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MICROBIAL COMMUNITIES OF RECIRCULATING AQUACULTURE FACILITIES: INTERACTION BETWEEN HETEROTROPHIC AND AUTOTROPHIC BACTERIA AND THE SYSTEM ITSELF

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MICROBIAL COMMUNITIES OF RECIRCULATING AQUACULTURE FACILITIES: INTERACTION BETWEEN HETEROTROPHIC AND AUTOTROPHIC BACTERIA AND THE SYSTEM ITSELF

- JURY -

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CHAPTER 1

Introduction and problem statement

It is actually recognized that most of the world's fishing areas have reached their maximal potential for capture fisheries production, while demand for seafood worldwide is steadily increasing (Blancheton, 2000), and that the expansion of the aquaculture sector will probably fill the gap.

Aquaculture, the fastest growing agro-food industry, actually produces almost 50 % of the world's food fish and has the greatest potential to meet the growing demand for aquatic food (FAO, 2006). For these reasons technology applied to the aquaculture field evolved rapidly, both in modifying existing facilities, as water recirculation for land based installations, and in developing new concepts, as off-shore sea cages.

Recirculating Aquaculture Systems (RAS) may be considered an alternative to pond aquaculture technology (by consuming lower amounts of water producing similar yields of crop) or can be integrated in a sort of "production chain" with net pen systems (Blancheton, pers. comm.).

The interest in RAS is due to their intrinsic advantages like the reduction of land and water requirements, the high degree of environmental control allowing year-round growth at optimum rates, the possibility to produce in close proximity to markets. Moreover, the high level of control improves risk management linked with bacterial and parasites infection and predator pests. Finally, waste production (both particulate and soluble) can be controlled and minimized contributing to make these systems ecologically sustainable.

Commercial-size RAS have been introduced in many parts of the world since many years for various purposes, but the development has largely been delayed due to many constraints, including economic costs and technical solutions yet to be improved. Nevertheless, aquaculture in inland waters and in protected marine coastal areas in the future must be practiced with waste treatment of effluents in order to reduce environmental pollution. Two kinds of approaches exist in the water reuse systems: ecological and mechanical approaches (Kim and Jo, 2002). The former is the recycling of the water in the rearing tank itself by natural nutrient recycling, and the latter treats waste water in a separate treatment system and return the treated water to the rearing tanks.

With the increasing of the reared biomass, all kinds of intensive aquaculture facilities carry certain risks as any other agricultural operation. High stocking densities, variations of breeding cycles, routine operations on the system and other manipulations impose stressors to the reared fish that can become more sensible to disease outbreaks due to opportunistic infectious organisms.

Today the fish rearing systems are intensifying and the study of the bacterial flora joined to the fish rearing has become increasingly important to avoid the outbreak of pathology. However, the majority of microbial species colonizing RAS are most likely benign or beneficial to the animals being cultured and the influence of bacterial activity in a recirculating system is of the utmost importance (Blancheton, 2000).

Nowadays, there is a few knowledge on the microbial ecology within Recirculating Aquaculture Systems, and a better understanding on the microbial populations will be helpful to optimize the growing protocols and the welfare of reared animals. In particular, it is vital to acquire information on the dynamic nature of the prokaryotic populations, the relative abundance of pathogens versus probionts and the interaction between heterotrophic bacteria and nitrifiers.

For these reasons researches on heterotrophic bacterial compartment in RAS are necessary in order to acquire the capacity to “pilot” these populations for at least three main reasons: trying to manage microbial mechanisms that cause a degradation of rearing water (oxygen consumption, metabolites by–products secretion, etc.), reducing their often negative interactions with autotrophic bacteria and exploiting the potentiality of these population to be used as biocontrol agents (Léonard *et al*., 2000, 2001; Verschuere *et al*., 2000).

Present thesis was aimed to the study of the heterotrophic bacterial communities inhabiting a marine RAS. In particular, their abundance, distribution and species composition were investigated. A study was carried out in order to investigate the relationship between available organic carbon in the rearing water and the biofilm heterotrophic community structure and activity and on the nitrification process. The heterotrophic bacteria (both total and cultivable) inhabiting the rearing water and the biological filter were characterized by molecular tools. Finally, data obtained from present work and those issued from previous researches, were used to create a conceptual model describing the main process governing the heterotrophic bacterial community in a marine RAS biofilter.

CHAPTER 2

Litterature review

2.1. Marine aquaculture: between increasing production and environmental impact

Marine living resources are a fundamental source of protein in many countries around the world but about 70% of the world conventional species are fully exploited, overexploited, depleted or in the rebuilding process following depletion.

In this context world aquaculture has dramatically grown during the last fifty years from less than a million tons produced in the early 1950s to 59.4 million tons in 2004, with an average annual rate of 8.8% from 1950 to 2004 (FAO, 2006).

In 2004, aquaculture production from mariculture was 30.2 million tons, representing 51% of the global total. Freshwater aquaculture contributed 25.8 million tonnes, or 43.4%. The remaining 3.4 million tons, or 5.7%, came from production in brackish environments (FAO, 2006) (Figure 2.1).

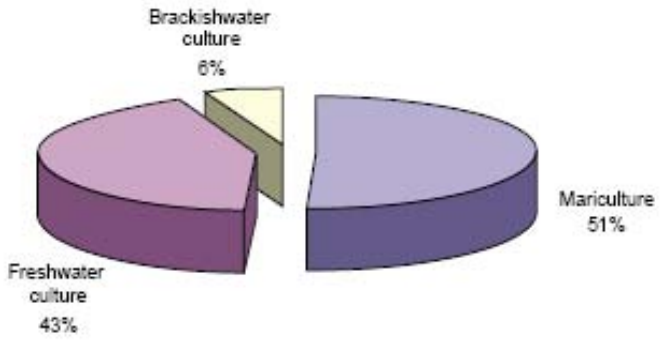

Figure 2.1: Aquaculture production by environment in 2004 (source FAO, 2006).

Aquaculture is facing challenges created by population growth and the resulting competition for water, land, and other natural resources. In some cases, these challenges are being met by intensifying the culture operations. The tendency to intensify fish culture, as for other agriculture industries, is an attempt to obtain higher yields for a given critical resource. How intensification is implemented depends on the type of aquaculture operation (Piedrahita, 2003).

Aquaculture, like many other human activities, produces high amounts of wastes which may negatively affect the environment. In particular, intensive aquaculture,

produces a considerable amount of particulate and soluble organic wastes, containing feces and uningested feed. It may generate a localized pollution by magnifying the chemical and biological oxygen demand (COD and BOD), reducing the water transparency and often generating anaerobic conditions of the seabed, resulting in the outgassing of hydrogen sulphide, ammonia and methane (Mc Caig *et al.*, 1999; Thoman *et al.*, 2001). The negative impacts of such effluents are greatest in low hydrodynamic aquatic environments where the high nitrogen and phosphorous content of effluent may lead to eutrophication and other ecosystem changes (e.g. algal blooms and low dissolved oxygen levels) (Thoman *et al.*, 2001; Li *et al.*, 2003). Moreover, nitrogen in its free ammonia and nitrite forms are toxic to fish and invertebrate animals (Mc Caig *et al.*, 1999; Kloep *et al.*, 2000).

2.2. Recirculating Aquaculture Systems (RAS)

The growing concerns and regulations associated with a clean environment encourage the aquaculture industry to adapt a recirculation system to reduce the wastewater discharged from aquaculture facilities (Kim *et al.*, 2000).

The increasing of environmental constraint and the land and water costs have led to the development of aquaculture systems designed to maintain a high biological carrying capacity, using less water than pond, raceway and relatively little space and minimizing wastes (Twarowska *et al.*, 1997; Skjølstrup *et al.*, 1998; Blancheton *et al.*, 2000; Malone and Beecher, 2000; Thoman *et al.*, 2001; Gutierrez-Wing and Malone, 2006).

The development of commercial scale Recirculating Aquaculture Systems (RAS) for fish production has dramatically increased over last years. If the initial investment cost is still elevated, the renewed interest in RAS is due to several advantages they have over traditional aquaculture, including proximity to the market, consistent quality products, greatly reduced land and water requirements, high degree of environmental control allowing year-round growth at optimum rates, and shorter production cycle due to controlled environment and improved feed conversion (Singh *et al*., 1999).

A recirculating aquaculture production unit is a configuration of several chemical, biological, and mechanical processes. It is constituted by one or more fish culture tanks, a biological filter for ammonia oxidation into nitrates, a mechanical filter for particles removal, a pump for water circulation and an oxygenation device. Moreover, the

treatment loop is constituted by several other purification devices as UV (ultraviolet) or ozone reactors, to reduce the bacterial concentration, to improve water clarity and to reduce disease transmission. Finally, the RAS treatment loop is completed by a CO_2 stripping device, pH and temperature control systems and other control apparatuses in order to maximize the security and to optimize the rearing performances (van Rijn, 1996; Blancheton and Canaguier, 1995; Blancheton *et al.*, 1997; Skjølstrup *et al.*, 1998; Singh *et al.*, 1999; Blancheton, 2000) (Figure 2.2).

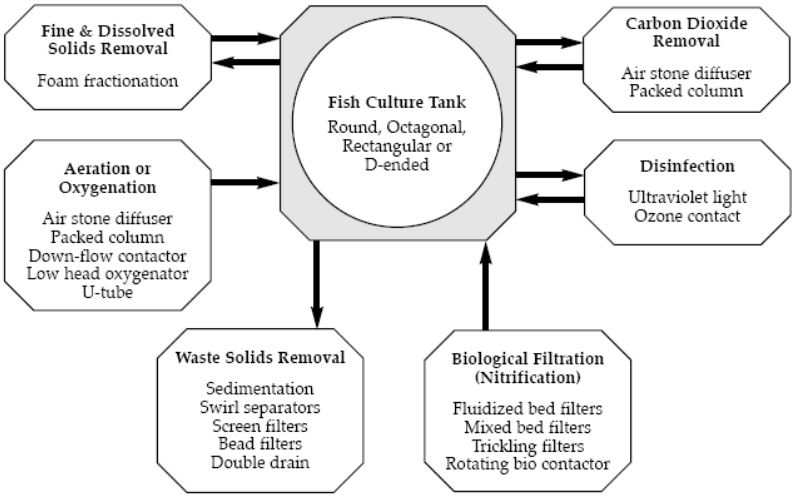

Figure 2.2: Required unit processes and some typical components used in recirculating aquaculture production systems (from Losordo *et al.*, 1999b).

2.3. RAS rearing water degradation and management

For recirculation systems, in which reused water must be of an adequate quality to maintain the culture organisms in a healthy and fast-growing condition, it is especially important to remove the waste products from water (Cripps and Bergheim, 2000). Generally the water quality criteria needed for reuse systems are two: criteria for the culture species and criteria for the operation of unit processes and unit operations (Colt, 2006).

In RAS, rearing water is rapidly degraded by the by-products released by fish and bacteria metabolism, including dissolved and particulate organic matter (DOM and POM), nutrients, such as nitrogen (N) and phosphorus (P), and specific organic or inorganic compounds, such as therapeutants (Piedrahita, 2003) and the use of biological, chemical, and mechanical filtration is useful to maintain proper water

quality. However, some water exchange via the inflow of purified water and the subsequent discharge of effluent is necessary, even in the most efficient recycling aquaculture systems (Twarowska *et al.*, 1997), in order to minimize the build-up and accumulation of some deleterious compounds (Colt, 2006).

Solids deriving from rearing tanks are composed of feces, uneaten feed and bacterial colonized particles (Piedrahita *et al*., 1996), while a typical treatment loop produces bacterial biomass and retains an unknown quantity of particulate matter. Large particles, above 100 microns, will settle out quite easily directly in the particle separator at the outlet of the rearing tank. Particles above 30-40 μm can be filtered out with a screen. Management of particulate matter consists of a rapid elimination of suspended and sedimentable materials from the rearing water, but, as particle size decreases, the efficiency of the solid removal devices decreases and biofouling increases. Hence, the control of adverse effects of particulate matter and the possibility of removing it from RAS before its degradation are challenges for aquaculture engineers (Franco-Nava *et al.*, 2004a).

If the fish are resistant to high levels of inorganic solids (Colt, 2006), the build-up of fine solids in an aquaculture system can lead to a decline in culture water quality that will increase the stress on the culture organisms and may compromise the fish gill function (Cripps and Bergheim, 2000; Summerfelt and Penne, 2005). Moreover, as particle size decreases, the efficiency of the typical solid removal processes decreases and biofouling increases (Franco-Nava *et al.*, 2004a). Particles are important components in the turnover, decomposition and sinking flux of both organic and inorganic matter and elements in aquatic systems (Grossart *et al.*, 2003). Quantitative information on the impacts of specific size fractions of fecal solids and uneaten feed on growth and fish health is lacking (Colt, 2006).

Particulate organic matters contribute to biochemical oxygen demand (BOD) and it provides substrate for growth of heterotrophic bacteria (Singh *et al*., 1999; ; Léonard *et al.*, 2001; Franco-Nava, 2003; Franco-Nava *et al*. 2004a, 2004b; Summerfelt and Penne, 2005).

There are many different types of mechanical filters: i) sand filters; ii) sock filters; iii) drum filters; iv) disk filters. These systems "screen" the particles and the size of particles removed depends on the size of the screen or sieve. Other type of particulate filter is the floatation or bubble separator, commonly known as protein skimmers. In

this device, air is bubbled into a column and the fine particles become attached to the surface of bubbles. For a review of different removal devices see Cripps and Bergheim (2000).

According to Léonard (2000), in a marine RAS the DOM is mainly constituted by humic substances and a recent work demonstrated that formation of these colour compounds is dependent on the content of processed fish meal (Colt, 2006).

The chemistry of aquatic humic and fulvic acids is complex and in some natural tropical waters, the pH may be controlled by humic and fulvic acids instead of the carbonate system. Detailed information on the potential impact of these compounds is lacking (Colt, 2006).

The TAN is the principal product of the fish catabolism (Hagopian and Riley, 1998). For the sea bass between 80% and 87% of the total nitrogen excretion is ammonia and the rest is mainly urea (Forster *et al*., 1969 and Guerin-Ancey, 1976 cited in Léonard, 2000). At this amount it must be added a fraction comprised between 13 and 78% of feces nitrogen, leached by the action of water (Dosdat *et al*., 1996 cited in Léonard, 2000).

Ammonia (NH_3) is very toxic to fish even at low concentrations but the ionized form (NH_4^+) is 100 time less toxic. The proportion of unionized ammonia relative to ionized ammonia (NH_4^+), for a given total ammonia (NH_3 and NH_4^+) concentration in an aqueous solution, depends on temperature, pH and salinity. Lower pH and temperatures increase the percentage of ionized ammonia at the expense of unionized ammonia. Thus, the total ammonia nitrogen (TAN) concentration instead of ammonia nitrogen is often used as a key limiting water quality parameter in intensive aquaculture systems design and operation (Losordo and Westers, 1994).

One of the critical processes in a recirculating system is ammonia removal from the circulating water (Meade, 1985; Avnimelech *et al*., 1995; Avnimelech, 1999; Kim *et al*., 2000). In fact, the accumulation of ammonium is an important limiting factor in rearing systems and the biological conversion of toxic ammonium into the relatively harmless nitrates must be well managed. Biological filtration is the most efficient system used in many field where the elimination of ammonia is needed as drinking water production or wastewater treatment systems (Gilmore *et al*., 1999) and it is a key-process in the water treatment of a recirculating system (Kamstra *et al*., 1998). Biological treatment is relatively cheap and produces no unwanted side-products

(Abeysinghe *et al.*, 1996; Hargrove *et al.*, 1996). Compared with domestic wastewater, aquaculture effluents have a relatively low concentration of pollutants and thus bacterial biomass yield in treatment systems is also low (Eding et al., 2006).

The concept of biofiltration is to supply to nitrifying bacteria a good and stable environment and a substrate with a high specific surface area (large surface area per unit volume) to colonize (Cohen, 2001). Commonly used biofilter substrates include gravel, sand, plastic beads and plastic rings. Depending upon nutrients, water flow rate and temperature, a biofilter typically takes 40-60 days to establish the required bacterial population. A perfect biofilter would remove all of the ammonia nitrogen of the effluent, produce no nitrite, support dense nitrifying bacterial populations on an inexpensive support material and require low maintenance. Unfortunately, no biofilter type can meet all of these objectives, but each biofilter type has there own advantages and limitations. In addition, different factors considered in biofilter selection can shift in relative importance depending upon production system requirements (Summerfelt, 2006).

For biological water treatment , there are many different biofilm systems in use, such as trickling filters, rotating biological contactors (RBC), fixed media submerged biofilters, granular media biofilters, fluidised bed reactors, etc. They all have their advantages and disadvantages (Rusten *et al.*, 2006). Moreover, because of differences in performance, operating characteristics and system requirements, it is difficult to rationally select the “best” filter for a given application. Many systems are selected and sized on the basis of personal experience, marketing promises, and other non-technical criteria. Biofilter performance studies are difficult to conduct due to the large number of parameters that must be controlled and the number of measurements that must be completed (Sandu *et al.*, 2002; Drennan *et al.*, 2005; Colt *et al.*, 2006).

Biofilters can be divided in *fixed film*, that are more stable, and *suspended growth* bioreactors. Because of their lower nitrification efficiency, suspended cell bioreactors, are infrequently employed in aquaculture industry. In contrast, fixed-film processes require much less management or maintenance, and the bacterial attachment provides sufficient time for the slow-growing nitrifiers to reproduce. Because of its advantages, biofilm nitrification has become the standard treatment method for Recirculating Aquaculture Systems (Golz, 1995). In a fixed film biological process, nutrients are transported by diffusion into the biofilm, which coats a Packing media: rock, shells,

sand or plastic are commonly used to support these bacterial films (Malone and Pfeiffer, 2006).

Filters are designed to maximize the oxygen transfer directly over the media. In the case of the tricking filter, transfer is achieved by water falling over the media, whereas rotating biological contactors create the same effect by rotating the media in and out of the water (Malone and Pfeiffer, 2006).

Submerged filters presume that sufficient oxygen can be transported with the ingoing water, by using high recirculation rates, internal recycling, or through oxygen enrichment of influent waters. These filters are distinguished by the strategies used to manage their biofilm accumulations: a potential problem is that the presence of large amounts of carbonaceous solids encourages the growth of heterotrophs that outcompete nitrifying microorganisms (Malone and Pfeiffer, 2006).

2.4. Principal bacterial compartment in a marine RAS

In an environment like a RAS, the importance and the influence of bacterial communities are of the utmost importance and of the same order of magnitude than fish in terms of biomass and processes directly linked to their activities. For instance, in high fish density recirculating systems, bacteria and fish consume similar quantities of oxygen per kg of fish produced (Blancheton, 2000).

Water recirculating systems support large populations of bacteria, protozoa, and micrometazoa. Some of these microorganisms metabolize the waste organic matter found within the system and other microorganisms metabolize dissolved wastes that include dissolved organic compounds, ammonia, nitrites and nitrates (Sharrer *et al.*, 2005). Many of these microorganisms live in biofilms that are located on all humid surfaces (Costerton, 1999) and in particular in the biofilm packing media, but they are also present within the water column.

Bacteria present in RAS can be divided in two main groups: the Autotrophic Bacteria (AB), including bacteria that derive carbon from CO_2 and energy from oxidation of an inorganic nitrogen, sulphur, or iron compound, and the Heterotrophic Bacteria (HB), including bacteria that use organic compounds such as carbohydrates, amino acids, peptides and lipids for their metabolism (Sharrer *et al.*, 2005). However, many other

bacterial groups can be found in RAS, as anammox (Tal *et al.*, 2003, 2006) and denitrifiers (van Rijn *et al.*, 2006).

The Autotrophic nitrifying bacteria (AB) must remove ammonia at a sufficient rate to maintain water quality at a level adequate to prevent exposure to the fish (Zhu and Chen, 1999, 2001a, 2001b). As a result, aquaculture nitrification biofilters operate at a lower TAN concentration compared with those used in industrial wastewater treatment (Zhu and Chen, 1999). Moreover, in some case, TAN concentrations in such systems are so low that become the rate-limiting factor of biological nitrification (Wheaton *et al.*, 1994, cited in Zhu and Chen, 1999).

The nitrifying bacteria (Figure 2.3) are chemolithoautotrophs: they oxidize ammonia nitrogen to nitrite and nitrate (Hovanec and DeLong, 1996; Logemann *et al.*, 1998). The nitrification process is accomplished in two steps by two distinct groups of bacteria not phylogenetically related. For both groups the only carbon source is CO_2 and they are strictly aerobes (Aoi *et al.*, 2000).

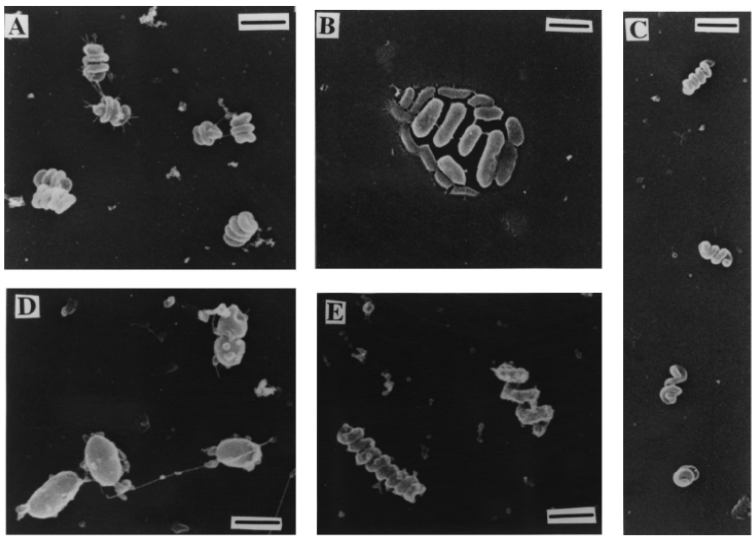

Figure 2.3: Scanning electron micrographs (SEM) of ammonia oxidizing bacteria (from Aakra *et al.*, 1999).

Firstly, the ammonia nitrogen is oxidized to nitrite by ammonia oxidizing bacteria (AOB) classified in two phylogenetic groups. One group is *Nitrosococcus*, belonging to the β-subclass of the Proteobacteria, represented by two described marine species (Koops and Pommerening-Röser, 2001), while the second group, belonging to the γ-subclass of the Proteobacteria, is represented by the *Nitrosospira* and the *Nitrosomonas* clusters. All members of the three genera of the *Nitrosospira* cluster are very closely

related to each other, whereas the *Nitrosomonas* cluster reveals at least five distinct lineages of descent (Purkhold *et al.*, 2000; Koops and Pommerening-Röser, 2001).

In the second step, nitrite is oxidized to nitrate by a distinct group of microrganisms, the Nitrite-Oxidizing Bacteria (NOB), that have been classified into four groups (Egli, 2003). The major group, which belongs to the α-subclass of the Proteobacteria, is represented by a single genus, *Nitrobacter*, including four described species. Two marine species, *N. mobilis* and *N. gracilis*, were assigned to the γ and the δ subclasses of the Proteobacteria, respectively (Koops and Pommerening-Röser, 2001). The two species of the genus *Nitrospira*, *Nitrospira marina* and *Nitrospira moscoviensis* (Ehrich *et al.*, 1995), are members of a distinct phylum close to the δ subclass of the Proteobacteria.

Nitrifying bacteria typically have a very long generation time (0.026 h^{-1} for *Nitrosomonas* and 0.012 h^{-1} for *Nitrobacter* (Hagopian and Riley, 1998). This is due to the fact that 80% of the product energy is used to fix CO_2 *via* the Calvin cycle and only the rest is used for the growth.

The oxidation of ammonia nitrogen is described by the following equation:

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O + 84\ \text{kcal mol}^{-1}$$

Ammonia is firstly oxidized to hydroxylamine and then to nitrite. Two enzymes are involved in this process: the ammonium monoxygenase (AMO) and a hydroxylamine oxido-reductase (HAO) (Tsang and Sukuki, 1982; Bock *et al.*, 1991). Hydroxylamine is the first oxidation product of aerobic ammonium oxidation, but also the reduction product of nitrite in the anammox process (van de Graaf *et al.*, 1996).

The conversion of the nitrite to nitrate follows this equation:

$$NO_2^- + 1/2\ O_2 \rightarrow NO_3^- + 17.8\ \text{kcal mol}^{-1}$$

In this case the enzymatic complex involved is the nitrite oxydoreductase (NOR), cytochromes a1 and c1, a quinine and a NADH dehydrogenase (Bock *et al.*, 1986, 1990).

Studies on the microbial communities performed onto wastewater treatment systems showed that *Nitrosomonas* strains constitute one of the most important populations. Three species (*Nitrosomonas europaea*, *Nitrosomonas oligotropha/urea*, and *Nitrosomonas communis*) are routinely observed as dominant organisms in wastewater treatment plants but also in biofilm systems (Gieseke *et al.*, 2001). However, little

information is available about how and which nitrifying bacteria compete with other related nitrifiers and with heterotrophs in complex systems (Gieseke *et al.*, 2001).

In contrast to textbook knowledge, *Nitrospira*-like bacteria and not *Nitrobacter* spp., are the dominant nitrite oxidizers, both in full-scale wastewater treatment plants and in laboratory scale reactors (Daims *et al.*, 2000).

Heterotrophic bacteria (HB) of RAS constitute an important factor in terms of oxygen consumption, metabolic by–products they release after cellular lyses, the diseases they may cause in fish and, finally, for the competition that may have with autotrophic bacteria for oxygen and space (Léonard *et al.*, 2001; Nogueira *et al*., 2002).

Because the biological filter environment is full of different microniches it can support the growth of a variety of heterotrophic microorganisms including pathogenic and opportunistic bacteria, that may also colonize various external and internal body surfaces of fish (Hansen and Olafsen, 1999). Several alternative strategies to the use of antimicrobials in disease control have been proposed and have already been applied very successfully in aquaculture; for example, biosecurity procedures can be implemented to reduce the introduction of pathogenic organisms into recirculating systems or the use of probiotics and bioaugmentation for the improvement of aquatic environmental quality (Gatesoupe, 1999; Gram *et al.*, 1999; Irianto and Austin, 2002). The antagonism among microbes is a naturally occurring phenomenon trough which pathogens can be killed or reduced in number in the aquaculture environment: this method is called biological control or biocontrol (Maeda *et al.*, 1997).

An intensive search for bacteria that can protect against fish diseases has occurred continuously for the past 10 years (Ringo and Gatesoupe, 1998; Verschuere *et al*., 2000). These researches were focused on finding some harmless bacteria (probiotics) that help the well being of the fish and contribute, directly or indirectly to protect the host animal against harmful bacterial pathogen. Nevertheless, it is well accepted that all microbial population can be implicated in maintaining a good and stable rearing environment (shelter effect), probably by releasing chemical substrates that have a bactericidal or bacteriostatic effect on other microorganisms or outcompeting for chemical and available energy (Gatesoupe, 1991; Vershuere *et al.*, 1997, 2000).

Among the heterotrophic bacteria that can inhabit a RAS the denitrifiers must be mentioned. Biological nitrate removal, that may occur under anaerobic, microaerophilic, and occasionally aerobic conditions (Egli, 2003), is conducted by a

wide variety of organisms, by either assimilatory or dissimilatory pathways, belonging to species related to the genera *Pseudomonas*, *Paracoccus*, *Ralstonia* and *Rhodobacter*, that are able to perform denitrification. The Pseudomonas especially represent one of the largest assemblies of denitrifying bacteria, favoring their use as model organisms (Baumann, 1997 cited in Egli, 2003).

Around 50 genes are required within a single bacterium for the synthesis of the biochemical denitrification apparatus (Zumft, 1997). Numerous environmental factors can influence the denitrification activity, such as nitrate and nitrite concentrations, pH, aeration, temperature, carbon availability, and relative activities of NO^- and N_2O reductases (Bergsma *et al.*, 2002 cited in Egli, 2003).

Finally, the biological filters often contain anaerobic regions where the newly described anaerobic ammonia oxidizing (*anammox*) bacteria can develop (Tal *et al.*, 2003, 2006).

Such bacteria combine ammonia and nitrites (nitrite as electron acceptor) to form molecular nitrogen, energy and biomass (Strous *et al.*, 1997, 1999; Schmidt et al., 2002;).

Nowadays only three genera of anammox bacteria have been discovered: *Brocadia, Kuenenia* and *Scalindua*. The first two have been found in wastewater treatment systems. The latter, *Scalindua*, has also been detected in many marine ecosystems, such as the Black Sea. The three genera share a common ancestor, but are evolutionally different (less than 85% sequence similarity on the 16S level) (Jetten *et al.*, 2001).

