Aquaculture 277 (2008) 125–137

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

The basics of bio-flocs technology: The added value for aquaculture

P. De Schryver, R. Crab, T. Defoirdt, N. Boon, W. Verstraete *

Laboratory Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 2 October 2007
Received in revised form 6 February 2008
Accepted 6 February 2008

Keywords:
Aquaculture
Bio-flocs technology
Bacterial aggregates
Fish feed
C/N-ratio

ABSTRACT

The expansion of the aquaculture production is restricted due to the pressure it causes on the environment by the discharge of waste products in the water bodies and by its dependence on fish oil and fishmeal. Aquaculture using bio-flocs technology (BFT) offers a solution to both problems. It combines the removal of nutrients from the water with the production of microbial biomass, which can *in situ* be used by the culture species as additional food source. Understanding the basics of bio-flocculation is essential for optimal practice. Cells in the flocs can profit from advective flow and as a result, exhibit faster substrate uptake than the planktonic cells. The latter mechanisms appear to be valid for low to moderate mixing intensities as those occurring in most aquaculture systems (0.1–10 W m^{-3}). Yet, other factors such as dissolved oxygen concentration, choice of organic carbon source and organic loading rate also influence the floc growth. These are all strongly interrelated. It is generally assumed that both ionic binding in accordance with the DLVO theory and Velcro-like molecular binding by means of cellular produced extracellular extensions are playing a role in the aggregation process. Other aggregation factors, such as changing the cell surface charge by extracellular polymers or quorum sensing are also at hand. Physicochemical measurements such as the level of protein, poly-β-hydroxybutyrate and fatty acids can be used to characterize microbial flocs. Molecular methods such as FISH, (real-time) PCR and DGGE allow detecting specific species, evaluating the maturity and stability of the cooperative microbial community and quantifying specific functional genes. Finally, from the practical point of view for aquaculture, it is of interest to have microbial bio-flocs that have a high added value and thus are rich in nutrients. In this respect, the strategy to have a predominance of bacteria which can easily be digested by the aquaculture animals or which contain energy rich storage products such as the poly-β-hydroxybutyrate, appears to be of particular interest.

Contents

* Corresponding author. Tel.: +32 9 264 59 76; fax: +32 9 264 62 48.
E-mail addresses: Peter.Deschryver@ugent.be (P. De Schryver), Roselien.Crab@ugent.be (R. Crab), Tom.Defoirdt@ugent.be (T. Defoirdt), Nico.Boon@ugent.be (N. Boon), Willy.Verstraete@ugent.be (W. Verstraete).

0044-8486/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2008.02.019

1. Introduction

The current worldwide growth rate of the aquaculture business (8.9–9.1% per year since the 1970s) is needed in order to cope with the problem of shortage in protein food supplies, which is particularly situated in the developing countries (Gutierrez-Wing and Malone, 2006; Matos et al., 2006; Subasinghe, 2005). However, environmental and economical limitations can hamper this growth. Especially intensive aquaculture coincides with the pollution of the culture water by an excess of organic materials and nutrients that are likely to cause acute toxic effects and long term environmental risks (Piedrahita, 2003). For long, the most common method for dealing with this pollution 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 fresh water per kg shrimp produced (Wang, 2003). For an average farm with a production of 1000 kg shrimp ha^{-1} yr^{-1} and total pond surface of 5 ha, this corresponds with a water use of ca. 270 m^3 day^{-1}. 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 (Gutierrez-Wing and Malone, 2006). The amount of water that needs to be replaced on a daily basis generally is reduced to about 10% of the total water volume (Twarowska et al., 1997). However, this technique is costly in terms of capital investment. While capital investment costs for normal flow-through ponds systems are ca. 1.3 € kg^{-1} annual production, they may increase to 5.9 € kg^{-1} in recirculating systems (Gutierrez-Wing and Malone, 2006). Operation of RAS furthermore increases energy and labour costs, so that taking all costs into consideration (investment plus operation costs) it can be estimated that unsustainable pond production can be performed at two thirds of the costs of RAS (Gutierrez-Wing and Malone, 2006).

A relatively new alternative to previous approaches is the bio-flocs technology (BFT) aquaculture (Avnimelech, 2006). In these systems, a co-culture of heterotrophic bacteria and algae is grown in flocs under controlled conditions within the culture pond. The system is based on the knowledge of conventional domestic wastewater treatment systems and is applied in aquaculture environments. Microbial biomass is grown on fish excreta resulting in a removal of these unwanted components from the water. The major driving force is the intensive growth of heterotrophic bacteria. They consume organic carbon; 1.0 g of carbohydrate-C yields about 0.4 g of bacterial cell dry weight-C; and depending on the bacterial C/N-ratio thereby immobilize mineral nitrogen. As such, Avnimelech (1999) calculated a carbohydrate need of 20 g to immobilize 1.0 g of N, based on a microbial C/N-ratio of 4 and a 50% C in dry carbohydrate.

In integrated aquaculture systems using bacteria as additional nutrient trapping stage, the increase in retention by the use of bacteria is rather small. Schneider et al. (2005) stated that hardly 7% of the feed nitrogen and 6% of the feed phosphorus were retained by conversion in microbial biomass. However, when carbon and nitrogen are well balanced in the water solution and microbial assimilation of the ammonium is efficiently engineered, a complete retention can be obtained. A concentration of about 10 mg NH_4^+–N L^{-1} could almost completely be removed within 5 h after the addition of glucose at C/N-ratio 10, and this without the accumulation of nitrite and nitrate (Avnimelech, 1999). This transformation, achievable by adding different types of organic carbon source, resulted in a production of microbial proteins that could be reused as fish food. As such, nitrogen recovery in the form of tilapia biomass from a tilapia breeding facility could be increased from 23% in normal operation to 43% when the system was operated with BFT. This increase was based on the internal recirculation of nutrients through the formation of new microbial biomass, which was subsequently grazed by the fish (Avnimelech, 2006).

It has been established that the removal of nitrogen from the culture water by means of BFT can be regulated by balanced addition of carbon. The added value that bio-flocs may bring to the aquaculture systems however still remains largely unknown. In this review, we strive to give an overview of the basics of the bio-flocs aggregation within the ponds and how this is influenced by the different pond operation parameters. Since most insight is related to floc formation in activated sludge systems, the latter are interpreted in terms of their use in aquaculture. The relationship between the different parameters is described and also suggestions for additional research are made. Throughout the text, the quality of the bio-flocs is emphasized in terms of their morphological characteristics and nutritional composition.

