FISH WASTE MANAGEMENT BY CONVERSION INTO HETEROTROPHIC BACTERIA BIOMASS

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Dit onderzoek is uitgevoerd binnen de onderzoekschool
Wageningen Institute of Animal Sciences (WIAS)

FISH WASTE MANAGEMENT BY CONVERSION INTO HETEROTROPHIC BACTERIA BIOMASS

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Proefschrift

Ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van Wageningen Universiteit

Prof. dr. M. J. Kropff

in het openbaar te verdedigen

op woensdag 24 mei 2006

des namiddags te vier uur in de Aula

Schneider, O.
Fish waste management by conversion into heterotrophic bacteria biomass

PhD Thesis, Wageningen University, The Netherlands
With ref.- With summary in English, and Dutch
ISBN: 90-8504-413-8

To my parents

Contents

Chapter 1
Introduction

Wherever animals are kept, waste is produced. Waste is basically the difference between the feed intake and weight gain plus other productions, such as milk. Waste production depends on species, breed, animal size, feed composition, nutrient availability, husbandry system and other factors (Kim et al., 1998; Eding and van Weerd, 1999; Lupatsch et al., 2001; Burton and Turner, 2003, Jongbloed and Kemme, 2005; Kemme et al., 2005). The waste, which is formed by non-retained nutrients, is excreted either as faecal or as non-faecal losses. Faecal losses are basically the non-absorbed nutrients and non-faecal losses the metabolites, which are excreted by the animal. The waste production can be quantified by nutrient balances, which present the fractions of retained and non-retained nutrients. Table 1 gives an example for the Dutch farming industry for nitrogen (N) and phosphorus (P), which are two important nutrients wasted by the animal. Fish, e.g. African catfish, and chicken are more efficient in retaining N than cows and pigs. However African catfish is less effective in P retention than pig or chicken. Other fish species, which are less efficient in N and P retention, such as sea bream, will produce even more non-faecal losses per kg feed (Lupatsch and Kissil, 1998; Eding and van Weerd, 1999). Such comparisons, however, are always limited by the factors mentioned above.

Land animal's faecal and non-faecal losses account for more than 93 Mio. MT N and 21 Mio MT P per year (Sheldrick et al., 2003). Aquaculture waste production can hardly be estimated, because of the high variety of aquaculture systems, such as ponds, flow through systems, cages, and recirculation aquaculture systems (RAS), and of fish species, such as herbivore, omnivore, carnivore, and of the different types of feed used, such as natural production, agriculture by-products, trash fish, high energy pellets, low protein feeds, and animal or human waste. Estimations are, therefore, limited to well observed sectors, such as the production of African catfish in the Netherlands in RAS. The waste production can be projected with 130MT N and 36MT P for 2005 for a production of 3900MT fish and a waste production based on Table 1.

Table 1: Estimation of nutrient retention and waste production in meat production for beef, pork, chicken and African catfish for the Dutch production sector for 2006 based on van Weerd et al., 1999, Eding and van Weerd, 1999, Jongbloed and Kemme, 2005, Kemme et al., 2005, and own data. The fish waste production is estimated for a commercial feed (Biomeerval, Skretting, France). FCR= feed conversion ratio, N= nitrogen, P=phosphorus.

	Weight		Time	FCR	Feed		N balance		P balance	
	Initial	Final			N	P	Retention	Waste	Retention	Waste
	kg	kg	d	kg/kg	g/kg feed	g/kg feed	g/kg feed (%)	g/kg feed (%)	g/kg feed (%)	g/kg feed (%)
Cow	46	625-700	525-588	4.1-4.5	12.5-34.0	2.0-7.0	4.3-4.4 (23)	14.3-14.9 (77)	~1.2 (36-37)	2.0-2.2 (63-64)
Pork	26	114	113	2.57	23.6-27.1	4.6-4.8	9.7 (39)	14.9 (61)	2.1 (45)	2.6 (55)
Chicken	0.04	2.1	42	1.71	30.9-34.6	4.6-6.2	15.9 (50)	15.9 (50)	2.5 (50)	2.5 (50)
African catfish	0.06	0.94	112	0.75	78.4	17.0	32.8 (42)	45.6 (58)	4.7 (28)	12.3 (72)

Animal waste: Hazard or valuable resource

Waste produced by land animals can be divided into two classes: gaseous losses and manure. Gaseous waste typically consists of ammonia, carbon dioxide, hydrogen sulphite, dinitrous oxide, organic compounds, such as methane, and others emissions (Burton and Turner, 2003). These gases are either released directly to the atmosphere or treated by e.g. chemical scrubbing, absorption and biological methods (Melse and Mol, 2004; Sheridan et al., 2002; Rappert and Mueller, 2005). Gaseous wastes contribute to the green house effect. Methane emissions of ruminants and animal waste were estimated with 16-20% of the global emission. CO_2 emission of the total agricultural sector was expected to be 5% and N_2O >50% (Wuebbles and Hayhoe, 2002, Tamminga, 2003).

Manure can be subdivided into two categories: slurry (liquid manure) and solid manure (Table 2). Slurry or liquid manure is a mixture of animal dung, urine, water and liquids drained from the solid manure. Solid manure is typically a mixture of animal excreta and beddings. The composition of the manure is next to animal related factors, highly depending on the husbandry and manure collection systems and the applied bedding (Petersen et al., 1998, Zahn et al., 2001; Burton and Turner, 2003). Similar to gaseous waste these liquid or solid waste fractions can result in environmental damage. Solid or dissolved N, P and potassium emissions lead to environmental pollution and eutrophication (Tamminga, 2003; Ekholm et al., 2005; Oenema et al., 2005).

Table 2: Waste composition in animal production for different land animals (cattle, pigs, poultry after Burton and Turner, 2003) and African catfish (RAS effluent stream, own data) in g/l manure. TAN (total ammonia nitrogen).* ortho-phosphate-phosphorus.

	Slurry or liquid manure	Solid manure	Fish slurry
Dry matter	15-300	140-700	2-7
Total nitrogen	1.2-18.0	2.0-58.0	0.1-0.7
TAN	1.0-7.8	0.3-60.0	0.000-0.005
Phosphate	0.2-15.0	1.0-39	0.006-0.040*

There are similarities between land animal and fish waste productions. Fish produce waste as faecal loss, organic matter (undigested protein, fat, carbohydrates) and ash, and as non-faecal losses ammonia, urea, ortho-phosphate, and carbon dioxide. The waste products are released in the surrounding water body and have to be removed to maintain water quality in acceptable ranges for the fish to survive and to grow optimally. Fish waste is, therefore, more diluted than land animal manure. Even if the highly concentrated effluent stream of a RAS is considered, the waste concentrations are magnitudes lower than for land animals (Table 2). However, fish waste products, such as N, P and carbon dioxide, are hazardous to

the fish, if they are not removed, and a risk to the environment (eutrophication and greenhouse effect), if they are released. There are different options to manage problems of land animal waste production, either to limit waste production by nutritional improvements or to manage the resulting waste. Nutritional improvements might minimize waste production (Hof et al., 1997; Jongbloed and Lenis, 1998). These improvements are limited. Ruminants, for example, emit about 85% of their total methane production due to their maintenance requirements. That means not feed improvements alone, but the reduction of ruminant numbers would lead to emission reductions (Tamminga, 2003). Waste management might minimize waste discharge to the environment, such as manure land application as fertilizer. However, this application is not entirely unproblematic (Figure 1). Furthermore waste production can exceed the local soil carrying capacity. In that case the manure has to be transported within a feasible distance to land with nutrient deficits (Janzen et al.1999; Adhikari et al., 2005).

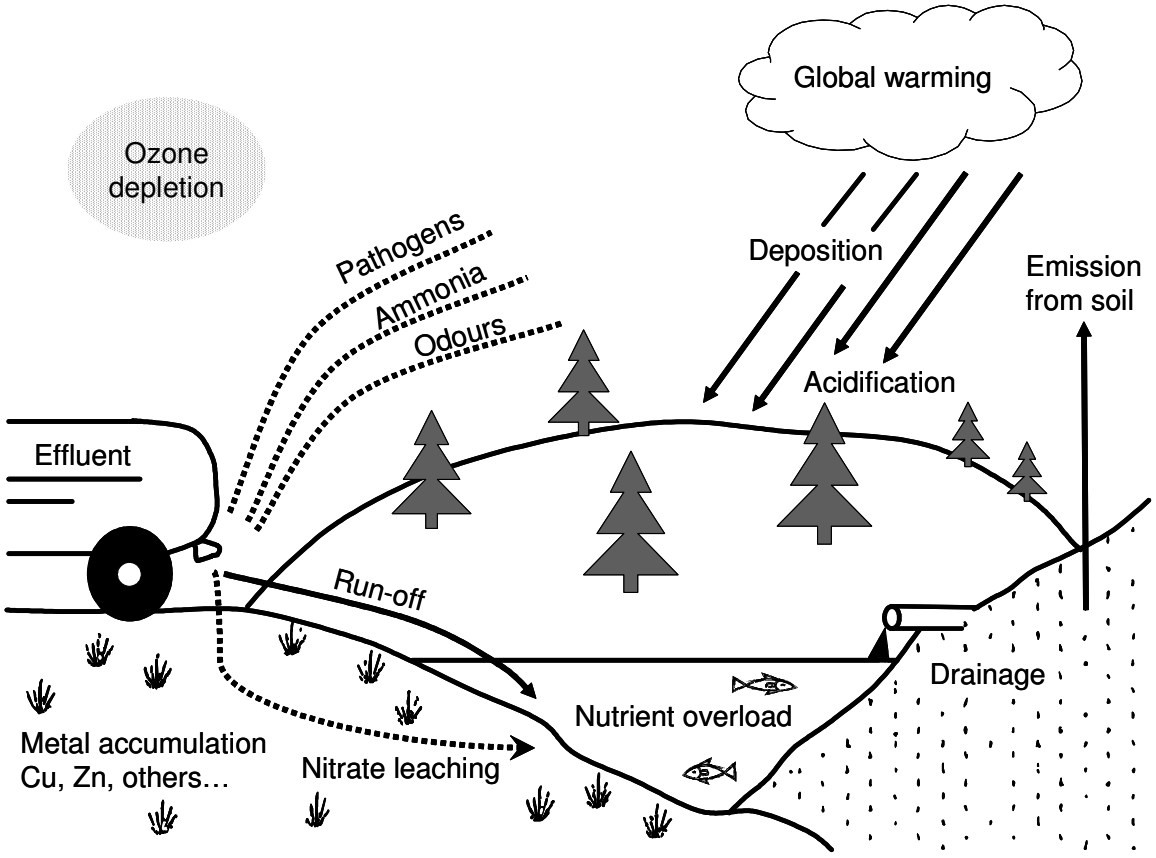

Figure 1: Issues associated with manure land application (after Burton and Turner, 2003).

If manure is managed and efficiently spread, it can meet a significant amount of European fertilizer and organic matter demand. Next to simple spreading on fields, manure can be processed into fertilizer on industrial scale. However, such production seems only economically viable for centralized production units (Burton and Turner, 2003). There are

different possibilities to treat or manage manure: mixing (to obtain a homogenized material, to allow for easy spreading), separation of coarse solids and liquids (to allow for a better fermentation of the solid fraction, and an easier spreading and penetrating of the liquid fraction during field applications), aerobic treatment (to reduce in ammonia, pathogens and odors) or anaerobic treatment (to produce e.g. biogas) and composting (to condition soil). Applying these methods changes manure treatment to management: from discharge/destruction or basic applications to re-cycle and re-use.

In aquaculture, similar problems exist, nowadays, as in land animal manure management. In the past, aquaculture waste production was not an issue. In integrated pond culture, fish are even the final sink for waste of land animals or humans. In such systems, a complex food web (algae, bacteria and others) is converting the fish and waste from the outside in fish feed (Li, 1986; Edwards, 1993; Kestemont, 1995). This loop is comparable to waste application on land as fertilizer and re-using the resulting plants as feed source. However, aquaculture production has changed dramatically. It has developed from a production at low trophic levels (1-3; 2=herbivore) towards high trophic levels (3-5; 5=highly carnivore, based on Froese and Pauly, 2005 and Shatz, 2005, Figure 2, Figure 3). These species are often produced in monoculture, and their systems are not self cleaning in contrast to integrated systems. Fish are, therefore, exposed to accumulating waste inside the system. This requires adequate treatment and management methods.

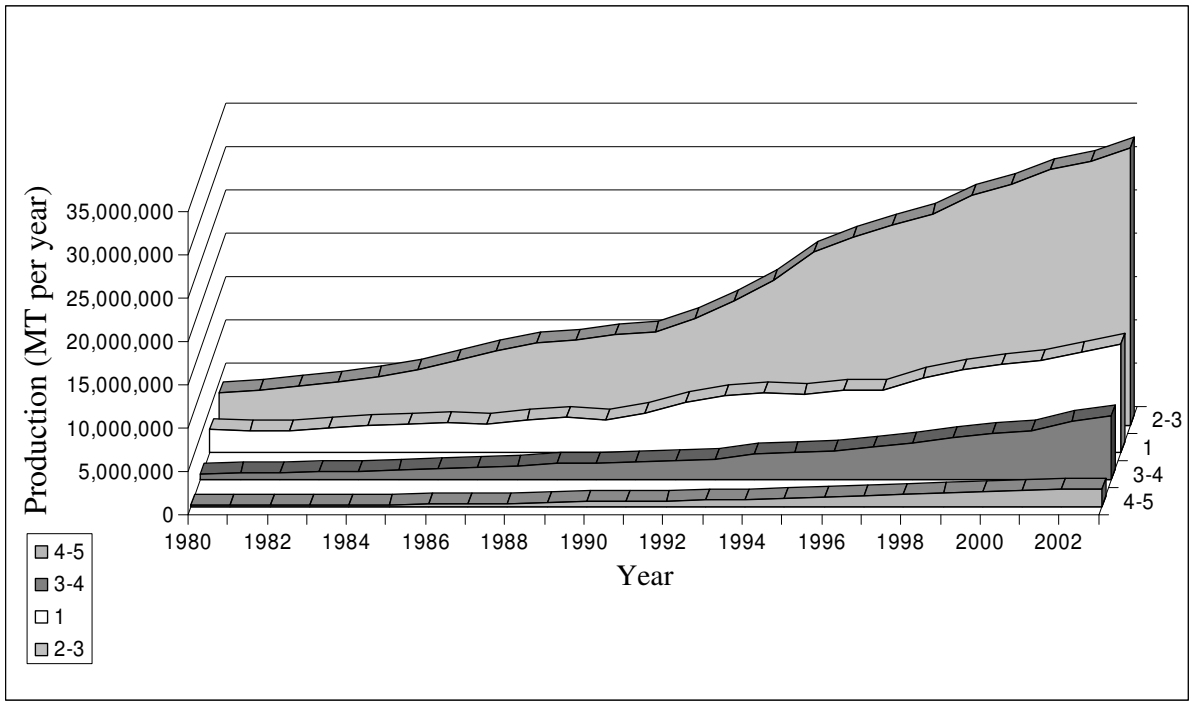

Figure 2: Development of fish production, separating fish species by trophic levels. Plants have a trophic level = 1, herbivores = 2,..,5 = purely carnivores fish (based on Froese and Pauly, 2005 and Shatz, 2005).

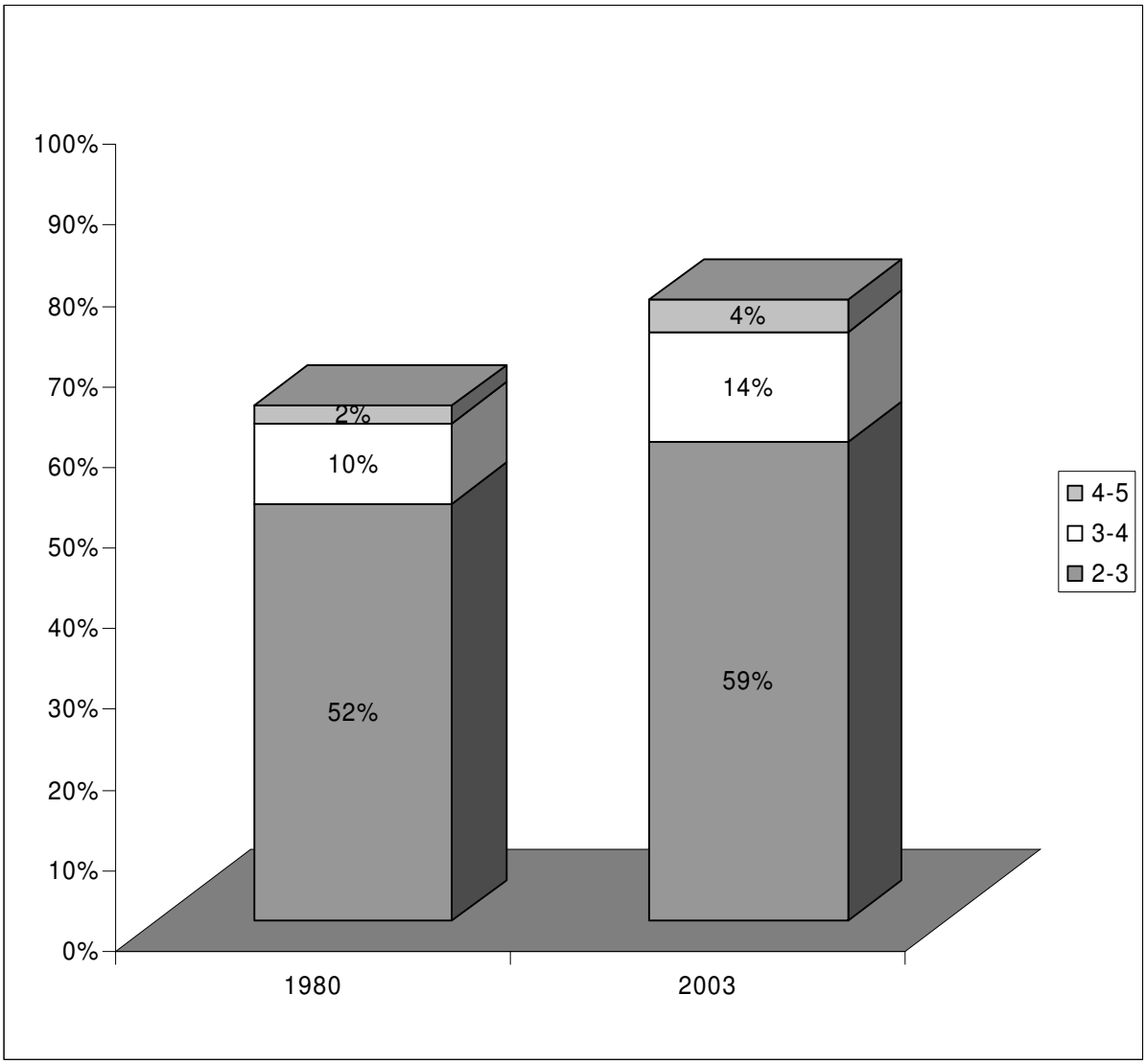

Figure 3: Comparison of fish production as relative part of total production (fish+plants) for 1980 (4.7Mio MT) and 2003 (42.3Mio MT), separating fish species by trophic levels. (herbivores = 2,..,5 = purely carnivores fish (based on Froese and Pauly, 2005 and Shatz, 2005).

In aquaculture similar attempts to land animal farming have been made to minimize and manage waste production. Improvements in feed digestibility, extending knowledge in fish physiology and bioenergetics, resulted in diets that were less polluting (Tacon, 1990; Sugiura et al., 1998; Bureau and Cho. 1999). Furthermore possibilities to improve solid waste characteristics were investigated by changing fish diets. Such improvements should result in easier removal of solid waste from the systems (Amirkolaie, 2005). The mantra of aquaculture waste treatment has been for a long time “the solution to pollution is dilution”. Such treatment is still practiced for the majority of cage farms and flow through systems. RAS have been able to lower water use and to concentrate solid and dissolved waste in one effluent stream (Table 3). However, RAS treatment units are only purifying the rearing water by solid removal and nitrification and are often not managing their waste (Figure 4).

Table 3: Water use, waste discharge, productivity and treatment approach of three different aquaculture production systems (after Verdegem et al., 1999; Schneider and Eding, 2001; Eding and Kamstra, 2002; Edwards, 2004, Verdegem et al. 2006).

System type	Water use (l/kg fish)	Waste Discharge (gCOD/kg fish)	Productivity (MT/ha/year)	Treatment approach
Pond	2000	286	10-15	Ecological
Flow-Through system	14500-210000	780	variable	None
RAS	100-900	150	300-2500	Technical

The main RAS developments were focusing on the conversion of ammonia in less hazardous nitrate by nitrification, and on destructive techniques, such as denitrification and solid capture (Bovendeur et al., 1987; Chen et al., 1997; van Rijn et al., in press). In such systems, solids and nitrogenous and phosphorus waste leaves the system in a slurry and carbon dioxide is stripped to the air and dissolved N is eventually converted into gaseous nitrogen. Due to the water purification, the waste is not an issue inside the production system. It first gets problematic at the system's border line as effluent stream to the outside environment.

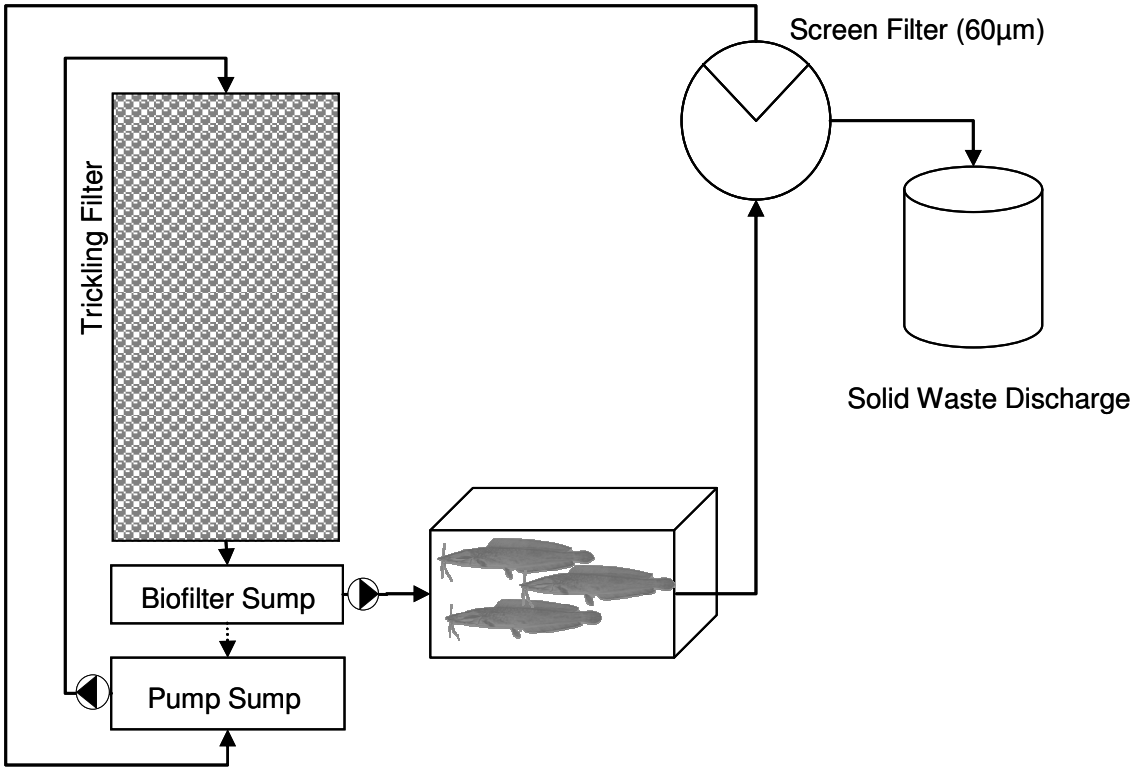

Figure 4: Simplified systematic overview of an African catfish RAS. Arrows are indicating water flows (modified after Bovendeur et al., 1987).

Normally RAS' effluent stream has been either directly discharged to the environment, digested in lagoons or septic tanks, thickened and/or applied as fertilizer for

land based agriculture (Chen et al., 1997; Losordo et al., 2003). These methods were eventually combined with flocculation to minimize the waste volume (Kamstra et al., 2001; Ebeling et al., 2003; Ebeling et al., 2005). Alternative re-use of the obtained solids, N and P, in horticulture, for algae, or for biogas production were recently under investigation (Rakocy, 1998; Brune et al., 2003; Gebauer, 2004; Neori et al., 2004). These waste management methods are comparable to land animal waste management. They all take place outside the husbandry system and use partly the same methodology. However, alternatively waste cannot only be treated but as well be managed and re-used inside the husbandry system. One method is the waste conversion into bacteria biomass. This biomass can be reutilized as aquatic feed source. Such processes are already applied in aquaculture, e.g. in integrated and activated ponds, but not in RAS. In such ponds, waste conversion does not only improve pond water quality but as well feed conversion ratios, because the produced bacteria biomass and other phototrophic and heterotrophic proto- and metazoans contribute as food (Avnimelech et al., 1989; Edwards, 1993; Burford et al., 2003; Hari et al., 2004). In RAS suspended bacteria growth processes have been applied as activated sludge treatments for water purification only (Knoesche and Tscheu, 1974; Meske, 1976). This system was not successful, since it affected the overall RAS performance and was subsequently abandoned by the RAS industry. Yet, the concept may still be valid if the overall RAS performance is not disturbed. If the high productivity of a RAS, its low land and water use would be combined with waste conversion in bacteria biomass and re-use as feed, then a system with potentially high sustainability emerges. It is, therefore, needed to investigate bacteria production potential using RAS' effluents as substrate. This would result in true waste management, under the condition that the effluent stream is not only converted but the obtained bacteria biomass is re-usable as fish feed. This would create a loop from the feed to the fish over waste and bacteria conversion back to feed inside the culture system.

Study objectives

The integration of such an alternative method to treat, manage and re-use fish waste inside the culture system can be studied in five consequent steps (Figure 5). Based on this procedure the study objectives were derived.

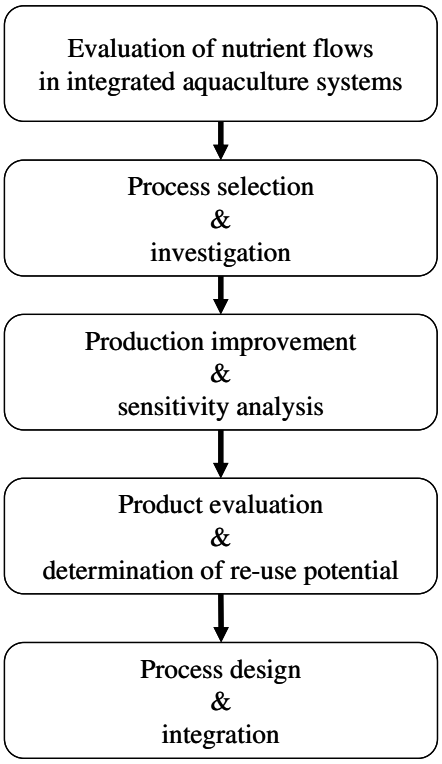

Figure 5: Five consequent steps to investigate the potential of an alternative waste management process in recirculation aquaculture systems.

Several fish waste treatment and management processes in integrated aquaculture systems have been investigated and reported in literature. However, there has been no inventory of these processes and no evaluation of their contribution to increased nutrient retention in intensive aquaculture systems. As first objective, it was, therefore, necessary to review the existing work. From such a study bottlenecks have to be identified that limit the integration of waste management and re-use inside the aquaculture system. Furthermore, only after such an evaluation the potential of the bacteria conversion process can be compared to processes reported in literature. The second objective was to investigate the potential and bottlenecks for fish waste conversion and bacteria production, to assess bacteria growth kinetics and nutrient conversions under different conditions. After investigating the conversion process as such, the third objective was to study the sensitivity of the process for different conditions (such as carbon and nitrogen sources, carbon supplementation levels, hydraulic retention). The aim was to improve bacteria conversion and production by manipulating the bacteria substrate. The fourth objective was to evaluate the conversion product. The bacteria community was expected to change under different culture conditions. It was, therefore, necessary to observe bacteria community changes if production parameters were manipulated and to assess the potential pathogenic risk for a re-use as aquatic feed. Next to the analysis of the bacteria community, the attraction of the bacteria biomass as aquatic

feed had to be studied. If the produced biomass would have been not attractive as feed, its re-use might be limited. The last objective focused on the integration of the experimental data from the first four objectives to determine critical process variables and the design for a bacteria reactor integrated in a fish farm.

Thesis Outline

General study aim was to investigate the potential of heterotrophic bacteria production integrated in a RAS to convert fish waste into bacteria biomass. This goal included that the bacteria biomass should be re-used as aquatic feed. The study outcome should deliver knowledge on waste conversion and management in intensive aquaculture systems in general and specifically on the heterotrophic bacteria production, on nutrient conversion rates, on the sensitivity of the process for various conditions, on the resulting bacteria community, on the attractance of the bacteria as aquatic feed and on the reactor design characteristics. In chapter 2, several processes were inventoried and evaluated, which can be applied for waste management inside intensive aquaculture systems. These processes convert waste released by the fish into harvestable or directly re-used biomass. Nutrient conversions and system nutrient retention were compared and the limitations of the different conversions discussed. This discussion served as starting point for chapter 3. There, as selected conversion process heterotrophic bacteria production was investigated for different carbon supplementation levels and hydraulic retention times utilizing the drum filter effluent of a RAS as bacteria substrate. To improve the obtained production rates and yields, the influence of ammonia and nitrate as nitrogenous substrate on heterotrophic bacteria production were tested in chapter 4. To investigate the effect of different carbon sources on bacteria production rates, in chapter 5, experiments were reported using different molasses supplementation levels instead of the model substance sodium acetate (chapter 3 and 4). In chapter 6 the re-use potential of the produced bacteria biomass was investigated. The bacteria community obtained with the reactor broth for various conditions and substrates was analyzed. In chapter 7 the re-use potential was furthermore investigated by feeding the biomass to shrimps in a feed preference test. In chapter 8, the reactor design for a 100MT African catfish farm and the related bacteria kinetics were determined, based on the integration of experimental data obtained in earlier studies.

Chapter 2
Analysis of nutrient flows in integrated intensive aquaculture systems

Abstract

This paper analyses nutrient conversions, which are taking place in integrated intensive aquaculture systems. In these systems fish is cultured next to other organisms, which are converting otherwise discharged nutrients into valuable products. These conversions are analyzed based on nitrogen and phosphorous balances using a mass balance approach. The analytical concept of this review comprises a hypothetical system design with five modules: (1) the conversion of feed nutrients into fish biomass, the "Fish-Biomass-Converter"; (2) the separation of solid and dissolved fish waste/ nutrients; the "Fish-Waste-Processor"; (3) the conversion of dissolved fish waste/nutrients, the "Phototrophic-Herbivore-Converter"; (4 and 5) the conversion of solid fish waste, the "Bacterial-Waste-Converter", or the "Detrivorous–Converter". In the reviewed examples, fish culture alone retains 20-50% feed N and 15-65% feed P. The combination of fish culture with phototrophic conversion increases nutrient retention of feed nitrogen (N) by 15-50% and feed phosphorus (P) by up to 53%. If in addition herbivore consumption is included, nutrient retention decreases by 60-85% feed N and 50-90% feed P. This is according to the general observation of nutrient losses from one trophic level to the next. The conversion of nutrients into bacteria and detrivorous worm biomass contributes only in smaller margins (e.g. 7% feed N and 6% feed P and 0.06% feed N $0.03x10^{-3}$% feed P, respectively). All integrated modules have their specific limitations, which are related to uptake kinetics, nutrient preference, unwanted conversion processes and abiotic factors.

*Schneider, O., V. Sereti, E. H. Eding, J. A. J. Verreth (2004). "Analysis of nutrient flows in integrated intensive aquaculture systems." Aquacultural Engineering **32**(3/4): 379-401.*

Introduction

Future development of the aquaculture industry is limited by resources, such as water, land, fishmeal, and by other factors, such as environmental pollution (IWMI, 2000; Naylor et al., 2000; Westers, 2000). Nitrogen (N) and phosphorus (P) are the two main pollutants of intensive aquaculture (Hakanson et al., 1998; Lemarie et al., 1998). In contrast to cage, pen and raceway systems both recirculation aquaculture systems (RAS) and integrated pond systems allow to recycle parts of the non-retained nutrients. In RAS, these nutrients are partly liberated from their organic matrix and either immobilized in bacterial biomass or volatized. In integrated systems, nutrients are converted into harvestable products. These two systems result in reduced waste discharge and resources use. RAS are mainly applied in the Western hemisphere. They reuse water and are less competitive for land and water (Losordo, 1998). Compared to an integrated pond system, RAS show relatively low retention of nutrients within its production (Verdegem et al., 1999). Integrated pond systems are applied traditionally in Asia. Their higher nutrient retention is a result of nutrient re-use by primary and secondary producers (Liu and Cai, 1998). In an integrated pond system, however, fish production is only 10-15 MT/ha (Edwards, 2004) compared to a RAS with a recalculated production of 300-2500 MT/ha for turbot, eel, or African catfish (Eding and Kamstra, 2002). Integrated pond systems and RAS comprise several nutrient conversion processes. In an integrated pond system, waste serves as nutrient for phototrophic and detrivorous/heterotrophic conversion into plants, bacteria, and invertebrates, on which different fish are feeding (Li, 1986; Riise and Roose, 1997; Liu and Cai, 1998). In a RAS, waste coming from the fish is processed into a solid and dissolved waste stream. These waste streams are either directly discharged (solid waste flow), or converted into less harmful products and volatilized by bacterial conversion (dissolved waste flow). The purified water is subsequently recirculated (Bovendeur et al., 1987; Eding and van Weerd, 1999).

When the conversion processes of both systems are combined, a new intensive and integrated production system emerges. From a theoretical point of view such intensive integrated systems can be conceptualized as consisting of five different modules: (1) the conversion of feed nutrient into fish biomass, the “Fish-Biomass-Converter”; (2) the separation of solid and dissolved fish waste/nutrients; the “Fish-Waste-Processor”; (3) the conversion of dissolved fish waste/nutrients, the “Phototrophic-Herbivore-Converter”; (4) & (5) the conversion of solid fish waste, the “Bacterial-Waste-Converter”, or the “Detrivorous–Converter” (Figure 6). This paper reviews nutrient conversions taking place in such integrated

modules for their N and P balances. It discusses furthermore constraints of intensive integrated systems, based on the mentioned conceptual framework.

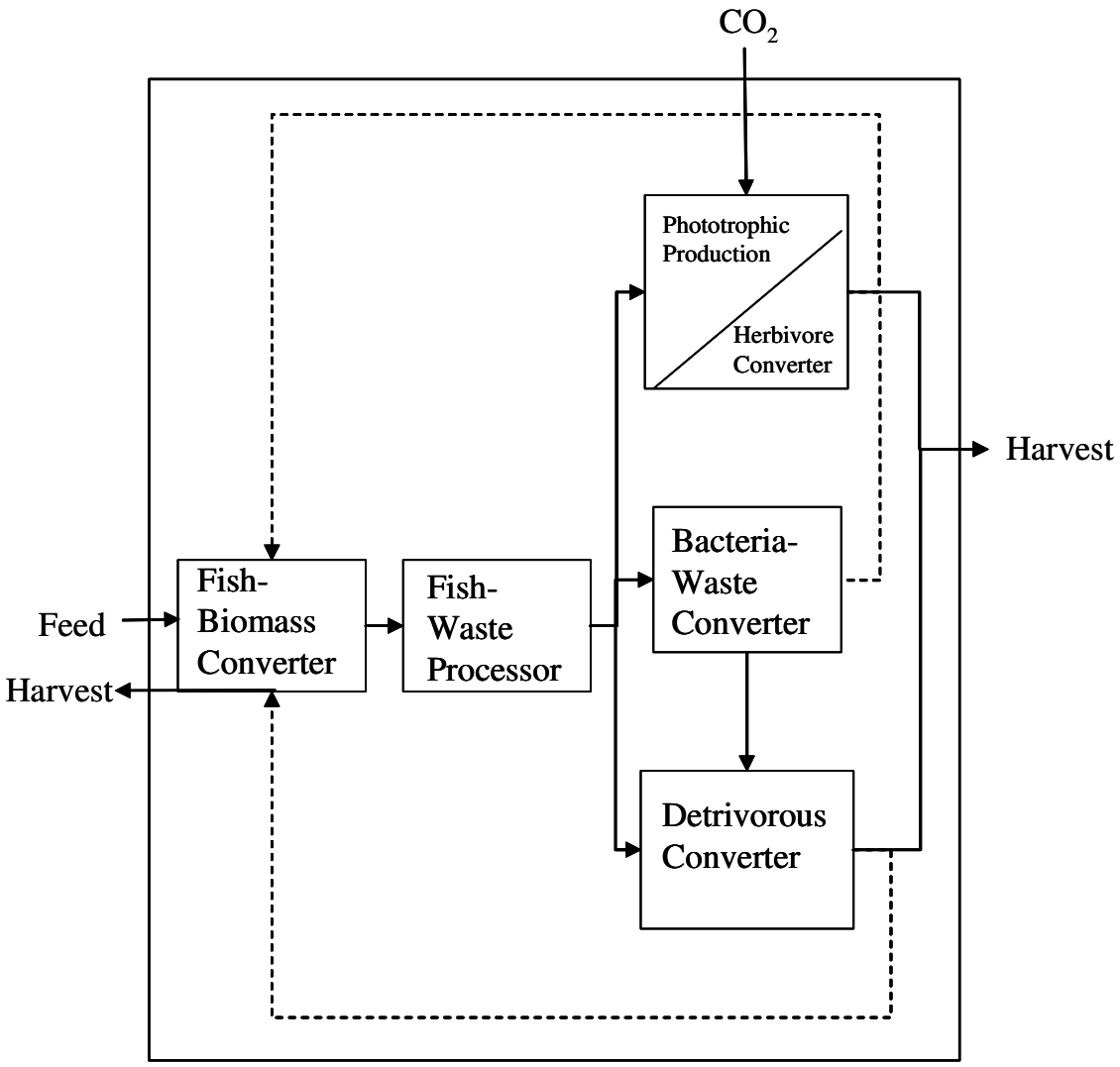

Figure 6: Simplified structure of the system concept illustrating macronutrient flows (N and P), and identifying the five modules of an integrated intensive aquaculture system. Solid lines are indicating nutrient flows in existing integrated intensive systems, dotted lines are representing potential nutrient flows in future recirculation system designs. Blocks represent processes in separate culture modules.

Methodology

Existing examples of integrated intensive farming processes were analyzed in relation to the introduced concept, which comprises five modules: Fish-Biomass-Converter, Fish-Waste-Processor; Phototrophic-Herbivore-Converter, Bacterial-Waste-Converter, and Detrivorous–Converter (Figure 6). The process division of an integrated intensive farming system over these five modules allows examining each conversion process separately for its characteristics. Furthermore, if nutrient flows are subsequently connected from one of these modules to another one, overall system nutrient retention and balances can be estimated.

Sometimes reviewed systems focus on one conversion process only. In those situations, literature information was used to calculate the modules and to extend the system. Because the Fish-Biomass-Converter has a central role in all conversion processes, it served as starting point of the nutrient flow analysis. The related nutrient flows are then followed through the system (Figure 6). The N and P flows and their retentions are calculated using mass balances, based on the concept: output = input – retention. This retention can be expressed as g / kg feed (wet weight) or as fraction of the total nutrient given with the feed to the fish (% feed nutrient). The nutrient discharges (output) from the converters/modules serve as input in the subsequent module.

The retention of N and P is estimated based on proximate composition of the cultured organism, feed conversion ratios (FCR), and production rates. In the case of the P balance, feed phosphorous content had to be estimated based on commercial feeds, because feed phosphorus contents were not given for the reviewed system examples.

Results

Fish-Biomass-Converter

In the Fish-Biomass-Converter, fish transforms feed into fish biomass and in dissolved and un-dissolved waste. N and P retention in fish biomass varies, and is highly dependent on fish species, feeding level, feed composition, fish size, and temperature. Table 4 presents different examples of fish converting conventional diets into fish biomass. Nutrient retention varies between 20-50% feed N and 15-65% feed P. The amount and composition of the produced waste reflect these differences (Table 4). Non-faecal loss is approximately 30-65% feed N and up to 40% for feed P, and faecal loss is 10-30% feed N and 30-65% feed P.

Fish-Waste-Processor

Table 5 provides an overview of different Fish-Waste-Processors that are applied in aquaculture systems. Nutrient degradation and leaching should be reduced as much as possible by this separation process. Nutrient degradation or the destruction of bigger waste particles in the Fish-Waste-Processor will lead to a loss of nutrients for other conversions. Micro-screen filtration, e.g. drum filtration, and eventually swirl separation serve best to separate the nutrient flows in solid and dissolved fractions. If, for example, a drum filter is applied as Fish-Waste-Processor, the total suspended solid concentration influences the efficiency of the filtration process (Summerfelt, 2001). If an average efficiency of 65% is assumed, roughly two third of the solid waste coming from the fish tanks is captured within the solid waste flow.

Table 4: Nitrogen (N) and Phosphorus (P) retention in fish biomass for different fish species fed conventional diets. (Weight in g; FCR=kg feed/kg gain; Feed content, retention, excretion, and faecal loss g/kg feed).

Species	Initial - Final Weight	Feeding Level	FCR	N Feed	N Retention	N Excretion	N Faecal Loss	P Feed	P Retention	P Excretion	P Faecal Loss	Source
Trout	24-55	Ad. Lib.	1.13	67	23	34[a]	10[a]	13	4	4[b]	5[b]	Kim et al., 1998
Trout	156-238	Ad. Lib.	1.25	67	21	36[a]	10[a]	13	2	5[b]	6[b]	Kim et al., 1998
Sea Bream	1-400	Ad. Lib.	1.79	74	16	45	13	14	4	3	7	Lupatsch and Kissil, 1998
Sea Bream	18-50	Ad. Lib.	1.24	89	26	48[c]	15[c]	17	---	---	---	Company et al., 1999
Sea Bream	40-470	2.9% $kg^{0.8}$BW per day[d]	2.00	72	14	35[d]	23[d]	---	---	---	---	Neori et al., 2000
Sea Bream	50-300	1-4%/kg BW per day	3.00	64	17	41[e]	6[e]	---	---	---	---	Shpigel et al., 1993
African Catfish	47-144	1.27%/$kg^{0.8}$BW per day	0.71	69	35	24	10	16	6	0	10	van Weerd et al., 1999
African Catfish	15-899	2.5%BW	0.80	78	39	27	12	---	---	---	---	Eding and van Weerd, 1999
Tilapia	23-49	2%BW	1.12	72	32	26[f]	14[f]	---	---	---	---	Alasgah and Ali, 1994
Tilapia	54-128	1.24%/$kg^{0.8}$BW per day	1.12	44	21	14	9	12	8	0	4	Own observation

BW=Body weight in g; Ad. Lib.= ad libitum, FCR=Feed Conversion Ratio; [a] Based on an estimated digestibility of 85% for N. [b] Concluded after Cripps (1995), Coloso et al. (2003), that particulate P losses from trout hatcheries are accounting for 30-50% of soluble P. [c] Estimated after Lupatsch and Kissil (1998). [d] Estimated after Neori et al., 2000. [e] Estimated after Shpigel et al., 1993. [f] Estimated after Verdegem et al. (2000) and own data

The solid waste flow is directed to the Bacteria- or to the Detrivorous-Waste-Converter, while the remaining waste, comprising one-third of the solid waste and the dissolved waste, is directed towards the Phototrophic-Herbivore-Converter. If N and P are homogenously distributed in the solid waste particles (Kamstra, 2001), 3-10% of feed N and 10-20% of feed P (based on Table 4) will be additionally directed towards the Phototrophic-Converter. The total waste flow towards the Phototrophic- and towards the Bacteria- or Detrivorous-Converter is then approximately 40-70% feed N and 10-55% feed P, and 5-25% feed N and 25-45% feed P, respectively.

Table 5: Typical techniques applied in aquaculture systems for suspended solid (SS) removal, summarized and modified after Chen et al. (1997), Summerfelt (2001) and Timmons et al. (2001).

Technique	Solid Size Removed	SS removed	Advantages for integrated systems	Disadvantage for integrated systems
	µm	%		
Sedimentation	>100	40-60		Nutrient leaching and digestion Long sludge retention time No removal of fine particles
Rotary Mirco-Screen (e.g. drum filter)	>60	22-80	Short sludge retention time No nutrient loss by bacteria activity	Removal efficiency is depending on total solid load
Swirl Separation (e.g. hydroclone)	>50	<87 for > 77µm	Short sludge retention time	Poor removal of fine particles Potential nutrient loss by bacteria activity if sludge is not constantly removed
Granular Media	>30	20-95	High removal efficiency	Nutrient loss by bacteria activity Cacking Nutrient leaching
Porous Media	<1	>90	High removal efficiency	Clogging Nutrient leaching
Foam Fractionation	<30	<50 turbidity removal		Easily affected by chemical water and solid properties Low overall removal efficiency

Phototrophic-Herbivore-Converter

The Phototrophic-Herbivore-Converter comprises two sub modules: a phototrophic part, containing photosynthetic organisms, and an herbivore part, containing herbivorous organisms.

Phototrophic-Converter

Phototrophic conversion can be distinguished by its focus on macroalgae, microalgae, and macrophytes.

