	0.3 ± 0.4^{AB}	0.2 ± 0.3^{A}	0.5 ± 0.5^{AB}	0.6 ± 0.5^{B}
NO_2^--N (mg/L)	0.8 ± 0.3^{a}	0.3 ± 0.3^{b}	0.3 ± 0.3^{b}	0.4 ± 0.3^{b}	0.5 ± 0.4^{b}
Temperature (°C)	28 ± 1^{x}	28 ± 0^{x}	27 ± 1^{x}	28 ± 1^{x}	28 ± 1^{x}

Mean values in the same row with a different superscript differ significantly ($p < 0.05$).

4.3.2.3 Shrimp performance

In contrast to the first trial, the feed of the shrimp was only partially replaced by bioflocs in the second trial. The survival of shrimp fed glucose flocs (with and without *Bacillus*) was again lower than that of shrimp fed artificial feed or glycerol flocs (with and without *Bacillus*), although the differences were less pronounced than in the first trial (Table 4.7). In contrast to the first trial, there were no differences in growth performance between the different treatments (Table 4.7).

During the experiment once more some abnormalities were observed and therefore the substitution level of the artificial feed was adjusted on day 14 from 50% to 10 and 30% for the glucose and glycerol treatments, respectively. Furthermore, the Total *Vibrio* Count of the shrimp rearing water was determined on day 20 to check for pathogens. Vibrios were found to be present in the culture water of all treatments, with the highest levels in the control treatment (Table 4.8). The lowest *Vibrio* counts were found in the treatments with *Bacillus* containing flocs, with 10-fold lower levels in the glucose + *Bacillus* treatment than in the control treatment.

Table 4.7 Survival, average body weight (ABW), specific growth rate (SGR) and cumulative specific growth rate (SGRc) of shrimp in feed trial 2. The shrimp were fed either an artificial feed or artificial feed partially replaced by bioflocs. Bioflocs used as feed were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

	Control			Glucose			Glucose + Probiotics			Glycerol			Glycerol + Probiotics		
DOC (day)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGRc (% BW/d)	SR (%)	ABW (g)	SGR (% BW
7	100 ± 0	17.2 ± 0.3		100 ± 0	17.0 ± 0.1		100 ± 0	17.1 ± 0.5		100 ± 0	17.0 ± 0.4		100 ± 0	16.7 ± 0.2	
14	100 ± 0	17.7 ± 1.1		100 ± 0	16.8 ± 0.4		85 ± 7	17.1 ± 0.5		100 ± 0	17.3 ± 0.3		95 ± 7	17.2 ± 0.0	
21	100 ± 0	18.9 ± 2.1		83 ± 0	17.2 ± 0.2		79 ± 16	18.1 ± 0.1		100 ± 0	18.3 ± 0.2		92 ± 12	17.6 ± 0.2	
28	100 ± 0^{a}	19.9 ± 1.6^{A}	0.8 ± 0.2^{x}	79 ± 6^{ab}	18.2 ± 0.7^{A}	0.4 ± 0.1^{x}	67 ± 23^{b}	19.0 ± 0.1^{A}	0.5 ± 0.0^{x}	100 ± 0^{a}	19.6 ± 1.1^{A}	0.7 ± 0.2^{x}	83 ± 0^{ab}	18.1 ± 0.7^{A}	0.4 ± (

Mean values on day 28 with a different superscript differ significantly ($p < 0.05$).

Table 4.8 Total *Vibrio* counts in the shrimp culture water at day 20 of feed trial 2 (mean ± standard deviation of plate counts on TCBS agar). The shrimp were fed either an artificial feed or artificial feed partially replaced by bioflocs. Bioflocs used as feed were either grown on glucose or glycerol, with and without inoculation of a probiotic *Bacillus* mixture.

Treatment	*Vibrio* counts (x 10^3 CFU mL^{-1})
Artificial feed	5.7 ± 0.4
Glucose	1.5 ± 0.2
Glucose + *Bacillus*	0.6 ± 0.1
Glycerol	1.7 ± 0.1
Glycerol + *Bacillus*	1.1 ± 0.1

4.4 Discussion

Bioflocs technology aims at improving water quality in aquaculture systems by carefully balancing carbon and nitrogen. In this study, we studied the effect of bioflocs grown on two different carbon sources (glucose and glycerol) for white shrimp (*Litopenaeus vannamei*). In addition, the effect of initial inoculation of the biofloc reactors with a commercial probiotic mixture consisting of different *Bacillus* strains on the biofloc composition and hence performance of the shrimp was also studied. The nutritional properties of the bioflocs differed depending on the carbon source used. The highest protein content was noted in the (glycerol + *Bacillus*) bioflocs, followed by the glucose and (glucose + *Bacillus*) bioflocs, whereas glycerol bioflocs showed the lowest protein content in both trials. These results confirm the findings of Crab et al. (2010b) where bioflocs grown on (Glycerol + *Bacillus*) showed a higher crude protein content when compared to acetate, glycerol and glucose grown bioflocs. In general, the nutritional parameters of the bioflocs were within the appropriate range for shrimp feed and therefore, the acceptance, palatability and digestibility of the bioflocs were studied in two feeding trials with complete or partial replacement of shrimp feed by bioflocs.

In the first feeding trial the uptake of bioflocs by the shrimp was manifested by the light color of their digestive tract (Figure 4.1). Survival, average body weight and cumulative specific growth rate of the shrimp in the different treatments was compared after 28 days of culturing. Survival of shrimp fed glucose-grown bioflocs was lower than that of shrimp fed artificial feed or glycerol-grown bioflocs (Table 4.3). The lower survival of the shrimp fed glucose-grown bioflocs was likely due to starvation because the animals stopped eating from the second week of culture onward. This observation stresses the importance of the accessibility and the palatability of the feed. The shrimp fed an artificial diet showed a better growth performance than shrimp fed bioflocs. Moreover, some abnormalities (tail biting) were observed in the shrimp, especially those fed with glucose-grown bioflocs (Figure 4.2). Therefore, a second feeding trial was performed in which the artificial feed was only partially replaced by bioflocs. Partial replacement of the artificial feed by glucose bioflocs yielded good shrimp survival, but their growth performance was considerably lower than in shrimp fed an artificial diet (Table 4.7). The floc structure or taste of the glucose bioflocs might have caused a lower uptake or digestibility towards the shrimp. In the glycerol biofloc treatment, where 30% of the artificial feed was replaced, survival and growth performance of the shrimp did not significantly differ from survival and growth of shrimp fed an artificial diet (Table 4.7).

In all other treatments, the cumulative specific growth rate was about a factor 2 lower than in the treatments with artificial diet and glycerol-grown bioflocs.
Since the uptake of neither artificial feed nor bioflocs was quantified, it is not conclusive to tell whether the observed effects were derived from the biofloc differences or intake. It is possible that the shrimp preferentially ate the artificial feed.

In feeding trial 1, the highly unsaturated fatty acids (HUFA) profile was measured for shrimp and bioflocs. The shrimp of all biofloc treatments showed a lower content in HUFA than the shrimp of the artificial feed treatment, except for arachidonic acid (20:4(n-6), AA) (Table 4.4). The bioflocs profiles revealed that they contain a higher concentration of total n-6 than total n-3 HUFA. The findings in the study of Wouters et al. (2001) indicate the importance of n-3 HUFA for larval development. These nutrients cannot be synthesized de novo by shrimp, and should be included at high levels in the diet. HUFA such as arachidonic (20:4(n-6), AA) eicosapentaenoic (20:5(n-3), EPA) and docosahexaenoic (22:6(n-3), DHA) acids are important nutrients and are considered essential fatty acids because of the limited ability of shrimp to elongate and desaturate shorter-chain polyunsaturated fatty acids (PUFA) to HUFA (Gonzalez-Felix et al., 2002). Bioflocs are characterized by the lack of the n-3 marine fatty acids EPA and DHA, which are essential for the growth and survival of marine shrimp (Amaya et al., 2007) and might also have a suboptimal composition of other essential nutrients such as vitamins, minerals, cholesterol, indispensable amino acids, attractants and unidentified growth factors. Hence, these essential nutrients need to be supplied through artificial feed and bioflocs can only be used as a partial replacement for artificial feed. This notion is consistent with the feed trials performed in this study, in which partial replacement of the artificial diet with bioflocs resulted in better survival and performance of white shrimp than complete replacement.

Finally, the effect of inoculating the biofloc reactors with a probiotic *Bacillus* mixture on *Vibrio* levels in the shrimp cultures was examined. The mixture did not result in increased performance of the shrimp. However, the *Vibrio* levels were lower in both *Bacillus* treatments than in the other treatments (Table 4.8). These results indicate that inoculating biofloc reactors with probiotic bacteria might have a biocontrol effect towards *Vibrio* spp., but this field of research needs further in depth investigation.

In conclusion, the results presented in this study demonstrate that fishmeal can be partially replaced using bioflocs as an alternative protein source without compromising survival and performance of white shrimp (*Litopenaeus vannamei*). Hence, by applying bioflocs technology in white shrimp culturing, a lower quantity of (artificial) feed would be required to obtain the same growth performance, thereby maintaining a good water

quality. This would lead to higher nutrient efficiency and lower costs, together with a lower environmental impact on ecosystems receiving the effluents.

In addition to *Macrobrachium rosenbergii* and *Litopenaeus vannamei*, there are other species that can benefit from the use of BFT in their culture systems. A specific case studied in the following chapter is that of tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) during overwintering. This study performed in Israel, looked at the advantage of using BFT as a means to control water quality in low water exchange greenhouse ponds so that low pond water temperatures were prevented during winter.

CHAPTER 5

BIOFLOCS TECHNOLOGY APPLICATION IN OVERWINTERING OF TILAPIA

A 50-day experiment was conducted to investigate the effectiveness of the bioflocs technology for maintaining good water quality in overwintering ponds for tilapia hybrid fingerlings (*Oreochromis niloticus* x *Oreochromis aureus*). To preserve adequate water temperatures in the ponds, they were covered with polyethylene sheets and the water exchange rate was minimized to increase pond water temperature. To avoid water quality deterioration, starch was added to the ponds to stimulate the formation of bioflocs. Temperature in the covered ponds could easily be controlled and was 0.4 – 4.9°C higher than the influent water. Adjusting the C/N ratio in the ponds by adding starch or increasing the amount of carbohydrates added through the feed limited the presence of inorganic nitrogen species when the C/N was about 20, even at high stocking densities of 20 kg/m^3 at harvest. Fish survival levels were excellent, being 97 ± 6% for 100 g fish and 80 ± 4 for 50 g fish. Moreover, at harvest the condition of the fish was good in all ponds with a fish condition factor of 2.1 – 2.3. Overall, these findings can help to overcome overwintering problems, particularly mass mortality of fish due to low temperatures in the ponds.

Redrafted after:

Crab, R., Kochva, M., Verstraete, W. and Avnimelech, Y., 2009. Bioflocs technology application in over-wintering of tilapia. Aquacultural Engineering 40, 105-112.

5.1 Introduction

Tilapiine fishes (family Cichlidae) originate from the tropical and subtropical parts of Africa and colonized into the Middle East through the Great Rift Valley (Fryer and Iles, 1972). Presently, tilapia spp. are grown in many countries in Europe, North and South America. In temperate and some subtropical regions, their culture is highly affected by sensitivity to low water temperatures leading to poor growth and mass mortality during overwintering (Charo-Karisa et al., 2005). More than 50% of tilapia grown in Israel died during a cold spell in January 2008. The Bet Shaan Regional Council estimated the damage to be worth 11.3 million € (Lappin, 2008). In China, observers said that the production early 2008 declined by as much as 80% and tilapia traders estimated that there will be a scarcity in supply in 2008 which could last for at least 8 – 12 months (AQUA Culture AsiaPacific Magazine, 2008). Similar catastrophic events occurred previously and in other countries (Hsieh et al., 2004; Sifa et al., 2002). In Israel, as well as in many temperate regions, these temperature restrictions limit the grow-out period between 6 and 8 months (Hofer and Watts, 2002; Sarig and Arieli, 1980; Zohar et al., 1985). In order to maximize the grow-out season and to get market size fish, juveniles are grown up to a weight of 50 – 100 g in June – July before the winter months (Charo-Karisa et al., 2005). The juveniles need to be stocked during winter after which they are transferred to production ponds in the spring to get market size fish during the summer. Larger fish also need to be kept along the cold season so as to supply fish to the market along the winter, or to start fattening them in the spring to get market size fish in the early spring and summer season (Dan and Little, 2000b; Charo-Karisa et al., 2005).

Successful overwintering of tilapia in ponds has been conducted using heated facilities, geothermal water and greenhouses insulated with plastic sheet covers (Dan and Little, 2000a). A practical way to provide a proper temperature in the ponds is to cover the ponds with plastic sheets to preserve the temperature in the water and to absorb the solar radiation for heating (Rothbard and Peretz, 2002). However, an intensive utilization of the pond is required to justify the investment. In addition, water exchange should be kept to a minimum in order not to loose heat when the replacement water is cold. Holding dense fish populations with limited water exchange gives rise to a substantial build-up of waste, comprising uneaten feed and feces, as well as other metabolic residues including ammonia excreted by the fish (Read and Fernandes, 2003; Piedrahita, 2003; Sugiura et al., 2006). Even low concentrations of ammonia and nitrite are toxic to most aquaculture species (Timmons et al., 2002). Therefore, control of

ammonia and nitrite at an adequate concentration while keeping water exchange minimal is vital.
The bioflocs technology (BFT) seems to be a practical solution for overwintering of tilapia in conjunction with densely stocked greenhouse ponds with limited water exchange. The BFT is the development and control of dense heterotrophic microbial bioflocs in the water column by adding carbohydrates to the pond. This suspended growth consists of phytoplankton, bacteria, aggregates of living and dead particulate organic matter, and grazers of the bacteria. If carbon and nitrogen are well balanced, ammonium in addition to organic nitrogenous waste will be converted into bacterial proteinaceous biomass (Avnimelech, 1999; Schneider et al., 2005). Hence, adding carbohydrates enhances the water quality in the pond and in addition, the bioflocs can be consumed and hereby act as a source of feed by the cultivated aquatic organism (Burford et al., 2003; Burford et al., 2004; Hari et al., 2004; Avnimelech, 2005).

The objectives of this study were motivated by the urgent need to develop a sustainable method for overwintering of tilapia fingerlings. This work represents a concentrated effort to propose the proper technology within a single season. The study started with a series of experiments aimed at evaluating the rate of nitrogen enrichment in the water under the conditions of fish growing at relatively low temperature and limited water exchange in ponds with a high and low organic carbon to nitrogen (C/N) ratio. The next stage was to investigate the effect of the proper elevated organic C/N ratio, induced by starch additions or increasing the amount of carbohydrates added through the feed, on the nitrogen balance in the ponds and the ability of the BFT system to prevent inorganic nitrogen accumulation during winter conditions. The study included 2 sizes of fingerlings, being 50 and 100 g, to cover both extremes of the size interval of fingerlings typically used for overwintering. It has to be emphasized that due to time and space limitations, there were in course corrections and adaptations of treatments, aimed at targeting the work on the more important treatments. Parts of the treatments, mostly the extreme, seemingly un-practical ones, were not replicated.

The part of the work reported here in detail was based upon periods along the growing cycle when water exchange was completely stopped and nitrogen balances and dynamics were monitored. The processes shown in these sub-treatments gave some insight on the effects of organic C/N ratio on nitrogen dynamics and can serve as a guideline to minimize cold season damages in tilapia culturing. Further refinement will be needed in the future.

5.2 Materials and methods

5.2.1 Experimental design

The experiments were conducted during the winter period of 2008, from the13th of January till the 4th of March. The pond experiments were carried out in 10 circular concrete ponds, 1 meter deep and an area of 50 m^2 each, in the Genosar Research Station (Genosar, Israel). A paddlewheel aerator of 0.75 kW aerated each pond and a 0.37 kW upward flow aerator was placed in the centre of each pond to keep fine particles in suspension. Ponds were stocked with 100 g tilapia hybrids (*Oreochromis niloticus* x *Oreochromis aureus*) at a stocking density of about 16 kg/m^3, except for three ponds that were stocked with 50 g fingerlings (Table 5.1).

Table 5.1 Stocking of ponds and fish growth, survival and condition factor at harvest.

Pond No.	At stocking		At harvest		
	No. of fish	Mean weight (g)	Survival (%)	Daily growth (g/fish)	Condition factor
1	8 130	98.4	102.4	0.26	2.2
2	7 583	105.5	89.0	0.35	2.3
3	9 195	87.0	94.6	0.25	2.2
4	8 602	93.0	96.7	0.28	2.2
5	8 602	93.0	105.8	0.31	2.2
6	9 195	87.0	91.8	0.27	2.1
7	9 195	87.0	95.7	0.28	2.1
8	16 000	50.0	84.6	0.28	2.2
9	16 000	50.0	78.7	0.28	2.2
10	16 000	50.0	78.1	0.25	2.1

Convex polyethylene greenhouses covered each pond to preserve temperature in the water and to absorb solar radiation for heating. The daily water exchange rate used in the ponds was initially about 24%. Water for the ponds was extracted from Lake Kinneret (Sea of Galilee) and was characterized by a temperature of 14.4 ± 0.5°C and a salinity of 250 mg Cl/L. At the onset of the experiment on the 13th of January, daily water exchange was lowered to 10%.

In a first series of experiments the effect of an organic C/N ratio ranging from 18 to 22 was explored. This series of experiments indicated an optimal organic C/N ratio of about 20 to control inorganic nitrogen at the present winter temperatures (data not

shown). For verifying the effects of the organic C/N ratio on the evolution of the total, inorganic and organic suspended nitrogen concentration, water exchange was eliminated for a period of 5 days, from the 3rd till the 7th of February. Two treatments with their respective control treatment were monitored in this assay, based upon feeding with either 23 or 30% protein pellets with the addition of starch (23% P + STARCH and 30% P + STARCH, respectively) and without addition of starch (23% P and 30% P, respectively). The amounts of 23% and 30% protein pellets were dosed to provide all ponds with the same protein input (1.5 kg protein/day) and the starch was added daily after the addition of the feed. The organic C/N ratios were 10.8 and 14 in the treatments without starch and 20.4 and 20.5 in the treatments with starch. The detailed feeding regime and starch addition is given in Table 5.2. Feeding started at 8 AM and the feed was dosed every 30 minutes and this in 3 subsequent doses. Starch addition was carried out at 10 AM.

Table 5.2 Different treatments varying in protein content of the pellet feed added and the amount of starch applied to the pond. This results in different organic C/N ratios in the pond water.

Treatment	Feed protein (% DW)	Feed added (kg/day)	Starch addition (kg/day)	C/N	Pond No.
30% P	30	5	0	10.8	1
30% P + STARCH	30	5	4.5	20.4	3, 5, 8
23% P	23	6.5	0	14.0	2, 9
23% P + STARCH	23	6.5	3	20.5	4, 6, 7, 10

The water quality and the development of the bioflocs were followed up more into detail in the ponds for all different treatments and this during 24 hours at different days along the experiment. These results can give more insight into the distribution of the different inorganic nitrogen species in the different treatments. Results were similar for treatments 23% P and 30% P, and 23% P + STARCH and 30% P + STARCH. In this paper the results are represented for the measurements carried out on the 29th and 30th of January and this in pond 2 and 4, representing treatment 23% P and 23% P + STARCH, respectively. Measurements started at 7 AM and were carried out every 3 hours till 7 PM and a final measurement was made at 7 AM the next day.

Feeding rates were adjusted to 30 – 40% of the conventional feeding rate, since the feed utilization is minimal during overwintering. Feeding rates were lowered when there were residues of uneaten floating pellets. This was only the case when temperature in the ponds was low and did not occur regularly.

5.2.2 Water analysis

Oxygen concentrations and water temperature were determined once each day at 7 AM. Initially, more measurements were performed (every 3 hours for 12 hours), and it was noted that the dissolved oxygen (DO) concentration and the temperature were rather stable over 24 hours and that the measurement at 7 AM is an indicative value of the average oxygen concentration and water temperature.
Water from the ponds was sampled periodically. Both a filtered (10 mL) and unfiltered (250 mL) sample was collected and kept at -20°C. The filtered water samples were analyzed for ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N). Unfiltered samples were analyzed for total suspended solids (TSS). The chemical properties of the water samples were determined following "Standard Methods for the examination of water and wastewater" (APHA, 1998). Nitrogen and organic carbon content of the suspended matter were determined in material collected after filtering with a GF/A filter. Dichromate and persulphate oxidation were used to determine organic carbon and nitrogen, respectively (Raveh and Avnimelech, 1972).
The volume of the bioflocs was determined using an Imhoff cone, registering the volume taken in by the flocs in 1 L of pond water after 15 – 20 minutes sedimentation. The sludge volume index (SVI) is defined as the volume in milliliters occupied by 1 g of TSS after settling (APHA, 1998).

5.2.3 Fish harvest

Fulton's condition factor was calculated at the end of the experiment according to the equation $CF = 100W/L^3$, where W is the wet body weight of fish expressed in grams and L is the standard body length expressed in centimeters (Ricker, 1975; Charo-Karisa et al., 2006a; Ali et al., 2008). This ratio is conventionally used to describe the condition of fish. The amount fed per tank was recorded daily, allowing calculation of food conversion ratio (FCR), being the food fed/biomass gain (Duston et al., 2004).

5.2.4 Molecular analysis

Biofloc samples for DNA extraction were taken from all experimental ponds, except from pond 9, on February 24th and sent to LabMET (Ghent, Belgium). Bioflocs were harvested from 250 mL of pond water taken at 7 AM and biomass was collected by

centrifugation (10 min, 8000 g). Pond 9 belongs to treatment 23% P. In this pond there is minimal stimulation of the BFT, hence, not enough biofloc biomass could be recovered. Total DNA was extracted from 2 g wet weight of the bioflocs suspension by a protocol reported previously by Boon et al. (2000), yielding 9 DNA samples for pond 1-8 and 10 (Grommen et al., 2005). To obtain DNA for further analysis of the total bacterial community by DGGE, a single round PCR was performed using a Taq polymerase kit (Fermentas GmbH, St-Leon Rot, Germany). The mastermix contained the following components: 10X reaction buffer with 15 mmol/L $MgCl_2$, 200 µmol/L of each deoxynucleoside triphosphate, 0.2 µmol/L of each primer, 2.5 U/(100 µL) of Taq DNA polymerase (Promega), 400 ng/µL of bovine serum albumin (Hoffman-La Roche, Basel, Zwitzerland), and DNase- and RNase-free filter-sterilized water (Sigma-Aldrich Chemie, Steinheim, Germany). The primers 338f-GC and 518r used in this study were as described by Muyzer et al. (1993), amplifying the V3 region of the 16S rRNA gene. In the PCR 1 µL of DNA was added to 24 µL of the PCR master mixture. After the PCR, the size of the amplicon was verified by running it next to a low range DNA Massruler^{MT} (Fermentas, Burlington, ON, Canada) on a 1% agarose gel. DGGE based on the protocol of Muyzer et al. (1993) was performed using the Bio-Rad DGene^{TM} system (Bio-Rad, Hercules, CA). PCR fragments were loaded onto an 8% (w/v, prokaryotic) polyacrylamide gel in 1 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4). To separate the amplified DNA fragments, the polyacrylamide gel was made with denaturing gradients ranging from 45% to 60% (100% denaturing contains 7 mol/L urea and 40% formamide). On the gel, a homemade marker of different PCR fragments was loaded, as required for processing and comparing the different gels. The electrophoresis was run for 16 hr at 60°C and 38 V. Staining of the gel was performed as described previously (Boon et al., 2002).

The obtained DGGE patterns were subsequently normalized and analyzed using the BioNumerics software version 3.5 (Applied-Maths, St-Martens-Latem, Belgium). Clustering was carried out using Pearson correlation and using UPGMA linkage created dendrograms. Relevant and non relevant clusters were further separated by the cluster cut-off method (BioNumerics Manual 2.5; Applied Maths, Sint-Martens-Latem, Belgium). Microbial community diversity analysis was based on the method of Lorenz (1905). Pareto curves describe the equality of distribution within a population and have been shown to be good estimators of species evenness (Mertens et al., 2005; Wittebolle et al., 2008; Marzorati et al., 2008). A Pareto curve is obtained as follows: abundances of the operational taxonomic units (OTUs) are ranked from high to low. The cumulative proportion of OTUs is used as x-axis and the cumulative proportion of abundances of OTUs represents the y-axis. This yields a convex curve. For Pareto curves applied to DGGE data the abscissas are the cumulative proportions of bands and the ordinates the cumulative proportions of the peak heights of the densiometric curves. The curves were evaluated by a horizontal y-axis projection of their intercepts with the vertical 20% x-axis line. The more the Pareto curve deviated from the 45° diagonal (the theoretical

perfect evenness line), the higher the projected y-value, thus the less evenness is observed in the structure of the studied community. The latter means that a smaller fraction of the different species was present in dominant numbers. These approaches of species evenness and richness were used to assess microbial diversity.