2. Selective forces for bacteria to live in floc structures

2.1. Bacteria living in floc structures

Microbial flocs (Fig. 1A) consist of a heterogeneous mixture of micro-organisms (floc-formers and filamentous bacteria), particles,

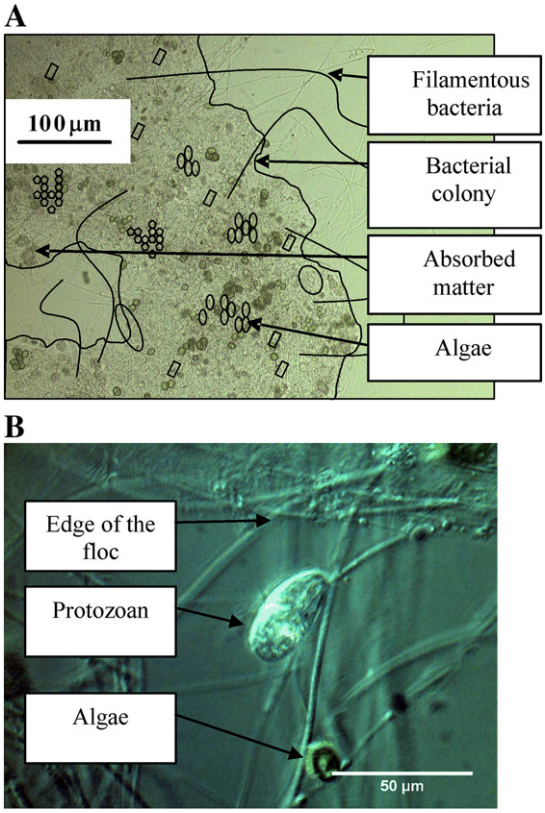


Fig. 1. A. Image of a floc structure within a BFT-system and its composition; B: a protozoan that is grazing at the edge of a floc removes the cells that tend to leave the floc.

colloids, organic polymers, cations and dead cells (Jorand et al., 1995) and can reach more than 1000 µm in size. Typical flocs are irregular by shape, have a broad distribution of particle sizes, are fine, easily compressible, highly porous (up to more than 99% porosity) and are permeable to fluids (Chu and Lee, 2004b). Several parameters can be used to characterize the flocs. A distinction can be made between the parameters that describe the physical characteristics of the flocs and the ones that describe the chemical composition of the flocs (Table 1). Only 2–20% of the organic fraction of sludge flocs are believed to be living microbial cells while total organic matter may be 60–70% and total inorganic matter 30–40% (Wilen et al., 2003). Densities of the microbial biomass average slightly above 1.0 g wet weight mL^{-1} floc aggregate; hence they tend to sink rather slowly (1–3 m h^{-1}) (Sears et al., 2006).

Efficient aggregation is of paramount importance in conventional activated sludge systems (AS), since their operational success depends heavily on good settling sludge (Bossier and Verstraete, 1996). In the clarifier, the aggregated organisms settle to the bottom of a conic tank and are recycled to an aeration tank where new nutrient rich water is awaiting. Non- or poorly settling flocs, mainly dominated by filamentous organisms, are washed out at this stage and are lost from the system. The continuous recycle and repetition of the settling process selects for those organisms living in flocs and having access to the food supply.

In aquaculture systems, the capacity to settle may offer a selective advantage to the extend that the flocs may escape detrimental impact of light, avoid grazing by the higher organisms feeding in the top layer or acquire more food near the bottom sediment layer. These aspects all warrant closer investigation. Good settling flocs are not necessarily lost as food by sedimentation since the aeration devices keep them in suspension.

2.2. Acquisition of food at the cellular level: physical constraints

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 parameters 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 1.0 µm diameter (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^{-1}) at a speed of 1000 µm s^{-1} (Vs) (Mitchell and Kogure, 2006) results in a Reynolds number of 1.0×10^{-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 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).

Organisms are considered to counter the nutrient diffusion problem by growing in amorphous aggregates or microbial flocs, as

Table 1
Main parameters and methods of importance for the characterization of the flocs present in bio-flocs technology aquaculture systems

	Definition and units	Determination*	Suggested range for bio-flocs technology aquaculture	Remarks
Physical characterization				
– Suspended solids (SS)	The amount of particulate matter present in a pond sample (g SS L^{-1})	The particulates are separated out of the water sample either by filtration or centrifugation and dried overnight at 100 °C.	0.2–1.0 g L^{-1}	
– Volatile suspended solids (VSS)	The amount of organic matter in particulate form in a pond sample (g VSS L^{-1})	After drying, the suspended solids are ashed at 600 °C. The SS minus the ash yields the VSS value.	N.D.	
– Floc volume index (FVI)	The volume occupied by 1.0 g floc-VSS (mL g^{-1})	Calculated from the floc volume after 30' of sedimentation in an Imhoff cone and the VSS value	>200 mL g^{-1}	In activated sludge systems, this value is 40–60 for good settling sludge and >200 for bulking sludge.
– Porosity	The space within a floc that is not occupied by bacterial biomass but free for water and/or gas	Determined by image analysis (Perez et al., 2006) or indirect by measuring floc settling velocity (Chung and Lee, 2003)	N.D.	The porosity value ranges between 0 and 1 with a higher value representing a higher porosity.
– Floc size distribution	An overview of the sizes of the flocs as well as their relative frequency of incidence	Determined automatically e.g. by the Malvern MastersizerS or the Galai CIS-100 Particle Analyzer (Govoreanu et al., 2004)	N.D.	
Chemical characterization				
– Chemical oxygen demand (COD)	Amount of oxygen required to chemically oxidize the organic matter in a pond sample (g L^{-1})	The determination is based on the reaction of organic carbocompounds with an excess of $K_2Cr_2O_7$, a strong oxidizing compound, in acidic solution.	N.A.	For monitoring the carbon source in the water, the dissolved COD can be determined (after filtration, 0.45 µm). It can be stated that 1.0 g carbohydrate or 1.0 g protein equals about 1.0 g COD.
– Biological oxygen demand (BOD)	Amount of oxygen that is used by micro-organisms to biochemically convert organic matter into metabolites (g L^{-1})	Measured by means of an Oxitop bottle during a five day test (therefore often referred to as BOD_5)	N.A.	Gives an indication of the rapidly biodegradable part of organic matter in the pond BOD_5 = biodegradable COD × 0.65 (Verstraete and Van Vaerenbergh, 1977)

N.D.: no data available.
N.A.: not applicable.
* The determination of the parameters is performed by standard methods according to Greenberg et al. (1992) unless stated otherwise.

is the case in BFT. Originally, the mass transfer within flocs has been attributed to molecular diffusion. Models however revealed that individual cells within a dense bacterial floc could not reach a higher substrate uptake rate by diffusion relative to dispersed planktonic cells (Logan and Hunt, 1988). This means that aggregated cells have a mechanism resulting in nutritious advantage for which they may have to invest energy in order to sustain floc formation (Li and Ganczarczyk, 1988). The answer can be found within the highly porous internal structure of aggregated microbial communities. The permeability of the flocs allows advective flow to pass through the pores since the water tends to follow the path of least resistance (Chu and Lee, 2004a). As a result, the amount of nutrients supplied to the micro-organisms in the flocs by mixed flow is considered to be higher as compared to the amount supplied by laminar flow to an individual cell. The substrate availability can thus increase up to a factor two (Bossier and Verstraete, 1996).