Macroalgae

Macroalgae culture has been integrated in intensive land-based aquaculture systems combining fish-macroalgae (Cohen and Neori, 1991; Neori et al., 1991), fish-bivalve-macroalgae (Shpigel and Neori, 1996), fish-macroalgae-shellfish (Neori et al., 2000, Schuenhoff et al., 2003), and fish-microalgae-bivalves-macroalgae (Shpigel et al., 1993). An extensive review on general aspects of seaweed biofiltration in mariculture is given elsewhere (Neori et al., 2004). An integrated system with *Ulva* is able to retain between 20 and 30% feed N (Shpigel et al., 1993; Neori et al., 2000) and potentially 1-7% feed P (Ventura et al., 1994), if a feed phosphorous content of 0.9% (DAN-EX 2446, Danafeed, Denmark) is assumed.

Microalgae

Aquaculture systems, such as the "partitioned aquaculture system" (PAS), integrate microalgae culture, using high-rate algae pond culture techniques, and aquaculture production (Brune et al., 2003). This system comprises catfish, tilapia, *Scenedesmus* and other green algae. According to Brune et al. (2003) algae production in this system was 3.7g/m² per day (1.9gC/m²/d) with a N retention of 38% feed N. The related P retention is about 30% feed P, if the P feed content is estimated to be 0.9-1% (Cho and Lovell, 2002). Other existing systems integrate shrimp, algae, and oyster production (Wang, 2003). In the latter study 1kg of shrimp feed produces 0.8kg of dry weight algae, retaining 50% of feed N and 53% feed P for an estimated feed phosphorous content of 1.8% (*L. vannamei* grow-out feed, VDS, Belgium) and algae P content of 1.2% (Brune et al., 2003).

Macrophytes

Macrophytes, such as willow, hyacinth, or duckweed have been used in wastewater treatment (Culley and Epps, 1973; Oron, 1994; Smith and Moelyowati, 2001). For the integration with fish culture, however, water hyacinths are less favorable than duckweed due to their intolerance to low temperatures and difficulties in harvesting and processing (Oron, 1994). Furthermore, duckweed can have a high protein content of up to 50%, a nutritionally

valuable amino acid pattern (Mbagwu and Adeniji, 1988), and a high digestibility of about 60% dry matter (Castanares, 1990; El-Shafai, 2004). Own observations showed an increase in nitrogen retention from 42% for tilapia alone to 57% feed N in a tilapia recirculation system where the trickling filter was replaced by a duckweed reactor and the duckweed was harvested. This equals an additional retention in duckweed of about 17% feed P (Table 6), based on a P feed content of 1.56% (TI 2 Tilapia Start Pellets, Trouvit, The Netherlands).

Table 6: Range of production, nitrogen (N) and phosphorus (P) for selected macro-, and microalgae, and macrophyta, which can be cultured on wastewater. (dm=dry matter).

Group	Species	Production	N content	P content	Reference
		g dm/m²/day	g/kg dm	g/kg dm	
Macroalgae	*Ulva*	40-52	33-46	1	Ventura et al., 1994 del Rio et al., 1996 Neori et al., 2000
	Falkenbergia rufolanosa	60	---	---	Luening et al., 2002
Microalgae	*Chlorophycea*	5-22	70-90	---	Cromar and Fallowfield, 1997
	Scenedesmus & Chlorella	25	67	---	Chowdhury et al., 1995 Hammouda et al., 1995
	Chlorophycea	3.7	88	12	Brune et al., 2003
	Chaetoceros	---	32-91	---	McCausland et al., 1999 Renaud et al., 2002 Wang, 2003
Macrophyta	*Lemna*	3-35	22-80	5-11	Alaerts et al., 1996; van der Steen et al., 1998 Casal et al., 2000

Herbivore-Converter

Depending on the available plant species either abalone, crustaceans, oysters or finfish might be cultured as herbivorous organisms (Table 7).

Macroalgae abalone

Abalones grazes on *Ulva* with a feed conversion ratio of 5-25kg wet weight *Ulva*/kg wet bodyweight gain (Shpigel and Neori, 1996; Neori et al., 2000). Production are as high as 31kg/m² per year (Neori et al., 2000). Abalone contains 16 gN/kg wet weight and 0.47-0.84gP/kg wet weight including shell (Mai et al., 1995; Neori et al., 1998; Neori et al., 2000; Tan et al., 2001). For an average feed conversion ratio of 15kg wet weight *Ulva*/kg wet bodyweight gain, the N and P retention in abalone are 7-13% feed N and 2-3% feed P, respectively, depending on N content in *Ulva* and realized feed conversion ratio (Table 7).

Micro algae-fish/oyster/shrimps

Micro algae represent either a valuable product by themselves (Stromme et al., 2002), or they can be fed to fish, oysters or shrimp (Shpigel et al., 1993; Brune et al., 2003; Wang, 2003). In the PAS system, algae and heterotrophic production, expressed as volatile solids (VS), were converted into tilapia biomass with a conversion factor of 2.2kgVS/kg fish, which comprise 60% algae and 40% bacteria biomass (Brune et al., 2003). If bacteria and algae N content is 12 and 8.7%, respectively, and their phosphorus content is 2% and 1.2%, respectively (Brune et al., 2003; Tchobanoglous et al., 2003), and tilapia N content is 2.6% (van Dam and Penning de Vries, 1995; own data) and P content is 0.6% (Rectenwald and Drenner, 2000), then N retention in fish is 9% feed N and P retention is 10% feed P. Also oyster production can be integrated with microalgae. For a FCR of 2kg algae dry weight/kg fresh weight oyster meat, 16% meat content, and 2.3gN/kg oyster and 0.5gP/kg oyster (Anthony et al., 1983; Wang, 2003), 7% feed N and 7% feed P are retained in the oysters. Algae N content was estimated here as 5% (Wang, 2003) and P content as 1.2% (Brune et al., 2003). In a similar approach using a chain of fish-microalgae/heterotrophic production-bivalves, 15% feed N and 22% feed P, assuming a similar oyster composition for N and P as in the previous example, would be retained in the bivalves (Shpigel et al., 1993).

Macrophytes-fish

Duckweed can be fed as sole feed or as supplemental feed ingredient to finfish, such as tilapia. Quantitative information on optimal feeding rates and feed conversion ratios are scarce. Gaigher et al. (1984), Hassan and Edwards (1992) and El-Shafai (2004) reported FCRs of 1-2.3 for feeding trials with tilapia. For 1kg of dry duckweed (30% protein) fed to tilapia with an FCR of 2.3, a nutrient retention of 3.5% feed N and 4% feed P (Table 6; Table 7) can be obtained.

Table 7: Comparison of different integrated aquaculture systems for intensive production and waste/nutrient conversion into a harvestable product.
[a] Estimated based on calculations made by Knoesche and Tscheu (1974). [b] Estimated based on a protein body content of 175g/kg fish and an FCR=2. [c] This product will not be harvested and directly re-used within the system, therefore, it is not included in the total harvest. [d] Fish protein content is estimated with 16% wet weight based on van Dam (1995) and own data (unpublished). [e] using an FCR of 5 (Neori et al., 2000).

System	Species	Feed	Harvestable Product	Harvestable Product	Harvested Product	Reference
		gN per kg feed	gN Retention/kg feed	%N Retention	kg wet weight/kg feed	
Fish	*Sparus aurata*	72	14	20	0.5	Neori et al., 2000
Marco-Alga	*Ulva lactuca*		23	32	2.8	
Total			**37**	**52**	**3.3**	
Fish	*Sparus aurata*	72	14	20	0.5	Neori et al., 2000
Macroalgae	*Ulva lactuca*		23[c]	32[c]		
Abalone	*Haliotis discus hannai*		9[e]	12	1.7	
Total			**23**	**32**	**0.22**	
Fish	*Ictalurus punctatus*	58	14	25	1	Brune et al., 2003
Microalgae	*Scenedesmus* and other green algae		22[c]	37.5[c]		
Bacteria	Div bacteria		22[c]	37.5[c]		
Fish	*Oreochromis niloticus*		5[d]	9	0.2	
Total			**19.4**	**34**	**1.2**	
Shrimp	*Penaeus vannamei*	80	17	21	1	Wang, 2003
Microalgae	*Chaetoceros sp.*		40[c]	50[c]		
Oyster	*Crassostrea virginica*		5.8	7	2.5	
Total			**23**	**28**	**3.5**	
Fish	*Spaurus aurata*	64	16.6	26	0.3	Shpigel et al., 1993
Bivalves	*Crassostrea gigas/Tapes semidecussatus*		9.3	14.5	1.7	
Macroalgae	*Ulva lactuca*		14.2	22.4	1.0	
Total			**40.1**	**62.9**	**3.0**	
Fish	*Oreochromis niloticus*	72	30[d]	42	1.3	Own observation
Macrophyta	*Lemna minor*		11	15	3.8	
Total			**41**	**57**	5.1	
Fish	*Cyprinus carpio*	54[a]	14[b]	26[a]	0.6[a]	Knoesche and
Heterotrophics	div. bacteria		4	7	0.05	Tscheu, 1974
Total			**18**	**33**	**0.65**	
Fish	*Dicentrarchus labrax*	67	19	28.4	0.6	Bischoff, 2003
Worms	*Nereis diversicolor*		0.04	0.06	0.003	
Total			**19**	**28.5**	**0.6**	

Bacterial-Waste-Converter

In a RAS, nutrients are not re-used, they are in fact destroyed and discharged in a harmless form by nitrification, denitrification and heterotrophic degradation (van Rijn and Shnel, 2001; Eding et al., 2003). Although these kinds of processes successfully decrease the amount of discharged nutrients, such systems do not increase the retention of nutrients. Instead of destructing and or volatilizing or storing nutrients, nutrients can also be converted into bacteria biomass and re-used as single cell protein (SCP). If carbon and N are well balanced in the bacterial substrate, ammonia in addition to organic nitrogenous waste will be converted into bacteria biomass (Henze et al, 1996). This conversion is an additional sink for ammonia and contributes to dissolved waste conversion. Knoesche et al (1974) already adopted the idea of intensive heterotrophic bacteria growth in aquaculture systems and could retain 7% feed N (Table 7) and 6% feed P (estimated from 1% P feed, KarpiCo Supreme-7Ex, Coppens International, The Netherlands). He used an activated sludge process to treat water in a recirculation system, and proposed to mix produced sludge with grains for later re-use as fish feed for carps. A comparable approach for activated sludge reuse was as well proposed by Tacon (1979) for trout culture. In pond systems, use of bacteria production was suggested by Avnimelech et al. (1988) and Avnimelech (1999). Tilapia showed better performance in pond cultures, when they were fed on a low protein diet in combination with SCP produced in the pond than tilapia, which were fed with a high protein diet. However, detailed data on the nutrient balances for SCP/sludge consumption and its specific contribution to the nutrient balance are not available, although SCP has frequently being tested as protein source in fish feeds (Tacon, 1979; Oliva-Teles et al., 1998; Storebakken et al., 1998; El-Sayed, 1999, Schneider et al., 2004).

Detrivorous-Converter

In the Detrivorous-Converter, solid waste is fed to invertebrate organisms after separation from the rearing water in the fish waste processor. Recent first trials with integrated sea bass and *Nereis diversicolor* culture, showed a nutrient retention of 0.06% feed N and 0.03 $x10^{-3}$% feed P (Bischoff, 2003; Waller et al., 2003).

Discussion

Nutrient balance

After integration of all five modules into one integrated intensive system, an overall nutrient balance could be established. In this concept, the Fish-Biomass-Converter retains 20-50% feed N and 15-65% feed P. This means that 50-80% feed N and 35-85% feed P are

discharged as waste from this converter. This waste is then divided into two flows towards the Phototrophic-Herbivore-Converter (40-70% feed N and 10-55% feed P) and the Bacteria- or Detrivorous-Converter (5-25% feed N and 25-45% feed P). Parts of these nutrients are either retained in the Phototrophic-Herbivore-Converter (4-15% feed N and 2-22% feed P) as abalone, oyster or tilapia, or in the Bacteria-Waste-Converter (7% feed N and 6% feed P) as bacteria biomass or in the Detrivorous-Converter (0.06% feed N and 0.03 $x10^{-3}$% feed P) as worms.

Overall nutrient retention of integrated systems is depending on their specific configuration. If sea bream-*Ulva*-abalone are cultured, total nutrient retention increases from 20 to 32%N, for catfish-algae/bacteria-tilapia from 25 to 34%N, for shrimp-algae-oyster from 21 to 28%N, and for tilapia-duckweed-tilapia from 42 to 45.5%N (Table 8). Thus, integration of the different modules into one integrated intensive systems increases nutrient retention substantially. From the reviewed integrated intensive systems, a fish-microalgae-bivalves-macroalgae system shows the highest overall N retention, 63%. This high overall retention is due to the fact, that the cultured *Ulva* is not fed to an herbivorous organism, but harvested. One limitation of this system is that it is based on a hypothetical design. Integration of herbivores generally lowers the additional nutrient retention achieved by phototrophic production by 60-80% for N because of their conversion efficiency. This decrease in retention follows the general ecological principle that energy retention decreases by a factor 10 from one trophic level to the next.

Integrated systems using bacteria and detrivores show generally smaller increases (7%N and 0.06%N, respectively) in overall nutrient retention compared to the other modules. However, the latter systems focus on the re-use of the solid waste stream, which to date has hardly been re-used for aquatic production.

The phosphorous balances could only be estimated based on the combination of available data from the existing systems and data from, e.g. nutritional research, feeding companies and proximate analysis. These balances are, therefore, not evaluated in detail. For a better evaluation of the P balances more accurate data will be required in the future. In general, all nutrient retentions and balances have to be interpreted carefully.

Table 8: Nitrogen (N) and phosphorus (P) mass balances for selected integrated system configurations. No values for P for the Fish-Biomass-Converter were available for the selected examples. P balances remain therefore incomplete. Values are given as g/kg feed. Values are taken from Table 7 or resulting from the calculations presented in the text. Numbers in parenthesis are % of feed nutrient; [a] = converted nutrients are used in a subsequent converter and therefore not included in the sum of retained nutrients.

Input		Fish-Biomass Converter		Phototrophic Converter		Herbivore Converter		Bacterial-Waste-Converter		Detrivorous-Converter		Not retained
N	72	*Sparus aurata*	14 (20)	*Ulva lactuca*	23[a] (32)	*Haliotis discus hannai*	9 (12)					49 (68)
P	9		---		0.1-0.6[a] (1-7)		0.2-0.3 (2-3)					---
N	58	*Ictalurus punctatus*	14 (25)	*Scenedesmus* & other green algae	22[a] (38)	*Oreochromis niloticus*	5 (9)	div. bacteria	22[a] (38)			39 (67)
P	9		---		3[a] (30)		0.9 (10)		3.6[a] (40)			---
N	80	*Penaeus vannamei*	17 (21)	*Chaetoceros* spec.	40[a] (56)	*Crassostrea gigas*	6 (7)					57 (71)
P	18		---		9.5[a] (53)		1.3 (7)					---
N	72	*Oreochromis niloticus*	30 (42)	*Lemna minor*	11[a] (15)	*Oreochromis niloticus*	2.5 (3.5)					39.5
P	16		---		2.7[a] (17)		0.6 (4)					---
N	54	*Cyprinus carpio*	14 (26)					div bacteria	4 (7)			36 (67)
P	10		---						0.6 (6)			---
N	67	*Dicen-trarchus labrax*	19 (28)							*Nereis diver-sicolor*	0.04 (0.06)	48 (72)
P	13		---								<0.001 (0.03×10^{-3})	---

The calculations, especially of the Phototrophic-, the Bacteria-Waste-, and the Detrivorous-Converter, are based on highly different systems. The fish species and sizes, feed compositions, feed loads, system dimensions, related waste loads and waste/nutrient concentrations, and environmental conditions differ between the reviewed systems. In order to compare the system nutrient retentions and their conversion processes more accurately in the future, it is necessary, to compare them in a hypothetical integrated system design. This design should be based on a standardized feed composition and feed load, comparable fish production and waste loads, and apply the related nutrient conversion kinetics.

Limitations

The integration of different culture modules into one system results in higher nutrient retention, but is limited by different factors.

Fish-Biomass-Converter

Nutrient retention and nutrient discharge from the Fish-Biomass-Converter is limited by the nutritional value of the feed, and the specific nutritional demands of the cultured fish species. Unbalanced fish feeds lead to higher faecal and non-faecal losses for N and P from the fish (Brunty et al., 1997; Satoh et al., 2003). Conversion efficiencies and nutrient retention have an impact on module's water quality and are, thereby, indirectly affecting fish growth and the design of all subsequent modules. To achieve an efficient nutrient retention in the overall system, optimized nutrient loads at each module are needed. In integrated systems, this can be partly achieved by adjusting the composition of fish feeds (Brunty et al., 1997; Satoh et al., 2003). However, from a feed formulation point of view, such desired feed adjustments might not be easily attainable. For example, to reduce the excessive P supply to the system, it would be advisable to replace fishmeal by other ingredients in the diet. This is not easy to achieve without serious economic and nutritional consequences.

Fish-Waste-Processor

The application of Fish-Waste-Processors is limited by two factors: the efficiency of the separation process, and the prevention of nutrient degradation. Fish waste should be separated as efficient as possible in a solid and dissolved fraction. This avoids a diminished water quality in the modules connected to the dissolved waste stream and prevents unwanted bacterial activity and suboptimal function of these modules. The solid waste should preferably be transported exclusively to the Bacteria- and the Detrivorous-Waste-Converter, where solid nutrient conversion takes place under controlled and optimal conditions. Because nutrient degradation or digestion should be prevented, Fish-Waste-Processors with long hydraulic or

sludge retention times are not applicable. Table 5 summarizes the limitations of different Fish-Waste-Processors. However, in alternative system designs, with limited or even without bacteria or detrivorous conversion, these limitations could be applied in a positive way, e.g. dissolving the available nutrients through leaching. This would reduce the nutrient loads towards the solid waste converters in the system and increase the nutrient loads to the phototrophic converter. Such higher loads of dissolved nutrients might be preferred, because of the high nutrient retention in Phototrophic-Converter.

Phototrophic-Converter

Several factors, such as micro-, and macronutrient ratios, concentrations and fluxes, preferences for N sources, light regime, hydraulic retention time, temperature, and nutrient loss to different sinks will strongly determine the success of phototrophic production. The N/P ratio in plant tissue shows the different requirements and retentions by phototrophic conversion (Table 6). If N or P is offered in excess, the other macronutrient will become a limiting production factor. The excessively available nutrient is released unconverted from the module and accumulates in the culture system, and needs finally to be discharged into the environment. Ammonia uptake efficiency follows a Michaelis-Menten-type saturation curve (Cohen and Neori, 1991). TAN fluxes of 8.1g TAN/ m² per day resulted in an uptake efficiency of 40%, while a flux of about 2.0g TAN/ m² per day resulted in an uptake efficiency of 90%. Differences in wastewater COD (chemical oxygen demand) loading can influence biomass composition in high rate algal ponds (HRAP). Cromar et al. (1992) found that at low COD loadings (around 100kg COD/ha per day) green algae are dominant while cyanobacteria become dominant at higher loadings. In intensive algae culture systems, additional carbon dioxide might be required. An algae production of 3.7g/m²/d dry matter algae fixates 1.8gC/m²/d (Brune et al., 2003). This amount of CO_2 has to be supplied to be the conversion module. If the supply from fish and air is not sufficient due to either high algae productions or because pH values get unfavorable for algae growth additional carbon has to be added (Richmond, 1986). Successful algae culture requires also the availability of micro-nutrients in the right concentration and in the right ratio (de la Noüe and de Pauw, 1988). The form of nitrogen, ammonia or nitrate influences phototrophic production, as the here discussed aquatic plants prefer ammonia over nitrate (Richmond, 1986; Skillicorn et al., 1993; Runcie et al., 2003).

Light is a key factor in phototrophic production, as light intensity and dark-light-cycle influences production. The dependence of *Ulva* on light is described in different models

(Ellner et al., 1996; Coffaro and Sfriso, 1997). In HRAP optimal mixing and flow patterns will expose the algae to favorable sunlight conditions, maximizing algae production and avoiding photoinhibition (Mihalyfalvy et al., 1997). *Chlorella vulgaris* is already inhibited at light intensities of 200-300µE/m^2/s, which occur already at 10% of full sunlight. This inhibition can be prevented by rotating the suspended cells from the light to the dark to recover their photosynthetic apparatus (Mihalyfalvy et al., 1997). Duckweed is photo-inhibited at light intensities above 1200µE/m^2/s (Wedge and Burris, 1982).

Hydraulic retention time is a major design factor for Phototrophic-Converters and their integration in aquaculture systems. The relation between retention time and nutrient inflow and nutrient uptake has been documented for *Ulva* biofilter systems (Cohen and Neori, 1991; del Rio et al., 1996). In HRAPs, where retention time was increased from 4 to 7 days, a remarkable shift in algal species composition from *chlorophycea* to cyanobacteria was observed (Cromar and Fallowfield, 1997). A stable HRAP performance can be maintained at retention times of 2-10 days depending on light, temperature, and nutrient concentrations (Picot et al., 1992; Brune et al., 2003). Phototrophic reactor dimensions and flow rates are, therefore, critical design criteria to meet a balance of species composition, biomass production, nutrient conversion, and purified water volume. Temperature is another important factor in outdoor systems. Growth variations of plants depending on temperature are reported for HRAP, macroalgae filter, and duckweed reactors (Martínez et al., 1999; Pagand et al., 2000; Smith and Moelyowati, 2001; Schuenhoff et al., 2003).

Nitrogen and P might be lost in aquatic plant production systems to other sinks than algae biomass. Nitrification and denitrification are reported for almost all types of Phototrophic-Converters (Neori, 1996; Cromar and Fallowfield, 1997; Koerner and Vermaat, 1998). Another sink is ammonia stripping and ortho-phosphate precipitation due to increasing pH values and calcium concentrations in HRAPs (Nurdogan and Oswald, 1995). In some HRAPs ammonia stripping is the most dominant nitrogen removal process. If pH values rise due to bioremediation, ammonia removal by stripping becomes dominant. In a HRAP studied by Voltolina et al. (1993) over 76% of the total removed nitrogen was stripped, while the pH rose from 8.9 to 10.4 within 2-3 h. It is important to understand that Phototrophic-Converters contain not only plants but also bacteria. Cromar et al. (1992) mentions that in a HRAP 60-80% of the N was assimilated by floccular and bacterial biomass and not by algae. Similar data have been provided for duckweed with a share of 35-46% of the total N removal due to bacteria activity in the system (Koerner and Vermaat, 1998). Also in the PAS waste conversion of 6.2g VS (volatile solids) /m² per day comprises two fractions: 3.67gVS algae

and 2.57gVS heterotrophic production (Brune et al., 2003). A side aspect of limits to successful culture can be the choice for the optimal species and/or optimal system configuration. One study focused on the integration of microalgae production with sea bass for effluent treatment (Pagand et al., 2000). During the experiment, macroalgae out-competed the microalgae. At the end of the experiment macroalgae production was 8.5g dry matter/m² per day versus 0.5g dry matter/m² per day for microalgae. System configuration influences algae production and vice versa. The occurrence and control of, e.g. epiphytes depends on system configuration. Epiphytes are a biological threat of macroalgae production (Pickering et al., 1993). They can over-shade their host plants and drag their currents, which can lead to heavy production losses. Epiphytes can be reduced either mechanically, by chemicals or if N is given in pulses and not continuously. Pulsing N results in a major system configuration change: from continuous to fed-batch operation mode. This might result in limitations of the desired conversion processes, because effluent streams from the fish can then not be treated continuously anymore.

Herbivore-Converter

Conversion of produced plants by herbivore organisms is limited by the nutritional value of the product, harvestability and potential nutrient deficiencies. Several animals are lacking the necessary enzymes to digest the cellulose plant cell wall (Anupama and Ravindra, 2000). Therefore, higher digestibility is achieved, if the cell wall is broken prior to digestion. The low dry weight of fresh plant material is another issue, as roughly 20 times more material has to be consumed by the fish compared to a commercial feed pellet for the same amount of dry matter intake (Gaigher et al., 1984). Some algae are deficient for some nutrients. For example *C. vulgaris* is Vitamin B12 deficient for *Brachionus* culture, if it is fed as solely feed, and has to be enriched (Maruyama et al., 1997). Efficient harvesting of algae and aquatic plants appears to be difficult and costly (de la Noüe and de Pauw, 1988; Poelman et al., 1997).

Direct harvesting and consumption by herbivore organisms within the same culture module as practiced, i.e. in the PAS (Brune et al., 2003), requires an ecological balance between nutrient input for the phototrophic production, the phototrophic production itself and the consumption by the herbivorous fish. Another approach is to separate phototrophic and herbivorous conversion. This separation still requires a balance between nutrient inputs and production inside the converters; however, those separated converters might be more controllable for nutrient inputs and production. In addition, culture conditions, such as hydraulic retention time, reactor mixing, reactor depth can be optimized for requirements of

the intended culture organism. A shallow HRAP of 30 cm (Picot et al., 1992), providing good culture conditions for microalgae, might not be suitable for intensive fish production, because fish require deeper waters. For reduced water refreshment rates, such as realized in RAS, nutrient deficits might occur especially for shellfish production. The growth of shellfish requires calcium and other elements, which are not scarce in open marine systems (Tan et al., 2001). However, the availability can be depleted, if shellfish are harvested and calcium is not replaced due to, e.g. too low water refreshment rates. One kilogram of a mollusk shell contains 98% calcium carbonate (Tan et al., 2001). The removal of 1kg shells equals a removal of 400g calcium, an amount that is contained in $1m^3$ sea water (Kennish, 1990).

Bacteria-Waste-Converter and SCP re-use

SCP production and its re-use might be limited for different factors related to the production and to the nutritional value. SCP production is limited by nutrient ratio, oxygen availability and problems with harvesting techniques. To optimize production and, therefore, the retention of nutrients in bacteria biomass, a C/N ratio in the substrate of ± 15gC/gN is required (Henze et al., 1996). Most commercial fish feeds are protein rich but relatively low in carbohydrates. Consequently C/N ratios in the fish waste are lower than 15gC/gN (Avnimelech, 1999). Low protein fish feeds are one possibility to achieve favorable C/N ratios in fish waste. However, a lower dietary protein content might result in a lower fish production. It would be a challenge to counterbalance this growth reduction by conversion of produced SCP into fish biomass (Avnimelech, 1999). If high protein feeds are applied in the Fish-Biomass-Converter, SCP production requires additional C sources (Schneider et al., 2003). Endogenous SCP production inside the Fish-Biomass-Reactor is limited by oxygen availability (Knoesche, 1994; McIntosh, 2001) and requires extensive aeration and oxygenation. Harvesting of SCP is an additional obstacle, because of the high costs involved (Tacon, 1979). The nutritional value of SCP is limited by a high content of nucleic acids (Rumsey et al., 1991), possible toxins and pathogens (Tacon, 1979; Anupama and Ravindra, 2000; Tacon et al., 2002), low digestibility due to heteropolysaccharides and exopolysaccharides (Tacon, 1979), and deficits in essential amino acids, especially methionine and cystine (Anupama and Ravindra, 2000). Although, solid waste conversion into SCP and its reuse might increase the overall nutrient retention in the system, the practical integration of such a module remains difficult.

Detrivorous-Converter

Worm production per unit surface area is relatively low compared to other conversion processes (1.1kg worms fresh weight/m^2 per year, Meyering, 2003) and the nutrient retention is less significant than for other conversion processes (Table 7), however, such worm production systems convert otherwise discharged nutrients into a valuable product. The microbial activity, which is enhanced by the worm's activity, might become a drawback in such converters (Riise and Roose, 1997) because nutrients are degraded and excluded from a potential re-use through the worms. On the other hand, nutrients might be upgraded by this bacteria production and, therefore, become a better food source for the worms. A balance of constraints and perspectives of such processes is not available yet.

Conclusion

The combination of fish culture with subsequent phototrophic and herbivorous conversion increases nutrient retention in the culture system (e.g. 20-42% feed N to 29-45% feed N). This relative small increase is due to the nutrient retention of the next higher trophic level, the herbivores. Herbivorous conversion decreases the nutrient retention achieved by phototrophic conversion substantially by 60-85% feed N and 50-90% feed P. Future research will be needed focusing on factors to increase nutrient retention in those secondary production and to re-utilize released nutrients from these conversion processes. The conversion of nutrients into bacteria or worm biomass contributes only in smaller margins (e.g. 7% feed N or 0.06% feed N) to the increased overall nutrient retention, however bacteria and detrivorous conversion are hardly integrated into intensive aquaculture systems, and their potential might be underestimated. Their converter design and conversion-processes require, therefore, more attention in the future. A general limitation of the reviewed system examples is the scarce re-use of nutrients, which are excreted during conversion processes, and nutrients, which could not be retained by those processes. If, in the future, recirculation systems should be developed without nutrient discharge, the accumulation of unconverted nutrients in the culture system has to be avoided. The prevention of such accumulation starts again at the Fish-Biomass-Converter, where nutritionally balanced fish feeds are required, which reduce fish waste production and result in more favorable nutrient ratios for the Phototrophic-Converter and Bacteria-Waste-Converter. These better nutrient ratios will lead then to a higher overall nutrient retention in the culture system, because fewer nutrients will be discharged unused from these converters and, therefore, accumulation will be less. A future comparison evaluating nutrient balances of such integrated intensive systems should be based on a

hypothetical system design, using comparable fish production and waste loads. This will deliver a more transparent picture of nutrient retentions in different modules, their design criteria and of modules' limitations. A general limitation of integrated systems is the potential nutrient accumulation of either not retained or released nutrients. These nutrients need to be reintegrated into the nutrient cycle to increase overall nutrient retentions further. Reviewing the calculated balances, and limitations of intensive integrated aquaculture systems, the perspectives of such integration are very promising, as these systems require fewer nutrients in relation to overall production, and reduce nutrient discharge by re-utilization.

Chapter 3

Heterotrophic bacteria production utilizing the drum filter effluent of a RAS: Influence of carbon supplementation and HRT

Abstract

The drum filter effluent from a recirculation aquaculture system was used as substrate to produce heterotrophic bacteria in suspended growth reactors. Effects of organic carbon supplementation (0, 3, 6, 8g/l sodium acetate) and of hydraulic retention times (11-1h) on bacteria biomass production and nutrient conversion were investigated. Bacteria production, expressed as volatile suspended solids (VSS) was enhanced by organic carbon supplementation, resulting in a production of 55-125g VSS/ kg fish feed (0.2-0.5gVSS/g carbon). Maximum observed crude protein production was ~100g protein / kg fish feed. The metabolic maintenance costs were 0.08Cmol/Cmol h^{-1}, and the maximum growth rate was 0.25- 0.5h^{-1}. 90% of the inorganic nitrogenous and 80% of ortho-phosphate-phosphorus were converted. Producing bacteria on the drum filter effluent results in additional protein retention and lower overall nutrient discharge from RAS.

Schneider, O., V. Sereti, M. A. M. Machiels, E. H. Eding and J. A. J. Verreth (submitted). "Heterotrophic bacteria production utilizing the drum filter effluent of a RAS: Influence of carbon supplementation and HRT." Water Research.

Introduction

Reuse of fish waste inside aquaculture systems focuses on dissolved substances (Troell et al., 1999; Neori et al., 2004). Non-dissolved waste is often discharged as sludge, leaving a significant amount of nutrients un-used (Chen et al., 1996). These discharged nutrients, mainly organic carbon (C), nitrogen (N), and phosphorus (P) lead to environmental pollution. This sludge can also be digested inside the system or used for composting or landfill (Shnel et al., 2002; Losordo et al., 2003). Inside the culture system, heterotrophic bacteria could convert these nutrients into bacterial biomass. This biomass can potentially be used as fish feed, thereby reducing waste discharge. Heterotrophic bacteria production, is applied in pond aquaculture systems culturing tilapia (Avnimelech et al., 1989), shrimps (Burford et al., 2003; Burford et al., 2004), or catfish and tilapia together (Brune et al., 2003). To date, only one attempt is known, where bacteria grown on fish waste in recirculation aquaculture systems (RAS) were envisaged as feed ingredient (Knoesche and Tscheu, 1974). In the latter system, activated sludge was used to purify the water and produce bacteria biomass. However, this system had many disadvantages and was subsequently abandoned by the RAS industry. Yet, the idea to produce bacteria biomass using suspended growth reactors may still be valid if the RAS performance is not disturbed. One solution is to connect the reactor to the drum filter effluent, so that interaction with the system is avoided.

A major constraint for producing heterotrophic bacteria is the C:N ratio in fish waste. In RAS, sludge C:N ratios are usually lower than the optimal ratios needed for bacteria production (Lechevallier et al., 1991; Avnimelech, 1999). Theoretically, when only feces are used, nearly the optimal C:N ratios (12-15g/g) can be obtained. However, under practical conditions, fish feces are in contact with the system water, which contains high concentrations of dissolved N, resulting in much lower C:N values of the slurry (2-3). Carbon supplementation can restore a proper C:N ratio, enabling solid waste conversion into bacteria biomass. Such effects have been achieved in activated pond systems, where organic C was supplemented. The farmed tilapia or shrimps were growing more efficient, because they consumed additional biomass (Avnimelech, 1999; McIntosh, 2001).

The present study focused on intensive bacteria production utilizing solid fish waste derived from the drum filter in a RAS with African catfish. The objectives were to investigate 1) the potential for bacteria production on fish waste by using different C supplementation levels and hydraulic retention times (HRTs) and 2) to assess bacteria growth kinetics, such as yields and maximum growth rate, specific substrate consumption rates and metabolic

coefficients; and to estimate the nutrient conversion of N and ortho-phosphate-P into bacteria biomass and the related Kjeldahl-N production.

Material and Methods

System set up

Two bacteria reactors were connected to a flow equalizer which was receiving the backwash flow of a screen filter (60µm mesh). The screen filter was part of a RAS, which consisted of four culture tanks, a biofilter and two sumps (Figure 7). In the sludge collector the slurry was aerated and agitated. This sludge collector was integrated into the system to allow for constant waste flows towards the bacteria reactor and thus acted as flow equalizer, because the screenfilter backwashes in pulses. The HRT of the drum filter effluent in the flow equalizer was 4h and the drum filter backwash about 120-140 l/kg feed.

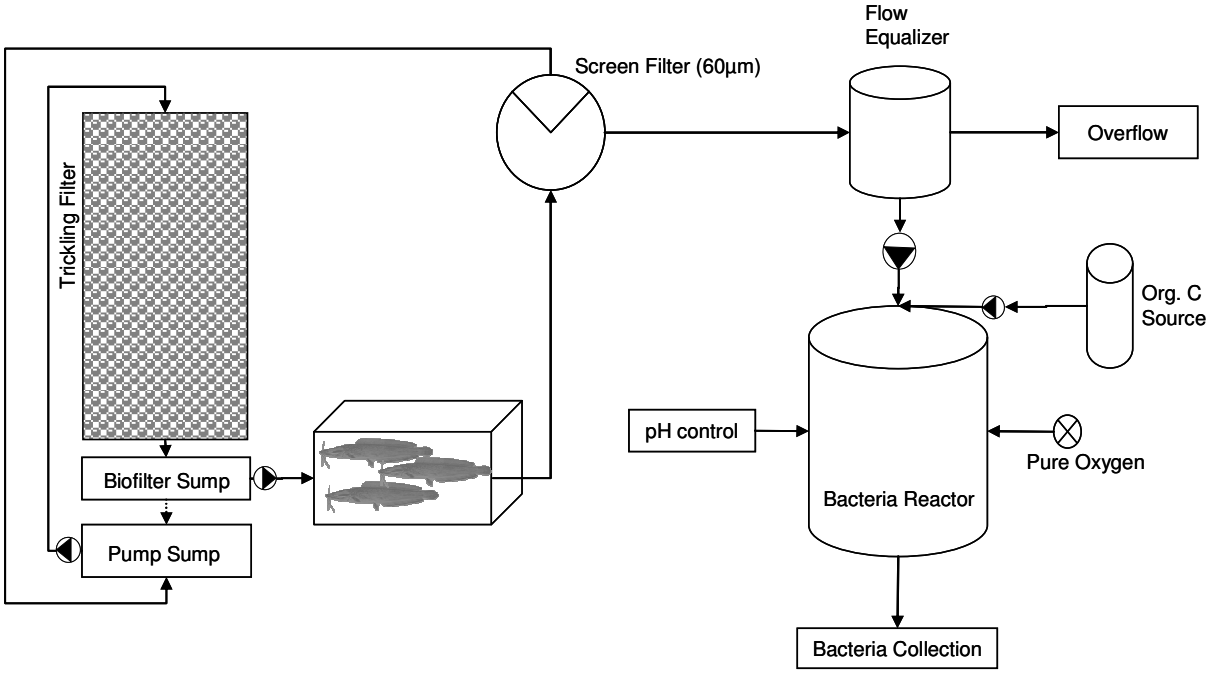

Figure 7: Simplified experimental set-up, comprising a semi-commercial African Catfish system and bacteria reactor connected to the screen filter effluent.

Fish and fish waste

Fish were obtained from a commercial hatchery (Fleuren and Nooijen, The Netherlands). At the beginning of the experiment, four cohorts were stocked with an individual weight of 70g, 170g, 320g, and 560g into the four tanks (Figure 7). Each 28 days the oldest cohort was harvested. The emptied tank was restocked with 140 fish (55-90g). Each tank was thus harvested completely after a production cycle of 112 days. The harvest weight ranged between 823-1038g. The applied procedure mimicked the stocking and harvesting of commercial farms. Fish were fed a commercial diet (Biomeerval, Skretting, France),

containing, according to the producer, 7% moisture, 49% crude protein, 11% crude fat, 22% carbohydrates, of which 2% crude fiber, 11% crude ash and 1.7% P. The realized feeding level was 16-19g per kg metabolic body weight ($W^{0.8}$)/d administered during a feeding period of 24h/d. The daily feed load was calculated based on feed consumption rates given by Eding and van Weerd (1999). At stocking, the initial feed load was 2.7 and increased to 3.7kg/d at the time of harvest. The obtained feed conversion ratios varied (0.70-0.84kg/kg). Diurnal waste fluctuations were minimized by applying a 24h feeding period. The monthly harvesting/restocking scheme minimized changes in biomass within the system and in feed load. This production strategy assured minimal fluctuations of waste production during a production cycle.

Bacteria reactors

From the flow equalizer the slurry was continuously pumped into two bacteria reactors, using a peristaltic pump (PD5101, Heidolph, Germany). The applied flow rates were 10.4±0.3 l/d, when different C supplementation levels were tested and 7.4-81.6 l/d when different HRTs were applied.

The reactors were made of glass in the workshop of Wageningen University, The Netherlands. The reactors had a working volume of 3.5 l and were equipped with baffles to improve the hydrodynamics (Figure 8). Pure oxygen was diffused by air-stones to maintain aerobic conditions in the reactors above 2mg/l. Oxygen was monitored online using pH/Oxi 304i combi-meters (WTW, Germany) connected to a PC. When C supplementation levels were evaluated, oxygen flows were increased by hand if concentrations dropped below 2ml. For the HRT evaluation oxygen flows were controlled by a PC, reacting on a set-point concentration of 3mg/l oxygen inside the broth. The pH levels were maintained at 7.0-7.2 by addition of acid or base (HCl, NaOH, 0.5-1M) stirred by a pH controller (Liquisys M, Endress-Hauser, Germany). The reactor temperature was maintained by a water bath at 28°C. The reactors were continuously agitated by a rotor (RZR 2102, Heidolph, Germany). When C supplementations levels were tested, agitation speed was increased during the culture progress from 100-350rpm to allow for optimal oxygen diffusion. When different HRTs were applied the agitation speed was fixed at 350rpm.

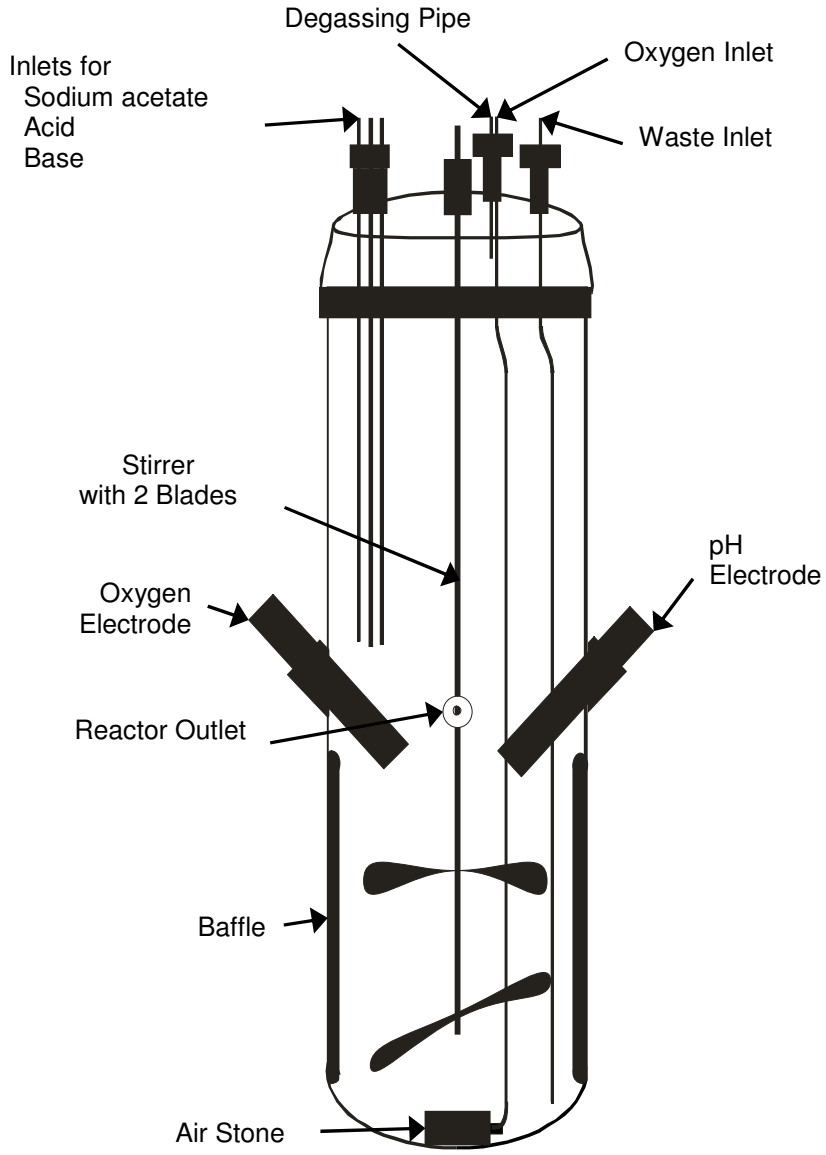

Figure 8: Schematic drawing of the bacteria reactor.

Experimental design and conditions

The initial waste composition in the flow equalizer effluent was analyzed (Table 9). Four different organic C supplementation levels (0, 3, 6, 8 g/l sodium acetate) were chosen based on preliminary batch experiments. Sodium acetate (anhydrous, Assay>98.5%, Fluka, Germany) was dissolved in distilled water at concentrations of 105, 203, 280g/l. This resulted in reactor inflow concentrations of 3, 6, and 8g/l sodium acetate (Table 10). For the 0g/l treatment, distilled water only was pumped. Sodium acetate solution was pumped into the reactors using a peristaltic pump (PD5001, Heidolph, Germany).

During the first trial, different supplementation levels were tested one after the other. First 6g/l was tested in both reactors, followed by 3 and 8g/l, which were tested parallel to each other (one level per reactor) and repeated by switching the reactor treatment assignment.

Finally, 0g/l and a repetition of the 6g/l were tested each time with two reactors. Data from the two trials, comprising each two reactors with 6g/l, were analyzed first separately for differences in VSS concentration at steady state. This procedure accounted for potential performance changes of the reactors over time due to aging or other effects. Data from the two reactors for each concentration of 0, 3 and 8g/l were pooled to obtain representative datasets.

Table 9: Waste composition of the reactor influent (averages ± standard deviation, (minimum-maximum), N= number of samples. TAN = total ammonia nitrogen, NO_2-N = nitrite-N, NO_3-N = nitrate-N, Kjd-N = Kjeldahl nitrogen corrected for TAN concentrations, TOC = total organic carbon, ortho-P-P = ortho-phosphate phosphorus, TS= total solids, TSS = total suspended solids, VSS = volatile suspended solids.

		C supplementation trial		HRT trial	
		Concentration	N	Concentration	N
TAN	mg/l	0.9±0.3 (0.3-1.4)	20	1.4±0.8 (0.6-3.7)	34
NO_2-N	mg/l	3.1±0.9 (2.3-5.5)	19	3.3±0.5 (2.5-4.4)	34
NO_3-N	mg/l	239±79 (176-419)	20	152±12 (130-165)	34
Kjd-N	mg/l	62±37 (24-170)	25	62±51 (13-261)	31
TOC	g/l	0.5±0.2 (0.1-0.7)	10	0.4±0.2 (0.1-0.9)	27
ortho-P-P	mg/l	19.5±6.9 (10.5-40.1)	20	8.6±1.0 (6.2-10.6)	34
Ash	g/l	1.6±0.3 (1.1-2.2)	17	1.9±0.9 (0.9-5.0)	31
TS	g/l	3.6±0.7 (2.3-4.8)	17	3.6±1.3 (1.9-7.3)	31
TSS	g/l	1.6±0.6 (0.9-2.8)	17	1.6±1.3 (0.2-5.8)	31
VSS	g/l	1.1±0.5 (0.6-2.2)	17	0.6±0.4 (0.04-1.5)	31
optical density$_{660nm}$		1.0±0.3 (0.6-1.7)	61	0.9±0.3 (0.4-1.7)	61

In the second trial, different HRT were tested. First the longest HRT (11h) was evaluated. Afterwards flow rates were increased gradually lowering the HRT on steps of 1h (Table 10). In this second trial, the supplementation level was fixed to 6g/l.

Table 10: Flow rates to the bacteria reactors, hydraulic retention time (HRT), realized sodium acetate concentration, and carbon (C):nitrogen (N) ratio of the reactor influent. (TOC=total organic carbon).