5.3 Results

5.3.1 Temperature and oxygen control

From mid-January to the end of February, the water temperature at the intake from Lake Kinneret was 14.4 ± 0.5°C down to a minimum of 13.3°C (Figure 5.1). Water temperature in the covered ponds was appreciably higher. The lowest recorded temperature was 14.2°C, but during most of the cold period, it was above 15°C and the average temperature was 18 ± 2°C. The temperatures in Lake Kinneret and in the experimental ponds are shown in Figure 5.1. Due to relatively higher air temperature and solar radiation at the later parts of the winter, water temperature rose to above 20°C, an un-favorable temperature for overwintering. Since the green houses did not have a ventilation option, heating of the water in the covered ponds was prevented by raising the water exchange rates in warm days to keep water temperature below 20°C.
The experimental ponds (50 m^3) were continuously aerated at a total capacity of 22.4 W/m^3. The oxygen concentration along the experimental period was in the range of 9 – 10 mg O_2/L in all ponds.

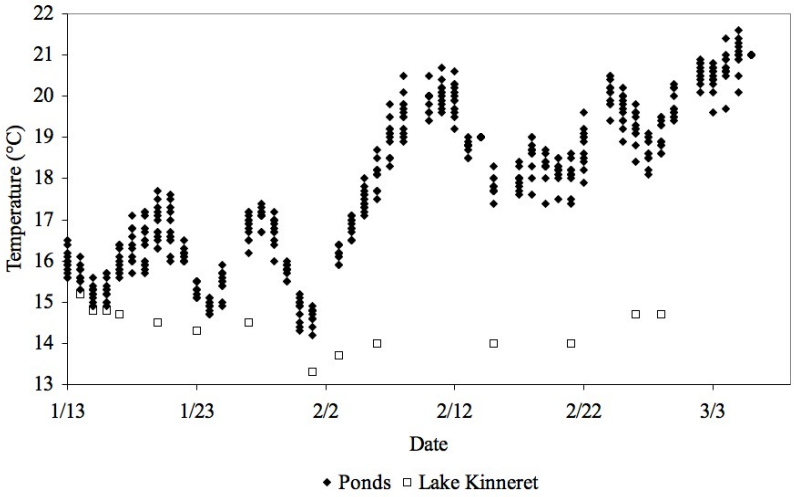


Figure 5.1 Water temperature in all experimental ponds provided with polyethylene covers and in Lake Kinneret.

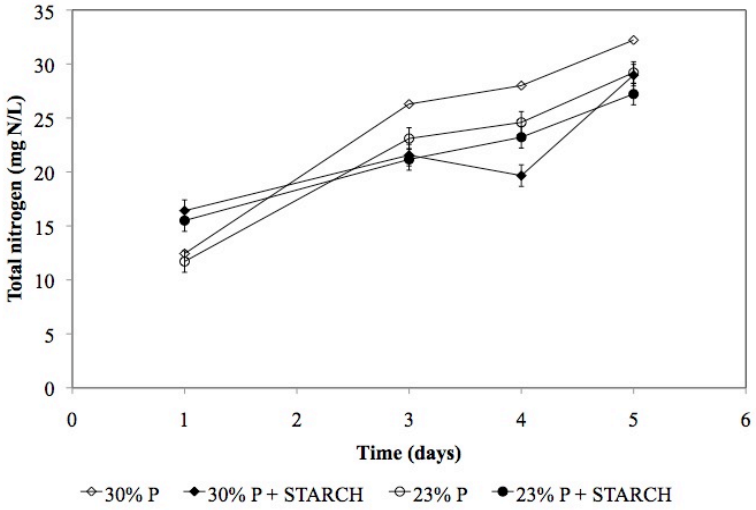
5.3.2 Nitrogen dynamics

From February 3rd till February 7th, the water exchange was interrupted for a period of 5 days. The changes in time in total nitrogen, inorganic nitrogen (the sum of ammonium, nitrite and nitrate concentrations) and organic suspended nitrogen concentrations are given in Figure 5.2 (a – c). Using a paired t-test the data was analyzed for significant changes over time. The total nitrogen concentration increased significantly in time in a similar manner in all experimental ponds, due to the equal amounts of nitrogen originating from the feed ($p < 0.05$). When comparing different treatments with each other, an independent two sample one-tailed t-test was carried out to distinguish significant changes. The ponds 30% P + STARCH and 23% P + STARCH, showed a significantly smaller increase in inorganic nitrogen species than the control pond 23% P ($p < 0.05$). A similar trend was observed towards the control pond 30% P, but could not be confirmed by statistical analysis since this treatment only consists out of 1 pond or has no repetitions. On the other hand, suspended organic nitrogen increased significantly in ponds 30% P + STARCH and 23% P + STARCH ($p < 0.05$), while in control ponds 23% P no significant increase was measured. Suspended organic nitrogen in treatment 30% P showed a similar trend as in treatment 23% P.

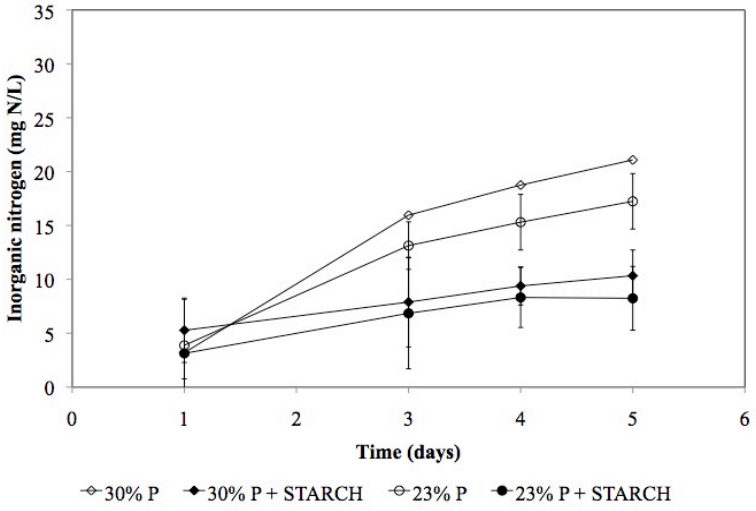
The water quality and the bioflocs development in ponds with different treatments were assessed during 24 hours on the on 29th and 30th of January. Water exchange was 0% during this period. Figure 5.3 (a – d) shows the variation in time of the different nitrogen species in the water for treatment ponds 2 and 4, representing treatment 23% P and 23% P + STARCH, respectively. Similar results were obtained in treatment ponds 30% P and 30% P + STARCH, hence, these results are not presented here. The evolution of the inorganic nitrogen species is illustrated more into detail and the changes in nitrogen species composition are specified. The concentrations of ammonium, nitrite and nitrate in pond 23% P without starch addition showed an increase, while in pond 23% P + STARCH there was a decrease in ammonium and nitrite and only a small increase in nitrate. When looking at the differences over 24 hours in inorganic, organic and total nitrogen (Figure 5.3 d), it is observed that the change in total nitrogen (inorganic + organic nitrogen) is almost equal. In the ponds receiving 23% protein pellets without starch, the increase in inorganic and organic nitrogen species is similar. When starch was added a small increase in inorganic nitrogen was observed. The change of total N was mainly due to the increase in organic nitrogen. Total suspended solids concentration decreased in 23% P (with 18 mg/L), while it increased (with 29 mg/L) in 23% P + STARCH.

The sludge volume indexes in all treatments were above 200 mL/g TSS with maximum values up to 500 mL/g TSS.

a)



b)



c)

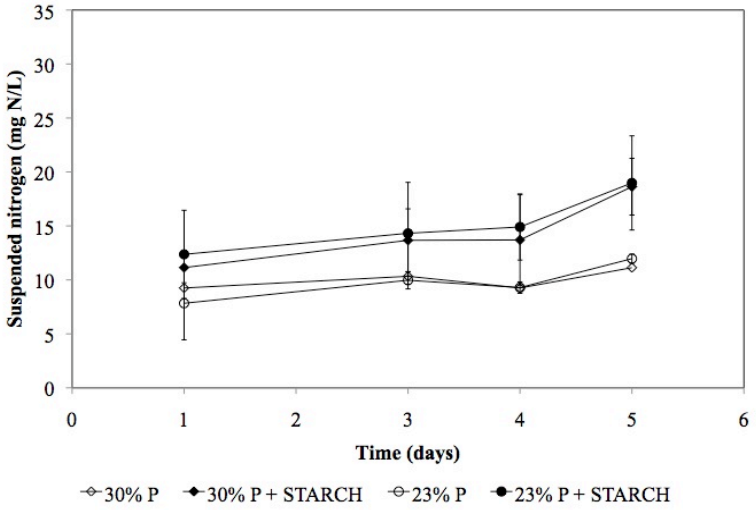
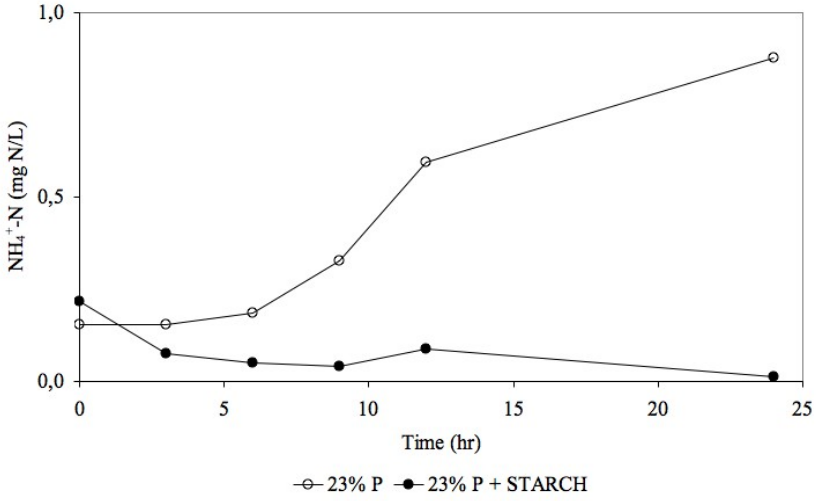
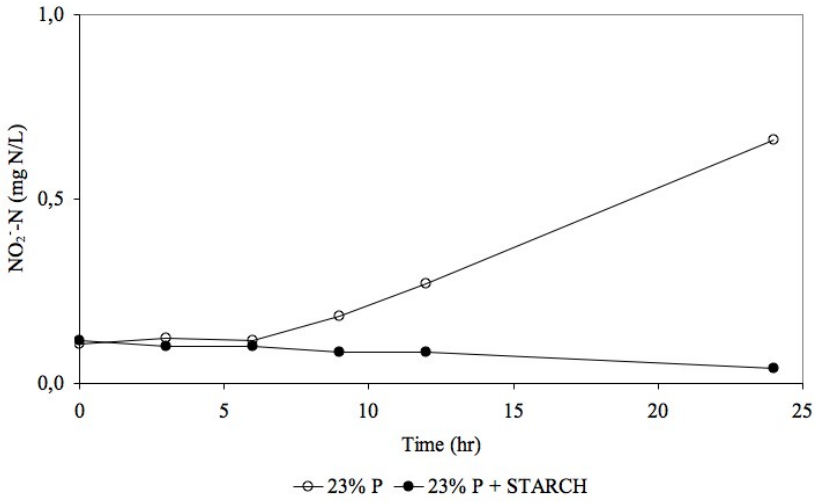


Figure 5.2 a) Changes over time of total nitrogen concentration in ponds fed with 23 or 30% protein pellets, with or without starch addition. b) Evolution over time of inorganic nitrogen concentration in ponds with 4 different treatments. c) Changes in suspended nitrogen concentration over time in different treatment ponds.

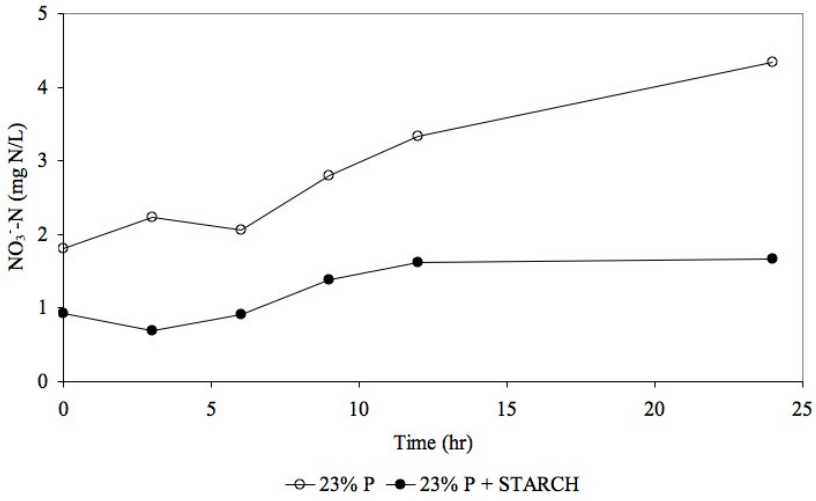
a)


1,0
0,5
0,0
NH_4^+-N (mg N/L)
0
5
10
15
20
25
Time (hr)
23% P
23% P + STARCH

b)


1,0
0,5
0,0
NO_2^--N (mg N/L)
0
5
10
15
20
25
Time (hr)
23% P
23% P + STARCH

c)


5
4
3
2
1
0
NO_3^--N (mg N/L)
0
5
10
15
20
25
Time (hr)
23% P
23% P + STARCH

d)

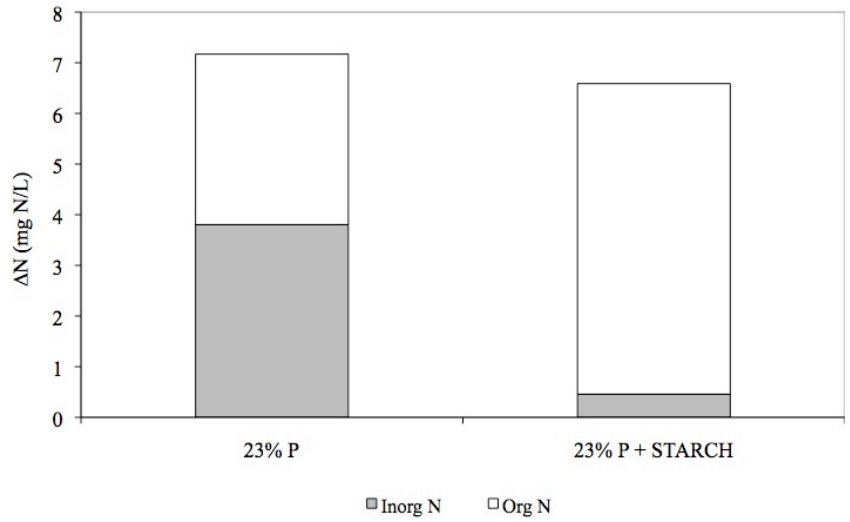


Figure 5.3 Changes of nitrogen species along a 24 hours period with no water release in ponds fed with 23% protein pellets without starch addition (23% P, C/N = 14.0) and with starch addition (23% P + STARCH, C/N = 20.5). a) Evolution of the ammonium concentrations. b) Evolution of the nitrite concentrations. c) Evolution of the nitrate concentrations. d) The change in inorganic, organic and total nitrogen over a 24 hr period.

5.3.3 Survival, growth and condition factor

The major goal in the overwintering of tilapia fingerlings is to obtain a high survival of the fish and to keep the fish in good condition for future growth in the production ponds. The survival, growth and condition factor of the fish at harvest are given in Table 5.1. Significant differences in survival, daily growth and condition factor between 50 and 100 g fish were checked with an independent two tailed sample one-tailed t-test. The survival of the 100 g fish stocked at a density of 16 kg/m^3 had an excellent average survival of 97 ± 6 %. The smaller fish of 50 g had a significantly lower survival, i.e. 80 ± 4 % ($p < 0.05$). The average daily growth was rather high, 0.29 ± 0.03 and 0.27 ± 0.02 g per fish stocked at a size of 100 and 50 g, respectively. The feed conversion ratio (FCR) did not significantly differ in between treatments and the overall FCR was 1.9 ± 0.4 kg feed/kg fish produced. The condition factor for fish of 100 and 50 g was 2.19 ± 0.07 and 2.17 ± 0.06, respectively.

5.3.4 DGGE results

The result of the cluster analysis of the DGGE is given in Figure 5.4. Molecular analysis was performed on all ponds, except pond 9 because of minor floc volume at time of sampling. When looking at the DGGE pattern (Figure 5.4 a) one sees some bands that are present in almost all ponds.

a)

b)

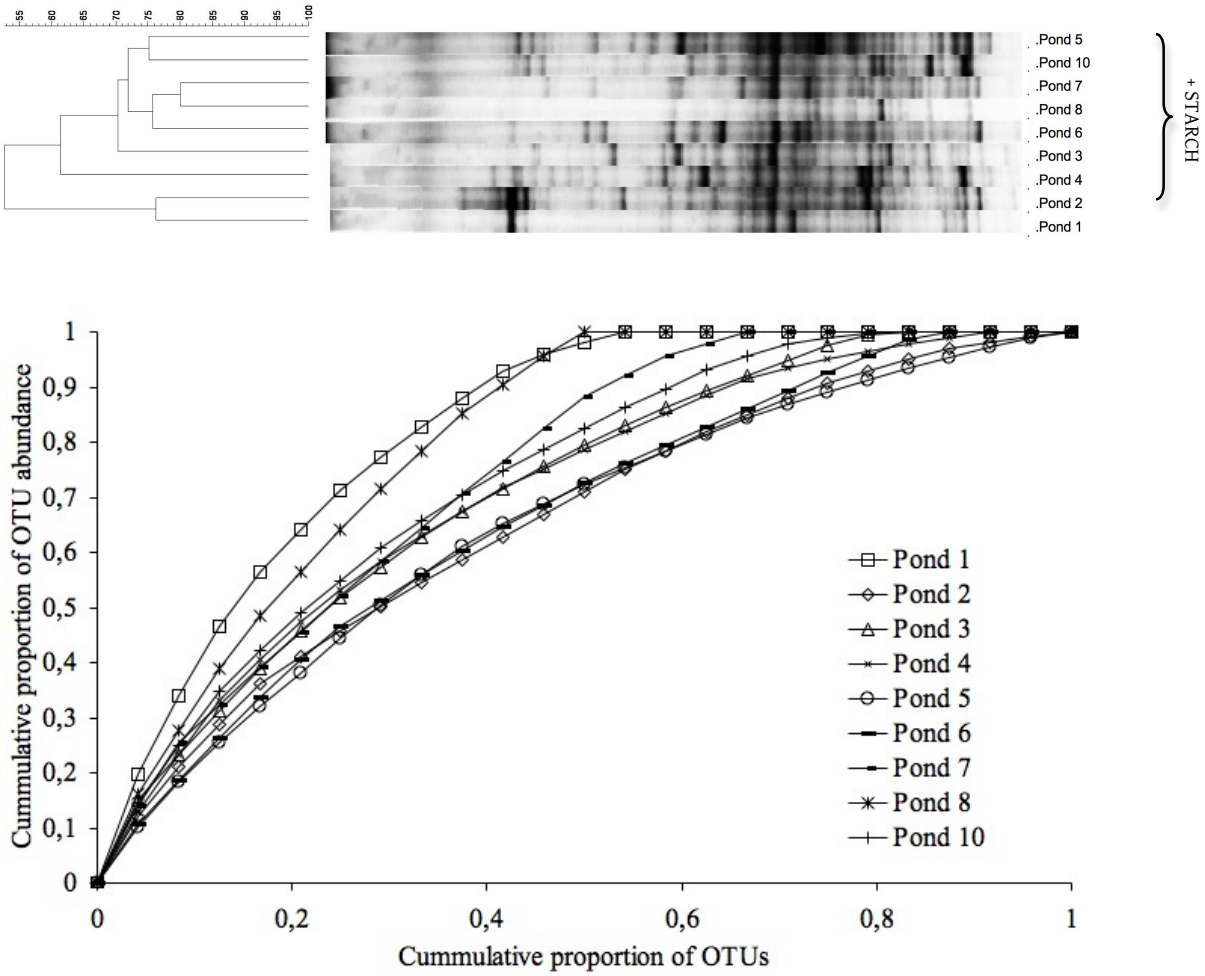


Figure 5.4 Molecular analysis of the bioflocs. a) Pearson correlation of microbial communities in experimental ponds based on the obtained DGGE patterns and dendograms were created by using UPGMA linkage. b) Pareto curves, where the cumulative normalized number of bands is represented on the x-axis and their respective cumulative normalized intensities is represented on the y-axis (OTU: Operational Taxonomic Unit).

The Pearson correlation indicates that in between the treatments without starch addition, there is a similarity of about 75%, while between the treatments with starch addition, a similarity of about 61% was noted. The clustering appeared to be influenced

by the absence or presence of starch addition and not by the protein content of the feed, nor the size of the stocked fish. Overall, there is 55% similarity between all ponds. When looking at the functional organization of the bacterial communities based on DGGE analysis (Figure 5.4 b), Pareto curves of all ponds are in the area of the 45% Pareto curve, from 35 to 62%. The 45% Pareto curve is the curve where the y-axis projection of the intercept with the vertical 20% x-axis line is 45%.

5.4 Discussion

During 2008, an exceptionally cold winter occurred in Israel. A series of cold spells persisted from mid-January till the end of February. Water temperature at the water intake from Lake Kinneret, the source of water for the experimental ponds, was on average 14.4°C. Tilapia demands a temperature between 25 and 28°C for optimal growth. Reproduction stops at 22°C and normal feed intake below 20°C (Wohlfarth and Hulata, 1983). Temperatures of 10 – 13°C showed to be lethal when exposed to these temperatures for more than a few days (Chervinski, 1982). The temperature in the ponds could successfully be controlled during the winter period by covering the ponds with convex greenhouses made out of plastic sheet covers and by decreasing the water exchange rate in the ponds. During the complete experimental period higher temperatures were measured in the ponds than in the water from Lake Kinneret, used as influent for the ponds. Temperatures were 0.4 – 4.9°C higher in the ponds than in Lake Kinneret (Figure 5.1). The temperature in the ponds was above the lethal temperature needed for the survival and well being of the fish. Although temperatures were below 20° C, feeding still occurred but at very low feeding rates, more specifically at 30-40% of the conventional feeding rate levels. Applying high exchange rates to maintain a low concentration of inorganic nitrogen included the risk of lower temperatures in the ponds down to critical values. It is clear that enclosing the ponds in plastic covered structures and limiting the exchange with cold fresh water enabled to keep the water temperature significantly above those of both the ambient air and the water in the lake. Other techniques of overwintering, e.g. using heating facilities and geothermal water, have constraints. The use of heating facilities is restricted due to high energy costs and providing relatively warm water has limitations due to the need for a warm water source (thermal effluents, geothermal springs or cooling water used by some industries) plus the need for large volumes of water to maintain adequate temperatures in exposed ponds (Kirk, 1972; Cruz and Ridha, 1995; Gelegenis et al., 2006). These problems could be overcome by the use of greenhouses insulated with plastic sheet covers.

In contrast to the relatively low temperatures during the first part of the experimental period, the air temperature and the solar radiation at the later parts of the experiment were above the average for the winter season. This led to the heating of the water in the covered ponds, reaching temperatures of around 19°C in the early morning hours. The covered ponds in the Genosar station were not provided with openings enabling ventilation to cool the water. Therefore, to keep water temperature below 20°C the water exchange rate was raised to 10% during warm days. It can be suggested that vents to allow air ventilation are needed in covered ponds.
Oxygen concentrations were always high, within the range of 9–10 mg O_2/L and good for tilapia culture (Popma and Masser, 1999). It seems that fish density could easily be maintained at 16 kg/m^3 without problems of oxygen depletion. Obviously, excessive aerator capacity was used to mix the water rather than to supply enough oxygen and possibly a higher fish biomass could be kept. In intensive aquaculture systems the average power input to the fluid is around 1 – 10 W/m^3, where in this experiment the power input was 22.4 W/m^3 (Boyd, 1998; McGraw et al., 2001; Schuur, 2003).