The advantage due to bio-flocculation can be represented by the relative difference in mass transfer rate towards cells inside flocs and towards dispersed cells used as reference. This is expressed by the relative uptake (γ) that is defined as the ratio of the uptake rate by cells growing in flocs over the uptake rate by cells dispersed in the fluid. Here, both the floc and dispersed cells are in the same fluid mechanical environment. If the relative uptake factor is larger than one, living in flocs is advantageous and micro-organisms will organize themselves into aggregates (Logan and Hunt, 1988).

The relative uptake factor is function of the power input into the water for aeration and mixing. This means that to obtain flocs, the optimal power input can be determined. In general, it is applied in aquaculture systems in the order of 0.1–10 W m^{-3} (Boyd, 1998). When the relative uptake factor is calculated according to Logan and Hunt (1988) in function of the power input for cells of 1 μm organized in a permeable floc, it is observed that in the low mixing ranges, aggregated cells have a distinct advantage relative to planktonic ones (Fig. 2). The maximal fluid shear rate that may be present in this specific case is about 90 W m^{-3}. Beyond this value, the dispersed cells will outcompete the cells living in flocs. For aquaculture ponds, applied values for the shear rate (0.1–10 W m^{-3}) generally result in flow regimes in which natural floc formation will have a selective advantage. Yet, it must be noted that these considerations are based on approximate unit values and theoretical calculations. In practice, the optimal power input will have to be established for each individual culture unit. Assessing floc formation at different power inputs will allow to determine a range for optimal bio-floc growth and each power input within the range will result in a different floc size distribution. Which floc size distribution is desired will mainly depend on the culture species. Adult species will be able to feed on larger flocs whereas these organisms in a juvenile life stadium will prefer/need smaller flocs. In case of filter feeders, e.g. clogging of the gills will be a determining factor. As described further, the power input is strongly related with other factors like mixing intensity and dissolved oxygen concentration. Obviously, experimental validation of the concept of advantageous advective flow in the context of BFT is warranted.

2.3. Protection against protistan grazing: biological stressor

It is postulated that grazing by protozoa (unicellular eukaryotic micro-organisms sized 2.0–2000 μm) is one of the major causes of bacteria removal in soil, freshwater and marine ecosystems (Matz and Kjelleberg, 2005) (Fig. 1B). 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).

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^{-1} that can considerably decrease capture (Matz and Jurgens, 2005)) or the attachment to surfaces that enhances survival all 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 on the outer layers are susceptible to predation by grasping feeders (Matz and Kjelleberg, 2005).

3. 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

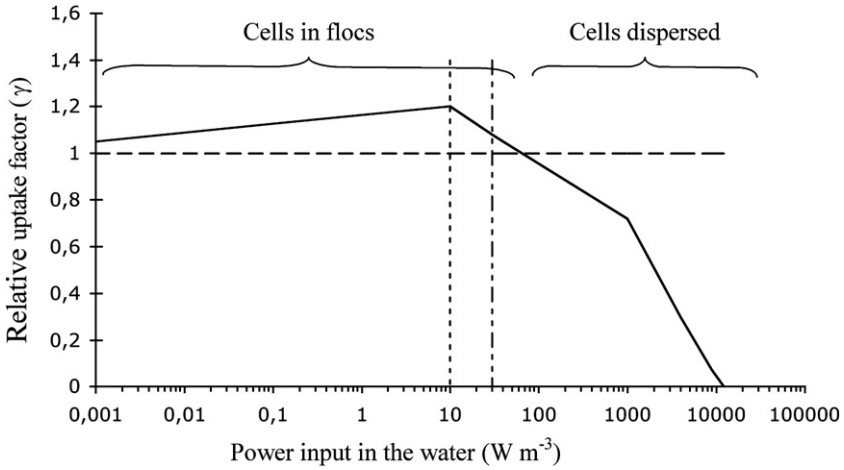


Fig. 2. Predicted relative uptake for microbial cells within permeable flocs (Logan and Hunt, 1988). A shear rate of 10 W m^{-3} represents the mixing of the sea (·····); a shear rate of about 30 W m^{-3} represents the mixing in aerated activated sludge (–··–··) and a shear rate of 0.1–10 W m^{-3} corresponds to the mixing in most aquaculture systems.

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 out of polysaccharides, protein, 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 like e.g. nitrogen (Steiner et al., 1976).

3.1. 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^{-1} 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) (Fig. 3). 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) (Fig. 3). 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 (Fig. 3). 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.

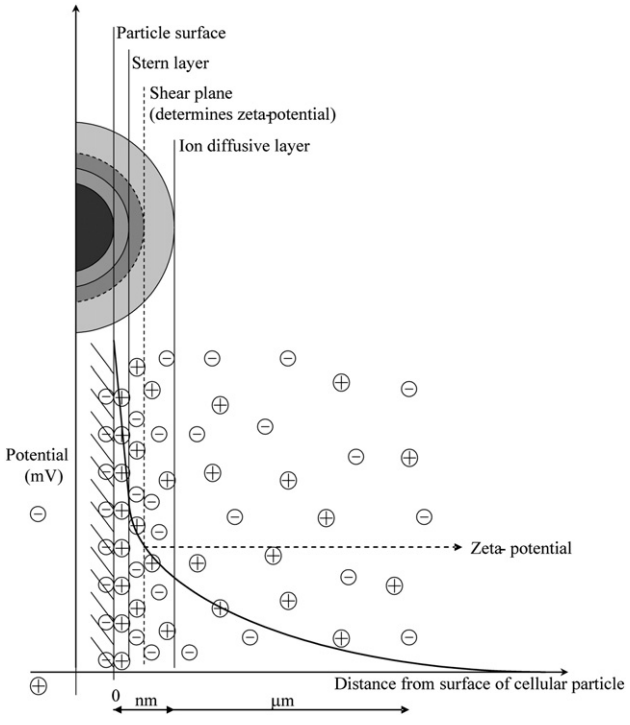


Fig. 3. Schematic view of a charged cellular particle with its counter charges and the potential in the area of a particle surface.