Sodium acetate	Waste Flow	Sodium acetate Flow	Total Flow	HRT	Sodium acetate-C	TOC	C:N
g/l	l/d	l/d	l/d	H	g/l	g/l	g/g
0	10.3	0.32	10.6	8	0.01	0.51	2.3
3	10.0	0.32	10.3	8	0.94	1.50	5.9
6	10.4	0.32	10.7	8	1.70	1.97-2.17	5.0-8.8
8	10.0	0.32	10.3	8	2.52	3.09	12.6
6	7.4	0.22	7.6	11	1.73	2.26	8.58
6	9.0	0.27	9.3	9	1.73	2.17	9.09
6	10.3	0.30	10.6	8	1.70	2.11	9.04
6	11.7	0.35	12.1	7	1.73	2.09	9.86
6	13.6	0.40	14.0	6	1.71	2.15	9.31
6	16.3	0.48	16.8	5	1.71	2.07	7.91
6	20.5	0.60	21.1	4	1.70	2.11	8.86
6	27.2	0.80	28.0	3	1.71	1.88	11.43
6	40.7	1.20	41.9	2	1.71	2.06	12.68
6	81.6	2.40	84.0	1	1.71	2.17	9.81

Experimental procedure

Inoculum Preparation

1200ml slurry tapped from the flow equalizer was equally divided in six 500ml Erlenmeyer flasks. In each of these flasks 1.2g sodium acetate was added. The flasks were incubated in a water bath (Julabo SW20-C, Julabo Labortechnik, Germany, 28˚C) for 24h, and constantly shaked (110rpm). From all flasks the broth was pooled and used as inoculum for the bacteria reactors. The initial and final optical density of these cultures ranged between 0.6-0.9 and 1.2-2.2, respectively.

Reactor operation mode

Slurry (3.15 l), obtained from the flow equalizer, and inoculum (0.35 l) were added to the reactors. Sodium acetate was added, according to treatment concentration. Reactors were operated in batch mode until bacteria growth was detected by optical density measurements. Reactors were then switched to a flow through mode by pumping fish waste from the flow equalizer and sodium acetate solution into the reactor. Reactors were operated in continuous flow mode during the consecutive exponential and steady state growth phase of the bacteria.

Chemical analysis

Samples were collected as grab samples from the center of the flow equalizer or by siphoning from the bacteria reactors.

Total solids (TS) were analyzed according to APHA-Method 2540.B using a volume of 7ml. Total suspended solids (TSS) were analyzed after APHA-Method 2540.D. 5ml were

filtered through 0.45µm preweighted sodium acetate filters (Millipore, MF 0.45µm HA). Fixed suspended solids and VSS were analyzed using APHA-Method 2540.E (Clesceri et al., 1998). The VSS fraction is considered as a measure of bacteria concentration (Tchobanoglous et al., 2003).

Optical density (OD) was used as a measure of bacteria concentration and correlated linearly to VSS concentrations. OD was measured using a photometer at 660nm (cuvette-size 15mm diameter, round shape, Photometer SQ118, Merck, Germany). The obtained samples (10ml) were diluted in case OD values exceeded 0.3.

In the C supplementation test, filtrate (0.45µm) was stored at -20˚C and later analyzed by an autoanalyser (SAN, Skalar, The Netherlands) for total ammonia nitrogen (TAN), nitrite-N, nitrate-N, and ortho-phosphate-P concentrations, using the methods 155-006, 461-318, 467-033, 503-317 from Skalar, dating from 1993 and 1999. In the HRT trial, a 20ml sub-sample of a grab sample was centrifuged at 4000rpm for 10minutes and then stored at 4˚C for further analysis as above. Kjeldahl nitrogen was determined in unfiltered grab samples which were acidified (H_2SO_4) and stored at -20˚C prior to analysis using a Tecator 2020 Digestor (400°C) for 4h and distillation by Tecator Kjeltec Autosampler system 1035 (Tecator AB, Hoganas, Sweden) according to ISO 5983. To obtain organic N concentrations, the measurements were corrected for TAN concentrations.

A filtrated or the supernatant of a centrifuged sample was stored at -20˚C and analyzed for sodium acetate content using a gas chromatograph (HRGC Mega 2, Fisons, Italy, packed 6 feet column (inside diameter 2mm), Chromosorb 101 (80-100Mesh) nitrogen as carrier gas saturated with formic acid, FID detector). The injection temperature was 185°C, the column temperature 190°C and the detection temperature 225°C. Results were analyzed with Chromcard 2.2 (Fisons, Italy). Total organic carbon (TOC) concentration of grab samples from the reactor and flow equalizer were stored (-20˚C) and analyzed photometrically by using the Dr. Lange test LCK 381 (Hach Lange, Germany).

Calculations

VSS concentrations were checked for steady state by regressing measured values for each supplementation level or HRT linear with subsequent F and t test, using SPSS 11.5 (SPSS Inc., USA). Steady state was accepted if the obtained regression was non-significantly different from a regression with a slope of 0 ($p>0.05$). VSS concentrations were further compared using ANOVA and Tukey's post hoc test to determine differences caused by supplementation levels or different HRTs. Furthermore a paired t-test ($t<0.05$) was used to

detect differences between reactors for the same HRT, in the second trial. In the C supplementation experiment, bacteria growth was calculated using a logistic model (Brown and Rothery, 1993). This model served to calculate yields and to compare those results with measured yields.

$$VSS = \frac{VSS_{\text{Steady State}}}{1 + e^{-k(t-h)}} \quad (1)$$

VSS=concentration at time t (gVSS/l); $VSS_{SteadyState}$=mean concentrations during steady state (gVSS/l); h= time, when 50% of $VSS_{SteadyState}$ is reached (h); t=time (h); k= steepness (h^{-1})

After $VSS_{SteadyState}$, h, and k were estimated separately, the overall model was re-estimated using the estimated values for k and h plus 10% as initial guesses, while $VSS_{SteadyState}$ was constrained. The model fit was tested for each dataset obtained from a single reactor using the Runs test to detect non-randomness (SPSS 11.5, SPSS Inc. USA, $z<0.05$).

Yields of bacteria production were calculated as $gVSS/gC_{\text{Sodium acetate converted}}$. VSS yields for different HRTs were tested using ANOVA and Tukey's Post Hoc test to compare values of the same reactor and using a paired t-test to compare the performance of the two reactors at parallel moments. Nutrient conversion and VSS production in g/kg feed at steady state were analyzed by one-way ANOVA using SPSS 11.5. The means were compared by a Tukey's Post hoc test ($p < 0.05$). In addition nutrient conversions were tested by paired t-test to compare differences in reactor performance at the same time. The maximum yield was estimated based on the maintenance concept (Tijhuis et al., 1993; Tijhuis et al., 1994). The observed growth rate was calculated based on differences in VSS production over time at steady states (equation 1). The results were applied in equation 2:

$$q_s = \frac{1}{Y_{\max}} * \mu + m \quad (2)$$

q_s=biomass-specific substrate consumption rate (gC/gVSS/h); Y_{max}=true biomass yield (gVSS/gC); μ=specific growth rate (h^{-1}); m=maintenance coefficient (gC/gVSS/h)

Furthermore relations between supplementation levels and yields, and substrate flux and specific substrate consumption rate or growth rate for different HRTs were related with linear or non-linear regressions (NLREG 4.1, Sherrod Software, USA) or Microsoft Excel 2003 (Microsoft, USA).

Results and Discussion

VSS concentrations and yields at steady state for different C supplementation levels

No differences in steady state VSS concentrations of the two 6g/l repetitions were observed (p=0.981), and data of these treatments were pooled. VSS steady state concentrations showed significant differences (p<0.001) between treatments: 8g/l showed the highest and 0g/l showed the lowest VSS concentration, even lower than the average values in the flow equalizer (Table 11 and Figure 9). Bacteria growth was estimated using the model (equation 1, Figure 9, Table 12). Because in the 0g/l treatment, VSS seemed degraded, no growth model was established for this concentration. The model fit was better for 6g/l and 8g/l compared to 3g/l. The datasets for 3g/l might have been biased by the fluctuating VSS concentrations in the flow equalizer (Table 9, Table 11). These concentrations were partly exceeding reactor VSS concentrations. The non randomness test yielded significant results (z=0.010) for one of the four 6g/l treatments. The means were not randomly distributed around the model curve, but these differences between predicted and measured values can be neglected. The difference mean was 0.08±0.05gVSS/l for the negative and ±0.1gVSS/l for the positive values. Compared to an average concentration of 1.68gVSS/l, this difference can be considered as very small. Yields were biased by fluctuating VSS inflow concentrations from the flow equalizer. Therefore, besides the measured yields, also yields based on the growth model were calculated (Table 12).

Table 11: Mean concentration and standard deviation (SD) for volatile suspended solids (VSS) inside the bacteria reactors during steady state calculated based on optical density (OD) measurements[1,2]. p is related to a mean comparison of VSS concentration for the different treatments (ANOVA). a-f are indicating significant differences among treatments (Tukey's Post Hoc test, p<0.05). Differences among reactors for the same HRT were detected by paired t-test. Sod. =sodium; HRT=hydraulic retention time; N=number of sample points.

Carbon supplementation				HRT							
					Reactor 1			Reactor 2			
Sod. acetate	VSS (g/l) [1]	N	SD	HRT	VSS (g/l) [2]	N	SD	VSS (g/l) [2]	N	SD	t
0g/l	0.49^{a}	6	0.02	11	0.66^{a,b}	7	0.16	0.63^{a}	7	0.09	0.312
3g/l	1.27^{b}	32	0.13	9	1.14^{c}	7	0.07	1.18^{b}	7	0.08	0.375
6g/l	1.68^{c}	29	0.13	8	1.12^{d,c}	4	0.19	1.11^{b}	4	0.07	0.914
8g/l	2.13^{d}	18	0.19	7	0.94^{e,d,c}	4	0.14	1.13^{b}	4	0.21	0.339
				6	0.87^{b,e,d}	3	0.10	0.89^{c}	3	0.04	0.044
				5	0.93^{e,d,c}	4	0.08	0.84^{c}	4	0.06	0.055
				4	0.82^{b,e}	5	0.06	0.25^{d}	5	0.07	0.000
				3	0.72^{a,b,e}	4	0.06	---	---	---	---
				2	0.55^{a}	4	0.05	---	---	---	---
				1	0.27^{f}	4	0.05	---	---	---	---
p	0.000				0.000			0.000			

[1] $VSS_{concentration}$=0.8493*OD-0.1 (R^2=0.69, n=41); [2] $VSS_{concentration}$=0.447*OD-0.1832 (R^2=0.43, n=48)

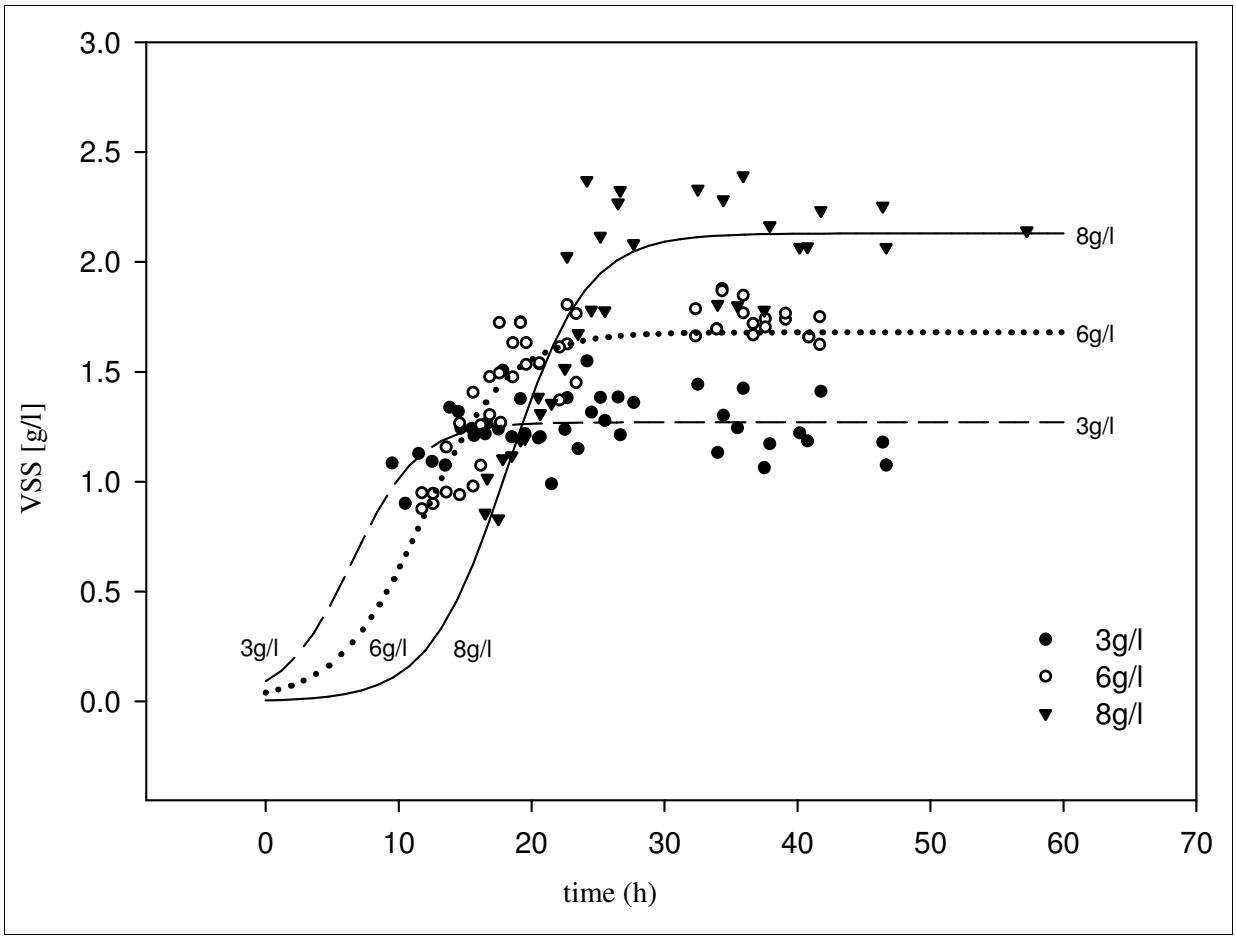

Figure 9: Volatile suspended solid (VSS) concentration over time for 3, 6, 8g/l sodium acetate and as predicted by the growth model.

Table 12: Model parameters estimated by linear and non-linear regression (equation 1). Yields (g VSS/g $C_{Sodium\ acetate}$) were either based on model or on measurements. k_p is related to the function estimation (non-linear regression) for k. h_{final} and k_{final} result from the re-estimated non-linear model. Runs test (z) for the single treatments per reactor. () indicating numbers of observations.

	k_p	h_{final}	k_{final}	R^2	z	Yields	
						Measured	Model
3g/l	<0.001	6.5	0.39	0.20	0.783 (18) 0.135 (24)	0.61	0.21
6g/l	<0.001	11.9	0.31	0.80	0.431 (7) 0.363 (7) 0.095 (18) 0.010 (18)	0.39	0.35
8g/l	<0.001	18.2	0.34	0.83	0.797 (15) 0.094 (18)	0.32	0.42

VSS concentration at steady state for different HRTs

VSS concentrations at steady state decreased with shorter HRT up to the moment the bacteria were flushed out and the critical dilution rate was exceeded (Figure 10). For both reactors this happened at different moments. Reactor 1 exceeded the critical dilution rate at 1h and reactor 2 at 4h HRT. The maximum relative growth rate was assumed as reciprocal to the

shortest HRT, where bacteria growth was still detected. Maximum relative growth rate was 0.2-0.5 h^{-1}. This is in agreement with literature in environmental biotechnology or wastewater treatment studies, e.g. 0.2-0.5 per h for aerobic heterotrophic growth (Henze et al., 1996; Rittmann and McCarty, 2001). The differences in maximum growth rate between the two reactors may have been caused by different factors. The established cultures were open mixed cultures, which were not controlled for any specific bacteria strain. Small differences in available bacteria, growth performance or environmental conditions may lead to the observed differences.

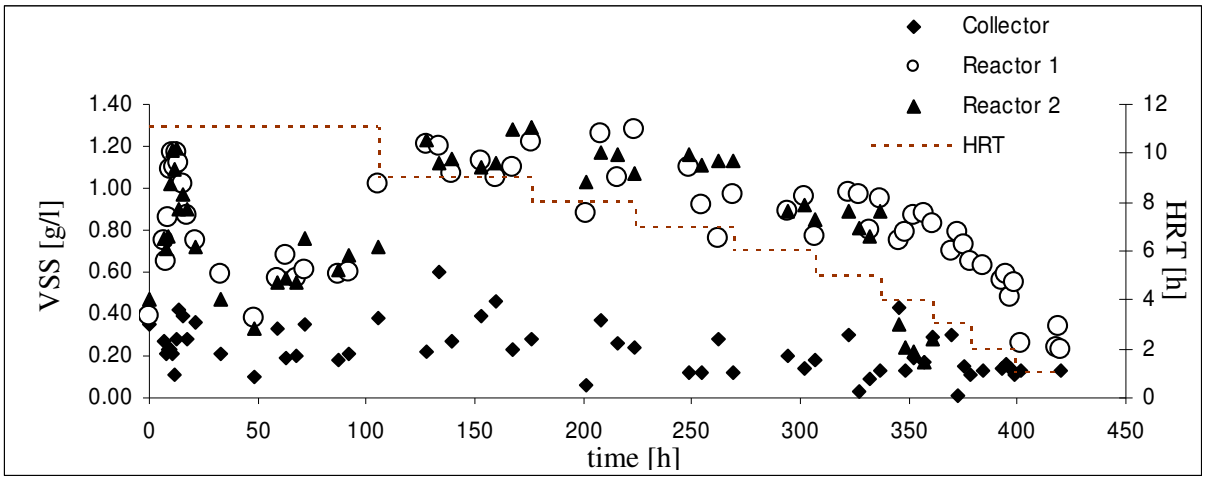

Figure 10: Bacteria biomass development expressed as volatile suspended solids (gVSS/l) over time during different hydraulic retention times (HRT, indicated by the dotted line) for the two reactors and the flow equalizer.

Yields

For every HRT, a yield was calculated (0.3-0.5gVSS/gC, Figure 11) and in addition the maximum yield, (equation 2), was determined (0.49gVSS/gC, Figure 12). The obtained yields were generally at the lower range of yields found in literature (Table 13). Three main factors might have caused these lower yields: Firstly in the established open cultures bacteria strains may not have been adapted to the applied substrates. Secondly water conductivity might have reduced the yields. The conductivity of the rearing water was about 2000-3000µS/cm, much higher than in domestic waste water (~1200µS/cm, Henze et al., 1996). At such salinities osmotic pressure on the bacteria is high, resulting in higher maintenance costs and possibly limiting growth (Rittmann and McCarty, 2001). Evidence for the latter hypothesis was found by comparing the metabolic costs found in the present study with those reported in literature. The determined metabolic costs were 0.04gC/gVSS/h (Figure 12).

When converted to Cmol/Cmol/h, this equals 0.08Cmol/Cmol/h (Tchobanoglous et al., 2003) Literature values range between 0.017-0.05mol/Cmol/h (Atkinson and Mavituna, 1991; Tijhuis et al., 1994). A third reason might be the unaccounted amount of extracellular

material. Different studies report that 30-40% of the volatile solids can be accounted as extracellular polymeric substances (Frolund et al., 1998) or that 10-15% of the organic C was found in this fraction, if biofilms were investigated (Jahn and Nielsen, 1998). Evidence was found supporting this hypothesis, as TOC productions in the broth, were on average 135% of the theoretical C production based on VSS production (Tchobanoglous et al., 2003). Despite the factors mentioned above, methodological issues influenced calculated yields. The yields were based on the concentration differences between in- and out-flowing VSS and $C_{Sodium\ acetate}$. While C was identifiable as sodium acetate, VSS was based on APHA determination procedures. This did not permit to distinguish between bacteria and other organic particular matter and might have resulted in misjudging the true yield.

VSS production was increasing from 1g/l/d (11h HRT) to about 5g/l/d (2h HRT). This value was much higher than the production determined for organic matter decomposition in in-vitro ponds (Beristain, 2005) or in continuous flow reactors (Rittmann and McCarty, 2001). In the present study, recalculated VSS production was 1000-5000g/m^3/d for loading rates of 3.6-20.4gC/l/d. This was much higher than 15-36g/m^3/d found in the mentioned in-vitro ponds at a loading of 1.2gC/l/d or 333gVSS/m^3/d at a loading of 0.4gC/l/d for aerobic continuous flow reactors (CSTR), fed with sodium acetate (Beristain 2005; Rittman et al. 2001). Apparently, in the in-vitro ponds nutrient conversions, growth rates and yields were much lower. In CSTR the yield was higher but the productivity was lower than the one observed in the experiment. The main reasons for these differences were probably organic C degradability (fish feed versus sodium acetate) for the in-vitro ponds and the discussed differences in culture conditions for the CSTR, which would have a higher production for similar loading rates.

Increasing C fluxes resulted in linearly increasing specific substrate consumption rates (Figure 13). This illustrates that substrate was not given in excess, and no increase in substrate residue was found. In contrast increasing C fluxes resulted in logarithmically increasing observed growth rates (Figure 14). This shows that the efficiency of the growth process was leveling off. Observed growth rate increased still linearly for fluxes between 0.25 and 0.35gC/l/h. In our experimental set-up this equals 5-6h HRT, which is above the threshold of 4h, where reactor production was reliable.

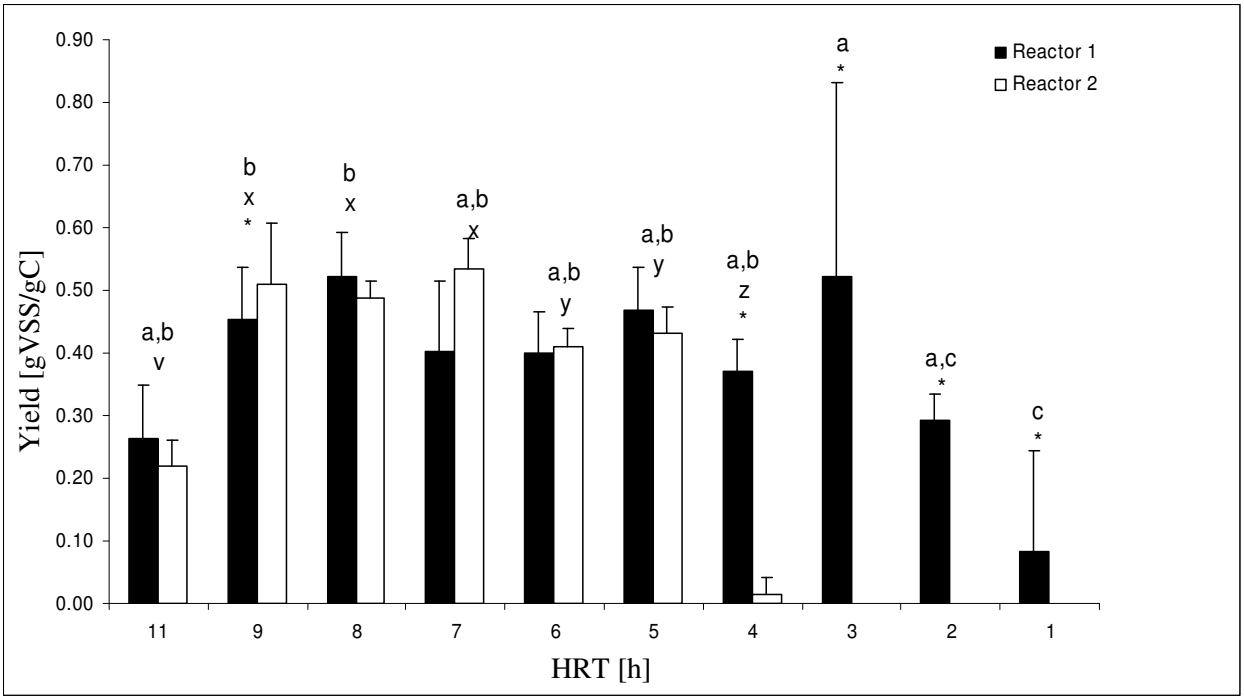

Figure 11: Volatile suspended solid yields (gVSS/gC) as determined for different hydraulic retention times (HRT) for the two bacteria reactors. * indicates significant differences between reactors for the same HRT determined (paired t-test (t<0.05)); a-c for reactor 1 and v-z for reactor 2 indicate significant differences between HRT means (ANOVA and Tukey's post hoc test; p<0.05).

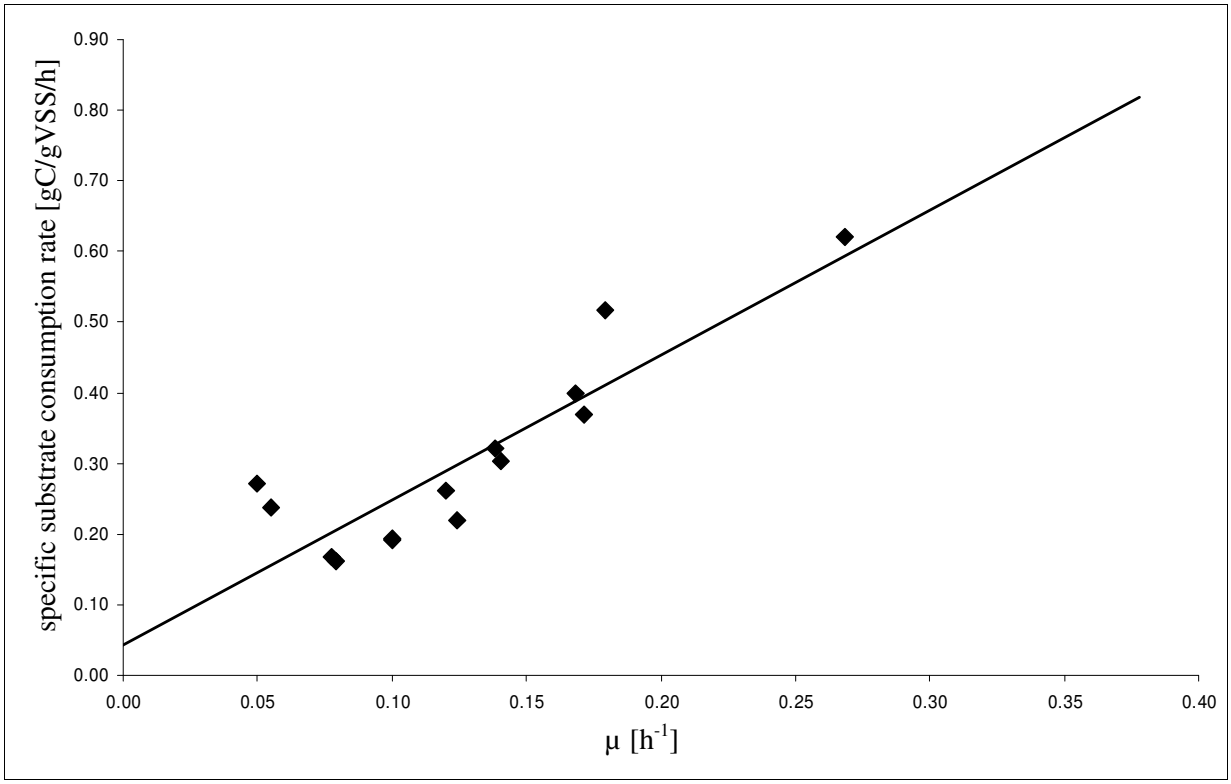

Figure 12: Metabolic plot of growth rate (μ) versus specific substrate consumption rate (gC/gVSS/h). The intercept on y-axis equals the metabolic coefficient (m), and the reciprocal of the slope the maximum yield (equation 2). y=2.05*x+0.04, R^2=0.78.

Table 13: Yields in gVSS/gC for aerobic or sequencing reactors reported in literature.

Yield	Substrate	Remark	Reference
0.57*	organic compound	common yield coefficient	Tchobanoglous et al., 2003
0.62*	glucose	aerobic growth model	Marazioti et al., 2003
0.44-0.88*	acetate	different sources	Atkinson and Mavituna, 1991
0.91*	organic matter	aerobic heterotrophic growth	Henze et al., 1996
0.26-0.71*	acetate	sequencing Batch Reactor	Aulenta et al., 2003
0.93-0.97*	acetate (90%)	fungus culture in a chemostat	van der Westhuizen and Pretorius, 1996
0.71-1.2*	sodium acetate	examples of aerobic heterotrophic yields	Rittmann and McCarty, 2001
0.74-0.92*	acetate	biofilm growth study on small suspended particles	Tijhuis et al., 1994

*recalculated: VSS or organic matter converted to COD (1.42g COD/gVSS (Henze et al., 1996), 0.78gCOD/g sodium acetate); VSS calculated from Cmol (1374gVSS/mol,C content 60molC/VSSmol (Tchobanoglous et al., 2003))

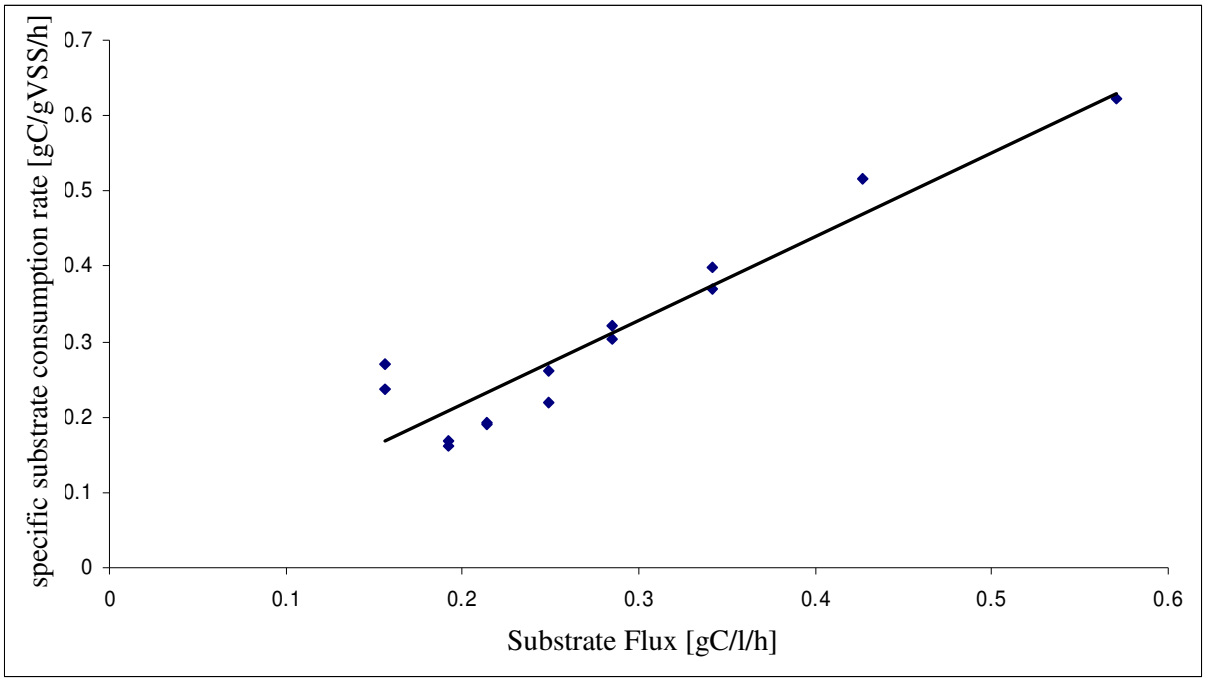

Figure 13: Substrate flux in gC/l/h versus specific substrate consumption rate (gC/gVSS/h). y=1.10*x-0.005, R^2=0.88.

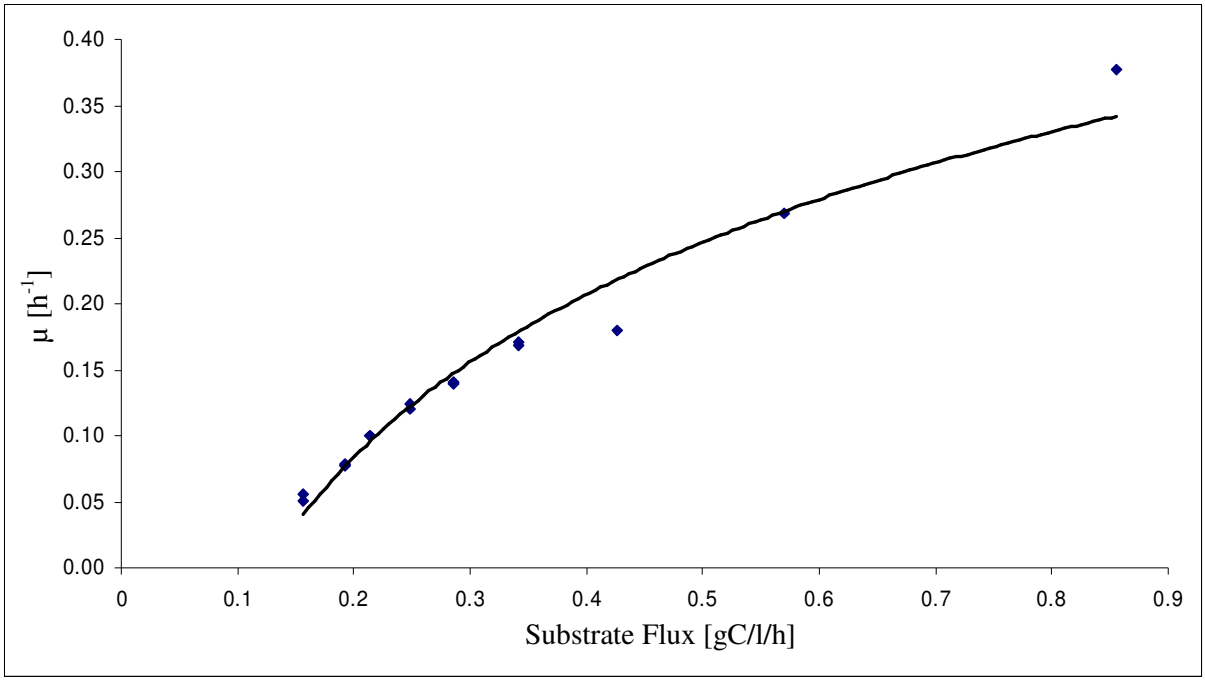

Figure 14: Substrate flux (gC/l/h) versus observed growth rate μ (h^{-1}). y=0.178ln(x)+0.370, R^2=0.97.

Nutrient Conversion

Based on the nutrient concentrations (Table 14) nutrient conversions were calculated (Table 15). Small differences were detected for HRTs ranging between 5 and 9h. The resulting reactor volume for such HRTs would be 28-51 l per kg feed. On average 90% of the inorganic N was converted and 80% of the ortho-phosphate-P (Table 9, Table 14).

At 11, 6, 3 and 2h HRT, crude protein production was higher than VSS production (Table 15). This can be explained by two reasons: The related Kjeldahl-N concentration in the broth was 14-20% of the VSS concentration. This concentration was higher than the range for N comprised in VSS of 6-12% found in literature (Rittmann and McCarty, 2001; Tchobanoglous et al., 2003). This can be due to free Kjeldahl-N in the culture broth, such as free amino acids and other substances (Frolund et al., 1996; Jahn and Nielsen, 1998). A not distinguished fraction of the Kjeldahl-N should, therefore, not be accounted as crude protein comprised inside the bacteria biomass. Secondly, because bacteria, which are dividing at high rates, have high nucleic acids contents, e.g. 13-34%, using the universal factor 6.25 to convert Kjeldahl-N into crude protein leads to overestimations of the crude protein content and production (Shuler, 2001; Vriens et al., 1989; Anupama and Ravindra, 2000).

Table 14: Mean concentrations±standard deviation for total ammonia nitrogen (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N), Kjeldahl-N (KjD-N), ortho-phosphate-phosphorus (ortho-P-P) and Sodium acetate-C (C=Carbon), total organic carbon (TOC) measured during steady state in the flow equalizer and in the bacteria reactors. N=number of samples. [1] **based on n=2 due to analytical error.**

	TAN	NO2-N	NO3-N	Kjd-N	ortho-P-P	Sodium acetate-C	TOC	N
	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	
Flow equalizer	1.4±0.8	3.3±0.5	152.0±11.6	61.7±51.2	8.6±1.4	1.5±3.1	398±161	30-34
Reactor 1								
11h	0.5±0.4	4.0±4.6	32.4±31.3	109.3±18.0	4.2±1.9	1.5±0.3	904±28	4
9h	0.2±0.1	3.1±2.3	17.4±10.8	160.5±11.9	4.7±0.5	0.4±0.4	1039±145	4-6
8h	0.2±0.0	1.8±1.5	6.6±4.7	174.4±5.2	0.2±0.0	0.5±0.5	1110±149	3-6
7h	0.2±0.0	5.3±2.0	22.6±16.8	137.3±8.2	0.2±0.1	1.1±1.7[1]	799±132	3-6
6h	0.4±0.0	1.6±0.9	34.9±5.5	177.5±6.9	0.9±0.9	0.8±0.8	810±29	3-6
5h	0.8±0.2	0.6±0.4	14.0±1.9	175.1±3.9	0.4±0.3	0.2±0.3	840±228	3-6
4h	0.8±0.1	0.6±0.4	37.9±8.3	159.2±7.6	0.2±0.1	1.1±1.1	622±8	3-6
3h	0.1±0.0	0.5±0.4	6.7±5.0	138.0±6.6	0.3±0.0	372±445	782±295	3
2h	0.1±0.0	2.1±0.9	16.3±0.8	94.7±3.6	0.3±0.0	361±18	757±164	3
1h	0.2±0.17	4.4±1.0	67.9±6.5	62.8±12.3	7.9±1.8	1089±76	1413±326	3-4
Reactor 2								
11h	0.5±0.3	1.7±0.8	54.9±11.2	122.4±4.7	4.3±1.9	1.2±0.2	833±95	4
9h	0.2±0.1	3.3±2.3	9.9±12.9	162.3±12.8	5.4±0.6	0.5±0.8	1014±152	4-6
8h	0.1±0.0	6.2±0.4	33.0±3.7	161.8±4.4	4.1±0.1	0.3±0.3	799±95	3-6
7h	0.1±0.2	4.6±1.3	34.6±3.6	131.5±3.6	1.9±0.2	1.8±2.6	896±184	3-6
6h	0.3±0.1	2.7±0.8	38.2±1.0	137.9±8.2	0.8±0.4	1.9±3.3	783±65	3-6
5h	1.5±1.6	3.7±3.4	36.5±9.6	124.9±4.8	0.2±0.1	36.1±62.1	848±104	3-6
4h	0.1±0.0	7.8±0.1	40.0±9.1	67.4±67.3	9.6±0.4	969.2±32.7	1413±169	3-6

Table 15: Conversions of ortho-phosphate-P (ortho-P-P) and nitrate-N (NO_3-N), production of crude protein (CP) and volatile suspended solids (VSS) in g/kg feed for different HRTs in the reactors (R1 & R2). p values are given for ANOVA, superscripts a-d indicate significant differences (Tukey's post hoc test (p<0.05)), + and - are indicating differences detected with paired t-test (t<0.05).

	ortho-P-P		NO_3-N		CP		VSS	
	Conversion (g/kg feed)				Production (g/kg feed)			
	R1	R2	R1	R2	R1	R2	R1	R2
11h	1.5^{a}_{+}	1.2^{b}_{-}	14.2	12.3^{a}	$43.0^{a,b}_{+}$	54.7_{-}	$56.6^{a,b}_{+}$	52.6^{b}_{-}
9h	1.5^{a}	1.1^{b}	19.3	20.2^{b}	$74.0^{a,b}$	75.7	112.0^{c}	$18.5^{c,d}$
8h	4.4^{b}	2.9^{c}	21.4	19.6^{b}	96.7^{b}	82.3	124.5^{c}_{+}	$23.2^{c,d}_{-}$
7h	4.8^{b}	$4.2^{c,d}$	20.2	19.1^{b}	$82.3^{a,b}$	80.0	109.7^{c}	136.5^{d}
6h	3.8^{b}	$3.9^{c,d}$	19.1	18.8^{b}	99.0^{b}_{+}	64.3_{-}	$98.6^{b,c}$	100.6^{c}
5h	4.2^{b}	4.3^{d}	20.6	19.1^{b}	$68.7^{a,b}$	25.0	110.8^{c}	98.9^{c}
4h	4.2^{b}_{+}	-0.4^{a}_{-}	19.9	19.9^{b}	76.7^{b}_{+}	-3.7_{-}	$82.4^{b,c}_{+}$	2.0^{a}_{-}
3h	3.8^{b}	---	18.8	---	$104.0^{a,b}$	---	$81.2^{b,c}$	---
2h	3.9^{b}	---	18.3	---	$67.0^{a,b}$	---	$56.2^{a,b}$	---
1h	0.5^{a}	---	21.7	---	-29.3^{a}	---	19.3^{a}	---
Std. error of the mean	0.3	0.3	0.6	0.7	9.4	11.3	5.5	8.2
p	<0.000	<0.000	0.160	<0.000	0.042	0.332	<0.000	<0.000

The values for ortho-phosphate-P conversion related to VSS production (0.9 to 6.9%, 3.5% on average) exceeded the range expected for bacteria biomass of 2.3% (Rittmann and McCarty, 2001; Tchobanoglous et al., 2003). Similar arguments as for crude protein conversion can be brought forward, as it remains unclear how much phosphorus is included in extracellular material.

Conclusions

Bacteria production using fish waste as substrate was enhanced by organic C supplementation, whereby resulting VSS concentrations in the reactor were clearly depending on supplementation levels. Measured and calculated yields were at the lower range, compared to values found in literature. During the experiments bioenergetic and kinetic parameters were determined, such as the metabolic maintenance costs (0.08Cmol/Cmol/h) and the maximum growth rate (0.2-0.5h^{-1}). Based on the yields, nutrient conversion and growth rate, it is recommended to apply a HRT of 5-9h. This resulted in a calculated reactor volume of 28-51 l/kg feed. If bacteria biomass would be harvested, 100g bacterial protein/kg feed was produced. In addition the overall conversion of inorganic N waste was on average 90% and of the ortho-phosphate-P about 80%. Producing bacteria on the drum filter effluent may, therefore, produce additional protein and lower the overall nutrient discharge from RAS and increase RAS sustainability; under the condition that bacteria biomass is harvested.

Chapter 4

TAN and nitrate yield similar heterotrophic bacteria production on solid fish waste under practical RAS conditions

Abstract

The drum filter effluent from a recirculation aquaculture system (RAS) can be used as substrate for heterotrophic bacteria production. This biomass can be re-used as aquatic feed. RAS effluents are rich in nitrate and low in total ammonia nitrogen (TAN). This might result in 20% lower bacteria yields, because nitrate conversion into bacteria is less energy efficient than TAN conversion. In this study the influence of TAN concentrations (1, 12, 98, 193, 257mgTAN/l) and stable nitrate-N concentrations (174±29mg/l) on bacteria yields and nitrogen conversions was investigated in a RAS under practical conditions. The effluent slurry was supplemented with 1.7gC/l sodium acetate, due to carbon deficiency, and was converted continuously in a suspended bacteria growth reactor (hydraulic retention time 6h). TAN utilization did not result in significantly different observed yields than nitrate (0.24-0.32gVSS/gC, $p=0.763$). However, TAN was preferred compared to nitrate and was converted to nearly 100%, independently of TAN concentrations. TAN and nitrate conversions rates were differing significantly for increasing TAN levels ($p<0.000$ and $p=0.012$), and were negatively correlated. It seems, therefore, equally possible to supply the nitrogenous substrate for bacteria conversion as nitrate and not as TAN. The bacteria reactor can, as a result, be integrated into an existing RAS as end of pipe treatment.

Schneider, O., V. Sereti, E. H. Eding and J. A. J. Verreth (submitted). "TAN and nitrate yield similar heterotrophic bacteria production on solid fish waste under practical RAS conditions." Bioresource Technology.

Introduction

In aquaculture systems aerobic heterotrophic bacteria convert nitrogen (N) and phosphorous (P) originating from dissolved and solid fish waste into bacteria biomass. This biomass can be re-utilized as aquatic feed. This re-use of otherwise wasted nutrients increases system sustainability. This approach has been applied in intensive shrimp ponds, activated tilapia ponds and recirculation aquaculture systems (RAS) (Knoesche and Tscheu, 1974; Avnimelech et al., 1989; Burford et al., 2003; Hari et al., 2004). Such heterotrophic bacteria conversion requires carbon/nitrogen (C: N) ratios of 12-15 (w/w) for optimal biomass production (Henze et al., 1996; Lechevallier et al., 1991). In the case of RAS, where drum filters are used to separate solid and dissolved waste, the C: N ratios of the drum filter effluent are only 3 or lower. This is due to the high nitrate content of the slurry. Under such conditions organic C must be supplemented if the slurry should be utilized for bacteria production. An example of such a C donor is sodium acetate. However, earlier experiments showed bacteria yields which were lower compared to yields reported in literature (Henze et al., 1996; Rittmann and McCarty, 2001, Schneider et al., submitted). These lower yields can be caused by the fact that, in RAS, N is mainly available as nitrate instead of TAN (total ammonia nitrogen). Nitrate conversion into bacteria biomass requires more energy than TAN conversion. In addition TAN is the preferred N source for bacteria compared to nitrate (Vriens et al., 1989; Rittmann and McCarty, 2001). In earlier experiments, the maximum observed yield for the conversion of fish waste utilizing nitrate and sodium acetate was about 0.5gVSS/gC. For this yield, an energy-transfer efficiency of 0.35 can be calculated as the ratio between cell synthesis and electron-acceptance. This ratio represents the energy loss by electron transfers. It is, therefore, a measure of bioenergetic factors limiting the bacteria growth (Rittmann and McCarty, 2001). In a similar approach, using the same energy-transfer efficiency of 0.35, but now replacing nitrate by TAN as N source, a yield of 0.6gVSS/gC can be calculated. This is 20% higher than the nitrate yield:

$$0.125CH_3COO^- + 0.17O_2 + 0.016NH_4^+ \rightarrow 0.016C_5H_7O_2N + 0.059CO_2 + 0.109HCO_3^- + 0.109H_2O$$

$$0.125CH_3COO^- + 0.15O_2 + 0.014NO_3^- + 0.014H^+ \rightarrow 0.014C_5H_7O_2N + 0.057CO_2 + 0.125HCO_3^- + 0.084H_2O$$

This yield difference can only be utilized in a RAS, if all nitrogenous waste is provided as TAN. To realize that goal, RAS have to de designed without a nitrifying biofilter (Figure 4, page 15).