One of the key enzymes of anaerobic ammonium oxidation is the hydroxylamine oxidoreductase (Schalk *et al*., 2000). This enzyme is located exclusively in a membrane-bounded region of the cell cytoplasm of anammox (Lindsay *et al.*, 2001): the anammoxosome (van Niftrik *et al.*, 2004). Ammonia is oxidized with hydroxylamine as the most probable electron acceptor, and hydrazine is the first detectable intermediate. Subsequently, the hydrazine is oxidized to dinitrogen gas. During the conversion of ammonia, some nitrate is formed from nitrite.

Inside biological filters (of a RAS but also of industrial and drinking water treatment facilities), faster growing heterotrophs are able to develop at the outer biofilm layer of the biofilm, where oxygen and substrate concentrations are highest, while autotrophic nitrifiers bacteria (that are very slow growers) are placed in deeper biofilm layers. In

this case they can be limited for oxygen and ammonia (Van Benthum *et al.*, 1997; Nogueira *et al.*, 2002; Lee *et al.*, 2004) (Figure 2.4).

Spatial distribution of microbes affects mass transfer reactions and thus the stability and performance of biofilm reactors. These biofilms are characterized by rapidly reproducing heterotrophs while the filters' performance is determined by a much more sensitive subpopulation of chemoautrophs, the nitrifiers. (Fdz-Polanco *et al.*, 2000; MacDonald and Brözel, 2000; Watnik and Kolter, 2000).

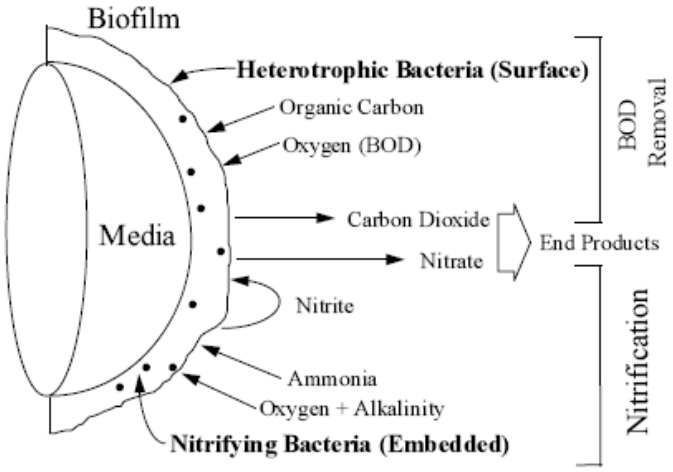

Figure 2.4: Heterotrophs exist on the surface because of their higher growth rates while the slower growing nitrifiers become embedded in the biofilm (Golz, 1995).

2.5. Aim of the work and objectives

Until now, microbiological researches in RAS have been focused on the nitrifying bacteria and the parameters for the biological nitrogen removal in biological filter, the non-pathogen heterotrophic bacteria received only few studies (Léonard *et al.* 2000, 2001).

The high amount of biodegradable organic matter in the rearing water strongly supports the growth of heterotrophic bacteria, which compete with autotrophic nitrifiers in multispecific biofilms for oxygen, nutrients and space (Nogueira *et al.*, 2002; Tsuno *et al.*, 2002). However this competition is a key factor that negatively affects the nitrification process (Blancheton *et al.*, 1997; Logemann *et al.*, 1998), data on the

relationships between nitrifiers, heterotrophs and available carbon remain scarce (Zhu and Chen, 2001a).

On the other hand, few data are available for the heterotrophic bacterial populations species composition and activity in the RAS. A good knowledge of the total heterotrophic microflora of associated to the biofilters could give information on the presence/absence of potential fish pathogens and of potential biocontrol bacteria and their accumulation in the systems, depending to the rearing conditions (Austin *et al*., 1995; Skjermo *et al.*, 1997; Smith and Davey, 1993; Vadstein *et al.*, 1993; Gram *et al*., 1999; Rombaut *et al*., 1999).

Finally, collecting data on the heterotrophic bacterial population activity and evolution, in order to create a mathematical model, can provide some precious elements to try to manage such populations, reducing their negative influences (as competition with nitrifiers, oxygen over-consumption, water quality degradation) and enhancing their potentialities in maintaining a good and stable environment, suitable for the success of the rearing system.

Experimental work has been divided into four objectives:

Objective 1: Evaluating the effects of various organic carbon concentrations on biofilms of a biological filter and on the nitrification efficiency (Chapter 3). Experiments for this objective were run on four pilot scale biological filters, receiving four different influents (four C/N ratios). Analyses were carried out in order to asses the nitrification efficiency (TAN removal rate, NO_3 production rate), the bacterial abundance (total and cultivable counts) both in the biofilter effluent and on the Biofilter Packing Media and the bacterial community composition and physiological status (CLPP, T-RFLP).

Objective 2: Characterizing the heterotrophic bacterial population of a marine RAS (Chapter 4). The aim of this work was to characterize the bacterial communities of a marine RAS by using molecular tools as restriction analysis (ARDRA) and 16S rDNA cloning and sequencing. Analyses were conducted both on cultivable bacteria (isolated on Marine Agar 2216) and on clone libraries by sampling (1) the water at different spots of the rearing system and (2) the biofilter packing media. On the basis of the restriction analysis, isolates and clones were divided onto specific groups (Operational Taxonomic

Units, OTUs). For each cluster obtained, two templates were randomly selected for sequencing. Moreover, bacterial abundance were investigated by direct counting (DAPI).

Objective 3: Evaluating the stability of the bacterial communities in terms of abundance and community structure composition of a marine RAS, focusing in particular on the biofilter packing media biofilms (Chapter 6). Two identical marine RAS were run in parallel. One received a massive introduction of a commercial Bacterial inoculum, supposed to be able to decrease rearing water concentrations of particulate matter (AQUACET LB/M, Ceetal, France) and the second was used as control. The two systems were monitored in terms of chemical dynamics (concentrations of NH_4, NO_2, NO_3, Suspended Solids, etc.), bacterial abundance (DAPI counts) and community structure (T-RFLP, Clones).

Objective 4: Describing a conceptual model of the bacterial compartment of a biological filter of a marine RAS (Chapter 7). By using data issued from the literature and from results obtained from present thesis work, the conceptual bacterial model of Léonard (2000) was implemented and modified. Some specific points of the model were investigated by performing lab scale *ad hoc* experiments, in order to allow, in future works, the design of a mathematical model useful to manage the bacterial compartment of such systems.

CHAPTER 3

Effect of Particulate Organic Carbon concentrations on multispecific biofilms of biological filters and on nitrification efficiency

3.1. Introduction

Heterotrophic bacteria are growing five times faster and have a yield two to three times higher than nitrifiers (Hagopian and Riley, 1998; Aoi *et al*., 2000), and this allows them to use more oxygen and space. Therefore, in multi-species biofiltration process where both types of bacteria coexist, the nitrification could be significantly inhibited when oxygen supply and the space become limited (Zhu and Chen, 2001a). Chen and Chen (1994, cited in Zhu and Chen, 2001a) reported that nitrification process in fluidized bed reactors was inhibited when sucrose was added. Moreover, Ohashi *et al.* (1995, cited in Zhu and Chen, 2001a), found that the percentage of ammonia-oxidizers and nitrite-oxidizers decreased with an increasing of C/N ratio (Organic Carbon/Inorganic Nitrogen) and heterotrophs became dominant. Okabe *et al.* (2000, 2003) showed that for C/N=0, heterotrophs and nitrifiers coexisted in the outermost biofilm and heterotrophs dominated in the innermost biofilm. At C/N=1.5, heterotrophs outcompeted nitrifiers for dissolved oxygen and space. In this case heterotrophs dominated in the outermost biofilm and were present in the innermost biofilm, where they coexisted with nitrifiers. An increase of the C/N ratio resulted in a stronger stratification of microbial species and in an inhibition of nitrification. Finally, Zhu and Chen (2001a), using a laboratory scale reactor, found that the nitrification process was strongly inhibited by the heterotrophic processes when organic carbon (sucrose) was added. For C/N=1 or 2 the TAN removal rate was reduced by 70% as compared with a pure nitrification process (C/N=0). Moreover, the potential of heterotrophic inhibitory impact on nitrifiers decreased with the increase in organic carbon concentration when C/N was more than 1.

Actually it is well accepted that organic matter is a major factor affecting the nitrification performance, but quantitative information regarding the degree of impact in biofilters used in aquaculture systems is still scarce (Zhu and Chen, 2001a).

Although some strategies have been developed for total nitrogen removal in submerged biofilters, the effects of an excessive growth of heterotrophs within

nitrification filters and previously colonized by nitrifiers, need further study in pilot scale reactors (Rogalla and Bourbigot, 1990, cited in Fdz-Polanco *et al.,* 2000). Many studies tried to investigate the role of organic matter on the nitrification rate but quantitative information on the impact of particulate organic matter on the biological filtration efficiency remain scarce. The C/N ratio in the environment links the availability and the competition for ammonia and the organic carbon source. If the C/N ratio increases, heterotrophic bacteria are subjected to nitrogen limitation and out-compete with nitrifiers for the available resources. However, the critical C/N ratio affecting the nitrification rate is variable between different systems and it is related to the quality of the available organic carbon.

Mathematical models which predict the spatial distribution inside a biofilm of autotrophs and heterotrophs as a result of oxygen and space competition already exist (Furumai *et al*., 1999; Furumai and Rittman, 1994a; 1994b). Moreover, the physical structure of biofilms in relation with mass transport and microbial distribution has also been experimentally described. However, the existing knowledge is mostly qualitative and the existing models are based on assumptions that have been scarcely verified in pilot scale biofilm reactors (Fdz-Polanco *et al.,* 2000).

Engineers continuously search for the most efficient and stable conditions to maximize the efficiency of the nitrifiers populations in water treatment systems. It is also clear that developing a better understanding of the biology and ecology of all the microbial populations in biological filter reactors is vital to the development and the optimization of efficient and economic systems in general.

The aim of present work was to evaluate the effects of different C/N ratios on both the microbial biofilms and the nitrification efficiency of a biological filter. Experiments were run on four pilot scale biological filters, receiving four different influents.

Researches were focused on the Particulate fraction of the Organic Carbon (POC) that seems to be the main source of carbon available in such systems (Léonard *et al.*, 2000, 2001).

Analyses were carried out in order to asses the nitrification efficiency and the bacterial abundance (total and viable counts), both in the effluent and in the Packing Media (PM) of the biofilter. In addition, the bacterial community composition and

physiology on the PM biofilm was investigated by the T-RFLP, CLPP and FISH methods.

3.2. Material and methods

3.2.1 Experimental facilities

A series of four identical pilot scale biological filters filled with a pre-colonized packing media (Biogrog) was used (Annex 1). The characteristics of each filter are reported in Table 3.1. The filters functioned in parallel and received the same ingoing water quality, constituted from heated (20°C ± 1), sand-filtered and UV disinfected seawater. pH was maintained between 7.5 and 8.

Table 3.1: Pilot scale biofilter specifications.

	Symbol	Value
Diameter column	∅	11 cm
Surface column	π x r²	95 cm²
Height column	h	85 cm
Volume column	S x h	8075 cm^3
Flow	Q=V/t	0.121 m^3/h
Water retention time	t=V/Q	4.03 min.
Water velocity	v=Q/S	12.74 m/h

POM, containing on average 175 mg of organic carbon *per* gram of dry matter (Franco-Nava, 2003), and ammonia (ammonium chloride, NH_4Cl) were continuously added at the inlet of each filter with a peristaltic pump. Input of ammonia was fixed to rise a concentration of 2 mg/l. Filters were enriched for at least 4 weeks by continuously feeding in order to allow the formation of a steady–state biofilm before starting the experiment. Inputs of POM were calculated in order to obtain $C_{organic}$ /$N_{inorganic}$ ratios of 0, 0.5, 1 and 2. Each enrichment step had a duration of three weeks and was performed in duplicate. At the end of each step, filters were washed and let running 1 week only with seawater and ammonia.

During the final week of each C/N step, samples were collected for chemical, physical and microbiological analyses

3.2.2. Physico-chemical analyses

Physical and chemical analyses were performed according to current protocols and to in house methods (Table 3.2). In particular ammonia, nitrite and nitrate were analyzed with a Technicon® Autoanalyser II as described by Treguer and La Corre (1974).

The nitrification performance was expressed as volumetric Total Ammonia Nitrogen (TAN) removal rate and calculated with the following expression:

$$\text{TAN removal rate} = [\text{TAN}]_{in} - [\text{TAN}]_{out} \times Q / V$$

where $[\text{TAN}]_{in}$ and $[\text{TAN}]_{out}$ are the TAN concentration at the inflow and at the outflow of the filter respectively (g m^{-3} day), Q is the water flow rate (m^3 day^{-1}) and V is the volume of packing media of the biofilter (m^3). For Nitrate production rate and oxygen consumption rates, similar calculations were used. Each nitrification parameter was expressed as percentage of the maximal value found.

DOC was analyzed with a Shimadzu TOC5000 analyzer, and Suspended Solids (SS) were determined by filtering a known volume of water on a pre-weighted and pre-combusted (450°C for 30') glass fiber filter (Whatman GF/C, Springfield Mill, UK). Filters were dried at 65°C for two days and weighted again.

Table 3.2: Chemical analyses.

Parameter	**Instrument/methods**
Oxygen and temperature	YSI (Yellow Springs Instrument) Model 52
pH	CONSORT ION EC/METER C733
Suspended Solids	Protocol NF T90– 105 AFNOR 1994
NH_3 /NH_4	Alliance Autoanalyser Evolution II (Protocol NF T90–015) (AFNOR, 1994)
N– NO_2	Alliance Autoanalyser Evolution II (Protocol NF T90–012) (AFNOR, 1994)
N– NO_3	Technicon Autoanalyser II (Protocol NF T90– 012) (AFNOR, 1994)
P– PO_4	Alliance Autoanalyser Evolution II (Protocol NF T90–023) (AFNOR, 1994)
TOC	Schimadzu TOC– 5000 Analyzer

3.2.3. Sampling procedures

Free-living HB were sampled by using sterile bottles. An aliquot was diluted in the detachment buffer and a soft ultrasonic treatment was performed in order to detach bacteria associated to particles (Mermillod–Blondin *et al.*, 2001).

A specific preliminary experiment was performed to define the best bacterial detachment method from the packing media.

Packing media subunits were collected from an operating biological filter of rearing system in a sterile becker and pre-weighted.

Six different detachment buffer were tested: 1) seawater, 2) seawater and Tween 80 (0.1% vol/vol), 3) seawater and Triton X-100 (0.1% vol/vol), 4) PBS (Phosphate Buffer Saline (130mM NaCl, 10mM $NaHPO_4$ and 10mM NaH_2PO_4, pH 7.4), 5) PBS and Tween 80 (0.1% vol/vol) and 6) PBS and Triton X-100 (0.1% vol/vol).

All solutions were supplemented with 0.1% of Sodium Pyrophosphate and sterilized by autoclaving (121°C, 1 atm. for 20'). Samples were pre-incubated in 100ml of each sterile detachment buffer at 4°C for 10'. Then, subunits were scraped with a sterile iron brush mounted on a mini-drill, until the entire surface appeared clean.

Finally samples received tree different ultrasonic treatment in an ice bath: 1) 5' at 20 KHz, 2) 10' at 20 KHz, 3) 3' at 47 KHz. An aliquot of this mixture was collected and plated on Marine Agar 2216 and incubated as described below.

As shown in Figure 3.1, the detachment buffer constituted by PBS and 0.1 % of sodium pyrophosphate resulted as the most efficient. No differences were found between the sonication treatment at 20 KHz for 10' and 47 KHz for 3'.

From these results, the protocol, which was applied to study the different characteristics of biofilm bacteria, was the following. Attached heterotrophic bacteria (HB) were sampled from four subunits of packing media at every sampling point. Subunits were collected in a sterile becker, weighted and pre-incubated in 100ml of sterile detachment buffer constituted by phosphate buffer saline (130mM NaCl, 10mM Na_2HPO_4 and 10mM NaH_2PO_4, pH 7.4) and 0.1% of Sodium Pyrophosphate at 4°C for 10'. Then, subunits were scraped with a sterile iron brush mounted on a mini-drill, until all the surface appeared clean. Finally samples received an ultrasonic treatment (10' at 20 KHz, in an ice bath). An aliquot of this mixture was collected and used for microbiological analysis.

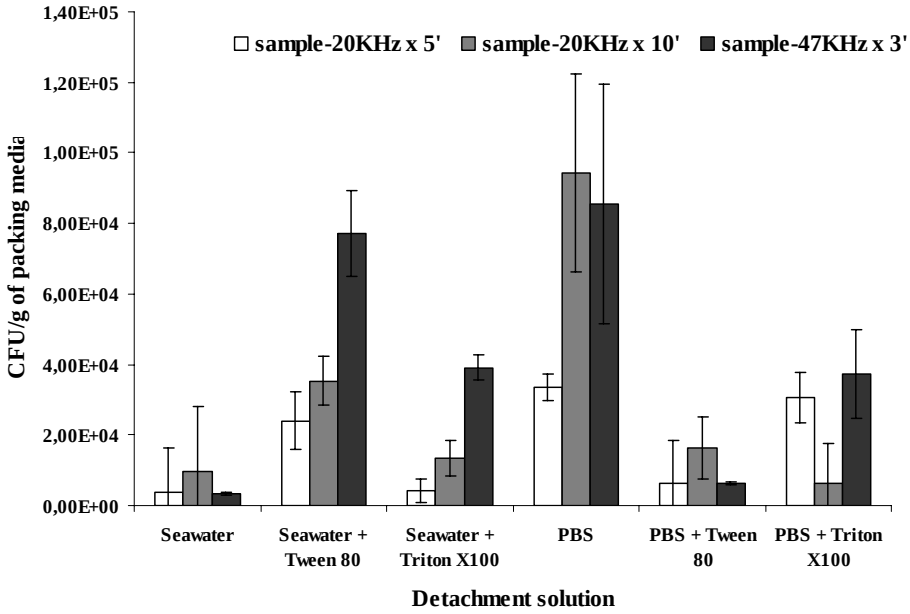

Figure 3.2: Efficiency of the different detachment protocols.

3.2.4. Microbiological analyses

Cultivable cell abundance. Count of cultivable heterotrophic free living and attached bacteria were performed by spread plate method on Marine Agar (Difco 2216) sterilized by autoclaving (121°C, 1 atm for 20'). Dilutions were performed in 34g l^{-1} sterile sodium chloride solution. Plates were set up in duplicate for each dilution. Only plates having a number of colonies comprised between 20 and 200 were considered. Incubation time was 10 days at 25°C. Bacterial concentration in the biological filter was expressed as colony forming units (CFU) per ml for free living bacteria and as CFU per gram of wet packing for attached ones (Leonard *et al*, 2000), because the surface area of the packing material was not well definite. Incubation was performed at 20°C for 10 days.

The abundance of presumptive *vibrios* (PV), usually present in the marine environment, was evaluated by plating a subsample on TCBS (thiosulphate, citrate, bile and sucrose) agar plate (Difco).

Total cell abundance. After detachment, samples for direct enumeration of free living bacteria were fixed in 2 % (w/v) formalin (final concentration) and stored at 4°C until processing. For free bacteria an aliquot of sample was directly filtered on a 25 mm diameter, 0.2 µm pore size polycarbonate black filter, under a vacuum of <5 mm Hg

and stained with DAPI (4',6– diamidino–2–phenilindole, 10µg/ml, final concentration, in distilled water 0.2 µm filtered) according to Porter and Feig (1980). Cells were visualized by an epifluorescence microscope (Axioplan, Zeiss). For attached bacteria, in order to overcome excessive interference with particles, formalin fixed samples were diluted 50 fold with sterile phosphate buffer saline (130mM NaCl, 10mM Na_2HPO_4 and 10mM NaH_2PO_4, pH 7.4). Then Tween 80 (0.1% vol/vol) was added to the diluted samples that were incubated 15' at room temperature. Finally, they were pre-filtered on 3 µm membranes. After this treatment, samples were prepared as previously described for free bacteria.

Specific phylogenetic group abundances. In situ characterization of different microbial groups by the Fluorescent In Situ Hybridisation (FISH) method was carried out according to Nogueira *et al.* (2002) and to MacDonald and Brözel (2000).

Sample fixation was done by the method of Glockner *et al.* (1999). Cells were concentrated from water samples (1 to 3 ml) or from detachment solution on white polycarbonate filters (diameter, 25 mm; pore size, 0.2 mm). They were subsequently fixed by overlaying the filter with 3 ml of a 4% paraformaldehyde PBS solution (130mM NaCl, 10mM Na_2HPO_4 and 10mM NaH_2PO_4, pH 7.4) freshly prepared, for 30 min at room temperature. The fixative was removed by applying vacuum, and the filter was subsequently washed with 3ml of phosphate-buffered saline for two times and finally with distilled water. Filters were air dried and stored in a sterile Petri dish at -20°C until processing.

The probe sequences, hybridization conditions, and references are given in Table 3.3. CY3-labeled probes were purchased from MWG (M-Medical, Italy). Hybridization procedures were carried out as described by Glöckner *et al.* (1999). Each filter was cut into 4 sections, that were placed on glass slides and covered with 20 µl of hybridization buffer containing the suitable concentration of NaCl and formamide (Table 3.4), 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulphate and 50 ng of CY3-labeled probe and incubated at 46°C for 90' in an equilibrated chamber. The filters were transferred to a vial containing 50 ml of prewarmed (48°C) washing solution (70 mM NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.01% sodium dodecyl sulfate) and incubated without shaking at 48°C for 15 min. The filter sections were dried on Whatman 3M paper (Whatman) placed back on a glass slide, and covered with 50 µl of DAPI (10µg/ml,

final concentration, in distilled water 0.2 µm filtered) for 5 min at room temperature in the dark. Subsequently, they were gently washed in 50 ml of 0.2 µm filtered distilled water, dried on Whatman 3M paper, and mounted on glass slides with Citifluor AF1 (Citifluor Ltd., Canterbury, United Kingdom). Glöckner *et al.* (1999) have shown previously that due to firm adhesion of the cells to the polycarbonate filters, cumulative cell losses are below 10%.

The filter sections were visualized with an epifluorescence microscope (Axioplan, Zeiss) equipped with specific filter sets for DAPI and CY3. For each sample and probe, more than 10-20 field and 500 cells were enumerated

Table 3.3 Probes used for FISH analysis.

Probe and Target Organisms	Sequences	FA%	NaCl	Ref.
EUB 338 I - Most but not all Bacteria	5'-GCTGCCTCCCGTAGGAGT-3'	20	225	1-2
EUB 338 II - Planctomycetes	5'-GCAGCCACCCGTAGGTGT-3'	20	225	1
EUB 338 III - Verrucomicrobiales	5'-GCTGCCACCCGTAGGTGT-3'	20	225	1
Nso1225 - Ammonia oxidizing bacteria	5'-CGCCATTGTATTACGTGTGA-3'	35	80	1-2
NIT2- Nitrobacter spp.	5'-CGGGTTAGCGCACGCCT-3'	40	56	2-3
NIT3- Nitrobacter spp.	5'-CCTGTGCTCCATGCTCCG-3'	40	56	2-3

FA%: % of Formamide in hybridization buffer – NaCl: NaCl concentration (mM) in washing buffer

References: [1] Egli *et al.*, 2003; [2] Kloep *et al.*, 2000; [3] Gieske *et al.*, 2001;

Direct genomic DNA extraction. For the DNA extraction, 50 ml to 100 ml of detaching buffer for each sample were filtered on sterile 47-mm diameter, 0.22 µm pore-size (Nuclepore) membranes and subsequently stored at -20 °C until processing. Then, membranes were pretreated with 150 µl of a 5 mg/ml lysozyme solution for 10 min. and minced filters were putted in a sterile 2 ml eppendorf tube and subjected to the DNA extraction by using the RNA/DNA extraction kit (Qiagen) following the manufacter's instructions. Finally, DNA was precipitated by adding 0.7 volumes of 100 % isopropanol, followed by a wash with ice-cold 70 % ethanol and, after air-drying, resuspended in 50 µl of deionizated sterile water. The quantity and quality of the DNA were checked by agarose gel electrophoresis (1 %, w/v) in TAE buffer.

Genetic diversity of bacterial communities. Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu *et al.*, 1997; Moeseneder *et al.*, 1999; Dunbar *et al.*,

2001) was used to estimate the genetic diversity of bacterial communities. This technique represents a relatively fast and easy way to provide a semiquantitative 'snapshot' of community diversity. It permits quick comparisons of different communities, providing a minimum estimate of the number of different taxa in the sample and some idea about the evenness of their distribution.

The genomic DNA were amplified by using the primers 27F-FAM (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'), which give a 1,503-bp product of the 16S rDNA. 27F-FAM was 5' end labeled with phosphoramidite fluorochrome 5-carboxyfluorescein (5' 6-FAM), which was synthesized by MWG (Germany).

The PCR mixture (50-µl volume) contained both primers at 0.2 µM, 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate (Fermentas), and 2.5 U of *Taq* polymerase (QIAGEN). The PCR protocol was: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Cycling was completed by a final extension at 72°C for 7 min. PCR products were purified with the Qiaquick PCR Purification Kit (Qiagen) and then quantified on an agarose gel (0.8%, wt/vol) by comparing them with a DNA standard. 300ng of PCR product were digested with 10 U of the tetrameric restriction enzyme *Hha*I (Fermentas) and the respective restriction buffer Tango and was filled up to a final volume of 20 µl with autoclaved Milli-Q water. Incubation was done at 37°C for 3 h and reaction was stopped at 65°C for 15'. The digested product was purified by precipitation with isopropanol and sodium acetate and washing with 70% ethanol. Samples were analysed by the Fragment Analysis Service of Genlab Laboratory (Italy).

Only profiles with a cumulative peak height ≥5,000 fluorescence units were used for the analysis. The size of T-RFs was estimated by reference to the internal standard and only peaks with peak height >50 fluorescent units were analyzed. The percentage abundance (Ap) of each T-RF was calculated as:

$$Ap = n_i/N \times 100$$

in which n_i represents the peak area of one distinct T-RF and N is the sum of all peak areas in a given T-RFLP pattern (Lukow *et al.*, 2000, Osborn *et al.*, 2000). Samples were aligned by using the web-based program T-Align (Smith *et al.*, 2005).

After standardization, **T-RFLP** profiles were normalized so that the cumulative peak height in each profile was 10,000 fluorescent units. This allowed for comparison of

profiles based on relative peak heights (peak height divided by the cumulative peak height for a profile). Normalized data were then subjected to statistical analysis (Horz *et al .*, 2000).

Physiological diversity of bacterial communities. The Community-level physiological profiles (CLPPs) of potential substrate used by the bacterial populations were determined with Biolog-GN™ (original type) 96-well microtiter plates (Garland and Mills, 1991; Garland, 1996a, b; 1997; Garland *et al.*, 1997; Grover and Chrzanowski, 2000; Grove *et al*., 2004). Biolog plates contain 95 different carbon substrates, with the 96th well serving as a blank. Each well also contains a redox-sensitive tetrazolium dye that is reduced to a colored compound if respiratory metabolism occurs in the well.

For the study of bacterial populations associated to the biofilter Packing Media microplates were inoculated with 150µl of the same cells suspension (in detaching buffer), used for DAPI counts (before formalin fixation), and were kept at room temperature (25 ± 1°C). For the determination of the physiological profile of bacteria present in rearing water, an aliquot of sample was concentrated by centrifugation at 10,000 rpm at R.T. Suspensions were pre-incubated over night in order to allow microbial utilization of any soluble organic carbon derived from the Packing Media that could interfere in the sole-C-source-use response (Gomez *et al.*, 2004).

For each sample Biolog plates were set up in triplicate and followed for one week by daily determining the optical density (OD), by using an automatic microplate reader, at 595 nm (OD595) and data were electronically recorded.

Absorbance values for the wells with C sources were blanked against the control well. Negative values were considered as 0 in subsequent data analyses. Then the average (blank-corrected) well color development (AWCD) as the average OD of all wells on a single plate at a single reading time was calculated (Garland, 1997). For each sample, data to construct the CLPP were selected from the first reading time at which AWCD for all 3 replicate plates in a sample exceeded an OD of 0.4.

The data-set obtained consists in 95 readings of blank-corrected OD, corresponding to wells with different carbon substrates. CLPP, the fingerprint of the metabolic activities in each sample, was compared among the soil samples using multivariate statistics (principal component analysis, PCA).

3.2.5. Data analysis

Statistical analyses. Results from different experiments were analyzed using analysis of variance (ANOVA). Comparison between groups for a significant difference of mean or ranks values were performed after normality and variance tests. Data were analyzed with one-way ANOVA or ANOVA on ranks (Krustal-Wallis method) and the relative importance of each treatment group was investigated by a Pairwise Multiple Comparison procedure (Student-Newman-Keuls or Dunn's method). ANOVA were computed by using SigmaStat software for Windows, version 3.1 (Copyright 1992-1995 Jandel Corporation).