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, with the amount of protein rising considerably with increasing sludge age (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 (≥0.1 M), it can be assumed that the binding of micro-organisms 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 micro-organisms and substrata (Rijnaarts et al., 1999). At low ionic strengths (<0.001 M), the binding is hindered by the DLVO-type 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 bio-flocculation 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). Bio-flocculation may also be steered by the *choice of organic compound* used as food for the

bio-flocs. 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 Shojaosadati, 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.

3.2. Quorum sensing as biological control

The grouping of micro-organisms 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, signaling 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 signaling 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 rhlR*/*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 micro-colonies (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 bio-flocs, 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 *P. aeruginosa* (Lynch et al., 2002; Shrout et al., 2006). A clear relationship such as e.g. the excretion of signaling molecules by micro-organisms 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 *T. 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) recently 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 micro-organisms is far from complete, it is difficult for use in the control of BFT. However, some interesting application prospective and related research certainly exists. For example, 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 bio-control 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.

4. Factors influencing floc formation and floc structure in bio-flocs 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 2 may need adjustment to obtain good aggregation and high quality of the bio-flocs together with optimal growth conditions

Table 2
Overview of the main operational parameters for bio-flocs 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 and 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)	– Type of organic carbon source	– Organic loading rate – Dissolved oxygen
Organic loading rate	– Microbial floc composition (filamentous vs. floc forming bacteria) – Chemical floc composition (cellular reserves like polyhydroxyalkanoates)	– Feeding strategy (continuous feeding or regular interval feeding)	– Dissolved oxygen
Dissolved oxygen (DO)	– Microbial floc composition (filamentous vs. floc forming bacteria) – Floc structure and floc volume index	– Choice of power input (W m^{-3}) – Aeration device – Floc production in the pond vs. floc production in external unit	– Mixing intensity – Organic carbon source – Organic loading rate
Temperature	– Floc structure and activity	– Addition of heat	– Dissolved oxygen
pH/ionics	– Stability of the flocs	– Addition of acid/base; mono- or polyvalent ions	– Alkalinity – Conductivity

The interrelation between the parameters is indicated.

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 bio-flocs quality. Therefore, the following can be seen as an overview of possible research topics within the BFT aquaculture.

4.1. 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 0.1–10 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 an average velocity gradient (or *G*-value) of 19.4 s^{-1}, the stable floc size was ca. 130 μm whereas this was decreased to ca. 20 μm for a velocity gradient of 346 s^{-1}. The relationship between floc size and mixing intensity has been represented by Parker et al. (1972) with the power law relationship $d=CG^{-x}$, 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. 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).

4.2. 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^{-1}, occurred at low DO values (0.5–2.0 mg L^{-1}) compared to settling at higher DO values (2.0–5.0 mg L^{-1}) where the SVI was ca. 100 mL g^{-1}. 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^{-1} 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 bio-flocs with a higher floc volume index (FVI) are produced at lower DO levels in the bio-flocs ponds. We suggest, although experimental values are lacking, that the FVI should be higher than 200 mL g^{-1} 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^{-1} results in a decrease of food consumption by the fish, a lower average body weight and a decreased net production (Torrans, 2005).

4.3. Organic carbon source

The dosing of an *organic carbon source* to the culture water in bio-flocs 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 bio-flocs 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 (Fig. 4). 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 an SBR resulted mainly in poly-β-hydroxybutyrate 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).

4.4. 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 day^{-1}). 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 poly-β-hydroxybutyrate under conditions of excess nutrient availability with which the micro-organisms can bridge the periods of nutrient shortage. As described further, the storage products may be of high importance to the added value that bio-flocs 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 are also known to influence floc characteristics but are more difficult to change.

4.5. 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

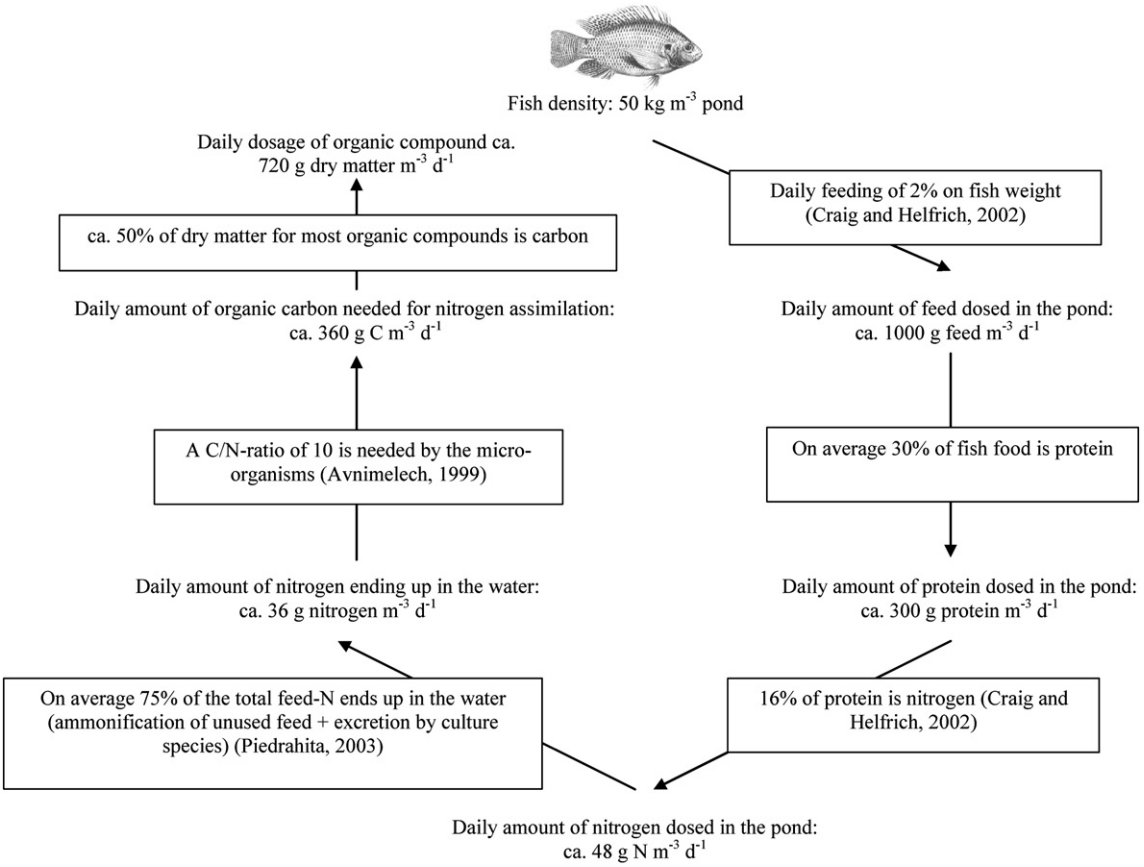


Fig. 4. Schematic calculation of the daily amount of organic carbon needed by bio-flocs to remove the nitrogen excreted in an intensive aquaculture pond of 50 kg fish m^{-3}.