A potential obstacle in the maintenance of dense fish biomass without an intensive water replacement is the accumulation of toxic ammonia. A number of tests to evaluate the effects of the organic carbon to nitrogen (C/N) ratio to avoid the accumulation of inorganic nitrogen and to promote the build up of organic suspended nitrogen (bioflocs) were conducted; some of them are reported here. A test along a 5 days period when water exchange was completely interrupted (3rd till 7th February) enabled to get a long-range follow up. In contrast with the similar evolution of the total nitrogen concentrations, there were clear differences among treatments in the evolution of inorganic and suspended organic nitrogen. The inorganic nitrogen increased most in 30% P (C/N = 10.8) followed by 23% P (C/N=14), both without starch addition. The increases of inorganic nitrogen in the two treatments with starch addition, 30% P + STARCH and 23% P + STARCH (with a C/N ratio of 20.4 and 20.5, respectively), were significantly lower. The reverse was evident as to the changes in concentrations of the organic nitrogen. A significant increase was found for the low C/N treatments in contrast to the high C/N treatments where practically all added nitrogen was accumulated as organic nitrogen. The amount of nitrogen added by the feed was approximately the same in all ponds, equivalent to a dose of 4.8 mg N/L.day. The lower total nitrogen build up in the starch-amended treatments can be due to an increased harvesting of the bioflocs by the fish, possibly higher in these treatments. The SVI values in all ponds was > 200 mL/g DW and hence the flocs had the capacity to remain suspended in the water column without excessive settling (De Schryver et al., 2008). For this reason, bioflocs can easily be harvested by the fish since they are accessible in the water column and do not tend to sink and build up at the bottom of the ponds. For intensive systems generally a protein feed is used, which contains 25 to 32 percent

protein on a dry weight basis (Lim and Webster, 2006). Fishmeal and fish oil are one of the prime constituents of feed for commercial aquaculture: approximately 79000 tonnes fishmeal and 15800 tonnes fish oil were used for production of tilapia feed in 2003, which implicates that for tilapia production the pelagic input per unit of output is around 0.23-0.28 (Naylor et al., 2000; FAO, 2006). Moreover, the purchase of feed in fish culture has a portion of at least 50% in the production costs; this is predominantly due to the cost of the protein component (Bender et al., 2004). Furthermore, only about 25% of the feed nutrients are converted into harvestable products (Avnimelech and Lacher, 1979; Boyd, 1985; Muthuwani and Lin, 1996). By applying the BFT an additional inexpensive feed source is created that results in a higher efficiency of nutrient conversion and increases the sustainability of the BFT system as compared to conventional pond systems. With respect to feeding, this technique operates at "neutral cost", because it upgrades starch to protein. Moreover, one does not need to invest in an external water treatment system (Crab et al., 2007). This transformation resulting in a production of microbial protein that can be reused as fish food is achievable by adding different types of organic carbon source. The road to go for BFT is the use of organic carbon sources that are considered low-value products in other processing units as e.g. glycerol, which is a by-product from bio-diesel production (Dube et al., 2007; De Schryver et al., 2008).

The ponds fed with 23% protein pellets without starch addition (C/N = 14.0) showed a better water quality regarding the distribution among the different nitrogen species than the ponds fed with 30% protein pellets without starch addition (C/N = 10.8). These findings are confirmed by the results from 24 hours sampling. In general, ponds operated at higher organic C/N ratios showed a better performance in maintaining good water quality at zero water exchange. The data confirms that when the C/N ratio of feed is adjusted to circa 20 by adding starch, the water quality could be controlled by the bioflocs growing in the ponds. This corresponds with the theoretical predictions made by Avnimelech (1999). At this C/N ratio, the inorganic nitrogen was converted into organic nitrogen, due to assimilation by the dense floc cultures. Moreover, the ability to control and minimize inorganic nitrogen build up even without water exchange indicates that the decision to replace daily 10% of the water was probably over-cautious. It was demonstrated that it is possible to completely avoid water exchange during cold days and to lower the daily water exchange rate.

When the temperature is maintained above 13°C, the fish experiences no irreversible harm, such as losing equilibrium, cessation of respiration or cessation of food consumption (Sifa et al., 2002). However, diseases more easily infect tilapia during winter. Sifa et al. (2002) describe a tilapia farm at Zhejiang province (China) where fish exposed to a water temperature of 14°C during the winter had a survival level of only 20

to 30%. In the current experiment, influent water had an average temperature of about 14°C, but water temperature in the ponds could be controlled to an average of 17.7°C. High survival levels of the fish were noted, 97 and 80% for 100 and 50 g fish, respectively (Table 5.1). The average survival level under good temperature conditions of hybrid tilapia of about 100 g was 99% in a study by Siddiqui and Al-Harbi (1995). In the same study, the survival level of smaller fish of about 30 g was reported to be 98%. However during the winter period growth and survival of all hybrid tilapia was lowered. Several studies are in compliance with the experience of farmers and observations that smaller fish are more vulnerable during winter months (Charo-Karisa et al., 2006b).
The average daily growth was high in all ponds with 0.29 and 0.27 g per fish for the fish stocked at a size of 100 and 50 g, respectively (Table 5.1). Nile tilapia in general do not grow at temperatures below 16°C and since temperatures in the pond were on average 17.7°C we observed growth (Chervinski, 1982).
The physiological condition of the fish and the absence of stress following the winter are essential for good growth when fish are stocked in production ponds in the spring. The condition factor is an accepted criterion for the well being of the fish. The results obtained here resulted in a uniform condition factor of 2.2 (Table 5.1). This indicates that the fish were in good condition with minimal stress symptoms. Experience with the tilapia hybrid (*O. niloticus* x *O. aureus*) in Israel indicates that a CF < 1.8 is an indication for poor conditions, while a CF > 2 indicates that the fish are in a good physiological state (Fish Breeding Association, 2003).

Molecular analysis of the bioflocs revealed that species rich communities developed in all ponds. Overall, 55% similarity was noted in all bacterial communities (Figure 5.4). Analysis of DGGE data followed by Pareto curve fitting showed that in all communities about 20% of the microbial community counted up for about 45% of the abundances. The 45% Pareto curve and the area around it represents dominant species are present in high numbers while the majority (the remaining 80% on the x-axis) is present in decreasing lower amounts (Marzorati et al., 2008). Due to the elevated concentration of some species and the availability of many others, the community can potentially deal with changing environmental conditions and preserve its functionality (Marzorati et al., 2008). The distribution of dominant microorganisms and resilient ones assures the competence of counteracting the effect of a sudden exposure to disturbance. These preliminary molecular results indicate that the field of molecular analysis of the BFT needs further exploration.

The major goal of the present work was to develop and evaluate a method for the overwintering of tilapia. Covering the ponds with either plastic sheets or glass helps to collect and preserve solar heating with minimization of water exchange rates. Fish ponds were operated at an initial fish density of 16 kg/m^3. When the organic C/N ratio

in the ponds was adjusted to ca. 20 by adding starch or elevating the carbon content of the feed, the water quality was assured by the bioflocs growing in the ponds. Resulting from the increase in the organic C/N ratio, the inorganic nitrogen was converted into organic nitrogen, due to assimilation by the dense floc cultures. The latter can be harvested by the fish and be used as an additional feed source. Both fish survival and fish condition at harvest were excellent. The water quality and more than adequate oxygen concentration indicates that fish biomass can be easily raised by the BFT approach. Future research should focus on the effects of bioflocs technology used during overwintering on the subsequent fish life cycle. The further development of fish survival, growth and condition after redistribution of the fish in the grow-out ponds needs in depth investigation. Other culture species might also profit from the beneficial effects of overwintering in greenhouse ponds with application of bioflocs technology. Candidate species need to be selected and research regarding the effect of the implementation of this technique together with exposure to lower temperatures must be conducted.
The use of bioflocs technology was shown to be effective in overwintering of tilapia by maintaining appropriate water temperature, good water quality and high fish survival in low/no water exchange, greenhouse ponds. Quite a number of fish farmers in Israel plan to use this technique rather than to risk loss of fish and fingerlings during cold winter days. If one considers that there was an economical loss of about 11.3 million € during a cold spell in January 2008 in Israel, it is clear that lower temperatures during winter can have devastating effects on the aquaculture industry, concomitantly, a country's economy.

Besides overwintering, diseases can cause unpredictable massive mortalities in aquaculture systems due to the proliferation of pathogenic and opportunistic microorganisms in culture systems. Pathogens are considered to be the industry's single most important cause of economic losses. Although pathogens more easily infect tilapia during winter, this was not observed in the bioflocs technology study discussed here. This observation together with observations of farmers that bioflocs technology may have an effect on fish and shrimp health, raised the question whether bioflocs possibly have a biocontrolling effect towards pathogens. Therefore, the potential use of bioflocs as an anti-infective strategy towards *Vibrio harveyi* and *Vibrio campbellii* was investigated in Chapter 6.

CHAPTER 6

BIOFLOCS PROTECT GNOTOBIOTIC BRINE SHRIMP (*ARTEMIA FRANCISCANA*) FROM PATHOGENIC *VIBRIO HARVEYI*

The search for alternative anti-infective strategies is driven by the enormous economic losses in aquaculture. The bioflocs technology possesses possible extra added value features regarding pathogen control. In this research, glycerol-grown bioflocs were investigated for their antimicrobial and antipathogenic properties against the opportunistic pathogen *Vibrio harveyi* BB120 and the more virulent strain *Vibrio campbellii* LMG21363. The bioflocs did not produce growth-inhibitory substances. However, luminescence of *Vibrio harveyi* BB120 decreased significantly after biofloc application. This suggested that the bioflocs had biocontrol activity against this pathogen since quorum sensing regulates virulence of Vibrios towards different hosts. Interestingly, the addition of live bioflocs significantly increased the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged to *Vibrio harveyi* BB120. Autoclaved bioflocs, bioflocs supernatants and autoclaved biofloc supernatants did not exert the same protective effect. In contrast, survival of the *Vibrio campbellii* LMG21363 infected nauplii was not affected by addition of bioflocs. This research indicates that in addition to water quality control and extra *in situ* feed production, bioflocs grown on glycerol as carbon source inhibit quorum-sensing regulated bioluminescence in *Vibrio harveyi* and protect brine shrimp larvae from Vibriosis.

Redrafted after:

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., Verstraete, W., 2010. The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. Journal of Applied Microbiology, In press.

6.1 Introduction

Aquaculture is a rapidly growing food producing sector, but its growth is not without problems. The production process generates substantial amounts of polluted effluent, containing uneaten feed and feces that release ammonia and nitrite, which is toxic in elevated concentrations for both fish and shellfish (Crab et al., 2007). Conventional techniques used to overcome this problem are photoautotrophic algae-based systems, frequent water exchange and the use of biofilters (Sinha et al., 2008). These techniques however are lacking economical, technical, sustainable and practical competence. A technique that overcomes these shortcomings is the bioflocs technology. By adding carbohydrates into the aquaculture system in order to increase the C/N ratio (< 10), bacterial growth is stimulated and nitrogen uptake through the production of microbial proteins takes place (Avnimelech, 1999; Crab et al., 2007). The microorganisms tend to form amorphous aggregates called bioflocs and can be used by fish and shrimp as an additional source of protein.

Recent research questions focus on the possible extra added value of bioflocs, more specifically regarding pathogen control, since infectious diseases burden the aquaculture industry. These diseases are considered to be the industry's single most important cause of mass mortalities and economic losses. Knowing that in 2001 seafood export products were worth 43.9 billion € and that losses in the same year were estimated to be about 6.3 billion €, which is roughly 14% of the world's farmed fish and shellfish value, it is clear that it is essential to address this socio-economic problem. While under certain circumstances antibiotics can provide a useful means of helping to control some bacterial diseases, there are many problems associated with their use. Improper use of antibiotics has resulted in the development and spread of (multiple) antibiotic resistance (Defoirdt et al., 2006). As a consequence, there is an urgent need for alternative, more sustainable control techniques.

The Gram-negative bacterium *Vibrio harveyi* and closely related species, such as *Vibrio campbellii* are reputed for causing mass mortalities in penaeid shrimp, although they can affect almost all types of cultured animals, causing disease often referred to as luminescent Vibriosis (Phuoc et al., 2009; Defoirdt et al., 2007). *Vibrio harveyi* and *Vibrio campbellii* are genetically related species with a DNA similarity value of 69% and a 16S rRNA similarity higher than 97% (Gomez-Gil et al., 2004). Virulence of *Vibrio* species has been shown to be linked to quorum sensing or bacterial cell-to-cell communication with small signal molecules (Milton, 2006; Defoirdt et al., 2007). Quorum sensing is a

mechanism by which bacteria coordinate the expression of certain genes in response to the presence or absence of small signal molecules (Defoirdt et al., 2007). Phenotypes that were found to be controlled by quorum sensing in *in vitro* grown luminescent Vibrios include bioluminescence and the production of several virulence factors such as a type III secretion system, extracellular toxin, metalloprotease and a siderophore (Bassler et al., 1993; Henke and Bassler, 2004; Manefield et al., 2000; Mok et al., 2003; Lilley and Bassler, 2000). Moreover, quorum sensing has been shown to regulate *in vivo* virulence of these bacteria towards different hosts (Defoirdt et al., 2008). Because quorum sensing regulates the virulence of pathogenic bacteria, its disruption has been proposed as a new anti-infective strategy for aquaculture (Defoirdt et al., 2004).

The use of gnotobiotically grown aquatic animals gives the opportunity to better understand the mechanisms involved when evaluating a possible new treatment of disease control. Here, a model with gnotobiotic brine shrimp (*Artemia franciscana*) was used. Through elimination of all microorganisms present in and around the host, one can look at the effect of a defined component, like the bioflocs, on the host regarding amongst others its performance after infection with a pathogen. The use of this model increases the control over certain variables and enhances the reproducibility of the results (Marques et al., 2006). Although the models are technical complex and certain skills and experience are necessary, they can elucidate the host-microbial interactions and nutritional factors in absence of all microorganisms.

This study aimed at determining the effect of bioflocs on growth and luminescence (as readout for quorum sensing activity) of *Vibrio harveyi* BB120 and *Vibrio campbellii* LMG21363. In addition, the use of bioflocs as new biocontrol agents for aquaculture was studied in a model system with gnotobiotic brine shrimp (*Artemia franciscana*).

6.2 Materials and methods

6.2.1 Biofloc reactor

Biofloc reactors were operated as described previously (Crab et al., 2010a). The bioflocs were grown in a rectangular reactor with a water volume of 5 L (360 x 210 x 145 mm). The synthetic aquaculture water continuously flowed in the tank at a flow rate of 0.21 L/hr and the excess of reactor water was removed by means of an overflow resulting in a hydraulic residence time (HRT) of 1 day. The reactor was continuously aerated. The

influent contained about 25.0 mg TAN/L and 3.6 mg PO_4^{3-}P/L to simulate an aquaculture effluent from an intensive system. The carbon source, glycerol, was added once a day in an amount corresponding with a C/N ratio of 10. At start up, 0.25 L of drum filter slurry originating from an intensive tilapia aquaculture farm, VitaFish (Dottignies - Mouscron, Belgium), was supplied to the reactor.

6.2.2 Bacterial strains and growth conditions

The bacterial strains used in this study were *Vibrio harveyi* BB120, *Vibrio campbellii* LMG21363 and *Aeromonas hydrophila* LVS3. *Vibrio harveyi* BB120 is a wild type used as model strain in quorum sensing studies, *Vibrio campbellii* LMG 21363 was isolated from the lymphoid organ of diseased shrimp (*Penaeus* spp.) juveniles (Philippines) and *Aeromonas hydrophila* LVS3 is an isolate that enhances growth and survival of *Artemia* (Bassler et al., 1997; Soto-Rodriguez et al., 2003; Gomez-Gil et al., 2004; Verschuere et al., 1999). All strains were stored in 40% glycerol at -80°C. Of these stored cultures, 10 µL was inoculated in Marine Broth and incubated at 28°C under constant agitation (100 RPM). Spectrophotometry at OD_{545} was used to measure growth. Bioluminescence was measured with a Lumac Biocounter M2500 luminometer.

6.2.3 Axenic hatching of *Artemia*

All experiments were performed with high quality hatching cysts of *Artemia franciscana*. Two hundred mg of cysts (INVE België nv, Baasrode, Belgium) were hydrated in 18 mL of tap water for 1 hr. Sterile cysts and nauplii were obtained via decapsulation, adapted from Marques et al. (2004), by adding 660 µL of NaOH (32%) and 10 mL of NaOCl (50%). After 2 min the decapsulation was stopped by adding 14 mL of $Na_2S_2O_3$ (10 g/L). During the reaction, 0.22 µm filtered aeration was provided. The decapsulation was followed by a washing step with filtered (0.22 µm) and autoclaved artificial seawater containing 35 g/L of Instant Ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were resuspended in a 50-mL tube containing 30 mL of filtered and autoclaved artificial seawater and hatched for 24 hr on a rotor (4 RPM) at 28°C with constant illumination (approximately 2000 lux). After hatching, groups of 20 nauplii were transferred to sterile 50-mL tubes that contained 30 mL of filtered and autoclaved artificial seawater. The nauplii were subjected to feeding, challenge and feeding tests.

6.2.4 Bioluminescence and growth experiment

The effect of the presence of (autoclaved) bioflocs on growth and bioluminescence of *Vibrio harveyi* BB120 and *Vibrio campbellii* LMG21363 were investigated. Therefore, 1 mL of (autoclaved) biofloc was added to 9 mL of Marine Broth medium and inoculated with 10^6 CFU/mL of the *Vibrio* strains. The test tubes were incubated on a rotor at 28°C. After 24 hr incubation the bioluminescence was measured using a Lumac Biocounter M2500 luminometer and the viable *Vibrio* cell number was determined by plating on TCBS agar plates. The number of colonies was counted after 24 hr of incubation at 28°C. Every treatment was carried out in triplicate.

6.2.5 Feeding tests

As standard feed *Artemia* were provided with autoclaved (20 min, 121°C) *Aeromonas hydrophila* LVS3 bacteria, grown in Marine Broth as described above. Before autoclaving, *Aeromonas hydrophila* LVS3 cells were washed twice with filtered and autoclaved artificial seawater (Defoirdt et al., 2006). In order to determine the concentration of LVS3 bacteria needed to obtain a similar survival of *Artemia* as when fed with (autoclaved) bioflocs, the *Artemia* nauplii were fed a 10-fold dilution series of an autoclaved *Aeromonas hydrophila* LVS3 suspension. The final concentration in the *Artemia* culture water was 0, 10^5, 10^6, 10^7, 10^8 and 10^9 cells/mL respectively. Bioflocs grown on glycerol were also tested regarding their ability to serve as a feed for the *Artemia* nauplii. The bioflocs were harvested directly from the biofloc reactors and fed both live and after autoclavation to the *Artemia* nauplii in 3 different dosages of 0.179, 0.358 and 0.537 g/L suspended solids, respectively. The survival and length of the *Artemia* nauplii was measured after 48 hr incubation on an illuminated rotor at 28°C. Every treatment was carried out in triplicate.

6.2.6 Challenge tests

Challenge tests were performed as described in Defoirdt et al. (2006). After incubation, the Vibrios were washed twice in filtered and autoclaved artificial seawater and the suspensions were diluted to an OD_{545} of approximately 0.1. The pathogens were inoculated into the *Artemia* culture water at 10^5 CFU/mL. Autoclaved LVS3 bacteria were added once at the start of the challenge test under LVS3 fed conditions at a final

concentration of 10^7 CFU/mL of *Artemia* culture water. Challenge tests were also performed under non-LVS3 fed conditions to avoid an increased feeding effect on the host and its susceptibility to infection through the possible feeding of (autoclaved) bioflocs by the *Artemia*. The survival of *Artemia* was scored 48 hr after the addition of the strains. Each treatment was performed in triplicate.

6.2.7 Toxicity test

To check the effect of glycerol on *Artemia* nauplii, the carbons source was added to the *Artemia* culture water at the same concentration as in the biofloc reactor, i.e. 250 mg C/L. The test was performed under non-feeding conditions. The percentage survival was measured after 48 hr. The control and glycerol treatment were both performed in triplicate.

6.2.8 Methods used to verify axenicity

In each *Artemia* test, axenicity of the control tubes was verified. Of the *Artemia* culture water, 1 mL was transferred to a test tube with 9 mL of fresh Marine Broth and the mixture was incubated for 2 days at 28°C. If a control tube would have been found to be contaminated (as manifested by increasing turbidity of the Marine Broth), the data from the corresponding experiment would not have been considered and the experiment would have been repeated.

6.2.9 Calculation and statistical methods

Pearson correlations were calculated and independent samples t-tests were performed using the SPSS software version 16.0.

6.3 Results

6.3.1 Feeding tests

In a first experiment, the effect of live and autoclaved bioflocs and untreated and autoclaved biofloc supernatants on survival and growth of *Artemia franciscana* nauplii was studied. The survival of *Artemia* nauplii was measured after 48 hr incubation. In the control treatment the survival was low (48 ± 19%) (Figure 6.1), which was expected since no feed (autoclaved *Aeromonas hydrophila* LVS3 suspension) neither (autoclaved) bioflocs, were added to the test tube. Comparable survival percentages were noted in the animals fed with 10^5, 10^6 and 10^7 cells LVS3/mL, 52 ± 26%, 43 ± 13%, 52 ± 3%, respectively. Significantly higher survival was measured in the LVS3 fed treatments when added in a concentration of 10^8 and 10^9 cells/mL, 97 ± 3% and 95 ± 5%, respectively. When bioflocs were added to the *Artemia* nauplii, a significantly higher survival was measured when compared to the control for all biofloc concentrations, with no difference between live and autoclaved bioflocs.

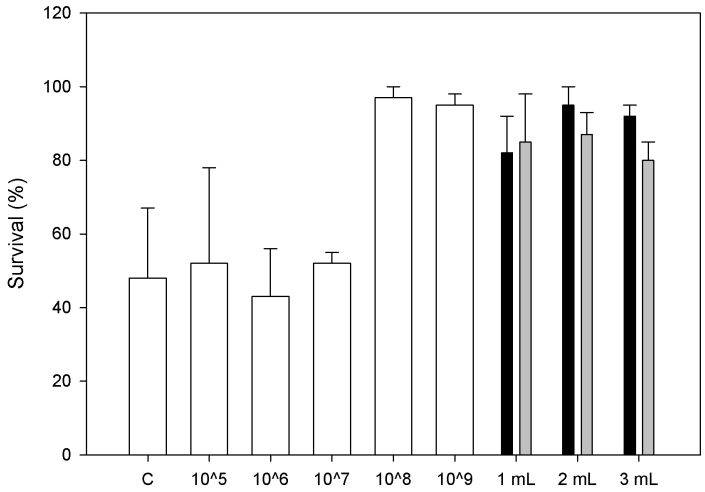


Figure 6.1 Percentage survival of *Artemia* nauplii (mean ± standard deviation of three replicates) after 48 hr after addition of C: no feed, 10^5: 10^5 cells LVS3/mL, 10^6: 10^6 cells LVS3/mL, 10^7: 10^7 cells LVS3/mL, 10^8: 10^8 cells LVS3/mL, 10^9: 10^9 cells LVS3/mL, 1 mL: 0.179 g SS bioflocs/L, 2 mL: 0.358 g SS bioflocs/L, 3 mL: 0.537 g SS bioflocs/L. The black bars represent live bioflocs addition and the grey bars autoclaved bioflocs addition.

In addition to survival, the length of the surviving *Artemia* nauplii was determined as a measure for growth (Figure 6.2). Significant higher lengths were measured when compared to the control in the treatments with 10^8 and 10^9 cells LVS3/mL and the

treatments with autoclaved bioflocs. When live bioflocs were added to the organisms, no significant difference in length as compared to the control could be noted.

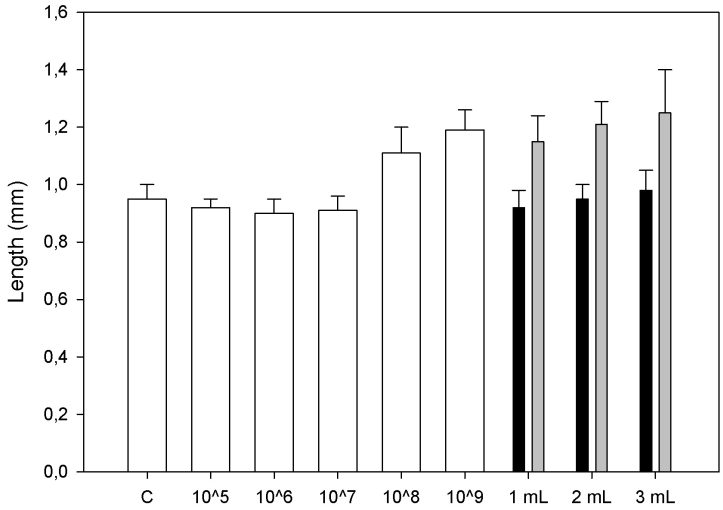


Figure 6.2 The length of *Artemia* nauplii measured after 48 hr after addition of C: no feed, 10^5: 10^5 cells LVS3/mL, 10^6: 10^6 cells LVS3/mL, 10^7: 10^7 cells LVS3/mL, 10^8: 10^8 cells LVS3/mL, 10^9: 10^9 cells LVS3/mL, 1 mL: 0.179 g SS bioflocs/L, 2 mL: 0.358 g SS bioflocs/L, 3 mL: 0.537 g SS bioflocs/L. The black bars represent live bioflocs addition and the grey bars autoclaved bioflocs addition.