For T-RFLP profiles, richness was calculated as the number of different T-RFs detected in each sample. Shannon diversity was computed on the basis of peak heights of each T-RF used as estimates of relative abundance of each detected genotype.

Aligned T-RFLP data were analyzed by nMDS: a binary matrix that considered the presence or absence of individual T-RFLP peaks (T-RFs) was constructed and used to calculate a distance matrix using the Jaccard similarity coefficient. The coefficient vary between 0 (identical samples) and 1 (the two samples have no T-RFs in common). The Jaccard matrix was used to construct a nonmetric multidimensional scaling (nMDS) diagram (Mummey and Stahl, 2003; Denaro *et al.*, 2005). A cluster analysis was also carried out.

Data from CLPPs analysis were analyzed by Principal components analysis (PCA) that is a mathematical technique that allows multivariate data to be characterized by a smaller number of variables.

Statistical calculations were performed by using SigmaStat software for Windows, version 3.1 (Copyright 1992-1995 Jandel Corporation). The PAST software (PAlaeontological STatistics, version 1.15, http://folk.uio.no/ohammer/past) and the PRIMER 6 (version 6 β R6) were used to compute the PCA, the nMDS and the cluster analysis.

Diversity indices. In order to evaluate structural diversity between samples, Shannon-Weaver diversity index (*H'*), Pielou Evenness (J'), Dominance (D) and the reciprocal of Simpson's index (1/*D*), were computed

The PAST software (PAlaeontological STatistics, version 1.15, http://folk.uio.no/ohammer/past) was used to compute the diversity indices.

3.3. Results and discussion

3.3.1. Nitrification efficiency and bacterial abundance

In the biofilter enriched with nitrogen as sole substrate (C/N=0) the TAN removal rate was maximal. As shown in Figure 3.2, the nitrification efficiency was strongly impacted by the addition of the POM. The reduction of TAN removal rate was significantly lower at higher C/N ratios than at C/N=0 (P=0.006).

From C/N=1 to C/N=2 the lost of nitrification efficiency (7 %) was less significant than from C/N=0 (maximal TAN removal rate) to C/N=1 (45 %), suggesting that the impact of POM on nitrification became less and less important while the carbon concentration increased (Zhu and Chen 2001). The nitrate production rate underwent a decrement of 24 % for C/N=0.5, 56 % for C/N=1 and 73 % for C/N=2 compared to C/N=0. In this case the inhibitory effect of POM remained comparable between each

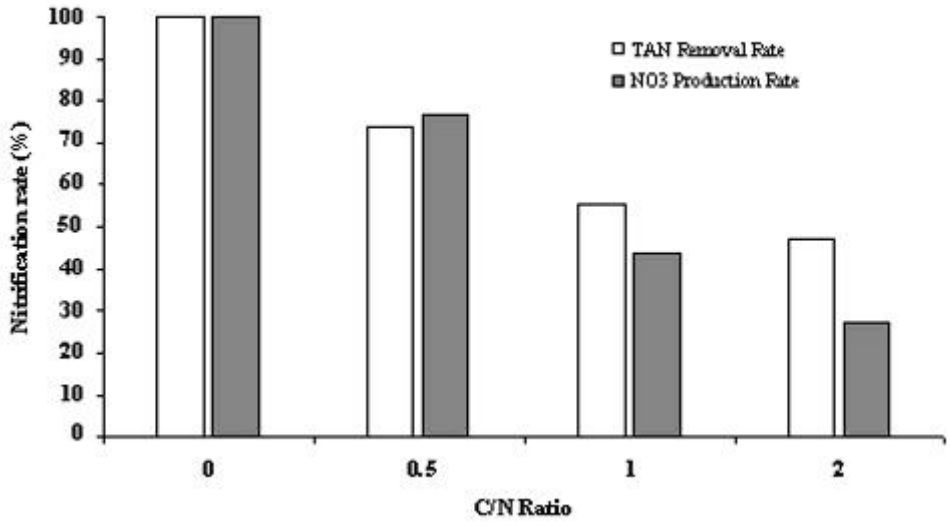

Figure 3.2: Nitrification performance indicators expressed as a percentage of the maximum at different C/N ratios.

C/N ratio tested. Concentrations of nitrites were always very low (data not shown).

The easily biodegradable organic carbon enrichment supporting the heterotrophic activity establishes inside the multispecific biofilms a competition between chemoautotrophic nitrifiers and heterotrophs for oxygen, nutrients and space reducing nitrification rates.

This competition between the different microbial groups can result in a spatial distribution of microorganisms within the biofilm matrix. Spatial distribution of

microbes affects mass transfer reactions and thus the performance of nitrification (Fdz-Polanco *et al.*, 2000). Okabe *et al.* (2000) showed that for C/N=0, heterotrophs and nitrifiers coexisted in the outermost biofilm. At C/N=1.5 heterotrophs dominated in the outermost biofilm and nitrifiers were present only in the deeper biofilm. Finally, an increase of the C/N ratio resulted in a stronger stratification of microbial species and in an inhibition of nitrification.

In addition with the competition mechanisms, the nitrification efficiency can be directly inhibited by the organic matter. In this case compounds having a structural homology with ammonia can create an interference with the active site of the ammonia monoxygenase and metal binding compounds can reduce the availability of copper, inactivating by this way enzymes involved in the nitrification pathway (Bedard and Knowles, 1989).By comparing the total Dissolved Inorganic Nitrogen concentration at the inlet of the filters (DIN_{in}) and the DIN at the outlet (DIN_{out}) a loss of nitrogen was observed (Figure 3.3).

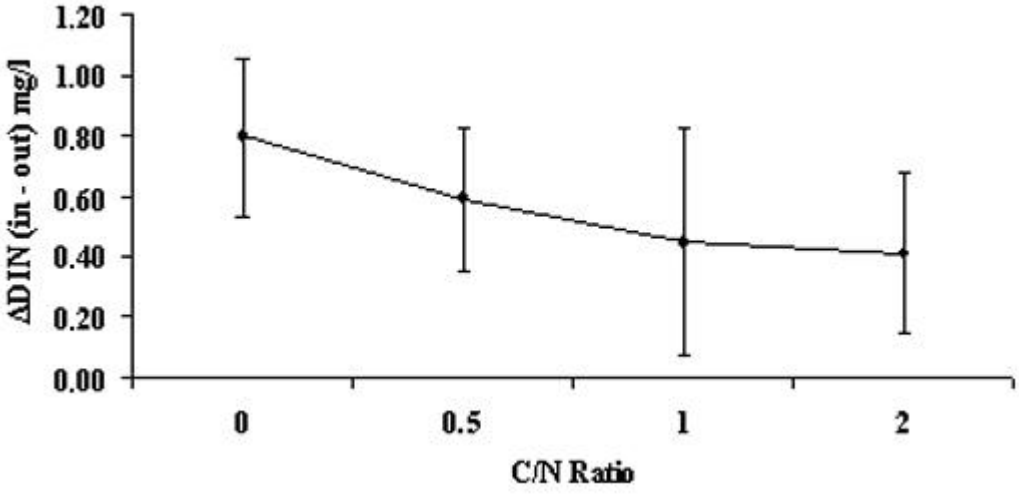

Figure 3.3: Difference between inlet and outlet concentrations of dissolved inorganic nitrogen (DIN) at different C/N ratios. The error bars represent the standard deviations.

It is well known that heterotrophic bacteria can efficiently use ammonia, if an appropriate source of carbon is available, and the difference in energetic costs of using ammonia instead of proteins as nitrogen source is very low (Russel and Cook, 1995). Although the heterotrophic ammonium uptake can be significant in several environments (Kirchman, 1994), the total bacterial biomass production in the filters, evaluated by direct microscopic enumeration (Figures 3.4 and 3.5), did not appear

sufficient to ascribe the unbalance of the DIN concentration only to an heterotrophic nitrogen uptake.

Another possible explanation for this gap could be a dissimilatory utilization of dissolved nitrogen compounds by denitrification microorganisms. In the biolfilters filled with a microporous mineral packing media hypoxic or totally anoxic micro-niches are certainly present. Thus, the POM used for the enrichment contributed to emphasize them. In this kind of environment the anaerobic denitrification could play an important role. Bacteria that in presence of organic hydrogen donors can reduce Nitrates and Nitrites to molecular Nitrogen are present in all water habitats, and they include species belonging to the most common genera like *Vibrio*, *Pseudomonas*, *Acinetobacter Flavobacterium-Bacterioides-Cytophaga* group, etc. As they are facultative anaerobes, they occur in oxygen rich environments or anoxic ones. These kinds of bacteria present the complete respiratory system and only when oxygen is lacking the enzyme system for nitrate respiration is induced. Moreover, the newly discovered anaerobic ammonia oxidizing (*anammox*) bacteria (Strouss *et al.*, 1997, 1999) can contribute to ammonia removal; their presence inside biofilters has been examined only recently (Tal *et al.*, 2006).

There was a good relation between the concentration of POC and the heterotrophic bacteria abundance, both for attached and free-living bacteria (Figures 3.4 and 3.5).

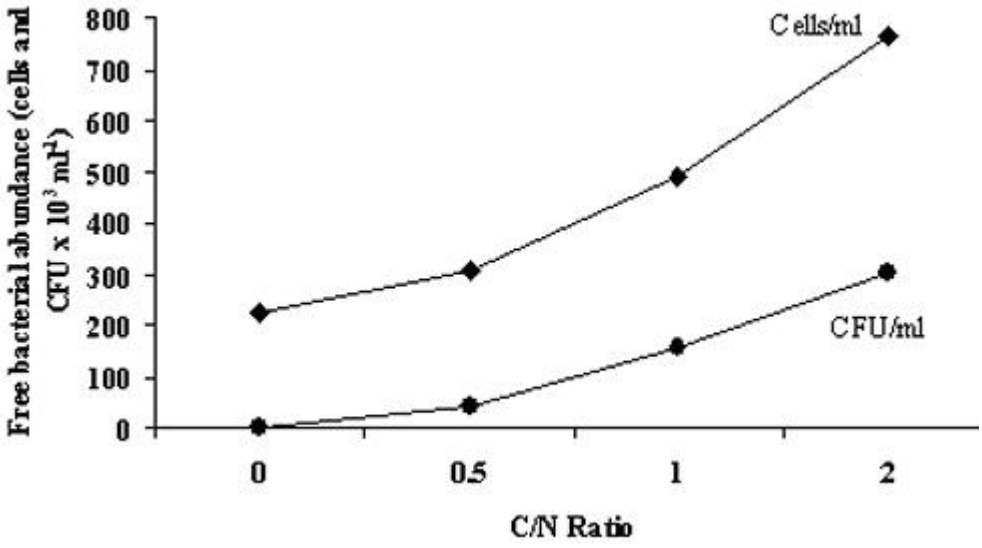

Figure 3.4: Total number of bacteria (Cells/ml) and cultivable heterotrophic bacteria (CFU/ml) found in the biofilter effluent at different C/N ratios.

The concentration of free heterotrophic bacteria entering in the biofilter was low ($1.63x10^2 \pm 1x10^2$ CFU/ml, M ± SD) and total abundance was $5x10^3 \pm 1.6x10^3$ cells/ml (M ± SD). Free living bacteria increased with the CN ratio from $4.92x10^2 \pm 1x10^2$ CFU/ml for C/N=0 to $3x10^5 \pm 1.3x10^5$ CFU/ml for C/N=2 (Figure 3.6). In the same way the total biomass increased from $2.26x10^5 \pm 6x10^4$ cells/ml for C/N=0 to $7.69x10^5 \pm 3.2x10^4$ cells/ml for C/N=2 (Figure 3.4).

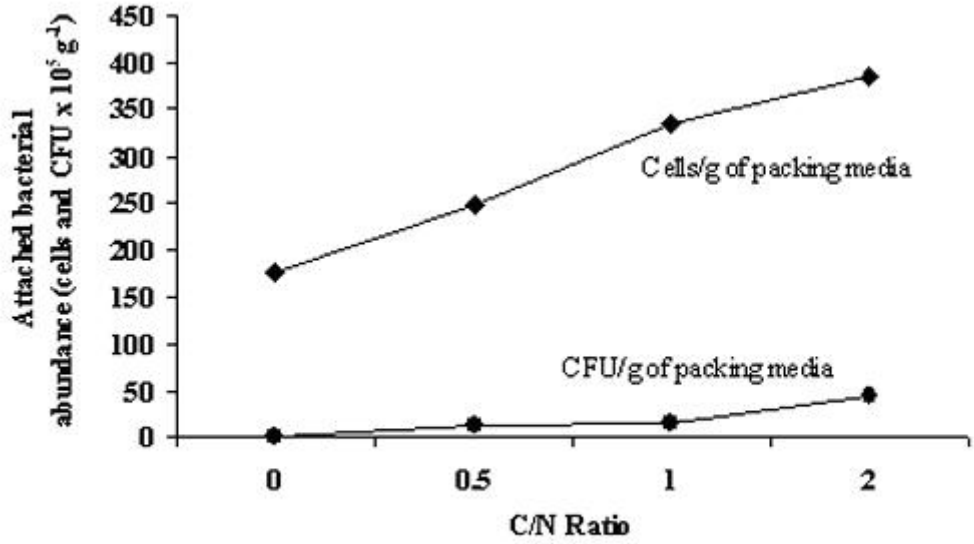

Figure 3.5: Total number of bacterial cells (Cells/g) and cultivable heterotrophic bacteria (CFU/g) attached to the packing media at different C/N ratios.

Figure 3.5 shows the abundance of attached bacteria on mineral PM. Results are expressed as Colony Forming Units *per* gram of PM (CFU/g $_{p.m.}$) and cells per gram of packing media (cells/g $_{p.m.}$) for the Heterotrophic Cultivable Bacteria (HCB) and for the total bacterial abundance respectively. The amount of HCB increased from $5x10^4 \pm 2x10^4$ (M ± SD) CFU/g $_{p.m.}$ for C/N=0 to $1.36x10^6 \pm 0{,}6x10^6$, $2.7x10^6 \pm 1.3x10^5$ and $4.4x10^6 \pm 1.6x10^6$ for CN=0.5, 1 and 2 respectively. As expected, total bacteria abundance was higher than cultivable one: in this case $1.8x10^7 \pm 9.3x10^5$ (M ± SD) cells/g were enumerated for C/N=0, $2.48x10^7 \pm 1.273x10^6$ for CN=0.5, $3.35x10^7 \pm 7.66x10^5$ for CN=1 and $3.85x10^7 \pm 1.02x10^6$ for CN=2. In this study the number of CFU was comparable with results obtained by Léonard *et al.* (2000) who found $7.3x10^6 \pm 7.25x10^6$ CFU/g of PM, for an average C/N ratio of 0.8 (Franco-Nava, 2003).

As shown in Figure 3.6 there was a linear relationship between the concentration of ingoing Suspended Solids (SS) and the SS production (out - in) of filters. Excepted that

for low ingoing SS concentrations, where the biofilters seemed to play also a mechanical separation role, the production of SS increased with the increasing of ingoing SS concentration. In the same figure the relation existing between ingoing Suspended Solids (SS) and the Total Bacterial Abundance production (out - in) of filters is shown. It is realistic to assume that a large amount of SS released from the filters is constituted by bacterial biomass that uses the ingoing POM to build new biomass.

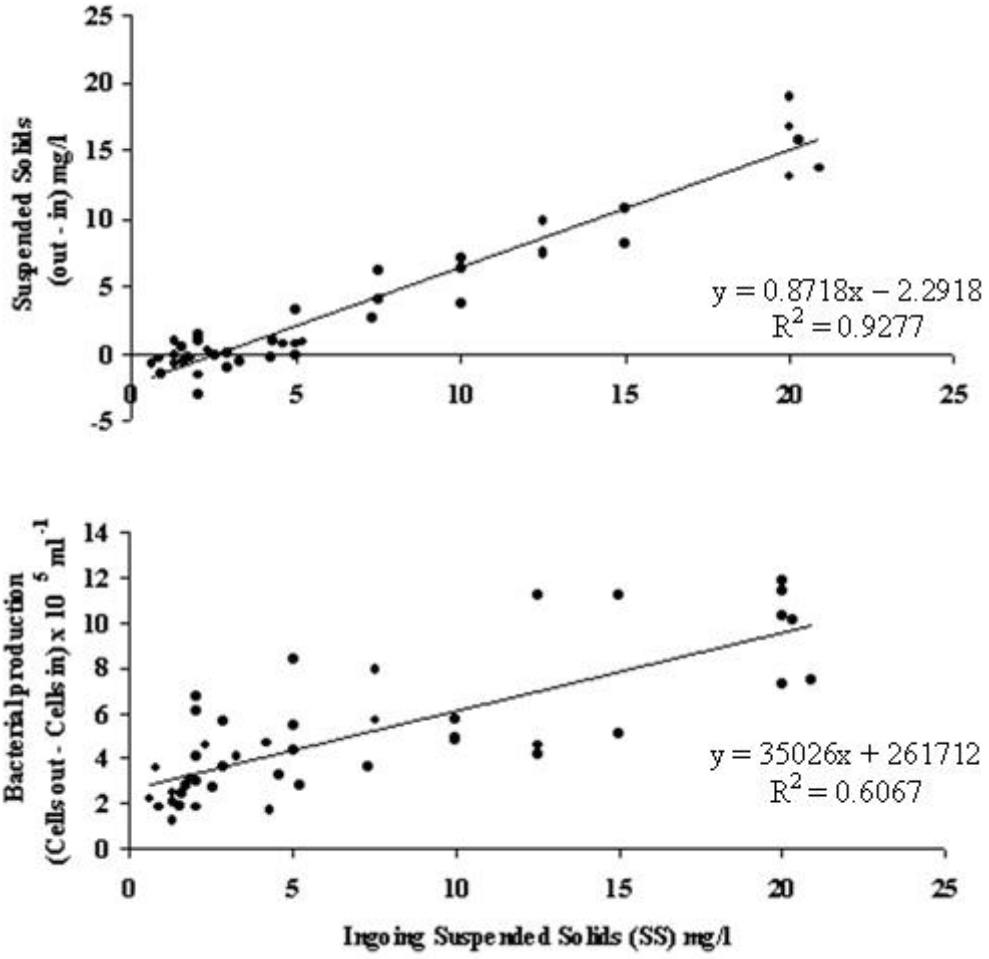

Figure 3.6: Suspended solids (SS) production (Out – In) and total number of bacteria added to the water as it flowed through the biofilters (Out – In) as functions of influent suspended solids concentrations.

As shown in Figure 3.7, there was a good relation between total bacterial biomass attached on the packing media and free living sheared from the filters. This relationship confirmed the results obtained by Lèonard *et al.* (2000) that found the same relation when considering the sole cultivable heterotrophic bacteria.

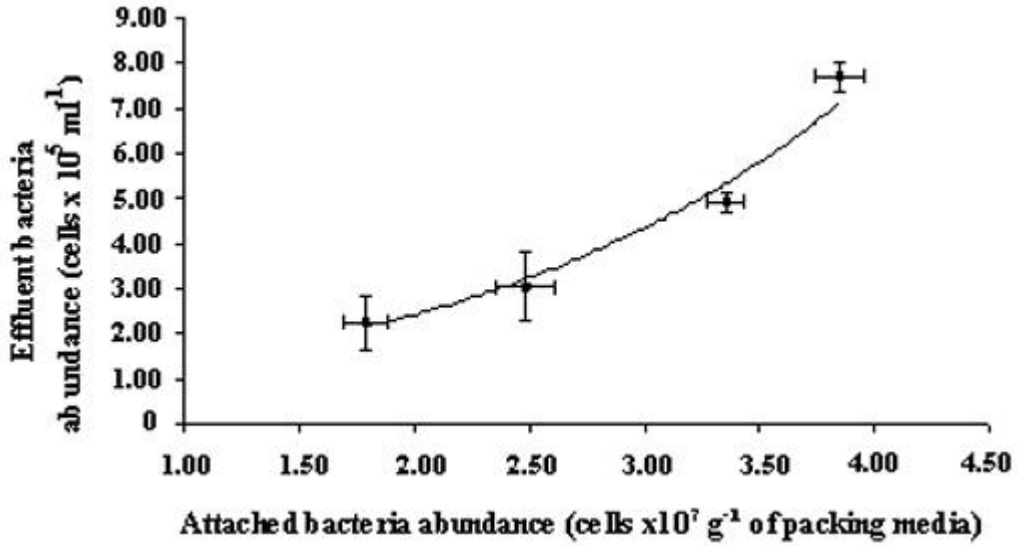

Figure 3.7: Relationship between the numbers of bacterial cells attached to the packing media and those present in the biofilter effluent at different C/N ratios.

The bacterial production of filters increased more dramatically for the cultivable bacteria than for total abundance (Figure 3.8). This suggests that the cultivability could be boosted by carbon addition. The ratio of cultivable bacteria increased from 0.2% for C/N=0 (both for attached and free bacteria) to 11% and 40% for C/N=2 for attached and free bacteria, respectively (Figure 3.9).

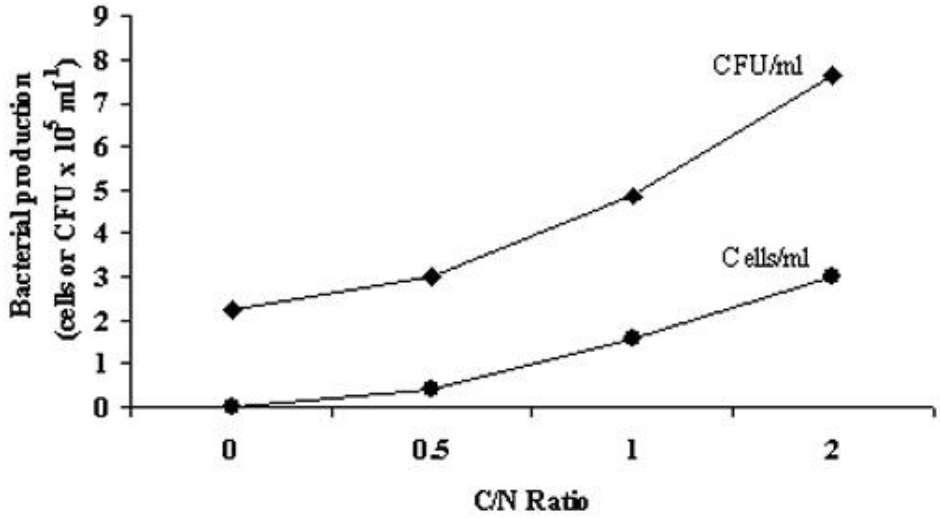

Figure 3.8: Production of bacteria (Out – In) in the biofilters at different C/N ratios, expressed as total number of bacteria (cells/ml) and cultivable heterotrophic bacteria (CFU/ml).

The magnification of the cultivability ratio with the increasing of the POM concentration could be due to a shift toward a selected bacterial population particularly adapted to this kind of substrate, or to a simply bacterial activation.

Although the high quantity of organic matter may mask many cells during epifluorescence enumeration, the difference between cultivable heterotrophic bacteria and total bacterial abundance was high. It may be considered that autotrophic cells were also enumerated in the total abundance. This overvalues the ratio cultivable/total abundance. No clear evidences were found in literature to evaluate the percentage of nitrifiers inside this kind of multispecific biofilms.

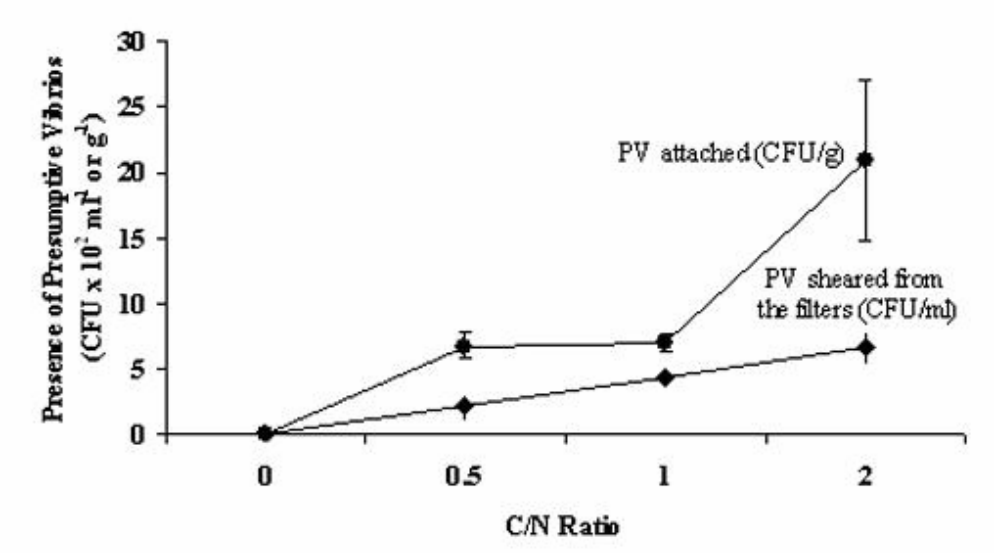

Figure 3.10: Presumptive vibrios (PV) bacteria present in the biofilter effluent (CFU/ml) and attached to the biofilter media (CFU/g) at different C/N ratios.

Only few cultivable presumptive vibrios (PV) were found in water inflow (less than 20 per ml). In Figure 3.10 are presented results obtained for the shared and for the attached PV. If the number of shared PV constantly increased for each C/N ratio tested, the PV heavily proliferated at the highest C/N ratio.

3.3.2. Fluorescent In Situ Hybridization (FISH)

It must be pointed that probably there was an underestimation of the number of bacteria stained with fluorescent probes due to the large amounts of suspended solids present on the membranes. Moreover, the unavailability of a Confocal Laser Microscope (Tolker-Nielsen and Molin, 2000), avoided the possibility to study the disposition of cells inside the biofilms.

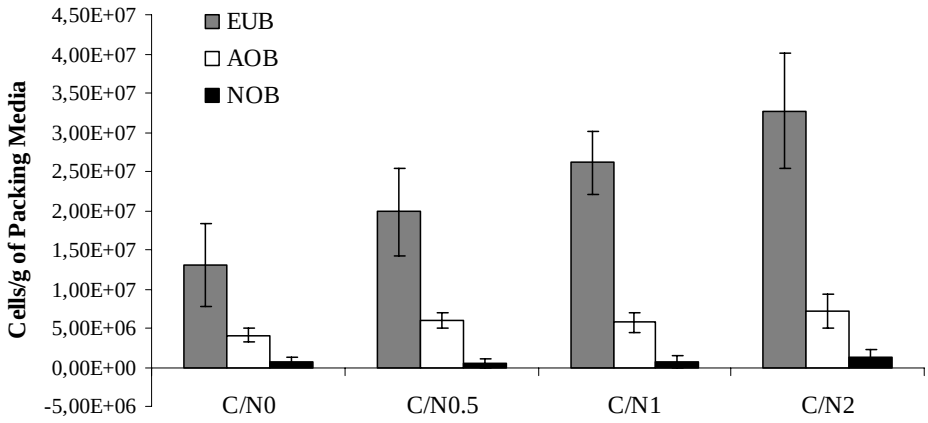

Figure 3.11: FISH analysis at different C/N ratios. EUB: Eubacteria; AOB: Ammonia oxidizing bacteria; NOB: Nitrite oxidizing bacteria.

Results (Figure 3.11 and Table 3.4) shows that the increasing of the C/N ratio determins an increasing in the number of cells hybridized with the probes for EUB and AOB. The One Way Analysis of Variance revealed a statistically significant difference between treatments ($P = <0.001$) confirmed by the subsequent Pairwise Multiple Comparison (Student-Newman-Keuls Method) ($P<0.05$). On the contrary, the NOB hybridized cells didn't show a well definite pattern among the four treatments: there was a statistically significant difference (ANOVA, $P=0.044$) but the Student-Newman-Keuls Multiple Comparison showed a statistically difference between C/N1 and C/N2 ($P<0.05$).

By using the EUB mix probe, FISH resulted in the detection of a large fraction of the microbial community associated to the packing media. The percentage of hybridized cells was comprised between 73 and 83 % of DAPI stained cells. Such detection yields are comparable with those for activated sludge but are higher than those for seawater (LLobet-Brossa *et al.,* 1998), suggesting a high cellular rRNA content due to the high amounts of available carbon that enhanced the bacterial abundance and activity.

Table 3.4: Cells/g of PM stained with the three probes used.

	EUB	**AOB**	**NOB**
C/N0	$1.30x10^7 \pm 5.27x10^6$	$4.09x10^6 \pm 8.40x10^4$	$7.56x10^5 \pm 5.24x10^5$
C/N0.5	$1.98x10^7 \pm 5.56x10^6$	$6.04x10^6 \pm 1.00x10^6$	$5.21x10^5 \pm 5.70x10^5$
C/N1	$2.61x10^7 \pm 4.04x10^6$	$5.72x10^6 \pm 1.22x10^6$	$7.04x10^5 \pm 8.56x10^5$
C/N2	$3.27x10^7 \pm 7.33x10^6$	$7.19x10^6 \pm 2.12x10^6$	$1.23x10^6 \pm 9.39x10^5$

3.3.3. Terminal Restriction Fragment Length Polymorphism

The suitability of the T-RFLP technique to study the bacterial community structure has been well documented (Denaro *et al*., 2005). According to published literature, the distribution of TRF has been used to compute diversity indices and evenness as a measure of bacterial diversity. Nevertheless, in most of the published articles, there is a lack in relating the obtained diversity values to the adopted sampling strategies (Denaro *et al*., 2005).