Two scenarios are conceivable to design such RAS: including the bacteria reactor within the RAS system or adding it as post treatment of the RAS effluent stream. The first design would lead to bigger bacteria reactor volumes than the second one, as all water leaving the fish tanks would have to be treated. In the second scenario, flows could be more concentrated and flow rates be based on solids control. However, this results in high TAN concentrations in the system water, which are potentially hazardous for fish. Alternatively, if the waste stream volumes are based on non hazardous TAN concentrations, water discharge of such RAS would be unreasonably high and the related bacteria reactor volume big. Therefore, both alternative scenarios are considered as non prospective. The solution can be found, if the conversion of TAN and nitrate would produce similar yields. In that case the bacteria reactor can be inserted in the RAS system after the drum filter.

The study objectives were, therefore, to evaluate if under practical RAS conditions TAN as nitrogenous substrate results in higher observed yields than nitrate, and furthermore to confirm if TAN is preferred compared to nitrate as nitrogenous substrate by the bacteria.

Material and Methods

System set up

In this experiment a RAS, composed of four culture tanks, a drum filter (60µm mesh size), a biofilter and two sumps was used (Figure 4, page 15). This system was extended with a flow equalizer and a bacteria reactor at the drum filter outlet, where normally the waste stream is discharged. The bacteria reactor was connected to the flow equalizer which was receiving the backwash flow of the drum filter. In the flow equalizer the slurry was aerated and agitated. The flow equalizer was integrated into the system to allow for constant waste flows towards the bacteria reactor. This was important because the drum filter backwashes in pulses, depending on its automated flushing cycle. The hydraulic retention time (HRT) of the drum filter effluent in the flow equalizer was 4h and the drum filter backwash volume was about 136 liters per kg feed.

Fish husbandry

Fish were obtained from a commercial African catfish hatchery (Fleuren and Nooijen, The Netherlands). Fish were stocked initially in four different cohorts of 140 fish each (70g, 170g, 320g, and 560g individual average weight) into the four tanks. Every 28 days the oldest cohort was harvested. The emptied tank was restocked with 140 fish of about 70g. The final fish weight ranged between 917-1025g. Therefore, a complete production cycle from 70 to about 1000g lasted 112 days. Fish were fed a commercial diet (Biomeerval, Skretting,

France), containing 7% moisture, 49% crude protein, 11% crude fat, 22% carbohydrates, of which 2% crude fiber, 11% crude ash and 1.7% phosphorous (based on manufacturer information). The realized feeding level was between 16 and 19g per kg metabolic body weight ($W^{0.8}$) per day. Diurnal waste fluctuations were minimized by applying a 24h feeding period. The monthly harvesting/restocking scheme minimized changes in biomass within the system and then also in feed load. This stocking and feeding strategy assured minimal fluctuations of waste production during a production cycle.

Bacteria reactor

The reactor was made of glass in the workshop of Wageningen University. The reactor had a working volume of 3.5 liters and was equipped with baffles to improve the hydrodynamics (Figure 8, page 43). From the flow equalizer the slurry was continuously pumped into the bacterial culture reactor at a flow rate of 13.0 l/d by a peristaltic pump (Masterflex L/S, Masterflex, USA). The resulting HRT was 6h. Pure oxygen was diffused by air-stones to maintain aerobic conditions in the reactor (>2mg/l). Oxygen was monitored online using pH/Oxi 304i meters (WTW, Germany) connected to a PC. This PC controlled then the oxygenation, reacting on a set-point concentration of 3mg/l oxygen inside the broth. pH levels were maintained between 7.0 and 7.2 by addition of acid or base (HCl, NaOH, 0.5-1M) stirred by a pH controller (Liquisys M, Endress-Hauser, Germany). The reactor temperature was 28°C, fixed by a water bath. The reactor was continuously agitated by a rotor (RZR 2102, Heidolph, Germany) and the agitation speed was fixed to 350rpm.

Initial waste composition and experimental set-up

The initial waste composition in the flow equalizer effluent was analyzed (Table 16). Five different treatments expressed as additional TAN levels were tested one after the other: no addition of TAN or an addition of about 10, 100, 200 and 250 mg TAN/l, whereby nitrate-N concentration remained unchanged. These levels were chosen because TAN concentrations of up to 10mg/l and higher are common in African catfish farms. Concentrations between 100 and 250mg/l would occur if the whole nitrogen budget of the recirculation system would only be regulated by the drum filter effluent and no nitrification would occur. Therefore, they represent a theoretical maximum concentration for a commercial RAS without nitrification unit. The C supplementation level of 1.7gC/l was to counteract the low C: N ratios of the drum filter effluent. Sodium acetate (anhydrous, Assay>98.5%, Fluka, Germany) was used as organic C source, because it is easily degradable and served already in earlier experiments as model substrate. Sodium acetate and ammonia chloride (analytical, Merck, Germany) were

mixed into one solution and the resulting total inflow concentrations in the reactor for TAN were: 1.3, 12, 98, 193 and 257mg/l. The supplementation flow rate was 0.4 l/d, maintained by a peristaltic pump (PD5001, Heidolph, Germany). This set-up was preferred above other set-ups, in which the ratio of TAN to nitrate would be changed, but the total inorganic N content would remain constant. Such designs would require to use either artificial waste or to eliminate the nitrate fraction from the slurry by pre-treatment. Such waste compilation would not reflect practical RAS conditions anymore and in addition would impair the complex waste matrix.

Table 16: Waste composition measured in the flow equalizer. Values are given as averages ± Standard deviation, minimum and maximum in parenthesis, and N = number of samples. TAN = total ammonia nitrogen, NO_2-N = nitrite-N, NO_3-N = nitrate-N, Kjd-N = Kjeldahl nitrogen corrected for TAN concentrations, TOC = total organic carbon, ortho-P-P = ortho-phosphate phosphorus, TS= total solids, TSS = total suspended solids, VSS = volatile suspended solids.

		Flow equalizer	N
TAN	mg/l	1.7±1.0 (0.8-4.8)	14
NO_2-N	mg/l	2.8±0.7 (0.7-3.7)	14
NO_3-N	mg/l	174±29 (76-202)	14
Kjd-N	mg/l	47±33 (23-161)	14
TOC	g/l	0.41±0.01 (0.37-0.47)	5
Ortho-P-P	mg/l	24.1±1.7 (21.1-26.6)	14
pH		7.6-7.9	14
Ash	g/l	1.7±0.6 (1.3-3.5)	15
TS	g/l	3.4±0.7 (2.9-5.4)	15
TSS	g/l	1.1±0.7 (0.5-3.0)	15
VSS	g/l	0.5±0.2 (0.2-1.7)	15
optical density$_{660nm}$*		1.0±0.6 (0.5-3.1)	15

*Samples were diluted prior measurement

The different TAN supplementation levels were tested one after the other, without stopping the bacteria production. When the supplementation level of TAN was changed, the reactors were not sampled for a period of 24h to allow for steady state re-establishment. The reactor and the flow equalizer were sampled by siphoning three times from their centers for grab samples during a period of 18h: six hours after the new steady state level was assumed, six hours later and again six hours later at the end of the steady state period, afterwards supplementation levels were changed.

Experimental procedure

Inoculum Preparation

About 1 Liter slurry was tapped from the equalizer. From this three times 200ml, were transferred in three 500ml Erlenmeyer flasks. Sodium acetate was added (1.7gC/l). The flasks were incubated in a water bath (Julabo SW20-C, Julabo Labortechnik, Germany) at 28˚C for 24h and were continuously shaked at 110rpm. The obtained cultures from all three flasks were pooled and used as inoculum for the bacteria reactor.

Reactor operation mode

Slurry (3.15 Liter), obtained from the equalizer, and inoculum (0.35 l) were added to the reactor. Subsequently sodium acetate was added (1.7gC/l). The reactor was operated in batch mode until bacteria growth was detected by observing differences in optical density. The reactor was then switched to flow through mode by pumping fish waste from the equalizer and sodium acetate/TAN solution into the reactor. The reactor was operated in continuous flow mode during the consecutive exponential bacteria growth phase and the steady states.

Chemical Analysis

Total solids, total suspended solids, VSS

Total solids (TS) were analyzed directly according to APHA-Method 2540.B using a volume of 7ml. Total suspended solids (TSS) analysis was following APHA-Method 2540.D; whereby a total volume of 5ml was filtered through 0.45µm filters (Millipore, MF 0.45µm HA). Fixed and volatized suspended solids (VSS) were analyzed using APHA-Method 2540.E (Clesceri et al., 1998).

Optical density

Optical density (OD) was measured using a photometer at 660nm (cuvette-size 15mm diameter, round shape, Photometer SQ118, Merck, Germany). The obtained samples of about 10ml were diluted in case OD values exceeded 0.3.

TAN, nitrite-N, nitrate-N, and ortho-phosphate

Samples were centrifuged at 4000rpm for 10minutes and then stored at 4 ˚C for further analysis by an autoanalyser (SAN, Skalar, The Netherlands) for TAN, nitrite-N, nitrate-N, and ortho-phosphate-phosphorus concentrations, using the methods 155-006, 461-318, 467-033, 503-317 from Skalar (1993 and 1999).

Kjeldahl-N

Kjeldahl nitrogen was determined in unfiltered grab samples which were acidified with H_2SO_4 and stored at -20˚C prior analysis. Samples were analyzed using a Tecator 2020 Digestor at 400°C for 4h and distillation by Tecator Kjeltec Autosampler system 1035 Analyzer (Tecator AB, Hoganas, Sweden) according to ISO 5983 procedures. The measurements were corrected for TAN concentrations to obtain organic N concentrations.

Organic Carbon

Total organic carbon (TOC) concentration of grab samples from the reactor and flow equalizer were stored at -20˚C and analyzed photometrically using the Dr. Lange cuvette test LCK 381 (Dr. Lange, Hach Lange, Germany).

Sodium acetate concentration was analyzed from a sample, which was separated from suspended solids and stored at -20˚C using a gas chromatograph (HRGC Mega 2, Fisons, Italy, packed 6 feet column (inside diameter 2mm), Chromosorb 101 (80-100Mesh) nitrogen as carrier gas saturated with formic acid, FID detector). The injection was automatically and the injection temperature was 185°C, the column temperature 190°C and the detection temperature 225°C, respectively. Results were analyzed with Chromcard 2.2 (Fisons Instruments, Italy).

Calculations and Statistics

Productions and conversions were calculated based on mass balances (In-Out), yields based on VSS production and the amount of sodium acetate removed and fluxes based on nutrient loads over reactor volume and time. VSS concentrations were checked for steady state by linear regression of measured values against time, using SPSS 11.5 (SPSS Inc., USA). Steady state was accepted if the slope of the regression line was not significant different from 0 ($p>0.05$). Means were compared using one-way ANOVA (SPSS 11.5) and subsequent Tukey's post hoc test ($p<0.05$). Linear regressions of fluxes versus various parameters were executed using Microsoft Excel (version 2003, Microsoft, USA).

Results

Mean VSS concentrations and means of dissolved inorganic nutrients, Kjeldahl-N and TOC at steady state are presented in Table 17. Significant differences have been detected for VSS, TAN, nitrate and ortho-phosphate concentrations. No significant differences were detected for VSS production and VSS yields. Therefore, TAN and nitrate based effluents produce similar yields.

Table 17: Mean concentrations ± standard deviation for total ammonia nitrogen (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N), Kjeldahl-N (KjD-N), ortho-phosphate-phosphorus (ortho-P-P) and sodium acetate-C (C=Carbon), total organic carbon (TOC), volatile suspended solids (VSS) concentration (g/l), VSS production (g/l/d), and yields as measured during steady state in the bacteria reactor (n=3). [1]based on n=2.

$TAN_{Initial}$	TAN	NO_2-N	NO_3-N	Kjd-N	ortho-P-P	Acetate-C	TOC	VSS		Yield
mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	g/l	g/l/d	gVSS/gC
1	0.3±0.2[a]	1.8±2.6	117±11[a]	109±11	11.6±1.5[a]	1.1±0.4	794±85	0.9±0.1[a]	1.8±0.1	0.26±0.02
12	0.2±0.2[a]	1.9±2.4	118±4[a]	101±3	11.9±0.7[a]	1.9±0.3[1]	870±220	0.9±0.1[a]	2.2±0.2	0.32±0.03
98	0.1±0.1[a]	2.2±0.9	124±9[a]	118±8	8.0±2.4[a,b]	1.7±0.3	1390±215	1.0±0.2[a,b]	2.1±0.7	0.31±0.11
193	0.5±0.3[a]	5.0±1.1	189±18[b]	126±9	6.9±0.7[b]	1.5±0.5	894±341	1.3±0.1[b]	2.2±0.8	0.32±0.12
257	14.7±2.1[b]	3.4±1.8	155±36[a,b]	186±74	2.3±2.0[c]	1.8±0.2	1238±483	0.9±0.2[a,b]	1.7±0.6	0.24±0.09
p value	<0.000	0.261	0.004	0.071	<0.000	0.095	0.134	0.018	0.791	0.763

To evaluate, if TAN or nitrate was preferred by the bacteria, data were analyzed in two steps: first the inorganic and organic N conversions were calculated and then the quantitative relations investigated. Nitrite-N conversions are not shown, as the conversions were insignificant and too small to be plotted (p=0.637, 3-8mg/l/d). At low TAN concentrations, nitrate-N was converted into Kjeldahl-N. For increasing TAN concentrations, more TAN and less nitrate was used by the bacteria. For the two highest TAN concentrations nitrate production was detected (Figure 15). Kjeldahl-N production was non-significantly different between all treatments (p=0.114), in contrast to TAN and nitrate-N conversion (p<0.000 and p=0.012). Absolute TAN and relative TAN and nitrate-N conversions were linearly related with TAN flux (Figure 16). For relative TAN conversion no R^2 was calculated, as the regression slope was not different from 0 (p=0.559).

Furthermore a negative correlation between TAN and nitrate-N conversion rates was detected (Nitrate-N conversion = -0.5952*TAN conversion + 279.5, R^2=0.951). In addition, total inorganic N conversion was influencing ortho-phosphate-phosphorus conversion (ortho-phosphate-P conversion = 0.101*total N conversion + 18.371, R^2=0.95). The maximum ortho-phosphate-P conversion was 90% of the inflowing ortho-phosphate-P.

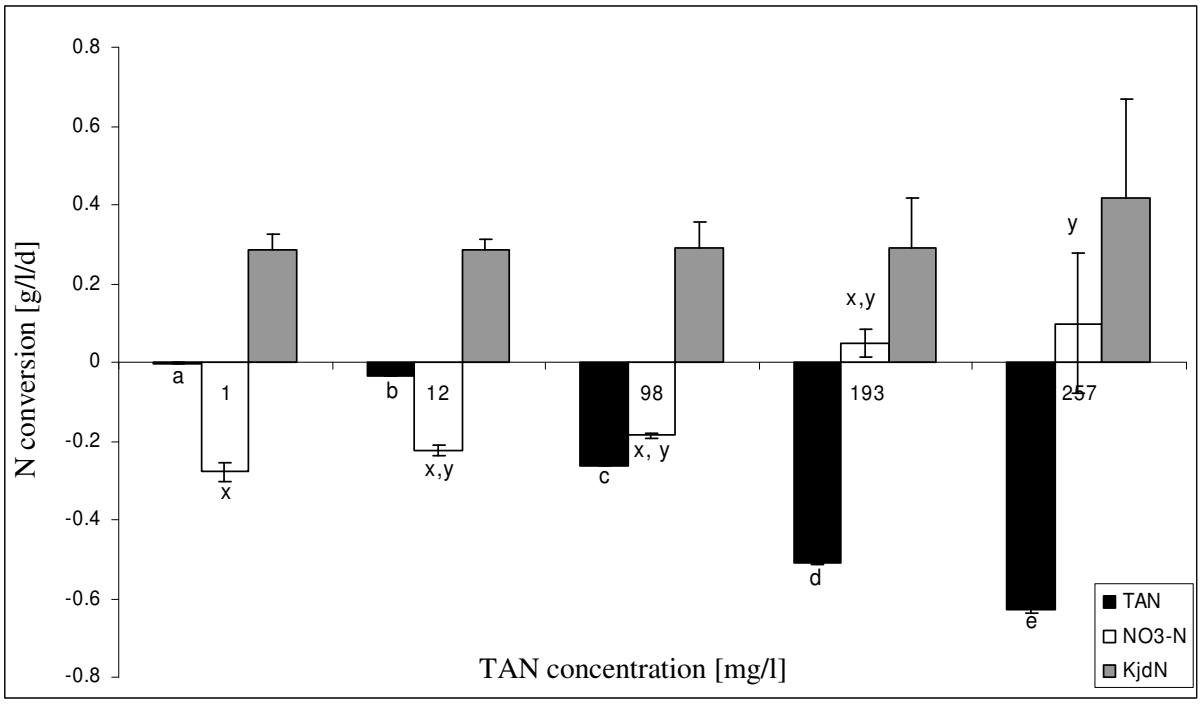

Figure 15: Nitrogen conversions of TAN (total ammonia nitrogen), NO_3-N (nitrate-N) and Kjeldahl nitrogen (Kjd-N) for the different TAN concentrations applied (mg/l). Positive values equal production and negative values removal. Indices a-e and x, y are indicating homogenous subsets (p>0.05).

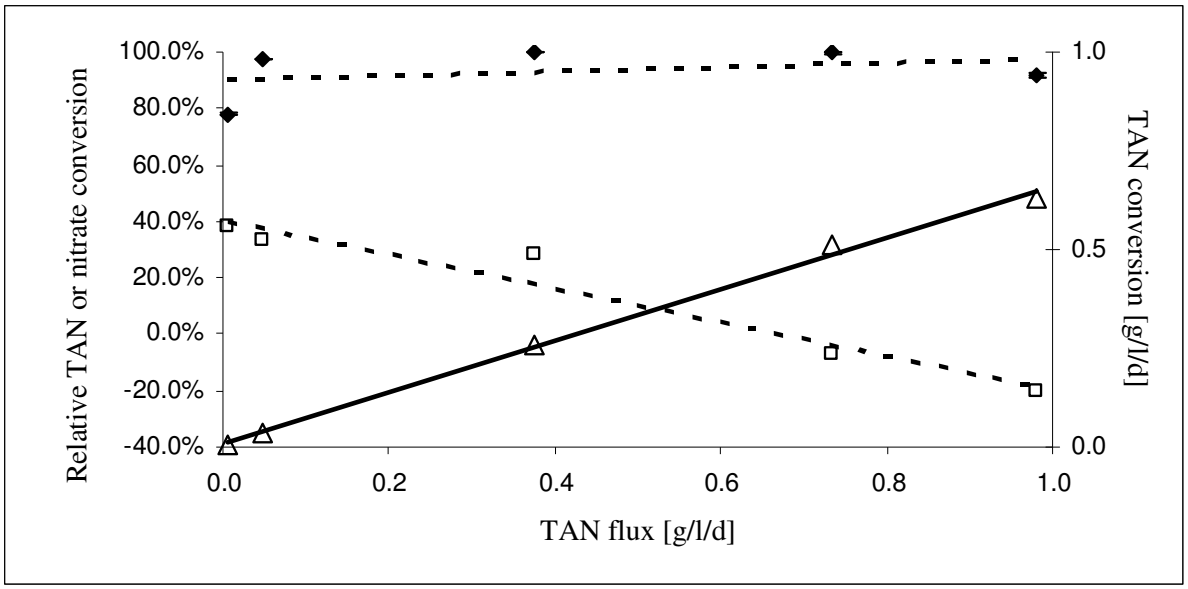

Figure 16: Relation of TAN (total ammonia nitrogen) Flux (g/l/d) and TAN conversion (g/l/d) (bold line and △) and relative conversion of TAN (dotted line and ♦) and nitrate (dotted line and ❑) in %, based on inflow and outflow concentrations. Linear regression based on averaged values. Absolute TAN conversion = 0.654*flux + 0.0074, R^2=0.996; Relative TAN conversion= 0.077*flux+0.9013; Relative nitrate-N conversion=-0.602*flux+0.403, R^2=0.949.

Discussion

VSS productions and yields did not change significantly with increasing TAN concentrations (Table 17). Hence the present results did not yield an apparent advantage of using TAN instead of nitrate. This is in contrast to theory, which predicted 20% yield improvement for TAN use (Rittmann and McCarty, 2001). The measured yields had a high variance. Seeing the practical RAS conditions in the present study, the variation in yields was mainly caused by fluctuating VSS concentrations in the flow equalizer. These concentrations were insufficiently controllable and were disabling, therefore, a more precise yield detection.

Detected yields (0.25-0.32gVSS/gC) were low compared to those found in literature (0.4-1.2gVSS/gC, Atkinson and Mavituna, 1991; Henze et al., 1996; Rittmann and McCarty, 2001; Tchobanoglous et al., 2003). Three main reasons might have caused these lower yields: Possibly in the established open cultures bacteria strains, which were present in an environment of high NO_3 concentration, may not have adapted to the increasing levels of TAN. The low yields might also be explained by the water conductivity in the RAS (2000-3000µS/cm), which is much higher than the values for concentrated domestic waste water (~1200µS/cm, Henze et al., 1996). Conductivity might have led to increased maintenance costs of the bacteria and, therefore, reduced yields (Rittmann and McCarty, 2001). Another reason might be the unaccounted amount of extracellular material. Different studies report that between 30 to 40% of the volatile solids can be accounted as extracellular polymeric

substances (Frolund et al., 1998) or that 10-15% of the organic carbon can be found in this fraction if biofilms were investigated (Jahn and Nielsen, 1998). Observed TOC productions rates support this hypothesis. They were on average two times higher than the theoretical C production based on VSS production (Tchobanoglous et al., 2003).

Despite the factors mentioned above, also methodological issues influenced the calculated yields. The yields were based on the concentration differences between in- and out-flowing VSS and C. While C was identifiable, VSS was based on standard APHA determination procedures. This did not permit to distinguish between bacteria and other organic particular matter and might have resulted in misjudging the true yield.

TAN uptake was preferred over nitrate (Figure 15, Figure 16, Table 17), and increased TAN flux and TAN conversion were correlated with decreased nitrate conversion. This was in agreement with literature (Vriens et al., 1989). Nearly all TAN was consumed by the bacteria, independently of the applied concentration (Figure 16). Only the highest TAN concentration resulted in a small TAN residue, because the provided TAN exceeded the optimal ratio of converted C: TAN (Figure 17, Lechevallier et al., 1991). The ratio of converted carbon was not linearly related to converted inorganic nitrogen (Figure 17). It dropped from 160:7:1 to 98:8:1 (C: N: P) from the lowest to the highest TAN supplementation level ($p=0.000$). That means more inorganic nitrogen was converted for higher supplementation levels than for the lower levels. No clear explanation can be given for this. Because VSS and Kjeldahl-N productions and yields were not changing with increased TAN conversion, theses sinks can be excluded. One explanation might be that an amount of converted inorganic nitrogen was not found back in either produced Kjeldahl-N or in the produced nitrate. This non detected fraction was 13-19% of the converted inorganic nitrogen for the three highest TAN levels. This nitrogen might have been converted into extracellular material and subsequently be lost as foam, which was forming above the broth during the experiment. Another fraction might have been denitrified by the bacteria even though the broth was maintained aerobic. The ratio of converted inorganic nitrogen and phosphorus was not significantly different for all treatments ($p=0.726$).

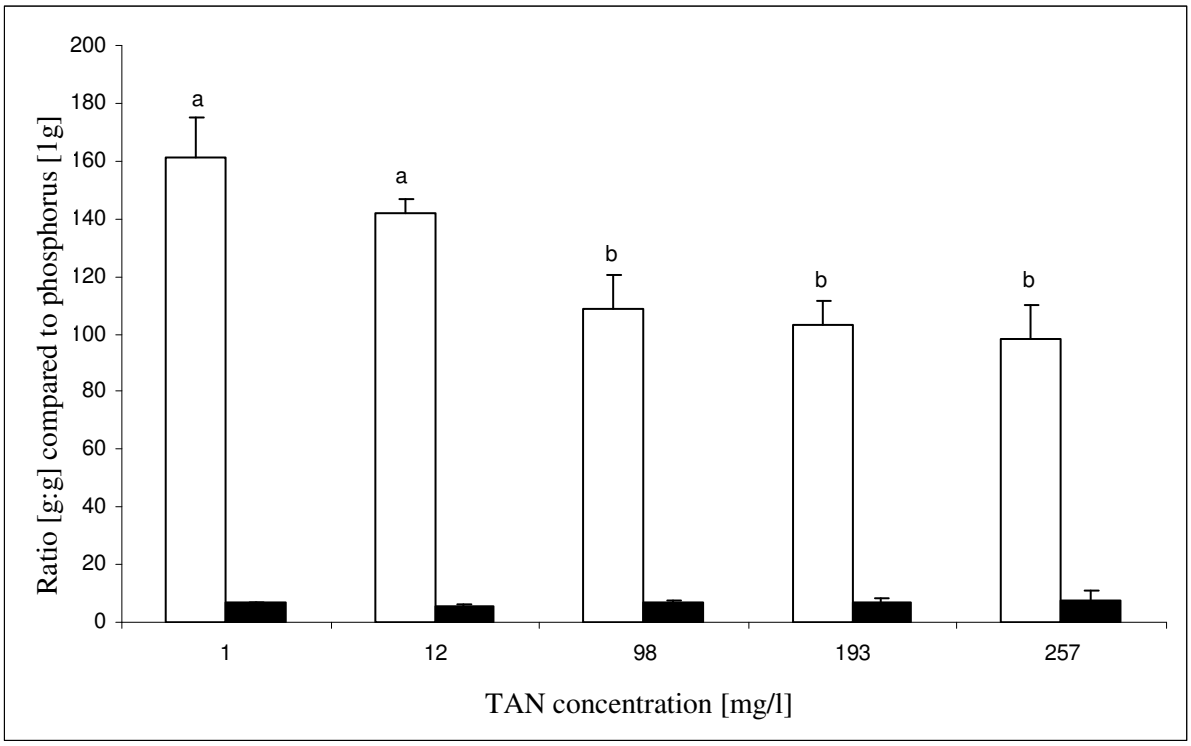

Figure 17: Ratio of carbon to nitrogen to phosphorus converted (C: N: P) on weight/ weight basis, relatively calculated to the base of 1g converted for phosphorus. C in white blocks, N in black blocks, P is not shown as it is the base of all values and equals, therefore, constantly 1. TAN= total ammonia nitrogen

Conclusion

The increase in TAN conversion compared to nitrate when TAN was used as the main nitrogenous substrate for heterotrophic bacteria did not result in a detectable higher VSS production and higher yields under practical RAS conditions. This is in contrast to one of the major hypothesis of this study. Even though this hypothesis could not be validated, the preference of TAN over nitrate by the bacteria was confirmed. TAN flux was linearly related to TAN conversion and increasing TAN conversions resulted in negatively correlated nitrate-N conversions.

Seen the insignificant changes in VSS production and yields, it seems equally possible to supply the nitrogenous substrate for bacteria conversion as nitrate and not as TAN. This allows integrating the conversion process into an existing RAS as end of pipe treatment, thereby converting the solid effluent stream utilizing nitrate, ortho-phosphate and solid waste. If the produced bacteria biomass is then subsequently re-used as fish feed, RAS sustainability would be increased.

Chapter 5
Molasses as C source for heterotrophic bacteria production on solid fish waste

Abstract

The drum filter effluent from a recirculation aquaculture system (RAS) can be used as substrate for heterotrophic bacteria production. This biomass can be re-used as aquatic feed. In this experiment, the solid waste produced in a pilot RAS with African catfish was used as substrate for growing heterotrophic bacteria. One bacteria growth reactor (3.5 l) was connected to the drum filter (filter mesh size 60µm) outlet of a recirculation system in a continuous flow. The hydraulic retention time in the bacteria reactor was 6h. Because fish waste was organic carbon deficient due to nitrogen accumulation in the system, different supplementation levels of molasses were tested, equivalent to carbon fluxes of 0.0, 3.2, 5.8, 7.8, 9.7gC/l/d (C: N ratios: 3.4, 6.4, 9.4, 13.0 and 16.5). For the maximum flux, the VSS and crude protein production were about 168gVSS and 95g crude protein per kg feed. The maximum conversion of nitrate and ortho-phosphate was 24g NO_3-N and 4gP/kg feed, a conversion of 90% of the inorganic nitrogenous waste and 98% of the ortho-phosphate-P. Furthermore the maximum substrate removal rate and the K_s were determined (1.62gC/l/h and 0.097gC/l respectively). The maximum specific removal rate was 0.31gC/gVSS/h and the related half saturation constant was 0.008gC/l. The observed growth rate reached a maximum for C fluxes higher than 8g/l/d. The present integration of heterotrophic bacteria production in RAS represents, therefore, an innovative option to reduce waste discharge and to increase system's ecological sustainability.

Schneider, O., V. Sereti, E. H. Eding and J. A. J. Verreth (submitted). "Molasses as C source for heterotrophic bacteria production on solid fish waste." Aquaculture.

Introduction

Conversion of fish waste into heterotrophic bacteria biomass is highly depending on carbon (C): nitrogen (N) ratios. Optimal C: N ratios for heterotrophic bacteria production are about 12-15g:1g (Lechevallier et al., 1991; Henze et al., 1996; Avnimelech, 1999). In that respect, fish, which are receiving high protein diets, are producing C deficient waste due to high levels of excreted N. For example, the faecal loss of African catfish has a C: N ratio of approximately 12-13: 1 (g/g) under commercial conditions. As consequence of nitrate accumulation in the system water, this ratio drops in recirculation aquaculture systems (RAS) to only 3:1. For other fish species that are less effective in their N retention than African catfish, this ratio can be even lower. In earlier experiments a RAS effluent stream was supplemented with sodium acetate as an easily degradable model substrate. The bacteria production, expressed as volatile suspended solids (VSS) was 100gVSS per kg feed and 18g/kg fish feed of nitrogenous waste and 4.8g/kg fish feed of ortho-phosphate-phosphorus (P) were converted (Schneider et al., submitted).

Even though sodium acetate is widely used as model substrate for bacteria production, under practical conditions adding sodium acetate may economically not be interesting. Sodium acetate costs about 1.5$ per kg or 4.8$ per kg C (Jarchem Industries, USA, pers. com., 2005). Molasses might serve as alternative C source. It costs only about 0.3$ per kg, 1.3$ per kg C (NASS, 2005). It has widely been used as C source for denitrification, anaerobic fermentation, aerobic conversion and been applied in aquaculture (Kargi et al., 1980; Burford et al., 2003; Jimenez et al., 2004; Quan et al., 2005). The composition of molasses is favorable because it hardly contains any N, ash and fiber (Curtin, 1993, Ugalde and Castrillo, 2002).

Using RAS as a system model, the present study tried to verify that heterotrophic bacteria production rates and resulting yields and nutrient conversion rates, obtained with molasses, were comparable to those obtained with sodium acetate. C: N ratio would then be the major factor influencing bacteria production and not the C source. Furthermore, determining microbiological kinetics would provide additional knowledge on activated ponds, where bacteria production rates have hardly been quantified.

Material and Methods

System set up

In this experiment a recirculation aquaculture system, which consists of four culture tanks, a drum filter (60µm mesh size), a biofilter and two sumps was used (Figure 7, page 41

and Figure 8, page 43). This system was extended with one bacteria reactor and a flow equalizer at the drum filter outlet. The bacteria reactor was connected to the flow equalizer which was receiving the backwash flow of the drum filter. In the flow equalizer the slurry was aerated and agitated. The flow equalizer was integrated into the system to allow for constant waste flows towards the bacteria reactor, because the drum filter backwashes in pulses, depending on its automated flushing cycle. The hydraulic retention time (HRT) of the drum filter effluent in the flow equalizer was 4h and the drum filter backwash volume was about 136 l per kg feed.

Fish husbandry

Fish were obtained from a commercial African catfish hatchery (Fleuren and Nooijen, The Netherlands). Fish were stocked initially in four different cohorts of 140 fish each (70g, 170g, 320g, and 560g individual average weight) into the four tanks. Every 28 days the oldest cohort was harvested. The emptied tank was restocked with 140 fish of about 70g. The final fish weight ranged between 917-1025g. Therefore a complete production cycle from 70 to about 1000g lasted 112 days. Fish were fed a commercial diet (Biomeerval, Skretting, France), containing 7% moisture, 49% crude protein, 11% crude fat, 22% carbohydrates, of which 2% crude fiber, 11% crude ash and 1.7% phosphorous (based on manufacturer information). The realized feeding level was between 16 and 19g per kg metabolic body weight ($W^{0.8}$) per day. Diurnal waste fluctuations were minimized by applying a 24h feeding period. The monthly harvesting/restocking scheme minimized changes in biomass within the system and thus also in feed load. As a consequence waste production showed minimal fluctuations during the experiment.

Bacteria reactor

The reactor was made of glass in the workshop of Wageningen University, The Netherlands. The reactor had a working volume of 3.5 l and was equipped with baffles to improve the hydrodynamics (Figure 7, page 41 and Figure 8, page 43). From the flow equalizer the slurry was continuously pumped into the bacteria reactor at a flow rate of 13.0 l/d by a peristaltic pump (Masterflex L/S, Masterflex, USA). The resulting HRT was 6h. Pure oxygen was diffused by air-stones to maintain aerobic conditions in the reactor above 2mg/l. Oxygen was monitored online using pH/Oxi 304i meters (WTW, Germany) connected to a PC. This PC controlled then the oxygenation, reacting on a set-point concentration of 3mg/l oxygen inside the broth. pH levels were maintained between 7.0 and 7.2 by addition of acid or base (HCl, NaOH, 0.5-1M) stirred by a pH controller (Liquisys M, Endress-Hauser,

Germany). The reactor temperature was 28°C, fixed by a water bath. The reactors were continuously agitated by a rotor (RZR 2102, Heidolph, Germany) and agitation speed was 350rpm.

Initial waste composition, experimental design and sampling

The initial waste composition was analyzed in the flow equalizer (Table 18). Five different organic C supplementation levels of 0, 0.8, 1.5, 2.1 and 2.5gC/l were chosen based on earlier experiments with sodium acetate (Schneider et al., submitted). To obtain those concentrations, molasses (bulk product, Research Diet Services, Netherlands) was diluted with distilled water. Molasses solution was pumped into the reactor at a flow rate of 0.4 l/d (PD5001, Heidolph, Germany). When the supplementation level was changed, the reactor was not sampled for a period of 24h to allow re-establishment of steady state. The treatment tested first was 1.5gC/l, then 2.1, 2.5, 0.8 and last 0gC/l. This equals C: N ratios of, 9.4, 13.0, 16.5, 6.4 and 3.4 respectively. The reactor and the flow equalizer were sampled by siphoning three times from their centers for grab samples during a period of 18h: six hours after the new steady state level was assumed, six hours later and again six hours later, afterwards supplementation levels were changed.

Experimental procedure

Inoculum Preparation

About 1 l slurry was tapped from the equalizer. From this, three times 200ml were transferred in three 500ml Erlenmeyer flasks. Molasses were added, until a concentration of 1.5gC/l was reached. The flasks were incubated in a water bath (Julabo SW20-C, Julabo Labortechnik, Germany) at 28˚C for 24h and were permanently shaked at 110rpm. The obtained cultures from all three flasks were pooled and used as inoculum for the bacteria reactor.

Reactor operation mode

Slurry (3.15 l), obtained from the flow equalizer, and inoculum (0.35 l) were added to the reactor. Molasses were added, until a concentration of 1.5gC/l was reached. The reactor was operated in batch mode until bacteria growth was detected by observing differences in optical density. The reactor was then switched to flow through mode by pumping fish waste from the equalizer and molasses solution into the reactor. The reactor was operated in continuous flow mode during the consecutive exponential bacteria growth phase and the steady states.

Acetate control treatment

A control reactor, similar to the molasses reactor was started at the same moment and connected to the same system in a similar experimental set-up using the same flow rates and equipment as the molasses reactor. The reactor was inoculated, handled and sampled in the same way as the molasses reactor with the exception that the inoculum was prepared and the reactor was fed by using sodium acetate (1.7gC/l, anhydrous, Assay>98.5%, Fluka, Germany) as substrate. The reactor was sampled during the first supplementation period of the molasses trial.

Chemical Analysis

Total solids, total suspended solids, VSS

Total solids were analyzed directly according to APHA-Method 2540.B using a volume of 7ml. Total suspended solids analysis was following APHA-Method 2540.D; whereby a total volume of 5ml was filtered through 0.45µm filters (Millipore, MF 0.45µm HA). Fixed and volatized suspended solids (VSS) were analyzed using APHA-Method 2540.E (Clesceri et al., 1998).

Optical density

Optical density (OD) was measured using a photometer at 660nm (cuvette-size 15mm diameter, round shape, Photometer SQ118, Merck, Germany). The obtained samples of about 10ml were diluted in case OD values exceeded 0.3.

TAN, nitrite-N, nitrate-N, and ortho-phosphate

Samples were centrifuged at 4000rpm for 10minutes and then stored at 4 ˚C for further analysis by an autoanalyser (SAN, Skalar, The Netherlands) for total ammonia nitrogen (TAN), nitrite-N, nitrate-N, and ortho-phosphate-phosphorus concentrations, using the methods 155-006, 461-318, 467-033, 503-317 from Skalar, dating from 1993 and 1999.

Kjeldahl-N

Kjeldahl N was determined in unfiltered grab samples which were acidified with H_2SO_4 and stored at -20˚C prior analysis. Analysis was done using a Tecator 2020 Digestor at 400°C for 4h and distillation by Tecator Kjeltec Autosampler system 1035 Analyzer (Tecator AB, Hoganas, Sweden) according to ISO 5983 procedures. The measurements were corrected for TAN concentrations to obtain organic N concentrations.

Organic Carbon

Total organic carbon (TOC) concentration of grab samples from the reactors and collector were stored at -20˚C and analyzed photometrical using the Dr. Lange cuvette test LCK 381 (Dr. Lange, Hach Lange, Germany).

Table 18: Waste composition measured in flow equalizer. Values are given as averages ± Standard deviation, minimum and maximum in parenthesis, and N = number of samples. TAN = total ammonia nitrogen, NO_2-N = nitrite-N, NO_3-N = nitrate-N, Kjd-N = Kjeldahl nitrogen corrected for TAN concentrations, TOC = total organic carbon, ortho-P-P = ortho-phosphate phosphorus, TS= total solids, TSS = total suspended solids, VSS = volatile suspended solids.

		Flow equalizer	N
TAN	mg/l	1.7±1.0 (0.8-4.8)	14
NO_2-N	mg/l	2.8±0.7 (0.7-3.7)	14
NO_3-N	mg/l	174±29 (76-202)	14
Kjd-N	mg/l	47±33 (23-161)	14
TOC	g/l	0.41±0.01 (0.37-0.47)	5
ortho-P-P	mg/l	24.1±1.7 (21.1-26.6)	14
pH		7.6-7.9	14
Ash	g/l	1.7±0.6 (1.3-3.5)	15
TS	g/l	3.4±0.7 (2.9-5.4)	15
TSS	g/l	1.1±0.7 (0.5-3.0)	15
VSS	g/l	0.5±0.2 (0.2-1.7)	15
optical density$_{660nm}$*		1.0±0.6 (0.5-3.1)	15

*Samples were diluted prior measurement

Molasses concentrations were determined by hydrolyzing the sugars to monosaccharides. Proteins were precipitated with Carrez I and II solutions. The obtained sugars were reducing copper, which formed with neocuproine a colored complex, which's extinction was measured at 460nm. The applied procedure is based on the protocol CE-45-025 version 7 of the chemical and endocrinological laboratories, Wageningen University, The Netherlands.

Acetate concentration was analyzed from a sample, which was separated from suspended solids and stored at -20˚C using a gas chromatograph (HRGC Mega 2, Fisons, Italy, packed 6 feet column (inside diameter 2mm), Chromosorb 101 (80-100Mesh) nitrogen as carrier gas saturated with formic acid, FID detector). The injection temperature was 185°C, the column temperature 190°C and the detection temperature 225°C, respectively. Results were analyzed with Chromcard 2.2 (Fisons Instruments, Italy).

Calculations and Statistics

Productions and conversions were calculated based on mass balances (In-Out), yields based on VSS production and the amount of supplemented carbon removed and fluxes based

on nutrient loads over reactor volume and time. VSS concentrations were checked for steady state by linear regression of measured values against time, using SPSS 11.5 (SPSS Inc., USA). Steady state was accepted if the slope of the regression line was not significantly different from 0 ($p>0.05$). Means were compared using one-way ANOVA (SPSS 11.5) and subsequent Tukey's post hoc test ($p<0.05$). Linear regressions of fluxes versus various parameters were executed using Microsoft Excel (version 2003, Microsoft, USA).

Results

The VSS concentrations at steady state were increasing with higher molasses supplementation level. For 0.8 and 1.5gC/l molasses treatments and the control, resulting VSS concentrations were not significantly different (Table 19). Although, VSS production rate increased with higher supplementation levels, no significant differences were detected for 0.8, 1.5 and 2.1gC/l and the control (Table 20). The 0gC/l level was not included in the ANOVA analysis. At this level, values were negative with a high standard deviation, which resulted in a less sensitive ANOVA analysis. Yields of all treatments were not differing significantly from the control, but differences were detected among treatments. Carbon flux correlated linearly with VSS production (Figure 18).

For higher supplementation levels inorganic N and ortho-phosphate-P concentrations were declining, and Kjeldahl-N and TOC levels were increasing. For the supplementation levels of 0.8 and 1,5gC/l and the control, nitrate-N and ortho-phosphate-P conversion rates were not significantly different. The conversions of inorganic nitrogen and ortho-phosphate-P were linearly related with C flux (Figure 19). Kjeldahl-N conversion was only significantly different from 0gC/l supplementation (Table 19), but Kjeldahl-N conversion was increased with increasing C flux. The main nitrogen source for the Kjeldahl-N production was nitrate (Table 18, Table 19, and Figure 20).

Kinetics of the microbial growth and conversion process were obtained using Lineweaver-Burk plots. The maximum substrate removal rate and the K_s were determined, as 1.62gC/l/h and 0.097gC/l respectively (R^2=0.95). The maximum specific removal rate was 0.31gC/gVSS/h and the related half saturation constant was 0.008gC/l (R^2=0.80). The observed growth rate reached a maximum for C fluxes higher than 8g/l/d (Figure 21).

Table 19: Mean concentrations ± standard deviation for total ammonia nitrogen (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N), Kjeldahl-N (KjD-N), ortho-Phosphate-P (ortho-P-P) molasses-C (C=Carbon), total organic carbon (TOC) and volatile suspended solids (VSS) measured during steady state in the bacteria reactor (n=3). The control is based on a supplementation of 1.7gC/l with sodium acetate. Carbon residue refers to the carbon supplement. a,b,c are indicating differences among treatments (Tukey's Post Hoc test, p<0.05).

Molasses	TAN	NO_2-N	NO_3-N	Kjd-N	ortho-P-P	TOC	Carbon Residue	VSS
gC/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mgC/l	g/l
0	1.9±0.8^{a}	3.1±0.2	141±8^{a}	33±5^{a}	24.6±1.5^{a}	274±151^{a}	-0.6±0.5^{a}	0.2±0.1^{a}
0.8	0.2±0.2^{b}	1.3±0.9	78±15^{bd}	120±19^{b}	17.4±2.0^{b}	910±324^{ab}	8.8±1.2^{a}	0.8±0.2^{b}
1.5	0.5±0.4^{b}	0.9±0.6	102±25^{bc}	186±5^{c}	10.6±4.1^{c}	1351±96^{b}	16.2±1.3^{a}	1.1±0.1^{bc}
2.1	0.3±0.1^{b}	0.4±0.1	45±7^{d}	211±4^{cd}	2.0±0.6^{d}	1469±187^{b}	18.4±3.2^{a}	1.3±0.0^{c}
2.5	0.3±0.1^{b}	0.2±0.1	2±0.5^{e}	231±7^{d}	1.0±1.0^{d}	1417±547^{b}	44.4±18.1^{b}	1.7±0.2^{d}
Control	0.3±0.2^{b}	1.8±2.6	117±11^{ac}	109±11^{b}	11.6±1.5^{bc}	794±85^{ab}	1.1±0.4^{a}	0.9±0.1^{b}
p value	0.01	0.099	<0.000	<0.000	<0.000	0.001	<0.000	<0.000

Table 20: Mean conversions ± standard deviation for total ammonia nitrogen (TAN), nitrite-N (NO_2-N), nitrate-N (NO_3-N), Kjeldahl-N (KjD-N), ortho-Phosphate-P (ortho-P-P), and volatile suspended solids (VSS) production (g/l/d), and yields (gVSS/gC) measured during steady state in the bacteria reactor (n=3). The control is based on a supplementation of 1.7gC/l with sodium acetate. Negative conversions are indicating a removal, while positive values indicate productions. * not included in the ANOVA analysis. a,b,c are indicating differences among treatments (Tukey's Post Hoc test, p<0.05).