6.3.2 Bioluminescence and growth experiment

In the second experiment, we studied the effect of live and autoclaved bioflocs and untreated and autoclaved supernatants on growth and bioluminescence (as a readout for quorum sensing activity) of *Vibrio harveyi* BB120 and *Vibrio campbellii* LMG21363.
There was no significant effect of autoclaved bioflocs, bioflocs supernatants and autoclaved biofloc supernatants on the growth of both *Vibrio harveyi* BB120 and *Vibrio campbellii* LMG21363 (Figure 6.3). The biofloc treatment did affect the growth of *Vibrio* harveyi BB120, but in only to a small extend ($p = 0.04$), growth of *Vibrio campbellii* LMG21363 was not affected.
There is however a significant effect of the biofloc treatment on the luminescence of *Vibrio harveyi* BB120, which suggests that the bioflocs had quorum sensing-disrupting activity in this strain. Bioluminescence is one of the phenotypes that is regulated by quorum sensing in *Vibrio harveyi* (Bassler et al., 1997) and consequently, bioluminescence was monitored as a measure of quorum sensing activity. Also all three other treatments showed a significant decrease of the luminescence when compared to the control, but it was less pronounced than in the other treatments. These results suggest that the bioflocs produced (heat stable) extracellular compounds with quorum

sensing-disrupting activity. In contrast, for *Vibrio campbellii* LMG21363 there was no significant effect on bioluminescence.

6.3.3 Challenge test

Because quorum sensing is known to regulate the virulence of Vibrios towards different hosts, and therefore, in the third experiment, we determined the effect of bioflocs and biofloc supernatants on the survival of *Artemia franciscana* nauplii challenged with *Vibrio harveyi* BB120 and *Vibrio campbellii* LMG21363.

The percentage survival of LVS3 fed *Artemia* nauplii was determined after 48 hr challenge with the opportunistic pathogen *Vibrio harveyi* BB120 (Figure 6.4a and Figure 6.4b). Similar trends were observed under both fed and unfed conditions, with *Vibrio harveyi* BB120 causing significant mortality. In both cases, treatment with live bioflocs resulted in complete protection of the *Artemia* nauplii from the pathogen (no significant difference in survival with unchallenged *Artemia* nauplii). The treatments with autoclaved bioflocs and biofloc supernatants did not increase the survival of challenged *Artemia* nauplii.

A second set of challenge tests was executed with the opportunistic pathogen *Vibrio campbellii* LMG21363. The percentage survival of LVS3 fed *Artemia* nauplii after 48 hr challenge with *Vibrio campbellii* LMG21363 is depicted in Figure 6.5a. In Figure 6.5b the results are given for non-fed *Artemia* nauplii after 48 hr challenge with *Vibrio campbellii* LMG21363. Under fed conditions and in all treatments, survival between the unchallenged and challenged *Artemia* did not differ significantly. Only in the control treatment a significant decrease in survival could be measured in the challenged *Artemia* nauplii as compared to the unchallenged nauplii. These results however could not be repeated under non-fed conditions. Survival of *Artemia* in all challenged treatment groups is significantly lower as in according unchallenged *Artemia* nauplii. As opposed to the results obtained with *Vibrio harveyi* BB120, no complete protection of the *Artemia* nauplii from the pathogen *Vibrio campbellii* LMG21363 through treatment with live bioflocs could be noted as in none of the other treatments.

6.3.4 Toxicity tests

In order to verify whether direct application of glycerol to the culture pond might have detrimental effects on the cultured organisms, *Artemia* cultures were treated with glycerol at the same concentration as those added to the biofloc reactors and survival of

the nauplii was measured after 48 hr incubation. A significantly higher mortality was noted in glycerol-treated cultures (12 ± 13%) when compared to the control (72 ± 3%) (Figure 6.6).

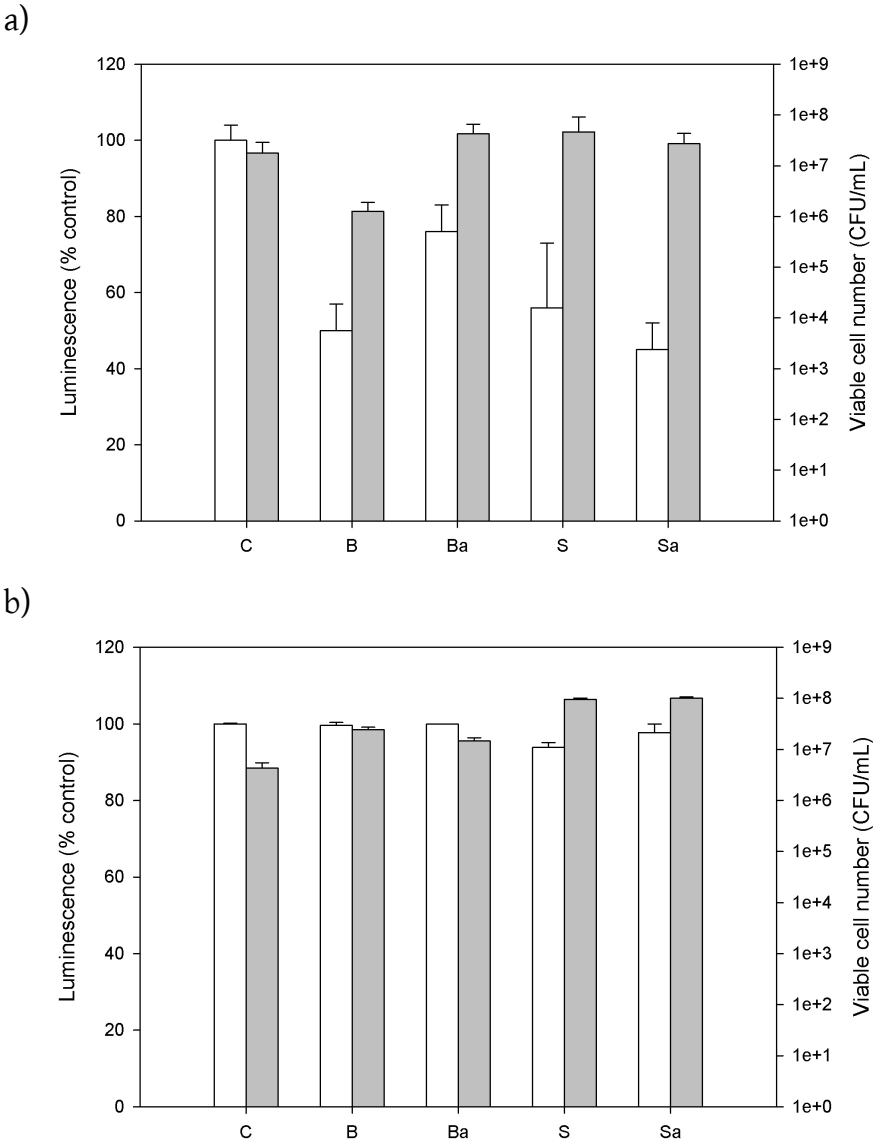


Figure 6.3 Luminescence (white bars) and viable cell number (grey bars) were measured (mean ± standard deviation of three replicates) for a) *Vibrio harveyi* BB120 and b) *Vibrio campbellii* LMG21363 after addition of C: no addition, B: glycerol biofloc, Ba: autoclaved glycerol biofloc, S: glycerol biofloc supernatants, Sa: autoclaved glycerol biofloc supernatants.

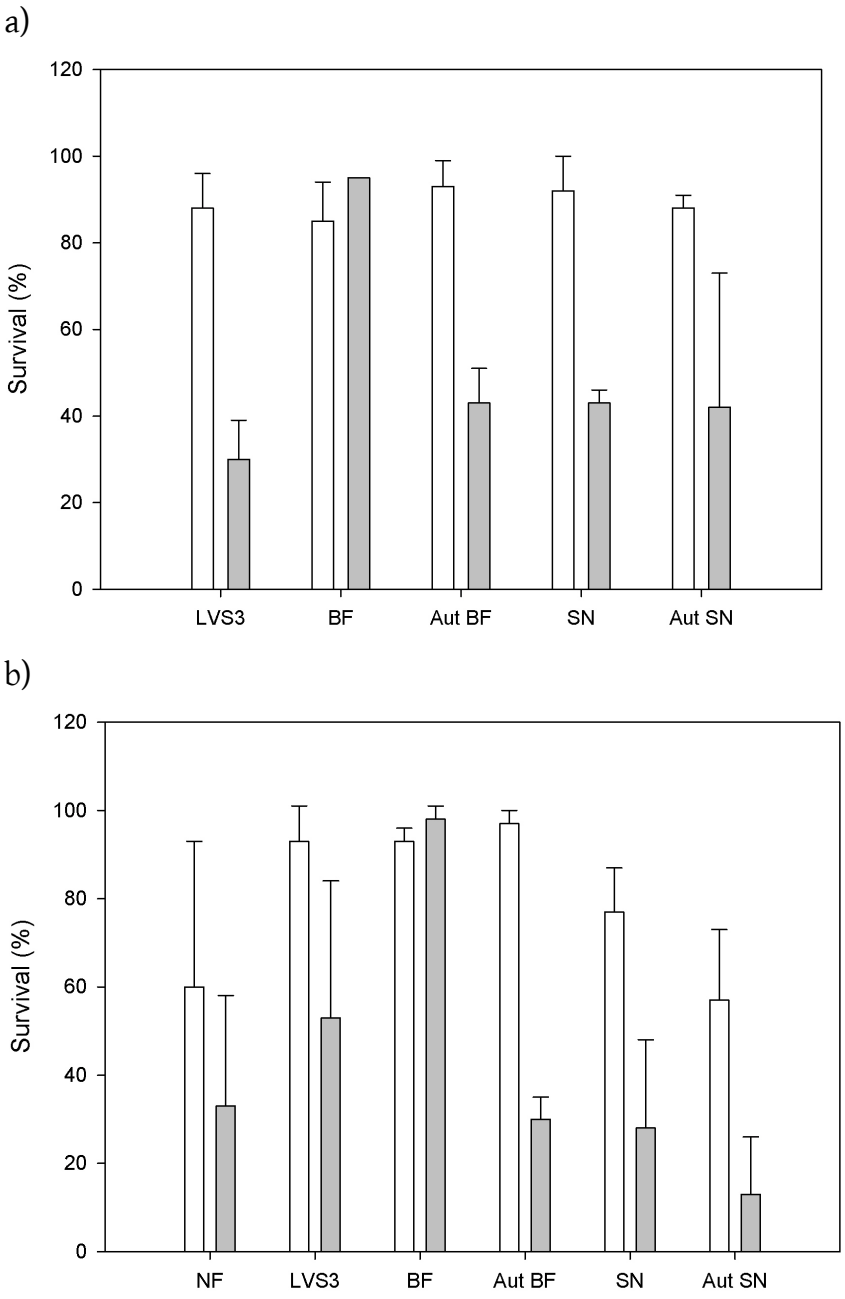


Figure 6.4 Survival percentage of *Artemia* nauplii (mean ± standard deviation of three replicates) after 48 hr challenge with *Vibrio harveyi* BB1120 under a) LVS3 fed conditions and b) non-fed conditions. The white bars represent the unchallenged organisms and the grey bars the *Vibrio harveyi* challenged organisms. The treatments include NF (no feed), LVS3 (*Aeromonas hydrophila* LVS3), BF (biofloc), Aut BF (autoclaved biofloc), SN (supernatants) and Aut SN (autoclaved supernatants) addition.

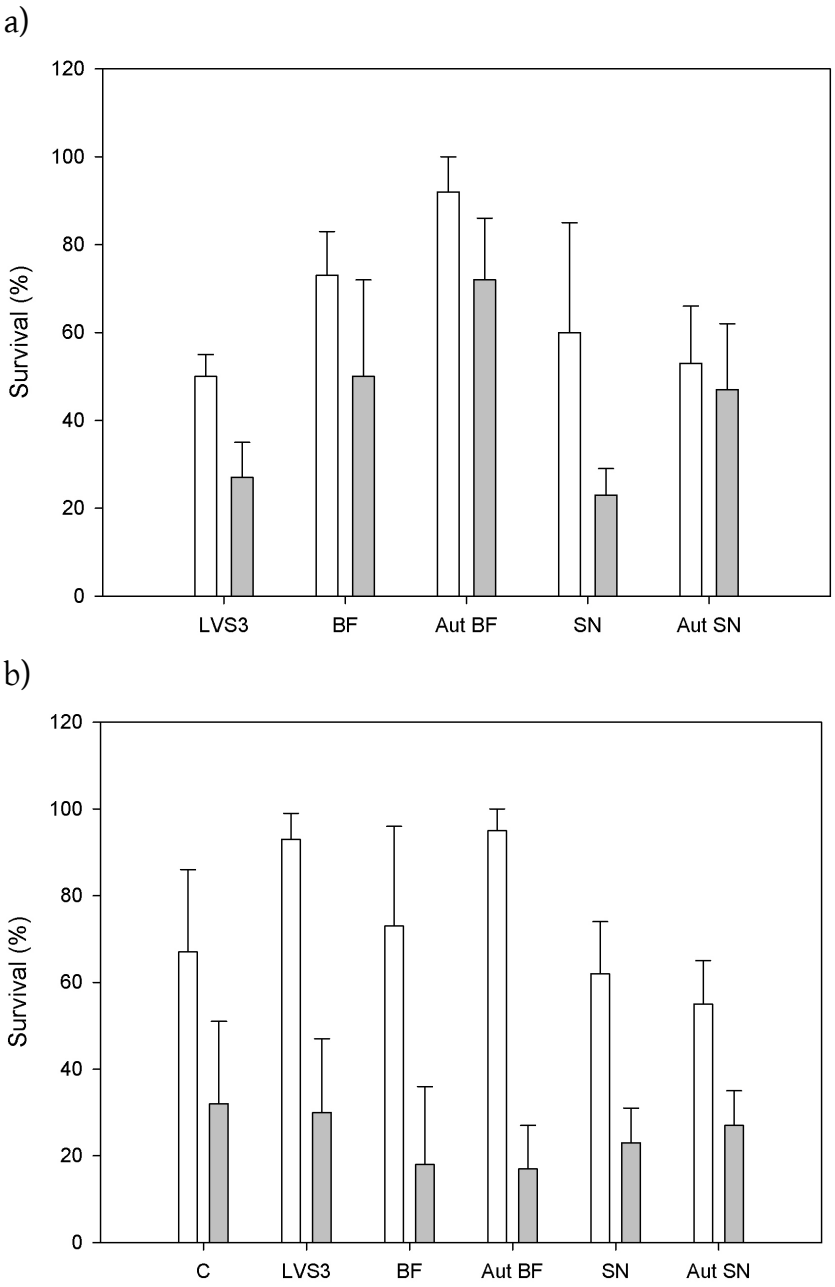


Figure 6.5 Survival percentage of *Artemia* nauplii (mean ± standard deviation of three replicates) after 48 hr challenge with *Vibrio campbellii* LMG21363 under a) LVS3 fed conditions and b) non-fed conditions. The white bars represent the unchallenged organisms and the grey bars the *Vibrio campbellii* challenged organisms. The treatments include NF (no feed), LVS3 (*Aeromonas hydrophila* LVS3), BF (biofloc), Aut BF (autoclaved biofloc), SN (supernatants) and Aut SN (autoclaved supernatants) addition.

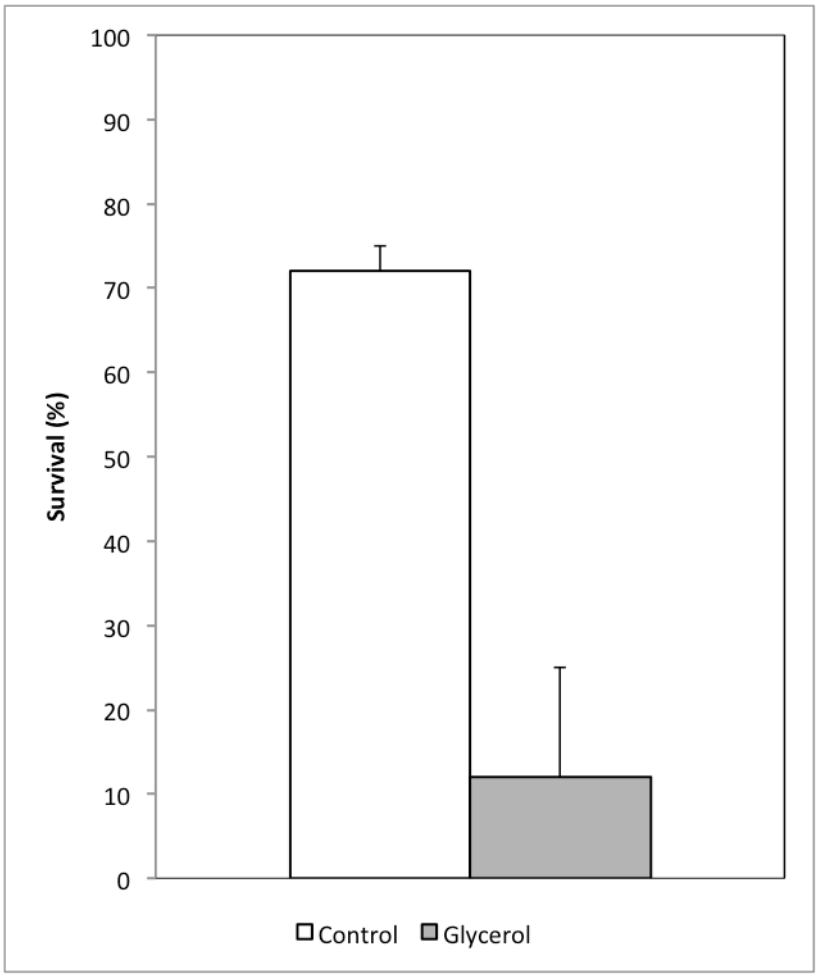


Figure 6.6 Percentage survival of *Artemia* nauplii (mean ± standard error of three replicates) after 2 days of incubation. In the Control, no feed was added to the organisms. In the Glycerol treatment glycerol was added at a concentration of 250 mg C/L without addition of LVS3.

6.4 Discussion

In this study, we aimed at determining the use of bioflocs as a feed and new biocontrol agents for aquaculture using a model system with gnotobiotic brine shrimp (*Artemia franciscana*). Previous research had shown that bioflocs could act as a feed for different culture species, e.g. Nile tilapia (*Oreochromis niloticus*), giant freshwater prawn (*Macrobrachium rosenbergii*), whiteleg shrimp (*Litopenaus vannamei*) (Azim and Little, 2008; Crab et al., 2009; Kuhn et al., 2009). Earlier biochemical analyses at our laboratories showed that glycerol-grown bioflocs have good nutritional properties and that they can be used as an additional feed source for giant freshwater prawn postlarvae (*Macrobrachium rosenbergii*) (Crab et al., 2010b). In this paper, we found that the bioflocs

can also be used by brine shrimp (*Artemia franciscana*) nauplii as a feed, here already at relatively low concentrations of 179 mg SS biofloc/L as compared to suspended solids values measured in shrimp ponds can go up to 300 mg SS/L and in fish ponds even up to 1000 mg SS/L (Avnimelech 2009). The survival of *Artemia franciscana* nauplii provided with live or autoclaved bioflocs was similar to the survival of nauplii fed 10^8 and 10^9 cells LVS3/mL (Figure 6.1). Also the length of the nauplii fed autoclaved bioflocs is similar to those of nauplii fed 10^8 and 10^9 cells LVS3/mL (Figure 6.2). Nauplii fed live bioflocs show a significantly lower length than the ones fed autoclaved bioflocs. Autoclaving might cause a change in structure of the bioflocs, which makes them more accessible for the nauplii to feed on, for example by influencing the size of the particles, or by making them easier to digest (e.g. by destroying extracellular polymers and cell wall material).

The bioflocs were further investigated for their effect on growth and bioluminescence of *Vibrio Harveyi* BB120 and *Vibrio campbellii* LMG21363. In addition to bioluminescence, these bacteria are known to regulate virulence by the process of cell-to-cell communication called quorum sensing and therefore, quorum sensing activity (as determined in this study by bioluminescence measurements) gives an indication of virulence of these pathogens (Defoirdt et al., 2008). Data in Figure 6.3 shows that the bioflocs do not produce substances that inhibit growth of *Vibrio harveyi* and *Vibrio campbellii*. The addition of live bioflocs had a slight growth-inhibitory effect towards *Vibrio harveyi* BB120. The effect was probably due to competition for nutrients (and not due to production of antibacterial compounds) because the biofloc supernatants and the autoclaved biofloc suspension did not inhibit growth.

The bioluminescence of *Vibrio harveyi* BB120 however was significantly inhibited by the addition of bioflocs and biofloc supernatants (resulting in a 45 to 55% decrease compared to the control). The bioluminescence was also affected after the addition of autoclaved bioflocs, however to a lesser extent. This implicates that there was a heat-stable extracellular compound or factor present in the biofloc suspension that affects the quorum-sensing-regulated bioluminescence of *Vibrio harveyi* BB120 without interfering with its growth. This component or factor has no effect on the luminescence biochemistry of *Vibrio campbellii* LMG21363 since the bioluminescence of this pathogen was not affected.

Quorum sensing is known to regulate virulence of *Vibrio harveyi* towards brine shrimp (Defoirdt et al., 2008) and because the bioflocs were able to block quorum sensing-regulated bioluminescence in *Vibrio harveyi* BB120, a challenge test was performed to verify whether addition of bioflocs to brine shrimp culture water could protect them from Vibriosis. The experiment revealed that live bioflocs can protect *Artemia fransciscana* nauplii from the pathogen since they significantly increased survival of

challenged nauplii up to non-challenged levels, both under fed and non-fed conditions (Figure 6.4). In contrast, although autoclaved bioflocs, biofloc supernatants and autoclaved supernatants also affected the bioluminescence of *Vibrio harveyi in vitro*, they did not enhance survival of the brine shrimp larvae. Our data suggest that the protective action of bioflocs against *Vibrio harveyi* infection is not due to growth inhibition of the pathogen, but that the effect can be assigned to disruption of the bacterial quorum sensing, however further research will need to elucidate the precise mode of action. The most common mechanisms of quorum sensing disruption are (1) the production of quorum sensing antagonists or (2) production of signal molecule-degrading enzymes by microorganisms (Defoirdt et al., 2004). The observation that only live bioflocs could protect *Artemia* from *Vibrio harveyi* might be explained by the fact that in this treatment release of the quorum sensing-disrupting compound could continue throughout the challenge test, whereas in the other treatments it was added only once at the start of the experiment.

From the results of the *Vibrio campbellii* LMG21363 challenge test one can conclude that they confirm the former growth and bioluminescence test, and no significant effect on the survival of *Artemia* nauplii was observed. The results of the fed challenge test are not conclusive, but when looking at the results of the non-fed challenge test, one can clearly see that the survival of the *Vibrio campbellii* LMG21363 infected nauplii is lower then the non infected control after addition of all biofloc components. The quorum sensing mechanisms of Vibrios vary in complexity and cellular output even though the components are quite similar, which can account for the different reaction of *Vibrio harveyi* BB120 on bioflocs addition than *Vibrio campbellii* LMG21363 (Milton, 2006).

The carbon source used in this study to grow the bioflocs, glycerol, showed a negative effect on the survival of *Artemia* nauplii (Figure 6.6). This test was performed to verify if direct application to the culture pond might have detrimental effects on the cultured organism. Hence, precaution will be needed when glycerol would be added directly to the pond. The highest non-toxic level of glycerol needs to be determined for the culture organism and this level should preferentially not be exceeded. On the other hand, once the carbon source is added to the culture water, it will be metabolized very quickly by the resident biofloc community using the waste nitrogen present in the water and producing proteinaceous flocs. A solution to overcome the toxicity problem is partitioned addition of lower levels of the carbon source to the culture pond instead of one single addition.