After the pretreatment and the alignment (as described in Materials and Methods section) a non-metric MDS were computed by a Jaccard (presence/absence) similarity matrix.

The MDS algorithm tries to construct a simple map whose inter-point distances have the same rank order as the corresponding Jaccard similarity matrix. Generally, MDS gives a better representation of data compared to, e.g. PCA analysis. Also, MDS does not assume linearity in correlations between parameters, a phenomenon rarely observed among biological relationships.

Results of T-RFLP analysis (by using the tetrameric restriction enzyme *HhaI*, Fermentas) revealed a shift of the population composition, assuming that each T-RF correspond to a distinct taxon (Figures 3.12 and 3.13).

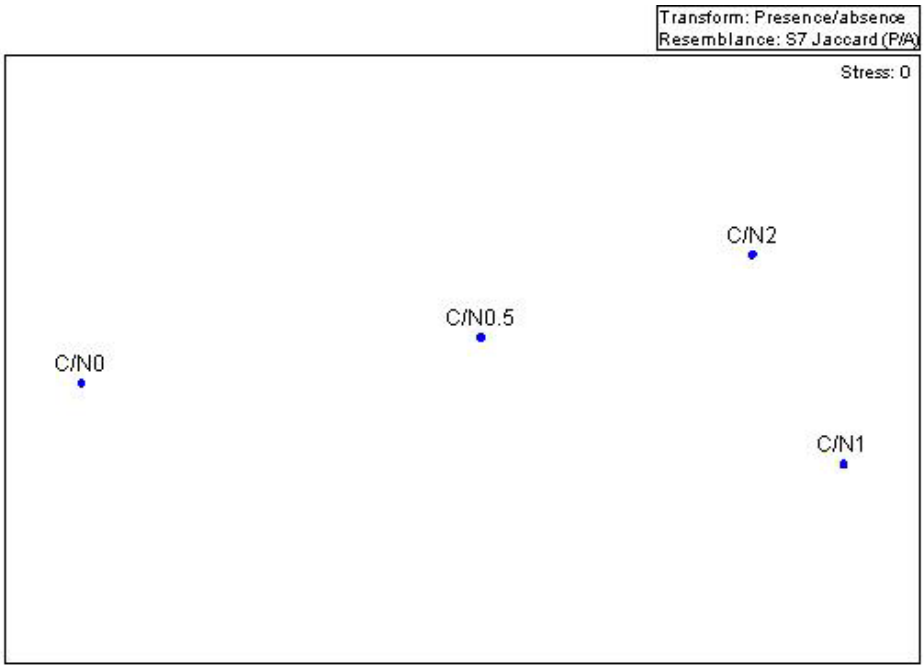

Figure 3.12: Non-metric multidimensional scale analysis (nMDS) by means of the Jaccard index of similarity for the T-RFLP patterns.

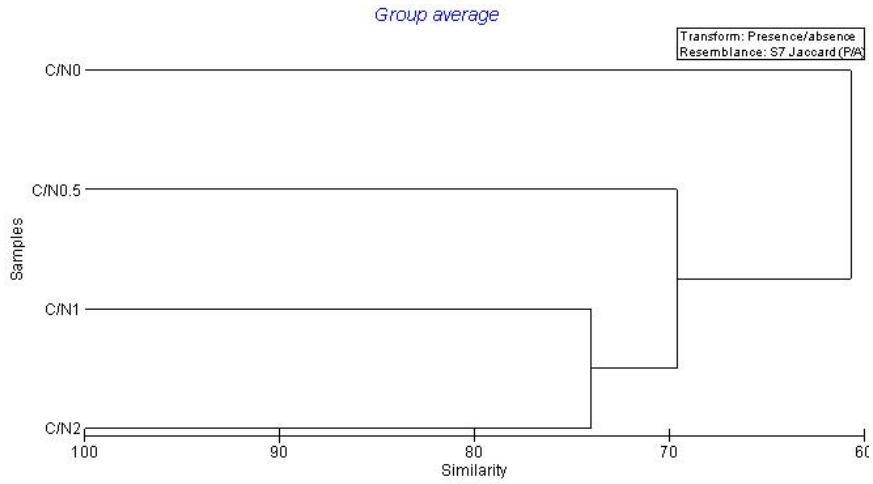

Figure 3.13: Cluster Analysis plot for T-RFLP patterns**.**

If the number of T-RF fragments decreased from C/N0 to C/N2, the different indices of community diversity remained stable, indicating the absence of dominant OTUs among populations, as confirmed by the Shannon diversity index (H'), that is more sensitive to changes in abundance of the rare groups, and the Simpson's (1/D) index, that measures both richness and relative abundance (Table 3.5) (Hill *et al*., 2003).

The values found were rather constant in all samples indicating that the general structure of the biofilter bacterial biofilms was quite similar and that the differences observed within the T-RF distributions could be due to a variation of the taxonomic composition among samples.

Shannon diversity index, ranging between 3.3 and 3.4, fell within the range found in a variety of ecosystems for other organisms (Schauer *et al*., 2000) and the richness values (number of different T-RFs) were, in general, comparable with the published data for marine coastal environments (Suzuki *et al*., 2001).

Table 3.5: Diversity indices of T-RFLPs.

	C/N0	C/N0.5	C/N1	C/N2
n° T-RF	53	43	42	45
Dominance D	0.042	0.040	0.043	0.042
Pielou Evenness J'	0.867	0.900	0.892	0.886
Shannon H'	3.443	3.397	3.335	3.372
Simpson 1/D	23.81	25	23.25	23.81

3.3.4. Community Level Physiological Profile

Community-level physiological profiling or CLPP is a technique for the analysis of bacterial community structure that use of microtiter plates with organic substrates (Garland and Mills, 1991; Garland, 1997). The pattern composed of the extent to which a community utilizes the different substrates, the so-called physiological fingerprint, is compared between samples using multivariate techniques. Based on the AWCD (see Material and Methods) the time selected for data analysis was 4 days.

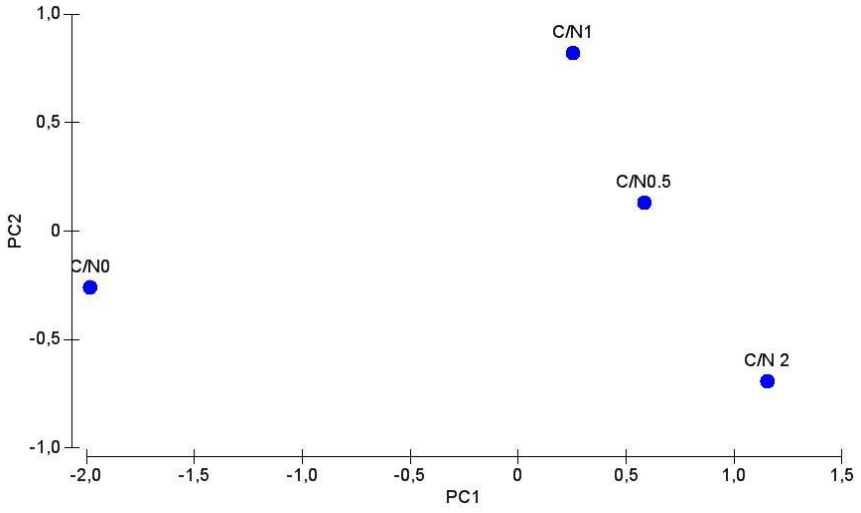

Figure 3.14: PCA plot of the community carbon source utilization at different C/N ratios.

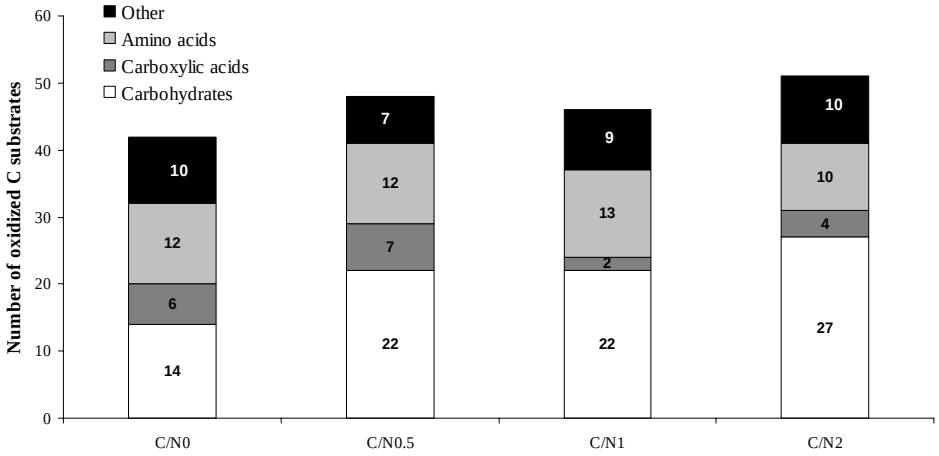

Figure 3.15: Number of Carbon sources oxidized for each C/N Ratio.

The CLPP indicated an interesting trend in the biofilter microbial community structure over different C/N ratios. Communities appeared to have two markedly different metabolic patterns: one at C/N0 and another for higher C/N ratios, as indicated by the wide spread of the data points (Figure 3.14). The cluster analysis (data not shown) confirmed that the carbon utilization pattern at C/N0 differed from the other three and that C/N0.5 and C/N1 were more closely related than all other C/N ratios.

The richness of the number of oxidized C substrates was quite similar for all the C/N ratios ranging from 42 oxidized substrates at C/N0 to 51 at C/N2 (Fig. 3.15). It must be underlined that at C/N0 the oxidation pattern was quite homogenously distributed among the different carbon sources, whereas at higher C/N ratios community seems to prefer carbohydrates than carboxylic acids. The number of oxidized aminoacids remained stable (Figure 3.15 and Table 3.6).

Table 3.6: C substrate patterns at different C/N ratios.

Well n°/ C Source	C/N 0	C/N 0.5	C/N 1	C/N 2	Well n°/ C Source	C/N 0	C/N 0.5	C/N 1	C/N 2
Carbohydrates					E9 Quinic acid				
A7 N-acetyl-D-galactosamine			+	+	E10 D-saccharic acid	+	+		
A8 N-acetyl-D-glucosamine	+	+	+	+	E11 Sebacic acid				
A9 Adonitol				+	E12 Succinic acid				
A10 L-arabinose		+	+	+	**Amino acids**				
A11 D-arabitol		+	+	+	F5 D-alanine	+			
A12 Cellobiose	+	+	+	+	F6 L-alanine	+	+	+	
B1 i-erythritol		+			F7 L-alanyl-glycine	+	+	+	+
B2 D-fructose	+	+	+	+	F8 L-asparagine	+		+	+
B3 L-fucose		+		+	F9 L-aspartic acid	+	+	+	+
B4 D-galactose	+	+	+	+	F10 L-glutamic acid	+	+	+	
B5 Gentobiose	+	+	+	+	F11 Glycyl-L-aspartic acid	+	+	+	+
B6 a-D-glucose	+	+	+	+	F12 Glycyl-L-glutamic ac.				
B7 m-inositol		+	+	+	G1 L-histidine				
B8 a-D-lactose				+	G2 Hydroxy-L-proline	+	+	+	
B9 Lactulose				+	G3 L-leucine	+	+	+	
B10 Maltose	+	+	+	+	G4 L-ornithine				
B11 D-mannitol	+	+	+	+	G5 L-phenylalanine				+
B12 D-mannose	+	+	+	+	G6 L-proline	+	+	+	+
C1 D-mellibiose		+	+	+	G7 L-pyroglutamic acid		+	+	+
C2 b-methyl-D-glucoside	+	+	+	+	G8 D-serine		+	+	+
C3 D-psicose					G9 L-serine	+	+	+	+
C4 D-raffinose	+	+	+	+	G10 L-threonine	+	+	+	+
C5 L-rhamnose		+		+	G11 D,L-carnitine			+	+
C6 D-sorbitol	+	+	+	+	G12 g-aminobutyric acid	+	+	+	
C7 Sucrose	+	+	+	+	**Others**				
C8 D-trehalose	+	+	+	+	H1 Urocanic acid				
C9 Turanose		+	+	+	H2 Inosine	+	+	+	+
C10 Xylitol			+	+	H3 Uridine	+		+	+
Carboxylic acids					H4 Thymidine			+	+
D1 Acetic acid					H5 Phenylethyl-amine				+
D2 Cis-acotinic acid		+		+	H6 Putrescine				
D3 Citric acid	+				H7 2-aminoethanol				
D4 Formic acid					H8 2,3-butanediol				
D5 D-galactonic acid lactone				+	H9 Glycerol	+	+	+	+
D6 D-galacturonic acid		+			H10 D,L-a-glycerol PO4				
D7 D-gluconic acid	+	+	+	+	H11 Glucose-1-PO4				
D8 D-glucosaminic acid					H12 Glucose-6-PO4	+			
D9 D-glucuronic acid		+	+	+	F1 Bromosuccinic acid				
D10 a-hydroxybutyric acid					F2 Succinamic acid				
D11 b-hydroxybutyric acid					F3 Glucuronamide				
D12 g-hydroxybutyric acid					F4 Alaninamide				
E1 p-hydroxyphenyl-acetic		+		+	C11 Methyl pyruvate	+	+	+	+
E2 Itaconic acid					C12 Monomethyl succ.				
E3 a-keto-butyric acid					A2 a-cyclodextrin	+	+	+	
E4 a-keto-glutaric acid					A3 Dextrin	+	+	+	+
E5 a-keto-valeric acid					A4 Glycogen	+	+	+	+
E6 D,L-lactic acid	+	+			A5 Tween 40	+		+	+
E7 Malonic acid					A6 Tween 80	+	+	+	
E8 Propionic acid	+								

3.4. Conclusions

The approach used here for bacteria monitoring has provided a basis for evaluating changes in bacterial abundance and community structure that can help to explain changes in biofilter performance associated with increased POM loading.

Results from the present study clearly showed a strong relationship between C/N ratio and bacterial abundance in biological filters but also with the carbon substrate utilization pattern and the community composition.

Results showed that there was a positive linear relationship between the number of bacteria fixed on the packing media and sheared from the filter. No differences were detected between the amount of POM entering and exiting the filter even though biofilm growth and bacteria production were positively influenced by the substrate loading. Further analyses should be carried out to investigate the composition of this influent and effluent POM.

The increasing of the C/N ratio, negatively affected the biofilter nitrification efficiency and stimulated the growth of bacteria. Results of FISH analysis revealed a magnification of the ratio between EUB stained cells and nitrifiers ones suggesting that the fast growing heterotrophs, normally found in the outer layers of a biofilm, reduced the availability of oxygen and the diffusion of ammonia to the deeper layers where the slow growing nitrifiers develop (Gieseke *et al.,* 2001; Nogueira *et al.*, 2002). As demonstrated by Aoi *et al.* (2000) the structure of a biofilm is strongly influenced by the C/N ratio loads and the nitrifiers resulted homogenously distributed into the biofilm matrix when C/N ratio was 0.

In this study, significant differences in community composition and activity were detected using genetic (T-RFLP) and physiological (CLPP) methods, underlining that the C/N ratio is the main regulator not only of the nitrification efficiency but also of the bacterial population in a biological filter (Grove *et al*., 2004; Denaro *et al*., 2005).

Results obtained by the T-RFLP technique highlighted that, while the general community structure, based on the diversity indices computation, was not affected, the community species composition evolved with the increasing of the C/N ratio. However, it is not easy and unambiguous to assess the taxonomy by using the T-RFLP technique (Moeseneder *et al.,* 1999; Liu *et al*., 1997; Dunbar *et al.,* 2001), and it could be argued that the large amount of carbon supplied to the system allowed the development of competitive advantages to microbial r-strategists instead of slow-growing organisms

that are particularly competitive at low resource abundance (K-strategist) as C/N0, where K-strategists might feed on relatively recalcitrant occluded organic matter (Baldock and Skjemstad, 2000).

Moreover, the CLPP results put on evidence that the heterotrophic bacterial community at C/N 2 was able to utilize almost all the carbohydrates supplied, while at C/N 0 it was able to utilize only the half of them and the oxidation pattern was quite homogenously distributed among the different carbon sources.

Even if the CLPP technique has been criticized (Grover and Chrzanowski, 2000), present results confirm that the shift in the physiology of the microbial community can be rapidly made using CLPP with BIOLOG GN-plates and that this method is valuable for the assessment of the stability of the microbial community in a biofilter as demonstrated by Grove *et al.* (2004). This technique could also be used for searching similarities and differences in the functional diversity of microbial communities in different biofilters operating under similar conditions.

Studying the microbial diversity (both species richness and evenness) in natural environments has significantly advanced after the introduction of molecular techniques. Different molecular methods are available and the choice of the right one would be dependent on the questions to be answered and on the amount of samples to be processed within a reasonable period of time (Casamayor *et al.*, 2002). An ideal technique for assessing microbial communities would be free of methodological biases, identify a large number of phenotypic and phylogenetic variants, and distinguish between dominant and rare populations (Grover and Chrzanowski, 2000).

The results obtained highlight the importance of the control and management of particulate organics in recirculating fish production systems, for example, by better mechanical filtration, for promoting the biofiltration efficiency. Moreover, the control of carbon concentrations in RAS can help in turn the control of the heterotrophic bacterial population proliferation (which consume high amount of oxygen and may contribute to the clogging of the filter and the development of opportunistic pathogens) and allow to use indigenous bacteria as a shelter against external pathogen introduction. On the other hand, a moderate heterotrophic layer could also have a positive effect on nitrification by protecting nitrifiers from detachment and grazing.

As previously reported, members of this bacterial group possess the dissimilative pathway for nitrates, but the aspect that must be underlined is that most of them are

potentially harmful against fish. For this reason the well control of the environment can reduce the risks linked to their proliferation. Nevertheless, it is well accepted that all microbial populations can be implicated in maintaining a good and stable rearing environment (shelter effect), probably by releasing chemical substrates that have a bactericidal or bacteriostatic effect on other microorganisms or outcompeting for chemical and available energy (Verschuere *et al.*, 2000).

CHAPTER 4

Phylogenetic characterization of heterotrophic bacterial communities of a RAS

4.1. Introduction

In Recirculating Aquaculture Systems (RAS) maintaining healthy fish greatly depends on adequate dissolved oxygen levels, the removal of solid wastes, an efficient ammonia nitrification, but also on a good management of the microbial populations colonizing the system itself.

A RAS presents many different microniches that can support the growth of a variety of microbial populations involved in nitrification, ammonification, nitrate reduction, denitrification, proteolysis and sulfate reduction (Itoi *et al.*, 2006). Thus, while it is known that the water quality of recirculating aquaculture systems is maintained by a diversity of microbial communities in the filter packing media, the difficulties associated with cultivation have meant that the majority of these microbes have not yet been investigated in detail (Amann *et al*., 1995).

The mineralization of the high amounts of organic matter, associated with the fish excretion and the decomposition of unconsumed feed, is an important parameter in recirculating aquaculture and can support the growth of large amounts of heterotrophic bacteria, including pathogens and/or opportunistic bacteria that may colonize various external and internal body surfaces of fish (Hansen and Olafsen, 1999). Heterotrophic bacteria constitute an important factor in terms of oxygen consumption, metabolic by–products they release after cellular lyses, the diseases they may cause in fish and, finally, for the competition that may have with autotrophic bacteria for oxygen and space (Léonard *et al.,* 2001). Moreover, the nitrogen in the proteins of organic material is decomposed to ammonia by proteases and deaminases produced by heterotrophic bacteria in the material of the filter, contributing to magnify the ammonia nitrogen concentration in the water (Itoi *et al.*, 2006).

Microbiological researches applied to aquaculture generally focused on the understanding of the deleterious effects caused by fish pathogens and the use of antibiotics to cure the fish, but a good knowledge of the total heterotrophic microflora of the rearing system can give some precious elements to detect the presence of potential fish pathogens and their accumulation depending on the rearing conditions.

Moreover, such investigation can provide information about the presence/absence of potential biocontrol bacteria in the systems (Gram *et al.*, 1999; Austin *et al*., 1995; Smith and Davey, 1993).

The antagonism among microbes is a naturally occurring phenomenon through which pathogens can be killed or reduced in number in the aquaculture environment: an intensive search for bacteria that can protect fish against diseases has occurred continuously during the past years (Verschuere *et al*., 2000; Ringo and Gatesoupe, 1998). These researches focused on harmless bacteria (probiotics) that help the well being of the fish and contribute, both directly and indirectly, to protect the host animal against harmful bacterial pathogens. Nevertheless, it is well accepted that all microbial populations can be implicated in maintaining a good and stable rearing environment (shelter effect), probably by releasing chemical substances that have a bactericidal or bacteriostatic effect on other microorganisms or outcompete for chemicals and available energy (Verschuere *et al.*, 2000).

Léonard *et al.* (2000; 2001), by using classical miniaturized tests, suggested that the bacterial community of the biofilter and the rearing water are widely comparable but these data must be confirmed by modern molecular tools. The heterotrophic bacterial community of a biological filter is directly released into the rearing water by the action of the flow and may colonize all the system. Moreover, according to Léonard *et al.* (2001), in a RAS the concentration of free and attached cultivable bacteria, expressed as Colony Forming Units *per* ml (CFU/ml), was constant for each system closure degree studied. In addition, the particulated organic matter (POM) seemed to be the real controlling factor for the heterotrophic development and the biological filter efficiency.
One of the main problems encountered in exploiting microbial diversity in complex environments, such RAS, is the unculturability by traditional methods of many important microorganisms (Amann *et al.*, 1995). The availability of molecular tools allows us to overcome this problem and to explore the microbial biodiversity with more precision.

Finally, it could be pointed out that one of the key aspects for improving the reliability and the sustainability of such intensive systems is acquiring the capacity to "pilot" these bacterial populations in order to exploit their potentiality and use them as biocontrol agents (Léonard *et al*., 2000, 2001; Verschuere *et al*., 2000). Few information are actually available on the bacteria present within these systems and

important questions, concerning the stability of the heterotrophic bacterial populations in terms of activity, species composition and factors influencing them, remain unanswered (Blancheton, 2000).

The results of a study using molecular and physiological fingerprinting of bacterial communities in biofilms of RAS submitted to different C/N treatments (chapter 4) has shown that while the structure of these communities was not significantly affected by the treatments, their genetic and physiological compositions were changed. However, theses methods do not allowed characterizing such changes in terms of bacterial populations.

The aim of the present work was to characterize the heterotrophic bacterial communities of a marine RAS by using culture-dependent (bacterial isolation) and -independent (clone libraries) molecular tools, in order to obtain more accurate information on the bacterial populations and to clarify the relationships existing between the bacterial flora in the rearing water and that associated to the biofilter biofilm. Finally, the role of the biofilter as main bacterial regulator in a RAS was investigated.

4.2. Material and methods

4.2.1 Experimental facilities

The experimental Recirculation Aquaculture System used for this work is located at the IFREMER research station of Palavas les Flots (France). It was equipped with three self cleaning fish tanks (1 m^3 each) equipped with particle separator, in which water replacement rate was 1m^3/h per tank. The recirculation loop (6 to 9 m^3 h^{-1}) was constituted of a drum screen filter (30 μm mesh), a pump tank where pH and temperature were regulated, a UV-disinfection unit, a submerged nitrifying biofilter (0.7 m^3), filled with a mineral packing media (Biogrog), a CO_2 stripping unit and an oxygenation unit (Figure 4.1 and Table 4.1).

The reared fish were sea bass (*Dicentrarchus labrax*) with an average weight of 80 ± 3g. The fish density was near 15 kg/m^3 and they were fed with commercial extruded pellets (Le Gouessant, Extra-Natura, 4.5 mm).

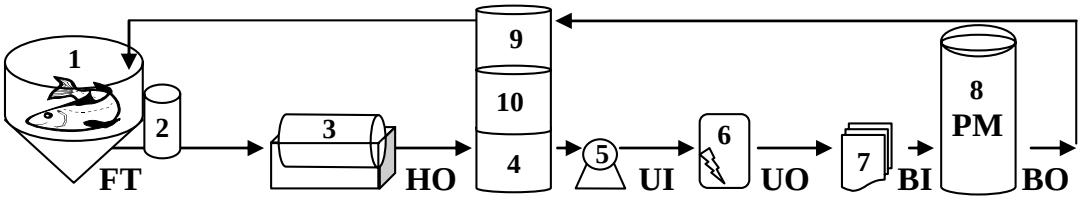

Figure 4.1: Simplified diagram of the Recirculating Aquaculture System and the Sampling Points.
System components (described in Table 1):
1) Fish tank; 2) Particle trap; 3) Mechanical filter; 4) Pump tank; 5) Pump; 6) UV lamp ; 7) Heat exchanger; 8) Biological filter; 9) CO_2 stripping;10) Surplus Storage tank.
Sampling Points:
FT) Fish Tank; HO) Mechanical filter Outlet; UI) Ultra Violet device Inlet; UO) Ultra Violet device Outlet; BI) Biofilter Inlet; BO) Biofilter Outlet; PM) Biofilter Packing Media.

Table 4.1: Description of the marine RAS used through this study.

Number in Fig.1	Components	Functions	Characteristics
1	Fish tank	Fish stocking	$1m^3$ x3
2	Particle trap	Feces and uneaten feed collection	
3	Mechanical filter	Removal of fine particles	Drum filter - 30μm mesh
4	Pump tank	pH regulation	pH range 7.0~7.5 with NaOH
5	Pump	Recirculation of water	$6~9m^3/h$
6	UV lamp	Bacteria control	$20\times10^{-3}J/cm^2$
7	Heat exchanger	Thermoregulation	18±1°C
8	Biological filter	Nitrification	$0.7m^3$, Mineral Microporous Packing Media[1]
9	Packed column	CO_2 stripping	Counter current air /water, packed column
10	Storage tank	Supersaturation DO	90%~100% saturation - bubbling of pure oxygen

[1] Biogrog®

4.2.2 Collection and preliminary treatment of samples

A simplified diagram of the sampling points following the water flow is given in Figure 4.1. Rearing water (RW) samples were collected in triplicate by using sterile polycarbonate 1L bottles at six different spots of the system: fish tank (FT), mechanical filter (HO), UV device inlet (UI), UV device outlet (UO), biofilter inlet (BI) and, finally, biofilter outlet (BO). The biofilter packing media (PM) was also sampled.

In addition, samples for bacterial isolation and for clone library construction were collected from BI, BO and from PM. The rearing water (RW; 500 ml) at BI and BO sampling points was collected by using sterile polycarbonate bottles (1 L volume); the

biofilter packing media subunits were collected in sterile becker and treated as described in Chapter 3. Both the RW and the PM packing media detaching buffer (100 ml) were filtered on sterile 47-mm diameter, 0.22 µm pore-size (Nuclepore) membranes and subsequently stored at -20 °C until processing.

4.2.3 Scanning Electron Microscopy (SEM)

In order to observe bacterial biofilms and to document the distribution of the cells on surface of the Packing Media, few Biogrog® subunits were sampled and fixed with glutaraldehyde (25%) in 0.1 % cacodylate buffer at pH 7.4 for 3 hours at 25°C and then for 17 h at 4°C. Finally, samples were washed 3 times for 5 min. in 0.1 M cacodylate buffer and then dehydrated by placing them in 25, 50, 70, 85, and 95% ethanol for 5 min. each and 3 times in 100% ethanol. Samples were stored at -20°C until processing.

Subsequent process (Critical Point Drying), manipulation and photography were carried out at the research station "A. Dorhn" of Naples by the Dr. Mariella Saggiomo, by using a SEM JEOL JSM-6500F (JEOL-USA Inc., Peabody, MA USA).

4.2.4. Microbiological analyses

Bacterial abundances and biomasses. Cultivable and total bacterial cell abundances were performed as described in Chapter 3 (Material and Methods section). Sizes of DAPI– stained cell were estimated using a photometric image video camera Axiocam (Zeiss) associated to an epifluorescent microscope (Axioplan, Zeiss). Images were captured and digitized on a personal computer by using the AXIOVISION 3.1 software. The subsequent morphometric analysis were carried out as described in La Ferla *et al.* (2004) on at least 200 cells. Bacteria were sized by using the measurement function of the software and were classified into five morphotypes: rods (elongated cells), cocci (spherical cells), coccobacilli and curved bacteria (C-shaped and S-shaped cells).