Molasses Concentration	**TAN**	**NO_2-N**	**NO_3-N**	**Kjd-N**	**ortho-P-P**	**VSS**	
gC/l	mg/l/d	mg/l/d	g/l/d	g/l/d	g/l/d	g/l/d	gVSS/gC
0	-1.3±9.1	3.0±5.2^{a}	0.04±0.18^{a}	-0.17±0.25^{a}	0.01±0.01^{a}	-2.2±2.35*	---
0.8	-7.0±2.0	-6.3±1.5^{a,b}	-0.38±0.04^{bc}	0.17±0.13^{b}	-0.03±0.00^{b}	0.2±0.9^{a}	0.07±0.28^{a}
1.5	-2.7±0.1	-6.7±3.2^{a,b}	-0.34±0.08^{bc}	0.40±0.04^{b}	-0.05±0.02^{b}	2.7±0.5^{b}	0.47±0.09^{b}
2.1	-4.0±2.0	-9.7±5.8^{b}	-0.50±0.02^{cd}	0.46±0.03^{b}	-0.09±0.01^{c}	3.5±0.2^{b,c}	0.45±0.03^{ab}
2.5	-4.0±2.0	-10.0±1.0^{b}	-0.66±0.01^{d}	0.41±0.07^{b}	-0.11±0.00^{c}	4.6±1.0^{c}	0.48±0.10^{b}
Control	-3.7±3.0	-3.7±9.2^{a,b}	-0.28±0.02^{b}	0.28±0.05^{b}	-0.04±0.01^{b}	1.8±0.1^{a,b}	0.26±0.02^{ab}
p value	0.688	0.0.43	<0.000	<0.000	<0.000	<0.000	0.022

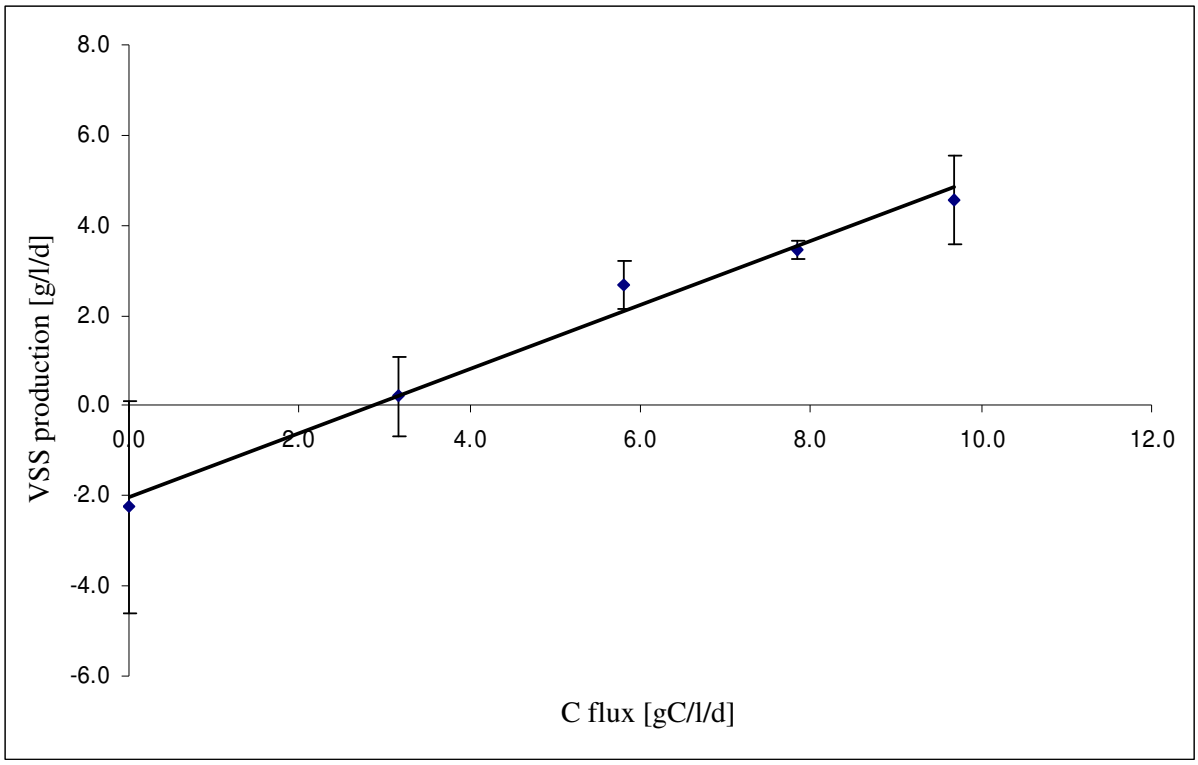

Figure 18: Volatile suspended solids (VSS) production as function of C flux. VSS production=0.711*C flux-2.02, R^2=0.98.

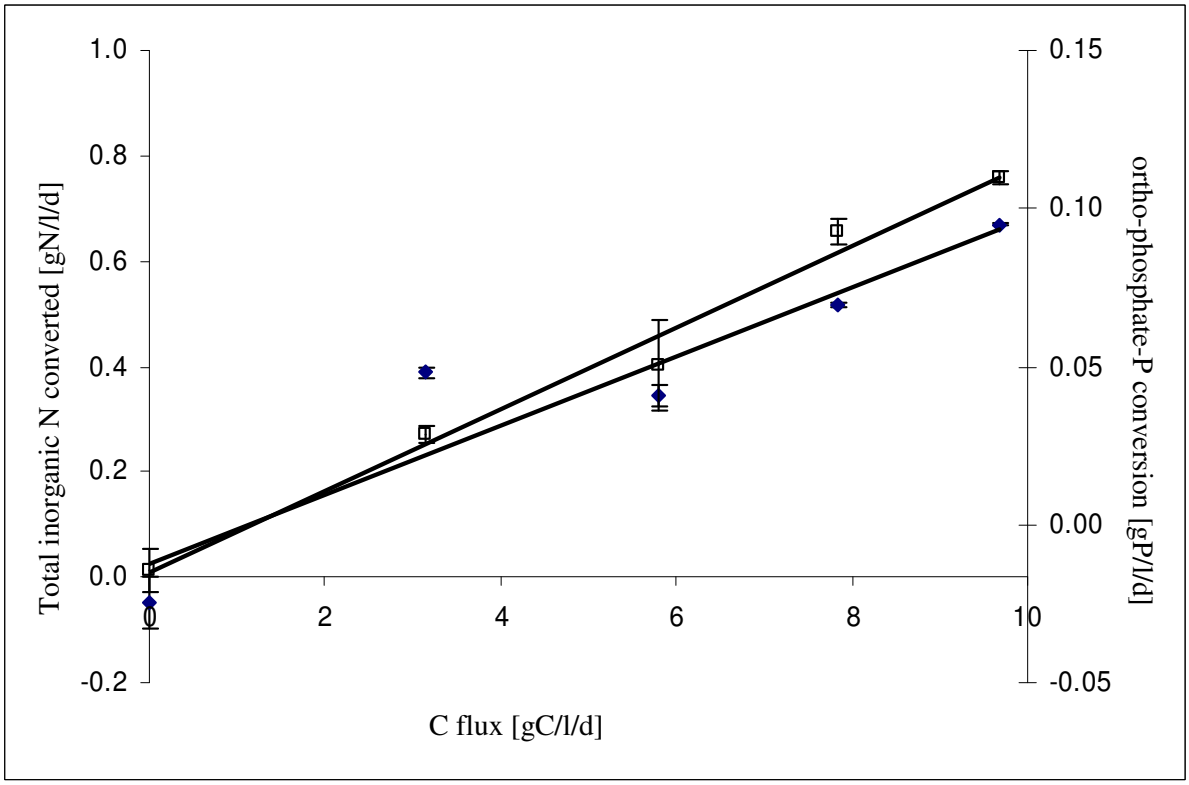

Figure 19: Relation between C (carbon) flux and the amount of total inorganic N converted (g/l/d, ♦) and the relation between gC/l/d given and the amount of ortho-phosphate-P converted (g/l/d, ❑). The linear regressions lines are Inorganic N conversion=0.0667*C Flux +0.0263, R^2=0.88; ortho-phosphate-P conversion = 0.0129 *C Flux - 0.0152, R^2 = 0.99.

Discussion

VSS production were comparable for the 0.8, 1.5, 2.1gC/l and the control. VSS production rates increased as a response to organic C supplementation and showed significant differences among each other. This result was expected, because increased C supplementation should lead to enhanced VSS production for the experimental conditions, as obtained in earlier studies (Schneider et al., submitted). Furthermore, because the control and the treatments yielded no different results, it is indicated that the C source was of less importance than the amount of supplemented C. For the maximum supplementation level VSS production was 4.6gVSS/l per d. This value was much higher than the production determined for organic matter decomposition in in-vitro ponds (Beristain, 2005). In the present study, recalculated VSS production was 4600g/m^3 per day for a loading rate of 9.6gC/l per day. This was much higher than 15-36g/m^3 per day found in the in-vitro ponds at a loading of 1.2gC/l per day. Apparently, in the in-vitro ponds nutrient conversions, growth rates and yields were much lower. The main reasons for these differences were probably the degradability of organic C: fish feed in the in-vitro ponds versus molasses in the present study.

The linear relation between VSS production, nutrient conversions and C fluxes (Figure 18 and Figure 19) illustrated that the VSS production was not N or P limited for the first four supplementation levels. Inorganic N and ortho-phosphate-P were converted by 98 and 90% at the supplementation level of 2.5gC/l (Figure 20). For the highest supplementation level, VSS production was possibly hampered by nutrient limitation, either of N or P, especially because a higher carbon residue was found compared to the lower treatments. N and P might have been limiting, because the ratio of supplemented C to inorganic N to ortho-phosphate-P was 100: 7.5: 0.4. This is lower than the optimal molar substrate ratio of C: N: P 100: 10: 1 (Lechevallier et al., 1991; Liu and Han, 2004). However this limitation must have been marginal since VSS production was increasing linearly with C flux (Figure 18 and Figure 19). The amount of supplemented C, molasses or sodium acetate, influences production rates, while the C source itself seems to be less important, provided that it is easily convertible. This is supported by the observation that VSS yields in the supplementation treatments and the control were not different. The observed yields for molasses (0.4-0.5gVSS/gC) were consistently in the lower range compared to yields found in literature. Poznanski et al. (1983) obtained a yield of 0.8-0.9g dry matter/gC for yeast and bacteria grown together on pig slurry and molasses mixtures. Other studies report yields for bacteria cultured on other organic C sources of 0.3-1.2gVSS/gC (Atkinson and Mavituna, 1991; Tijhuis et al., 1994; Henze et al., 1996; van der Westhuizen and Pretorius, 1996; Rittmann and McCarty, 2001; Aulenta et al.,

2003; Marazioti et al., 2003; Tchobanoglous et al., 2003). Three main factors might have caused these lower yields. Possibly in the established open cultures bacteria strains may not have been adapted to the applied substrates. Secondly the conductivity of the system water (2000-3000µS/cm) was high compared to e.g. domestic waste water (usually 1200µS/cm, Henze et al., 1996). This conductivity might have led to a high osmotic pressure and, therefore, may have increased bacteria maintenance costs. Another reason might be the unaccounted amount of extracellular material. Different studies report that between 30 to 40% of the volatile solids can be accounted as extracellular polymeric substances (Frolund et al., 1998) or that 10-15% of the organic C can be found in this fraction if biofilms were investigated (Jahn and Nielsen, 1998). Evidence was found to support this hypothesis. TOC productions in the broth (calculated as Out-In) were on average 3 times higher than the theoretical C production based on VSS production (Tchobanoglous et al., 2003). This comparison excludes one exceptionally high observation of the 0.8gC/l treatment. Despite the factors mentioned above, methodological issues influenced calculated yields. The yields were based on the concentration differences between in- and out-flowing VSS and $C_{Residue}$. While C was identifiable, analytical methods did not permit to distinguish between bacteria and other organic particular matter and might have resulted in misjudging the true yield.

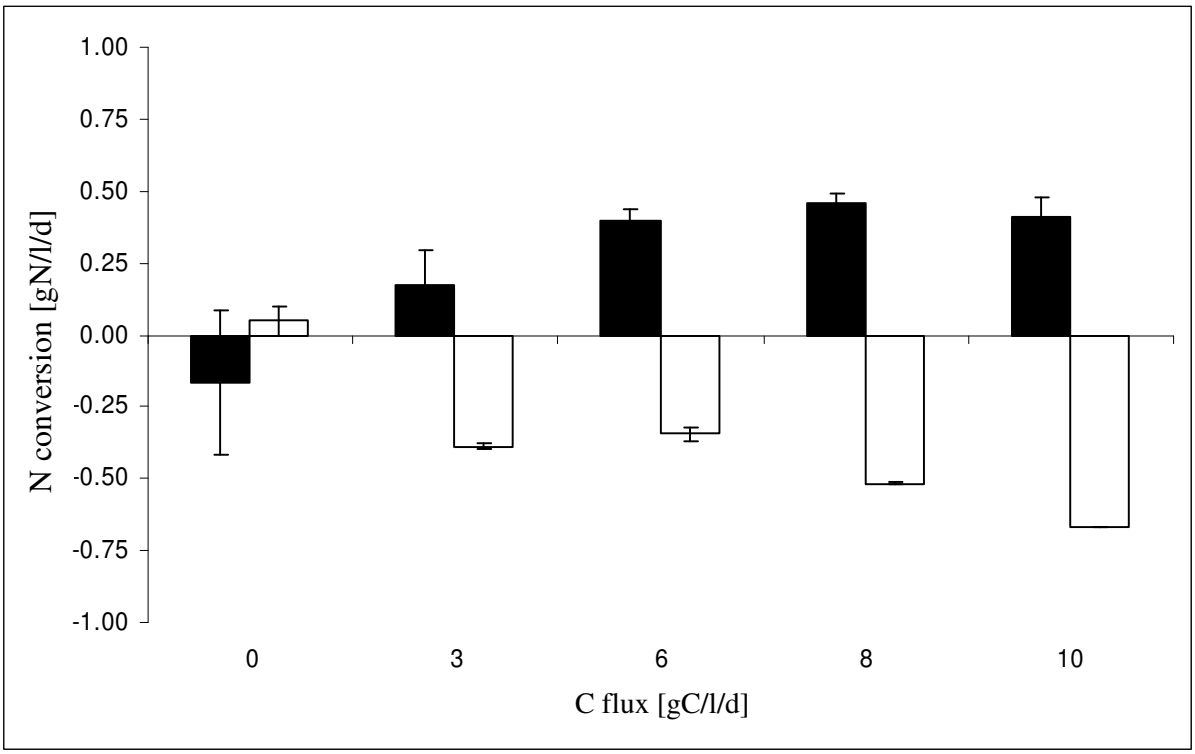

Figure 20: Kjeldahl-N (black) and inorganic N conversion (white) conversions. A negative conversion refers to the degradation of Kjeldahl-N or a conversion of inorganic N, positive values to a production of Kjeldahl-N or inorganic N.

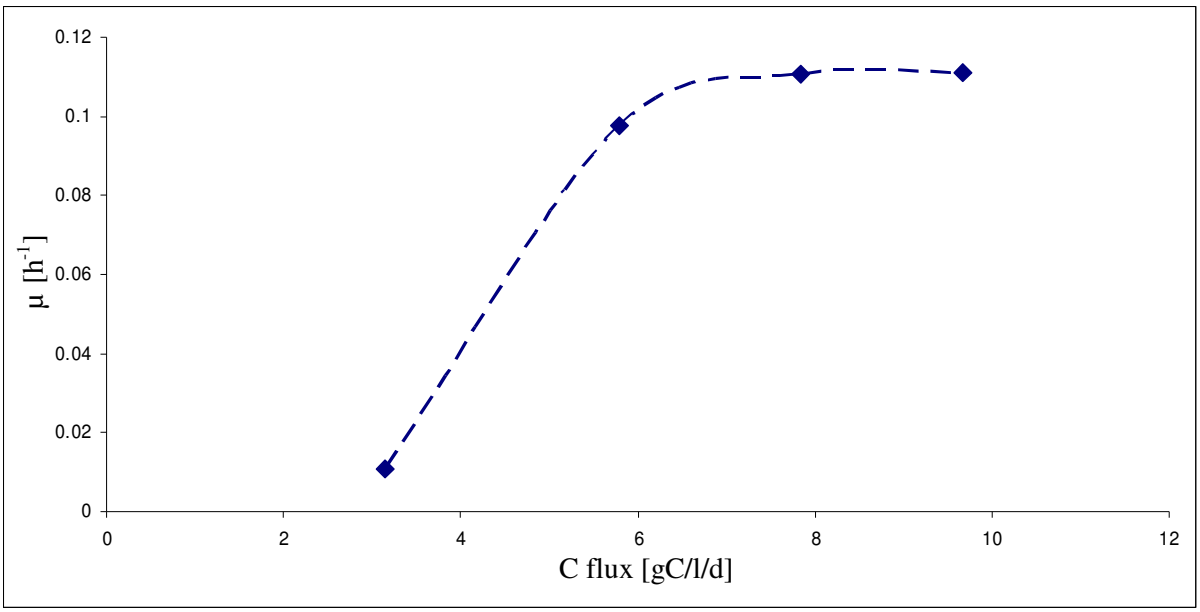

Figure 21: Carbon (C) flux versus relative growth rate. The dotted line was added as optical auxiliary.

Nitrate-N and ortho-phosphate-P conversion rates were significantly different between the treatments and linearly related with C flux (Figure 19). The 0.8 and the 1.5gC/l treatments were insignificantly different from the control level (1.7gC/l). This confirms an expected similarity of nutrient conversion rates, for cultures, which have similar VSS production rates and yields. The VSS and crude protein production were about 127gVSS and 105g crude protein per kg feed for 2.1gC/l supplementation. This VSS and crude protein production was comparable to 100gVSS and 112g crude protein/kg feed obtained in earlier experiments using sodium acetate and comparable C: N ratios of about 13 and fluxes of 7.5-7.8gC/l/d. Nitrate-N and ortho-phosphate-P conversions per kg feed were calculated (Figure 22). The maximum VSS production was 168gVSS/kg feed. The maximum conversion of nitrate-N and ortho-phosphate-P was 24g NO_3-N and 4gP per kg feed, a conversion of 90% of the inorganic nitrogenous waste and 98% of the ortho-phosphate-P. The fish waste supplementation with C can, therefore, be considered as a prospective mean to convert waste products of a RAS. One pitfall of C supplementation with molasses was the organic C residue compared to sodium acetate. Although, this residue was relatively small compared to the supplementation level, it represented an additional organic waste load, leaving the bacteria reactor. If such processes would be integrated in a RAS, the reactor volume per kg feed could be calculated as 34 l/kg feed based on a drum filter backwash of 136 l/kg feed and a HRT of 6h.

The specific substrate removal rate and the relative observed growth rate (0.23gC/gVSS/h, 0.10-0.12h^{-1}) were comparable to earlier experiments using sodium acetate supplementation (0.25gC/gVSS/h, 0.11h^{-1}, recalculated for comparable conditions for a flux

of 2.5gC/l, Schneider et al., submitted). The maximum specific substrate removal rate was double as high as values referred in literature (19.6gCOD/gVSS/d or 7.3gC/gVSS/d, 1.07gCOD/g glucose, compared to 2-10gCOD/gVSS/d, Tchobanoglous et al., 2003). The maximum observed relative growth rate (Figure 21 of 0.10-0.12h^{-1}) was lower than growth rates obtained for *Candida utilis* grown on sugar cane stillage (0.22-0.27h^{-1}, Cabib et al., 1983) or values referred in environmental biotechnology or wastewater treatment studies (e.g. 0.2- 0.5h^{-1} for aerobic heterotrophic growth, Henze et al., 1996; Rittmann and McCarty, 2001). Combining the results of lower yields, lower growth rates and higher COD (carbon) uptake rates than reported in literature, bacteria metabolic costs must have been higher than values presented there. Comparing the present carbon conversion rates with data reported in aquaculture literature was difficult. Degradation rates found in ponds were 0.011-0.013gC/l/d (recalculated from Avnimelech et al., 1992). If in those systems VSS yield was about 1gVSS/gC, then 0.022-0.026gC/l/d would have been converted. This is much lower than our total maximum substrate removal rate of about 38gC/l/d. This difference might be explained by the use of different C substrates. Avnimelech et al. (1992) used fish feed, which is less easily degradable than molasses. The present results indicate the potential of C, N and P conversion in in-vitro systems. It would be interesting to compare them with in-vivo data. Unfortunately these are hardly available, as those studies were reporting mostly on the altered fish or shrimp growth performance and water quality but not on the microbiological rates (Avnimelech, 1999; McIntosh et al., 2000; Velasco, 2000; Hari et al., 2004).

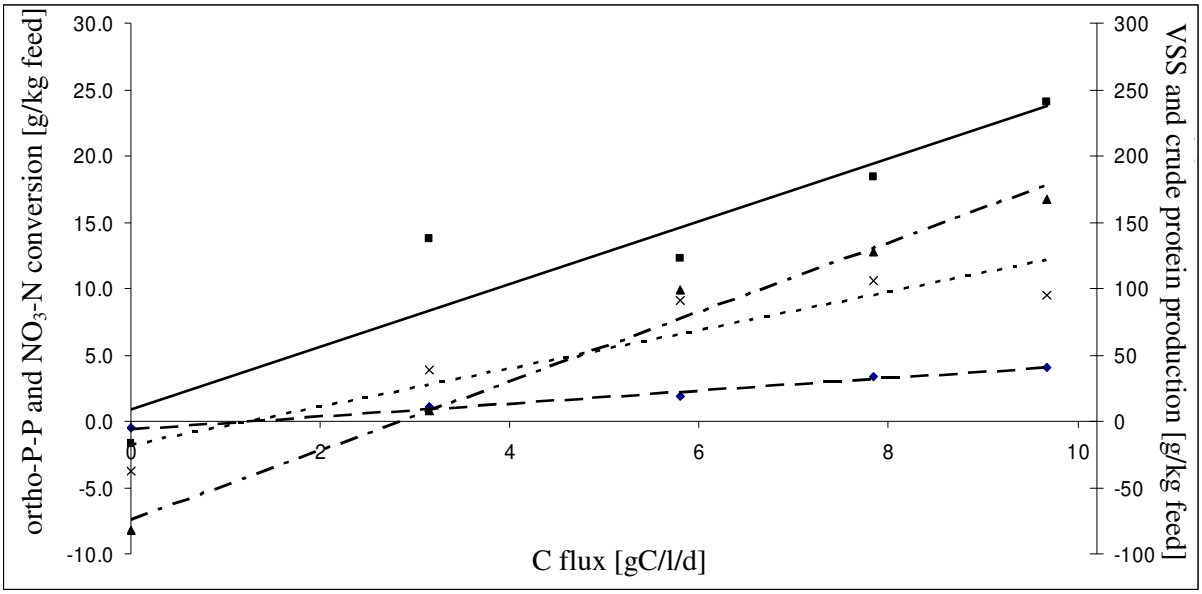

Figure 22: Production and conversions of various nutrients per kg feed in relation to C flux (g/l/d). (ortho-P-P=ortho-phosphate-phosphorus (♦, y=0.48x-0.56, R^2=0.99, NO_3-N=nitrate nitrogen (■, y=2.36x+0.88, R^2=0.89) VSS=Volatile suspended solids (▲, y=26.12x-74.32, R^2=0.98, Crude Protein (x, y=14,48x-18.06, R^2=0.86).

Conclusions

The present study showed that molasses can serve as C source to produce heterotrophic bacteria in suspended growth reactors with a production of up to 168gVSS per kg feed. Strong linear relations have been found for the conversion of inorganic N and ortho-phosphate-P with C fluxes. Inorganic nitrogenous waste and ortho-phosphate were eliminated from the waste stream with an efficiency of 90 and 98%, respectively. Conversions, growth rates and kinetics were comparable to those obtained for sodium acetate in this study and in earlier experiments. Production rates were generally lower than values referred in literature for waste water treatment. It is inferred that increased metabolic costs could explain this. Based on the comparison between molasses and acetate, it is concluded that the production of heterotrophic bacteria biomass on C supplemented fish waste is more dependent on C supplementation levels and resulting nutrient ratios than on the C source. Using RAS as a model system and molasses as easily degradable carbon source, bacteria production rates, nutrient conversions, and related bacteria kinetics could be determined in contrast to pond research, where often only fish or shrimp yield are investigated. Furthermore the present integration of heterotrophic bacteria production in RAS represents an innovative option to reduce waste discharge and to increase system's ecological sustainability. But ecological sustainability will only be achieved if the bacteria biomass is harvested and reused, which will require more attention in the future.

Chapter 6
HRT and nutrients affect bacterial communities grown on Recirculation Aquaculture System effluents

Abstract

In a recirculation aquaculture system the drumfilter separates the solid waste from the system water. Its effluent can be used as substrate for heterotrophic bacterial production, which can be recycled as aquatic feed. Because the produced bacterial biomass might contain pathogens, which could reduce its suitability as feed, it is important to characterize the obtained communities. Bacteria biomass was produced in bacteria growth reactors under different conditions, which affected its composition: 7h hydraulic retention time versus 2h, sodium acetate versus molasses (organic carbon supplement), and ammonia versus nitrate (nitrogen donor). Samples were analyzed by standard biochemical tests, by 16sRNA ribotyping and ribosomal RNA gene-targeted PCR-DGGE fingerprinting combined with clone library analysis. The community of the drumfilter effluent was different from the communities found in the reactors. However, all major community components were present in the effluent and reactor broths. Hydraulic retention times (7h versus 2h, HRT) influenced bacteria community resulting in a more abundant fraction of alpha proteobacterium Bioluz/ *Acinetobacter* at 2h HRT compared to 7h HRT (*Rhizobium*/ *Mezorhizobium*). The use of molasses instead of sodium acetate changed the bacteria community from *Rhizobium*/ *Mesorhizobium* to *Aquaspirillum* as major component. Providing TAN (total ammonia nitrogen) in addition to nitrate as nitrogenous substrate led to the occurrence of bacteria close to *Sphaerotilus*, *Sphingobacterium* and *Jonesia*. It was concluded from those results that a reactor operation regime of 6-7h HRT is recommended, and that the type of substrate (sodium acetate or molasses, TAN or nitrate) is less important, and results in communities with a comparable low pathogenic risk.

Schneider, O., M. Chabrillon-Popelka, H. Smidt, O. Haenen, V. Sereti, E. H. Eding and J. A. J. Verreth (submitted). "HRT and nutrients affect bacterial communities grown on Recirculation Aquaculture System effluents." FEMS Microbial Ecology.

Introduction

In recirculation aquaculture systems (RAS), feed is converted into fish and faecal and non faecal loss. These two waste sources comprise mainly of solid waste, and dissolved waste: ammonia and phosphate. The waste is treated by mechanical filtration to remove the solids and by biofiltration to nitrify ammonia to less hazardous nitrate. The effluent from the mechanical filter is the major discharge of such systems. It comprises solid and dissolved waste. The RAS' effluent is either directly discharged to the environment, or digested in lagoons or septic tanks, or thickened and/or applied as fertilizer for land based agriculture (Chen et al., 1997; Losordo et al., 2003). A possible alternative approach is to convert the waste into heterotrophic bacterial biomass. This biomass can be reutilized as aquatic feed. Such processes are already applied in integrated and activated ponds. In such ponds, waste conversion does not only improve water quality but also feed conversion ratios, because the produced bacteria biomass may be consumed by the fish (Avnimelech et al., 1989; Edwards, 1993; Burford et al., 2003; Hari et al., 2004). To produce bacterial biomass utilizing the effluent stream of the drum filter, a bacterial reactor has to be integrated after the drum filter (Figure 7, page 41). The nutrient ratios in the slurry coming from the filter are normally not ideal for bacteria production. Optimal C: N ratios for heterotrophic bacteria production are about 12-15g:1g (Lechevallier et al., 1991; Henze et al., 1996; Avnimelech, 1999). Fish, which are receiving high protein diets, are producing carbon deficient waste. This is due to the amount of nitrogen, which accumulates in the RAS system water. The resulting C: N ratio in the effluent is 2-3g:1g (Table 21). Therefore, the slurry requires organic carbon supplementation. Sources and levels of carbon supplementation, sludge composition (total ammonia nitrogen (TAN) or nitrate) and sludge and hydraulic retention time (SRT, HRT) are all factors influencing the bacteria community forming the produced biomass. Furthermore, the community composition depends also on the natural autochthonous microbiota from the sludge and system water. If the produced community is re-used as aquatic feed, it is important to evaluate the biomass for potential bacteria pathogens. The first study objective was to characterize the bacterial community in the system water, in the slurry coming from the flow equalizer, and of the produced bacterial biomass in the reactor by standard biochemical tests, by 16sRNA ribotyping and ribosomal RNA gene-targeted PCR-DGGE fingerprinting combined with clone library analysis The effect of different hydraulic retention times and the influence of different carbon and nitrogen sources were evaluated. The second objective was to assess if the produced bacteria biomass contains pathogens, which could reduce its

suitability as feed by comparing determined bacteria strains through comparison with those reported in literature as pathogenic.

Material and Methods

System set up

Two bacteria growth reactors were connected in parallel to a flow equalizer which received the effluent of a screen filter (60µm mesh size, Figure 7, page 41). The screen filter was part of a RAS, which was composed of four culture tanks, a biofilter and two sumps. In the equalizer the slurry was aerated and agitated. The equalizer was integrated into the system to allow for constant waste flows towards the bacteria reactor, because the screenfilter backwashes in pulses, depending on its automated flushing cycle. The HRT of the drum filter effluent in the equalizer was 4h and the drum filter backwash volume about 120-140 l per kg feed.

Fish husbandry

Fish were obtained from a commercial African catfish hatchery (Fleuren and Nooijen, The Netherlands). Fish were stocked initially in four different cohorts of 140 fish each (70g, 170g, 320g, and 560g individual average weight) into the four tanks. Every 28 days the oldest cohort was harvested. The emptied tank was restocked with 140 fish of about 70g. The final fish weight ranged between 823-1038g. Therefore a complete production cycle from 70 to about 1000g lasted 112 days. Fish were fed with commercial feed (Biomeerval, Skretting, France), containing 7% moisture, 49% crude protein, 11% crude fat, 22% carbohydrates, of which 2% crude fiber, 11% crude ash and 1.7% phosphorous (based on manufacturer information). The realized feeding level was between 16 and 19g per kg metabolic body weight ($W^{0.8}$) per day. Diurnal waste fluctuations were minimized by applying a 24h feeding regime. The monthly harvesting/restocking scheme minimized changes in both biomass within the system and in feed load. This stocking and feeding strategy assured minimal fluctuations of waste production during a production cycle.

Bacteria reactors

The reactors were made of glass in the workshop of Wageningen University. The reactors had a working volume of 3.5 liter and were equipped with baffles to improve the hydrodynamics (Figure 8, page 43). From the flow equalizer the slurry was continuously pumped into the bacterial culture reactor by a peristaltic pump (Masterflex L/S, Masterflex, USA). The SRT was equal to the HRT as no sludge was returned. Pure oxygen was diffused

by air-stones to maintain aerobic conditions in the reactor (>2mg/l). Oxygen was monitored online using pH/Oxi 304i meters (WTW, Germany) connected to a PC. This PC controlled then the oxygenation, reacting on a set-point concentration of 3mg/l oxygen inside the broth. pH levels were maintained between 7.0 and 7.2 by addition of acid or base (HCl, NaOH, 0.5-1M) stirred by a pH controller (Liquisys M, Endress-Hauser, Germany). The reactor temperature was 28°C, fixed by a water bath. The reactor was continuously agitated by a rotor (RZR 2102, Heidolph, Germany) and the agitation speed was fixed to 350rpm.

Table 21: Waste composition measured in the influent of the bioreactors. Concentrations as averages ± standard deviation (minimum and maximum). TAN = total ammonia nitrogen, NO_2-N = nitrite-N, NO_3-N = nitrate-N, Kjd-N = Kjeldahl nitrogen corrected for TAN concentrations, TOC = total organic carbon, ortho-P-P = ortho-phosphate phosphorus, TS= total solids, TSS = total suspended solids, VSS = volatile suspended solids.

	Waste Concentration
TAN	1.3±0.8 (0.3-4.8) mg/l
NO_2-N	3.3±1.3 (0.7-12.4) mg/l
NO_3-N	182±58 (76-419) mg/l
Kjd-N	59±43 (13-260) mg/l
TOC	0.4±0.2 (0.1-0.9) g/l
Ortho-P-P	15.1±7.7 (6.2-40.1) mg/l
Ash	1.8±0.7 (0.9-5.0) g/l
TS	3.5±1.0 (1.9-7.3) g/l
TSS	1.5±1.0 (0.2-5.8) g/l
VSS	0.7±0.5 (0.04-2.23) g/l
Conductivity	2000-3000µS/cm

Experimental designs and sampling

In this study, six bacterial communities corresponding to the content of bioreactors which operated under four different conditions were analyzed (Table 22). In addition the communities of the system water and flow equalizer were characterized. To achieve the different culture conditions two flows were combined in the reactor influent: the waste flow containing the fish waste from the flow equalizer and the supplement flow containing the three organic C supplements. In the fourth operation condition, TAN was added to the supplement flow. The supplements were mixed with distilled water and pumped by a peristaltic pump (PD5001, Heidolph, Germany) into the reactors at a flow rate which was about 5% of the total flow rate. These experimental conditions allowed comparing the effects of different HRTs, different C sources, and different N sources. Because bacteria prefer TAN above nitrate as nitrogen source, the effect of those two nitrogen sources could be investigated. Nitrate was available from the RAS effluent stream, but it was decreasingly taken up by the bacteria in the presence of increasing TAN concentrations. A more detailed

description of the experiments is provided in Schneider et al., (a,b,c) submitted. From the three sampling points (system water at the fish tanks influent, flow equalizer and bacteria reactor), samples were siphoned and either analyzed as aequous samples (50ml), or sample material was collected over time (10.5 l) and centrifuged at 10000 rpm for 20min (Table 22). The supernatant was discarded, and the solid fraction was freeze dried.

Table 22: Sample scheme for the four experimental conditions. Volumes are representing the original sample volume.

Sample	HRT (h)	Sample-ID	Biochemical analysis & 16SrRNA gene ribotyping	DNA isolation & PCR amplification
System Water		1	aqueous sample (50ml)	---
Equalizer		2	aequous sample (50ml)	lyophilized (10.5 l)
1.7gC/l sodium acetate	7h	3	aequous sample (50ml)	lyophilized (10.5 l)
1.7gC/l sodium acetate	2h	4	aequous sample (50ml)	lyophilized (10.5 l)
2.5gC/l molasses	6h	5	---	aequous sample (50ml)
1.7g/l sodium acetate plus 250mg/l TAN	6h	6	---	aequous sample (50ml)

Isolation and biochemical and 16S rRNA gene ribotyping of cultured bacteria

Aequous samples (1-4) were homogenized, and each homogenate was inoculated onto Brain Heart Infusion (BHI) agar with 5% sheep blood (home made at CIDC-Lelystad, The Netherlands), and in parallel onto Cytophaga agar (Oxoid), and incubated at 22ºC for five to seven days. After bacterial growth occurred, morphologically different colonies were randomly selected for further typing in a pure plate culture. These were cultured to a monoculture, using BHI with 5% sheep blood and identified according to standard biochemical tests (Bergey, 1984; Austin and Austin, 1987; Barrow and Feltham, 1993). If identification was not possible by these conventional methods, further typing was done by molecular methods, using the Microseq 500, 16srDNA bacterial identification kits (Applied Biosystems, USA), according to the method provided by the manufacturer.

DNA isolation and PCR amplification for molecular characterization of bacterial communities

In case of molecular analysis, DNA was isolated with the Fast DNASPIN kit (for soil, QBIOgene, Cambridge, United Kingdom). Briefly, 0.1g from each sample were placed in Lysing Matrix E Tubes with 122 µl of MT buffer and 978µl of PBS and processed three times for 30 seconds at setting 5.5. The rest of the protocol was carried out according to the manufacturer's instructions. PCR was performed with *Taq* polymerase kit (Invitrogen, Carlsbad, CA, USA) with the universal primer set 0968-a-S-GC-f (5'-

AACGCGAAGAACCTTA-3') and S-D-Bact-L1401-a-A-17 r (5-CGGTGTGTACAAGACCC-3') (Nübel et al., 1996), which amplify the V6 to V8 regions of the eubacterial 16S rRNA gene. The first primer has a 40 nucleotide GC rich sequence at the 5' end (CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G), which allows the detection of sequence variations of amplified DNA fragments by subsequent denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). Each PCR reaction mixture contained (final volume, 50µl) 20mM Tris-HCl (pH 8.4), 3mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.2mM, each primer at a concentration of 0.2µM, 1.25U of *Taq* polymerase, and 1µl of template DNA. Samples were amplified in a Whatman Biometra Thermocycler (Göttingen, Germany) using the following program: predenaturation at 95°C for 2min; 35 cycles of denaturation 95°C for 30s, annealing at 56°C for 40s, and extension at 72°C for 1min; and a final extension at 72°C for 5min. PCR products were verfied by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide.

DGGE analysis

Amplicons were separated by DGGE based on the protocol of Muyzer and Smalla (1998) using the Decode system (Bio-Rad Laboratories, Hercules, USA) with the following modifications. The polyacrylamide gels consisted of 8% (vol/vol) polyacrylamide (ratio of acrylamide to bisacrylamide: 37.5:1) and 0.5x Tris-acetate-EDTA buffer (pH 8.0). Denaturing acrylamide of 100% was defined as 7M urea and 40% formamide. The polyacrylamide gels were prepared with denaturing gradients ranging from 30 to 55% to separate the generated amplicons of the total bacterial communities. The gels were poured from the top using a gradient maker and a pump (Econopump; Bio-Rad Laboratories, Hercules, USA) set at a rate of 4.5 ml/min. Prior to polymerization of the denaturing gel (gradient volume, 28 ml), a 7.5ml stacking gel without denaturing chemicals was added. Electrophoresis was performed first for 5min at 200V and then for 16h at 85V in 0.5x Tris-acetate-EDTA buffer (pH 8.0) at a constant temperature of 60°C. The gels were stained with $AgNO_3$ according to the method of Sanguinetti et al. (1994) and dried overnight at 60°C. Gels were scanned at 400DPI, and analyzed with gel analysis software (Bionumerics 4.0, Applied Maths, USA).

Cloning of the PCR-amplified products

16S rRNA gene-targeted PCR amplicons (1500bp) were generated with the set of primers 27-f (5-GTTTGATCCTGGCTCAG-3) and S-D-Bact-1492-a-A-19 r (5-CGGCTACCTTGTTACGAC-3) (Lane, 1991) and were purified with NucloeSpin Extract II (Macherey-Nagel, The Netherlands) according to the manufacturer's instructions. PCR

products were cloned into *E. coli* XL1-Blue competent cells (Stratagene) using the Promega pGEM-T easy vector system (Promega, Madison, Wis.). Ligation and transformation reactions were performed according to the protocol described by the manufacturer. PCR was performed on cell lysates of ampicillin- resistant transformants by using vector specific primers T7 (TAATACGACTCACTATAGG) and Sp6 (GATTTAGGTGACACTATAG) to confirm the size of the inserts. A total of 96 amplicons of the correct size (per sample) were subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) using the restriction enzymes *Msp*I, *Cfo*I, and *Alu*I. From each sample, clones corresponding to a unique RFLP pattern were used to amplify V6-V8 regions of 16S rRNA genes with the primers 968f-GC-f and 1401r as described previously, and they were selected for subsequent sequence analysis according to their migration position in the DGGE gel compared to the amplicons of the original DGGE profile of the sample.

Sequence analysis

PCR amplicons (1.4 kb) of transformants selected by the above described ARDRA/DGGE screening procedure were purified with NucloeSpin Extract II (Macherey-Nagel, The Netherlands) according to the manufacturer's instructions. The samples were subjected to DNA sequence analysis (BaseClear Lab services, The Netherlands) with the primers SP6 and T7, yielding two partial sequences (5' and 3') per clone of ca. 500 nucleotides. Sequences were analyzed for similarity with sequences deposited in public databases using the BLAST tool (McGinnis and Madden, 2004) at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST). Alignment and further phylogenetic analysis of the sequences were performed using the ARB software package (Ludwig et al.. 2004). All sequences were added to the universal phylogenetic tree of the ARB database (release from February 2005) using the Maximum Parsimony procedures as implemented in ARB. Chimeric sequences were identified by comparison of phylogenetic affiliation of the two respective 5'- and 3- partial sequences.

Results

Isolation and biochemical and 16S rRNA gene ribotyping of cultured bacteria

The results from the biochemical and 16S rRNA gene ribotyping for the system water, the equalizer and different reactor broths are given in Table 23. While the system water and the flow equalizer contained five and seven different bacteria, only four and three different bacteria were detected in the samples.

Table 23: Results from the biochemical and 16S rRNA gene typing for the system water, the equalizer and different reactor broths. (C= carbon, HRT= hydraulic retention time). Method 1= Biochemical procedure; 2= 16S rRNA gene ribotyping.

	System water	Equalizer	1.7gC/l, HRT 7h	1.7gC/l, HRT 2h	% of matching (homology) by ribotyping	Method
Sample ID	1	2	3	4		
Bacillus sp.		+			---	1
Edwardsiella sp.		+			99	2
Proteus vulgaris		+			---	1
Aeromonas hydrophilia	+	+		+	---	1
Aeromonas sobria	+		+		---	1
Acinetobacter Iwoffi				+	---	1
Pseudomonas sp.			+	+	---	2
Comamonas sp.		+			99	2
Arcobacter butzlerii/sp.		+	+		99	2
Chryseobacterium sp.	+				100	2
Flavobacterium sp.					---	1
Myroides sp.	+	+	+		98 and 93	1,2
Sphingobacterium sp.	+				99	2

Molecular analysis of bacterial community structure for molecular characterization of bacterial communities

The phylogenetic affiliations of the clones corresponding to prevalent bands in the DGGE sample profile were determined by sequence analysis (Figure 23, Figure 24, Table 24). In the flow equalizer (sample 2), the predominant bands corresponded to sequences most closely related to *Sarcina, Flavobacterium* and *Rhodobacter* sp. (bands 2, 4, 5). Unfortunately, clones corresponding to bands 1 and 3 were found to be chimeric, prohibiting unambiguous identification. Nevertheless, partial sequences corresponding to the V6-V8 region used for DGGE analysis were most closely related to *Clostridium* (band 1) and *Salinococcus* (band 3), suggesting that both dominant populations belong to the low G+C Gram positive bacteria. In sample 3 (1.7gC/l sodium acetate, 7h HRT) and in sample 4 (1.7gC/l sodium acetate, 2h HRT) similar profiles were found. In sample 3 the microbial community consisted mainly of *Rhizobium/ Sinorhizobium/ Mesorhizobium* – related populations, and to a lesser extend bacteria related to *Acinetobacter lwoffi* and Gamma proteobacterium Bioluz, while in sample 4, the most predominant/abundant population was the *Acinetobacter lwoffi/* Gamma proteobacterium Bioluz – related population. *Rhizobium/*

Sinorhizobium/ Mesorhizobium were only detected as minor community components. In sample 5 (2.5gC/l molasses, 6h HRT), the most abundant bacteria was most closely related to *Aquaspirillum serpens. Rhizobium/ Sinorhizobium* was also present. The other bands have not been identified. In sample 6 (1.7gC/l sodium acetate, 250mgTAN/l and 6h HRT), the main identified components of the microbial community were populations related to *Jonesia quinghaiensis*, *Sphaerotilus* and *Sphingobacterium*. The phylogenetic relations between the detected bacteria and their closed matches are displayed in Figure 24.