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^{-1}) 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^{-1}. 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 linked 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.

4.6. pH

Changes in *pH* determine the stability of the bio-flocs 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 (vanGinneken 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.

5. Biological bio-floc monitoring technologies for aquaculture

The most obvious way to determine the presence and type of micro-organisms 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 FISH procedure is based on the binding of fluorescently labeled 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 micro-organism and thus allow to detect a certain species in a community. Since rRNA is only present in biologically active organisms, it only allows to detect 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 micro-organisms in a sample or to determine the 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.

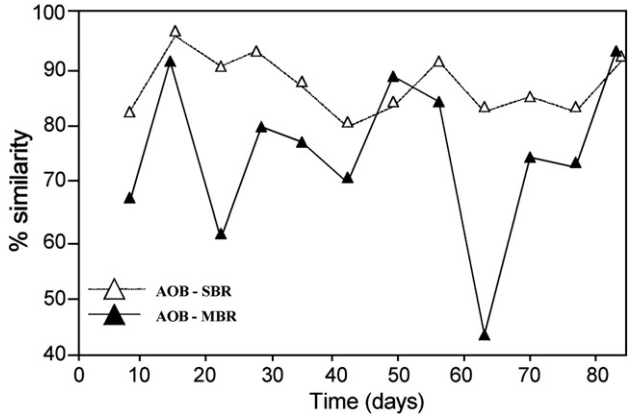


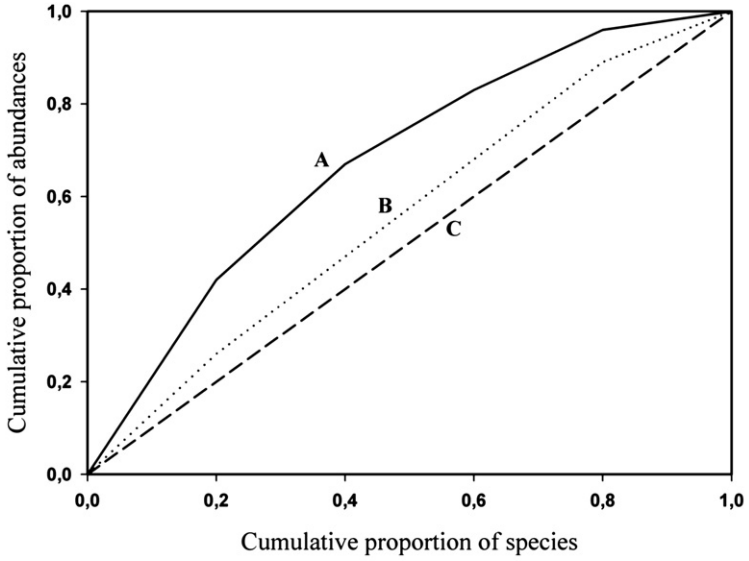
Fig. 5. Example of moving window analysis: moving window correlation on the DNA level of ammonia oxidizing bacteria in a sequential batch reactor (AOB-SBR) and in a membrane bioreactor (AOB-MBR). The variability between two consecutive sampling dates ($\Delta_{t(week)}$) was calculated based on the denaturing 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).

Denaturing gradient gel electrophoresis (DGGE) is a molecular approach that furnishes information concerning the genetic microbial diversity within any sample such as water, sludge, air, etc. (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 micro-organisms. The analysis of an environmental sample by means of DGGE results in a band pattern in which roughly each band represents a specific micro-organism.

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 (Fig. 5). 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–Lorenz 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–Lorenz curves (Fig. 6) (Lorenz, 1905;



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

Fig. 6. Example on how to calculate the Pareto–Lorenz 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.

Mertens et al., 2005). If every micro-organism (= DGGE band) is present in an equal amount (= DGGE band intensity), the curve reveals a perfect evenness (= the diagonal).

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.

6. 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 bio-flocs 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 1.0–5.0 kg of fish has to be caught in the oceans to be able to produce 1.0 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 (micro-algae 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 bio-flocs is of uppermost importance to economically produce a healthy, high quality product (Watanabe, 2002). 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, poly-unsaturated fatty acid (PUFA) and lipid content are the most important parameters determining the feasibility of the bio-flocs as feed in aquaculture.

Not only the nutritional value of the bio-flocs is important. Other internal compounds may also be beneficial to the aquaculture species. Short chain fatty acids as bio-control 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 a intracellular biodegradable polymer produced by a wide variety of micro-organisms 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 *A. 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.