Physiological diversity of bacterial communities. Community Level Physiological Profiles (CLPPs) was used as reported in Chapter 3 (Material and Methods section)

Bacterial strains isolation. Bacterial strains were isolated from the BI, BO and PM sampling points. Colonies were selected at random from those grown on MA plates. For

each sampling point, a minimum of 150 colonies were randomly picked-up and purified at least three times before being considered pure.

PCR amplification of 16S rDNA for bacterial isolates. A single colony of each strain was lysed by heating at 95°C for 10 min. Amplification of 16S rDNA was carried out *via* PCR as previously described (Michaud *et al*., 2004) and performed with an ABI 9600 thermocycler (PE, Applied Biosystems) using the forward primer P0 (5'-GAGAGTTTGATCCTGGCTCAG-3') and the reverse primer P6 (5'-CTACGGCTACCTTGTTACGA-3'). The results of the amplification reactions were analyzed by agarose gel electrophoresis (1 %, w/v) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), containing 1 μg/ml of ethidium bromide.

The reaction mixtures were assembled at 0°C and contained 1-10 ng DNA, 10X buffer, 1.5 mM $MgCl_2$, 150 ng of each forward and reverse primer, 250 μM dNTP, 0.5 units of PolyTaq polymerase (Polymed, Italy) and sterile distilled water to a final volume of 20 μl.

The PCR program was as follows: 3 min at 95°C, followed by 30 cycles of 1min at 94°C, 1 min at 50°C, 2 min at 72°C and a final extension step of 10 min at 72°C.

Direct genomic DNA extraction and cloning. Cloning experiments were carried out for the BI, BO and PM sampling points. The DNA extraction from frozen membranes was carried out by using the RNA/DNA extraction kit (Qiagen) following the manufacter's instructions as described in Chapter 3 (Material and Methods section).

PCR protocol and conditions were identical to these described above with the exception for the two primers: in this case the two universal primers used were the 530f (5'-GTGCCAGCGCCGCGG-3') and the 1492r (5'-GGTTACCTTGTTACGACTT-3').

The 16S rDNA fragments were cloned into the pGEM Easy Vector System (Promega) according to the manufacturer's instructions. The resulting ligation products were used to transform *E. coli* ElectroMAX DH10B cells (Invitrogen). One to two hundred white colonies inserts were subsequently PCR amplified from lysed white colonies with primers specific for the vector, M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3'), under the same PCR conditions previously described.

Amplified 16S rDNA Restriction Analysis (ARDRA). 5µl of each PCR mixture, containing approximately 1.5 µg of amplified 16S rDNA, were digested with 3 U of the restriction enzyme *Alu*I (Fermentas) in a total volume of 20 µl at 37°C for 3 h. The enzyme was inactivated by heating at 65°C for 15 min and the reaction products were analyzed by agarose (2.5 %, w/v) gel electrophoresis in TAE buffer containing 1 µg/ml of ethidium bromide (Vaneechoutte *et al.*, 1993, 1995; Michaud *et al*., 2004).

16S rDNA gene sequencing. On the base of ARDRA patterns, strains and clones were grouped into Operational Taxonomic Units (OTUs), assuming that one OTU was made up of strains and clones belonging to the same species.

One to three representative strains/clones, showing the identical ARDRA pattern, were randomly selected for sequencing. All singletons (strains or clones from those ARDRA groups containing a single member) were sequenced.

The amplified 16S rDNA fragments were directly purified from PCR reaction mixture by using the QIAquick PCR Purification Kit (Qiagen) according to the supplier's instructions. Automated sequencing was carried out by cycle sequencing using the dye terminator method (Sanger *et al.*, 1977). Sequences were obtained from the sequencing service of GENELAB (Italy).

4.2.5. Data analyses

Statistical analyses. Comparisons between groups for a significant difference of mean or rank values were performed after normality and variance tests. Data were analyzed with ANOVA on ranks (Krustal-Wallis method) and the relative importance of each treatment group was investigate by a Pairwise Multiple Comparison procedure (Dunn's method).

Statistical calculations were performed by using SigmaStat software for Windows, version 3.1 (Copyright 1992-1995 Jandel Corporation).

Sequences analyses. Sequences were edited and firstly corrected by using the Chromas Pro Version 1.34 software and then checked by using the CHECK-CHIMERA program, in order to avoid the presence of hybrid sequences (Maidak *et al*., 1997). Sequence analysis was performed by using the BLASTn (Basic Local Alignment Search Tool) option of the BLAST program (Altschul *et al*., 1997).

Diversity indices. In order to evaluate structural diversity among samples, Shannon-Weaver diversity index (*H'*), Pielou Evenness (J'), Dominance (D) and the reciprocal of Simpson's index (1/*D*), were computed

Rarefaction analysis was performed by plotting total number of obtained clones versus the number of clones representing each unique phylotype. Coverage values were calculated in order to determine how efficiently the clone libraries described the complexity of the original bacterial community. The coverage (Good, 1953) value is given as $C = 1 - (n1/N)$ where n1 is the number of clones which occurred only once in the library.

The PAST software (PAlaeontological STatistics, version 1.15, http://folk.uio.no/ohammer/past) was used to compute the diversity indices and the rarefaction analysis.

4.3. Results

4.3.1. Bacterial abundance and cellular carbon content

Results for the bacterial abundances (cultivable and total) and the average cell volumes and carbon contents for each sampling point are reported in Table 4.2. In particular, viable counts in the RW were comprised between $2.48 \times 10^3 \pm 3.94 \times 10^2$ CFU/ml (M ± SD) at the outlet of the UV reactor (UO) and $3.01 \times 10^5 \pm 5.20 \times 10^4$ CFU/ml at the outlet of the biological filter (BO). The total bacterial counts varied from $1.18 \times 10^6 \pm 3.77 \times 10^5$ cells/ml (M ± SD) at the outlet of the mechanical filter (HO) and $7.59 \times 10^5 \pm 2.42 \times 10^5$ cells/ml for UO. Cultivable and total abundance values for each sampling point showed a statistically significant difference based on the ANOVA results (Kruskal-Wallis) with $P<0.001$ and $P=0.003$, respectively. However, results of Pairwise Multiple Comparison procedure (Dunn's Method) for total abundance showed that there was only a statistically significant difference ($P<0.05$) between UO and BO and between BO and FT. On the contrary, for the cultivable counts the Pairwise Multiple Comparison procedure revealed that all the median values among the sampling point were statistically different, except between HO and UI. Abundances of bacteria associated to the PM of the biological filter were $7.77 \times 10^6 \pm 2.74 \times 10^6$ CFU/g (M ± SD) and $3.72 \times 10^7 \pm 3.37 \times 10^6$ cells/g. Values are expressed as CFU or cells per gram of dry packing media, because the surface area was not well defined.

The percentage of cultivability, calculated by comparing data about viable and total abundance, ranged between 0.33% for UO and 27.31% for BO.

The average cellular volumes at the different sampling points were statistically different (Kruskal-Wallis, P = <0.001) and were comprise between 0.037 ± 0.028 μm^3 for the inlet of the UV reactor (UI) and 0.114 ± 0.020 μm^3 for PM. In particular, the Mann-Whitney Rank Sum Test performed between the cellular volumes of PM attached bacteria (n=100) and the cellular volumes of all bacteria in the RW (n=318) revealed a statistically significant difference (P <0.001).

Table 4.2: Bacterial abundances and cell volumes in RAS (St.Dev.: Standard Deviation).

Sampling Point	CFU/ml		Cells/ml		CFU/Total Counts (%)	Cell Vol. (μm^3)	
	Mean	St. Dev.	Mean	St. Dev.		Mean	St. Dev.
FT	$4.39x10^4$	$6.70x10^3$	$8.07x10^5$	$1.27x10^5$	5.4	0.082	0.015
HO	$1.91x10^5$	$4.71x10^4$	$1.18x10^6$	$3.77x10^5$	16.1	0.078	0.020
UI	$1.30x10^5$	$3.55x10^4$	$8.72x10^5$	$1.78x10^5$	14.9	0.037	0.028
UO	$2.48x10^3$	$3.94x10^2$	$7.59x10^5$	$1.42x10^5$	0.3	0.041	0.035
BI	$4.46x10^4$	$1.10x10^4$	$8.39x10^5$	$1.73x10^5$	5.3	0.047	0.027
BO	$3.01x10^5$	$5.20x10^4$	$1.10x10^6$	$1.44x10^5$	27.3	0.081	0.012
	CFU/g		**Cells/g**		**CFU/Total Counts (%)**	**Cell Vol. (μm^3)**	
	Mean	**St. Dev.**	**Mean**	**St. Dev.**		**Mean**	**St. Dev.**
PM	$7.77x10^6$	$2.74x10^6$	$3.72x10^7$	$3.37x10^6$	20.9	0.114	0.020

4.3.2. Phylogenetic diversity of bacterial isolates

Isolation procedure allowed to achieve 289 strains: 84 from BI, 91 from BO and 114 from PM samples, respectively. They were clustered by ARDRA in 41 OTUs, distributed among the α- and γ-Proteobacteria (20 and 13 OTUs, respectively), the Firmicutes (4 OTUs), the Cytophaga-Flavobacterium-Bacteroidetes (CFB) group (2 OTUs) and the Actinobacteria (2 OTUs).

Comparative sequence analysis indicated that 263 of 289 isolates were closely related to known bacteria (similarity > 97 %), whereas two OTUs (namely, 2 and 50) shared <97 % 16S rRNA gene sequence similarity with previously described and may be considered as new species (Table 4.3).

Table 4.3: Phylogenetic affiliations of isolates.

Phylum or group	OTU	Strain	Nearest phylogenetic relative/ accession no.	%	Family	No. of Isolates		
						BI	PM	BO
α-Prot.	51	BI-27	*Roseobacter* sp. - AY690598	99	*Rhodobacteraceae*	2	2	0
	5	BO-61	*Roseobacter* sp. - AY536562	98	*Rhodobacteraceae*	7	15	5
	5b	BI-60	*Roseobacter* sp. YSCB-3 - AY955394	99	*Rhodobacteraceae*	1	2	0
	7	PM-10	*Roseobacter* sp. - AY576690	97	*Rhodobacteraceae*	2	6	0
	5c	BI-68	*Roseobacter* sp. ISM - AF098495	99	*Rhodobacteraceae*	3	5	1
	19	PM-54	Uncultured *Roseobacter* - AF245634	97	*Rhodobacteraceae*	2	4	0
	2	PM-11	*Stappia* sp. – DQ868686	96	*Rhodobacteraceae*	3	4	13
	14	PM-05	*Ruegeria atlantica* - AJ968648	99	*Rhodobacteraceae*	0	6	0
	52	BI26	*Sulfitobacter dubius* - AY180102	97	*Rhodobacteraceae*	2	0	0
	26	BO-15	*Sulfitobacter* sp. - AY258095	99	*Rhodobacteraceae*	0	0	3
	61	BI-16	*Sulfitobacter* sp. - AJ534244	99	*Rhodobacteraceae*	5	0	0
	43	BO-01	*Sulfitobacter* sp. - AY967725	99	*Rhodobacteraceae*	4	0	2
	5a	PM-43	*Rhodobacteraceae* bacterium - AJ810844	98	*Rhodobacteraceae*	4	3	2
	36	BI-06	*Rhodobacteraceae* bacterium - AF539789	96	*Rhodobacteraceae*	3	5	0
	1	PM-13	Marine bacterium - AY626827	99	*Rhodobacteraceae*	13	22	11
	1a	BO-16	*Hyphomonas* sp. - M83812	98	*Hyphomonadaceae*	0	0	8
	12	BI-12	*Sphingomonas* sp. - AY690679	97	*Sphingomonadaceae*	3	4	1
	16	PM-84	*Erythrobacter luteolus* - AY739662	97	*Erythrobacteraceae*	2	1	1
	60	BO-29	*Nitratireductor* sp. - AF534573	98	*Phyllobacteriaceae*	0	0	2
	50	BI-58	Alpha proteobacterium - DQ219355	96	nd	4	2	0
γ-Prot.	2a	PM-116	*Pseudomonas stutzeri* - AJ270453	98	*Pseudomonadaceae*	0	12	1
	3	PM-124	*Vibrio parahaemolyticus* - DQ164802	99	*Vibrionaceae*	1	5	1
	17	PM-09	*Vibrio harveyi* - AY750578	98	*Vibrionaceae*	0	4	0
	62	BO-02a	*Vibrio ichthyoenteri* - AM181657	97	*Vibrionaceae*	0	0	1
	41a	BO-39	*Vibrio* sp. - AF022409	98	*Vibrionaceae*	2	0	1
	6	PM-108	*Rheinheimera* sp. - AM110966	98	*Chromatiaceae*	1	1	0
	11	PM-100	*Erwinia* sp. - DQ303295	99	*Enterobacteriaceae*	0	2	0
	18	PM-91	*Marinobacter aquaeolei* - AY669169	99	*Alteromonadaceae*	0	3	0
	28	BI-65	*Alcanivorax* sp. - AY394865	99	*Alcanivoracaceae*	3	0	2
	72	BO-27	*Alcanivorax* sp. - AY307381	98	*Alcanivoracaceae*	1	0	1
	56	BO-23	*Halomonas venusta* - AY553074	99	*Halomonadaceae*	0	0	2
	41	BO-32	*Pseudoalteromonas* sp. - AJ551105	99	*Pseudoalteromonadaceae*	5	0	4
	42	BO-26	*Pseudoalteromonas* sp. - DQ218321	97	*Pseudoalteromonadaceae*	4	0	8
Fir	8	PM-103	*Enterococcus sanguinicola* - DQ411817	99	*Enterococcaceae*	0	6	0
	25	PM-66	*Oceanobacillus* sp. - AB167055	99	*Bacillaceae*	4	0	5
	29	BI-31	*Bacillus pseudofirmus* - AB201799	99	*Bacillaceae*	1	0	5
	33	BO-02	*Staphylococcus* sp. - AB167054	100	*Staphylococcaceae*	0	0	8
CFB	39	BI-23	*Vitellibacter vladivostokensis* - AB071382	97	*Flavobacteriaceae*	2	0	0
	46	BO-10	*Tenacibaculum* sp. - AY962294	99	*Flavobacteriaceae*	0	0	1
Act	57	BO-55	*Microbacterium schleiferi* - Y17237	98	*Microbacteriaceae*	0	0	1
	34	BO64	*Kocuria* sp. JL-33 - AY745865	99	*Micrococcaceae*	0	0	1
					Total Isolates	**84**	**114**	**91**

α-Prot. = α-Proteobacteria γ-Prot. = γ-Proteobacteria
Fir= Firmicutes Act= Actinobacteria
CFB= Cytophaga-Flexibacter-Bacteroidetes

OTU= Operational Taxonomic Unit
%= Similarity with the next relative
nd: not determined

Isolates were mainly assigned to the α-Proteobacteria (65.7 % of total isolates), with 162 (out of 190) strains belonging to the family Rhodobacteraceae (15 OTUs); in particular, 57 and 16 of them were affiliated to the *Roseobacter* clade and to *Sulfitobacter* spp., respectively. Addidional OTUs within the α-Proteobacteria were assigned to the families Hyphomonadaceae, Erythrobacteriaceae, Sphingomonadaceae and Phyllobacteriaceae.

Among the 65 γ-Proteobacteria strains (22.5 % of total isolates), 21 belonged to the genus *Pseudoalteromonas*, 15 to *Vibrio* spp. and 13 to the *Pseudomonas stutzeri* species.

Firmicutes, Actinobacteria and CFB were strongly less represented (10, 1.1 and 0.7 %, respectively).

4.3.3. Phylogenetic diversity of 16S rDNA clones

The 263 clones were clustered in 59 OTUs. Similar to results obtained for the isolates, clone OTUs were distributed among the α- Proteobacteria (18 OTUs), the CFB (12 OTUs), the γ-Proteobacteria (9 OTUs), the Actinobacteria (3 OTUs) and the Firmicutes (2 OTUs). Additionally, 4 OTUs were affiliated to the δ-Proteobacteria, 4 to the Planctomycetes and, finally, 2 to the Verrucomicrobia.

The phylogenetic assignment of clones (Table 4.4) showed that the α- Proteobacteria represented 28.1 % of all clones, mainly assigned to the family Rhodobacteraceae (8 OTUs).

The γ-Proteobacteria represented the 22.8 % of all screened clones. The genus *Pseudomonas* was the more abundant with *Pseudomonas stutzeri* as the dominant species (18 clones within the OTU 65).

Members of the CFB group amounted for 17.1 % of all clones, followed by Actinobacteria (11.4 %) and Planctomycetes (5.3 % of all clones), the latter mainly represented by the *Pirellula* genus (95 to 98 % similarity). The clone libraries allowed the detection of δ-Proteobacteria (10 clones; 3.8 % of all clones), assigned to *Idiomarina loihiensis* (96 % similarity), *Myxobacterium* sp. (94 % similarity) and *Desulfovivrio zosterae* (98 % similarity). The only three clones belonging to Firmicutes (1.1 % of all clones) were closely related to *Enterococcus seriolicida* (99 % similarity) and *Bacillus subtilis* (96 % similarity). The phylum Verrucomicrobia was also represented by only three clones clustered in two OTUs (1.1 % of all clones). Finally,

9.1 % of screened clones was classified as uncultured bacterium/clone, and not phylogenetically assigned. The sequences of only 11 clones (clustered in three OTUs, namely 16 and 43 among α- Proteobacteria, and 46 among CFB) were very similar to as many OTUs as isolates.

Table 4.4: Phylogenetic affiliation of clones.

Phylum or group	OTU	Clone	Nearest phylogenetic relative/accession no.	%	Family	No. of Clones		
						BI	PM	BO
α-Prot.	45	PM-10	Uncultured *Roseobacter* sp. - AY697886	99	*Rhodobacteraceae*	0	1	0
	12	PM-96	*Roseobacter* sp. - AJ534238	97	*Rhodobacteraceae*	0	3	0
	59	BI-154	*Roseobacter* sp. - AY167339	98	*Rhodobacteraceae*	1	5	1
	45	PM-92	*Sulfitobacter* sp. - AY967725	97	*Rhodobacteraceae*	0	1	0
	1a	BO-35	*Sulfitobacter mediterraneus* - Y17387	98	*Rhodobacteraceae*	1	0	4
	34	BO-17	*Methylarcula* sp. - AJ534223	99	*Rhodobacteraceae*	2	0	1
	74	PM-46	Mucus bacterium - AY654754	99	*Rhodobacteraceae*	0	3	0
	36	PM-54	Uncultured *Rhodobacteraceae* bacterium - DQ234245	95	*Rhodobacteraceae*	0	1	0
	23	BO-59	*Erythrobacter luteolus* - AY739662	94	*Erythrobacteraceae*	5	0	3
	39	BI-181	*Erythrobacter longus* - AF465835	98	*Erythrobacteraceae*	1	1	2
	32	BO-48	*Hyphomonas jannaschiana* - AJ227814	99	*Hyphomonadaceae*	1	0	1
	22	PM-55	*Hyphomicrobium sulfonivorans* - AY305006	95	*Hyphomicrobiaceae*	0	1	0
	39	PM-85	*Sphingomonas* sp. - AY947554	95	*Sphingomonadaceae*	0	3	0
	63	PM-65	Alpha proteobacterium - AB024595	96	nd	1	4	1
	9	BO-51	Uncultured alpha proteobacterium - DQ336985	92	nd	0	0	2
	56	PM114	Uncultured alpha proteobacterium - AY922245	97	nd	3	7	1
	1	BO-55	Uncultured marine alpha proteobacterium - AY794071	93	nd	1	0	6
	1b	BO-07	Uncultured SAR11 - AY868689	91	nd	2	0	4
γ-Prot.	75	PM-1	*Pseudomonas stutzeri* - AJ312163	99	*Pseudomonadaceae*	0	18	0
	20	PM-3	*Pseudomonas anguilliseptica* - AY771754	95	*Pseudomonadaceae*	0	1	0
	17	PM-8	*Pseudomonas plecoglossicida* - DQ140383	98	*Pseudomonadaceae*	0	1	0
	19	BO-56	*Pseudomonas* sp. - AM111063	98	*Pseudomonadaceae*	2	7	4
	44	BI-36	*Pseudoalteromonas* sp.- AY646155	96	*Alteromonadaceae*	4	3	1
	90	PM-56	*Coxiella* sp. - AE016828	96	*Coxiellaceae*	0	3	0
	20	BI-99	Uncultured gamma proteobacterium - AF424114	98	nd	2	1	1
	56	BI-140	Uncultured gamma proteobacterium - AF424165	95	nd	3	0	2
	66	BI-153	Gamma proteobacterium - AF505738	98	nd	1	4	2
δ-Prot	11	PM-72	*Idiomarina loihiensis* - AF288370	96	*Idiomarinaceae*	0	1	0
	38	BI-136	*Desulfovibrio zosterae* - Y18049	98	*Desulfovibrionaceae*	1	0	0
	55	BI-101	Myxobacterium SMH-27-4 - AB252740	94	nd	2	0	4
	80	BI-174	Uncultured delta proteobacterium - AY875880	92	nd	1	0	1
CFB	2	BO-50	*Cytophaga* sp. -AB0735570	98	*Flexibacteraceae*	2	0	1
	27	BO-11	*Cytophaga* sp. T-565 - AB073585	97	*Flexibacteraceae*	1	1	4
	57	BI-153	*Maribacter* sp. - AY576693	98	*Flavobacteriaceae*	1	0	1
	76	BI-179	*Winogradskyella thalassocola* - AY771730	97	*Flavobacteriaceae*	1	0	1
	3	PM-23	*Tenacibaculum litoreum* - AY962294	97	*Flavobacteriaceae*	0	1	0
	7	BO-15	*Saprospira* sp. - AB191039	98	*Saprospiraceae*	2	0	2
	13	BO-57	*Spirochaeta* sp. - AY337319	96	*Spirochaetaceae*	2	0	2
	72	PM-52	Bacteroidetes bacterium - AY372916	98	nd	0	3	0
	44	BO-33	Bacteroidetes bacterium clone - AY327883	97	nd	2	1	4
	67	BI-171	Bacteroidetes bacterium - DQ070811	95	nd	1	1	2
	5	BI-4	Uncultured Bacteroidetes bacterium AY947931	97	nd	4	1	1
	64	BI-107	Uncultured Bacteroidetes bacterium - AF507867	96	nd	2	0	1
Fir	9	PM-9	*Enterococcus seriolicida* - AF061005	99	*Streptococcaceae*	0	1	0
	11	PM-50	*Bacillus subtilis* - AM110921	96	*Bacillaceae*	0	2	0
Planc	8b	PM-61	*Pirellula* sp. - X81943	95	*Planctomycetaceae*	0	3	0
	19	BO-36	*Pirellula* sp. - AF099714	97	*Planctomycetaceae*	2	1	1
	20	BO-20	*Pirellula* sp. - X86388	98	*Planctomycetaceae*	2	0	1
	77	PM-26	Uncultured planctomycete - AB116415	95	nd	0	2	2
Act	33	BO-53	*Rhodococcus ruber* - AB218987	97	*Nocardiaceae*	2	0	1
	11	BO-85	*Micrococcus luteus* - AB079788	98	*Micrococcaceae*	7	1	7
Verr	20b	PM-15	Uncultured Verrucomicrobia - DQ302110	97	nd	0	1	0
	18	BO-18	Uncultured Verrucomicrobia bacterium - AF424512	95	nd	1	0	1
nd	18	PM-10	Uncultured bacterium clone - AY133397	98	nd	2	8	2
	17	BO-72	Uncultured bacterium clone - AY570641	94	nd	2	0	1
	48	BI-121	Uncultured bacterium - AB239761	94	nd	1	0	1
	40	BI-111	Uncultured proteobacterium - AY57789	99	nd	3	1	1
	42	PM120	Uncultured marine bacterium - DQ071119	97	nd	0	2	0
	11a	BO64	Uncultured spirochete - AY605176	96	nd	4	0	8
					Total Clones	78	99	86

nd: not determined α-Prot. = α-Proteobacteria
γ-Prot. = γ-Proteobacteria δ-Prot.= δ-Proteobacteria
CFB= Cytophaga-Flexibacter-Bacteroidetes
Verr= Verrucomicrobia Fir= Firmicutes Planc= Planctomycetes
Act= Actinobacteria OTU= Operational Taxonomic Unit
%= Similarity with the next relative

4.3.4. Distribution of the phylogenetic groups for bacterial isolates and clones

The number and the percent distribution of bacterial isolates and clones in samples from BI, BO and PM are summarized in Table 4.5.

Table 4.5: Number and percent distribution of bacterial isolates and clones in samples from BI, PM and BO.

Phylogenetic grouping	BI		PM		BO	
	Isolates	Clones	Isolates	Clones	Isolates	Clones
alfa-proteobacteria	60 (71.4 %)	18 (23.1 %)	81 (71.1 %)	30 (30.3 %)	49 (53.9 %)	26 (30.2 %)
gamma-proteobacteria	17 (20.2 %)	12 (15.4 %)	27 (23.7 %)	38 (38.4 %)	21 (23.1 %)	10 (11.6 %)
delta-proteobacteria		4 (5.1 %)		1 (1.0 %)		5 (5.8 %)
CFB	2 (2.4 %)	18 (23.1 %)		8 (8.1 %)	1 (1.1 %)	19 (22.1 %)
Firmicutes	5 (6.0 %)		6 (5.3 %)	3 (3.0 %)	2 (19.8 %)	
Actinobacteria		13 (16.7 %)		1 (1.0 %)	2 (2.2 %)	16 (18.6 %)
Verrucomicrobia		1 (1.3 %)		1 (1.0 %)		1 (1.2 %)
Planctomycetes		4 (5.1 %)		6 (6.1 %)		4 (4.7 %)
Not assigned		8 (10.3 %)		11 (11.1 %)		5 (5.8 %)

For bacterial isolates, those belonging to the α- Proteobacteria were present in all samples and were always dominant, ranging from 71.4 % in BI to 53.9 % in BO. The γ-Proteobacteria isolates were equally distributed among BI, BO and PM, as well as Firmicutes. Any CFB member was retrieved from PM. The only two Actinobacteria isolates, closely related to *Microbacterium schleiferri* (98 % similarity) and *Kocuria* sp. (99 % similarity), were isolated from BO.

Among bacterial clones, members of the α- Proteobacteria (dominating in BO, 30.2 %), the δ-Proteobacteria, the Planctomycetes and the Verrucomicrobia were quite homogenously distributed among the three sampling points. The γ-Proteobacteria were dominant in PM samples where they represented 39.4 % of screened clones. Members of the CFB group were more abundant in BI and BO (23.1 and 22.1 %, respectively) than in PM (8.1 %), as well as the Actinobacteria (18.6, 16.7 and 1 % in BO, BI and PM, respectively). Firmicutes clones were not detected in BO samples.

4.3.5. Analysis of diversity

For bacterial isolates, both the Shannon (H') and Simpson reciprocal (1/D) indices were higher in BI and BO (3.038 and 16.56 for BI; 2.868 and 13.50 for BO) than PM (2.726 and 11.44, respectively), while the Pielou index ranged from 0.880 and 0.9325 (Table 4.6).

The clone coverage was 80 %, 81 % and 77 % for the BI, PM and BO, respectively, suggesting that reasonably sufficient coverage of the libraries was achieved. The clone

libraries, as expected, revealed a higher OTU richness (59) than isolates (41). The diversity indices computed for BI and BO revealed a planktonic population characterized by a Simpson index (27.16 for BI and 23.86 for BO) not far from the maximum values (equivalent to the number of detected OTUs).

Table 4.6: Results for statistical analysis.

	Isolates			Clones		
Sampling Point	BI	PM	BO	BI	PM	BO
No of isolates/clones	84	114	91	78	99	39
No of OTUs	26	21	26	38	36	39
Dominance D	0.060	0.087	0.074	0.037	0.065	0.042
Pielou Evenness J'	0.932	0.895	0.880	0.955	0.881	0.931
Shannon H'	3.038	2.726	2.868	3.472	3.158	3.407
Simpson 1/D	16.56	11.44	13.50	27.16	15.39	23.86

4.3.6. Community Level Physiological Profile

The average (blank-corrected) well color development (AWCD), after 4 days of incubation, was 0.9. The CLPP analysis did not clearly revealed a different pattern in the carbon sources oxidation for the whole population associated to the biofilter packing media and the population of the reared water both ingoing and outgoing the filter unit (Figure 4.3). The number of oxidized carbon was maximal for PM (54) and minimal for BI (46).