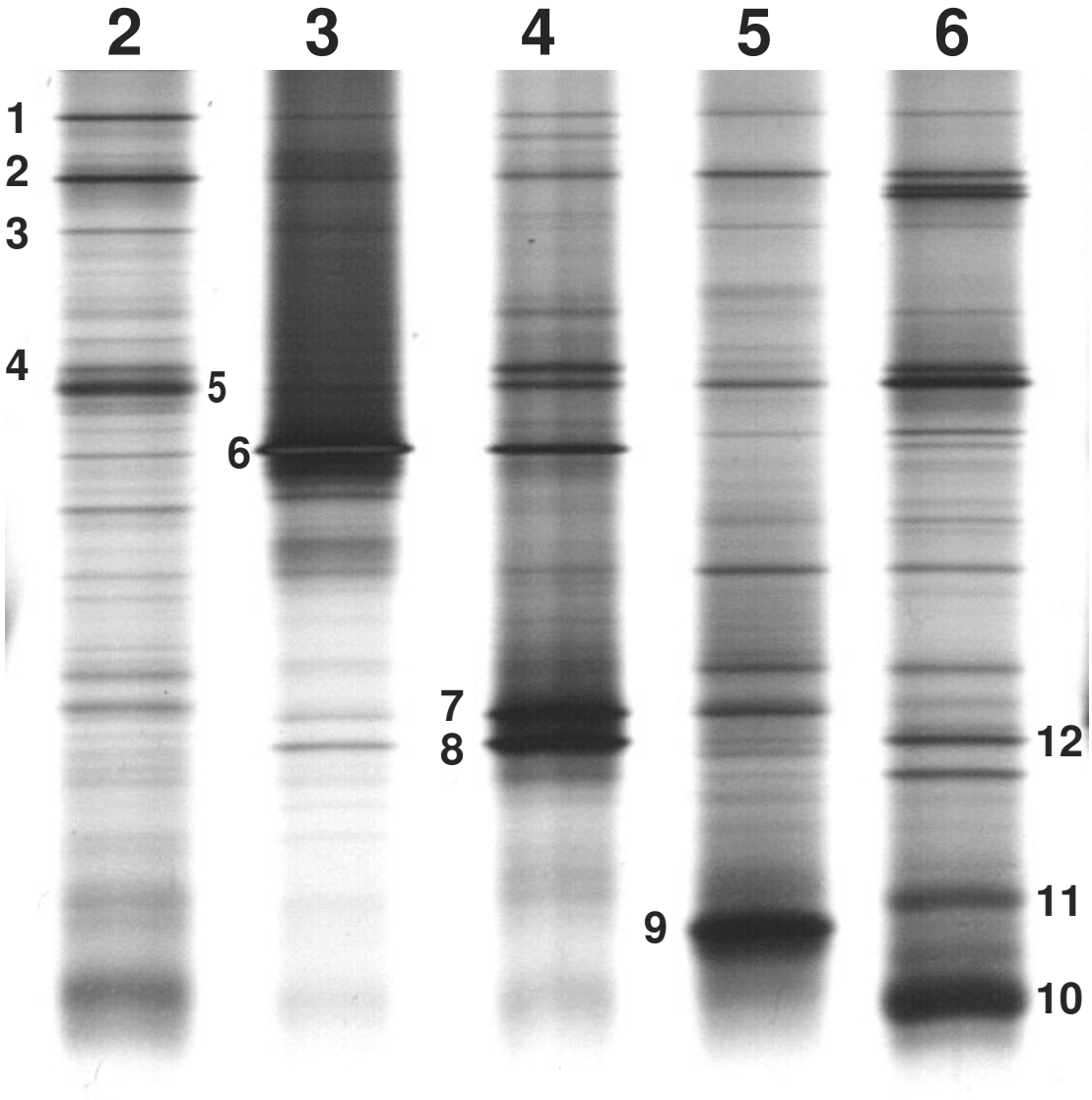

Figure 23: 16S rRNA gene-targeted PCR-DGGE analysis of bacterial communities in samples 2-6. 2= Equalizer; 3= 1.7gC/l sodium acetate, 2h HRT; 4= 1.7gC/l sodium acetate, 7h HRT; 5= 2.5gC/l molasses, 6h HRT; 6= 1.7g/l sodium acetate, 250mg/lTAN, 6h HRT. Identification of bands was done by DGGE analysis of clones. *1.chimeric,* 2. *Sarcina sp., 3.chimeric* 4.*Flavobacterium sp.,* 5.Uncultured freshwater Gram –bacterium, close to *Rhodobacter,* 6.gamma proteobacterium Bioluz, *7.Mesorhizobium 8.Rhizobium/Sinorhizobium, 9.Aquaspirillum serpens* 10.*Jonesia quinghaiensis 11.Sphaerotilus, 12. Sphingobacterium sp.*

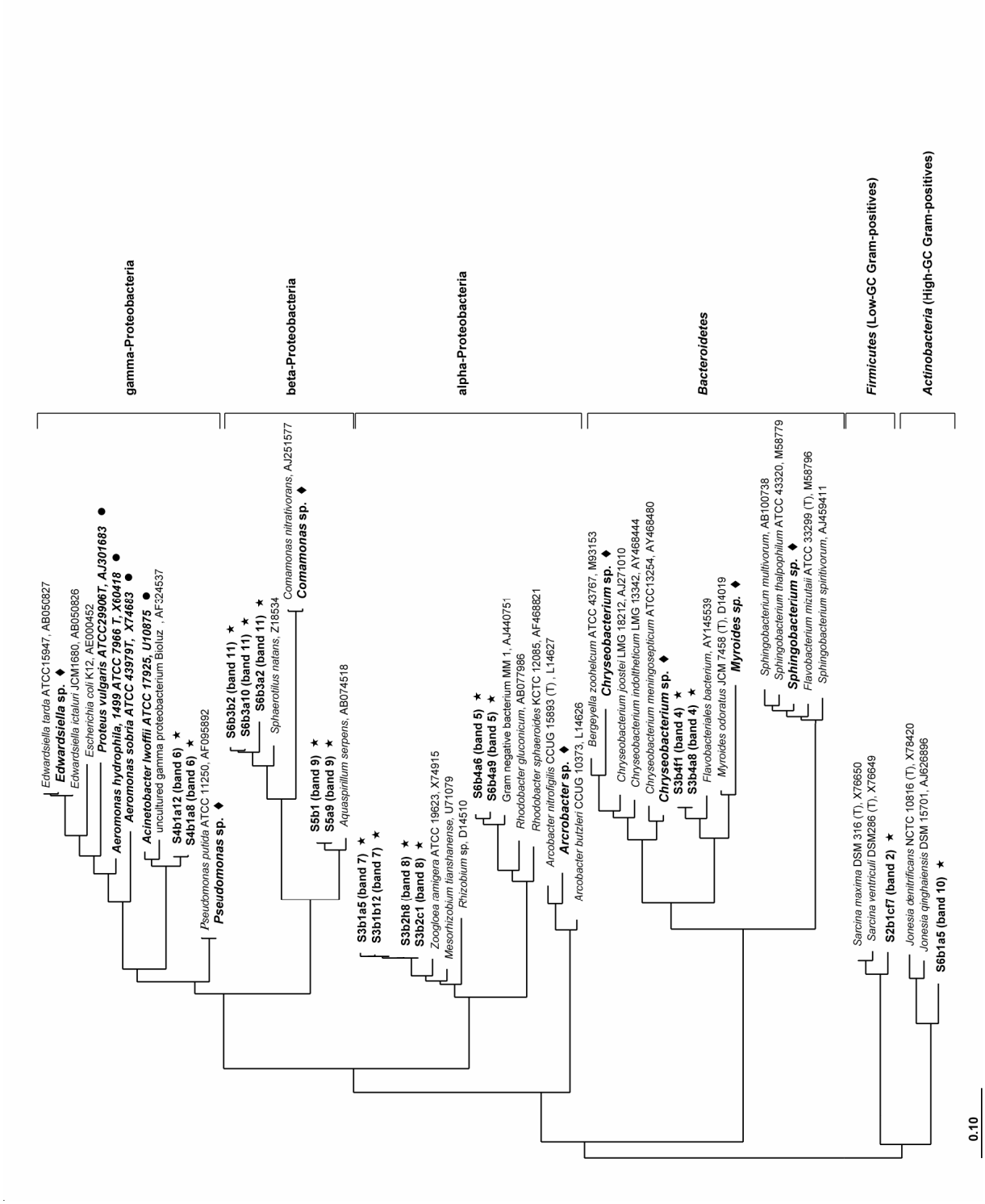

Figure 24: Phylogenetic tree of bacterial 16S rRNA sequences retrieved from the different samples and cultured isolates (♦ 16sRNA ribotyping, ● biochemical procedures, * PCR-DGGE). Sequences obtained in this study were added to a backbone tree of reference sequences by maximum parsimony procedures, using a bacterial filter, as implemented in ARB (Ludwig et al., 2004). Accession numbers of reference sequences are given in parentheses. The reference bar indicates 10% sequence divergence.

Table 24: Results from the DNA isolation and PCR amplification for the equalizer and different reactor broths. Named bacteria are the closet match to the analyzed sequences. + = identified as present in the sample, (+) = presence concluded from band similarity. (C= carbon, HRT= hydraulic retention time).

	Equalizer	1.7gC acetate/l HRT 7h	1.7gC acetate/l HRT 2h	2.5gC/l Molasses, HRT 6h	1.7gC/l, 250mgTAN/l, HRT 6h	Band
Sample ID	2	3	4	5	6	
Flavobacterium sp.	+	(+)	(+)	(+)	(+)	4
Gamma proteobacterium Bioluz		(+)	+	(+)	(+)	6
Sphaerotilus					+	11
Aquaspirillum serpens				+		9
Mesorhizobium		+	(+)	(+)	(+)	7
Rhizobium/Sinorhizobium (Zooglea)		+	(+)			8
uncultured fresh water bacterium, close to *Rhodobacter*	+	(+)	(+)	(+)	(+)	5
Sphingobacterium sp.					+	12
Sarcina sp.	+	(+)	(+)	(+)	(+)	2
Jonesia quinghaiensis					+	10

Discussion

The integrated application of complementary cultivation-dependent and biomolecular approaches allowed for the qualitative and semi-quantitative comparison of the bacteria communities present in the system water and the flow equalizer, and those that developed in bioreactors operated at four different conditions.

In general, only a limited number of bacterial populations were identified that were common to both system water and the flow equalizer. Examples were *Aeromonas* and *Myroides*. RAS configuration might have caused such differences in the two bacteria communities. The drum filter effluent originates from water with a higher organic waste load than the tank influent water, which was treated with the drum filter. This treatment can reduce the COD load in the system water with 50% (own unpublished data). This reduction affects bacteria numbers, namely by removal of those populations which grow in flocks and on solid particular waste, and of substrate, which are no longer available for bacteria growth. The

bacterial strains, found in the system water and the flow equalizer, contained pathogens at different levels (Table 23, Table 24 and Table 25). Despite the fact that pathogens were detected, during all experiments fish was healthy and performing well. The pathogenic bacteria had, therefore, no visible negative impact on fish health. In general, the bacteria found in the system water and flow equalizer are typical for aquatic, fish farm and wastewater environments (Table 25).

Table 25: List of the found or of those bacteria close to the found strains in the different samples, their habitat and growth conditions, their pathogenicity and the related references.

Bacteria	Habitat & growth conditions	Pathogenicity focusing on animals and fish	Reference
Bacillus sp.	saprophytic waste water, paper mill slime	some strains some strains, f.i. *Bacillus cereus* (in carp and striped bass), *Bacillus mycoides* (in channel catfish), and *Bacillus subtilis* (in carp)	Weber, 1997 Austin and Austin, 1999 Tchobanoglous et al., 2003 Oppong et al., 2003
Edwardsiella sp.	23-28 ºC aquatic habitats and especially fish, amphibians, reptiles, and birds	Some fish pathogenic enterobacteria: *Edw.tarda (*eel*),* *Edw.ictaluri* (channel catfish), different effects on various species, reaching from fatal to none	Austin and Austin, 1987 Abbott and Janda, 2001
Proteus vulgaris	saprophytic soil, water, integral part of gut flora	only few indication	Manos and Belas, 2001 Weber, 1997 Austin and Austin, 1987 Tanaka et al. 2004
Aeromonas hydrophilia	facultative anaerobic, 4-37ºC different salinities aquatic habitats, waste water found frequently at fish farms	facultative opportunistic found as well on healthy fish	Meyer-Reil and Koester, 1993 Weber, 1997 Austin and Austin, 1987 Kinne, 1984 Rice et al., 1984 Leonard et al., 2000
Aeromonas sobria	facultative anaerobic 4-37ºC different salinities aquatic habitats, waste water frequently on fish farms	facultative opportunistic or not necessarily attributed as pathogenic found as well on healthy fish	Meyer-Reil and Koester, 1993 Weber, 1997 Austin and Austin, 1987 Kinne, 1984
Acinetobacter Iwoffi	aerobic 20-30ºC different salinities	Facultative opportunistic , few indications	Meyer-Reil and Koester, 1993 Austin and Austin, 1987

Bacteria	Habitat & growth conditions	Pathogenicity focusing on animals and fish	Reference
	soil, aquatic habitats, waste water frequently on fish farms		Wagner and Loy, 2002 Fang et al., 2002 Rice et al., 1984
Pseudomonas sp.	Mesophilic temperatures Different salinities soils, water, sewage, animals, plants	Facultative opportunistic, or pathogenic: f.i. *Pseud.anguilliseptica* (in eel , sea bream and sea bass)	Austin and Austin, 1999 Palleroni, 1999 Adamse, 1968
Sphaerotilus sp.	aerobic/anaerobic Freshwater sludges, waste water	not reported	Pasveer, 1968 Adamse, 1968 Schonborn, 2003 Spring, 2002
Comamonas sp.	aerobic 20-37ºC waste water, activated sludge, animals' blood	rare opportunistic pathogens, no evidence of pathogenic effect on healthy people	Etchebehere et al., 2001 Gumaelius et al., 2001 Willems and de Vos, 2002
Aquaspirillum serpens (sp.)	aerobic different salinities denitrification reactors as well in marine recirculation systems	not reported	Thomsen et al., 2004 Payne, 1981 Tal et al., 2003 Pot et al., 1999
Rhizobium / Mesorhizobium sp.	facultative aerobic soil, denitrification reactors, culturable on wastewater sludge, aquatic systems, denitrification reactors	not reported	Payne, 1981 Batut and Boistard, 1994 Encarnacion et al., 1995 Rebah et al., 2001 O'Hara and Daniel, 1985 Sadowsky and Graham, 2000 Liu et al., 2005 Etchebehere et al., 2002
Zooglea ramigera	Aerobic Aquatic systems, domestic sewage and aerobic sewage-treatment systems	not reported	Dugan et al., 1999 Kargi and Karapinar, 1995
uncultured fresh water bacterium, close to *Rhodobacter* sp.	Fresh to salt water marine sludge	not reported	Cytryn et al., 2005 Cytryn et al., 2005 Kersters et al., 2003
Arcobacter butzlerii & sp.	aerobic 15ºC-37ºC gut fauna, surface & ground waters	possibly involved	Tanaka et al., 2004 Lehner et al., 2005 Moreno et al., 2003

Bacteria	Habitat & growth conditions	Pathogenicity focusing on animals and fish	Reference
	sewage and activated sludge		
Chryseobacterium sp.	aerobic different salinities soil, plants, aquatic habitat, activated sludge	Pathogenic, *f.i. Chr. scophthalmum* (in turbot), *Chr. balustinum* (in marine fish)	Urdaci et al., 1998 Austin and Austin, 1999 Jooste and Hugo, 1999 Mustafa et al., 2002 Bernardet et al., 2005 Bernardet and Nakagawa, 2000
Flavobacterium sp.	aerobic 5-42ºC salinity below 1% soil, aquatic habitat frequently at fish farms	Facultative, mostly found externally, may induce skin necrosis after stress found as well on healthy fish, some species are very pathogenic	Meyer-Reil and Koester, 1993 Murray et al., 1990 Austin and Austin, 1987 Kinne, 1984 Bernardet et al., 2005 Bernardet and Nakagawa, 2000
Myroides sp.	Aerobic 25-30 ºC Human intestine, soil, water	opportunistic	Gonzalez et al., 2000 Hugo et al., 2000
Sphingobacterium sp.	aerobic soil, activated sludge, gut fauna, liquid swine manure	not reported	Tanaka et al., 2004 Leung and Topp, 2001 Holmes, 1999
uncultured bacterium, close to *Sarcina ventriculi*	obligate anaerobic, but not oxygen sensitive 30-37 ºC Gut fauna	not reported	Goodwin and Zeikus, 1987 Jung et al., 1993 Snell-Castro et al., 2005
Jonesia quinghaiensis	aerobic 20-30 ºC different salinities mud	not reported	Schumann et al., 2004

The communities obtained from the bacteria reactor for the four different operation conditions were different from the community of the flow equalizer. For the bacteria determined with biochemical and 16S rRNA gene ribotyping only *Arcobacter* and *Myroides* were found in both the flow equalizer and in one reactor broth sample (sample 3). All bacteria present in the equalizer were also present in the reactor broth (Table 24). However, the major community components in the reactor were composed of other populations, which were not found in the equalizer. HRT seemed to have a minor effect on the bacterial community as is shown by the results of sample 3 and 4, which differed only in their HRT (7 versus 2h).

However, in sample 3, alpha-proteobacterial populations close to *Rhizobium/ Mesorhizobium/ Sinorhizobium* and *Zooglea* were the major community components. In sample 4 (2h HRT) the gamma proteobacterium Bioluz/ *Acinetobacter*-relative was the major component. This suggests that *Rhizobium/ Mesorhizobium/ Sinorhizobium* and *Zooglea* were out-competed at this low HRT. This corroborates data from Singleton et al. (1982), who reported growth rates for *Rhizobium* as 0.7-0.2 h^{-1} and 0.4–0.2 h^{-1} for water conductivities of 1200 and 6000µS/cm, respectively. The experimental conditions were in between this range (2000-3000µS/cm). In contrast, *Acinetobacter* grown on sodium acetate has higher growth rates of 0.2 to 0.8 h^{-1} at 25ºC compared to the high conductivity conditions (Oerther et al., 2002). Unfortunately water conductivity was not reported. To grow at a HRT of 2h a growth rate of at least 0.5h^{-1} is required, which is out of range for *Rhizobium* at high conductivities. Shorter HRT (e.g. 2h compared to 7h) might therefore bear the risk to culture mainly potentially pathogenic bacteria. A similar community as for sodium acetate (sample 3) was obtained for the reactor using molasses as substrate (sample 5). The major difference was a community shift from strains close to *Rhizobium/ Mesorhizobium* to those close to *Aquaspirillum serpens*, which was not detected as major component in sample 3. Such changes can occur, because both bacteria are utilizing similar substrates and can grow under similar conditions (Table 25). Furthermore was the molasses not sterile and bacteria other than those existing in the system environment might have been introduced. Whether the bacteria, close to *Aquaspirillum,* were superior to *Rhizobium/Mesorhizobium* in cultures with molasses as C donor as indicated by our results, has nevertheless not been reported elsewhere. When TAN was applied in addition to sodium acetate, the bacteria community changed significantly (sample 3 and 6). Nearly all bacteria, which were detected in sample 3 were also present in sample 6, but another three were also found in sample 6. These bacteria were close to *Sphaerotilus*, *Sphingobacterium* and *Jonesia* (Figure 24). For these three bacteria no pathogenicity has been reported (Table 25). *Sphingobacterium* grows well on swine manure, where TAN is a major N source (Leung and Topp, 2001). Furthermore, *Sphaerotilus* and *Jonesia*-related populations have been found in wastewater and mud (Table 25). All three might be then superior to *Rhizobium/ Mesorhizobium* in the utilization of TAN, resulting in higher growth rates.

Given the pathogenic risk associated with short HRTs, it is advisable to choose for HRTs of 6 to 7h. The choice of organic C donor seems of less importance, as the obtained communities in the presence of sodium acetate or molasses, respectively, did not change in their pathogenicity. Moreover, the addition of TAN did not increase the risk of potentially pathogenic populations, as revealed by the comparison of samples 3 and 6. Two

considerations have to be made: The “native” N source comprised in the RAS effluent stream is nitrate. To utilize this N species, the system design did not change and the reactor can easily be installed after the drum filter. If TAN should be used, the system would have to be modified to eliminate nitrification. The only advantage to use TAN might then be caused by a potentially higher nutritional value of the obtained bacteria biomass. This advantage would have to be confirmed by additional experiments. Generally, the pathogenic risk and nutritional value of all obtained bacterial material has to be further investigated in feeding trials, if the bacteria biomass should be used as aquatic feed. To compare the occurrence of bacteria found in the system water, the flow equalizer and in the bacteria reactor with bacteria found in RAS in general is difficult, because literature data is scarce. Because no biofilter material was investigated in this study, bacteria belonging to the nitrifying community were not identified. Those bacteria have been found in other studies, focusing on the system as a whole by investigating its components (Tal et al., 2003, Cytryn et al., 2005). Investigations of heterotrophic bacteria communities yielded some similar results, for e.g. *Pseudomonas*, *Aeromonas*, *Aquaspirillum* and others (Leonard et al., 2000; Tal et al. 2003). Anyway, it is unlikely to find complete identical bacteria communities in RAS, because of differences in their environmental conditions (marine versus freshwater), configurations (e.g. presence of UV, foam fractionators), and in the cultured animals.

Conclusion

The bacteria community found in the system water and in the flow equalizer contained some possible opportunistic pathogens, but did not result in severe disease symptoms or production losses during the fish culture operation. The community of the flow equalizer was semi-quantitatively different from the communities found in the bacteria reactors. However, all major community components were present in both equalizer slurry and reactor broths. Hydraulic retention times (7h versus 2h) influenced bacteria community resulting in a more abundant fraction of the potentially pathogenic alpha proteobacterium Bioluz/ *Acinetobacter* at 2h HRT compared to 7h HRT. At 7h bacteria close to *Rhizobium/ Mezorhizobium* were forming the major components of the community. The use of molasses instead of sodium acetate changed the bacteria community from *Rhizobium/ Mesorhizobium* to *Aquaspirillum* as major component. Providing TAN in addition to nitrate as nitrogenous substrate led to the occurrence of bacteria close to *Sphaerotilus*, *Sphingobacterium* and *Jonesia*. It was concluded from those results that a reactor operation regime of 6-7h HRT is recommended, and that the type of substrate (sodium acetate or molasses, TAN or nitrate) is less important. Considering

conventional RAS configurations, nitrate might be preferred over TAN. However for all the obtained bacteria communities, additional tests are required to investigate their pathogenic risk and nutritional values as aquatic feed in more detail.

Chapter 7
Bacteria or commercial diet: The preferences of *Litopenaeus vannamei*

Abstract

In this study, the produced bacteria biomass was fed to shrimps (*Litopenaeus vannamei*). In total three different diets were used in a variance of a T-maze test: a commercial shrimp feed, the bacteria biomass, which was produced in the suspended growth reactors on C supplemented fish waste under conditions, comparable to those reported in chapter 3, and slurry, which was anaerobically produced in a denitrification reactor. If the bacteria products would be attractive as diet, the nutrient retention of the RAS would be improved, resulting in a system, combining fish, bacteria and shrimp. The diet preference was interpreted as an expression of diet attractiveness. As a first result, shrimp were moving from an equal distribution before feeding (+/-50%, -2min), towards the feeding places (>50%, 2, 5, and 10 minutes after feeding). It was, therefore, reasoned, that all bacteria biomass and commercial feed combinations were basically attractive for the shrimp. This response was continuing and not limited to an instantaneous reaction. After feeding (2min) more than 80% of the shrimp were present at the feeding places and showed a significant preference for the commercial feed compared to the aerobically produced bacteria slurry. For the other diet combinations no significant differences could be detected for 2min. For 5 and 10min after feeding, shrimp behavior changed from the commercial feed to the aerobically and anaerobically produced bacteria biomass segments. It was concluded from this study that the bacteria slurries had attracted the shrimps, that the commercial diet was preferred above the aerobic slurry. There was no unambiguous conclusion to be made regarding the preference for aerobic or anaerobic produced slurry.

Schneider, O., T. L. Cong, V. Sereti, J. W. Schrama, E. H. Eding and J. A. J. Verreth (2006). "Bacteria or commercial diet: The preferences of Litopenaeus vannamei." Aquaculture Research 37: 204-207.Short Communication

In ponds, shrimp may not only eat the provided diet but also heterotrophic bacteria, derived from natural production (Tacon et al., 2002; Burford et al., 2004). Obviously such bacteria are easily obtained in pond systems, but they also occur in recirculation systems. In the latter systems, bacteria can be produced on solid waste derived from the fish; either aerobically using bacteria growth reactors (Schneider et al., 2003), or anaerobically in denitrification reactors (Eding et al., 2003). However, it is not clear whether such bacteria are attractive as diet for shrimp. Diet attractiveness is one of the factors determining whether the diet will be consumed and to which extend it will be consumed. If these bacteria products would be attractive as diet, the nutrient retention of the culture process would be improved, resulting in a system, combining fish, bacteria and shrimp. Adopting a behavioral model (Figure 25), diet preferences can be interpreted as an expression of diet attractiveness (Lee and Meyers, 1996). The objective of this study was to evaluate the attractiveness of bacteria slurry as diet compared to a commercial shrimp diet by scoring diet preference.

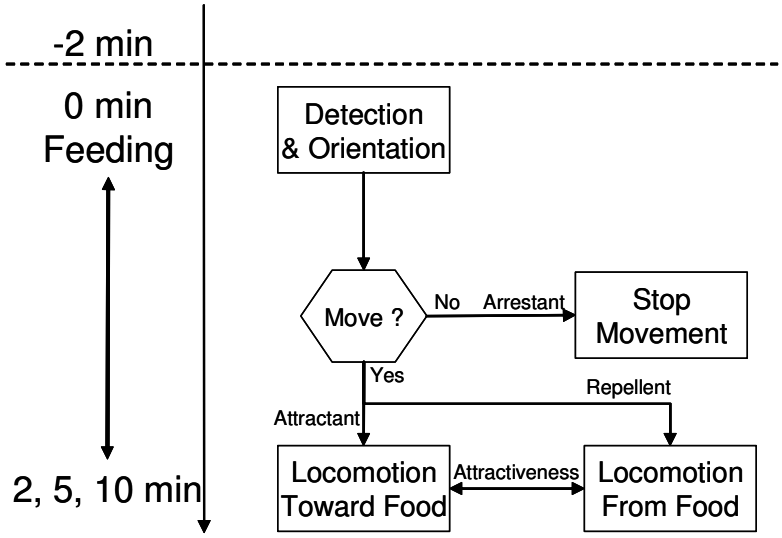

Figure 25: Feeding model for classifying crustacean chemical stimuli including a time axis illustrating the relation of feed timing and shrimp reaction (modified after Lee and Meyers, 1996).

Litopenaeus vannamei, were obtained from a farm, located in Germany. One week after arrival, shrimps with an initial weight of 6.7g +/- 0.3g were divided at random among 6 aquaria (45*90*45cm, 180 l) with an initial density of 5 shrimps per aquarium. These aquaria were connected to a recirculation system comprising aquaria, a sedimentation unit, a submerged biofilter, UV units, and a pump sump. Illumination was based on red light (12L: 12D). Shrimp were adapted to the experimental diets and feeding level during four days before diet preference scoring started. The water quality during the experimental period was:

temperature 27.5±0.4^{0}C, salinity 21±0.3‰, oxygen 6.8±1.3mg/l, pH 7.8-8.3, total ammonia nitrogen 0.05±0.03mg/l, nitrite nitrogen 0.8±2.1 mg/l and nitrate nitrogen 29.7±6.4mg/l.

The experimental diets consisted of a commercial diet (Marico Crumble premium EX 3.0mm, Coppens International, Helmond, The Netherlands) and two bacteria slurries. (Table 26). One slurry was produced aerobically in suspended bacteria growth reactors (Schneider et al., 2003), the other one was produced anaerobically in a denitrification reactor (Eding et al., 2003). These two slurries were selected as they are the products of two conventional processes, which can be integrated in a recirculation system to utilize solid fish waste. A difference in the shrimp's behavioral response toward these two bacteria slurries was expected, because of differences in their production process. The slurries were centrifuged, squeezed through a 40µm net and vacuumed to increase dry weight and to obtain a sinking paste. Afterwards the slurries were stored at -20°C. The slurries were not processed into a pellet directly comparable to the commercial pellet as by drying and processing, volatile substances might have been lost, which might influence shrimp behavior.

Table 26: Feed and slurry dry weight, crude protein, ash and energy content.

	Dry weight g/kg wet weight	**Crude protein** g/kg dry weight	**Ash** g/kg dry weight	**Energy** MJ/kg dry weight
Commercial diet (CMF)	920	624	113	23
Aerobically produced slurry (SCPA)	49	600	200	16.5
Anaerobically produced slurry (SCPAN)	90	419	250	15.6

In the diet preference test three diet combinations were tested: 1) aerobic produced bacteria (SCPA) and commercial diet (CMF), 2) anaerobic produced bacteria (SCPAN) and CMF and 3) SCPA and SCPAN. Six aquaria were randomly assigned to one of the three diet combinations, each combination in two replicates. In each aquarium, both diets were given simultaneously but each at another feeding place located in opposite corners at the same aquarium front end (Figure 26). Petri dishes were used as feeding places. Shrimp were fed by hand twice a day at 9am and at 4pm. Diet was administrated through tubes, which were mounted above the feeding places. The feeding ratio was fixed at 0.25g dry weight feed/shrimp per day. As a result of diets' dry matter content SCPA/CMF and SCPAN/CMF was given in a weight/weight ratio of 30:70, and 40:60 for SCPA/SCPAN. Five minutes prior feeding the aquarium aeration and water inflow was stopped and restarted after the observation period. This prevented that soluble attractants would have been spread over the system and would have influenced shrimp behavior across tanks. The total period without water flow was 15min. During this period, shrimp were scored 2 minutes before, and 2, 5 and

10 minutes after feeding on their presence in one of the four aquaria segments. The presence of shrimp in the one or other segment was scored as % of overall presence (Figure 26). The experiment lasted 42d, including the adaptation period to diet and feeding level of 4d. After 21d the diets within a treatment switched feeding place to avoid data bias. The first observation period was therefore 17d and the second 21d. Overall diet attractiveness was evaluated using ANOVA and Tukey's Post hoc test ($p<0.05$). Observations were averaged by aquaria and aquaria were then subsequently treated as experimental units. In contrast to this analysis, diet preference for an individual diet was evaluated using the Wilcoxon Signed Rank Test ($p< 0.05$, Field, 2000; SPSS 11.5) because for this test single observations were analyzed, which were repeated and not independent.

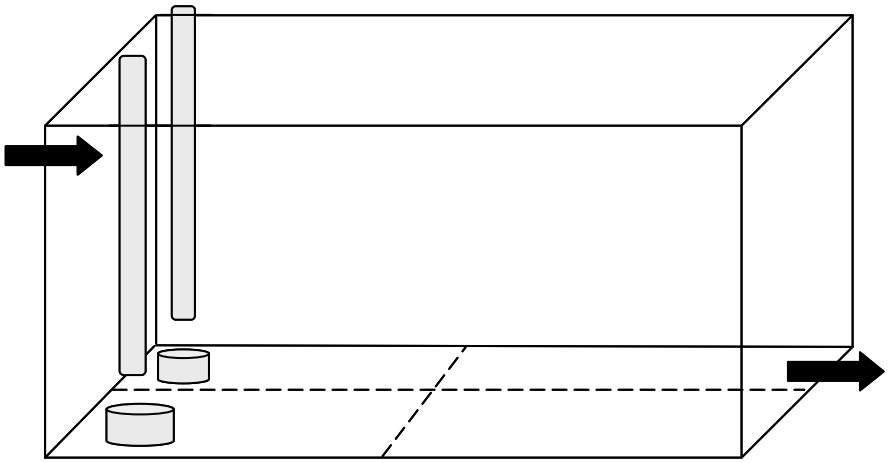

Figure 26: Schematic overview of an aquarium and its division into 4 segments. In two segments the round feeding places were located, which had tubes mounted above to drop the sinking feed on the feeding place. Arrows are marking the water flow direction from inlet to outlet. The outlet is simplified, as an outlet in U form was used, taking out the water at the bottom of the aquaria.

Because shrimp survival in the SCPA/SCPAN treatment was below 40% in both aquaria at day 37, no further observations of this treatment were included from that day onwards. The survival for the other two treatments was 90% for the whole period.

Figure 27 shows the presence of the shrimps for the two segments together, which contained the two feeding places. This illustrates the distribution change of shrimps over the aquarium as a response to diet supply. The expected shrimp distribution over all four aquaria segments at -2min is 25% for each segment and therefore 50% for the aquarium half with or without the feeding places. Figure 27 shows, that from the equal shrimp distribution before feeding (+/-50%, -2min), shrimp were moving towards the feeding places (>50%, 2, 5, and 10 minutes). It is, therefore, reasoned, that all diet combinations were basically attractive.

Moreover shrimp presence at 2, 5, and 10min at the feeding places remained above 50%. This suggested that the response was continuing and not limited to an instantaneous reaction.

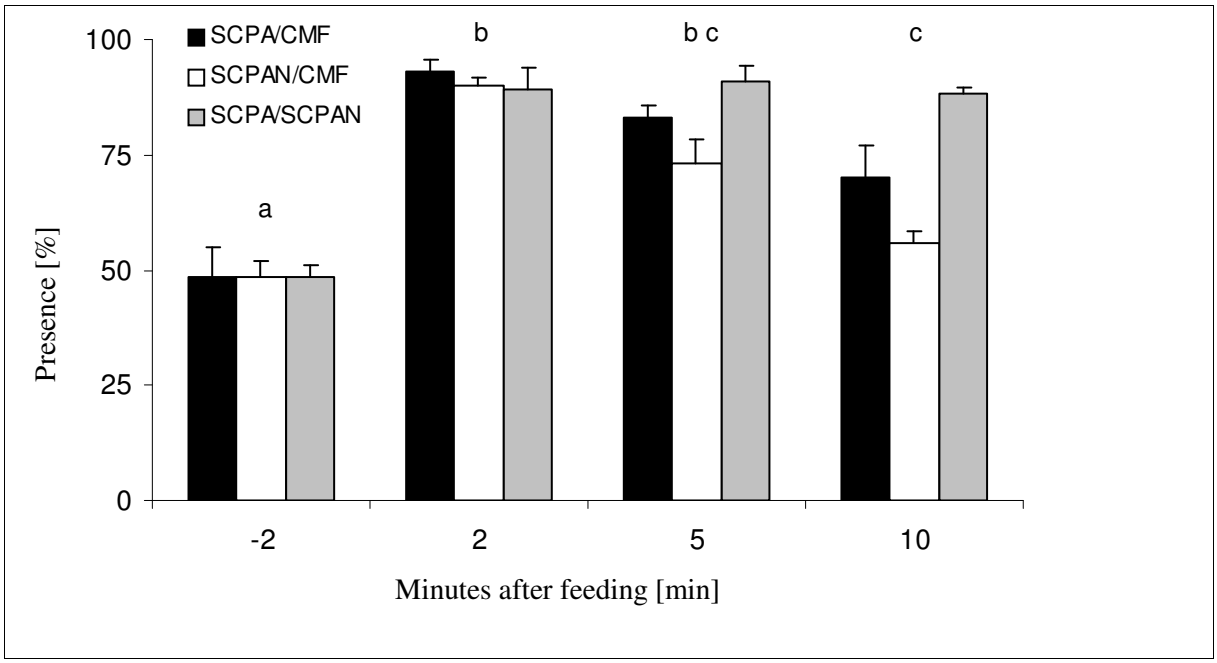

Figure 27: Presence of shrimps expressed as percentage of all shrimps present in the aquaria in the two segments containing the feeding places 2 minutes before, 2, 5 and 10 minutes after feeding, including standard deviation. a, b and c are indicating significant differences (ANOVA & Tukey's Post hoc test, $p<0.05$).

To evaluate shrimp preference in detail, the behavioral model of Lee and Meyers (1996) was adopted (Figure 25). The behavioral model was limited to observations of locomotion towards and from feeding places. In Figure 28**,** Figure 29 and Figure 30 the specific diet preference for the one or other diet is specified. At -2min preference for the segments, where the slurry was fed, was significantly higher than for the commercial diet segments and higher for SCPAN compared to SCPA for unknown reasons. After feeding (2min) more than 80% of the shrimp were present at the feeding places and showed a significant preference for CMF over SCPA (Figure 28). Following the behavior model of Lee and Meyers, (1996), the shrimps passed after detection and orientation, through a locomotion phase for the preferred diets. For the other diet combinations no significant differences could be detected for 2min. For 5 and 10min after feeding, shrimp behavior changed again. They changed from CMF to SCPA and SCPAN segments, resulting in a higher presence. This might be due to the fact that after 2min the CMF feed pellets were claimed by few shrimps and SCPA and SCPAN were still available.

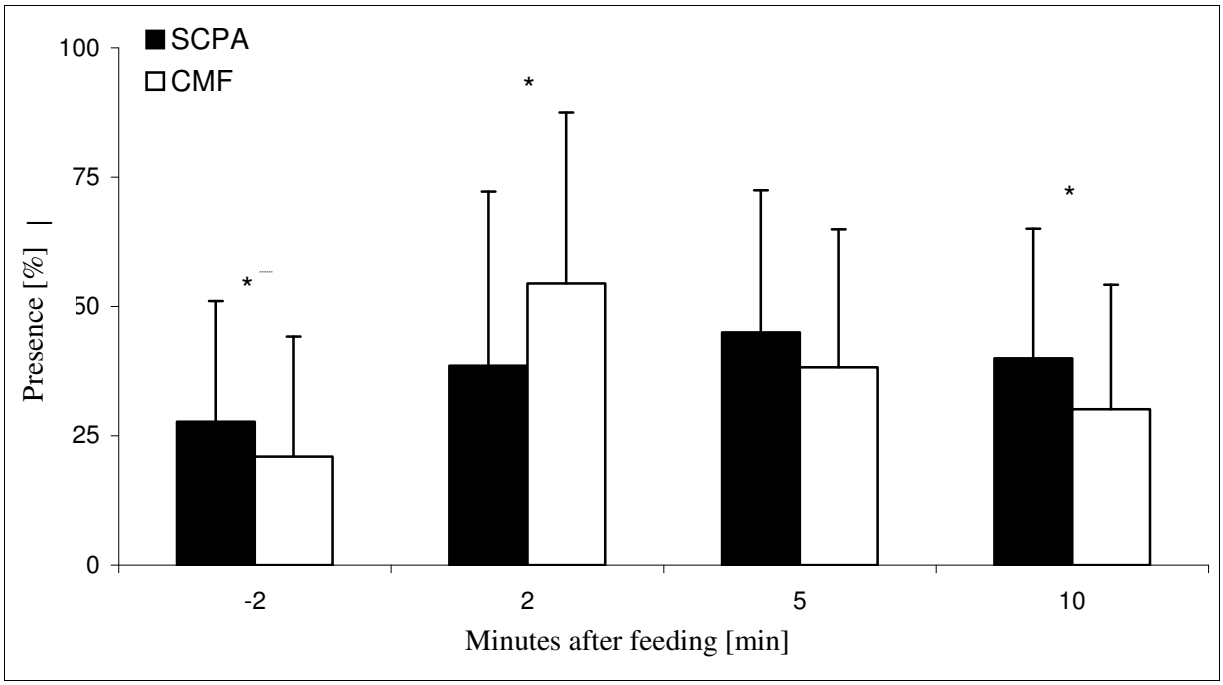

Figure 28: Presence of shrimps in the two segments comprising SCPA/CMF feeding places. Scored as percentage of all shrimps present +/- standard deviations. * = significant differences (Wilcoxon Signed Rang test, p <0.05).

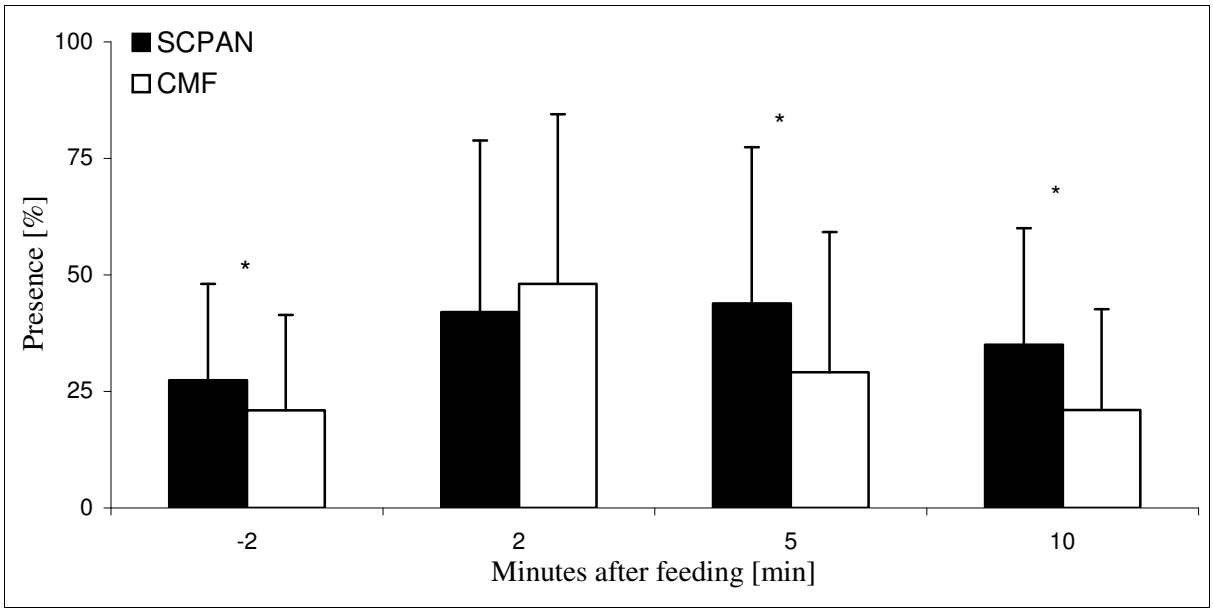

Figure 29: Presence of shrimps in the two segments comprising SCPAN/CMF feeding places. Scored as percentage of all shrimps present +/- standard deviations. * = significant differences (Wilcoxon Signed Rang test, p <0.05).

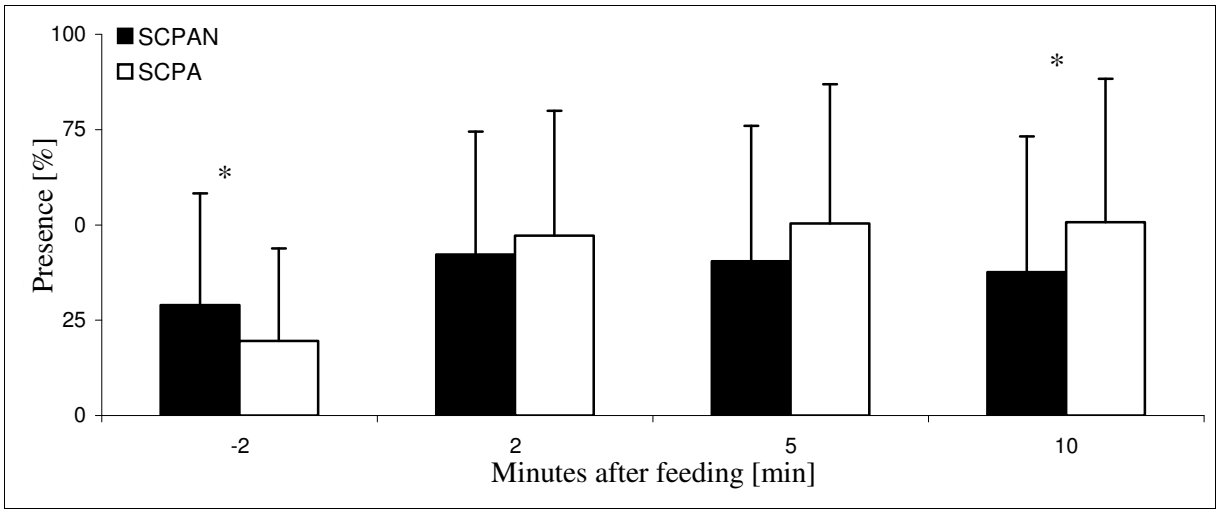

Figure 30: Presence of shrimps in the two segments comprising SCPAN/SCPA feeding places. Scored as percentage of all shrimps present +/- standard deviations. * = significant differences (Wilcoxon Signed Rang test, p <0.05).

In the SCPA/SCPAN treatment, the preference for SCPA was constantly higher than for SCPAN but differences were not significant with exception of 10min after feeding. With respect to the behavior model, shrimp continued to be present in the SCPA segment, but a significant change in distribution from SCPAN to SCPA occurred at 10min after feeding. Hence it is not possible to give a final conclusion whether the shrimp preferred anaerobically or aerobically produced bacteria.

It can be concluded from this study that the bacteria slurries a) had attracted the shrimps, b) that the commercial diet was preferred above the aerobic slurry, and c) that there is no unambiguous conclusion to be made regarding the preference for aerobic or anaerobic produced slurry. Even though the bacterial products (SCPA and SCPAN) were less attractive than CMF as diet for shrimp, they were still attractive as diet. We believe, therefore, that it may be worthwhile in the future to pursue and re-use bacterial slurries produced on the solid waste of a recirculation system, thereby creating a fish-bacteria-shrimp integrating system.

Chapter 8
Kinetics, design and biomass production of a bacteria reactor treating RAS effluent streams

Abstract

The kinetics and design of a suspended bacteria growth reactor, which can be integrated in a 100MT African catfish farm, were determined. Such a reactor converted nitrogen (N) and phosphorus (P) from RAS effluents into heterotrophic bacteria biomass. The determined kinetics were: Yield=0.537 gVSS/gC; endogenous decay coefficient=0.033h^{-1}; maximum specific growth rate=0.217h^{-1}; half-velocity constant=0.025g/l; and maximum rate of substrate utilization=0.404gC/gVSS*h. A reactor integrated in a 100MT farming facility would have a volume of 11m^3, based on a minimum HRT of 6h. The kinetics and reactor design were integrated in a model to predict the VSS production (volatile suspended solids as measure of bacteria biomass) and nutrient conversions. The VSS production was on average 187±2gVSS/kg feed and the inorganic nutrients (N and P) were removed with an efficiency of 85±3.0% and 95±2.5% respectively. A carbon (C) supplementation level of 455gC/kg feed was required to ensure optimal C:N ratios for heterotrophic bacteria production. The production of heterotrophic bacteria biomass is, therefore, a prospective tool to lower nutrient discharge and to increase nutrient retention and sustainability of RAS in the future.

Schneider, O., V. Sereti, E. H. Eding, J. A. J. Verreth and A. Klapwijk (submitted). "Kinetics, design and biomass production of a bacteria reactor treating RAS effluent streams." Aquacultural Engineering.

Introduction

In recirculation aquaculture systems (RAS) inorganic nitrogen (N) and phosphorus (P) are immobilized in bacterial biomass or volatized and/or discharged in a less hazardous form. The majority of the organic and inorganic waste is discharged though the effluent. This waste stream can be treated in lagoons, septic tanks, wetlands, and/or be used as fertilizer (Chen et al., 1987; Losordo et al., 2003). This waste management represents nutrient sinks outside the RAS. However, waste or nutrient management is also possible inside RAS. In contrast to the outside sinks, in RAS nutrients are converted into heterotrophic bacteria biomass, provided that the fish waste is supplemented with organic carbon (Schneider et al., submitted). This biomass can be re-utilized as an extra source of aquatic feed. Such an approach increases overall RAS nutrient retention. This philosophy has already been applied in activated pond culture. In these ponds, the produced bacteria biomass is consumed by fish or shrimp. As a result improved feed conversion ratios and water quality were observed (Avnimelech et al., 1989; Brune et al., 2003; Burford et al., 2004; Hari et al., 2004).