In conclusion, this research aimed at evaluating the bioflocs technology as a possible alternative measure to control luminescent Vibriosis in shrimp through the use of a model system with gnotobiotic brine shrimp, *Artemia franciscana*. Our data indicate that

in addition to being a tool for water quality and feed management, this technique possibly also has potential to protect the cultured organisms from infections with the pathogenic bacteria, *Vibrio harveyi*. There was, however, no protective action noted when *Artemia* was challenged with *Vibrio campbellii*. Although both pathogens are closely related, they were affected differently by the bioflocs. This opens a window for future work, where the underlying mechanisms need further in depth investigation and the effect on other pathogens should be evaluated. During this investigation, only the larval stage was examined; further research should extent to the juvenile and adult stages to validate on a wide range of the host under study. Moreover, also other parameters need further study to understand the mechanisms involved in the observed effects, such as the influence of bioflocs on the model host regarding bacterial community analysis, host gene expression analysis and biochemical analysis. A point to consider in all these possible research angles is that germ-free animals might react differently on the test conditions when not germ-free and under field conditions, due to the intrinsic characters of germ-free animals. Studies on the biocontrolling effects of biofloc technology in aquaculture systems are warranted.

CHAPTER 7

GENERAL DISCUSSION

With almost seven billion people now on earth, the demand for aquatic food carries on to increase and hence, expansion and intensification of aquaculture production are highly required. The prime goal of aquaculture expansion must be to produce more aquaculture products without significantly increasing the usage of the basic natural resources of water and land (Avnimelech, 2009). The second goal is to develop sustainable aquaculture systems that will not damage the environment (Naylor et al., 2000). The third goal is to build up systems providing an equitable cost/benefit ratio to support economic and social sustainability (Avnimelech, 2009). All these three prerequisites for sustainable aquaculture development can be met by the bioflocs technology.

7.1 The strength of bioflocs technology

The bioflocs technology makes it possible to minimize water exchange and water usage in aquaculture systems through maintaining adequate water quality within the culture unit, while producing low cost bioflocs rich in protein, which in turn can serve as a feed for aquatic organisms (Crab et al., 2007; Crab et al., 2009; Crab et al., 2010b, Crab et al., 2010c). Compared to conventional water treatment technologies used in aquaculture, the bioflocs technology provides a very economical alternative (decrease of water treatment expenses in the order of 30%), and additionally, a potential gain on feed expenses (protein utilization is twice as high in biofloc technology systems in

comparison with conventional ponds), making it a low-cost sustainable constituent to future aquaculture development (De Schryver et al., 2008; Avnimelech, 2009). Conventional technologies to manage and remove nitrogen compounds are based on either earthen treatment systems, or a combination of solids removal and nitrification reactors (Crab et al., 2007). These methods have the disadvantage of requiring frequent maintenance and in most instances the units can achieve only partial water purification. They generate secondary pollution and are often costly (Lezama-Cervantes and Paniagua-Michel, 2010). Bioflocs technology, on the other hand, is an economical technique, easy in operation and robust. One important aspect of the technology to consider is the high concentration of total suspended solids present in the pond water. Therefore, suitable aeration and mixing needs to be sustained for keeping particles in suspension and intervention might be needed when suspended solids concentration gets too high through either water exchange or drainage of sludge (Avnimelech, 2009). Scientific knowledge about selection and placement of aerators is still lacking and critical in bioflocs technology. Future research can address this issue and also look at new ideas such as the integration of bioflocs technology in raceways, which might prevent solids build up through its proper system configuration (Avnimelech, 2009). Construction aspects for bioflocs technology ponds merely deal with aeration. So improving and fine-tuning of the design of these ponds in terms of water mixing and sludge control is needed (Avnimelech, 2009).

Unlike the conventional techniques such as biofilters, bioflocs technology thus supports nitrogen removal even when organic matter and biological oxygen demand of the system water is high (Avnimelech, 2009). To establish bioflocs in aquaculture ponds, a certain start-up period is needed to obtain a well-developed system regarding water quality control. When using nitrifying bacteria in biofilters, they grow at a rate that is 10 times lower than that of heterotrophs, present in bioflocs (Crab et al., 2007). To establish the required microbial community in a biofilter one needs approximately 4 weeks, depending on nutrients, water flow rate and temperature (Avnimelech, 2009). Bioflocs can be established within a few weeks, depending on the nitrogen and organic load of the culture water and thus the intensity of the system. To even further shorten the start-up period of bioflocs technology, it might be interesting to look at the effect of adding nucleation sites, such as clay, to the water at start-up, which will stimulate floc formation. Also the inoculation with water from existing good performing bioflocs ponds might allow an accelerated start-up.

The strength of the bioflocs technology lies in its ‘cradle to cradle’-concept as described by McDonough and Braungart (2002) in which the very concept of waste does not exist. Translated in bioflocs terms, ‘waste’-nitrogen generated by uneaten feed and excreta from the cultured organisms is converted into proteinaceous feed available for those

same organisms. Instead of 'downcycling', a phenomenon often found in an attempt to recycle, the technique actually 'upcycles' through closing the nutrient loop. Hence, the water exchange can be decreased without deterioration of water quality and thus growth or survival and, consequently, the total amount of nutrients discharged into adjacent water bodies may be lessened (Lezama-Cervantes and Paniagua-Michel, 2010). In this context, bioflocs technology can also be used in the specific case of maintaining appropriate water temperature, good water quality and high fish survival in low/no water exchange, greenhouse ponds to overcome periods of lower temperature during winter (Crab et al., 2009). The study described in Chapter 5 regarding bioflocs technology application in overwintering of tilapia showed that fish survival levels were excellent, being 97 ± 6% for 100 g fish and 80 ± 4 for 50 g fish. Moreover, at harvest the condition of the fish was good in all ponds with a fish condition factor of 2.1 – 2.3. Besides winter periods, we need to be aware of the fact that future impacts of climate change on fisheries and aquaculture are still poorly understood and colder periods might be more often an issue to deal with in the future. The key to minimizing possible negative impacts of climate change on aquaculture and maximizing opportunities will be through understanding and promoting a wide range of inventive adaptive new technologies, such as the bioflocs technology combined with greenhouse ponds.

7.2 Implementation of the bioflocs technology in aquaculture

Nevertheless, no technique is without drawbacks and also the bioflocs technique is prone to obstacles. The major obstacle is to convince farmers to implement the technique, since the concept of bioflocs technology goes in against conventional common wisdom that water in the pond has to be clear (Avnimelech, 2009). Notwithstanding this impediment, several factors facilitate the implementation of the technique. Firstly, water has become scarce or expensive to an extent of limiting aquaculture development. Secondly, the release of polluted effluents into the environment is prohibited in most countries. Thirdly, severe outbreaks of infectious diseases led to more stringent biosecurity measures, such as reducing water exchange rates (Avnimelech, 2009). Experience regarding bioflocs technology and technical knowledge about the technique needs to be transferred to the farmers in a clear, practical and straightforward way, not forgetting to emphasis the economic benefit that comes along with this technique. Avnimelech (2009) recently published the handbook 'Biofloc Technology - A practical guide book' that is directed to aquaculturists, farmers,

students and scientists and is a first tremendous step forward in providing general information on this technology. A very important aspect in the implementation of bioflocs technology in aquaculture is monitoring of the ponds. Bioflocs technology is not yet fully predictable and can therefore be risky to implement at farm level. Possible monitoring tools are the concentration of total suspended solids or bioflocs, and the settleability of the biofloc, which can both be measured quickly and easily. Molecular monitoring can also provide much information on the condition of the bioflocs, but time and cost limitations might prevent the utility of this approach in real biofloc systems.

As soon as future research has fine-tuned the art of bioflocs technology and farmers are convinced to implement the technique, there is still the consumer that needs to be convinced to buy aquatic products originating from biofloc ponds. The simplified idea of recycling excreta of aquatic organisms into feed might frighten the consumers and prohibit them from buying these products. Despite this hitch, it is clear that with the growing human population, technological progress in aquaculture is needed to protect wild fish stocks and control fish prices (Jiang, 2010). Population growth pushes up fish prices as a result of a seafood shortage and increases pressure on wild fish stocks (Péron et al., 2010). In contrast, technological improvement tends to decrease fish prices and increases wild fish stocks by making the alternative fish product, farmed fish, relatively easier to produce. Therefore, bioflocs technology could alleviate the depletion of wild fish stocks and poverty, while improving social welfare through lowering the fish production prices, all beneficial for both farmer and consumer. Moreover, consumers now call for guarantees that their food has been produced, dealt with and commercialized in a way that is not hazardous to their health, respects the environment and addresses diverse other ethical and social considerations (FAO, 2009).

Besides bioflocs technology on its own, several researchers are looking at combinations of this method or other innovative techniques to control water quality in aquaculture and its effluents. Researchers are now investigating the combination of periphyton with carbon to nitrogen ratio control (Asaduzzaman et al., 2008; Asaduzzaman et al., 2010). Lezama-Cervantes and Paniagua-Michel (2010) investigated microbial mats that are able to adapt to large fluctuations in dissolved oxygen and pH and were able to remove and stabilize different organic and inorganic substrates partly due to the mixed autotrophic and heterotrophic communities that co-exist in the substrate matrix. Kumar and Lin (2010) investigated the use of short-cut nitrification-denitrification and anaerobic ammonium oxidation (anammox) for nitrogen removal. Their research indicated that these techniques could be useful and cost-effective especially for recirculating aquaculture systems with lower energy demand.

The use of bioflocs technology ponds integrated in a polyculture set-up is also an inventive and promising approach. Kuhn and co-workers included dried and processed bioflocs from tilapia ponds into shrimp feed and obtained about 1.6 times higher average weight gain per week than that obtained with commercial diets (Kuhn et al., 2009). Although this is an indirect form of polyculture, the more direct form – where the culture of fish or shrimp is integrated with vegetables, microalgae, shellfish and/or seaweed – can be very promising (Neori et al., 2004). This integrated intensive aquaculture strategy finds its origin in traditional extensive polycultures. Most of today's world aquaculture production is reared in semi-intensive and extensive systems. Nowadays, the interest in high technology intensive aquaculture systems increases with the increasing demand for aquaculture products. Bioflocs technology could be combined with polyculture ponds, further enhancing the water quality, natural food availability, dietary preference, growth and production in an intensive set-up (Rahman et al., 2008). At the University of the Virgin Islands, where researchers are currently looking at tilapia and shrimp polyculture in intensive, bacterial-based, aerated tanks. The multitrophic approach in polyculture of combining species with different specific feeding niches brings about a more complete use of resources than in the monoculture approach (Rahman et al., 2008).

7.3 The use of bioflocs as a feed for aquaculture species

In addition to the growing demand for seafood for human consumption, also the demand for production not dedicated to human consumption, but used by the industrial sector for conversion into fishmeal and fish oil products, increases (Péron et al., 2010). Fishmeal and fish oil are used as food for other human food supply systems, such as poultry, pigs and aquaculture. Hitherto, part of the aquaculture production relies on wild fish harvests, as fishmeal and fish oil are essential elements of the diet for aquaculture species, both carnivorous as herbivorous fish and shrimp. About 5-6 million tonnes of low-value/trash fish are used as direct feed in aquaculture worldwide either provided without processing or as part of farm-made feeds (FAO, 2009). FAO (2009) reported that the total amount of fishmeal and fish oil used in aquafeeds is estimated to have grown more than threefold between 1992 and 2006, from 0.96 million tonnes to 3.06 million tonnes and from 0.23 million tonnes to 0.78 million tonnes, respectively. For the 10 types of fish most regularly farmed, a mean of 1.9 kg of wild fish is required for every kilogram of fish reared on commercial feeds (Naylor et al., 2000). In terms of fishmeal, many intensive and semi-intensive aquaculture systems use 2 to 5 times more fish protein to feed the farmed species than is supplied by the farmed product (Naylor et

al., 2000). Therefore, research in recent times has focused on the development of feed substitution strategies with a minimal supply of fishmeal and fish oil, which are replaced by alternative and cheaper sources of protein such as plant proteins. In contrast to intensive and semi-intensive systems, extensive and traditional systems already use little or no fishmeal, farmers often supply nutrient-rich materials to the water to enhance growth of algae and other indigenous organisms on which the fish can feed (Naylor et al., 2000). This inspired researchers to develop the bioflocs technology, which is in addition to extensive and traditional systems also applicable to intensive and semi-intensive systems. With bioflocs technology, where nitrogenous waste generated by the cultivated organisms is converted into bacterial biomass (containing protein), *in situ* feed production is stimulated through the addition of an external carbon source (Schneider et al., 2005).

In this work, bioflocs were grown on glucose, starch, acetate and glycerol. An overview of the prime nutritional parameters measured for these bioflocs are presented in Table 7.1. This overview shows that, as stated before, the bioflocs technology is not yet fully predictable and although the same carbon sources were applied in the different studies, nutritional parameters differed. On the other hand, an important observation was made, being that glycerol bioflocs have overall a higher total n-6 PUFA content. Although the biofloc reactors were initiated with the same inoculum, drum filter slurry originating from an intensive tilapia aquaculture farm (VitaFish, Dottignies – Mouscron, Belgium), it might have had a different composition over time and hence, influenced the results of each study.

Table 7.1 Overview of nutritional parameters of the bioflocs, obtained in the different studies (Ch.: Chapter).

	Glucose			Glucose + *Bacillus*	Starch	Acetate		Glycerol		Glycerol + *Bacillus*	
	Ch.2	Ch.3	Ch.4	Ch.4	Ch.2	Ch.2	Ch.3	Ch.3	Ch.4	Ch.3	Ch.4
Crude protein (% DW)	40 ± 6	28 ± 3	19 ± 7	20 ± 4	21 ± 3	19 ± 8	42 ± 8	43 ± 1	15 ± 3	58 ± 9	22 ± 10
Crude Lipid (% DW)	41 ± 9	5.4 ± 0.6	nd	nd	17 ± 1	21 ± 11	2.3 ± 0.4	2.9 ± 0.9	nd	3.5 ± 0.7	nd
Ash (% DW)	5 ± 2	17 ± 1	nd	nd	3 ± 2	20 ± 10	27 ± 7	20 ± 3	nd	25 ± 5	nd
Carbohydrate (% DW)	14 ± 17	50 ± 4	nd	nd	59 ± 6	40 ± 13	29 ± 14	34 ± 3	nd	14 ± 11	nd
Gross energy (kJ/g DW)	27 ± 2	17.0 ± 0.2	nd	nd	21 ± 1	19 ± 1	15.5 ± 0.8	16.9 ± 0.5	nd	17.0 ± 0.6	nd
Total n-3 PUFA (mg/g DW)	1.0 ± 0.3	0.65 ± 0.07	0.3 ± 0.3	0.7 ± 0.3	0.30 ± 0.07	0.19 ± 0.08	0.4 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
Total n-6 PUFA (mg/g DW)	0.80 ± 0.03	12 ± 4	9 ± 11	28 ± 32	1.0 ± 0.1	0.6 ± 0.1	7 ± 2	20 ± 5	12 ± 16	27 ± 3	17 ± 16

Although bioflocs show an adequate protein, lipid, carbohydrate and ash content for use as an aquaculture feed, more research is needed on its amino acids and fatty acids

composition. Now, fishmeal and fish oil supply essential amino acids (such as lysine and methionine) that are deficient in plant proteins and fatty acids (eicosapentanoic acid and docosahexanoic acid) not found in vegetable oils (Naylor et al., 2000). Herbivorous, omnivorous and carnivorous finfish all necessitate about the same amount of dietary protein per unit weight, but herbivorous and omnivorous species utilize plant-based proteins and oils better and they require minimal quantities of fishmeal to supply essential amino acids (Naylor et al., 2000). Therefore, lowering the input of wild fish required for production of farmed carnivorous fish seems not feasible at this time. Nevertheless, compound feeds for omnivorous fish often exceed required levels (Naylor et al., 2000). As already discussed above, it is very important to inform the farmers clearly and thoroughly, at this juncture about feeding strategies and management. New initiatives by governments and funding organizations are needed that can act as incentives for aquaculture to augment farming of low trophic level with herbivorous diets in stead of high-value, carnivorous fish that increases the need for fishmeal and fish oil, which in turn could place even more stress on pelagic fisheries, resulting in high feed prices and damage to marine ecosystems (Naylor et al., 2000). Concomitantly, more research is needed regarding feed replacement strategies such as those handling substituting vegetable oils, meat byproducts and bioflocs technology. With bioflocs technology, one also needs to consider that one is dealing with high suspended solids concentrations, which affects the choice of cultivated species, since certain fish species are negatively affected by high suspended solids concentrations. Besides the effects for the farmer, this 'farming down the food web' will also affect the consumer and asks for sensitizing the consumer's awareness.

Under the concept of using bioflocs as an *in situ* produced feed, several studies were performed and they indicate that bioflocs can be taken up by aquaculture species depending on the species and feeding traits, fish size, floc size and floc density (Avnimelech, 2007; Crab et al., 2009; Crab et al., 2010b; Crab et al., 2010c). In this work *Macrobrachium rosenbergii*, *Litopenaeus vannamei* and tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) were all positively identified to take up bioflocs and profit from this additional protein source. This makes bioflocs technology applicable to both freshwater and seawater systems both to control water quality and as a surplus through *in situ* feed production. The potential gain on feed is estimated to be in the order of 10-20% (De Schryver et al., 2008). With this, production costs will decline considerably since food represents 40-50% of the total production costs (Craig and Helfrich, 2002).

While in general bioflocs meet proper nutritional standards to serve as a aquaculture feed, research has shown that depending on the carbon source used, the capacity of the technique to control the water quality in the culture system and the nutritional properties of the flocs are influenced (Crab et al., 2010a; Crab et al., 2010b; Crab et al.,

2010c). Each different organic carbon source used, stimulated specific bacteria, protozoa and algae, and hence influenced the composition and community organization of the bioflocs, both the prokaryotic and eukaryotic component (Crab et al., 2010a). Feeding experiments revealed that besides these characteristics, also the availability, palatability and digestibility towards the culture organisms were influenced (Crab et al., 2010b; Crab et al., 2010c). Overall, glycerol fed bioflocs gave the preeminent results in this work. Future research, however, should focus on the use of other possible low-cost non-conventional agro-industrial residues and hence upgrade waste to nutritious feed. Different carbon sources will stimulate the growth of the indigenous microbiota in another way and thus exert a distinctive effect on water quality, *in situ* feed production and utilization of the flocs by the culture organism. In addition, not only the carbon source, but also the indigenous microbiota present in the pond water will put forth a characteristic effect that needs to be considered. Downstream carbonaceous byproducts of local industry can provide a low cost external carbon source for application of bioflocs technology in nearby ponds, but will need preceding research before implementation. The problem might be that nowadays all carbon sources have a certain value and possible application, which raises the question whether it is acceptable to take a carbon source with a certain value to upgrade nitrogen from feces to microbial protein. These questions can be answered through field studies and case-by-case economical analysis. Moreover, this technique offers the choice of elevating the organic carbon to nitrogen ratio through addition of an external organic carbon source or through elevating the carbon content of the feed fed to the culture organism (Crab et al., 2009). The application of these lower protein pellets has the advantage of convenience and saving labor, as compared to separate application of feed pellets and an organic carbon source (Avnimelech, 2009). Another consideration to make in this decision process is the possible added features that are related to a specific carbon source. For example, glycerol bioflocs tend to have a higher n-6 fatty acids content as compared to acetate and glucose bioflocs (Crab et al., 2010b). An important factor here is to determine the role of algae and their interaction with the bacteria in the bioflocs. Crab et al. (2010c) showed that with *Litopenaeus vannamei*, glucose bioflocs lacked accessibility and palatability for good survival and growth. The latter opens an interesting field of research, where one can look at carbon sources that would increase attractiveness of the bioflocs towards fish and shrimp. A worthy carbon source to look at in this regard is molasses obtained during sugar processing of sugar beet (*Beta vulgaris* L. v. *altissima*), which contains glycine betaine, a known attractants used in aquaculture (Mäkelä et al., 1998; Felix and Sudharsen, 2004). All these findings and possible modus operandi emphasize the need for further study of floc composition in order to achieve a desired nutritional outcome, since different research groups obtain different results in respect to biofloc nutritional composition (Avnimelech, 2009).

7.4 The use of bioflocs as a biosecurity measure

Above and beyond all the advantages of the bioflocs technology discussed above, Crab et al. (2010d) have recently shown that bioflocs are a possible alternative measure to fight pathogens in aquaculture. Intensive aquaculture of crustaceans is one of the fastest growing sectors in aquaculture production (Wang et al., 2008). Despite its huge success, shrimp culture is facing severe outbreaks of infectious diseases, which have caused significant economic losses. Due to the haphazard mishandling of antibiotics in aquaculture, Vibrios are now resistant to numerous antibiotics and as a result, antibiotics are no longer effective in treating luminescent Vibriosis (Defoirdt et al., 2008). Defoirdt et al. (2004) proposed to control infections in aquaculture through disruption of quorum sensing, bacterial cell-to-cell communication, as a new strategy. Glycerol bioflocs were able to protect gnotobiotic brine shrimp (*Artemia franciscana*) against the pathogenic *Vibrio harveyi*, likely through interference with the pathogen's quorum sensing system (Crab et al., 2010d). The results presented in Chapter 6 show that survival of challenged nauplii increased 3-fold after addition of live glycerol-grown bioflocs. This complies with former research that revealed that primary production and promotion of *in situ* microbial populations, as is the case in bioflocs technology, were found to be beneficial for shrimp (Lezama-Cervantes and Paniagua-Michel, 2010). In contrast to the bioflocs protective action against *Vibrio harveyi*, this was not the case against *Vibrio campbellii* (Crab et al., 2010d). The exact mechanism of the protective action of the bioflocs and its selective action, however, needs further in depth investigation. Another interesting feature of bioflocs to further investigate in this regard is its poly-β-hydroxybutyrate content. Poly-β-hydroxybutyrate accumulating bacteria, likely present in the bioflocs, were shown to protect *Artemia franciscana* from *Vibrio campbellii*, only if it had a PHB content of at least 15% DW and if it had been subjected to freezing and thawing, resulting in a higher concentration of conglomerates smaller than 50 µm (Crab et al., 2010a; Halet et al., 2007). Untreated cultures had no effect on the survival of the challenged organism. In the work presented in Chapter 2 measured PHB levels were 0.5, 6.0 and 18% DW for the starch, glucose and acetate treatments, respectively.

Another important factor essential for growth and survival of aquaculture species are vitamins. Vitamin C content of the bioflocs was determined in this study and ranged from 0 to 54 µg/g DW. The measured values were below the required concentration for fish and shrimp. Besides vitamin C, other vitamins such as thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, biotin, folic acid, vitamin B12, inositol, choline, vitamin A, vitamin D3, vitamin E and vitamin K, are not synthesized by the culture organism or at a sufficient rate, and need to be supplied through the feed. Other

features, such as minerals (e.g. Calcium, Magnesium, Phosphorus, Iron and Zinc), growth factors (e.g. probiotics) and other micronutrients, need further research regarding their presence/absence in bioflocs, depending on the carbon source used.

The stimulated indigenous organisms present in bioflocs may contribute nutritionally and serve as a pre- or probiotics and/or unknown growth promoters. Numerous researches have denoted that shrimp are healthiest and grow best in aquaculture systems that have high levels of algae, bacteria and other natural biota (Kuhn et al., 2009). Probiotics, as mentioned above, are viable cells that have beneficial effects on health of a host by improving its intestinal equilibrium through improved feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, antimutagenic and anticarcinogenic actions, growth-promoting factors, and an increased immune response (Liu et al., 2010). In an attempt to produce probiotic bioflocs, we inoculated the biofloc reactors with a *Bacillus* mixture. Several research articles regarding the benefits of using *Bacillus* to improve shrimp growth performance, survival, immunity, and disease resistance in aquaculture have been published (Tseng et al., 2009). Decamp et al. (2008) studied the effect of a commercial probiotics product, which is a mixture of specific *Bacillus* strains, on *Vibrio* strains; the results showed that the probiotics were able to inhibit the growth of *Vibrio* resulting in an improved shrimp larvae survival rate. Also in the study of Crab et al. (2010c), preliminary results show that the water of shrimp tanks fed bioflocs inoculated with *Bacillus* had an on average 5 times lower *Vibrio* load as compared to the shrimp tanks fed artificial feed. These results indicate that inoculating biofloc reactors with probiotic bacteria might have biocontrol effect towards *Vibrio* spp., but this field of inoculating bioflocs with specific desired microorganisms needs further in depth investigation. Other interesting fields of research regarding this subject are possible immunostimulatory features of the bioflocs. Enhancement of the shrimp's innate immunity may provide broad-spectrum resistance to infections. Existing immunostimulants include bacteria and bacterial products, complex carbohydrates, nutritional factors, animal extracts, cytokines, lectins, plant extracts and synthetic drugs such as levamisole (Wang et al., 2008). Since bioflocs technology deals with bacteria and bacterial products, complex carbohydrates and nutritional factors, this might very likely be the case. Since the bacterial composition and hence bacterial products of the bioflocs is influenced by the carbon source used to develop the technology, it speaks for itself that through the choice of carbon source also the possible immunostimulatory features of the flocs will be influenced.