7. Overall added value of bio-flocs technology for aquaculture

The added value that BFT brings to aquaculture is represented by the reduced costs for water treatment that is not needed anymore. Crab et al. (2007) gave an overview of the costs for different treatment techniques ranging from 1.1 € kg^{-1} of annual fish production in case of rotating biological contactors to 0.2 € kg^{-1} annual fish production in case of fluidized sand bio-filters. Bio-flocs 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 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 bio-flocs technology, the feed conversion ratio can be taken 2.2 with 30% protein feed: Without flocs, 2.2 kg feed is dosed kg^{-1} fish produced (feed conversion ratio of 2.2)
  - ➔ 0.3×2.2=0.66 kg protein is dosed kg^{-1} fish produced (30% protein content in feed)
  - ➔ 0.25×0.66=0.17 kg protein is taken up kg^{-1} fish produced (25% of the feed is taken up by the fish). This is in accordance with the protein content of 14–17% on wet fish biomass for tilapia earlier reported (Hanley, 1991).
- In a system with bio-flocs, part of the feed will be recycled into flocs, which can also be used by the animals as feed source. Therefore, less 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 flocs, *F* kg feed is dosed kg^{-1} fish produced
  - ➔ (0.3×*F*) kg protein is dosed kg^{-1} fish produced
  - ➔ 0.3×(0.25×*F*)=0.075×*F* kg protein is taken up kg^{-1} fish produced
  - 75% of the conventional feed is thus unused and recycled into the flocs:
  - ➔ (0.75×*F*) kg feed is recycled
  - ➔ 0.3×(0.75×*F*)=0.23×*F* kg protein is recycled
  - Assume that the fish also take in only 25% of the flocs:
  - ➔ 0.25×(0.23×*F*)=0.06×*F* kg protein is taken up out of the flocs per kg fish produced
- Calculation of the amount of external feed needed when BFT is applied
  The total protein requirement by the fish is 0.17 kg protein kg^{-1} fish produced:
  - ➔ Total protein requirement=protein obtained from feed+protein obtained from the flocs=0.17
  - ➔ Total protein requirement=(0.075×*F*+0.06×*F*)=0.17
  - ➔ The amount of feed that still needs to be applied (*F*) is ca. 1.3 kg
- Calculation of the amount of organic carbon needed to grow the flocs:
  - ➔ 0.75×1.3=1.0 kg of the decreased feed amount (at 1.3 kg feed kg^{-1} fish produced) is unused by the fish (75% of the feed for fish is unused)
  - ➔ 0.3×1.0=0.3 kg protein is unused kg^{-1} fish produced (assumed protein content in feed is 30%)
  - ➔ 0.16×0.3=0.048 kg nitrogen is unused kg^{-1} fish produced (16% nitrogen content in protein) and is recycled into floc biomass
  - The flocs have a C/N-ratio of 4 (Avnimelech, 1999)
  - ➔ 4×0.048=0.19 kg C in floc biomass is produced kg^{-1} fish produced since all the excess nitrogen should be assimilated in the bio-flocs
  - The yield of bacterial biomass can be taken to be 0.5 (Avnimelech, 1999)
  - ➔ 0.19/0.5=0.38 kg C needs to be added in the water for the flocs to be able to assimilate the excess nitrogen kg^{-1} fish produced
  - In case acetate (40% C) is used as organic carbon source:
  - ➔ 0.38/0.4=0.95 kg acetate needs to be dosed to the water kg^{-1} fish produced

Table 3
Calculation of the relative difference in operation system costs for tilapia culture (€ kg^{-1} fish annually produced) between systems operated with a nitrifying trickling filter and systems operated with bio-flocs technology

Bio-flocs technology in the aquaculture pond	Nitrification in a recirculating aquaculture system by a trickling filter
1. Capital investment cost No extra reactor investment costs required	1. Capital investment cost The investment costs for a trickling filter can be estimated to be 5 100 000 € for the treatment of 5 000 m^3 water day^{-1} (=5 times the water volume) (USEPA, 2000). At a pay-back over 20 years at an interest of 10%, this corresponds to an annuity of 280 000 € yr^{-1}.
2. Operational costs * Cost for carbon supplementation: The removal of 13 140 kg NH_4^+–N yr^{-1},[a] requires 131 400 kg C yr^{-1} at a C/N-ratio of 10. In case of e.g. acetate (38% C based on weight), this yields an acetate requirement of 345 800 kg acetate yr^{-1}. At a unit cost of 0.4 € kg^{-1} acetate (Salehizadeh and Van Loosdrecht, 2004), this corresponds to 138 000 € yr^{-1}.	2. Operational costs * Cost for aeration: Stoichiometrically, nitrification requires 4.34 g O_2 g^{-1} N (Eding et al., 2006). An ammonium production of 13 140 kg N yr^{-1} requires 57 000 kg O_2 yr^{-1}. Aerators for aquaculture purposes have on average a standard aeration efficiency of 2.0 kg O_2 kW^{-1} h^{-1} (Boyd, 1998), resulting in an annual energy consumption of 28 500 kWh. At a cost of ca. 0.1 € kWh^{-1}, this correspond to a cost of ca. 2 850 € yr^{-1}. * Cost for water replacement: In RAS, an average daily water replacement of 10% of the water volume is required (Twarowska et al., 1997). At a pond volume of 1 000 m^3, this results in a water volume of 100 m^3 that needs to be replaced every day. At a cost of 0.28 € m^{-3} in e.g. Israel (Avnimelech, 2006), this results in a cost of ca. 10 000 € yr^{-1}
Overall costs in relation to (1) + (2) per amount of fish annually produced 0.28 € kg^{-1} fish live weight	Overall costs in relation to (1) + (2) per amount of fish annually produced 0.59 € kg^{-1} fish live weight

A tilapia farm with an annual production of 500 ton, a volume of 1 000 m^3 and operated at a fish density of 50 kg fish m^{-3} is represented.
[a] The amount of nitrogen that is produced in a tilapia farm at a fish density of 50 kg m^{-3} is based on the data presented in Fig. 4.

- Calculation of the cost saving for the tilapia breed by the application of BFT The costs for the production of 1 kg fish without BFT:

➔ 2.2 kg feed kg^{-1} fish produced × 0.6 € kg^{-1} feed (in Belgium) = 1.3 € kg^{-1} fish produced

The costs for the production of 1 kg fish with BFT:

➔ (1.3 kg feed kg^{-1} fish produced × 0.6 € kg^{-1} feed) + (0.95 kg acetate kg^{-1} fish produced × 0.43 € kg^{-1} acetate (Salehizadeh and Van Loosdrecht, 2004)) = 1.19 € kg^{-1} fish produced.

The gain thus appears to be in the order of 10% in terms of feed costs kg^{-1} fish produced. For an intensive culture system producing at e.g. 500 ton fish yr^{-1}, this represents a gain of 65 000 € yr^{-1}. 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%) must be compared to possible increase of costs 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). In Table 3, the difference in cost contribution to the fish price resulting from the application of a nitrifying trickling filter and limited water exchange is compared with that resulting from application of BFT. In this calculation, only the costs that are specific for each of the two techniques and thus result in a different price of the end product are taken into account. For example, the energy for the extra aeration required to sustain the microbial metabolism in the bio-flocs technology is considered to be of the same order of magnitude as the pumping energy requirements to supply the nitrification filter in RAS with water (Avnimelech, 2006). Therefore, these aspects will not yield a major difference in fish price and as such are not included in the cost calculations. Since this calculation is made for comparison, the actual cost for application of these techniques should also include labour, maintenance, etc. and thus will be higher than the represented values per kg fish live weight. In Table 3, it is estimated that the contribution resulting from BFT is about half of that resulting from the nitrifying trickling filter operation.

8. Conclusions

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 the aquaculture organisms. A good understanding of the microscopic mechanisms that are involved in bio-flocculation, 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 (protein, polyunsaturated fatty acids, lipids, poly-β-hydroxybutyrate,…) of the bio-flocs, although the latter can represent a major added value for aquaculture. The nutritional value of the bio-flocs, as well as their morphological characteristics, are dependent on a large set of operational parameters currently under development 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 bio-flocs 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 floc morphology and compositional and nutritional value of the flocs so that indeed it can replace both water treatment and protein supply based on fishery products.