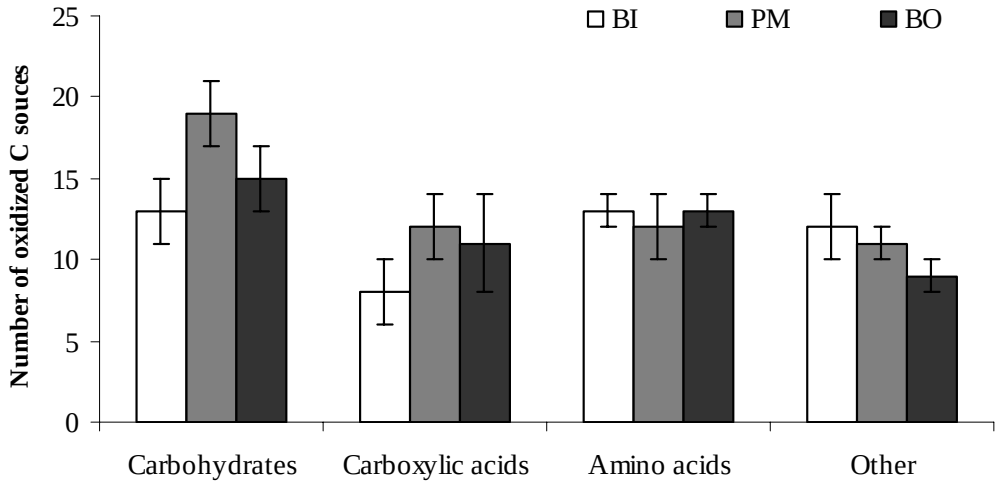

Figure 4.3: Number of oxidized carbon sources for Biofilter Inlet (BI), Packing Media (PM) and Biofilter Outlet (BO).

4.3.7. Packing media Scanning Electron Microscopy (SEM)

Few units of the packing media inside the biological filter were sampled to carry out microscopic observations, by using a Scanning Electron Microscopy (model JSM-6500F, JEOL-USA Inc.), of the bacteria associated to them. The Figure 4.4a shows the exopolysaccharide matrix of the biofilm, while in the Figure 4.4b bacteria colonizing the packing media, characterized by different cell morphologies, are put on evidence.

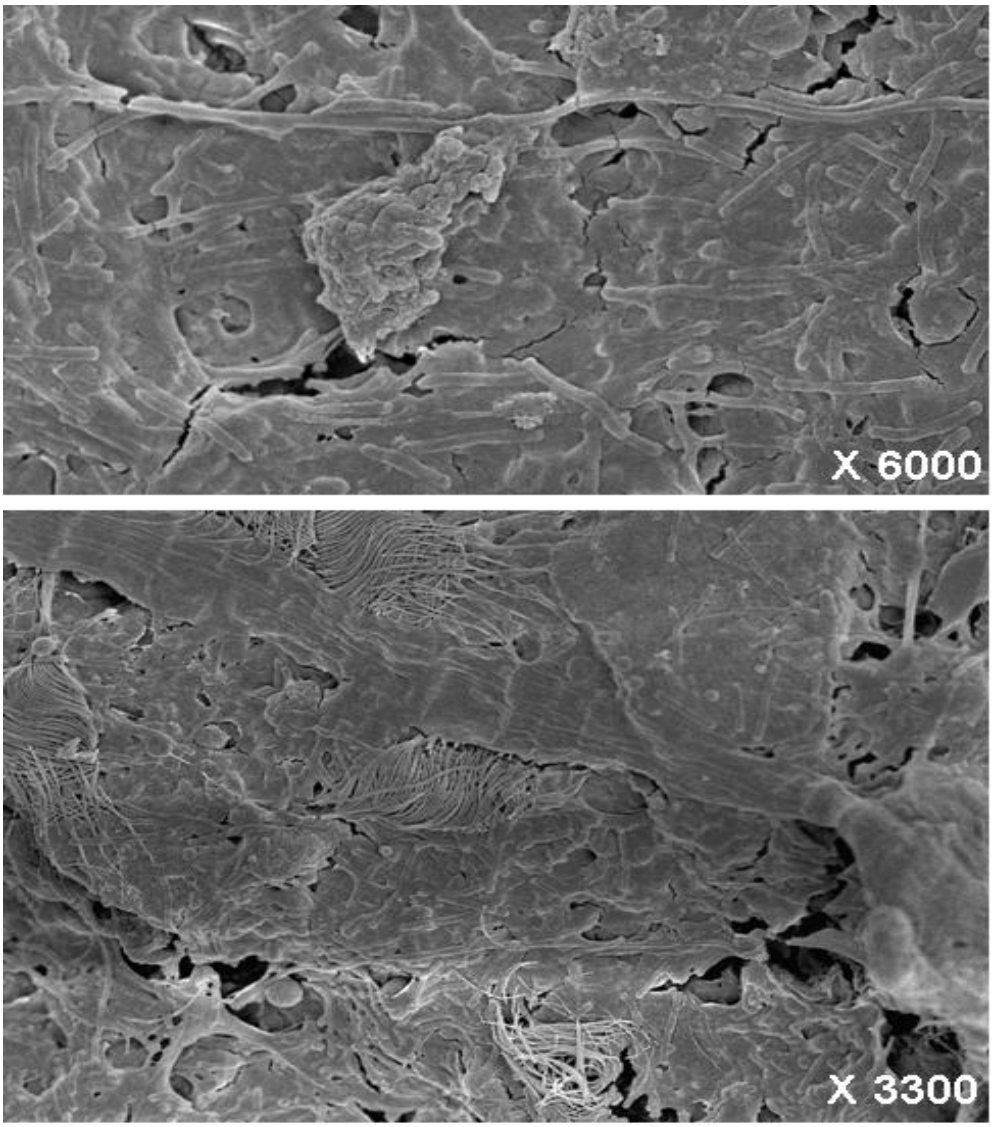

Figure 4.4 a: Scanning Electron Microscopy (SEM) of Packing Media associated biofilms.

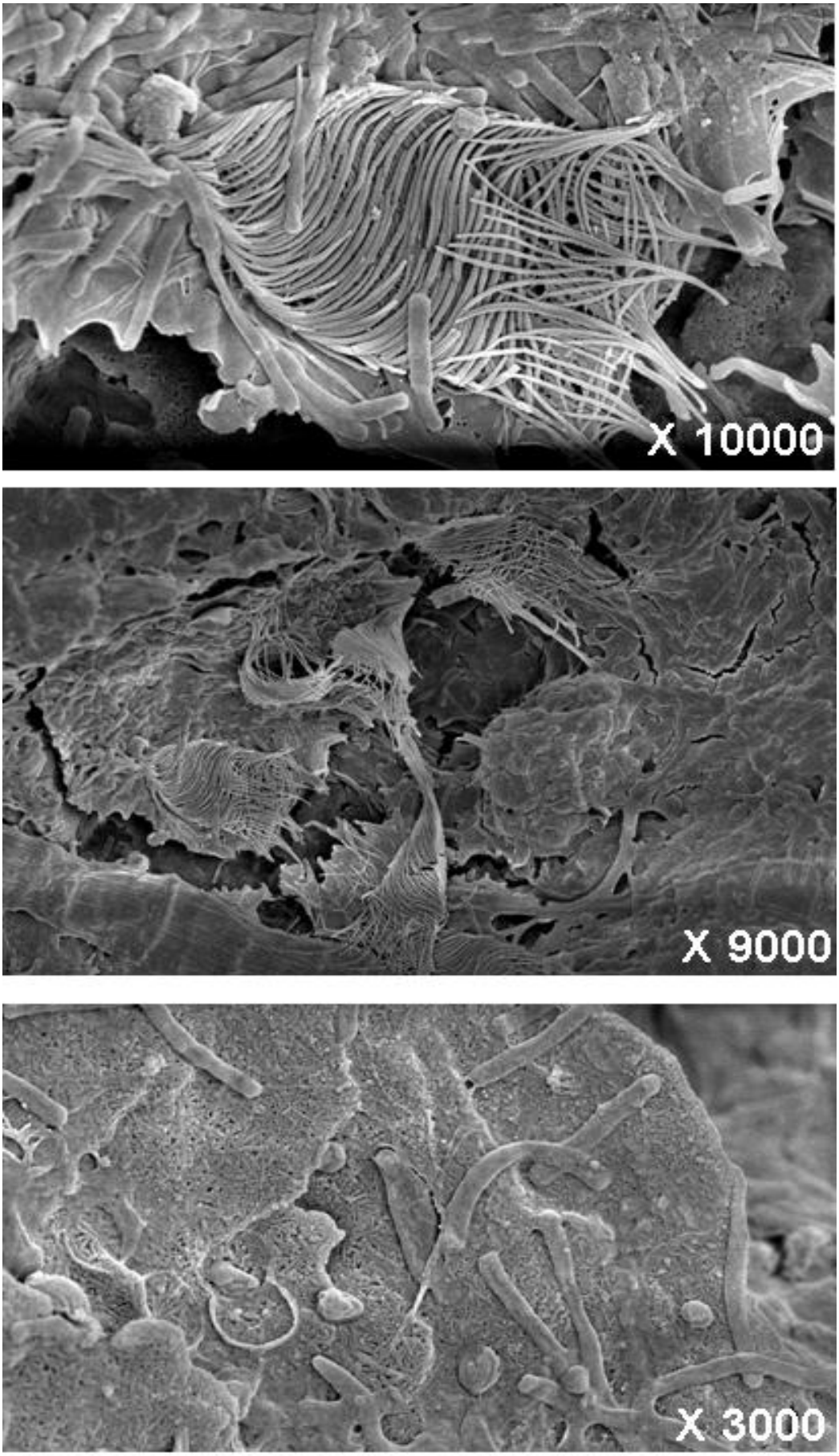

Figure 4.4 b: Scanning Electron Microscopy (SEM) of Packing Media.

4.4. Discussion

This study was aimed to provide information about the heterotrophic bacterial compartment of a marine RAS in terms of abundance and community species composition. Data obtained from the present work on bacterial abundance are comparable with those reported by Léonard *et al.* (2000) and Sugita *et al.* (2005). The former found that the cultivable bacteria abundance in a marine RAS ranged from $< 10^3$ (at the UV disinfection unit outlet) and $\geq 10^5$ CFU/ml (at the biofilter outlet), while 7.3 $\pm$ 7.25 x 10^6 CFU/g were present on the biofilter packing medium. The same authors individuated the biofilter as main bacterial producer in the system for cultivable bacteria, while for the total bacterial counting (DAPI) the main producer resulted the mechanical filter. Sugita *et al.* (2005), studying the filter material of freshwater recirculating systems, reported a bacterial density on the filter pebbles of 1.1 x 10^7 cells/g.

In addition, the high cultivability, compared with values normally reported in literature (e.g. Aman *et al.*, 1995), suggests a good physiological status of the bacterial populations, probably due to large amounts of available organic carbon sources and to a good oxygenation of the system.

The biofilter probably acts as a reactor not only by producing large amounts of bacteria, but also by improving the bacterial physiological status after their exposure to UV irradiation. At the exit of the UV device, the cultivable bacterial abundance decreased from 10^5 to 10^3 CFU/ml, while the total counts remain stable, suggesting an inactivation process more than a killing one. The cultivability felt at 0.3 % at the UV device outlet, but the microbial community was able to restore its physiological status in a very little time, considering that the cultivability increased (5.3 %) again after few meter run of the water at the biofilter inlet (BI) and was maximal at the outlet (27.3 %).

The cellular volumes calculated for FT (0.082 μm^3), HO (0.078 μm^3), BO (0.081 μm^3) and PM (0.114 μm^3) were higher than those (0.0593 μm^3) reported by other authors for marine bacteria (La Ferla *et al.*, 2004; Lee and Fuhrman, 1987). These high values of biovolumes are consistent with an environment where organic and inorganic substrates are not limiting.

Molecular analyses revealed that the large part of the microflora, both cultivable and total, was dominated by α- and γ-Proteobacteria, CFB and Actinobacteria. In terms of phylogenetic affiliation, the communities were widely comparable between RW (BI

plus BO) and PM, confirming the tight relationship among the attached and the planktonic bacterial communities within a RAS. Sugita *et al.* (2005), studying the microbial composition of the filter material of two freshwater recirculating systems (one for carp and the second for goldfish), showed that the sequenced clones were mainly assigned to the α-Proteobacteria, the β-Proteobacteria and the Actinobacteria but quite different to those found in the present study with regard to the species composition. Moreover, Tal *et al*. (2003), by applying a DGGE approach to a moving bed bioreactor, detected 10 different bacterial taxa, among which two belonged to the α- Proteobacteria and two to the Plantomycetes.

The difference in the Simpson reciprocal index between the RW and PM, both for isolates and for clones, showed that the general structure of the planktonic microbial community is different from the biofilm of the biological filter (where the lower Simpson index values were 11.44 and 15.39 for isolates and clones, respectively), suggesting the development of one or more dominant species in PM. In fact, on the biological filter biofilm, *Pseudomonas stutzeri* (12 strains and 18 clones), and *Roseobacter sp.* represented the more abundant strains and clones. *Pseudomonas stutzeri* (Ward *et al.*, 1993; Lalucat *et al.*, 2006) is a nonfluorescent, dinitrogen producer denitrifying bacterium, widely distributed in the environment. It has been isolated as an opportunistic pathogen from humans and it has also been proposed as a model organism for denitrification studies. This bacterium, as well as some others denitrification strains and clones, could explain the loss of ammonia when mass balances are calculated for such biofilters. Conversely, clones related to the Planctomycetes were detected in all samples, suggesting the presence of anammox bacteria, where nitrite and ammonia combine *via* microbial processes to form N_2 gas and water with no nitrate produced (Strouss *et al.*, 1997, 1999; Jetten, 1999; Jetten *et al.*, 1997, 2001). On the very irregular surface characterizing the porous mineral packing medium used for the biological filter (Biogrog), many microniches can be present allowing the establishment of anoxic microenvironments, supporting the growth of such organisms.

The second main group of isolates and clones detected on the PM was the genus *Roseobacter* and species affiliated with this group, forming the so-called *Roseobacter* clade, important members of the marine microbiota (Bruhn *et al*., 2005). These aerobic organisms, which may account for as much as 40 % of prokaryotic DNA from the

ocean, are excellent biofilm-forming organisms and are among the first and dominant colonizers of surfaces in all marine environments (Dang and Lovell, 2000).

A variety of fish bacterial pathogens were identified in this study, even if the rearing system was in an optimal sanitary status. This finding confirmed that pathogens or opportunistic pathogens are always present in the system. Most of them are ubiquitous in aquatic environments and the non-expression of their virulence could be ascribed to a good management of the system and to a good physiological status of reared fish. Moreover, the presence of bacteria previously described as probiotics or producers of inhibitory compounds, as members of *Roseobacter* clade and lactic bacteria (Gatesoupe, 1991, 1999; Irianto and Austin, 2002; Bruhn *et al*., 2005), suggests that the indigenous microbiota can control pathogenic organisms in fish rearing systems, the so-called "Shelter Effect". Several studies have demonstrated that probiotic bacteria may be used to larval rearing (Bruhn *et al*., 2005), and one of several promising candidates are bacteria of the marine *Roseobacter* clade. *Roseobacter* strains have also been isolated from turbot larval farms, and they were selected from this environment due to their strong anti-*Vibrio* activity (Hjelm *et al*., 2004).

4.5. Conclusions

In conclusion, our present data on bacterial compartment of a marine RAS, both planktonic and associated to biofilter packing media, underline that a good management of the rearing environment would benefit the maintenance of favorable water physicochemical parameters in these systems.

The influence of heterotrophic bacteria on the rearing water conditions is not clear and requires further investigation, but it is well known that microbes play a key role in aquaculture environments. The application of such modern methods for the study of microbial communities will make possible to correlate microbial systematic and dynamics with aquaculture management.

The aquaculture industry is heavily regulated with regards to the use of antibiotics and chemicals in the water. The proliferation of antibiotic resistant organisms is of great concern because the antibiotics currently legally available are becoming increasingly ineffective. Developing methods of pathogen reduction or elimination in RAS will require a better understanding of the viability of these pathogens inside indigenous

microbial communities and a deep investigation on the relationship and interaction between different taxa and between microbes and the systems itself.

Keeping facilities pathogen-free is an impossible task, but reducing levels of pathogens to below infective levels, by promoting the presence and the activity of probiotics bacteria, should decrease the chance of fish becoming clinically infected.

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CHAPTER 5

Molecular detection of anaerobic ammonium oxidizing anammox bacteria in a marine submerged aerobic biological filter

5.1. Introduction

In on-shore intensive aquaculture rearing systems the biofiltration is employed for the oxidation of highly toxic ammonia-nitrogen, excreted by fish gills, into relatively harmless nitrate, through the process of nitrification, and the nitrate is normally maintained at low levels by daily replacement of a portion of the water (van Rijn, 1996).

However, the strict environment protection regulations for nitrate concentration in industrial effluent is motivating the recirculating aquaculture industry to integrate a denitrifying biofiltration stage in the "normal" treatment loop, which results in the conversion of nitrate to nitrogen gas (Tal *et al.*, 2006).

The denitrification process is performed mainly by facultative anaerobic bacteria that utilize organic (heterotrophic denitrification) or inorganic (autotrophic denitrification) compounds as electron donor to reduce nitrate. So far, difficulties associated with the denitrification process, such as the high potential for toxic sulfide production, as well as the requirement for addition of an external electron source, have prevented its use in full-scale commercial marine recirculating systems (van Rijn *et al.*, 2006).

After the recent discovery of the existence of anaerobic ammonium oxidation (ANAMMOX) process, carried out by newly described bacteria deeply branched in the *Planctomycetes* phylum (Figure 5.1), nitrogen losses, which could only be explained by the anammox reaction, were reported in many wastewater treatment facilities (Mulder *et al.*, 1995, van de Graaf *et al.*, 1996; Schmid *et al.*, 2005).

Molecular techniques showed the presence of organisms affiliated with the anammox branch within the *Planctomycetes* in all these wastewater treatment plants (Schmid *et al.*, 2005). Anammox bacteria have been found in the anoxic water column of the Black Sea, the Costa Rica shoreline, an oceanic oxygen-minimum zone and sediments (Tal *et al.*, 2006). Moreover, evidence for anammox activity, has been obtained in a larger variety of engineered systems, such as a rotating biological contactor (Tal *et al.*, 2005), sequencing batch reactors (Strous *et al.*, 1997), and enrichments (Strous *et al.*, 1999; Egli *et al.*, 2001). This suggested that anammox bacteria can build reasonably sized

populations provided that the reactor conditions are suitable for the anammox process (Tal *et al.*, 2005).

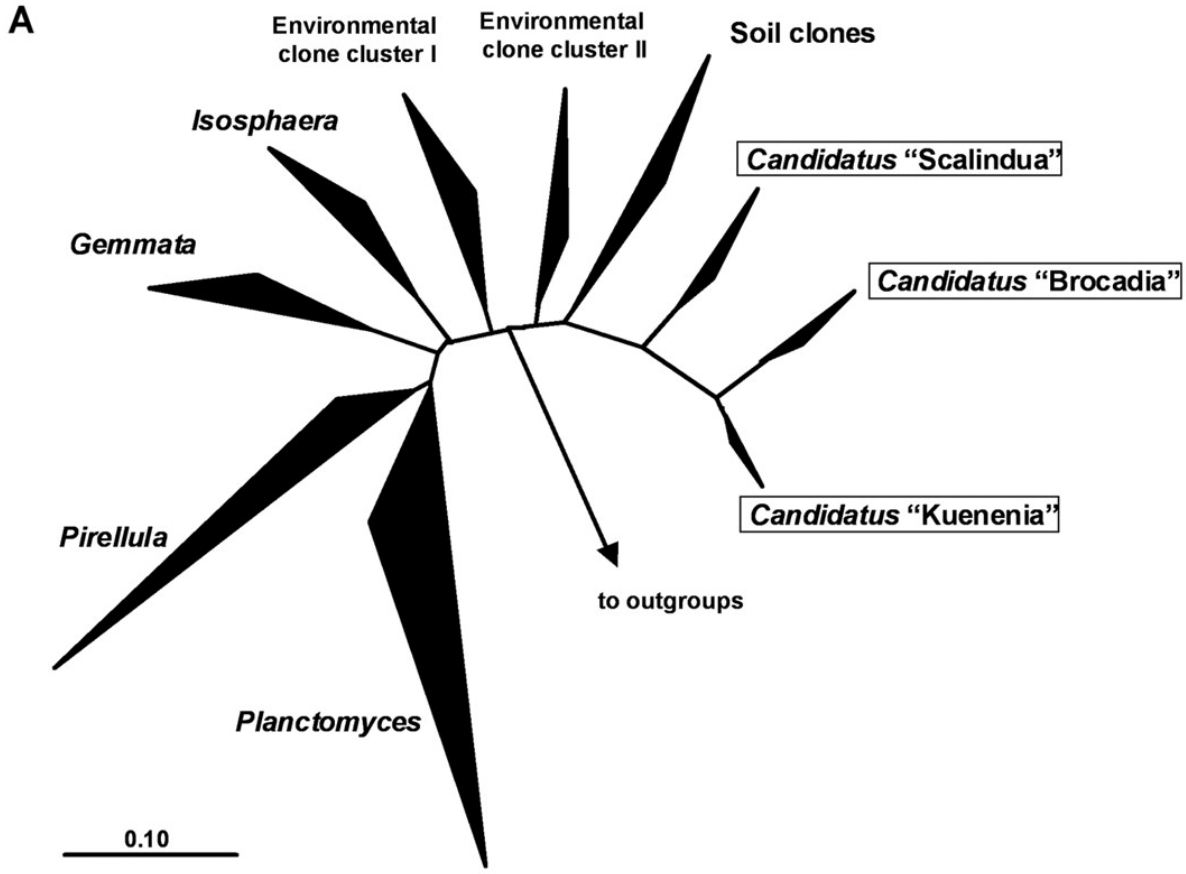

Figure 5.1: 16S rRNA gene-based phylogenetic tree of bacteria performing the anammox reaction (Schimd *et al.*, 2005).

In recirculating aquaculture systems, one option for closing the nitrogen cycle without exclusive reliance on denitrification is to integrate the anaerobic ammonium oxidation (anammox) process in recirculating systems (Tal *et al.*, 2006).

Investigating and improving the utilization of an anammox reactor in the treatment loop of a full-scale recirculating aquaculture facility can allow the achievement of two mains goals: (1) the reduction of the environmental impact of RAS and the improvement of their sustainability, and (2) the possibility to realize a totally closed reared system, that can be used for the so called "Urban aquaculture" (Tal *et al.*, 2006).

In the Chapter 3 it was put on evidence a loss of nitrogen from the inlet and the outlet of the biofilters used for the experiments (Figure 3.3). Such result was ascribed to the presence both of denitrifiers bacteria and anammox, making the hypothesis that

hypoxic and/or anoxic microniches, present on the mineral packing media, could support their growth and activity. The microbial characterization of heterotrophic bacterial communities of a marine RAS (Chapter 4), allowed to individuate some bacterial species able to perform denitrification, such as *Pseudomonas stutzeri.* Thus, the objective of this study was to preliminary investigate on the presence of anammox bacteria associated to the mineral packing media of an aerobic submerged biological filter.

5.2. Material and methods

5.2.1. Collection and preliminary treatment of samples

This experiment was carried out on a sub-sample of the same packing media collected for Chapter 4 experiment.

5.2.2. PCR-based detection of Anaerobic Ammonium-Oxidizing (Anammox) bacteria

Direct genomic DNA extraction and cloning. DNA extraction was performed as previously described in Chapter 3 (Material and Methods section), by using the RNA/DNA extraction kit (Qiagen) following the manufacter's instructions.

Nested-PCR. Because *Planctomycete*s, such as anammox organisms, are still underrepresented in general, 16S rRNA gene clone libraries, the approached used here was a nested-PCR in order to increase the relative amounts of 16S rRNA gene sequences. To identify anammox bacteria in the biofilter biofilms, a nested-PCR approach was employed, as described by Tal *et al.* (2005).

The first PCR step was carried out on extracted DNA by using a *Planctomycetales* specific 16S rRNA oligonucleotide primer (Pla46 5'-GAC TTG CAT GCC TAA TCC-'3) (Neef *et al.*, 1998) coupled with the universal bacterial primer 1492r (5'-GGTTACCTTGTTACGACTT-3'). The product of this reaction (1μl) was re-amplified using primer Pla46 and anammox-specific primer Amx820 (5'-AAA ACC CC TCT ACT TAG TGC CC-'3) (Schmid *et al.* 2005).

Products of this second PCR were then cloned into *Escherichia coli*, as previously described, and selected transformants were examined further by ARDRA and sequencing.

The PCR mixtures (50 μl) contained 5 μl of PCR buffer, 4 μl of dNTPs (25 mM each), 3 μl of $MgCl_2$ (25 mM), 0.1 μl of Platinum *Taq* polymerase (Invitrogen), 2 μl of DNA template (10 to 100ng), and 1 μl of each primer (100 to 200 ng). The reaction cycle parameters included an initial denaturation step consisting in 5 min at 95°C, followed by seven touchdown PCR cycles (Tal *et al.*, 2005) (30 s of denaturation at 94°C, 30 s of annealing at 62, 60, 59, 58, 57, 56, and 55°C, and 30 s of elongation at 72°C) and then 30 cycles consisting of 30 s of denaturation at 94°C, annealing for 30 s at 54°C, and elongation for 30 s at 72°C and a final extension step consisting of 5 min at 72°C.

The sizes and yields of PCR products were determined by using a 0.8% (w/v) agarose gel in TAE buffer (20 mM Tris-HCl, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 8.0).

Cloning. The rDNA fragments were cloned into the pGEM Easy Vector System (Promega) according to the manufacturer's instructions, as described in Chapter 4 (Material and Methods section).

Amplified 16S rDNA Restriction Analysis (ARDRA). Clones were clustered in Operational Taxonomic Units (OTUs), as described in Chapter 4 (Material and Methods section).

16S rDNA gene sequencing and phylogenic analysis. One to three representative strains/clones, showing the identical ARDRA pattern, were randomly selected for sequencing, as described in Chapter 4 (Material and Methods section). Sequences were obtained from the sequencing service of GENELAB (Italy) and edited and firstly corrected by using the Chromas Pro Version 1.34 software. Sequences were then checked by using the CHECK-CHIMERA program, in order to avoid the presence of hybrid sequences (Maidak *et al.*, 1997). Sequence analysis was performed by using the BLASTn (Basic Local Alignment Search Tool) option of the BLAST program (Altschul *et al.*, 1997). The ClustalW program (Thompson *et al.* 1994) was used to align the 16S rDNA sequences obtained with the most similar ones retrieved from the database. Each alignment was checked manually, corrected and then analyzed using the Neighbour-Joining method (Saitou and Nei 1987) according to the model of Jukes-Cantor

distances. Phylogenetic trees were constructed using the MEGA 3 (Molecular Evolutionary Genetics Analysis) software (Kumar *et al*. 1993). The robustness of the inferred trees was evaluated by 500 bootstrap re-samplings.

5.3. Results and discussions

The DNA amplification and subsequent cloning procedure allowed constructing a library of 80 positive clones. After the ARDRA screening they were clustered into 8 OTUs and one representative clone was sequenced.

Among the 8 sequenced clones 3 were artefacts (after the CHECK-CHIMERA program) and the 5 others were related to uncultured bacteria belonging to the *Planctomycetes* phylum (Figure 5.2).