7.5 Conclusion

The research presented in this work relates to a variety of features that can be ascribed to bioflocs technology, from water quality control to *in situ* feed production and some possible concomitantly extra added features. Bioflocs technology offers aquaculture a sustainable tool to simultaneously address its environmental, social and economical issues concurrent with its growth. Researchers are challenged to further develop this technique and farmers to implement it in their future aquaculture systems. The basis of the technology is there, but its further development, fine-tuning and implementation will need further research and development from the present and future generation of researchers, farmers and consumers to make this technique a keystone of future sustainable aquaculture.

REFERENCES

Abdel-Tawwab, M., Mousa, M.A.A.A., Ahmad, M.H., Sakr, S.F.M., 2007. The use of calcium pre-exposure as a protective agent against environmental copper toxicity for juvenile Nile tilapia, *Oreochromis niloticus* (L.). Aquaculture 264, 236-246.

Ali, A., Al-Ogaily, S.M., Al-Asgah, N.A., Goddard, J.S., Ahmed, S.I., 2008. Effect of feeding different protein to energy (P/E) ratios on the growth performance and body composition of *Oreochromis niloticus* fingerlings. Journal of Applied Ichtyology 24, 31-37.

Allan, G.L., Moriarty, D.J.W., Maguire, G.B., 1995. Effects of pond preparation and feeding rate on production *Penaeus monodon* Fabricius, water quality, bacteria and benthos in model farming ponds. Aquaculture 130, 329-349.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiol. Mol. Biol. Rev. 59, 143-169.

Amaya, E.A, Davis, D.A., Rouse, D.B., 2007a. Alternative diets for the Pacific white shrimp *Litopenaeus vannamei*. Aquaculture 262, 419-425.

Amaya, E.A., Davis, D.A., Rouse, D.B., 2007b. Replacement of fish meal in practical diets for the Pacific white shrimp (*Litopenaeus vannamei*) reared under pond conditions. Aquaculture 262, 393-401.

Amirkolaie, A.K., 2005. Dietary carbohydrate and faecal waste in the Nile tilapia (*Oreochromis niloticus* L.). PhD dissertation. University Wageningen, Wageningen, The Netherlands.

Anupama, P.R., 2000. Value-added food: Single cell protein. Biotechnol. Adv. 18, 459-479.

APHA, 1998. Standard methods for the examination of the water and wastewater (22nd ed.), American Public Health Association, Washington, DC (1998).

AQUA Culture AsiaPacific Magazine, 2008. May/June 2008, p. 4.

Asaduzzaman, M., Wahab, M.A., Verdegem, M.C.J., Adhikary, R.K., Rahman, S.M.S., Azim, M.E., Verreth, J.A.J., 2010. Effects of carbohydrate source for maintaining a high C:N ratio and fish driven re-suspension on pond ecology and production in periphyton-based freshwater prawn culture systems. Aquaculture 301, 37-46.

Asaduzzaman, M., Wahab, M.A., Verdegem, M.C.J., Huque, S., Salam, M.A., Azim, M.E., 2008. C/N ratio control and substrate addition for periphyton development jointly enhance freshwater prawn *Macrobrachium rosenbergii* production in ponds. Aquaculture 280, 117-123.

Avnimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176, 227-235.

Avnimelech, Y., 2005. Tilapia harvest microbial flocs in active suspension research pond. Global Aquaculture Advocate. October 2005.

Avnimelech, Y., 2006. Bio-filters: the need for an new comprehensive approach. Aquac. Eng. 34, 172-178.

Avnimelech, Y., 2007. Feeding with microbial flocs by tilapia in minimal discharge bio-flocs technology ponds. Aquaculture 264, 140-147.

Avnimelech, Y., 2009. Biofloc Technology – A practical guide book. The World Aquaculture Society, Baton Rouge, Louisiana, United States. 182 pp.

Avnimelech, Y., Kochva, M., Diab, S., 1994. Development of controlled intensive aquaculture systems with a limited water exchange and adjusted carbon to nitrogen ratio. Bamidgeh 46, 119-131.

Avnimelech, Y., Lacher, M., 1979. A tentative nutrient balance for intensive fish ponds. Bamidgeh, 31, 3-8.

Avnimelech, Y., Mokady, S., Schroeder, G.L., 1989. Circulated ponds as efficient bioreactors for single-cell protein production. Israeli J. Aquaculture Bamidgeh 41, 58-66.

Avnimelech, Y., Ritvo, G., 2003. Shrimp and fish pond soils: Processes and management. Aquaculture 220, 549-567.

Azim, M.E., Asaeda, T., 2005. Periphyton structure, diversity and colonization. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 15-33.

Azim, M.E., Beveridge, M.C.M., van Dam, A.A., Verdegem, M.C.J., 2005. Periphyton and aquatic production: an introduction. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 1-13.

Azim, M.E., Little, D.C., 2008. The biofloc technology (BFT) in indoor tanks: Water quality, biofloc composition, and growth and welfare of Nile tilapia (*Oreochromis niloticus*). Aquaculture 283, 29-35.

Azim, M.E., Milstein, A., Wahab, M.A., Verdegem, M.C.J., 2003a. Periphyton-water quality relationships in fertilized fishponds with artificial substrates. Aquaculture 228, 169-187.

Azim, M.E., Verdegem, M.C.J., Mantingh, I., van Dam, A.A., Beveridge, M.C.M., 2003b. Ingestion and utilization of periphyton grown on artificial substrates by Nile tilapia, *Oreochromis niloticus* L. Aquac. Res. 34, 85-92.

Azim, M.E., Verdegem, M.C.J., Singh, M., van Dam, A.A., Beveridge, M.C.M., 2003c. The effects of periphyton substrate and fish stocking density on water quality, phytoplankton, periphyton and fish growth. Aquac. Res. 34, 685-695.
Azim, M.E., Wahab, M.A., 2005. Periphyton-based pond polyculture. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 207-222.
Azim, M.E., Wahab, M.A., Biswas, P.K., Asaeda, T., Fujino, T., Verdegem, M.C.J., 2004. The effect of periphyton substrate density on production in freshwater polyculture ponds. Aquaculture 232, 441-453.
Azim, M.E., Wahab, M.A., van Dam, A.A., Beveridge, M.C.M., Verdegem, M.C.J., 2001. The potential of periphyton-based culture of two Indian major carps, rohu *Labeo rohita* (Hamilton) and gonia *Labeo gonius* (Linnaeus). Aquac. Res. 32, 209-216.
Azim, M.E., Wahab, M.A., Verdegem, M.C.J., van Dam, A.A., van Rooij, J.M., Beveridge, M.C.M., 2002. The effects of artificial substrates on freshwater pond productivity and water quality and the implications for periphyton-based aquaculture. Aquat. Living Resour. 15, 231-241.
Aziz, J.A., Tebbutt, T.H.Y., 1980. Significance of COD, BOD and TOC correlations in kinetic models of biological oxidation. Water Res. 14, 319-324.
Bassler, B.L., Greenberg, E.P., Stevens, A.M., 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. 179, 4043-4045.
Bassler, B.L., Wright, M., Showalter, R.E., Silverman, M.R., 1993. Intercellular signaling in *Vibrio harveyi* – sequence and function of genes regulating expression of luminescence. Mol. Microbiol. 9, 773-786.
Bender, J., Lee, R., Sheppard, M., Brinkley, K., Philips, P., Yeboah, Y., Wah, R.C., 2004. A waste effluent treatment system based on microbial mats for black sea bass *Centropristis striate* recycled-water mariculture. Aquaculture Engineering 31, 73-82.
Biggs, C.A., Lant, P.A., 2000. Activated sludge flocculation: on-line determination of floc size and the effect of shear. Water Res. 34, 2542-2550.
Bodík, I., Blstáková, A., Sedlácek, S., Hutnan, M., 2009. Biodiesel waste as source of organic carbon for municipal WWTP denitrification. Bioresour. Technol. 100, 2452-2456.
Boon, N., De Windt, W., Verstraete, W., Top, E.M., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol. Ecol. 39, 101-112.
Boon, N., Goris, J., De Vos, P., Verstraete, W., Top, E.M., 2000. Bioaugmentation of Activated Sludge by an Indigenous 3-Chloroaniline-Degrading *Comamonas testosteroni* Strain, I2*gfp*. Applied and Environmental Microbiology 66, 2906-2913.

Boujard, T., Gélineau, A., Covíès, D., Corraze, G., Dutto, G., Gasset, E., Kaushik, S., 2004. Regulation of feed intake, growth, nutrient and energy utilization in European seabass (*Dicentrarchus labrax*) fed high fat diets. Aquaculture 231, 529-545.

Boyd, C., Zimmerman, S., 2000. Grow-out systems – water quality and soil management, in: New, M.B., Valenti, W.C. (Eds.), Freshwater Prawn Culture – The Farming of *Macrobrachium rosenbergii*. Blackwell Science, Oxford, UK, pp. 221-238.

Boyd, C.E., 1985. Chemical budgets for channel catfish ponds. Trans. Am. Fish. Soc. 114, 291-298.

Boyd, C.E., 1998. Pond water aeration systems. Aquacult. Eng. 18, 9-40.

Brazil, B.L., 2006. Performance and operation of a rotating biological contactor in a tilapia recirculating aquaculture system. Aquacult. Eng. 34, 261-274.

Brune, D.E., Schwartz, G., Eversole, A.G., Collier, J.A., Schwedler, T.E., 2003. Intensification of pond aquaculture and high rate photosynthetic systems. Aquacult. Eng. 28, 65-86.

Burdman, S., Jurkevitch, E., Soria-Diaz, M.E., Serrano, A.M.G., Okon, Y., 2000. Extracellular polysaccharide composition of *Azospirillum brasilense* and its relation with cell aggregation. FEMS Microbiol. Lett. 189, 259-264.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., Pearson, D.C., 2003. Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. Aquaculture 219, 393-411.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., Pearson, D.C., 2004. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system. Aquaculture 232. 525-537.

Cammarota, M.C., Sant-Anna, G.L., 1998. Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. Biotechnol. Lett. 20, 1-4.

Cavalli, R.O., Lavens, P., Sorgeloos, P., 1999. Performance of *Macrobrachium rosenbergii* broodstock fed diets with different fatty acid composition. Aquaculture 179, 387-402.

Cavalli, R.O., Menschaert, G., Lavens, P., Sorgeloos, P., 2000. Maturation performance, offspring quality and lipid composition of *Macrobrachium rosenbergii* females fed increasing levels of dietary phospholipids. Aquacult. Int. 8, 41-58.

Chaignon, V., Lartiges, B.S., El Samrani, A., Mustin, C., 2002. Evolution of size distribution and transfer of mineral particles between flocs in activated sludges: an insight into floc exchange dynamics. Water Res. 36, 676-684.

Charo-Karisa, H., Komen, H., Reynolds, S., Rezk, M.A., Ponzoni, R., Bovenhuis, H., 2006a. Genetic and environmental factors affecting growth of Nile tilapia (*Oreochromis niloticus*) juveniles: Modeling spatial correlations between hapas. Aquaculture 255, 586-596.

Charo-Karisa, H., Rezk, M.A., Bovenhuis, H., Komen, H., 2005. Heritability of cold tolerance in Nile tilapia, *Oreochromis niloticus*, juveniles. Aquaculture 249, 115-123.

Charo-Karisa, H., Rezk, M.A., Bovenhuis, H., Komen, H., 2006b. Effects of rearing conditions on low-temperature tolerance of Nile tilapia, *Oreochromis niloticus*, juveniles. pp 30-41.
Chen, S., Ling, J., Blancheton, J.P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacult. Eng. 34, 179-197.
Chervinski, J., 1982. Environmental physiology of tilapias. In: Pullin, R.S.V., Lowe-McConnel, R.H. (Eds.), The Biology and Culture of Tilapias, ICLARM Conference Proceedings, vol. 7. International Centre for Living Aquatic Resources Management, Manila, pp. 119-128.
Cho, C.Y., Hynes, J.D., Wood, K.R., Yoshida, H.K., 1994. Development of high-nutrient-dense, low-pollution diets and prediction of aquaculture wastes using biological approaches. Aquaculture 14, 293-305.
Cho, C.Y., Kaushik, S.J., 1990. Nutritional energetic in fish: energy and protein utilization in rainbow trout (*Salmo gairdneri*). World Rev. Nutr. Diet 61, 132-172.
Chu, C.P., Lee, D.J., 2004. Multiscale structures of biological flocs. Chem. Eng. Sci. 59, 1875-1883.
Colt, J., 2006. Water quality requirements for reuse systems. Aquacult. Eng. 34, 143-156.
Correia, E.S., Pereira, J.A., Apolinario, M.O., Horowitz, A., Horowitz, S., 2002. Effect of pond aging on natural food availability and growth of the freshwater prawn *Macrobrachium rosenbergii*. Aquacult. Eng. 26, 61-69.
Correia, E.S., Pereira, J.A., Silva, A.P., Horowitz, A., Horowitz, S., 2003. Growout of freshwater prawn *Macrobrachium rosenbergii* in fertilized ponds with reduced levels of formulated feed. J. World Aquacult. Soc. 34, 184-191.
Cosson, P., Zullianello, L., Join-Lambert, O., Faurisson, F., Gebbie, L., Benghezal, M., van Delden, C., Curty, L.K., Kohler, T., 2002. *Pseudomonas aeruginosa* virulence analyzed in a *Dictostelium discoideum* host system. J. Bacteriol. 184, 3027-3033.
Coutteau, P., Sorgeloos, P., 1995. Intercalibration exercise on the qualitative and quantitative analysis of fatty acids in *Artemia* and marine samples used in mariculture. ICES Cooperative Research Report, N° 211, 30 pp.
Coyle, S., Tidwel, J.H., 2002. Research examines feeding regimes for freshwater prawns. Glob. Aquacult. Advocate 5, 28-29.
Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., Verstraete, W., 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1-14.
Crab, R., Boon, N., Defoirdt, T., Bossier, P., Verstraete, W., 2010a. Different organic carbon sources shape the nutritional properties and the microbial community composition of bioflocs. Aquaculture, Submitted.
Crab, R., Chielens, B., Wille, M., Bossier, P., Verstraete, W., 2010b. The effect of different carbon sources on the nutritional value of bioflocs, a feed for

Macrobrachium rosenbergii postlarvae. Aquac. Res. 41, 559-567.
Crab, R., Ekasari, J., Defoirdt, T., Verstraete, W., Bossier, P., 2010c. The effect of bioflocs on the survival and growth of white shrimp *Litopenaeus vannamei*. Aquaculture, Submitted.
Crab, R., Kochva, M., Verstraete, W., Avnimelech, Y., 2009. Bio-flocs technology application in over-wintering of tilapia. Aquacult. Eng. 40, 105-112.
Crab, R., Lambert, A., Defoirdt, T., Bossier, P., Verstraete, W., 2010d. Bioflocs protect gnotobiotic brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. J. Appl. Microbiol., In Press.
Craggs, R.J., Adey, W.H., Jessup, B.K., Oswald, W.J., 1996. A controlled stream mesocosm for tertiary treatment of sewage. Ecol. Eng. 6, 149-169.
Craig, S., Helfrich, L.A., 2002. Understanding Fish Nutrition, Feeds and Feeding (Publication 420-256). Virginia Cooperative Extension, Yorktown (Virginia). 4 pp.
Cruz, E.M., Ridha, M.T., 1995. Survival rates of tilapia, *Oreochromis spilurus* (Günther), fingerlings reared at high densities during winter using warm underground sea water. Aquaculture Research 26, 307-309.
D'Abramo, L.R., 1998. Nutritional requirements of the freshwater prawn *Macrobrachium rosenbergii*: comparisons with species of penaeid shrimp. Rev. Fish. Sci. 6, 153-163.
Dan, N.C., Little, D.C., 2000a. Overwintering performance of Nile tilapia *Oreochromis niloticus* (L.) broodfish and seed at ambient temperatures in northern Vietnam. Aquaculture Research 31, 485-493.
Dan, N.C., Little, D.C., 2000b. The culture performance of monosex and mixed-sex new-season and overwintered fry in three strains of Nile tilapia (*Oreochromis niloticus*) in northern Vietnam. Aquaculture 184, 221-231.
Das, S.K., Tiwari, V.K., Venkateshwarlu, G., Reddy, A.K., Parhi, J., Sharma, P., Chettri, J.K., 2007. Growth, survival and fatty acid composition of *Macrobrachium rosenbergii* (de Man, 1879) post larvae fed HUFA-enriched *Moina micrura*. Aquaculture 269, 464-475.
de Barros, H.P., Valenti, W.C., 2003. Food intake of *Macrobrachium rosenbergii* during larval development. Aquaculture 216, 165-176.
De Schryver, P., Crab, R., Defoirdt, T., Boon, N., Verstraete, W., 2008. The basics of bioflocs technology: The added value for aquaculture. Aquaculture 277, 125-137.
De Schryver, P., Verstraete, W., 2009. Nitrogen removal from aquaculture pond water by heterotrophic nitrogen assimilation in lab-scale sequencing batch reactors. Bioresource Technol. 100, 1162-1167.
Decamp, O., Moriarty, D.J.W., Lavens, P., 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquac. Res. 39, 334-338.

Defoirdt, T., Boon, N., Bossier, P., Verstraete, W., 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aquaculture 240, 69-88.
Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2008. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from *in vivo* work. The ISME Journal 2, 19-26.
Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W., 2006. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. Aquaculture 261, 804-808.
Defoirdt, T., Halet, D., Sorgeloos, Vervaeren, H., Boon, N., Van de Wiele, T., Sorgeloos, P., Bossier, P., Verstraete, W., 2007. The bacterial storage compound poly-β-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. Environ. Microbiol. 9, 445-452.
delos Reyes, A.A., Lawson, T.B., 1996. Combination of a bead filter and rotating biological contactor in a recirculating fish culture system. Auquacult. Eng. 15, 27-39.
Diab, S., Cochaba, M., Mires, D., Avnimelech, Y., 1992. Combined intensive – extensive (CIE) pond system. A: Inorganic nitrogen transformations. Aquaculture 101, 33-39.
Dias, J., Alvarez, M.J., Diez, A., Arzel, J., Corraze, G., Bautista, J.M., Kaushik, S.J., 1998. Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (*Dicentrarchus labrax*). Aquaculture 161, 169-186.
Díez, B., Pedros-Alio, C., Marsh, T.L., Massana, R., 2001. Application of denaturing gradient gel electrophoresis (DGGE) to study the diversity of marine picoeukaryotic assemblages and comparison of DGGE with other molecular techniques. Appl. Environ. Microbiol. 67, 2942-2951.
Dodds, W.K., 2003. The role of periphyton in phosphorus retention in shallow freshwater aquatic systems. J. Phycol. 39, 840-849.
Dube, M.A., Tremblay, A.Y., Lio, J., 2007. Biodiesel production using a membrane reactor. Bioresour. Technol. 98, 639-647.
Duston, J., Astatkie, T., Maclsaac, P.F., 2004. Effect of body size on growth and food conversion of juvenile striped bass reared at 16-28°C in freshwater and seawater. Aquaculture 234, 589-600.
Ebeling, J.M., Timmons, M.B., Bisogni, J.J. (2006). Engineering analysis of the stoichiometry of photoautotrophic, autotrophic and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346-358.
Eboigbodin, K.E., Newton, J.R.A., Routh, A.F., Biggs, C.A., 2006. Bacterial quorum sensing and cell surface electrokinetic properties. Appl. Microbiol. Biotechnol. 73, 669-675.
Eding, E.H., Kamstra, A., Verreth, J.A.J., Huisman, E.A., Klapwijk, A., 2006. Design and operation of nitrifying trickling filters in recirculating aquaculture: A review. Aquacult. Eng. 34, 234-260.

El Samra, M.I., Olàh, J., 1979. Significance of nitrogen fixation in fish ponds. Aquaculture 18, 367-372.
FAO, 2004. The state of world fisheries and aquaculture 2004. FAO, Rome.
FAO, 2005-2008. Text by New, M.B. In: FAO Fisheries and Aquaculture Department (online). Rome.
http://www.fao.org/fishery/culturedspecies/Macrobrachium_rosenbergii.
FAO, 2006. Use of fishery resources as feed inputs to aquaculture development: trends and policy implications. FAO Fisheries Circular No. 1018. Food and Agriculture Organization of the United Nations, Rome, 2006.
FAO, 2009. The state of world fisheries and aquaculture 2008. FAO, Rome.
Farzanfar, A., 2006. The use of probiotics in shrimp aquaculture. FEMS Immunol. Med. Microbiol. 48, 149-158.
Felix, N., Sudharsan, M., 2004. Effect of glycine betaine, a feed attractant affecting growth and feed conversion of juvenile freshwater prawn *Macrobrachium rosenbergii*. Aquacult. Nutr. 10, 193-197.
Fish Breeding Association, 2003. Technical Handbook 2003, Fish Breeding Association, Israel, 106 pp. (Hebrew).
Flemish government, 2005. Afvalwaterproblematiek op melkveebedrijven. Flemish government, Department Agriculture and Fisheries, Department Sustainable Agricultural Development, Belgium, 61 pp.
Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509.
Franco-Nava, M.A., Blancheton, J.P., Deviller, G., Charrier, A., Le-Gall, J.Y., 2004a. Effect of fish size and hydraulic regime on particulate organic matter dynamics in a recirculating aquaculture system: elemental carbon and nitrogen approach. Aquaculture 239, 179-198.
Franco-Nava, M.A., Blancheton, J.P., Deviller, G., Charrier, A., Le-Gall, J.Y., 2004b. Particulate matter dynamics and transformations in a recirculating aquaculture system: application of stable isotope tracers in seabass rearing. Aquacult. Eng. 31, 135-155.
Fryer, G., Iles, T.D., 1972. The Cichlid Fishes of the Great Lakes of Africa, Their Biology and Evolution. Oliver & Boyd, Edinburgh, Scotland, 641 pp.
Fux, C., Siegrist, H., 2004. Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitration/anammox: environmental and economical considerations. Water Science and Technology 50, 19-26.
Garatun-Tjeldsto, O., Ottera, H., Julshamn, K., Austreng, E., 2006. Food ingestion in juvenile cod estimated by inert lanthanide markers – effects of food particle size. Ices J. Mar. Sci. 63, 311-319.
Geets, J., de Cooman, M., Wittebolle, L., Heylen, K., Vanparys, B., De Vos, P., Verstraete, W., Boon, N., 2007. Real-time PCR assay for the simultaneous

quantification of nitrifying and denitrifying bacteria in activated sludge. Appl. Microbiol. Biotechnol. 75, 211-221.
Gelegenis, J., Dalabakis, P., Ilias, A., 2006. Heating of a fish wintering pond using low-temperature geothermal fluids, Porto Lagos, Greece. Geothermics 35, 87-103.
Ghaly, A.E., Kamal, M., Mahmoud, N.S., 2005. Phytoremediation of aquaculture wastewater for water recycling and production of fish feed. Environ. Int. 31, 1-13.
Gomez-Gil, B., Soto-Rodriguez, S., Garcia-Gasca, A., Roque, A., Vazquez-Juarez, R., Thompson, F.L., Swings, J., 2004. Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. Microbiology 150, 1769-1777.
Gonzalez-Felix, M.L., Lawrence, A.L., Gatlin, D.M., Perez-Velazquez, M., 2002. Growth, survival and fatty acid composition of juvenile *Litopenaeus vannamei* fed different oils in the presence and absence of phospholipids. Aquaculture 205, 325-343.
Greiner, A.D., Timmons, M.B., 1998. Evaluation of the nitrification rates of microbead and trickling filters in an intensive recirculating tilapia production facility. Aquacult. Eng. 18, 189-200.
Grommen, R., Dauw, L., Verstraete, W., 2005. Elevated salinity selects for a less diverse ammonia-oxidizing population in aquarium biofilters. FEMS Microbiol. Ecol. 52, 1-11.
Grommen, R., Van Hauteghem, I., Van Wambeke, M., Verstraete, W., 2002. An improved nitrifying enrichment to remove ammonium and nitrite from freshwater aquaria systems. Aquaculture 211, 115-124.
Gutierrez-Wing, M.T., Malone, R.F., 2006. Biological filters in aquaculture: trends and research directions for freshwater and marine applications. Aquacult. Eng. 34, 163-171.
Hahn, M.W., Hofle, M.G., 1999. Flagellate predation on a bacterial model community: interplay of size-selective grazing, specific bacterial cell size, and bacterial community composition. Appl. Environ. Microbiol. 65, 4863-4872.
Hahn, M.W., Hofle, M.G., 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiol. Ecol. 35, 113-121.
Halet, D., Defoirdt, T., Van Damme, P., Vervaeren, H., Forrez, I., Van de Wiele, T., Boon, N., Sorgeloos, P., Bossier, P., Verstraete, W., 2007. Poly-β-hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. FEMS Microbiol. Ecol. 60, 363-369.
Hanly, F., 1991. Effects of feeding supplementary diets containing varying levels of lipid on growth, food conversion, and body-composition of Nile tilapia, Oreochromis niloticus (L.). Aquaculture 93, 323-334.
Hantula, J., Bamford, D.H., 1991. The efficiency of the protein-dependent flocculation of *Flavobacterium* sp is sensitive to the composition of growth-medium. Appl. Microbiol. Biotechnol. 36, 100-104.