Acknowledgements

The authors would like to thank Dr. ir. Massimo Marzorati and Dr. ir. Marta Carballa for the help and the critical reading of the manuscript.

References

Abdel-Tawwab, M., Mousa, M.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 (1–4), 236–246.

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 (1), 143–169.

Avnimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176 (3–4), 227–235.

Avnimelech, Y., 2006. Bio-filters: the need for an new comprehensive approach. Aquac. Eng. 34 (3), 172–178.

Biggs, C.A., Lant, P.A., 2000. Activated sludge flocculation: on-line determination of floc size and the effect of shear. Water Res. 34 (9), 2542–2550.

Bossier, P., Verstraete, W., 1996. Triggers for microbial aggregation in activated sludge? Appl. Microbiol. Biotechnol. 45 (1–2), 1–6.
Boyd, C.E., 1998. Pond water aeration systems. Aquac. Eng. 18 (1), 9–40.
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 (2), 259–264.
Cammarota, M.C., Sant'Anna, G.L., 1998. Metabolic blocking of exopolysaccharides synthesis: effects on microbial adhesion and biofilm accumulation. Biotechnol. Lett. 20 (1), 1–4.
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 (3), 676–684.
Chu, C.P., Lee, D.J., 2004a. Advective flow in a sludge floc. J. Colloid Interface Sci. 277 (2), 387–395.
Chu, C.P., Lee, D.J., 2004b. Multiscale structures of biological flocs. Chem. Eng. Sci. 59 (8–9), 1875–1883.
Chung, H.Y., Lee, D.J., 2003. Porosity and interior structure of flocculated activated sludge floc. J. Colloid Interface Sci. 267 (1), 136–143.
Colt, J., 2006. Water quality requirements for reuse systems. Aquac. Eng. 34 (3), 143–156.
Cosson, P., Zulianello, 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 *Dictyostelium discoideum* host system. J. Bacteriol. 184 (11), 3027–3033.
Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., Verstraete, W., 2007. Nitrogen removal in aquaculture towards sustainable production. Aquaculture 270 (1–4), 1–14.
Craig, S., Helfrich, L.A., 2002. Understanding Fish Nutrition, Feeds and Feeding (Publication 420–256). Virginia Cooperative Extension, Yorktown (Virginia). 4 pp.
de Barros, H.P., Valenti, W.C., 2003. Food intake of *Macrobrachium rosenbergii* during larval development. Aquaculture 216 (1–4), 165–176.
Defoirdt, T., Boon, N., Bossier, P., Verstraete, W., 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aquaculture 240 (1–4), 69–88.
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 (2), 804–808.
Defoirdt, T., Halet, D., 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 (2), 445–452.
Dube, M.A., Tremblay, A.Y., Liu, J., 2007. Biodiesel production using a membrane reactor. Bioresour. Technol. 98 (3), 639–647.
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 (3), 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. Aquac. Eng. 34 (3), 234–260.
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 (2), 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 (1), 211–221.
Govoreanu, R., Saveyn, H., Van der Meeren, P., Vanrolleghem, P.A., 2004. Simultaneous determination of activated sludge floc size distribution by different techniques. Water Sci. Technol. 50 (12), 39–46.
Greenberg, A.E., Clesceri, L.S., Eaton, A.D., 1992. Standard Methods for the Examination of Waste and Wastewater. American Public Health Society, Washington. 1085 pp.
Gutierrez-Wing, M.T., Malone, R.F., 2006. Biological filters in aquaculture: trends and research directions for freshwater and marine applications. Aquac. Eng. 34 (3), 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 (11), 4863–4872.
Hahn, M.W., Hofle, M.G., 2001. Grazing of protozoa and its effect on populations of aquatic bacteria. FEMS Microbiol. Ecol. 35 (2), 113–121.
Hanley, 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 (4), 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 (1), 100–104.
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 (2–4), 248–263.
Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M., 1996. Real time quantitative PCR. Genome Res. 6 (10), 986–994.
Hermansson, M., 1999. The DLVO theory in microbial adhesion. Colloids Surf. B. 14 (1–4), 105–119.
Higgins, M.J., Novak, J.T., 1997. Characterization of exocellular protein and its role in bioflocculation. J. Environ. Eng.-ASCE 123 (5), 479–485.
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 (4), 355–360.
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 (2), 332–372.
Jendrossek, D., Handrick, R., 2002. Microbial degradation of polyhydroxyalkanoates. Annu. Rev. Microbiol. 56, 403–432.
Johnson, M.R., Montero, C.I., Conners, S.B., Shockley, K.R., Bridger, S.L., Kelly, R.M., 2005. Population density-dependent regulation of exopolysaccharide formation in the hyperthermophilic bacterium *Thermotoga maritima*. Mol. Microbiol. 55 (3), 664–674.
Jorand, F., Zartarian, F., Thomas, F., Block, J.C., Bottero, J.Y., Villemin, G., Urbain, V., Manem, J., 1995. Chemical and structural (2d) linkage between bacteria within activated-sludge flocs. Water Res. 29 (7), 1639–1647.
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 (4), 373–380.
Keiding, K., Nielsen, P.H., 1997. Desorption of organic macromolecules from activated sludge: effect of ionic composition. Water Res. 31 (7), 1665–1672.
Kjelleberg, S., Molin, S., 2002. Is there a role for quorum sensing signals in bacterial biofilms? Curr. Opin. Microbiol. 5 (3), 254–258.
Knights, B., 1983. Food particle-size preferences and feeding–behavior in warmwater aquaculture of European eel, *Anguilla anguilla* (L). Aquaculture 30 (1–4), 173–190.
Krishna, C., Van Loosdrecht, M.C.M., 1999. Effect of temperature on storage polymers and settleability of activated sludge. Water Res. 33 (10), 2374–2382.
Landman, 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 (5), 1061–1067.
Lazazzera, B.A., 2000. Quorum sensing and starvation: signals for entry into stationary phase. Curr. Opin. Microbiol. 3 (2), 177–182.
Li, D.H., Ganczarczyk, J., 1988. Flow through activated-sludge flocs. Water Res. 22 (6), 789–792.
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 (3), 237–273.
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 (1), 247–255.
Logan, B.E., Hunt, J.R., 1988. Bioflocculation as a microbial response to substrate limitations. Biotechnol. Bioeng. 31 (2), 91–101.
Lorenz, M.O., 1905. Methods of measuring the 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 (1), 18–28.
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 (4), 271–284.