The objective of the present study was to design a bacteria reactor integrated in a 100MT African catfish farm. In this reactor, N and P nutrients from the RAS effluent should be converted by heterotrophic bacteria into biomass. This procedure required knowledge of bacteria growth kinetics on fish waste. Up to now only experimental data were available, which focused on the influence of carbon (C) supplementation levels and hydraulic retention times (HRT) on bacteria production, but not on the related kinetics (Schneider et al., submitted). It was, therefore, necessary to evaluate those experimental data and to calculate the kinetics (yield, endogenous decay coefficient, maximum specific growth rate, half-velocity constant and maximum rate of substrate utilization) to enable and design the reactor.

Material and Methods

To design an integrated heterotrophic bacteria reactor treating effluents of a 100MT African catfish RAS, the feed load, RAS effluent characteristics, and the bacteria kinetics have to be determined. The resulting values and parameters were applied to design the bacteria reactor. This procedure followed design philosophies which have been commonly used in wastewater treatment (Tchobanoglous et al., 2003).

Feed load and effluent characteristics

A commercial 100MT RAS for African catfish production is composed of fish tanks, a drum filter for solids removal, screen mesh size 60µm, a nitrifying biofilter and a two sumps (Figure 4, page 15). For an annual 100MT African catfish production under commercial

operation conditions, feed loads fluctuated between 228-325kg feed per day (Figure 31, based on Eding and van Weerd, 1999). Fish are supplied with feed 24 hours a day. Per kg feed 130 l of drum filter backwash water was used (own observation) resulting in an effluent volume of 30-42m^3 per day.

The effluent composition of a 100MT African catfish farm was assumed to be equal to samples taken from a flow equalizer (4h HRT) collecting the effluent of a 1.5 MT/year pilot scale recirculation system (Schneider et al., a,b,c submitted). The effluent originated from a drum filter (60µm mesh size). The effluent composition and the theoretical waste load, based on nutrient mass balances, are presented in Table 27. The waste has a C:N ratio of 2-3g:1g (Table 27). Optimal C:N ratios for heterotrophic bacteria production are about 12-15g:1g (Lechevallier et al., 1991; Henze et al., 1996; Avnimelech, 1999). Therefore, organic C has to be added to the effluent in order to achieve these C:N ratios. The waste load per kg feed, based on the theoretical nutrient balance, was used in the later predictions of bacteria production.

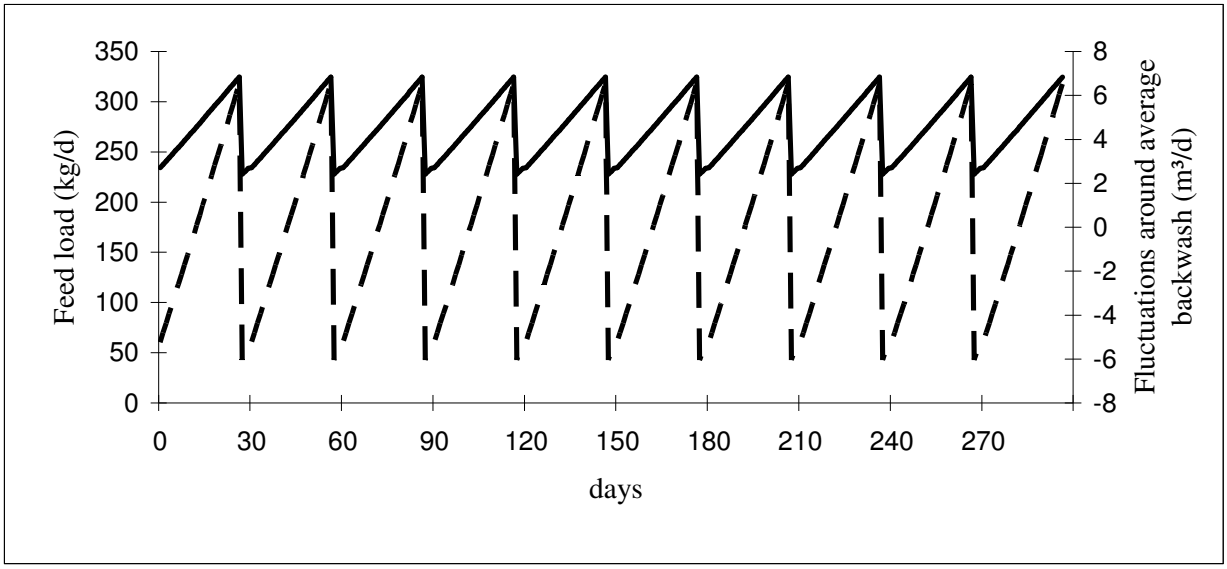

Figure 31: Feed load 228-325kg/d (bold line) and resulting fluctuations of the daily drum filter backwash flow rate compared to the average backwash flow rate of 36m^3/d (dotted line). Calculations based on Eding and van Weerd, 1999.

Table 27: RAS effluent composition measured in a flow equalizer (4h HRT) during different experiments (Schneider et al., a,b,c, submitted). Concentrations as averages ± standard deviation (minimum and maximum). Waste load was based on the theoretical waste production for African Catfish (own data, Eding and van Weerd, 1999; Machiels, 1987, using a FCR=0.85). TAN = total ammonia nitrogen, NO_2-N = nitrite-N, NO_3-N = nitrate-N, Kjd-N = Kjeldahl nitrogen corrected for TAN concentrations, TOC = total organic carbon, ortho-P-P = ortho-phosphate phosphorus, TS= total solids, TSS = total suspended solids, VSS = volatile suspended solids. [a] complete nitrification assumed.

	Waste Concentration measured during experiments	Waste Load theoretically calculated g/kg feed
TAN	1.3±0.8 (0.3-4.8) mg/l	--- [a]
NO_2-N	3.3±1.3 (0.7-12.4) mg/l	--- [a]
NO_3-N	182±58 (76-419) mg/l	40.4[a]
Kjd-N	59±43 (13-260) mg/l	7.8
TOC	0.4±0.2 (0.1-0.9) g/l	73.1
Ortho-P-P	15.1±7.7 (6.2-40.1) mg/l	5.5
Ash	1.8±0.7 (0.9-5.0) g/l	157
TS	3.5±1.0 (1.9-7.3) g/l	227
TSS	1.5±1.0 (0.2-5.8) g/l	182
VSS	0.7±0.5 (0.04-2.23) g/l	146
Drum filter effluent (60µm screen size)	130 l/kg feed	

Bacteria kinetics

Bacteria growth kinetics were derived from data, which were obtained from earlier experimental work (Schneider et al submitted). In this experiment, the solid waste stream was derived from an African Catfish farming unit (Figure 4, page 15), which was extended with a flow equalizer and a bacteria reactor (Figure 7, page 41 and Figure 8, page 43). In the experiment, different HRTs were evaluated (11 to 1h). The organic C supplementation level was constant (1.7gC/l), using sodium acetate. The environmental conditions were: temperature 28ºC, pH 7.0-7.2 and oxygen >2mg/l.

Based on the experimental data the following kinetic parameters were determined by regression analysis: yield, endogenous decay coefficient, and maximum specific growth rate, half-velocity constant and maximum rate of substrate utilization. The regressions were given by equations 1-5 (Pirt, 1975; Rittmann and McCarty, 2001; Tchobanoglous et al., 2003) and tested for significance ($p<0.05$, NLREG Version 4.1, Sherrod Software, USA).

$$q = \frac{Q * (S - S_0)}{V * X} \quad (1)$$

$$\mu = Y * q - k_d \quad (2)$$

$$q = \frac{1}{Y} * \mu + \frac{Y}{k_d} \quad (3)$$

$$\frac{V * X}{Q * (S_0 - S)} = \frac{Y * (K_S + S)}{\mu_{max} * S} \quad (4)$$

$$k = \frac{\mu_{max}}{Y} \quad (5)$$

q= specific substrate removal rate for carbon (M/M/T); Q= reactor flow rate (L^3/T); S= residual carbon substrate concentrations (M/L^3); S_0= initial carbon substrate concentration (M/L^3); V= reactor volume (L^3); X= biomass (VSS) concentration in the reactor (M/L^3); Y= yield (M/M); k_d= endogenous decay coefficient (1/T); μ= observed growth rate (1/T); μ_{max}= maximum growth rate (1/T); K_s= Half-velocity constant (M/L^3); k= maximum rate of carbon substrate utilization (1/T); M=mass; L = length; T=time

The conversion of inorganic N and ortho-phosphate-P is depending on the bacteria production and, therefore, its kinetics. The nutrient conversions (N and P) were linearly related with C consumption rates; whereby:

$$INCR = a * q + b \quad (6)$$

INCR=specific inorganic nutrient conversion rate (M/M/T); a,b= slope and intercept of the regression

Furthermore, oxygen consumption and carbon dioxide production were calculated based on the differences between the initial substrate concentration, the residual substrate concentration, and the amount of C retained in VSS production (equation 7 and 8, modified after Tchobanoglous et al., 2003). This approach ignores cell debris, because of the short SRTs, and nitrification. Since nearly no total ammonia nitrogen (TAN) was provided, it was assumed that neglecting nitrification was appropriate. The obtained kinetic parameters and rates were integrated into a model, combining all equations (Figure 32, Stella, Version 8.1.1, ISEE systems, USA).

$$CO_2 = ((S_0 - S) * Q - (VSS\_Production / VSS_{mol} * C_{mol/molVSS} * C_{mol})) / C_{mol} * (C_{mol} + O_{2\,mol}) \quad (7)$$

$$O_2 = CO_2 / (C_{mol} + O_{2\,mol}) * O_{2\,mol} \quad (8)$$

O_2=oxygen consumption (g/d); CO_2= carbon dioxide production (g/d); VSS_Production= volatile suspended solids production (g/d); VSS_{mol}=1374g/mol=1mol VSS; $C_{mol/molVSS}$=60mol carbon/ mol VSS; C_{mol}=12g carbon/mol carbon; $O_{2\,mol}$ =32g oxygen/mol oxygen (O_2)

The model was validated, using five independent datasets derived from another experiment, executed in a similar setup and using the same equipment and comparable conditions as previously described (HRT 7-9h, sodium acetate supplementation levels 1-3gC/l, Schneider et al., submitted). For model validation, the differences between model and experimental data were evaluated with an one-sample t-test (SPSS 11.5, SPSS, USA). The model was used to predict VSS production, N and P conversion, oxygen requirements and carbon dioxide production in a bacteria reactor integrated in a 100MT farm. In the model some assumptions were made for simplification reason: The waste loads (model input) were based on the theoretical waste loads (Table 27). Denitrification was excluded and all excreted non-faecal loss was considered as being available for the bacteria. Faecal loss in form of VSS (organic matter) was assumed to be removed from the RAS with an efficiency of 70% by the drum filter (estimated after Timmons et al., 2001). Therefore, 70% of the produced organic matter was entering the reactor as VSS. Nutrient leaching from the solid waste into the dissolved waste was ignored. Harvestability of bacteria biomass was assumed to be 100%. The reactor flow rates in such a farm fluctuated together with backwash flow (30-42m^3 per day, Figure 31).

Reactor design

A bacteria reactor (continuous-flow stirred-tank reactor, CSTR) was designed to convert the solid and dissolved waste in the effluent of a 100MT African catfish RAS into bacteria biomass (Figure 7, page 41). The CSTR volume was calculated using the minimum HRT (HRT=SRT, sludge retention time), because no sludge was returned. The minimum HRT was based on the highest flow rate and not on the average flow rate (Figure 31) and on bacteria kinetics. Otherwise flow rate fluctuations lead to HRTs shorter than the critical HRT and bacteria wash out (Pirt, 1975; Tchobanoglous et al., 2003, equation 9-12). A safety factor was integrated to accomplish that the minimum HRT was always longer than the critical HRT (Tchobanoglous et al., 2003).

$$HRT_{critical}=(\mu_{max}-k_d)^{-1} \quad (9)$$

$$HRT_{minimum}= HRT_{critical}*\text{safety factor} \quad (10)$$

$$Q = db \times pfl \quad (11)$$

$$V=Q/(24/HRT_{minimum}) \quad (12)$$

$HRT_{critical}$=critical hydraulic retention time, at which bacteria wash out occurs (h); $HRT_{minimum}$ = minimum hydraulic retention time (h); db= drum filter backwash (m^3/kg feed) pfl= peak feed load (kg/d)

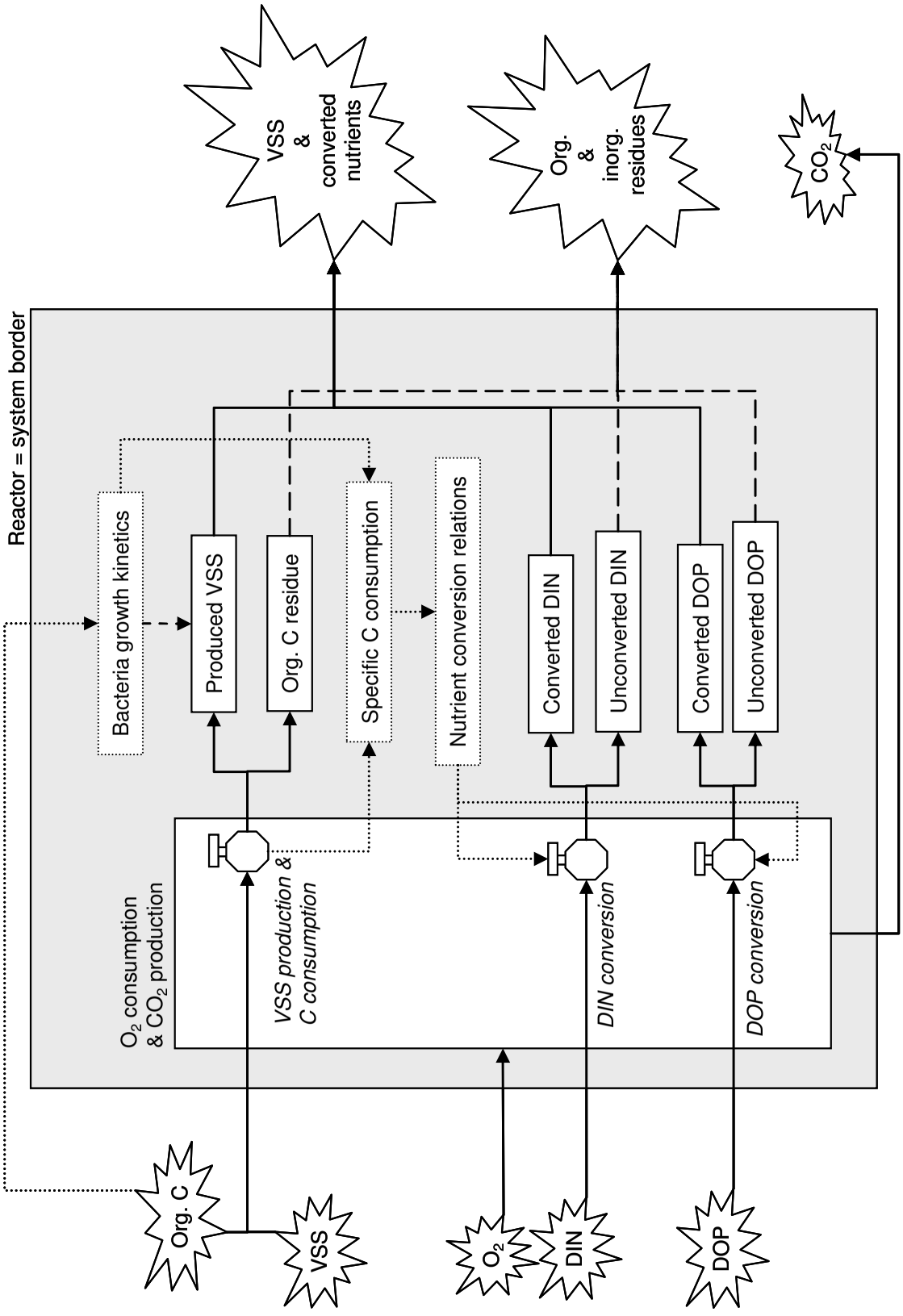

Figure 32: Schematic model overview, predicting bacteria growth, yield, VSS production, organic C, inorganic nitrogen and ortho-phosphate conversion into bacteria biomass. Bold lines and broken lines indicate matter flows, small dotted lines information flows. DIN=dissolved inorganic nitrogen, DOP=dissolved ortho-phosphate-phosphorus, O_2=oxygen, CO_2=carbon dioxide.

Results

Bacteria Kinetics

Bacteria growth kinetics were calculated based on the presented experimental dataset using regression analysis (Figure 33 and Figure 34). The obtained parameter values were integrated in the model (Table 28, appendix). The conversion rates of inorganic N and ortho-phosphate-P were linearly related with C consumption rates and yielded significant regressions ($p<0.05$, Figure 35). The resulting equations were integrated in the model (appendix).

Table 28: Bacteria kinetics as determined by experimental data. HRT (1-11h), C level = 1.7gC/l, Temperature 28ºC, pH = 7-7.2 and oxygen > 2mg/l.

Parameter		Dimension	Determined value
Yield	Y	gVSS/gC	0.537
Endogenous decay coefficient	k_d	h^{-1}	0.033
Maximum specific growth rate	μ_{max}	h^{-1}	0.217
Half-velocity constant	K_s	g/l	0.025
Maximum rate of substrate utilization	k	gC/gVSS *h	0.404

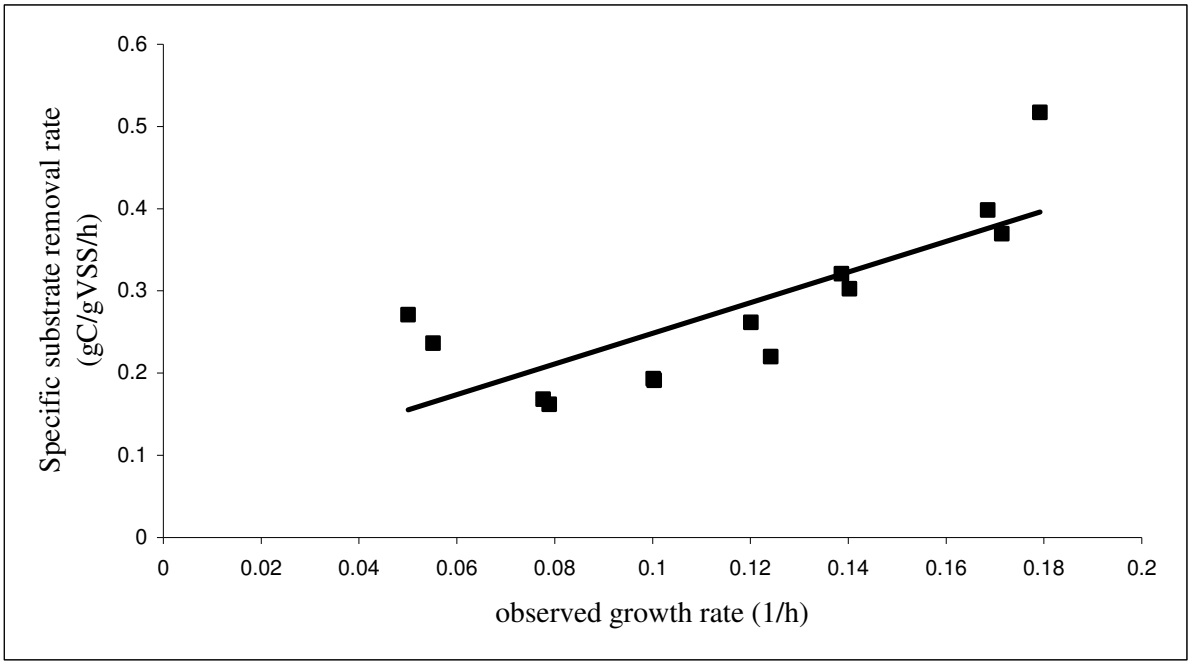

Figure 33: Regression of the observed growth rate versus the substrate removal rate (gC/gVSS/h). (y=1.863*x+0.0622, R^2=0.607, p<0.05).

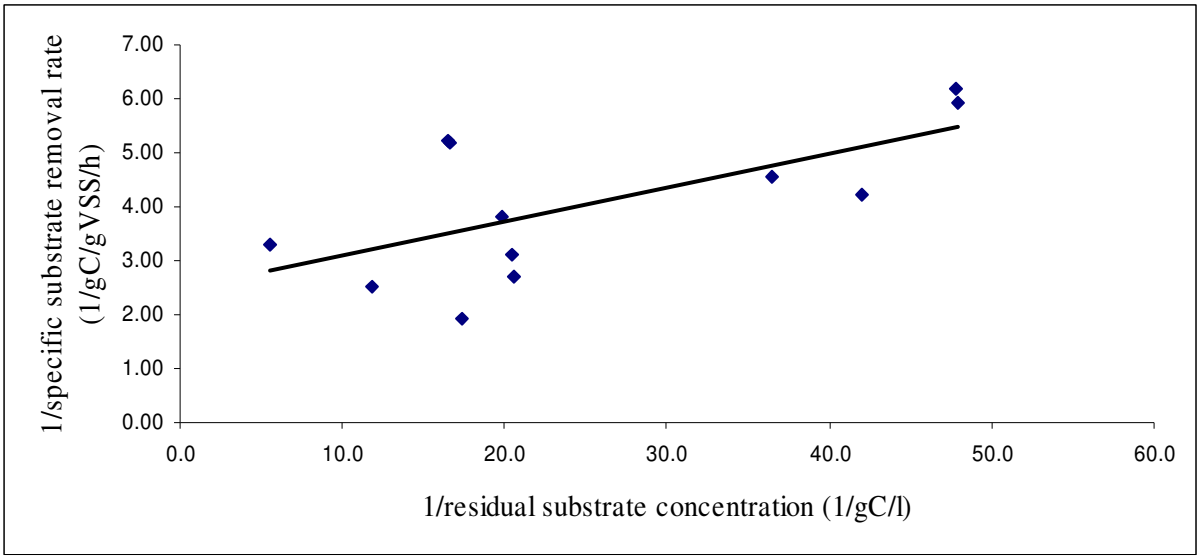

Figure 34: Regression of 1/residual substrate concentration (1/gC/l) versus 1/ specific substrate removal rate (1/gC/gVSS/h). (y=0.0628x+2.4731, R^2=0.43, p<0.05). One observation was eliminated from the dataset because of a very high S value.

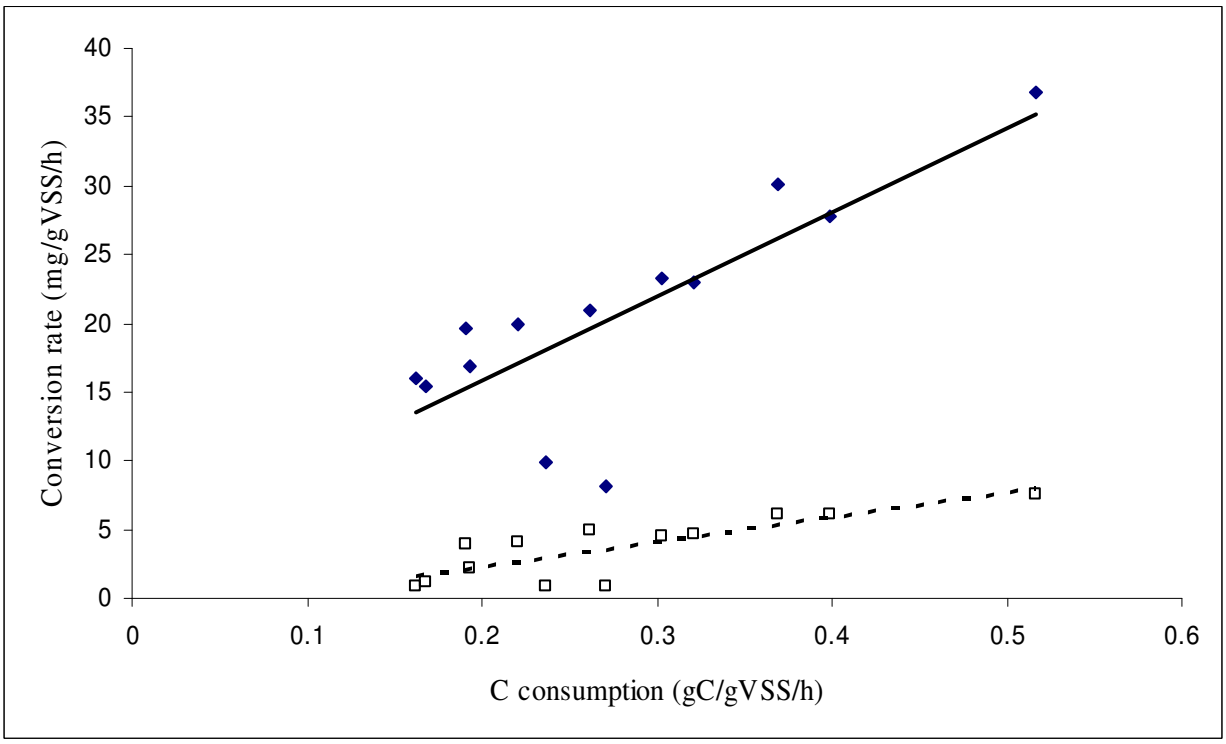

Figure 35: Specific carbon consumption rate (gC/gVSS/h) versus inorganic nitrogen (♦) or ortho-phosphate-phosphorus conversion (❑) in mg/gVSS/h. (Inorganic nitrogen conversion = 61.02*Cconsumption + 3.64, R^2=0.63, p<0.05; ortho-phosphate-P conversion= 17.78*Cconsumption -1.25, R^2=0.65, p<0.05)

To evaluate differences between values measured in the validation experiment and the predicted values by the model, the data were plotted against each other (Figure 36). There were no differences between predicted model and experimental data (t=0.785). The model

was therefore validated and assumed to have a sufficient overall fit, even though 2 points of the validation dataset were outside the 10% confidence interval of the line.

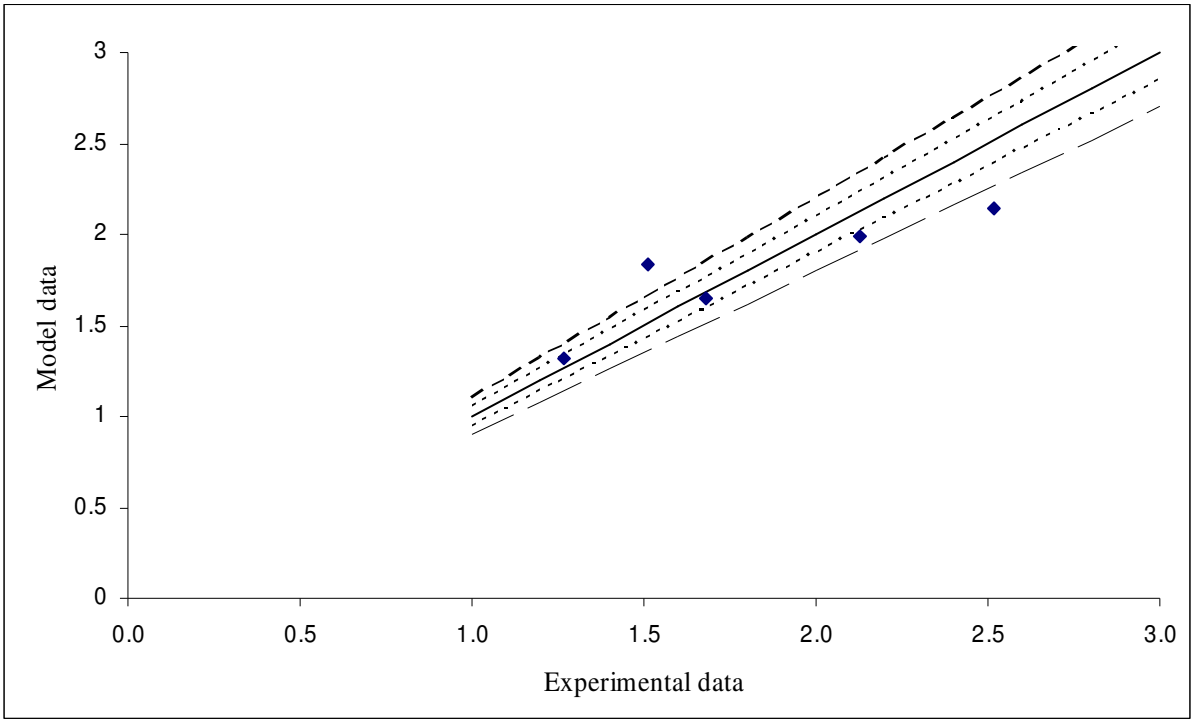

Figure 36: Model validation for VSS production (gVSS/l/d), bold line equals y=x and 5% and 10% confidence interval around this optimal line. Points reflect data from independent experiments.

Reactor design

The maximum growth rate and the decay coefficient were μ_{max}=0.217h^{-1} and k_d = 0.033h^{-1}. The related critical HRT would be 1/0.184 h^{-1}=5.5h. To avoid cell oxidation, HRT and SRT should be as short as possible. Therefore, based on the maximum flow rate, a safety factor of 1.1 was chosen (minimum HRT 6h), which equaled an additional period of 0.5h. If the maximum feed load in the system was 325kg feed and the reactor backwash was 130 l/kg feed and the minimum HRT was 6h, then a reactor volume of 11m^3 would be required (equations 9-12).

Model results (integrating kinetics and reactor design)

The model input and resulting output were presented in Table 29. For a C supplementation level of 3.5gC/l, 187gVSS/kg feed were produced. The residual concentration for dissolved inorganic N and ortho-phosphate-P was 47.4mg/l and 1.4mg/l and the conversion efficiencies 85 and 95% respectively. For C supplementation levels >3.5gC/l N and P conversions and VSS production were limited by the amount of available inorganic N and P resulting in conversions of more than 100% of the available inorganic N and P (data not shown).

Discussion

Reactor type and its position in the system

The selection of the CSTR depended on different aspects, such as bacteria kinetics, waste load, SRT and HRT (Tchobanoglous et al., 2003). The position of the bacteria reactor in the RAS had to ensure that the solid waste and the non-retained inorganic N and ortho-phosphate-P were accessible for conversion. Furthermore, no interferences with other RAS processes should occur and a continuous bacteria harvest was required, without stopping the system operation. Furthermore, no sludge should be returned into the reactor from its effluent, a contrast to activated sludge systems. This avoided unnecessary cell oxidation by long SRTs (Pirt, 1975; Henze et al., 1996; Tchobanoglous et al., 2003). Because of these considerations a CSTR with suspended growth and no sludge return was selected and integrated at the drum filter effluent (Figure 7, page 41 and Figure 8, page 43).

This reactor allowed for stable and reliable production of bacteria during several experiments without interfering with the normal system processes. The solid and dissolved waste discharged from the RAS were available without system disturbance (Schneider et al., a,b,c, submitted). This was an advantage compared to earlier systems (Knoesche and Tscheu., 1974, Meske, 1976), which applied heterotrophic bacteria production inside RAS' water flow. Those configurations were problematic, since these systems affected the overall RAS performance. The present design and operational conditions allowed harvesting the produced biomass with the effluent flow by collection. Nutrient concentrations in the drum filter effluent are relatively high (185mgN/l and 15mgP/l). For high nutrient concentrations, high bacteria biomass concentrations can be expected in a CSTR and therefore it is not necessary to use a CSTR with sludge recycle. Furthermore, the reactor operated at short SRTs, which equaled HRT (4-11h). This modus operandi was in contrast to activated sludge systems, which have a sludge return and do not aim for bacteria biomass production (Tchobanoglous et al., 2003). The reactor was inoculated with bacteria coming from the system's own micro fauna through the drum filter effluent. This practice is comparable with activated sludge systems or the conversion of nutrients by heterotrophic bacteria in aquaculture ponds, which were using open and mixed cultures and were not inoculated with specific bacteria strains (Avnimelech et al., 1989; Brune et al., 2003; Burford et al., 2004; Hari et al., 2004).

Table 29: Model Input and output of a bacteria reactor simulation. The reactor was connected to the hypothetical effluent stream of a 100MT African catfish farming unit. HRT= hydraulic retention time; VSS= volatile suspended solids; dt=model integration time. Output ± standard deviation.

Parameter	Dimension	Value	Source
INPUT			
Feed load	kg / day	228-325	Figure 31
Backwash volume	l/kg feed	130	Table 27
minimum HRT	h	6	own data
Reactor volume	m^3	11	design result
Dissolved inorganic nitrogen (reactor influent)	mgN/l	310	Table 27
Ortho-phosphate-phosphorus (reactor influent)	mgP/l	42	Table 27
Volatile suspended solids (reactor influent)	gVSS/l	0.7	Table 27
Organic C supplementation	gC/l	3.5	
dt	h	1	
OUTPUT			
VSS production	gVSS/kg feed	187±2	
Dissolved inorganic nitrogen (reactor effluent)	mgN/l	47.4±9.4	
Dissolved inorganic nitrogen (conversion efficiency)	%	85±3.0	
Ortho-phosphate-phosphorus (reactor effluent)	mgP/l	1.4±1.1	
Ortho-phosphate-phosphorus (conversion efficiency)	%	95±2.5	
Organic carbon use	gC/kg feed	455	
Carbon dioxide production	gCO_2/kg feed	1244±31	
Oxygen consumption	gO_2/kg feed	905±23	

Waste loads

The theoretical waste loads, which were used as model input, did not consider processes occurring in the fish culture system, such as denitrification, nutrient leaching and organic matter degradation. These processes were influencing the measured waste composition and resulted in lower waste loads then used here as model input (Table 27). By basing the reactor design and the predictions for VSS production and nutrient conversions on the theoretical waste loads, model input was related clearly with the fish waste production and not biased by processes occurring inside RAS. However, such processes have to be taken into account if the reactor is up-scaled. Differences among fish species and fish performance, such as lower or higher feed conversion ratios, or differences in nutrient retention, or different waste production caused by changes in feed compositions, influence waste loads and, therefore, reactor performance and design (Kim et al., 1998; Eding and van Weerd, 1999;

Lupatsch et al., 2001, Tchobanoglous et al., 2003). If the reactor would be up-scaled, process fine tuning will be required according to the local conditions and loading rates. It has to be noted that the model was established and validated based on reactor experiments using a reactor volume of 3.5 l. The results obtained from the model have, therefore, to be treated carefully.

Kinetic parameters

Kinetic parameters (yield, endogenous decay coefficient, maximum specific growth rate, half-velocity constant and maximum rate of substrate utilization) were determined. The calculated yield (0.5gVSS/gC) and bacteria growth rates were in the lower range compared to those reported in literature (0.3-1.0gVSS/gC, Atkinson and Mavituna, 1991; Tijhuis et al., 1994; Henze et al., 1996; van der Westhuizen and Pretorius, 1996; Rittmann and McCarty, 2001; Aulenta et al., 2003; Marazioti et al., 2003; Tchobanoglous et al., 2003). Three factors might have caused the low yields: insufficient adaptation of the bacteria strains to the substrate, differences in water conductivity, and the non-accounted amount of produced extracellular material (Schneider et al, submitted). The maximum relative growth rate (μ_{max}=0.22 h^{-1}) was in the lower range of values referred in environmental biotechnology or wastewater treatment studies, e.g. 0.2-0.5 per h for aerobic heterotrophic growth (Henze et al., 1996; Rittmann and McCarty, 2001). This supported the hypothesis that increased metabolic costs due to high water conductivity caused the lower growth rates and yields. The inorganic nutrient conversion rates were related linearly with C consumption rates in a ratio of C: N: P 100g:7g:2g. This equals a C: N ratio of 14g:1g, which is in the range of the expected ratio for optimal bacteria growth (12-15g:1g, Lechevallier et al., 1991; Henze et al., 1996; Avnimelech, 1999).

The predicted residual C substrate concentration was on average 0.12±0.07gC/l and even 0.3gC/l for 6h HRT. This was higher than the measured concentration in the experiment delivering the data for kinetic determination (0.05±0.04gC/l, 4-11h HRT, Schneider et al., submitted). However, the estimate of oxygen consumption and carbon dioxide production is still acceptable, if the initial substrate concentration (3.5gC/l) and the high removal (~3.4gC/l) were considered. The average oxygen consumption was 905gO_2/kg feed and the carbon dioxide production 1244gCO_2/kg feed, respectively (Table 29). The model input parameters require fine-tuning to predict the oxygen requirements and carbon dioxide production more accurately.

HRT, SRT and reactor volume

The minimum HRT included a safety factor of 1.1 (5.5h*1.1≈6h). Higher factors, such as 1.3-2, are applied in activated sludge systems, which do not aim for biomass production (Tchobanoglous et al., 2003). A safety factor of 30min was, furthermore, sufficient, because the realized HRTs (≥6h) would never be shorter than the minimum HRT (6h) or the critical HRT (<5.5h). Feed load fluctuations increased the realized HRT to an average of 7.2h +/- 0.8h. Using such a design prevented, therefore, bacteria wash out.

VSS production and organic C requirements

VSS production was 187gVSS/kg feed, applying a acetate-C supplementation level of 455 gC/kg feed This is lower but still comparable to the VSS production obtained in similar systems using molasses as C donor (228gVSS/kg feed, calculated after Schneider et al., submitted).

Reactor effluent characteristics and nutrient conversion efficiencies

The reactor effluent might be re-used as system water, considering the low residual concentrations for inorganic N and P (47.4 and 1.4mg/l respectively, Table 29). The N conversion efficiency equaled a maximum conversion rate of 1gN/l/d. This was comparable to average conversion rates given by van Rijn et al. (in press) for aquaculture recirculation systems of about 0.9g/l/d. However, the carbon consumption per g inorganic N removed was much higher for the present heterotrophic conversion than for denitrification (13gC/gN versus 2gC/gN, Henze et al., 1999). The P conversion efficiency was slightly higher than expected, as normally 2.3% P are contained in 1gVSS (Rittmann and McCarty, 2001; Tchobanoglous et al., 2003). In the present study 2.8% were retained in VSS production. However, it remains unclear how much of this P is included in extracellular material. The low residual concentrations and high conversion efficiencies for inorganic N and ortho-phosphate-P reflected the potential of heterotrophic bacteria conversion to retain inorganic nutrients in bacteria biomass.

Harvestability

An important aspect has not been included in this study: The harvestability and re-utilization of the bacteria as feed. Only if the bacteria biomass can be harvested efficiently from the reactor effluent, inorganic and organic waste are not only converted, but truly removed. If harvestability is efficient (100%), then 85 and 95% of the inorganic N and ortho-phosphate-P can be removed from the RAS effluent stream and low residual concentrations would remain. Possible harvest techniques, which still have to be tested, are: mechanical or

membrane filtration, sedimentation, centrifugation, flocculation, foam fractionation, evaporation or electrokinetic methods (Atkinson and Mavituna, 1991; Rittmann and McCarty, 2001). By these methods, the bacteria biomass must be made available for culture organisms as feed. It might as well be possible to feed the obtained bacteria biomass directly to filterfeeders, such as tilapia or shrimp (Avnimelech et al. 1989; McIntosh, 2001; Turker et al., 2003; Brune et al., 2003).

Conclusion

This study delivered the design of a reactor for the heterotrophic conversion of N and P nutrients from the effluent into bacteria of a 100MT African catfish RAS. The HRT of the CSTR would be 6h based on the kinetic parameters resulting in a volume of $11m^3$. For this conversion process the related kinetics and design were determined (Yield: 0.537gVSS/gC; endogenous decay coefficient: $0.033h^{-1}$; maximum specific growth rate: $0.217h^{-1}$; half-velocity constant: 0.025g/l; maximum rate of substrate utilization: 0.404 gC/gVSS*h). The kinetics and design were integrated together with nutrient conversion rates into a model, to calculate the VSS production (187gVSS/kg feed) and nutrient conversion efficiencies from the effluent (inorganic N 85%; ortho-phosphate-P 95%). The applied organic C supplementation level was 3.5gC/l or 455gC/ kg feed.

The production and potential re-use of heterotrophic bacteria biomass is, therefore, a prospective tool to lower nutrient discharge and to increase nutrient retention and sustainability of RAS in the future.

Appendix (Model Code)

Inputs

k = 0.404351

kd = 0.033387

Ks = 0.025393

N_rsu_intercept = 3.6408

N_rsu_slope = 61.017

P_rsu_intercept = -1.2481

P_rsu_slope = 17.776

S0 = 3.5

u_max = 0.217043

X_in = 0.7

Y = 0.5367

design_HRT = 6

design_kg_feed_per_day = 325

Feed_load_fluctuating = GRAPH(timer)

DIN_Drumfilter_Effluent = 310

DOP_Drumfilter_Effluent = 42

feed_switch = 1

design_drum_filter_backwash_per_kg_feed = 130

Auxiliary calculations

SRT = HRT

timer = time

kg_feed = if feed_switch =0 then 325 else Feed_load_fluctuating*24

design_backwash_volume_per_day =

design_drum_filter_backwash_per_kg_feed*design_kg_feed_per_day

design_Reactor_Volume = design_backwash_volume_per_day/(24/design_HRT)

Q = (design_drum_filter_backwash_per_kg_feed*kg_feed)/24

HRT = 24/(design_drum_filter_backwash_per_kg_feed*kg_feed/design_Reactor_Volume)

VSS_production_per_kg_feed = VSS_Production/(kg_feed/24)

DIN_concentration = DIN_reactor/design_Reactor_Volume

DIN_efficiency = (DIN_Reactor_Influent-DIN_Reactor_Effluent)/DIN_Reactor_Influent*100

DOP_Concentration = DOP_reactor/design_Reactor_Volume

DOP_efficiency = (DOP_Reactor_Influent-DOP_Reactor_Effluent)/DOP_Reactor_Influent*100

rg = Y*(k*X*S)/(Ks+S)-kd*X

rsu = u_max*X*S/(Y*(Ks+S))

S = (Ks*(1+kd*SRT))/(SRT*(Y*k-kd)-1)

u = rg/X_plus_Xin

X = Y*(S0-S)/(1+kd*SRT)

X_plus_Xin = VSS_Reactor/design_Reactor_Volume

observed_rsu = rsu/X_plus_Xin

observed_yield = VSS_Production/((S0-S)*Q)

inroganic_N_conversion__per_X_plus_Xin = N_rsu_slope*observed_rsu+N_rsu_intercept

orthoPP_conversion_per_X_plus_X_in = P_rsu_slope*observed_rsu+P_rsu_intercept

CO2_production = (((S0-S)*Q*24

(VSS_production_per_kg_feed*kg_feed/1374*60*12))/12*(12+32))/kg_feed

O2_consumption = CO2_production/(12+32)*32

Rates and States

DIN_reactor(t) = DIN_reactor(t - dt) + (DIN_Reactor_Influent - DIN_Reactor_Effluent - DIN_Uptake_by_VSS) * dt

INIT DIN_reactor = design_Reactor_Volume*DIN_Drumfilter_Effluent

INFLOWS:

DIN_Reactor_Influent = DIN_Drumfilter_Effluent*Q

OUTFLOWS:

DIN_Reactor_Effluent = DIN_reactor/design_Reactor_Volume*Q

DIN_Uptake_by_VSS = inroganic_N_conversion__per_X_plus_Xin*VSS_Reactor

DOP_reactor(t) = DOP_reactor(t - dt) + (DOP_Reactor_Influent - DOP_Reactor_Effluent - DOP_Uptake_VSS) * dt

INIT DOP_reactor = DOP_Drumfilter_Effluent*design_Reactor_Volume

INFLOWS:

DOP_Reactor_Influent = DOP_Drumfilter_Effluent*Q

OUTFLOWS:

DOP_Reactor_Effluent = DOP_reactor/design_Reactor_Volume*Q

DOP_Uptake_VSS = orthoPP_conversion_per_X_plus_X_in*VSS_Reactor

VSS_Reactor(t) = VSS_Reactor(t - dt) + (VSS_Reactor_influent + VSS_Production - VSS_Reactor_Effluent) * dt

INIT VSS_Reactor = IF X_in*design_Reactor_Volume = 0 then 0.000000000000001 else X_in*design_Reactor_Volume

INFLOWS:

VSS_Reactor_influent = Q*X_in

VSS_Production = rg*design_Reactor_Volume

OUTFLOWS:

VSS_Reactor_Effluent = VSS_Reactor/design_Reactor_Volume*Q

Chapter 9
Discussion

In recirculation aquaculture systems (RAS), treatment units are only purifying the rearing water by solids removal and by nitrification but are not managing the fish waste. Even in advanced RAS, solids and nitrogenous and phosphorous wastes leave the system as slurry. Carbon dioxide is stripped to the air and dissolved nitrogen (N) may eventually be converted into gaseous N (Bovendeur et al., 1987; van Rijn et al., in press). Due to water treatment and purification, the waste is not an issue inside the production system anymore, but it is on the outside as effluent. It is, therefore, needed to apply waste management in RAS. Comparable to terrestrial husbandry systems, waste can be managed outside the system boundaries. For this purpose, digestion, re-use as fertilizer and other techniques have been applied (Burton and Turner, 2003). However, waste can be managed also inside RAS. In the latter case, processes have to be selected to convert the waste into a re-usable product. In the present study, the scheme, presented in chapter 1 was followed: first an evaluation was made of nutrient flows and conversion processes in integrated aquaculture systems, second a specific conversion process was selected and studied, third options for process improvements and factors influencing the process sensitivity were investigated, fourth product suitability was evaluated, and fifth the design criteria were developed and the integration possibilities into RAS were studied.