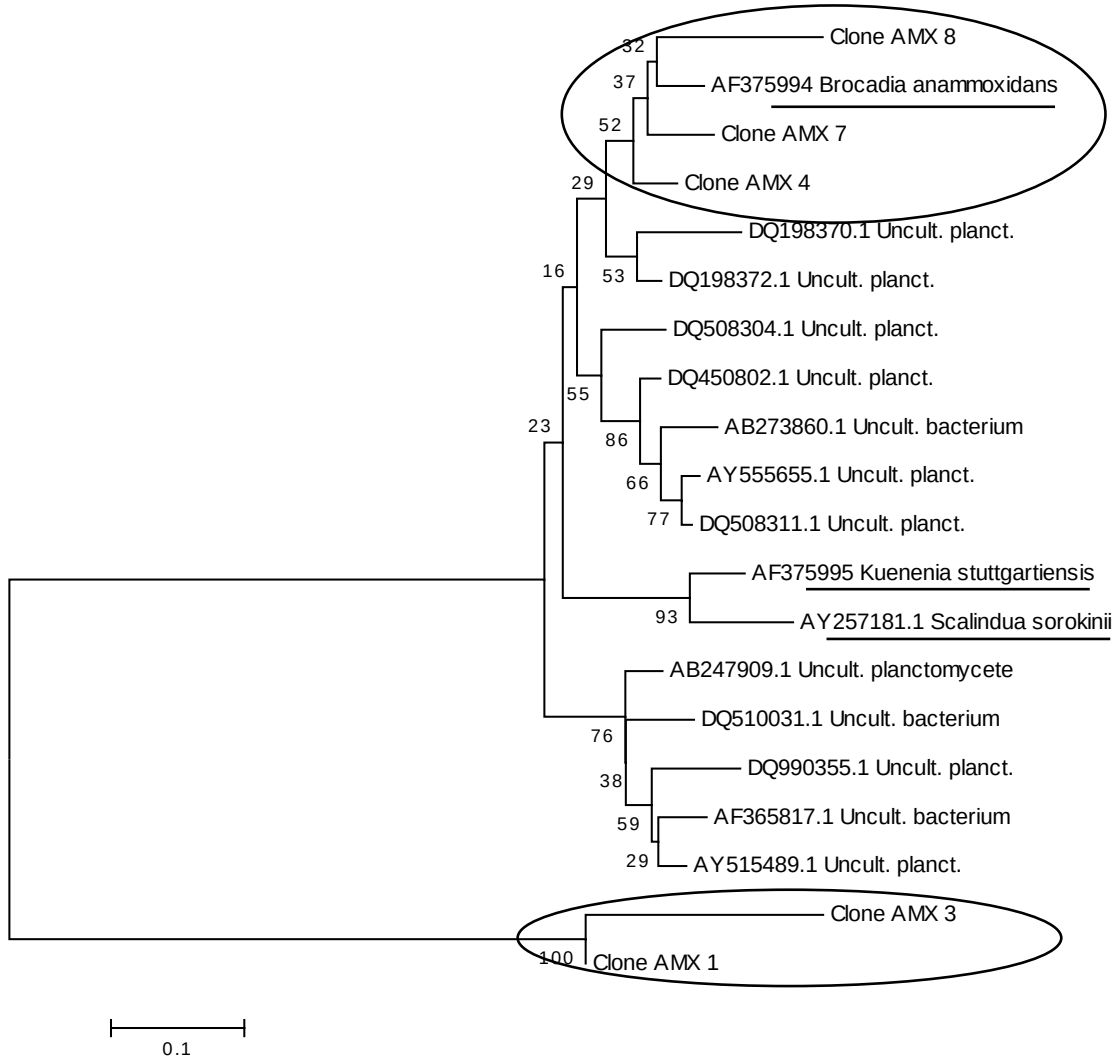

Figure 5.2: Phylogenetic tree of screened clones**.**

In particular, the OTUs AMX7 (12 clones), AMX8 (15 clones) and AMX4 (16 clones) presented a 92%, 90% and 93% similarity with the uncultured *Planctomycetes* (accession number AB247909) and 85 to 90% of similarity with the *Candidatus Brocadia anammoxidans* (accession number AF375994). Any clone was related to the two others known anammox bacteria (*Kuenenia stuttgartiensis* and *Scalindua* sp.). This could be due because of the Amx820 probe used for library screening is selective for a subgroup of anammox *Planctomycetales* (Tal *et al.*, 2006).

The two other OTUs, AMX3 (10 clones) and AMX1 (7 clones) were related to a not well specified uncultured soil bacterium clone L1A.12H03 (accession number AY989432).

5.4. Conclusions

Although the DNA homology between screened clones and the uncultured *Planctomycetes* found in the Basic Local Alignment Search Tool (BLAST) and the *Candidatus Brocadia anammoxidans* appeared low (under the 97% cut-off value for the species assignment, normally used), these findings strongly suggested that anammox bacteria are present in our biological filter, even if it is characterized by high oxygen concentrations (reaching 90-100% of the saturation).

The porous mineralized packing media used (Biogrog) can harbour many anoxic microniches where anaerobic microoorganisms, as denitrifiers, methanogen Archaea, sulphur bacteria and anammox can develop.

Results of present work are absolutely preliminary and must be confirmed and improved. A range of suitable methods is actually available for the detection and enumeration of anammox bacteria and their activity in natural and man-made ecosystems. For a proper evaluation of the contribution of the anammox process to nitrogen cycling in a particular system, the combination of different methods is crucial.

The search for anammox and for their utilization in biotechnological and industrial applications, as the association of their activity with the consortia of a denitrifying biofilter, is a promising field that can be useful to implement an environmentally sustainable land-based mariculture industry.

Although the real impact of anammox in aquaculture has not been well studied (Tal *et al.*, 2003), anammox process is a viable option for biological wastewater treatment

(Jetten *et al*., 1997, 1999; Strous *et al.*, 1997, 1999). Very recently, it was discovered that anammox makes a significant (up to 70%) contribution to nitrogen cycling in the World's oceans (Thamdrup and Dalsgaard, 2002).

The advantages of the anammox process over the traditional combination of nitrification and denitrification for wastewater treatment are lower oxygen demand, which is needed by nitrifiers for partial oxidation of ammonium to nitrite, and no requirement for organic carbon sources because the process is autotrophic (Egli *et al*., 2001).

CHAPTER 6

Dynamics of bacterial populations in a RAS

6.1. Introduction

In a RAS, the typical production loop produces high quantity of Particulate Organic Matter (POM), mainly constituted by fish feces, unconsumed feed and by bacterial biomass and bacterial colonized particles (Léonard *et al.*, 2001, 2002; Franco-Nava *et al.*, 2004a, 2004b). Because in intensive aquaculture the POM can cause many problems for the reared fish, by physically irritating gills, releasing toxic ammonia nitrogen or increasing the Biological Oxygen Demand (BOD), (Skjølstrup *et al.*, 1998; Kim *et al.* 2000; Franco-Nava *et al*., 2004a, 2004b), its removal is a main objective of virtually all recirculating treatment schemes. Several devices are currently used in RAS to reduce POM concentrations, as sedimentation and microscreens; they have proven effective for removal of larger particles but remain ineffective for fine solids of <50-40 μm (Losordo *et al.*, 1999b). The finer particles, that are not removed, can accumulate and constitute the majority of the organic solids within recirculating systems (Sharrer *et al.*, 2005).

As demonstrated by Léonard (2000) the POM represents the main available carbon source for heterotrophic bacterial metabolism in a marine RAS, where the dissolved carbon is mainly constituted by humic substances. Moreover, as demonstrated in the Chapter 3 of present Thesis, the C/N ratio strongly influences the biofilter biofilms in terms of abundance, activity and community composition. Thus, if the growth dynamics of every living population is linked to the environmental conditions and available carbon sources, the key factor for controlling the heterotrophic bacteria in a RAS is the management of POM, more than treatment such as ultraviolet light or ozone.

One of possible way to reduce the particles amounts in rearing water can be the introduction of a biological activator, based on a bacterial cocktail, as for instance AQUACEET LB/M (Laboratoires Ceetal, 42001 St. Etienne, France – www.ceetal.com).

The efficiency of such biological activator designed to improve the coagulation of the smallest particles into larger ones, in order to improve their removal from the water by the screen filter, must be investigated for aquaculture systems.

However, the remaining question is if the introduction of such amount of allochthonous bacteria can have an influence on the indigenous bacterial communities structure of the system and on the biofilter performances.

The objective of this work was to evaluate the effect of the AQUACEET LB/M® biological activator on the bacterial community abundance and structure in a RAS, by using T-RFLP fingerprinting technique both on the DNA and on the cDNA of the whole bacterial community.

6.2. Material and Methods

6.2.1 Experimental facilities

Two identical RAS were used for this experiment located at the IFREMER research station of Palavas les Flots (France). The experimental Recirculation Aquaculture Systems are detailed in the Chapter 4 (Material and Methods section, Figure 4.1 and Table 4.1). The first was subjected to the LB/M inocula, following the supplier instruction, and the second served as a control. Hereinafter, systems will be designed as "Blank" for the Control and "Treated" for the system receiving the inoculum.

The reared fish were sea bass (*Dicentrarchus labrax*) with an initial average weight of 160 ± 15g and a density of 22 kg/m^3. Fish were feed with commercial pellets Extra-Natura®, 4.0 mm (Le Gouessant, France), with an average composition of 45% protein, 1.04% phosphorus, 20 % fat, 2.4% crude fiber, 7.6% ash and 10% humidity. The R (replacement water/ supplied food) was kept at 1.0 ± 0.2 m^3/kg, the pH at 7.0-7.5, the temperature at 20 ± 1 °C and the dissolved oxygen at 90-100 % of the saturation.

6.2.2 Experimental procedures

The bacterial activator AQUACEET LB/M® tested in the experiment was supplied by the LABORATOIRES CEETAL. The bacterial composition remained confidential.

The activator was daily prepared following the supplier instructions: 5.6 ml of activator stock solution were diluted 100 times with sea water (0.2 μm filtered) and supplied with 0.56 g of the AQUACEET nutriment powder and 0.56 g of sucrose. This bacteria solution was incubated for 24h at 30°C with an air diffuser before using. As

suggested by the supplier, 8 ml of pre-activated solution, containing about 50 x 10^9 cells, were added every day in the storage tank of the system.

Water samples were collected weekly for the determination of both the chemical parameters and the bacterial abundance. Water samples were taken from inlet (BI) and the outlet (BO) of the biofilter of the two systems assuming that these two points were representative of the entire RAS (Table 6.1). At time 0 (corresponding to the day before the first LB/M inoculum) and at the end of the experiment, rearing water and biofilter packing media were sampled for bacterial abundance and community DNA/RNA extraction for T-RFLP.

Table 6.1: Rationale of experiment sampling.

Code	Sampling Points	Treatment Weeks						
		W1	**W2**	**W3**	**W4**	**W5**	**W6**	**W7**
PMB	Packing Media Blank	D/DNA						D/DNA
BIB	Biofilter Inlet Blank	D/DNA	D	D	D	D	D	D/DNA
BOB	Biofilter Outlet Blank	D/DNA	D	D	D	D	D	D/DNA
PMT	Packing Media Treated	D/DNA						D/DNA
BIT	Biofilter Inlet Treated	D/DNA	D	D	D	D	D	D/DNA
BOT	Biofilter Outlet Treated	D/DNA	D	D	D	D	D	D/DNA

Notes: W= experiment weeks; D= DAPI; DNA= DNA and RNA extraction

6.2.3 Collection and preliminary treatment of samples

Samples for chemical analyses and for bacterial abundance were collected as described in the Chapter 3 (Material and Methods section), while samples for DNA/RNA extraction for T-RFLP were collected as described in Chapter 4 (Material and Methods section).

6.2.4. Microbiological analyses

Bacterial abundance. Total bacteria abundance determination was performed as described in Chapter 3 (Material and Methods section).

Direct genomic DNA and RNA extraction. DNA and RNA extraction was carried out as described in Chapter 3 (Material and Methods section) by using the RNA/DNA extraction kit (Qiagen), following the manufacter's instructions.

RNA templates were treated with DNaseI (Invitrogen) according to the manufacturer's instructions, before performing the RT reaction. RNA was converted to cDNA using SuperScript(tm) II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. 16S rRNA was reverse-transcripted by using the universal primer 1492R (5′- GGTTACCTTGTTACGACTT -3′).

Genetic diversity of bacterial communities. Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu *et al.*, 1997; Moeseneder *et al.*, 1999; Dunbar *et al.*, 2001) was carried out as described in Chapter 3 (Material and Methods section).

6.2.5. Data analyses

Statistical analyses. Comparisons between groups for a significant difference of mean or rank values were performed after normality and variance tests. Data were analyzed with ANOVA on ranks (Krustal-Wallis method) and the relative importance of each treatment group was investigate by a Pairwise Multiple Comparison procedure (Dunn's method).

After the pretreatment and the alignment (as described in Chapter 3, Materials and Methods section) of the T-RFLP profiles, a non-metric MDS was computed using a Jaccard (presence/absence) similarity matrix.

Statistical calculations were performed by using SigmaStat software for Windows, version 3.1 (Copyright 1992-1995 Jandel Corporation).

Diversity indices. In order to evaluate structural diversity between samples, Shannon-Weaver diversity index (*H'*), Pielou Evenness (J'), Dominance (D) and the reciprocal of Simpson's index (1/*D*), were computed.

The PAST software (PAlaeontological STatistics, version 1.15, http://folk.uio.no/ohammer/past) was used to compute the diversity indices.

6.3. Results

6.3.1. Effect on rearing water chemical and physical parameters

During the experimentation period, water parameters (temperature, salinity, dissolved oxygen and pH) were maintained stable by the routine operations and the control devices (average values are reported in Table 4.1 of the Chapter 4).

The ammonium concentrations in the rearing water, at the inlet and at the outlet of the two biological filters, are plotted in Figure 6.1. Results showed any statistically significant differences (P>0.05) in the Total Ammonia Nitrogen removal rate between the two systems.

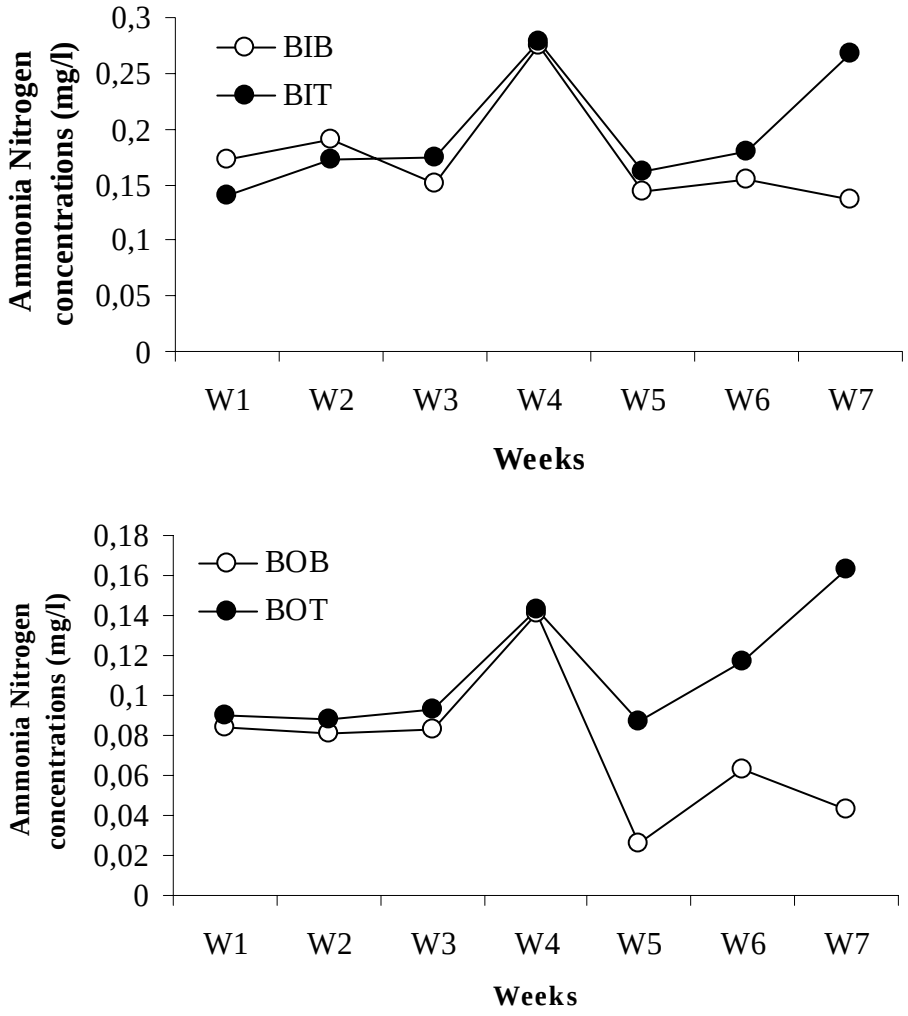

Figure 6.1: Ammonia Nitrogen evolution over time in the two rearing systems (BIB = Biofilter In "Blank"; BIT = Biofilter In "Treated"; BOB = Biofilter Out "Blank"; BOT = Biofilter Out "Treated".

Moreover, also the other chemicals concentration considered in this study (NO_2, NO_3 and PO_4), were not statistically significantly different between the two systems, as well as the solid removal rate from water (P>0.05) (data not shown).

6.3.2. Effect on the rearing water bacterial community abundance

The total bacterial abundance in the circulating water in the "Blank" system remained constant during all the experiment, with on average of 10.69 ± 1.98 x 10^5 and 10.11 ± 1.97 x 10^5 cells/ml for the inlet and the outlet of the biofilter, respectively (Figure 6.2). In the "Treated" system, during the first three weeks, the bacterial abundance was constant and comparable with the "Blank" (10.33 ± 1.41 x 10^5 cells/ml), but, from the week 4, the bacterial abundance increased to an average value of 16.70 ± 1.51 x 10^5 cells/ml.

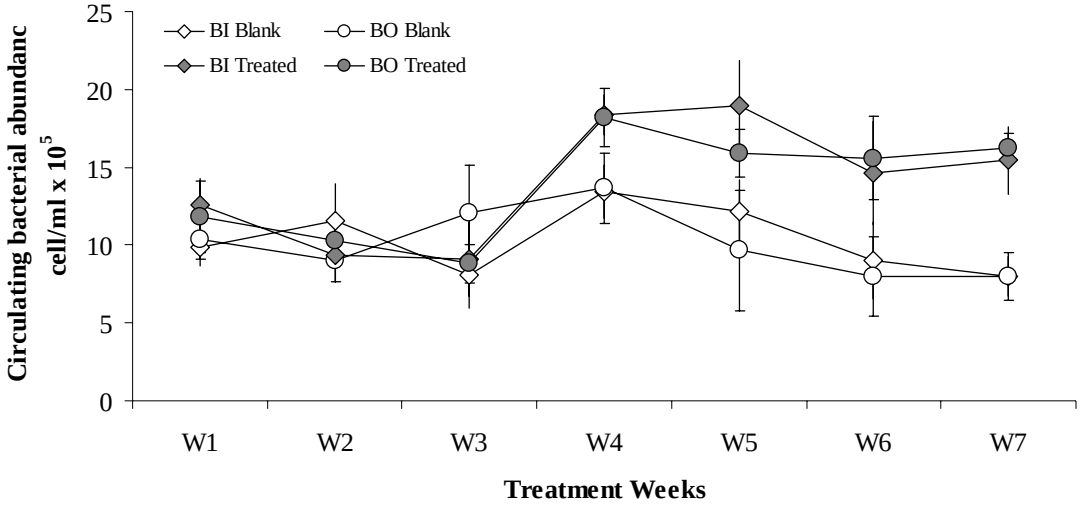

Figure 6.2: Bacterial abundance of Rearing Water for the two systems at the Biofilter Inlet (BI) and at the Biofilter Outlet (BO).

The One Way ANOVA analysis, firstly computed by comparing separately values week by week for the four sampling points (BIB, BOB, BIT and BOT), showed a statistically significant difference among the sampled weeks (P=<0.001), for both systems, even if the subsequent pairwise multiple comparison procedure (Student-Newman-Keuls method), revealed that only few pairwise comparisons, attaining the *P* value of <0.050, could be considered statistically significant different.

A second One Way ANOVA was computed between values for inlets and outlets of the two systems (BIB vs. BOB and BOB vs. BOT) week by week. Results showed a

statistically significant difference (P=<0.001) confirmed by the subsequent pairwise multiple comparison procedure (Student-Newman-Keuls method), only from the week 4 to the week 7 (end of the experiment).

6.3.3. Effect on the packing medium biofilm abundance

The bacterial abundance in the biofilms attached on the biofilter packing medium (Figure 6.3) ranged, for the "Blank" system, from 3.69 x 10^7 ± 7.78 x 10^6 at the beginning of the experiment (W1) to 3.33 x 10^7 ± 3.41 x 10^6 at the end of the experiment (W7).

The abundance inside the biofilter of the "Treated" system increased from 3.31 x 10^7 ± 4.50 x 10^6 (W1) to 2.13 x 10^8 ± 8.76 x 10^7 at the end of the experiment (W7).

There was a statistically significant difference between samples (ANOVA on Ranks, P=<0.001) and the Dunn's multiple comparison pointed out that only the bacterial abundance inside the biofilter at W7 was statistically different from all others (P<0.05).

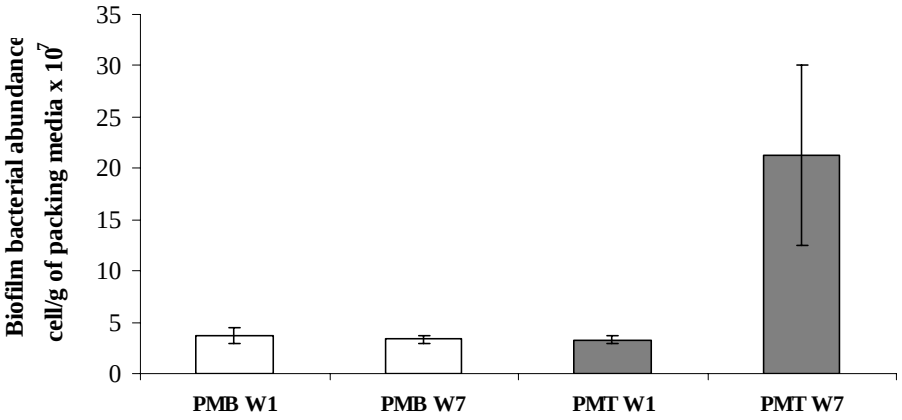

Figure 6.3: Bacterial abundance of biofilms fixed on the Packing Media (PM)of the two systems (B = "Blank"; T = "Treated") at the beginning (W1) and at the end (W7) of the experiment.

6.3.4. Effect on the rearing water bacterial community composition and structure

The T-RFLP analysis showed that the planktonic community composition of the "Treated" system was subjected to a more marked evolution than the "Blank" one (Figure 6.4).

Diversity indices computed for the relative abundance of each T-RF were quite similar in all samples (Table 6.2), suggesting that the planktonic community structure did not vary over time (in "Blank") and with the bacterial inoculum (in "Treated").

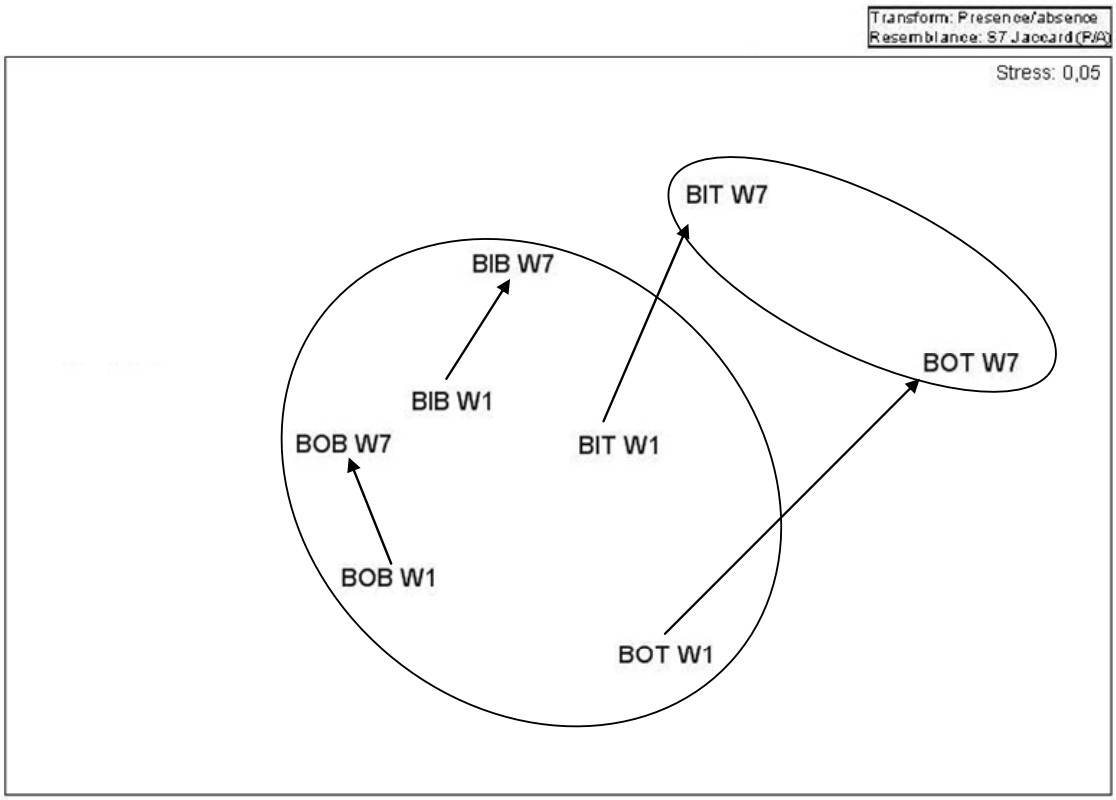

Figure 6.4: Non-metric multidimensional scale analysis (nMDS) for the T-RFLP patterns for the Rearing Water samples (BIB W1 = Biofilter In "Blank", week 1; BIB W7 = Biofilter In "Blank", week 7; BIT W1 = Biofilter In "Treated", week 1; BIT W7 = Biofilter In "Treated", week 7; BOB W1 = Biofilter Out "Blank", week 1; BOB W7 = Biofilter Out "Blank", week 7; BOT W1 = Biofilter Out "Treated", week 1; BOT W7 = Biofilter Out "Treated", week 7).

Table 6.2: Diversity indices of T-RFLPs.

	W1				**W7**			
	BIB	**BOB**	**BIT**	**BOT**	**BIB**	**BOB**	**BIT**	**BOT**
n° T-RF	47	45	52	34	40	45	53	47
Dominance D	0.042	0.045	0.033	0.053	0.050	0.035	0.036	0.037
Pielou Evenness J'	0.88	0.87	0.88	0.88	0.86	0.92	0.89	0.90
Shannon H'	3.39	3.32	3.51	3.10	3.19	3.5	3.56	3.50
Simpson 1/D	23.76	22.13	26.46	18.73	19.89	28.02	27.32	26.64

BIB W1 = Biofilter In "Blank", week 1 — BIB W7 = Biofilter In "Blank", week 7
BOB W1 = Biofilter Out "Blank", week 1 — BOB W7 = Biofilter Out "Blank", week 7
BIT W1 = Biofilter In "Treated", week 1 — BIT W7 = Biofilter In "Treated", week 7
BOT W1 = Biofilter Out "Treated", week 1 — BOT W7 = Biofilter Out "Treated", week 7

6.3.5. Effect on the packing medium biofilm community structure

The study of the biofilm structure was carried out both on the community DNA and RNA, in order to assess eventual changes in the whole and in the metabolically active bacterial community structure.

The nMDS of DNA (Figure 6.5) and cDNA (Figure 6.6) of the T-RFLP results, showed that, at time zero the two communities were comparable in T-RFs composition, both for total DNA and for the active fraction of the community. The two biofilter

communities composition evolved in two separate directions over experimentation weeks, suggesting that the bacterial activator strongly influenced the biofilter biofilms.

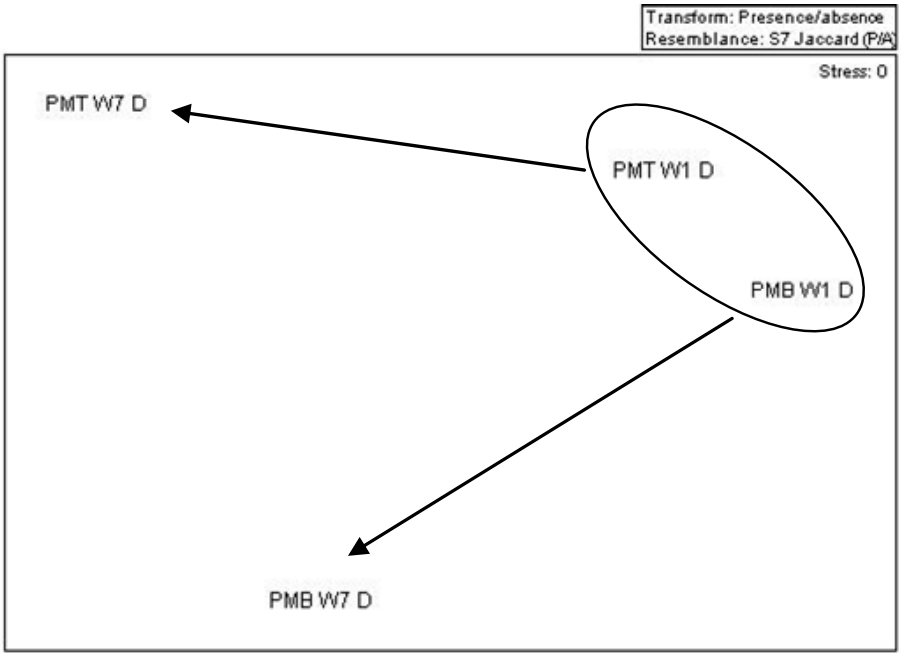

Figure 6.5: Non-metric multidimensional scale analysis (nMDS) for the T-RFLP patterns for the DNA Packing Media samples (PMB W1 D = Packing Media "Blank", Week 1, DNA; PMT W1 D = Packing Media "Treated", Week 1, DNA; PMB W7 D = Packing Media "Blank", Week 7, DNA; PMT W7 D = Packing Media "Treated", Week 7, DNA.