Hargreaves, J.A., 2006. Photosynthetic suspended-growth systems in aquaculture. Aquacult. Eng. 34, 344-363.
Hari, B., Kurup, B.M., 2002. Vitamin C (ascorbyl 2 polyphosphate) requirement for freshwater prawn *Macrobrachium rosenbergii*. Asian Fish. Sci. 15, 145-154.
Hari, B., Kurup, B.M., Varghese, J.T., Schrama, J.W., Verdegem, M.C.J., 2006. The effect of carbohydrate addition on water quality and the nitrogen budget in extensive shrimp culture systems. Aquaculture 252, 248-263.
Hari, B., Madhusoodana, K., Varghese, J.T., Schrama, J.W., Verdegem, M.C.J., 2004. Effects of carbohydrate addition on production in extensive shrimp culture systems. Aquaculture 241, 179-194.
Harpaz, S., 1997. Enhancement of growth in juvenile freshwater prawns, *Macrobrachium rosenbergii*, through the use of a chemoattractant. Aquaculture 156, 221-227.
Hassen, A., Mahrouk, M., Ouzari, H., Cherif, M., Boudabous, A., Damelincourt, J.J., 2000. UV disinfection of treated wastewater in a large-scale pilot plant and inactivation of selected bacteria in a laboratory UV device. Bioresource Technol. 74, 141-150.
Helfman, G.S., Collette, B.B., Facey, D.E., 1997. The diversity of fishes. Blackwell publishing, Malden, USA, 1006 pp.
Henke, J.M., Bassler, B.L., 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bacteriol. 186, 3794-3805.
Hepher, B., 1988. Nutrition of pond fishes. Cambridge Univ. Press, Cambridge, NY, USA, 388 pp.
Hermansson, M., 1999. The DLVO theory in microbial adhesion. Colloids Surf. B. 14, 105-119.
Hertrampf, J.W., Piedad-Pascual, F., 2000. Handbook on ingredients for aquaculture feeds. Kluwer Academic Publishers, Dordrecht, The Netherlands, 624 pp.
Higgins, M.J., Novak, J.T., 1997. Characterization of exocellular protein and its role in bioflocculation. J. Environ. Eng.-ASCE 123, 479-485.
Hofer, S.C., Watts, S.A., 2002. Cold tolerance in genetically male tilapia (GMT®), *Oreochromis niloticus*. World Aquac. 33, 19-21.
Hollender, J., van der Krol, D., Kornberger, L., Gierden, E., Dott, W., 2002. Effect of different carbon sources on the enhanced biological phosphorus removal in a sequencing batch reactor. World J. Microbiol. Biotechnol. 18, 355-360.
Hondzo, M., Wüest, A., 2009. Do microscopic organisms feel turbulent flows? Environ. Sci. Technol. 43, 764-768.
Hopkins, J.S., Hamilton, R.D.L., Sandifers, P.A., Browdy, C.L., Stokes, A.D., 1993. Effect of water exchange rate on production, water quality, effluent characteristics and nitrogen budgets of intensive shrimp ponds. J. World Aquacult. Soc. 24, 304-320.
Hossain, M.A., Paul, L., 2007. Low-cost diet for monoculture of giant freshwater

prawn (*Macrobrachium rosenbergii* de Man) in Bangladesh. Aquacult. Res. 38, 232-238.
Hovanec, T.A., DeLong, E.F., 1996. Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria. Appl. Environ. Microb. 62, 2888-2896.
Hsieh, S.L., Chang, H.T., Wu, C.H., Kuo, C.M., 2004. Cloning, tissue distribution and hormonal regulation of stearoyl-CoA desaturase in tilapia, *Oreochromis mossambicus*. Aquaculture 230, 527-546.
Huchette, S.M.H., Beveridge, M.C.M., Baird, D.J., Ireland, M., 2000. The impacts of grazing by tilapias (*Oreochromis niloticus* L.) on periphyton communities growing on artificial substrate in cages. Aquaculture 186, 45-60.
Huntingford, F.A., Adams, C., Braithwaite, V.A., Kadri, S., Pottinger, T.G., Sandoe, P., Turnbull, J.F., 2006. Current issues in fish welfare. J. Fish Biol. 68, 332-372.
Hussenot, J.M.E., 2003. Emerging effluent management strategies in marine fish-culture farms located in European coastal wetlands. Aquaculture 226, 113-128.
Jauncey, K., 1982. The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). Aquaculture 27, 43-54.
Jendrossek, D., Handrick, R., 2002. Microbial degradation of polyhdroxyalkanoates. Annu. Rev. Microbiol. 56, 403-432.
Jiang, S., 2010. Aquaculture, capture fisheries, and wild fish stocks. Resource Energy Econ. 32, 65-77.
Jiménez-Montealegre, R., Verdegem, M.C.J., van Dam, A., Verreth, J.A.J., 2002. Conceptualization and validation of a dynamic model for the simulation of nitrogen transformations and fluxes in fish ponds. Ecol. Model. 147, 123-152.
Jobling, M., 2001. Nutrient partitioning and the influence of feed composition on body composition. In: Houlihan, D., Boujard, T., Jobling, M., Editors, 2001. Food Intake in Fish, Blackwell, Oxford, pp. 354-375.
Johnson, M.R., Montero, C.J., Conners, S.B., Shockley, K.R., Bridger, S.I., Kelly, R.M., 2005. Population density-dependent regulation of exopolysaccharide formation in the hyperthermophilic bacterium *Thermotoga maritime*. Mol. Microbiol. 55, 664-674.
Joye, S.B., Hollibaugh, J.T., 1995. Influence of sulphide inhibition of nitrification on nitrogen regeneration in sediments. Science 270, 623-625.
Kamarudin, M.S., Roustaian, P., 2002. Growth and fatty acid composition of freshwater prawn, *Macrobrachium rosenbergii*, larvae fed diets containing various ratios of cod liver oil – corn oil mixture. J. Appl. Ichtyol. 18, 148-153.
Kamstra, A., van der Heul, J.W., Nijhof, M., 1998. Performance and optimisation of trickling filters on eel farms. Aquacult. Eng. 17, 175-192.
Kang'ombe, J., Likongwe, J.S., Eda, H., Mtimuni, J.P., 2007. Effect of varying dietary energy level on feed intake, feed conversion, whole-body composition and growth of Malawian tilapia, *Oreochromis shiranus* – Boulenger. Aquac. Res. 38, 373-380.

Kaushik, S.J., Oliva-Teles, A., 1985. Effect of digestible energy on nitrogen and energy balance in rainbow trout. Aquaculture 50, 89-101.
Kautsky, N., 2004. Aquaculture and the environment – Integrated aquaculture. Course, University Ghent, Belgium.
Keiding, K., Nielsen, P.H., 1997. Desorption of organic macromolecules from activated sludge: effect of ionic composition. Water Res. 31, 1665-1672.
Keshavanath, P., Gangadhar, B., 2005. Research on periphyton-based aquaculture in India. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 223-236.
Kirk, R.G., 1972. A review of recent developments in *Tilapia* culture, with special reference to fish farming in the heated effluents of power stations. Aquaculture 1, 45-60.
Kjelleberg, S., Molin, S., 2002. Is there a role for quorum sensing signals in bacterial biofilms? Curr. Opin. Microbiol. 5, 254-258.
Knights, B., 1983. Food particle-size preferences and feeding-behavior in warmwater aquaculture of European eel, *Anguilla Anguilla* (L.). Aquaculture 30, 173-190.
Körner, S., Das, S.K., Veenstra, S., Vermaat, J.E., 2001. The effect of pH variation at the ammonium/ammonia equilibrium in wastewater and its toxicity to *Lemna gibba*. Aquat. Bot. 71, 71-78.
Krishna, C., Van Loosdrecht, M.C.M., 1999. Effect of temperature on storage polymers and settleability of activated sludge. Water Res. 33, 2374-2382.
Kuhn, D.D., Boardman, G.D., Lawrence, A.L., Marsh, L., Flick, G.J., 2009. Microbial floc meals as a replacement ingredient for fish meal and soybean protein in shrimp feed. Aquaculture 296, 51-57.
Kumar, M., Lin, J.-G., 2010. Co-existence of anammox and denitrification for simultaneous nitrogen and carbon removal – Strategies and issues. J. Hazard. Mater., In press.
Kutnink, M.A., Hawkes, W.C., Schaus, E.E., Omaye, S.T., 1987. An internal standard method for the unattended high-performance liquid analysis of ascorbic acid in blood components. Anal. Biochem. 166, 424-430.
Lanman, M.J., Van Den Heuvel, M.R., Ling, N., 2005. Relative sensitivities of common freshwater fish and invertebrates to acute hypoxia. N. Z. J. Mar. Freshw. 39, 1061-1067.
Lappin, Y., 2008. Business in brief. Jerusalem Post, Economics, Feb 7, 2008, p. 17.
Lavens, P., Sorgeloos, P., 1984. Controlled production of *Artemia* cysts under standard conditions in a recirculating culture system. Aquacult. Eng. 3, 221-235.
Lazazzera, B.A., 2000. Quorum sensing and starvation: signals for entry into stationary phase. Curr. Opin. Microbiol. 3, 177-182.

Lee, D.J., Putnam, G.B., 1973. The response of rainbow trout to varying protein/energy ratios in a test diet. J. Nutr. 103, 916-922.
Lee, S.Y., 1996. Bacterial Polyhydroxyalkanoates. Biotechnol. Bioeng. 49, 1-14.
Lee, Y.-K., 2001. Microalgal mass culture systems and methods: Their limitation and potential. J. Appl. Phycol. 13, 307-315.
Lekang, O.-I., Kleppe, H., 2000. Efficiency of nitrification in trickling filters using different filter media. Aquacult. Eng. 21, 181-199.
Lezama-Cervantes, C., Paniagua-Michel, J., 2010. Effects of constructed microbial mats on water quality and performance of *Litopenaeus vannamei* post-larvae. Aquacult. Eng. 42, 75-81.
Lilley, B.N., Bassler, B.L., 2000. Regulation of quorum sensing in *Vibrio harveyi* by luxO and σ54. Mol. Microbiol. 36, 940-954.
Lim, C., Webster, C.D., 2006. Tilapia: Biology, Culture, and Nutrition. The Haworth Press, Inc., the United States, 678 pp.
Ling, J., Chen, S., 2005. Impact of organic carbon on nitrification performance of different biofilters. Aquacult. Eng. 33, 150-162.
Liu, J.R., Liu, C.T., Edwards, E.A., Liss, S.N., 2006. Effect of phosphorus limitation on microbial floc structure and gene expression in activated sludge. Water Sci. Technol. 54, 247-255.
Liu, K.-F., Chiu, C.-H., Shiu, Y.-L., Cheng, W., Liu, C.-H., 2010. Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune status of white shrimp, *Litopenaeus vannamei* larvae. Fish Schellfish Immun., In Press.
Liu, Y., Fang, H.H.P., 2003. Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge. Crit. Rev. Environ. Sci. Technol. 33, 237-273.
Logan, B.E., Hunt, J.R., 1987. Advantages to microbes of growth in permeable aggregates in marine systems. Limnol. Oceanogr. 32, 1034-1048.
Logan, B.E., Hunt, J.R., 1988. Bioflocculation as a microbial response to substrate limitations. Biotechnol. Bioeng. 31, 91-101.
Lorenz, M.O., 1905. Methods of measuring concentration of wealth. J. Am. Stat. Assoc. 9, 209-219.
Lynch, M.J., Swift, S., Kirke, D.F., Keevil, C.W., Dodd, C.E.R., Williams, P., 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ. Microbiol. 4, 18-28.
Lyssenko, C., Wheaton, F., 2006. Impact of positive ramp short-term operating disturbances on ammonia removal by trickling and submerged-upflow biofilters for intensive recirculating aquaculture. Aquacult. Eng. 35, 26-37.
Madigan, M.T., Martinko, J.M., 2006. Brock Biology of Microorganisms, 11th ed. Pearson Prentice Hall (Pearson Education, Inc), Upper Saddle River (New Jersey). 992 pp.

Maillard, V.M., Boardman, G.D., Nyland, J.E., Kuhn, D.D., 2005. Water quality and sludge characterization at raceway-system trout farms. Aquac. Eng. 33, 271-284.
Mäkelä, P., Jokinen, K., Kontturi, M., Peltonen-Sainio, P., Pehu, E., Somersalo, S., 1998. Foliar application of glycinebetaine – a novel product from sugar beet – as an approach to increase tomato yield. Ind. Crop Prod. 7, 139-148.
Mallasen, M., Valenti, W.C., 2006. Effect of nitrite on larval development of giant river prawn *Macrobrachium rosenbergii*. Aquaculture 261, 1292-1298.
Malone, R.F., Beecher, L.E., 2000. Use of floating bead filters to recondition recirculating waters in warmwater aquaculture production systems. Aquacult. Eng. 22, 57-73.
Malone, R.F., Pfeiffer, T.J., 2006. Rating fixed film nitrifying biofilters used in recirculating aquaculture systems. Aquacult. Eng. 34, 389-402.
Manefield, M., Harris, L., Rice, S.A., de Nys, R., Kjelleberg, S., 2000. Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists. Appl. Environ. Microbiol. 66, 2079-2084.
Manush, S.M., Pal, A.K., Das, T., Mukherjee, S.C., 2005. Dietary high protein and vitamin C mitigate stress due to chelate claw ablation in *Macrobrachium rosenbergii* males. Comp. Biochem. Physiol. A 142, 10-18.
Marques, A., François, J.-M., Dhont, J., Bossier, P., Sorgeloos, P., 2004. Influence of yeast quality on performance of gnotobiotically grown *Artemia*. J. Exp. Mar. Biol. Ecol. 310, 247-264.
Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P., Bossier, P., 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. J. Appl. Microbiol. 100, 903-918.
Martin, A.M.P., Heijnen, J.J., van Loosdrecht, M.C.M., 2003. Effect of dissolved oxygen concentration on sludge settleability. Appl. Microbiol. Biotechnol. 62, 586-593.
Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ. Microbiol. 10, 1571-1581.
Masser, M.P., Rakocy, J., Losordo, T.M., 1999. Recirculating aquaculture tank production systems – management of recirculating systems. SRAC Publication No. 452.
Matz, C., Bergfeld, T., Rice, S.A., Kjelleberg, S., 2004. Microcolonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environ. Microbiol. 6, 218-226.
Matz, C., Jurgens, K., 2005. High motility reduces grazing mortality of planktonic bacteria. Appl. Environ. Microbiol. 71, 921-929.
Matz, C., Kjelleberg, S., 2005. Off the hook – how bacteria survive protozoan grazing. Trends Microbiol. 13, 302-307.

McDonough, W., Braungart, M., 2002. Cradle to cradle: remaking the way we make things. North Point Press, New York, US, 193 pp.
McGraw, W., Teichert-Coddington, D.R., Rouse, D.B., Boyd, C.E., 2001. Higher minimum dissolved oxygen concentrations increase penaeid shrimp yields in earthen ponds. Aquaculture 199, 311-321.
McIntosh, P.R., 2000. Changing paradigms in shrimp farming. IV. Low protein feeds and feeding strategies. Global Aquac. Adv. 3, 44-50.
Meade, J.W., 1985. Allowable ammonia for fish culture. Prog. Fish. Cult. 47, 135-145.
Merchie, G., Lavens, P., Sorgeloos, P., 1997. Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture 155, 165-181.
Mertens, B., Boon, N., Verstraete, W., 2005. Stereospecific effect of hexachlorocyclohexane on activity and structure of soil methanotrophic communities. Environ. Microbiol. 7, 660-669.
Michaud, L., Blancheton, J.P., Bruni, V., Piedrahita, R., 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquacult. Eng. 34, 224-233.
Mikkelsen, L.H., Gotfredsen, A.K., Agerbaek, M.L., Nielsen, P.H., Keiding, K., 1996. Effects of colloidal stability on clarification and dewatering of activated sludge. Water Sci. Technol. 34, 449-457.
Miller, G.E., Libey, G.S., 1985. Evaluation of three biological filters suitable for aquaculture applications. J. World Maric. Soc. 16, 158-168.
Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55, 165-199.
Milstein, A., 2005. Effect of periphyton on water quality. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 179-190.
Milton, D.L., 2006. Quorum sensing in Vibrios: Complexity for diversification. Int. J. Med. Microbiol. 296, 61-71.
Mitchell, J.G., Kogure, K., 2006. Bacterial motility: links to the environment and a driving force for microbial physics. FEMS Microbiol. Ecol. 55, 3-16.
Mitra, G., Chattopadhyay, D.N., Mukhopadhyay, P.K., 2005. Nutrition and feeding in freshwater prawn (*Macrobrachium rosenbergii*) farming. Aqua Feeds: Formulation & Beyond 2, 17-19.
Mok, K.C., Wingreen, N., Bassler, B.L., 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. EMBO J. 22, 870-881.
Morgan-Sagastume, F., Boon, N., Dobbelaere, S., Defoirdt, T., Verstraete, W., 2005. Production of acylated homoserine lactones by *Aeromonas* and *Pseudomonas* strains isolated from municipal activated sludge. Can. J. Microbiol. 51, 924-933.

Muthuwani, V., Lin, C.K., 1996. Water quality and nutrient budget in intensive shrimp culture ponds. Book of abstracts, The 1996 annual meeting of the World Aquaculture Society. January 29 - February 2, Bangkok Thailand. p. 270.
Muyzer, G., de Waal, E.C., Uitterlinden, A., 1993. Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.
Naqvi, A.A., Adhikari, S., Pillae, B.R., Sarangi, N., 2007. Effect of ammonia-N on growth and feeding of juvenile *Macrobrachium rosenbergii* (De-Man). Aquacult. Res. 38, 847-851.
Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J, Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.
Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigel, M., Yarish, C., 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231, 361-391.
New, M.B., 2005. Freshwater prawn farming: global status, recent research and a glance at the future. Aquacult. Res. 36, 210-230.
Oehmen, A., Keller-Lehmann, B., Zeng, R.J., Yuan, Z.G., Keller, E., 2005. Optimisation of poly-beta-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. J. Chromatogr. A. 1070, 131-136.
Oehmen, A., Yuan, Z., Blackall, L.L., Keller, J., 2004. Short-term effects of carbon source on the competition of polyphosphate accumulating organisms and glycogen accumulating organisms. Water Sci. Technol. 50, 139-144.
Park, D., Lee, D.S., Young, J.Y., Park, J.M., 2005. Comparison of different bioreactor systems for indirect H_2S removal using iron-oxidizing bacteria. Process Biochem. 40, 1461-1467.
Parker, D.S., Asce, A.M., Kaufman, W.J., Jenkins, D., 1972. Floc breakup in turbulent flocculation processes. J. Sanit. Eng. Div.-ASCE 98, 79.
Peres, H., Oliva-Teles, A., 1999. Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (*Dicentrarchus labrax*). Aquaculture 179, 325-334.
Péron, G., Mittaine, J.F., Le Gallic, B., 2010. Where do fishmeal and fish oil products come from? An analysis of the conversion ratios in the global fishmeal industry. Mar. Policy, In press.
Phuoc, L.H., Corteel, M., Thanh, N.C., Nauwynck, H., Pensaert, M., Alday-Sanz, V., Van den Broeck, W., Sorgeloos, P., Bossier, P., 2009. Effect of dose and challenge routes of *Vibrio* spp. On co-infection with white syndrome virus in *Penaeus vannamei*. Aquaculture 290, 61-68.

Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture 226, 35-44.
Ponis, E., Robert, R., Parisi, G., 2003. Nutritional value of fresh and concentrated algal diets for larval and juvenile Pacific oysters (*Crassostrea gigas*). Aquaculture 221, 491-505.
Popma, T., Masser, M., 1999. Tilapia – Life history and Biology. Southern Regional Aquaculture Center Publication No. 283.
Portz, D.E., Woodley, C.M., Cech, J.J., 2006. Stress-associated impacts of short-term holding on fishes. Rev. Fish Biol. Fish. 16, 125-170.
Queck, S.Y., Weitere, M., Moreno, A.M., Rice, S.A., Kjelleberg, S., 2006. The role of quorum sensing mediated developmental traits in the resistance of *Serratia marcescens* biofilms against protozoan grazing. Environ. Microbiol. 8, 1017-1025.
Rahman, M.M., Nagelkerke, L.A.J., Verdegem, M.C.J., Wahab, M.A., Verreth, J.A.J., 2008. Relationships among water quality, food resources, fish diet and fish growth in polyculture ponds: A multivariate approach. Aquaculture 275, 108-115.
Raveh, A., Avnimelech, Y., 1972. Potentiometric determination of soil organic matter. Soil Science Society of America 36, 967-968.
Read, P., Fernandes, T., 2003. Management of environmental impacts of marine aquaculture in Europe. Aquaculture 226, 139-163.
Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. Bull. Fish. Res. Board Can. 191, 209-210.
Rijnaarts, H.H.M., Norde, W., Lyklema, J., Zehnder, A.J.B., 1999. DLVO and steric contributions to bacterial deposition in media of different ionic strengths. Colloids Surf. B. 14, 179-195.
Rittmann, B.E., McCarty, P.L., 2001. Environmental Biotechnology: Principles and Applications, McGraw-Hill, New York.
Rothbard, S., Peretz, Y., 2002. Tilapia Culture in NEGEV, The Israeli Desert. In: Guerrero, R.D., Guerrero – del Castillo, R. (Eds.), Tilapia Farming in the 21st Century, pp. 60-65.
Roustaian, P., Kamarudin, M.S., Omar, H., Saad, C.R., Ahmad, M.H., 2001. The effect of dietary lipid sources on the *Macrobrachium rosenbergii* larval performance, post larval production and fatty acid composition. J. Aquac. Trop. 16, 251-263.
Salahizadeh, H., Shojaosadati, S.A., 2001. Extracellular biopolymeric flocculants – recent trends and biotechnological importance. Biotechnol. Adv. 19, 371-385.
Salehizadeh, H., Van Loosdrecht, M.C.M., 2004. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. Biotechnol. Adv. 22, 201-279.

Sanin, F.D., Vatansever, A., Turtin, I., Kara, F., Durmaz, B., Sesay, M.L., 2006. Operational conditions of activated sludge: influence on flocculation and dewaterability. Dry. Technol. 24, 1297-1306.
Sarig, S., Arieli, Y., 1980. Growth capacity of tilapia in intensive culture. Bamidgeh 32, 57-65.
Sastry, B.N., delos Reyes, A.A., Rusch, K.A., Malone, R.F., 1999. Nitrification performance of a bubble-washed bead filter for combined solids removal and biological filtration in a recirculating aquaculture system. Aquacult. Eng. 19, 105-117.
Satoh, H., Okabe, N., Watanabe, Y., 2000. Significance of substrate C/N ratio on structure and activity of nitrifying biofilms determined by *in situ* hybridization and the use of microelectrodes. Water Sci. Technol. 41, 317-321.
Schneider, O., Sereti, V., Eding, E.H., Verreth, J.A.J., 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. Aquacult. Eng. 32, 379-401.
Schnel, N., Barak, Y., Ezer, T., Dafni, Z., van Rijn, J., 2002. Design and performance of zero-discharge tilapia recirculating system. Aquacult. Eng. 26, 191-203.
Schulz, H.N., Jorgensen, B.B., 2001. Big Bacteria. Annu. Rev. Microbiol. 55, 105-137.
Schuur, A.M., 2003. Evaluation of biosecurity applications for intensive shrimp farming. Aquacult. Eng. 28, 3-20.
Shrout, J.D., Chopp, D.L., Just, C.L., Hentzer, M., Givskov, M., Parsek, M.R., 2006. The impact of quorum sensing and swarming motility on *Pseudomonas aeruginosa* biofilm formation is nutritionally conditional. Mol. Microbiol. 62, 1264-1277.
Siddiqui, A.Q., Al-Harbi, A.H., 1995. Evaluation of three species of tilapia, red tilapia and a hybrid tilapia as culture species in Saudi Arabia. Aquaculture 138, 145-157.
Sifa, L., Chenhong, L., Dey, M., Gagalac, F., Dunham, R., 2002. Cold tolerance of three strains of Nile tilapia, *Oreochromis niloticus*, in China. Aquaculture 213, 123-129.
Simoni, S.F., Schafer, A., Harms, H., Zehnder, A.J.B., 2001. Factors affecting mass transfer limited biodegradation in saturated porous media. J. Contam. Hydrol. 50, 99-120.
Sinha, A., Baruah, K., Bossier, P., 2008. Horizon scanning: the potential use of biofloc as an anti-infective strategy in aquaculture – an overview. Aquaculture Health International, June 2008.
Sobeck, D.C., Higgins, M.J., 2002. Examination of three theories for mechanisms of cation-induced bioflocculation. Water Res. 36, 527-538.
Soto-Rodriguez, S.A., Simoes, N., Jones, D.A., Roque, A., Gomez-Gil, B., 2003. Assessment of fluorescent-labeled bacteria for evaluation of *in vivo* uptake of bacteria (*Vibrio* spp.) by crustacean larvae. J. Microbiol. Methods 52, 101-114.
Spicer, P.T., Pratsinis, S.E., 1996. Shear-induced flocculation: the evolution of floc structure and the shape of the size distribution at steady state. Water Res. 30, 1049-1056.