Martins, A.M.P., Heijnen, J.J., van Loosdrecht, M.C.M., 2003. Effect of dissolved oxygen concentration on sludge settleability. Appl. Microbiol. Biotechnol. 62 (5–6), 586–593.
Matos, J., Costa, S., Rodrigues, A., Pereira, R., Pinto, I.S., 2006. Experimental integrated aquaculture of fish and red seaweeds in Northern Portugal. Aquaculture 252 (1), 31–42.
Matz, C., Jurgens, K., 2005. High motility reduces grazing mortality of planktonic bacteria. Appl. Environ. Microbiol. 71 (2), 921–929.
Matz, C., Kjelleberg, S., 2005. Off the hook — how bacteria survive protozoan grazing. Trends Microbiol. 13 (7), 302–307.
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 (3), 218–226.
Mertens, B., Boon, N., Verstraete, W., 2005. Stereospecific effect of hexachlorocyclohexane on activity and structure of soil methanotrophic communities. Environ. Microbiol. 7 (5), 660–669.
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 (3–4), 449–457.
Miller, M.B., Bassler, B.L., 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55, 165–199.
Mitchell, J.G., Kogure, K., 2006. Bacterial motility: links to the environment and a driving force for microbial physics. FEMS Microbiol. Ecol. 55 (1), 3–16.
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 (11), 924–933.
Muyzer, G., Dewaal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Appl. Environ. Microbiol. 59 (3), 695–700.
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 (6790), 1017–1024.
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 (10), 139–144.
Parker, D.S., Asce, A.M., Kaufman, W.J., Jenkins, D., 1972. Floc breakup in turbulent flocculation processes. J. Sanit. Eng. Div.-ASCE 98 (NSA1), 79.
Perez, Y.G., Leite, S.G.F., Coelho, M.A.Z., 2006. Activated sludge morphology characterization through an image analysis procedure. Braz. J. Chem. Eng. 23, 319–330.
Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture 226 (1–4), 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 (1–4), 491–505.
Portz, D.E., Woodley, C.M., Cech, J.J., 2006. Stress-associated impacts of short-term holding on fishes. Rev. Fish Biol. Fish. 16 (2), 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 (6), 1017–1025.
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 (1–4), 179–195.
Salehizadeh, H., Shojaosadati, S.A., 2001. Extracellular biopolymeric flocculants — recent trends and biotechnological importance. Biotechnol. Adv. 19 (5), 371–385.
Salehizadeh, H., Van Loosdrecht, M.C.M., 2004. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. Biotechnol. Adv. 22 (3), 261–279.
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 (10), 1297–1306.
Schneider, O., Sereti, V., Eding, E.H., Verreth, J.A.J., 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. Aquac. Eng. 32 (3–4), 379–401.
Schulz, H.N., Jorgensen, B.B., 2001. Big bacteria. Annu. Rev. Microbiol. 55 (1), 105–137.
Sears, K., Alleman, J.E., Barnard, J.L., Oleszkiewicz, J.A., 2006. Density and activity characterization of activated sludge flocs. J. Environ. Eng.-ASCE 132 (10), 1235–1242.
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 (5), 1264–1277.
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 (1–2), 99–120.
Sobeck, D.C., Higgins, M.J., 2002. Examination of three theories for mechanisms of cation-induced bioflocculation. Water Res. 36 (3), 527–538.
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 (5), 1049–1056.
Spoering, A.L., Gilmore, M.S., 2006. Quorum sensing and DNA release in bacterial biofilms. Curr. Opin. Microbiol. 9 (2), 133–137.
Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101 (2), 87–96.
Stanley, N.R., Lazazzera, B.A., 2004. Environmental signals and regulatory pathways that influence biofilm formation. Mol. Microbiol. 52 (4), 917–924.
Steiner, A.E., McLaren, D.A., Forster, C.F., 1976. Nature of activated-sludge flocs. Water Res. 10 (1), 25–30.
Subasinghe, R.P., 2005. Epidemiological approach to aquatic animal health management: opportunities and challenges for developing countries to increase aquatic production through aquaculture. Prev. Vet. Med. 67 (2–3), 117–124.
Tay, J.H., Xu, H.L., Teo, K.C., 2000. Molecular mechanism of granulation. I: H+ translocation–dehydration theory. J. Environ. Eng.-ASCE 126 (5), 403–410.
Teo, K.C., Xu, H.L., Tay, J.H., 2000. Molecular mechanism of granulation. II: proton translocating activity. J. Environ. Eng.-ASCE 126 (5), 411–418.
Torrans, E.L., 2005. Effect of oxygen management on culture performance of channel catfish in earthen ponds. N. Am. J. Aquac. 67 (4), 275–288.
Trainer, M.A., Charles, T.C., 2006. The role of PHB metabolism in the symbiosis of *Rhizobia* with legumes. Appl. Microbiol. Biotechnol. 71 (4), 377–386.
Twarowska, J.G., Westerman, P.W., Losordo, T.M., 1997. Water treatment and waste characterization evaluation of an intensive recirculating fish production system. Aquac. Eng. 16 (3), 133–147.
USEPA, 2000. Wastewater Technology Fact Sheet — Trickling Filter Nitrification. United States Environmental Protection Agency, Washington, D.C. 7 pp.
Valle, A., Bailey, M.J., Whiteley, A.S., Manefield, M., 2004. *N*-acyl-L-homoserine lactones (AHLs) affect microbial community composition and function in activated sludge. Environ. Microbiol. 6 (4), 424–433.
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 (8), 1893–1897.
vanGinneken, V.J.T., vanEersel, R., Balm, P., Nieveen, M., vandenThillart, 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 (4), 795–806.
Verstraete, W., Van Vaerenbergh, E., 1977. BOD: begrip, betekenis en bepaling. Tijdschr. BECEWA 43, 50–62.
Wang, J.K., 2003. Conceptual design of a microalgae-based recirculating oyster and shrimp system. Aquac. Eng. 28 (1–2), 37–46.
Watanabe, T., 2002. Strategies for further development of aquatic feeds. Fish. Sci. 68 (2), 242–252.
Wilen, 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 (2), 391–400.
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 (2), 145–156.
Wilen, B.M., Jin, B., Lant, P., 2003. The influence of key chemical constituents in activated sludge on surface and flocculating properties. Water Res. 37 (9), 2127–2139.
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 (5), 997–1006.
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 (6), 389–394.
Yagci, N., Cokgor, E.U., Artan, N., Randall, C., Orhon, D., 2007. The effect of substrate on the composition of polyhydroxyalkanoates in enhanced biological phosphorus removal. J. Chem. Technol. Biotechnol. 82 (3), 295–303.
Young, K.D., 2006. The selective value of bacterial shape. Microbiol. Mol. Biol. Rev. 70 (3), 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 (9), 3041–3048.