Evaluation of nutrient flows in integrated aquaculture systems

In chapter 2, nutrient flows in integrated aquaculture systems were evaluated. In those systems different processes can convert aquaculture waste inside the aquatic system into a valuable product. In literature two possible routes for within-system treatment are reported: waste conversion by phototrophic and waste conversion by heterotrophic organisms. Directly harvestable products were found in both pathways: e.g. plants that are of direct use for the pharmaceutical industry (Luening et al., 2003) or e.g. worms, which can be utilized as baits or aquatic feed in other systems (Olive, 1999). This direct use of the primary conversion product ensures the highest increase in nutrient retention. Feeding the conversion product to other animals reduced the overall nutrient retention. For example, in a phototrophic-herbivorous chain, the gained nutrient retention decreased by 60-85% feed N and 50-90% feed phosphorus (P) compared to a setup, in which only fish and phototrophic production would be integrated

and the plants harvested. In a chain shrimp-algae-oyster, the N and P retention in oysters is only 6gN and 1.3gP, while in the consumed algae 40gN and 9.5gP were retained.

Phototrophic-herbivore chains have been reported repeatedly in literature and more often than the heterotrophic-bacterivore chain (chapter 2). However, issues related with phototrophic production justify a more detailed investigation of the heterotrophic conversion process. Such issues in phototrophic conversion are: excessive surface requirements, need for balanced supply of micro- and macronutrient, maintaining optimal water temperatures, providing sufficient light, but at the same moment avoiding photoinhibition, stabilizing pH, preserving culture purity, and ensuring algae harvestability. These difficulties were reported by various authors and evaluated in chapter 2. There are also disadvantages of heterotrophic conversions. The contribution of heterotrophic bacteria to overall system nutrient retention is low (7% of feed N, recalculated after Knoesche and Tscheu, 1974). The waste conversion by bacteria is limited by the amount of available organic carbon (C), by availability of oxygen, and by the nutritional value of the obtained bacteria biomass. Worms were also only contributing marginally to the overall system nutrient retention (0.06% feed N, chapter 2, Bischoff, 2003).

Despite the reported disadvantages of heterotrophic bacteria, it is still believed that they may offer a perspective tool for conversion of fish waste into a reusable product. In that case the design, integration, and operation of the bacterial reactor have to be handled differently from the past. To overcome problems from the past, the following points should be taken into account: (1) reactor size can be small, if HRTs are in the range of hours (Pirt, 1975; Rittmann and McCarty, 2001); (2) the process must be developed in such a way that it ensures easy control; (3) activated sludge processes and activated ponds constitute good examples where such processes occur (Avnimelech et al. 1989; Henze et al., 1996; Brune et al., 2003; Burford et al., 2003; Tchobanoglous et al., 2003; Hari et al., 2004) and (4) the feasibility of re-using the biomass as aquatic feed was demonstrated in several studies (Tacon, 1979, Perera et al., 1995; Schneider et al., 2004). Furthermore, the conversion process is light independent, which allows designing deeper reactors with smaller surfaces than required for phototrophic conversion (chapter 2).

Process selection and investigation

RAS offer a unique possibility to manage solid and dissolved waste streams together, when the bacteria reactor is integrated after the drum filter. Therefore, in the present study RAS design was conserved and, due to the position of the bacterial reactor in the drum filter

effluent, no interferences with other processes inside the system occurred. This was an advantage compared to earlier systems, which integrated the heterotrophic bacteria production inside the RAS. Those systems were not successful and were abandoned by the RAS industry (Knoesche et al., 1974, Meske, 1976). The example of activated sludge reactors and activated pond aquaculture (Avnimelech et al. 1989; Rittmann and McCarty, 2001; Brune et al., 2003; Burford et al., 2003; Tchobanoglous et al., 2003) gave the inspiration to focus on suspended bacteria growth processes. Therefore, as reactor type, a continuous-flow stirred-tank reactor (CSTR), allowing for waste conversion by suspended bacteria growth, was chosen.

Investigations of the fish slurry composition revealed that RAS effluents are deficient in organic C to allow good heterotrophic bacteria production due to N accumulation in the system water (2-3g:1g C:N). It was, therefore, necessary to enrich the fish waste with C, thereby providing optimal C: N ratios (12-15g:1g) for heterotrophic bacteria production (Lechevallier et al., 1991; Henze et al., 1996; Avnimelech, 1999). The effect of C supplementation levels (sodium acetate or molasses) on bacteria production was investigated in chapter 3 and chapter 4. Bacteria production rates increased in response to increased C supplementation levels. In chapter 3, also the effect of a decreasing HRT was evaluated. This was necessary to calculate kinetic parameters, which were used to design a reactor in chapter 8. The calculated yield (0.4-0.5gVSS/gC; VSS=volatile suspended solids) was lower than most of the ones reported in literature (0.3-1.2gVSS/gC, Atkinson and Mavituna, 1991; Tijhuis et al., 1994; Henze et al., 1996; van der Westhuizen and Pretorius, 1996; Rittmann and McCarty, 2001; Aulenta et al., 2003; Marazioti et al., 2003; Tchobanoglous et al., 2003). Three factors might have caused these low yields: high water conductivity and, therefore, increased metabolic costs; non sufficient adaptation of the bacteria to the substrate; and the non-accounted amount of produced extracellular material. Future investigations of these factors might result in yield improvements.

Production improvement and sensitivity

Production improvements and sensitivity of the conversion process were evaluated for different HRTs and for different C and N sources. In chapter 3, the sensitivity of bacteria waste conversion in response to decreasing HRTs was evaluated. Nearly no yield differences were detected unless the critical HRT was approached, and bacteria wash out occurred. Therefore, it can be concluded that HRT (11-2h) were not very important for bacteria yields However, at short HRTs (close to the critical HRT) the highest growth rates (0.2-0.5h^{-1}) were observed which allowed to produce bacteria in small reactor volumes (Pirt, 1975). Because

yields were rather low compared to values reported in literature, the effect of different N sources on bacteria yields was investigated. Theoretically, 20% yield improvement should occur if nitrate is replaced by total ammonia nitrogen (TAN) as N source (Rittmann and McCarty, 2002 and chapter 3). However, the experiments did not show differences in yields and VSS production. Only a preference for TAN over nitrate was detected, which is in agreement with literature (Vriens et al., 1989; Rittmann and McCarty, 2001). This result has significant consequences. In case replacement of nitrate by TAN would have improved yields, RAS design and reactor position would have had to be changed, eliminating nitrification. However, the present results suggest that the common RAS design can be maintained and that the reactor position after the drum filter is acceptable. A comparison of sodium acetate and molasses at comparable culture conditions showed no differences in yields and productions (chapter 5). Only the levels of C supplementation yielded a sensitive response in bacteria production, and the effect was similar for both C sources. It is, therefore, possible, to replace sodium acetate by other C sources, such as molasses, as long as C degradability is similar.

During the experiments several factors were fixed which may influence the conversion process. Those factors were the oxygen concentration in the broth (>2mg/l), the pH (7.0-7.2), the agitation speed (350rpm) and temperature 28°C. Those factors influence culture conditions and affect VSS production rates and yields (Pirt, 1975; Rittmann and McCarty, 2001; Tchobanoglous et al., 2003). Their influence on waste conversion in heterotrophic bacteria remains unclear in this study.

Product evaluation and determination of re-use potential

The composition of the bacteria community, which was produced in the reactors, was influenced by the culture conditions (chapter 6). Although nearly all bacteria in the flow equalizer were also found in the reactor broth, the community forming these broths differed in its major components, both qualitatively and quantitatively. The most important bacteria were not pathogenic (HRT 6-7h). When HRTs were shorter a more abundant fraction of the potentially pathogenic alpha proteobacterium Bioluz/ *Acinetobacter* appeared. At 7h, bacteria close to *Rhizobium*/ *Mezorhizobium* were forming the major components of the community. The use of molasses instead of sodium acetate changed the bacteria community from *Rhizobium*/ *Mesorhizobium* to *Aquaspirillum*. Providing TAN in addition to nitrate as nitrogenous substrate led to the occurrence of bacteria close to *Sphaerotilus*, *Sphingobacterium* and *Jonesia*. Because the major community components were associated with no pathogenic risks, bacteria biomass was evaluated in a nutritional study (chapter 7).

The results of the study revealed that even though the slurry was less attractive than the tested commercial feed for shrimp, it was still attractive enough for consumption.

The produced bacteria biomass was collected, and analyzed for its proximate composition (chapter 7). The material obtained from the broth had a higher ash (20% versus 13%) and a lower dry matter content (49% versus 93%) than a commercial African catfish diet (Biomeerval, Skretting, France). The crude protein content was higher (60% versus 52%) and the energy content (17 versus 20 MJ/kg) was comparable to this feed. The high water and ash content, however, might reduce the suitability of the bacteria biomass as feed. Parts of the slurry composition remained unanalyzed, such as nucleic acids, and they may have great influence on the nutritional value (Tacon, 1979). Furthermore, fatty acids and amino acid profiles, vitamins and other micronutrients were not analyzed yet and this information is also needed to make a full appraisal of the nutritional quality of the product.

If the determined bacteria composition and production (chapter 7 and 8) was considered then ~140g crude protein/kg feed were produced:

$$Protein_{Production}(g/kg\ feed)=Protein_{Biomass}(g/kg\ dm)/(1000g\text{-}Ash_{Biomass}(g/kg\ dm))*187gVSS/kg\ feed$$

Protein=crude protein; Dm=dry matter

In chapter 8, the conversion efficiency of inorganic N was 85% for 455gC/kg feed. If all N would have been converted into crude protein, then ~210g crude protein per kg feed would have been produced (40.4g/kg feed of inorganic N not retained in the fish*0.85*6.25g crude protein/gN). This means 140-210g crude protein/kg feed would have been made available as aquatic feed from converted waste. If this biomass would be fed to tilapia (40% assumed protein efficiency, Schneider et al., 2004) then ~55-85g crude protein would be retained in fish biomass. This, theoretically, results in a weight gain of ~350-530g per kg feed and improves FCR by ~0.4-0.5. This would increase the N retention of the RAS by ~30-40% (from ~30gN/kg feed for African catfish alone to ~39-43gN/ kg feed for African catfish and tilapia together). These assumptions, however, require direct bacteria harvesting (100% efficiency) and bacteria consumption by the fish. The calculated FCR improvement agrees with results reported for shrimps or tilapia which grew more efficiently if the heterotrophic production inside the pond or aquarium was stimulated and consumed (Avnimelech, 1999; Velasco, 2000). In the future, a more detailed evaluation of the bacteria biomass is required, to characterize the nutritional value in vitro and in vivo.

Process design characterization and integration

In chapter 8, the design of a bacteria reactor integrated in a 100MT African catfish farm was calculated. This required combining fish production data, the effluent characteristics and the bacteria kinetics. Experimental data from chapter 3 were used to determine the relevant kinetic parameters and conversion rates (yield, endogenous decay coefficient, and maximum specific growth rate, half-velocity constant and maximum rate of substrate utilization, nutrient conversion rates, oxygen consumption, and carbon dioxide production). The kinetics and rates were integrated into a model. This validated model was used to predict bacteria production and nutrient conversion in the designed reactor. Based on the simulation the inorganic nutrient (N and P) removal efficiencies and the C supplementation level were determined. Fish waste was converted with an efficiency of 85 and 95% for N and P, respectively, into bacteria biomass (187gVSS/kg feed) in a reactor volume of 11m^3 (HRT 6-9h, 455gC/kg feed). In the designed CSTR, sludge was not returned and sludge retention time equaled HRT to prevent unnecessary cell oxidation (Pirt, 1975). This modus operandi was in contrast to activated sludge systems, which have a sludge return and do not aim for biomass productions (Tchobanoglous et al., 2003).

RAS design had not to be changed and no interferences with other processes inside the system occurred. This was in contrast to earlier systems as described before (Knoesche and Tscheu., 1974, Meske, 1976). The designed reactor was efficient in inorganic nitrogen removal similar to denitrification reactors with about 1gN/l/d (van Rijn et al., in press). However, the carbon consumption per g inorganic N removed was much higher for the present heterotrophic conversion than for denitrification (13gC/gN versus 2gC/gN, Henze et al., 1999).

In conventional RAS designs all nutrients that are not retained by the fish are transferred or lost to the outside environment. The solid waste is treated outside the system boundaries with long SRTs or HRTs, such as in septic tanks or lagoons (HRT ~15d, Chen et al., 1997). Alternatively, when composting and/or anaerobic fermentation would be applied, it would lead to a net loss of nutrients due to bacteria activity and would result in odor and greenhouse gas emissions (Chen et al., 1997, Burton and Turner, 2003). When the solid waste would be treated as terrestrial waste (manure) and destined as fertilizer for agricultural land, it can result into long transport distances, due to limitations in soil carrying capacity around the fish farm (Janzen et al.1999; Adhikari et al., 2005). The present approach avoids or reduces these negative impacts. Furthermore, nutrients were made available for re-use inside the aquatic system. All this makes the proposed heterotrophic bacteria reactor an interesting

alternative for current solid waste treatment systems in RAS. A concern, however, is the required organic C supplementation to provide optimal C: N ratios (455gC/kg feed, chapter 8). Furthermore, it is unclear, to which extent the converted nutrients will be really retained in a bacterivore organism. Other aspects, which have not been covered in this study, have to be investigated in the future. These aspects concern the chain feed-fish-waste-bacteria, the harvestability and the up scaling of the reactor to farm size. In the present study only one fish species, fed with a specific commercial diet, was used as waste producer. However, fish waste composition is highly depending on fish species, fish size, environmental conditions and feed type. If only one of these factors is changed, the starting values of the conversion process change. Analytical issues made it difficult to determine exactly how much of the waste VSS entering the reactor was converted into bacteria biomass. To obtain more precise data, alternative analytical methods must be applied. If the exact fractions of converted and unconverted VSS are known, the conversion process can be optimized and fine-tuned to decrease the fraction of unconverted VSS.

In the future, harvest techniques must be evaluated, such as mechanical or membrane filtration, sedimentation, centrifugation, flocculation, foam fractionation, evaporation or electrokinetic methods (Atkinson and Mavituna, 1991; Rittmann and McCarty, 2001). By these methods, the bacteria biomass can be made available for culture organisms as feed. It might also be possible to feed the obtained bacteria biomass directly to filter feeders, such as tilapia or shrimp (Avnimelech et al. 1989; McIntosh, 2001; Turker et al., 2003; Brune et al., 2003).

Reactor up-scaling will impact the reactor's hydrodynamics, the oxygenation design, the agitation requirements, the pH management, the C supplementation and the harvest techniques. Based on the experiments with a small-scale reactor, it is recommended to follow a two step approach in the future, (1) integrating a reactor on semi-farm level (1.5MT fish/production per year) to investigate the influence of reactor volume (from 3.5 l to about 110 l) on the conversion process, to fine tune the kinetic parameters and to evaluate the feasibility to feed the bacteria biomass to shrimp or fish; (2) up-scaling to full farm level and to set-up a pilot system, integrating fish-bacteria and a secondary crop, such as shrimp or tilapia.

Conclusions

From this study the following conclusions have been drawn: Fish waste management inside a RAS is an alternative mean to waste management outside RAS. Waste can be

converted into heterotrophic bacteria biomass inside RAS in a specifically designed reactor. This conversion is less sensitive for changes in hydraulic retention time (11-2h) than for organic C supplementation levels (0-3.5gC/l). The organic C source (sodium acetate or molasses) has thereby no detectable effect, provided that it is easily degradable. The form of nitrogenous waste (TAN or nitrate) had no effect on bacteria yields either. Furthermore, bacteria kinetics were derived from the conducted experiments (yield: 0.537gVSS/gC; endogenous decay coefficient: $0.033h^{-1}$; maximum specific growth rate: $0.217h^{-1}$; half-velocity constant: 0.025g/l; and maximum rate of substrate utilization: 0.404 gC/gVSS*h). By applying these kinetics in a model, the bacteria production of a reactor integrated in a 100MT African catfish farm was calculated (187gVSS/kg feed). The removal efficiency of dissolved inorganic N and P was 85 and 95% respectively, assuming a bacteria harvestability of 100%. The produced bacteria, mainly *Rhizobium* and *Mezorhizobium* strains, could not be associated with a pathogenic risk. The biomass, however, was less preferred by shrimps than a commercial feed but accepted as diet. If the bacteria biomass would have been fed to tilapia, a theoretical maximum improvement of 0.4 to 0.5 in FCR could be obtained which equaled an increased N retention of 30-40% in the RAS. The integration of heterotrophic bacteria conversion to manage the waste effluent of a RAS together with the integration of a bacterivore secondary crop is, therefore, a prospective tool to increase RAS sustainability in the future.

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Summary

Just as all other types of animal production, aquaculture produces waste. This waste can be managed outside the production system, comparable to terrestrial husbandry systems. However, particularly recirculation aquaculture systems (RAS) are suited to manage waste within the system. In this case, processes have to be selected to convert the waste into a re-usable product. Dissolved and solid waste conversion by heterotrophic bacteria is one of these processes. In the present study, the potential of the latter process was investigated. An operational scheme was followed, which contained five steps: (1) to evaluate nutrient flows in integrated aquaculture systems, (2) to select and to investigate a conversion process, (3) to improve the process and analyze its sensitivity, (4) to evaluate the product suitability, (5) to derive the kinetics, reactor design, and to determine the integration possibilities into RAS.

In *chapter 2* nutrient flows, conversions and waste management were evaluated, which are taking place in integrated intensive aquaculture systems. In these systems, fish is cultured next to other organisms, which are converting nutrients, which would be otherwise discharged. These conversions were evaluated based on nitrogen (N) and phosphorous (P) balances using a mass balance approach. In the reviewed examples, fish culture alone retained 20-50% feed N and 15-65% feed P. The combination of fish culture with phototrophic conversion increased nutrient retention of feed N by 15-50% and of feed P by up to 53%. If in addition herbivore consumption was included, then the gained nutrient retention decreased by 60-85% feed N and 50-90% feed P. The conversion of nutrients into bacteria and detrivorous worm biomass contributed only to a smaller extent (e.g. 7% feed N and 6% feed P and 0.06% feed N $0.03x10^{-3}$% feed P, respectively). All integrated modules had their specific limitations, which were related to uptake kinetics, nutrient preference, unwanted conversion processes and abiotic factors and implications.

Chapters 3 to 5 focused on the experimental production of heterotrophic bacteria biomass on carbon (C) supplemented fish waste under different operational conditions. The results covered step two and three in the operational scheme.

In *chapter 3*, the drum filter effluent from a RAS was used as substrate to produce heterotrophic bacteria in suspended growth reactors. Effects of organic C supplementation (0, 0.9, 1.7, 2.5gC/l as sodium acetate) and of hydraulic retention times (HRT: 11-1h) on bacteria biomass production and nutrient conversion were investigated. Bacteria production, expressed

as VSS (volatile suspended solids) was enhanced by organic C supplementation, resulting in a production of 55-125gVSS/kg fish feed (0.2-0.5gVSS/gC). Maximum observed crude protein production was ~100g protein/kg fish feed. The metabolic maintenance costs were 0.08Cmol/Cmol h^{-1}, and the maximum growth rate was 0.25-0.5h^{-1}. Approximately, 90% of the inorganic nitrogen and 80% of ortho-phosphate-phosphorus were converted.

The influence of nitrogenous waste on bacteria yields was investigated in *chapter 4*. RAS effluents are rich in nitrate and low in total ammonia nitrogen (TAN). This might result in 20% lower bacteria yields, because nitrate conversion into bacteria is less energy efficient than TAN conversion. In this chapter, the influence of TAN concentrations (1, 12, 98, 193, 257mgTAN/l) and stable nitrate-N concentrations (174±29mg/l) on bacteria yields and N conversions was investigated in a RAS under practical conditions. The effluent slurry was supplemented with 1.7gC/l sodium acetate, due to C deficiency, and was converted continuously in a suspended bacteria growth reactor (6h HRT). TAN utilization did not result in different yields compared to those for nitrate (0.24-0.32gVSS/gC, p=0.763). However, TAN was preferred compared to nitrate and was converted to nearly 100%, independently of TAN concentrations. TAN and nitrate conversion rates differed significantly for increasing TAN levels ($p<0.000$ and $p=0.012$), and were negatively correlated. It seems, therefore, equally possible to supply the nitrogenous substrate for bacteria conversion as nitrate or as TAN. Because in RAS, nitrate is the predominant N form in the waste, the bacteria reactor can safely be integrated into an existing RAS as end of pipe treatment.

In *chapter 5*, sodium acetate, which was used in *chapter 3 and 4* was replaced by molasses as organic C supplement. The effect of molasses as alternative C source on bacteria productions and yields was investigated. One bacteria reactor (3.5 l) was connected to the drum filter (filter mesh size 60µm) outlet of a recirculation system in a continuous flow (HRT: 6h). The different supplementation levels of molasses were 0.0, 3.2, 5.8, 7.8, 9.7gC/l/d. For the maximum flux, the VSS and crude protein production were about 168gVSS and 95g crude protein per kg feed. The maximum conversion of nitrate and ortho-phosphate was 24g NO_3-N and 4gP/kg feed, a conversion of 90% of the inorganic nitrogenous waste and 98% of the ortho-phosphate-P. Furthermore the maximum substrate removal rate and the half saturation constant (K_s) were determined (1.62gC/l/h and 0.097gC/l respectively). The maximum specific removal rate was 0.31gC/gVSS/h and the related K_s was 0.008gC/l. The observed growth rate reached a maximum for C fluxes higher than 8g/l/d.

Chapter 6 and 7 were focusing on the fourth step of the operational scheme (product evaluation and determination of re-use potential).

Because the produced bacteria biomass might contain pathogens, which could reduce its suitability as feed, it was important to characterize the obtained bacteria communities under different conditions (*chapter 3 to 5, reported in chapter 6*). The operation conditions were: 7h hydraulic retention time versus 2h, sodium acetate versus molasses (organic C supplement), and ammonia versus nitrate (N donor). Samples were analyzed by standard biochemical tests, by 16sRNA ribotyping and ribosomal RNA gene-targeted PCR-DGGE fingerprinting combined with clone library analysis. The community of the drum filter effluent was different from the communities found in the bacteria reactors. However, all major community components were present in both the drum filter effluent and reactor broths. HRTs (7h versus 2h) influenced bacteria community resulting in a more abundant fraction of alpha proteobacterium Bioluz/ *Acinetobacter* at 2h HRT compared to 7h HRT (*Rhizobium/ Mezorhizobium*). The use of molasses instead of sodium acetate changed the bacteria community from *Rhizobium/ Mesorhizobium* to *Aquaspirillum* as major component. Providing TAN in addition to nitrate as nitrogenous substrate led to the occurrence of bacteria close to *Sphaerotilus*, *Sphingobacterium* and *Jonesia*. From those results, it was concluded that 6-7h HRT is recommended, and that the type of substrate (sodium acetate or molasses, TAN or nitrate) is less important, and results in communities with a comparable low pathogenic risk.

In *chapter 7*, the produced bacteria biomass was fed to shrimps (*Litopenaeus vannamei*). In total three different diets were used in a variance of a T-maze test: a commercial shrimp feed, the bacteria biomass, which was produced in the suspended growth reactors on C supplemented fish waste under conditions, comparable to those reported in *chapter 3*, and slurry, which was anaerobically produced in a denitrification reactor. If the bacteria products would be attractive as diet, the nutrient retention of the RAS would be improved, resulting in a system, combining fish, bacteria and shrimp. The diet preference was interpreted as an expression of diet attractiveness. As a first result, shrimp were moving from an equal distribution before feeding (+/-50%, -2min), towards the feeding places (>50%, 2, 5, and 10 minutes after feeding). It was, therefore, inferred, that all bacteria biomass and commercial feed combinations were basically attractive for the shrimp. This response was not instantaneous. After feeding (2min) more than 80% of the shrimp were present at the feeding places and showed a significant preference for the commercial feed compared to the aerobically produced bacteria slurry. For the other diet combinations no significant differences could be detected for 2min. For 5 and 10min after feeding, shrimp behavior changed from the commercial feed to the aerobically and anaerobically produced bacteria

biomass segments. From this study it was concluded that although the commercial diet was preferred above the aerobic slurry, the bacteria slurries had also attracted the shrimps. There was no unambiguous conclusion to be made regarding the preference for aerobic or anaerobic produced slurry.

In *chapter 8*, the design of a suspended bacteria growth reactor integrated in a 100MT African catfish farm was determined. This study integrated results from the earlier chapters to calculate the bacteria kinetics (yield=0.537gVSS/gC; endogenous decay coefficient=0.033h^{-1}; maximum specific growth rate=0.217h^{-1}; half-velocity constant=0.025g/l; and maximum rate of substrate utilization=0.404gC/gVSS*h). As part of the study a model was developed and validated. This model was used to calculate the VSS production and nutrient conversion by heterotrophic bacteria conversion for a 100MT African catfish farm. The VSS production was 187gVSS/kg feed and the inorganic nutrients (N and P) were removed with an efficiency of 85 and 95% for a C supplementation level of 3.5gC/l (455gC/kg feed). A reactor integrated in a 100MT farming facility would have a volume of 11m^3, based on a minimum HRT of 6h.

The production and potential re-use of heterotrophic bacteria biomass is, therefore, a prospective tool to lower nutrient discharge and to increase nutrient retention and sustainability of RAS in the future.

Samenvatting

Vergelijkbaar met alle andere typen van dierlijke produktie, produceert aquacultuur afval. Dit afval kan buiten het produktiesysteem behandeld worden, zoals bij houderij-systemen op het land. Recirculatie aquacultuur systemen (RAS) zijn echter bij uitstek geschikt om afval in het systeem zelf te behandelen. In dit geval zullen processen aangewend moeten worden om het afval om te zetten in een produkt dat opnieuw gebruikt kan worden. De omzetting van opgelost en vast afval door heterotrofe bacteriën is één van deze processen. In dit proefschrift is de potentie van dit laatstgenoemde proces onderzocht. Een operationeel schema is gevolgd, bestaande uit vijf stappen: (1) het evalueren van nutriëntenstromen in geïntegreerde aquacultuursystemen, (2) het kiezen en onderzoeken van een omzettingsproces, (3) het proces verbeteren en de gevoeligheid ervan analyseren, (4) de geschiktheid van het produkt evalueren, (5) het afleiden van de kinetiek, reaktor ontwerp, en de mogelijkheden bepalen voor integratie in het RAS.

In *hoofdstuk 2* zijn nutriëntenstromen, omzettingen en afval-management geëvalueerd die plaatsvinden in geïntegreerde intensieve aquacultuursystemen. In deze systemen wordt vis gekweekt naast andere organismen die nutriënten omzetten die anders afgevoerd zouden worden. Deze omzettingen zijn geëvalueerd op basis van stikstof (N) en fosfor (P) balansen, daarbij gebruikmakend van een massa balans benadering. In de bekeken voorbeelden werd door viskweek alléén 20-50% voer-N en 15-65% voer-P behouden. De combinatie van viskweek met fototrofe omzetting verhoogde het behoud van nutriënten van voer-N met 15-50% en van voer-P tot 53%. Als hier nog herbivore consumptie aan werd toegevoegd, daalde het toegenomen nutriëntenbehoud met 60-85% voer-N en 50-90% voer-P. De omzetting van nutriënten in bacteriën en detrivore worm-biomassa droeg in mindere mate bij (bijv. 7% voer-N en 6% voer-P en 0.06% voer-N en $0.03*10^{-3}$% voer-P, respectievelijk). Alle geïntegreerde modules hadden hun specifieke beperkingen die gerelateerd waren aan opname kinetiek, voorkeur voor nutriënten, ongewilde omzettingsprocessen en abiotische factoren en implicaties.

Hoofdstukken 3 tot 6 gingen nader in op de experimentele produktie van heterotrofe bacteriële biomassa op koolstof (C)-gesupplementeerd visafval onder verschillende operationele condities. De resultaten zijn gerelateerd aan stappen twee en drie van het operationele schema.

In *hoofdstuk 3* is het effluent van het drum filter van een RAS gebruikt als substraat om in suspensie zijnde heterotrofe bacteriën in groei-reaktoren te produceren. De effekten van organische C toevoeging (0, 0.9, 1.7, 2.5g C/l als natriumacetaat) en van hydraulische retentietijden (HRT: 11-1h) op bacteriële biomassa produktie en nutriënten omzetting zijn onderzocht. Bacteriële produktie, uitgedrukt als VSS (vluchtige opgeloste vaste stof) was verhoogd door organische C toevoeging, resulterend in een produktie van 55-125g VSS/kg visvoer (0.2-0.5g VSS/g C). De maximale waargenomen ruwe eiwit produktie was ongeveer 100g eiwit per kg visvoer. De metabolische onderhoudskosten waren 0.08Cmol/Cmol h^{-1}, en de maximale groei snelheid was 0.25-0.5h^{-1}. Ongeveer 90% van het inorganische stikstof en 80% ortho-fosfaat-fosfor waren omgezet.

De invloed van stikstofhoudend afval op de opbrengst van bacteriën is onderzocht in *hoofdstuk 4*. Effluent van RAS is rijk aan nitraat en arm aan totaal ammonia stikstof (TAN). Dit kan resulteren in 20% lagere bacterie-opbrengst, omdat omzetting van nitraat in bacteriën energetisch minder efficiënt is dan TAN-omzetting. In dit hoofdstuk zijn invloeden van TAN concentraties (1, 12, 98, 193, 257 mg TAN/l) en stabiele nitraat-N concentraties (174±29 mg/l) op bacterie-opbrengsten en N-omzettingen onderzocht in een RAS onder praktijkomstandigheden. De effluent smurrie was aangevuld met 1.7g C/l natriumacetaat, vanwege C-deficiëntie, en werd continu omgezet in een groei reaktor met bacteriën in suspensie (6h HRT). Vergeleken met nitraat leidde verbruik van TAN niet tot verschillende opbrengsten (0.24-0.32gVSS/g C, $p=0.763$). Echter, TAN werd verkozen boven nitraat en werd bijna 100% omgezet, onafhankelijk van TAN concentraties. De omzettingssnelheden van TAN en nitraat verschilden significant bij toenemende TAN concentraties ($p<0.000$ en $p=0.012$) en waren negatief gecorreleerd. Het lijkt daarom evengoed mogelijk om het stikstofhoudend substraat voor bacterie-omzetting als nitraat of als TAN toe te voegen. Aangezien nitraat de voornaamste N-bron in afval van RAS is, kan de bacterie-reaktor prima in een bestaande RAS worden geïntegreerd als het einde van de behandelingsstap.

In *hoofdstuk 5* is natriumacetaat, dat gebruikt is in *hoofdstukken 3 en 4*, vervangen door melasse as organisch C supplement. Het effect van melasse als alternatieve C-bron op bacteriële produktie en opbrengst is onderzocht. Een bacterie-reaktor (3.5l) was verbonden met het afvoerkanaal van een drum filter (filter maaswijdte 60 μm) van een recirculatiesysteem in een continue stroom (HRT: 6h). De verschillende toegevoegde melasse concentraties waren 0.0, 3.2, 5.8, 7.8, 9.7g C/l/d. Voor de maximale flux waren de VSS en ruwe eiwit produktie ongeveer 168g VSS en 95g ruw eiwit per kg voer. De maximale omzetting van nitraat en ortho-fosfaat was 24g NO_3-N en 4g P/kg voer, i.e. een omzetting van

90% van het inorganische stikstofhoudende afval en 98% van het ortho-fosfaat-P. Verder zijn de maximale snelheid van substraatverwijdering en de half-verzadigingsconstante (Ks) bepaald (1.62g C/l/h en 0.097g C/l, respectievelijk). De maximale specifieke verwijderingssnelheid was 0.31g C/g VSS/h en de gerelateerde K_s was 0.008g C/l. De waargenomen groeisnelheid bereikte een maximum voor C-fluxen hoger dan 8g/l/d.

Hoofdstukken 6 en 7 gingen nader in op de vierde stap van het operationele schema (produkt evaluatie en bepaling van het hergebruik potentieel).

Aangezien de geproduceerde bacteriële biomassa ziekteverwekkers kan bevatten die de geschiktheid als voer kunnen verminderen, is het belangrijk om de verkregen bacteriepopulaties onder verschillende omstandigheden te karakteriseren (*hoofdstuk 3 tot 5, weergegeven in hoofdstuk 6*). De operationele condities waren: 7h hydraulische retentietijd versus 2h, natriumacetaat versus melasse (organisch C supplement), en ammonia versus nitraat (N donor). Monsters zijn geanalyseerd door standaard biochemische testen, met behulp van 16sRNA ribotyping en ribosomaal RNA gene-targeted PCR-DGGE fingerprinting gecombineerd met clone library analysis. De populatie van het drum filter-effluent verschilde van de populaties in de bacterie-reaktors. Echter, alle belangrijke populatie-componenten waren aanwezig in het drum filter effluent en reaktor soep. HRTs (7h versus 2h) beïnvloedden de bacteriepopulatie, resulterend in een toegenomen fractie van alpha proteobacterium Bioluz/ *Acinetobacter* bij 2h HRT vergeleken met 7h HRT (*Rhizobium/Mezorhizobium*). Het gebruik van melasse in plaats van natriumacetaat veranderde de bacteriepopulatie van *Rhizobium/Mezorhizobium* naar *Aquaspirillum* als belangrijkste component. Het toevoegen van TAN bovenop nitraat als stikstofhoudend substraat leidde tot bacteriën gerelateerd aan *Sphaerotilus*, *Sphingobacterium* en *Jonesia*. Naar aanleiding van deze resultaten is geconcludeerd dat 6-7h HRT wordt aangeraden en dat het type substraat (natriumacetaat of melasse, TAN of nitraat) minder belangrijk is en resulteert in populaties met een vergelijkbaar laag risico op ziekteverwekking.

In *hoofdstuk 7* is de geproduceerde bacteriële biomassa gevoerd aan garnalen (*Litopenaeus vannamei*). Drie verschillende voeders zijn gebruikt in een variatie op de T-maze test: een commercieel garnalen voer, de bacteriële biomassa geproduceerd in de groei-reaktoren op C-toegevoegd visafval onder omstandigheden vergelijkbaar als in *hoofdstuk 3*, en anaëroob geproduceerde smurrie in een denitrificatie reaktor. Indien de bacterie-produkten aantrekkelijk zouden zijn als voer, zal de nutriënten retentie van het RAS verbeterd zijn, resulterend in een systeem dat vis, bacteriën en garnalen combineert. De voorkeur voor voer was uitgelegd als een maat voor aantrekkelijkheid van het voer. Als eerste resultaat bewogen

de garnalen zich vanuit een gelijke verdeling vóór het voeren (+/-50%, -2 min) naar de voerplaatsen (>50%, 2, 5, en 10 minuten na voeren). Hieruit werd geconcludeerd dat alle combinaties van bacteriële biomassa en commerciële voeders aantrekkelijk waren voor de garnaal. Deze respons was niet onmiddellijk te zien. Na het voeren (2 minuten) was meer dan 80% van de garnalen aanwezig bij de voerplaatsen en lieten een significante voorkeur zien voor het commerciële voer vergeleken met de aëroob geproduceerde bacteriële smurrie. Voor de andere voercombinaties werden geen significante verschillen waargenomen gedurende 2 minuten. Bij 5 en 10 minuten na het voeren veranderde het gedrag van de garnalen op het commerciële voer naar de aëroob en anaëroob geproduceerde bacteriële biomassa segmenten. Uit dit experiment werd geconcludeerd dat ondanks het feit dat het commerciële voer verkozen werd boven de aërobe smurrie, de bacteriële smurries ook aantrekkelijk waren voor de garnalen. Er kon niets geconcludeerd worden met betrekking tot de voorkeur voor aëroob of anaëroob geproduceerde smurrie.

In *hoofdstuk 8* is het ontwerp van een groei reaktor met bacteriën in suspensie geïntegreerd in een 100MT Afrikaanse meerval kwekerij bepaald. Deze studie gebruikte resultaten van eerdere hoofdstukken om de bacteriële kinetiek te berekenen (opbrengst=0.537g VSS/g C; endogene decay coefficiënt=0.033h^{-1}; maximale specifiek groeisnelheid=0.217h^{-1}; halve-snelheidsconstante=0.025g/l; en maximale snelheid van substraatverbruik=0.404g C/g VSS*h). Als onderdeel van deze studie is een model ontwikkeld en gevalideerd. Dit model is gebruikt om de VSS produktie en de omzetting van nutriënten door heterotrofe bacteriën te voorspellen voor een 100MT Afrikaanse meerval kwekerij. De VSS produktie was 187g VSS/kg voer en de inorganische nutriënten (N en P) werden verwijderd met een efficiëntie van 85 en 95% bij een C-supplementatie concentratie van 3.5g C/l (455g C/kg voer). Een reaktor die geïntegreerd is een 100MT kwekerij zou een volume hebben van 11 m^3, gebaseerd op een minimum HRT van 6 uur.

De produktie en potentieel hergebruik van heterotrofe bacteriële biomassa is daarom een te verwachten middel om de nutriënten uitstoot te verlagen en het behoud van nutriënten en duurzaamheid van RAS voor de toekomst te verhogen.

Acknowledgements

It would have been impossible to begin and to finish this thesis without the help and friendship of big number of people. I am grateful to my supervisors: Prof. Dr. Johan Verreth, Ing. Ep Eding, Dr. Vicky Sereti and Dr. Marcel Machiels for their help, their time and their support during the last years. Ep, especially many thanks to you! I will always remember our discussions lasting sometimes up to five in the morning. Your advices and your helpfulness make you a unique person.

I would like to thank all my colleagues and friends at the department, which I could meet during the last five years: my room mates (Titu, Miriam and Beatriz), and all my fellow colleagues and friends in the AFI group and at ZODIAC, which were always there for me: Rodrigo, Pablo, Samad, Mohammed-Ali, Catarina, Paula, Marc, Neil, Iyob, Yonas, Harrison, An, Patricia, Goncalo, Hanh, Hans, Karin, Rob, Bernado, Sander, Pascal, Ana, Ajay, Ekram, Nanh, Marc, Ronald, Johan, Roel, Leo, Paul, Geertje, Bram, Jascha (Many thanks for the Dutch translation!!), Anne, Hans, Helene, Gerrie, Netty, and Lies. It was a pleasure to work, discuss and celebrate with you.

There are some special people, without their support this thesis would have remained nothing but fiction: Many thanks for all their help to the AFI staff Menno, Ronald, Tino and Rolf, to the ANU staff Saskia, Dick, and Huug, to Hetty from ATO, Sietze, Wian, Truus, Sander and Aart from the hatchery, to Eric, Olaf, Evert and Hans from the workshops, to Marianne from the library, to Gab and Marianne from WIAS, Peter from Repro and to Chris, Gerald and Jeroen from IT. Furthermore there are some people that had always an open ear for me, if I needed to discuss and was seeking information and advice. Many thanks, therefore, to Wiebe Koops, Martin Verstegen, Thomas van der Poel, Bram Klapwijk, Eddy Bokkers, Olga Haenen, Hauke Smidt, Mariana Chabrillon-Popelka, Jaap van Rijn, Yoram Avnimelech and Raul Piedrahita.

Special thanks go the European Union for funding the ZAFIRA project, in which frame my research was conducted, especially to Dr. Cornelia Nauen. Furthermore many thanks to the whole project team (Amos Tandler, Michal Ucko, Ingrid Lupatsch, Noam Mozes, Uwe Waller, Jaime Orellana, Wang Ji-Qiao, Liu Chang-Fa, Xue-Jun He, Si-fa Li) for your cooperation, for a really good time and for unique impressions during my travels to you.

I am grateful for the contribution of five students, which I could supervise during my research: Jordi Vera, Tuan Le Cong, Likang Deng, Adrian Bischoff, João Carvalho and Job Munten.

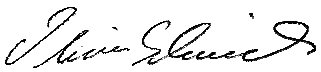
Bobby Unser said once: “Success is where preparation and opportunity meet.”. I have to thank my mentors Prof. Dr. Dr. h.c. mult. Harald Rosenthal, Prof. Dr. Hans Uhlarz, Prof. Dr. Rainer Kollmann and Dr. Karl-Ronald Otto which did both: prepared me and gave me the opportunity to walk my way.

But life would have been incomplete without my friends outside the workgroup: Oliver, Arne, Liesbeth, Piter, Vincent, Eelco, Erik-Jan, Ana, Maaike, Lotte, Nienke, Juan, Sebastien, Andreas, Margriet, Maarten, Kirsten, Maaike, Ulrich, and of course my volleyball team mates.

This thesis is dedicated to my parents. Many thanks to you for the trust in my decisions, the support, and for being my safe harbor when waves were pounding!

Ainhoa, my love, I do not know, how to thank you for your support and love during the last years. You have been my sunshine, when the days were grey and my shade, when the sun shined too much on my forehead. A big kiss!

Many thanks to all of you!



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Training and Supervision Plan

Training and Supervision Plan		**Graduate School WIAS**
Name PhD student	Oliver Schneider	
Project title	Heterotrophic bacteria production on solid fish waste in recirculation aquaculture systems	
Group	AFI	
Daily supervisor(s)	Ep Eding, Vicky Sereti	
Supervisor(s)	Johan Verreth	
Project term	from 01/01/2002 until 01/06/2006	
Submitted	01/02/2006 first plan / midterm / **certificate**	

EDUCATION AND TRAINING (minimum 30 credits)		
		credits *
The Basic Package (minimum 3 credits)	year	
WIAS Introduction Course (mandatory, 1.5 credits) (22-25 February 2005)	2005	
Course on philosophy of science and/or ethics (mandatory, 1.5 credits) (8 March -19 April 2005)	2005	
Subtotal Basic Package		**3**
Scientific Exposure (conferences, seminars and presentations, minimum 8 credits)	year	
International conferences *(minimum 3 credits)*		
World Aquaculture Conference, Beijing (23-27 April 2002), oral presentation	2002	
European Aquaculture Meeting, Trondheim (8-12 August 2003), poster presentation	2003	
European Aquaculture Meeting, Barcelona (20-23 October 2004), oral and poster presentations	2004	
World Aquaculture Conference, Bali (9-13 May 2005), oral presentation	2005	
Seminars and workshops		
WIAS Science Day (2004), Wageningen	2004	
WIAS Science Day (2005), Wageningen, oral presentation	2004	
Unesco IHE Topic day on nitrification, Delft	2003	
WIAS Seminar of ZAFRIA/INREF Pond, Wageningen	2003	
WIAS Seminar Vitality of fish, Wageningen	2005	
WIAS Workshop of Ifremer/RIVO/AFI, Wageningen	2005	
WIAS Seminar on food for brain, Wageningen	2003	
Aquainnovation Workshop, Szarvas (26-30 September 2005), 2 presentations	2005	
Subtotal International Exposure		**18**
In-Depth Studies (minimum 6 credits, of which minimum 4 at PhD level)	year	
Disciplinary and interdisciplinary courses		
Uncertainty Analysis (S02, Inst. Environment and Climate Research) (January-February 2004)	2004	
WIAS advanced Statistic Course (25-27 November 2002)	2002	
Aquatic Animal Disease Diagnostics (15-20 January 2006)	2006	
Advanced statistics courses *(optional)*		
Basic and Advanced Statistics (December-February 2002/2003)	2002/2003	
Subtotal In-Depth Studies		**10**

Professional Skills Support Courses (minimum 3 credits)	year	
Course Techniques for Scientific Writing (2-5 July 2002)	2002	
Use of Laboratory Animals (2-13 September 2002)	2002	
Subtotal Professional Skills Support Courses		**6**
Research Skills Training (optional)	year	
Preparing own PhD research proposal (maximum 6 credits)	2002	
Special research assignments *(apart from PhD project)*		
ZAFIRA project (Research cooperation with international partners)	2002-2005	
Subtotal Research Skills Training		**14**
Didactic Skills Training (optional)	year	
Lecturing		
Fish and Fish Production	2004/2005	
PGSO Course	2002/2003	
Aqualabs I (Recirculation Aquaculture)	2006	
National Course on Recirculation Tech. (13-26 March 2002, Temuko, Chile)	2002	
Supervising practicals and excursions		
Fish and Fish Production (Practical)	2002-2004	
National Course on Recirculation Tech. (13-26 March 2002, Temuko, Chile)	2002	
Supervising MSc theses *(maximum 2 credits per major, 1.5 credits per minor)*		
4 major and 1 minor	2002-2005	
Preparing course material		
Nat. Course on Recirc. Technology (Practicals/Reader,13-26 March 2002, Temuko, Chile)	2002	
Subtotal Didactic Skills Training		**21**
Management Skills Training (optional)	year	
Organisation of seminars and courses		
ZAFIRA workshops & meetings	2002-2006	
Membership of boards and committees		
WAPS Council Member & Wageningen PhD Student Council Member (WPC)	2003-2004	
Subtotal Management Skills Training		**8**
Education and Training Total (minimum 30 credits)		**80**

* one ECTS credit equals a study load of approximately 28 hours

About the author

Oliver Schneider was born on September the 17th, 1973 in Muenster, Germany. After finishing high school and fulfilling his civil services, he studied biology at the Christian-Albrechts-University in Kiel, Germany from 1994 to 2001. During his study he specialized in aquaculture and fisheries, zoology, organic chemistry and marine biology. His major thesis was investigating nutrient and energy flows in recirculation aquaculture systems. He completed the study in 2001 with a Diploma in Biology (MSc equivalent) and obtained in 2003 the MSc in Aquaculture from Wageningen University, The Netherlands. After working in different functions at the Aquaculture and Fisheries Group of Wageningen University between 2000 and 2001, he carried out his PhD research within the ZAFIRA-Project (Zero discharge Aquaculture by Farming in Integrated Recirculating Systems in Asia) in the same chair group, since 2002. This research resulted in the present thesis. From June 2006 onwards, Oliver Schneider is working for IMARES (Institute for Marine Resources & Ecosystem Studies, The Netherlands).

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This research was funded by the European Union in the frame of the ZAFIRA project (Zero discharge Aquaculture by Farming in Integrated Recirculating Systems in Asia)
ICA4-CT-2001-10025