Spoering, A.L., Gilmore, M.S., 2006. Quorum sensing and DNA release in bacterial biofilms. Curr. Opin Microbiol. 9, 133-137.
Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87-96.
Stanley, N.R., Lazazzera, B.A., 2004. Environmental signals and regulatory pathways that influence biofilm formation. Mol. Microbiol. 52, 917-924.
Steiner, A.E., McLaren, D.A., Forster, C.F., 1976. Nature of activated-sludge flocs. Water Res. 10, 25-30.
Sugiura, S.H., Marchant, D.D., Wigins, T., Ferraris, R.P., 2006. Effluent profile of commercially used low-phosphorus fish feeds. Environ. Pollut. 140, 95-101.
Summerfelt, S.T., 2003. Ozonation and UV irradiation – an introduction and examples of current applications. Aquacult. Eng. 28, 21-36.
Summerfelt, S.T., 2006. Design and management of conventional fluidized-sand biofilters. Aquacult. Eng. 34, 275-302.
Tacon, A.G.J., 1990. Standard methods for the nutrition and feeding of farmed fish and shrimp, Argent Laboratories Press, Washington DC.
Tawfik, A., Klapwijk, B., Van Buuren, J., El-Gohary, F., Lettinga, G., 2004. Physico-chemical factors affecting the *E. coli* removal in a rotating biological contactor (RBC) treating UASB effluent. Water Res. 38, 1081-1088.
Tay, J.H., Xu, H.L., Teo, K.C., 2000. Molecular mechanism of granulation. I: H+ translocation-dehydration theory. J. Environ. Eng.-ASCE 126, 403-410.
Teo, K.C., Xy, H.L., Tay, J.H., 2000. Molecular mechanism of granulation. II proton translocating activity. J. Environ. Eng.-ASCE 126, 411-418.
Thompson, F.L., Abreu, P.C., Wasielesky, W., 2002. Importance of biofilm for water quality and nourishment in intensive shrimp culture. Aquaculture 203, 263-278.
Timmons, M.B., 1984. Use of foam fractionation in aquaculture. Dev. Aquac. Fish. Sci. 27, 247-279.
Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating aquaculture systems, 2nd edition. NRAC Publication, vol. 01-002.
Timmons, M.B., Holder, J.L., Ebeling, J.M., 2006a. Application of microbead biological filters. Aquacult. Eng. 34, 332-343.
Timmons, M.B., Summerfelt, S.T., 1998. Application of fluidized sand bed biofilters to aquaculture. In: Libey, G.S., Timmons, M.B. (Eds.), Recent advances in aquaculture engineering; proceedings of the second international conference on recirculating aquaculture. Virginia Polytechnic Institute and State University, Roanoke, VA, pp. 342-354.
Timmons, N., Timmons, M.B., Ebeling, J.M., 2006b. Recirculating Aquaculture System (RAS) technologies – Part 2. Aquacult. Mag. September/October 2006, 32-39.
Torrans, E.L., 2005. Effect of oxygen management on culture performance of channel catfish in earthen ponds. N. Am. J. Aquac. 67, 275-288.

Torres-Beristain, B., Verdegem, M., Kerepeczki, E., Verreth, J., 2006. Decomposition of high protein aquaculture feed under variable oxic conditions. Water Res. 40, 1341-1350.
Trainer, M.A., Charles, T.C., 2006. The role of PHB metabolism in the symbiosis of *Rhizobia* with legumes. Appl. Microbiol. Biotechnol. 71, 377-386.
Tseng, D.-Y., Ho, P.-L., Huang, S.-Y., Cheng, S.-C., Shiu, Y.-L., Chiu, C.-S., Liu, C.-H., 2009. Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. Fish Shellfish Immun. 26, 339-344.
Turker, H., Eversole, A.G., Brune, D., 2003. Comparative Nile tilapia and silver carp filtration rates of Partitioned Aquaculture System phytoplankton. Aquaculture 220, 449-457.
Twarowska, J.G., Westerman, P.W., Losordo, T.M., 1997. Water treatment and waste characterization evaluation of an intensive recirculating fish production system. Aquacult. Eng. 16, 133-147.
Uddin, M.S., Farzana, A., Lipidema, M.K., Azim, M.E., Wahab, M.A., Verdegem, M.C.J., 2007. Technical evaluation of tilapia (*Oreochromis niloticus*) monoculture and tilapia-prawn (*Macrobrachium rosenbergii*) polyculture in earthen ponds with or without substrates for periphyton development. Aquaculture 269, 232-240.
UN, 2003. Wastewater treatment technologies: a general review. Economic and social commission for Western Asia, United Nations, New York, 122 pp.
Valle, A., Bailey, M.J., Whiteley, A.S., Manefield, M., 2004. N-acyl-ι- homoserine lactones (AHLs) affect microbial community composition and function in activated sludge. Environ. Microbiol. 6, 424-433.
van Dam, A.A., Beveridge, M.C.M., Azim, M.E., Verdegem, M.C.J., 2002. The potential of fish production based on periphyton. Rev. Fish Biol. Fisher. 12, 1-31.
van Dam, A.A., Verdegem, M.C.J., 2005. Periphyton: systems ecology perspective. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 91-111.
van Ginneken, V.J.T., van Eersel, R., Balm, P., Nieveen, M., van den Thillart, G., 1997. Tilapia are able to withstand long-term exposure to low environmental pH, judged by their energy status, ionic balance and plasma cortisol. J. Fish. Biol. 51, 795-806.
Van Loosdrecht, M.C.M., Lyklema, J., Norde, W., Schraa, G., Zehnder, A.J.B., 1987. The role of bacterial-cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53, 1893-1897.
van Rijn, J., 1996. The potential for integrated biological treatment systems in recirculating fish culture – a review. Aquaculture 139, 181-201.
van Rijn, J., Tal, Y., Schreier, H.J., 2006. Denitrification in recirculating systems: theory and applications. Aquacult. Eng. 34, 364-376.

Velasco, M., Lawrence, A.L., Neill, W.H., 1998. Effects of dietary phosphorus level and inorganic source on survival and growth of *Penaeus vannamei* postlarvae in zero-water exchange culture tanks. Aquat. Living Resour. 11, 29-33.
Venero, J.A., Davis, D.A., Rouse, D.B., 2007. Variable feed allowance with constant protein input for the pacific white shrimp *Litopenaeus vannamei* reared under semi-intensive conditions in tanks and ponds. Aquaculture 269, 490-503.
Vermaat, J.E., 2005. Periphyton dynamics and influencing factors. In: Azim, M.E., Verdegem, M.C.J., van Dam, A.A., Beveridge, M.C.M. (Eds.), Periphyton – Ecology, exploitation and management. CABI Publishing, Wallingford, pp. 35-49.
Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P., Verstraete, W., 1999. Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. Appl. Environ. Microbiol. 65, 2527-2533.
Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biocontrol agents in aquaculture. Microbiol. Mol. Biol. Rev. 64, 655-671.
Villaverde, S., Fdz-Polanco, F., García, P.A., 2000. Nitrifying biofilm acclimation to free ammonia in submerged biofilters. Start-up influence. Water Res. 34, 602-610.
Walsh, P.J., Wright, P.A., 1995. Nitrogen metabolism and excretion. CRC press, Florida, USA, 352 pp.
Wang, J.-C., Chang, P.-S., Chen, H.-Y., 2008. Differential time-series expression of immune-related genes of Pacific white shrimp *Litopenaeus vannamei* in response to dietary inclusion of β-1,3-glucan. Fish & Shellfish Immun. 24, 113-121.
Wang, J.-K., 2003. Conceptual design of a microalgae-based recirculating oyster and shrimp system. Aquacult. Eng. 28, 37-46.
Watanabe, T., 2002. Strategies for further development of aquatic feeds. Fish. Sci. 68, 242-252.
Watten, B.J., Sibrell, P.L., 2006. Comparative performance of fixed-film biological filters: Application of reactor theory. Aquacult. Eng. 34, 198-213.
Ways, P., Hanahan, D.J., 1964. Characterization and quantification of red cell lipids in normal man. J. Lipid Res. 5, 318-328.
Wilen, B.M., Nielsen, J.L., Keiding, K., Nielsen, P.H., 2000. Influence of microbial activity on the stability of activated sludge flocs. Colloid Surf. B. 18, 145-156.
Willen, B.M., Balmer, P., 1999. The effect of dissolved oxygen concentration on the structure, size and size distribution of activated sludge flocs. Water Res. 33, 391-400.
Wittebolle, L., Boon, N., Vanparys, B., Heylen, K., De Vos, P., Verstraete, W., 2005. Failure of the ammonia oxidation process in two pharmaceutical wastewater treatment plants is linked to shifts in the bacterial communities. J. Appl. Microbiol. 99, 997-1006.
Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., De Vos, P., Verstraete, W., Boon, N., 2009. Initial community evenness favours functionality under selective stress. Nature 458, 623-626.

Wittebolle, L., Vervaeren, H., Verstraete, W., Boon, N., 2008. Quantifying community dynamics of nitrifiers in functionally stable reactors. Appl. Environ. Microb. 74, 286-293.
Wohlfarth, G.W., Hulata, G., 1983. Applied Genetics of Tilapias. ICLARM Studies and Review 6, Manila, Philippines, 26 pp.
Wood, C.M., Franklin, N.M., Niyogi, S., 2006. The protective role of dietary calcium against cadmium uptake and toxicity in freshwater fish: an important role for the stomach. Environ. Chem. 3, 389-394.
Wouters, R., Lavens, P., Nieto, J., Sorgeloos, P., 2001. Penaeid shrimp broodstock nutrition: an updated review on research and development. Aquaculture 202, 1-21.
Yagci, N., Cokgor, E.U., Artan, N., Randall, C., Orhon, D., 2007. The effect of substrate on the composition of polyhydroxyalkanoates in enhanced biological phospohorus removal. J. Chem. Technol. Biotechnol. 82, 295-303.
Young, K.D., 2006. The selective value of bacterial shape. Microbiol. Mol. Biol. Rev. 70, 660-703.
Zita, A., Hermansson, M., 1994. Effects of ionic-strength on bacterial adhesion and stability of flocs in a waste-water activated-sludge system. Appl. Environ. Microbiol. 60, 3041-3048.
Zohar, G., Rappaport, U., Sarig, S., 1985. Intensive culture of tilapia in concrete tanks. Bamidgeh 37, 103-111.

APPENDIX A - SUMMARY

Future development of intensive aquaculture must deal with its impacts on the environment in the form of water pollution and the use of fish oil and fish meal. The bioflocs technology simultaneously addresses both problems co-occurring with the further expansion of the industry. While maintaining good water quality within the aquaculture systems it produces additional feed for the cultured animals. In contrast to conventional water quality control techniques, the bioflocs technology offers a sustainable, economical and easy-to-implement alternative. **Chapter 1** gives an overview of the literature concerning nitrogen removal techniques in aquaculture and bioflocs technology.

In **Chapter 2**, the impact of the carbon source on the performance of biofloc reactors was studied. The carbon source influenced the capacity of the technique to control the water quality in the biofloc reactors and the nutritional properties of the flocs. The carbon source also affected the eukaryotic and prokaryotic community composition of the bioflocs, which offers great possibilities for fine-tuning of the technique, more specifically concerning water quality control, feed production and/or costs.

This prime importance of the choice of carbon source was confirmed in two further studies (**Chapter 3** and **Chapter 4**) in which bioflocs grown on different substrates were fed to giant freshwater prawn (*Macrobrachium rosenbergii*) postlarvae and white shrimp (*Litopenaeus vannamei*), respectively. In both studies, glycerol-grown bioflocs showed better results than glucose-grown bioflocs. The potential significance of these results calls for further studies on the use of bioflocs as a feed in aquaculture, both in freshwater and saline systems. Parameters to consider in the future are accessibility, palatability or attractiveness of the bioflocs towards the animals, amino acid composition, essential fatty acids content and cost of the used carbon source as well as

the overall cost of the technology (especially compared to conventional biofilter systems and feeding costs).

In addition to the environmental, economical and sustainable considerations addressed above, a more specific problem was studied in **Chapter 5**, where aquaculture animals are exposed to lower temperatures during winter, possibly leading to mass mortality in industrial ponds. Covering the ponds with either plastic sheets or glass allowed solar heating of the culture water (thereby reducing the temperature decrease) and permitted to minimize water exchange. The application of bioflocs technology resulted in maintenance of good water quality, concomitantly providing additional feed to the animals, tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) without compromising survival, growth and condition factor of the cultured species.

At this moment, the aquaculture industry is most importantly faced with mass mortalities due to infectious diseases. To conclude this work, a potential extra added value feature of the bioflocs technology was studied in **Chapter 6**. In this study, bioflocs were found to be able to protect brine shrimp (*Artemia franciscana*) larvae from pathogenic *Vibrio harveyi*. These results indicate that in addition to water quality control and extra *in situ* feed production, the technique also has potential to protect the cultured animals from infections with pathogenic bacteria, which are responsible for major economic losses in aquaculture.

To conclude, the last chapter (**Chapter 7**) provides a brief discussion of the performed studies. Directions for future in depth studies are raised based on the studies performed in this work that might contribute to further sustainable development of aquaculture.

APPENDIX B - SAMENVATTING

De toekomstige ontwikkeling van de intensieve aquacultuur dient de daarmee gepaard gaande problemen, met name milieueffecten in de vorm van watervervuiling en het gebruik van visolie en –meel, aan te kaarten en tegen te gaan. De biovlok-technologie pakt gelijktijdig beide problemen aan. Terwijl een goede waterkwaliteit in de aquacultuursystemen wordt gehandhaafd, is er gelijktijdig voedselproductie voor de gekweekte dieren. In tegenstelling tot de conventionele waterzuiveringtechnieken, biedt de biovlok-technologie een duurzaam, economisch interessant en gemakkelijk te hanteren alternatief. In **Hoofdstuk 1** wordt er een overzicht gegeven van de literatuur omtrent stikstofverwijdering in de aquacultuur en de biovlok-technologie.

In **Hoofdstuk 2** werd het effect van het type koolstofbron op de prestaties van de biovlok-reactoren bestudeerd. De koolstofbron beïnvloedde de capaciteit van de techniek om de waterkwaliteit in de biovlok-reactor te controleren en de voedingswaarde van de biovlokken. Daarnaast nam men ook een effect van de koolstofbron waar op de samenstelling van de eukaryote en prokaryote microbiële gemeenschap van de vlokken, wat talloze mogelijkheden biedt voor de verdere verfijning van de techniek, namelijk aangaande waterkwaliteitsbeheersing, voederproductie en/of kosten.

Het primaire belang van het type koolstofbron werd bevestigd in twee volgende studies (**Hoofdstuk 3** en **Hoofdstuk 4**) waarin biovlokken, geproduceerd met verschillende substraten, gebruikt werden als voeder voor de reuze zoetwatergarnaal (*Macrobrachium rosenbergii*) en de witte garnaal (*Litopenaeus vannamei*). Uit beide studies bleek dat de met glycerol geproduceerde biovlokken betere resultaten opleverden dan de met glucose geproduceerde biovlokken. Deze resultaten onderstrepen het potentiële belang van biovlokken als voeder in de aquacultuur, en dat zowel in zoet- als zoutwater systemen. Om dit te bevestigen zijn de toegankelijkheid, aanvaardbaarheid en aantrekkelijkheid

van de biovlokken voor de kweekdieren, de aminozuursamenstelling en het gehalte essentiële vetzuren van de biovlokken en de kosten van de technologie (vooral in vergelijking met de conventionele biofiltersystemen en voederkosten), allen belangrijke parameters die toekomstig onderzoek vereisen.

Naast de reeds behandelde milieu-, economische en duurzame overwegingen, werd er ook een specifieker probleem bestudeerd in **Hoofdstuk 5**, namelijk de blootstelling van de kweekdieren aan lagere temperaturen tijdens de winter, wat vaak leidt tot massale mortaliteit in industriële vijvers. Het bouwen van plastieken of glazen koepels op de vijvers zorgt ervoor dat de zon het water kan opwarmen waardoor de temperatuurdaling van het cultuurwater wordt beperkt. Het toepassen van de biovlok-technologie in dergelijke systemen maakt het mogelijk een goede waterkwaliteit te garanderen met gelijktijdige extra *in situ* voederproductie voor de kweekdieren, in dit onderzoek tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) zonder de overleving, groei of conditiefactor van de kweekvis negatief te beïnvloeden.

Op dit ogenblik heeft de aquacultuurindustrie in grote mate te kampen met massale mortaliteit toe te schrijven aan besmettelijke ziekten. Daarom werd deze studie beëindigd met een onderzoek naar de potentiële extra toegevoegde waarde van de biovlok-technologie (**Hoofdstuk 6**). In dit onderzoek werd vastgesteld dat biovlokken in staat waren de larven van pekelkreeftjes (*Artemia franciscana*) te beschermen tegen de pathogeen *Vibrio harveyi*. Deze resultaten wijzen erop dat naast waterkwaliteitsbeheersing en extra *in situ* voederproductie, de techniek ook het potentieel heeft om dieren te beschermen tegen besmetting met pathogene bacteriën, welke de oorzaak kunnen zijn van belangrijke economische verliezen in de aquacultuur.

Om te besluiten, wordt in het laatste hoofdstuk (**Hoofdstuk 7**) een korte bespreking van de uitgevoerde studies gegeven. Er worden eveneens aanwijzingen gegeven voor verder onderzoeken, gebaseerd op het hier uitgevoerde studies, die kunnen bijdragen tot een verdere duurzame ontwikkeling van de aquacultuur.

APPENDIX C – CURRICULUM VITAE

Roselien Crab

Vinçottestraat 11 B30
B-2140 Borgerhout
België
☎ +32 485 72 18 10
✉ Roselien.Crab@hotmail.be

Personalia

° 23 januari 1982, Antwerp, Belgium
Nationality: Belgian

Education

2004 – 2005	Thesis: The disruption of quorum sensing of pathogens in aquaculture Promotor: Prof. dr. ir. Willy Verstraete Co-promotor: Prof. dr. ir. Peter Bossier
2000 – 2005	Master of bioscience engineering – environmental technology Great Distinction Ghent University, Faculty of Bioscience Engineering
1994 – 2000	Secondary education: Science - Mathematics Koninklijk Atheneum, Antwerp

Additional training

2007 Training SAP
Ghent University, Ghent

2006 Advanced Laboratory Training in Aquaculture
AQUALAB Student Conference, Galway, Ierland

Professional record

2005 – 2009 Doctoral research with Prof. dr. ir. Willy Verstraete (Laboratory of Microbial Ecology and Technology, Ghent University) and Prof. dr. ir. Peter Bossier (Laboratory of Aquaculture and Artemia Reference Center, Ghent University) as scientific promotors. Research was supported by a grant of IWT-Vlaanderen.
Tutor of 4 students MsC in Bioscience Engineering, 1 student MsC in Environmental Sanitation and 1 student MsC in Aquaculture during their master's thesis.
Responsible for practical exercises 'Microbial Ecology and Environmental Sanitation' (2005 – 2007).
Participation in promotional campaign for Ghent University - http://www.communicatie.ugent.be/avs/2008/aflevering6.wmv

2009 – Now Forensic Expert DNA – Nationaal Instituut voor Criminalistiek en Criminologie (NICC)

International collaboration

2005 Research at Technion in collaboration with Prof. Yoram Avnimelech – Israel Institute of Technology (Civil and Environmental Engineering)

Publications in international peer-reviewed journals

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W. and Bossier, P. Quorum sensing-disrupting brominated furanones protect gnotobiotic brine shrimp *Artemia*

franciscana from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. Applied and Environmental Microbiology, Sept. 2006.

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P. and Verstraete, W. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270, 1-14.

Clauwaert, P., Toledo, R., Van der Ha, D., Crab, R., Verstraete, W., Hu, H., Udert, K.M., Rabaey, K., 2008. Combining biocatalyzed electrolysis with anaerobic digestion. Water Science and Technology 57, 575-579.

De Schryver, P., Crab, R., Defoirdt, T., Boon, N., Verstraete, W., 2008. The basics of bio-flocs technology: The added value for aquaculture. Aquaculture 277, 125-137.

Crab, R., Kochva, M., Verstraete, W., Avnimelech, Y., 2008. Bioflocs technology application in over-wintering of tilapia. Aquacultural Engineering 40, 105-112.

Crab, R., Chielens, B., Wille, M., Bossier, P., Verstraete, W., 2010. The effect of different carbon sources on the nutritional value of bioflocs, a feed for *Macrobrachium rosenbergii* postlarvae. Aquaculture Research 41, 559-567.

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., Verstraete, W., 2010. The application of bioflocs technology to protect brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. Journal of Applied Microbiology, In press.

Crab, R., Boon, N., Defoirdt, T., Bossier, P., Verstraete, W., 2010. Different organic carbon sources shape the nutritional properties and the microbial community composition of bioflocs. Aquaculture, Submitted.

Other publications

Verstraete, W., Defoirdt, T., Crab, R., De Schryver, P., 2006. Sustainable use of water and nutrients in aquaculture. Cursus Hergebruik, Universiteit Gent, gedoceerd door Prof. dr. ir. Willy Verstraete.

De Schryver, P., Crab, R., Defoirdt, T., Boon, N., Verstraete, W., 2008. The basics of bio-flocs technology: The added value for aquaculture. Panorama Acuícola Magazine, May/June 2008.

Avnimelech, Y., Crab, R., Kochva, M., Verstraete, W., 2008. BFT for over-wintering of tilapia. AQUA Culture Asia Pacific, Volume 4, Number 4 July/August 2008, Industry Review – Tilapia.

Contribution to international scientific congresses

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W. and Bossier, P. (2005). Disruption of quorum sensing protects gnotobiotic *Artemia* from pathogenic *Vibrio harveyi* and *Vibrio campbellii*. *Vibrio* 2005 – The Biology of Vibrios: Biodiversity, Genomics, Disease/Epidemiology, Ecology and Applications (November 6-8, Gent, Belgium).

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W. and Bossier, P. (2006). Quorum sensing-disrupting furanones protect gnotobiotic *Artemia* from pathogenic *Vibrio harveyi*, *Vibrio campbellii* and *Vibrio parahaemolyticus*. Aqua 2006 – Aquaculture Europe 2006 & World Aquaculture 2006 - European Aquaculture Society & World Aquaculture Society (May 9-13, Firenze, Italy).

Verstraete, W., De Schryver, P., Defoirdt, T., Crab, R. (2007). Added value of microbial life in flocs. Aquaculture 2007 – Bio-flocs technology primer session – Aquaculture Engineering Society (February 26 – March 2, San Antonio, Texas, US).

Crab, R., Kochba, M., Verstraete, W., Avnimelech, Y. (2009). Bio-floc technology application in over-wintering of tilapia. Special session on Biofloc Systems – World Aquaculture Society Meeting, World Aquaculture 2009 (September 25-29, Veracruz, Mexico).

Participation in international scientific congresses

Presentation

Crab, R., De Schryver, P., Verstraete, W., Avnimelech, Y. (2008). A comparison of municipal & aquaculture wastewater treatment. The Seventh International Conference on Recirculating Aquaculture (July 25-27, Roanoke, Virginia, US).

Poster

Crab, R., Kochva, M., Verstraete, W., Avnimelech, Y. (2008). Bio-flocs technology (BFT) for overwintering of tilapia. 14th PhD symposium in Applied Biological Sciences (September 15, Ghent, Belgium).