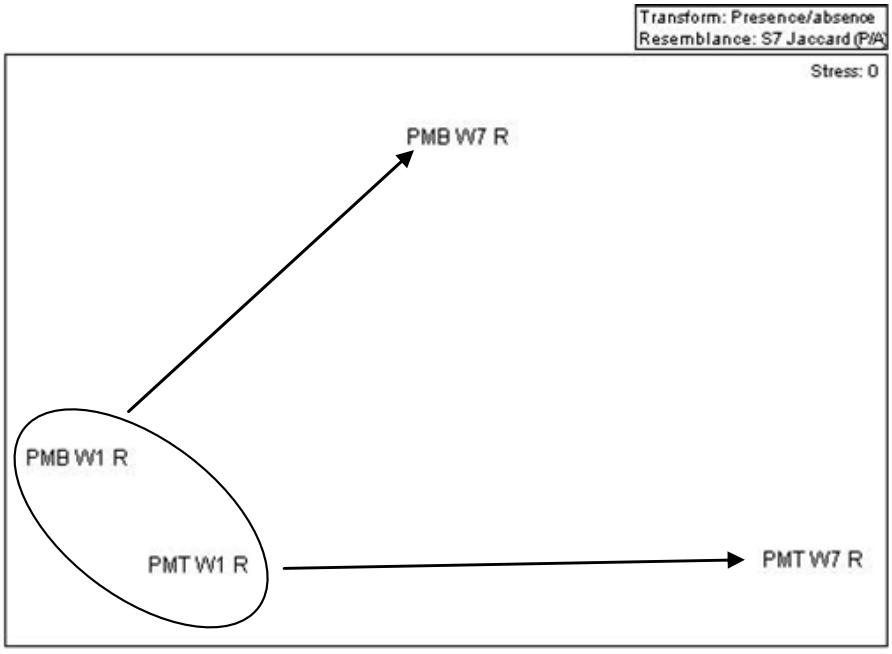

Figure 6.6 Non-metric multidimensional scale analysis (nMDS) for the T-RFLP patterns for the cDNA Packing Media samples (PMB W1 R = Packing Media "Blank", Week 1, RNA; PMT W1 R = Packing Media "Treated", Week 1, RNA; PMB W7 R = Packing Media "Blank", Week 7, RNA; PMT W7 R = Packing Media "Treated", Week 7, RNA.

Moreover, in both systems, the T-RFs richness increased over time as well as the Shannon index. The Simpson reciprocal index increased in all the samples, excepted for the "Treated" metabolically active fraction of the community, where also the Pielou index significantly decreased (Table 6.3).

Table 6.3: Diversity indices of T-RFLPs for biofilter biofilm DNA (D) and cDNA (R).

	PMB W1 D	**PMB W7 D**	**PMT W1 D**	**PMT W7 D**
n° T-RF	45	64	52	66
Dominance D	0.037	0.030	0.038	0.028
Pielou Evenness J'	0.911	0.906	0.92	0.89
Shannon H'	3.47	3.77	3.49	3.77
Simpson 1/D	33.87	33.01	35.44	35.81
	PMB W1 R	**PMB W7 R**	**PMT W1 R**	**PMT W7 R**
n° T-RF	44	67	41	65
Dominance D	0.03	0.03	0.03	0.04
Pielou Evenness J'	0.96	0.93	0.96	0.87
Shannon H'	3.62	3.89	3.58	3.64
Simpson 1/D	32.82	39.86	32.02	35.71

PMB W1 D = Packing Media "Blank", Week 1, DNA
PMB W7 D = Packing Media "Blank", Week 7, DNA
PMT W1 D = Packing Media "Treated", Week 1, DNA
PMT W7 D = Packing Media "Treated", Week 7, DNA
PMB W1 R = Packing Media "Blank", Week 1, RNA
PMB W7 R = Packing Media "Blank", Week 7, RNA
PMT W1 R = Packing Media "Treated", Week 1, RNA
PMT W7 R = Packing Media "Treated", Week 7, RNA

6.5. Discussion

The bacterial activator used in this work did not clearly show the expected effects on the suspended solid concentration in water. The chemical parameters monitored were quite constant during the entire experiment and additionally they were similar between the two rearing systems, suggesting that any negative or positive effect on biofilter performance can be ascribed to the LB/M activator.

Bacterial abundances in the rearing water remained stable over experimental weeks in the "Blank" system, confirming the results obtained by Léonard (2000) on the stability of the bacterial populations abundances in a marine RAS. On the contrary, in the "Treated" system, the planktonic bacterial abundances were stable and comparable with the "Blank" one, only for the three first weeks. Then, bacterial abundance increased, becoming statistically different between the two rearing systems. Moreover, as shown in Figure 6.3, in the biofilms of the two systems the bacterial abundance found at the beginning of the experiment was of 10^7, comparable with values reported in Chapter 4 and by Léonard *et al.*, (2001, 2002), and remained stable only within the

"Blank" biofilter. In the "Treated" biofilter it increases to 10^8, indicating an over-colonization of the packing media by the inoculum bacteria.

The moderate increasing of bacterial abundance in "Treated" rearing water could be ascribed to an increasing of the bacteria released from the biofilter, even if such increasing appeared not to be progressive. In Chapter 3 it was demonstrated that there was a relation between total bacterial biomass attached on the packing media and free-living bacteria sheared from the filters (Figure 3.7).

The T-RFLP results carried out on the rearing water bacteria of the "Blank" system indicated a stability in the community composition, while the "Treated" one was subjected to a moderate shift, suggesting a possible influence of bacterial inoculum. Moreover, the diversity indices computed, as well as the number of T-RFs, remained comparable over time. Shannon and Simpson reciprocal diversity index, ranging between 3.19 and 3.56, and from 18.73 and 28.02, fell within the range found in a variety of ecosystems for other organisms (Schauer *et al*., 2000). Values of the Simpson reciprocal index (1/D) and the Pielou one, suggested that the two communities were characterized by a low equitability.

The low impact of the AQUACEET LB/M® activator on the planktonic bacterial communities was probably due to the constant water renewal process (1/6 of the total volume *per* day on average), that allows the ingoing of bacteria from natural environment. As a consequence, the introduction of allochthonous bacteria was not able to produce a significant change in the structure of the autochthonous planktonic bacterial community. This hypothesis may be tested by using lower water renewal rates.

If the rearing water communities were quite stables and only scarcely affected by the AQUACEET LB/M® activator, the two biofilter biofilm were subjected to a marked shift over time, in term of composition. However such evolution appeared both in the "Blank" and in the "Treated", it followed two completely divergent directions. Thus, such changes cannot be unambiguously linked to the addition of the biological activator, as we do not have data on the potential natural divergence of bacterial community in biofilters. This point is a very critical one, and outlines the necessity to design further experiments that include replication at the biofilm level.

The biofilms were more dynamic than rearing water communities, changing their composition over time, both for total DNA and cDNA (active flora). The community structure of the "Blank" system, appeared quite stable, as suggested by the diversity

indices computed, and characterized by a high values of Pielou index, close to the maximum equitability. On the contrary, the “Treated” biofilm shifted toward a more dominated structure, as suggested by the decreasing of the Pielou index.

The shift of the bacterial community associated to biofilms could be linked with a normal evolution of filters, related to the change of environmental conditions, determined by the increasing of the fish biomass and on the accumulation of chemical substances in the rearing water during the rearing cycle. Unfortunately, no data are available to understand if the registered shift in community composition can be considered cyclical.

To summarize, this study do not allowed to conclude to a significant effect of the tested activator for the used experimental conditions. To better understand and parameterize the “buffering” capacities of the RAS system, further experiments are needed. These buffering capacities are mainly based on the equilibrium between processes (e.g., growth and grazing), which may regulate the bacterial population abundances and their activities. One very important forcing function of this equilibrium is the rate of water renewal, whose effects need to be further explored. The value used for this experiment appeared in line with these normally used (Léonard *et al.*, 2001, 2002). One can predict that decreasing this water renewal rate could lead to a greater influence of added activator, while increasing this rate in a large way will conduct to a null effect.

CHAPTER 7

Conceptual model of the heterotrophic bacterial compartment in a marine RAS biofilter

7.1. Introduction

One of the major goals in the future development of the recirculating aquaculture systems is the definition of a general mathematical model, describing the main biological and technical processes occurring inside it, in order to help engineers and producers to make predictions on the design and management of facilities operated in various situations in terms of fish species or technical-economic environment (Franco-Nava, 2003).

As suggested by Franco-Nava (2003) a model describing the functioning of a RAS may be composed by five sub-models: 1) Fish; 2) Dissolved Matter 3) Particulate Matter; 4) Nitrification and Autotrophic nitrifying bacteria and 5) heterotrophic Bacteria; (Figure 7.1).

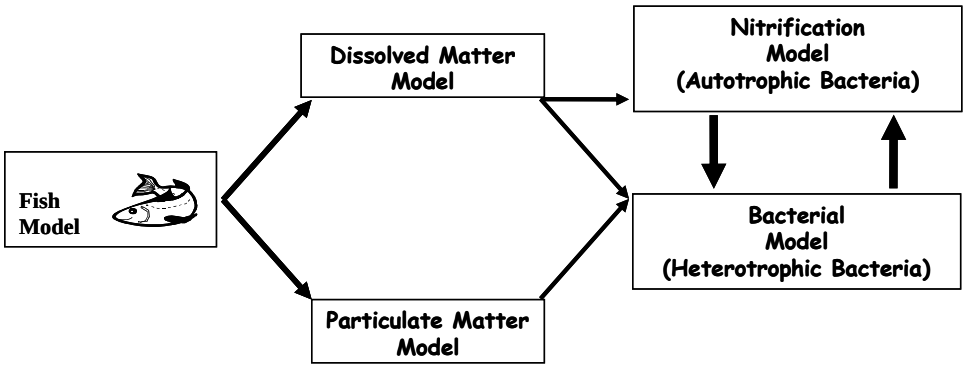

Figure 7.1: RAS sub-models.

Nowadays, the fish growth model is well known as well as the dissolved matter one (Pagand, 2000); the Particulate Matter model was described by Franco-Nava (2003), but it has to be calibrated and validated. The Nitrification has been widely investigated and many models already exist (Zhu and Chen, 2001a; 2001b). Finally, the conceptual basis of the heterotrophic bacterial model were proposed by Leonard (2000) and Quezada Jimenez (2001).

In aquaculture, microbiology has become increasingly important as production systems became more intensive and sophisticated. Today, it is well recognized that the

bacterial compartment is extremely important for the success and the sustainability of a RAS.

If the microbiology of nitrification has been widely investigated and related models have been described (Chen *et al.*, 2006), processes and dynamics of heterotrophic bacteria in recirculating aquaculture received much less attention.

However, the control over heterotrophic bacteria is essential to improve the productivity and the economic viability of intensive aquaculture. Any producer should manage his system in order to establish an environment suitable not only for the growth of reared fish, but also for maintaining a stable and active microbial flora.

Initially, microbiological researches in aquaculture were exclusively focused on the understanding of deleterious effects caused by pathogens and on the use of antibiotics to control bacteria. Recently, new approaches have been proposed, taking into account some principles of microbial ecology and current knowledge on the interactions among bacteria, and between bacteria and reared fish.

The aim of present work was to better describe different aspects of a conceptual model for the simulation of the dynamics of heterotrophic bacterial populations in a marine RAS, taking into account their main controlling factors. This chapter will integrate existing data (Léonard, 2000) with data obtained during the present Thesis work.

7.2. Model description

In Figure 7.2 is proposed a conceptual diagram of the principal processes linking heterotrophic bacteria (free and attached) and organic carbon sources (dissolved and particulate organic carbon) in a biological filter, based on the previous model described by Léonard (2000). The state variables and the main processes that can affect their dynamics are listed in Table 7.1 and 7.2, respectively.

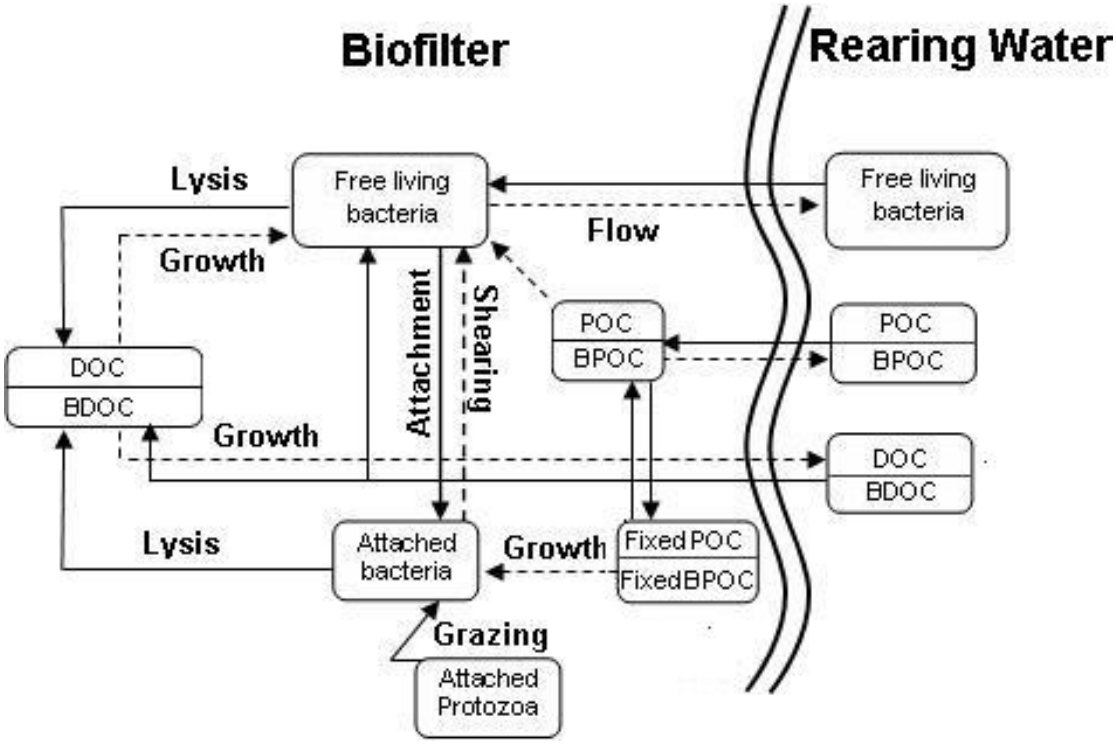

Figure 7.2: Conceptual diagram of the heterotrophic bacteria model.

Table 7.1: State variables

Ingoing the Biological Filter	**Symbol**	**Units**
Free bacteria in the Raring Water	FB_{RW}	cell l^{-1}
POC in the Rearing Water	POC_{RW}	g l^{-1}
POC biodegradable in the Rearing Water	$BPOC_{RW}$	g l^{-1}
DOC in the Rearing Water	DOC_{RW}	g l^{-1}
DOC biodegradable in the Rearing Water	$BDOC_{RW}$	g l^{-1}
Inside the Biological Filter	**Symbol**	**Units**
Free bacteria in the biological filter	FB_{BF}	cells l^{-1}
Attached bacteria in the biological filter	AB_{BF}	cells g^{-1}
Attached grazers in the biological filter	AG_{BF}	cells g^{-1}
POC in the Biological Filter	POC_{BF}	g l^{-1}
POC biodegradable in the Biological Filter	$BPOC_{BF}$	g l^{-1}
DOC in the Biological Filter	DOC_{BF}	g l^{-1}
DOC biodegradable in the Biological Filter	$BDOC_{BF}$	g l^{-1}
POC fixed on packing media	$APOC_{BF}$	g cm^{-2}
POC biodegradable fixed on packing media	$ABPOC_{BF}$	g cm^{-2}

Table 7.2: Main processes affecting the state varaible dynamics

Free Bacteria in the Rearing Water → $\delta FB_{RW}/\delta t$ = Growth + Ingoing (make-up water and biofilter shearing) – Outgoing (entering the biofilter) – Mortality;
POC in the Rearing Water → $\delta POC_{RW}/\delta t$ = Production (including biofilter shearing) –Elimination (mechanical filtration) – Ingoing the Biofilter –Dilution (make-up water);
Biodegradable POC in the Rearing Water → $\delta BPOC_{RW}/\delta t$ = Production (including biofilter shearing) – Utilization – Elimination (mechanical filtration) – Ingoing the Biofilter – Dilution (make-up water);
DOC in the Rearing Water → $\delta DOC_{RW}/\delta t$ = Production – Utilization – Dilution (make-up water);
Biodegradable DOC in the Rearing Water → $\delta BDOC_{RW}/\delta t$ = Production – Utilization – Dilution (make-up water);
Free Bacteria in the Biofilter → $\delta FB_{BF}/\delta t$ = Ingoing + Shearing + Growth – Attachment – Mortality – Outgoing;
Attached Bacteria in the Biofilter → $\delta AB_{BF}/\delta t$ = Attachment + Growth – Shearing – Grazing – Mortality;
Attached Grazers in the Biofilter → $\delta GB_{BF}/\delta t$ = Attachment + Growth – Shearing – Mortality;
POC in the Biofilter → $\delta POC_{BF}/\delta t$ = Ingoing + Shearing – Attachment – Outgoing;
Biodegradable POC in the Biofilter → $\delta BPOC_{BF}/\delta t$ = Ingoing + Shearing – Attachment – Utilization – Outgoing;
DOC in the Biofilter → $\delta DOC_{BF}/\delta t$ = Ingoing + Production – Utilization – Outgoing;
Biodegradable DOC in the Biofilter → $\delta BDOC_{BF}/\delta t$ = Ingoing + Production –Utilization – Outgoing;
POC fixed on the Biofilter packing media → $\delta APOC_{BF}/\delta t$ = Attachment – Shearing;
Biodegradable POC in the Biofilter packing media → $\delta ABPOC_{BF}/\delta t$ = Attachment - Shearing – Utilization;

The forcing variables of the model are the replacement water (NW, $m^3\ d^{-1}$), the fish feeding (Q_A, kg d^{-1}) and the fish feed conversion yield (IC, unitless) that are dependent to the reared fish.

Hereafter, we compile and partly complete the existing knowledge on these state variables and processes related to their dynamics in the two environmental compartments that constituted the water and biofilm subsystems and on their interactions.

7.2.1. Water subsystem

The circulating water flow (m^3 d^{-1}) carries large amounts of POC and DOC deriving from fish metabolism, but also planktonic bacteria.

POC and DOC. The quantity of the organic matter in the rearing system mainly depends on the quantity of feed distributed, fish feed conversion index, mechanical filtration efficiency. It was estimated that between 3 to 6 g of dissolved organic carbon per kg of fish feed and 30 to 54 g of POC per kg feed are released into the rearing water and available for the heterotrophic bacteria growth (Léonard, 2000).

Léonard *et al.* (2002) found that, in a marine RAS, cultivable heterotrophic bacterial population abundance was not correlated with the concentrations of dissolved carbon and demonstrated that the dissolved organic carbon (DOC) pool was principally composed by humic substances (HS) not easily degradable. They described the relationship between HS, DOC and system closure (R-ratio, water replacement/food supplied) as:

$$HS/DOC = 0.103 \times R + 0.50$$

for R<5; for R>5 all DOC was entirely composed by HS and HS/DOC=1. Moreover, the biodegradable DOC fraction (BDOC) can be estimated by the equation:

$$BDOC = -0.32 \times R^2 + 0.269 \times R + 2.04$$

for R<5; for R>5 BDOC = 0.5 (Léonard *et al.*, 2002).

Moreover, Léonard *et al.* (2002) suggested that heterotrophic bacteria mainly use for their metabolism the particulate organic matter (POM) because in RAS the DOC is mainly constituted by humic substances. Franco-Nava (2003) reported that in marine RAS, the POM contained on average 175 mg of carbon per gram. The biodegradable fraction of POC remains to be investigated.

Bacteria. Total bacterial abundance in the circulating water was evaluated to be around 10^5 cell/ml, while cultivable abundance was lower (10^4 CFU/ml) (see Chapters 4 and 6). Both the total and cultivable bacterial cell abundances were shown to increase

when the amount of particulate carbon increased, but not in the same way, leading to the hypothesis that increasing the organic carbon concentration both allowed the growth of planktonic bacteria and increased the proportion of active cells in the heterotrophic bacterial community.

However, in the circulating water of a RAS, planktonic bacteria are frequently exposed to UV treatment just before the biofilter inlet. The cultivable bacteria abundance falls down from 10^5 to 10^3 CFU/ml from the inlet and the outlet of the UV device, but the DAPI counts remain stable at 10^5 cell/ml. After few meter of water run, at the biofilter inlet, the cultivable bacteria are again 10^5 cell/ml, suggesting that the bacteria are simply inactivated by the UV exposure and able to reactivate in a very short time.

7.2.2. Biofilm subsystem

van Loosdrecht *et al.* (1995) suggested a conceptual model where the biofilm structure depends on the interactions between hydraulics and substrate surface load. At low substrate loads and under high shearing conditions, the biofilm becomes patchy due to detachment and erosion. At high substrate loads and under low shearing forces, the biofilm becomes porous, heterogeneous and may easily slough. The growth of the biofilm is known to be balanced by erosion caused by shear stress (Kugaprasatham *et al.*, 1992).

Until recently it was generally assumed that water only flows above the biofilm and that dissolved nutrients moves through the biofilm matrix by diffusion. However, the biofilm is an open structure with water flowing through the matrix and it presents a much more dynamic situation. Internal water flow within the biofilm implies that the influence of hydrodynamic drag on the detachment of individual cell clusters should be considered, in addition to the fluid shear stress (Horn and Hempel, 1997, 1997b)

POC fixation. The POC entering the system can be captured by the packing media and, as demonstrated in Chapter 3, cause a loss of nitrification efficiency (Figure 7.3). Such amount of ingoing carbon promotes the growth of heterotrophic biomass (Figure 7.4), which over-colonizes the biofilms, reducing the ammonia and oxygen diffusion in the biofilm matrix.

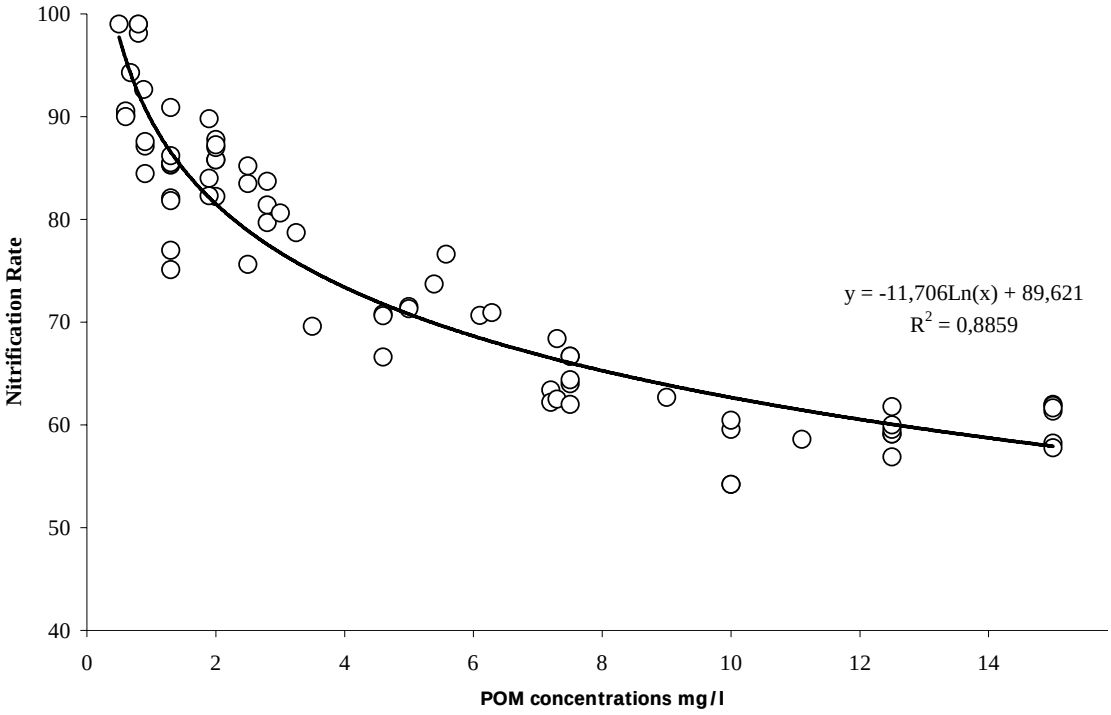

Figure 7.3: Effect of Ingoing POM on the nitrification rate.

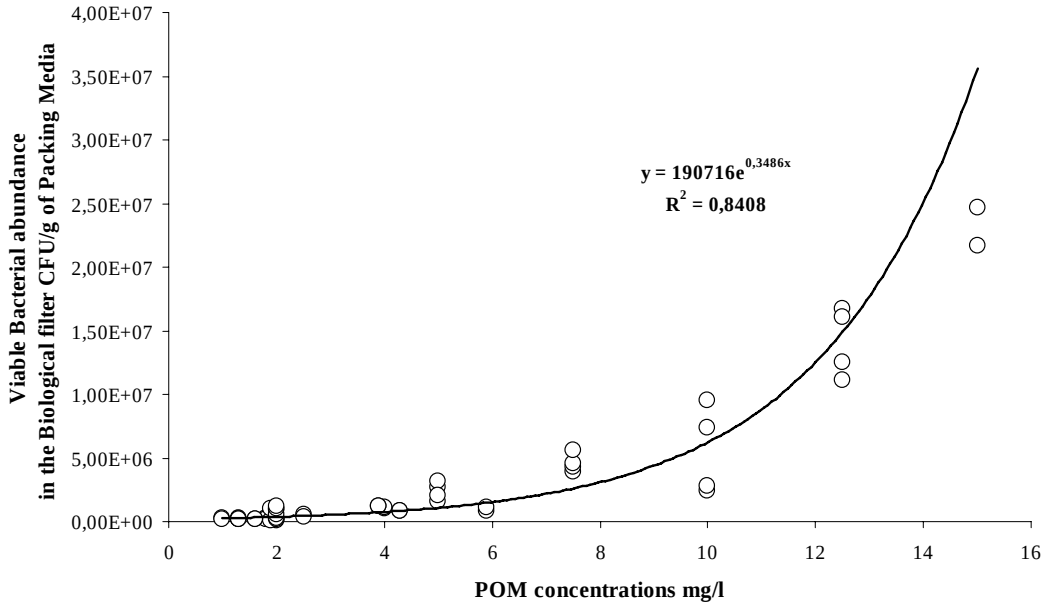

Figure 7.4: Relationship between suspended solids and packing media associated cultivable bacteria.

Bacteria shearing and fixation processes. Biofilms are dynamic structures with attaching and detaching cells by the erosion due to the water flow and the grazing activity (Morgenroth and Wilderer, 2000; Watnik and Kolter, 2000; Gheewala *et al.*, 2004; Mitchell *et al.*, 2004).

The biofilm bacteria are linked to the free bacteria of the recirculating water by the shearing and fixation processes. Léonard (2000), for a fish production system, reported shearing coefficient (Kshear) values between 0.6 and 0.9 h^{-1}, for water flow rates comprised between 0.006 and 0.09 m/s, and fixation coefficient (Kfix) values of 20 h^{-1} and 40 h^{-1} for water flow rates of 0.005 and 0.06 m/s. In particular, the Kfix was linked to water velocity by a logarithmic law (Léonard, 2000).

In addition, during operational routines and biofilter backwash, large particles of biofilm are certainly detached by the action of the water flow. Battin *et al.* (2003) showed that the flow significantly affected the biofilm development, yielding higher biomass under slower flows.

Biofilm bacteria growth. To our knowledge, no data are available in literature concerning the growth rates of biofilm bacteria in a marine RAS.

In order to assess the bacterial growth for such biofilms, two batch experiments were set up in duplicate: one series with a supplied C/N = 0 (C/N0) nutrients and the second with a C/N = 1 (C/N1). For each batch, 4 l of prefiltered (Whatman, GF/F Galss Microfibre) sterile seawater were aliquotated with 4 mg/l of NH_4 for C/N0, while for C/N1 4 mg/l of the same particulate organic carbon used for the experiments described in Chapter 3 (Material and Methods section) were added. 30 units of precolonized packing medium, aseptically collected from a biofilter, were put in the batches and incubated at 25°C in the dark for 10 days. Air (0.22 µm filtered) was blown in the batches to maintain oxygen concentrations close to saturation. At each sampling points, one packing medium subunit was sampled from each of the four batches and treated as described in Chapter 3 (Material and Methods section). Aliquots of the detachment buffer were serially diluted and used for Colony Forming Units determination over time.

In Figure 7.5 results of biofilm growth curves in batch experiments are plotted. Biofilms reached 10^6 CFU/g and 10^7 CFU/g after 120 hours of incubation for C/N0 and C/N1, respectively. The carbon supply allowed C/N1 biofilms to produce cell

abundances more rapidly (μ = 2.27 day^{-1}) than C/N0 (μ = 0.904 day^{-1}). The C/N1 biofilms reached the stationary phase in 48 h consuming 77 % of TOC, while C/N0 biofilms began the stationary phase after 96 h incubation, consuming 70 % of TOC (Figure 7.6).

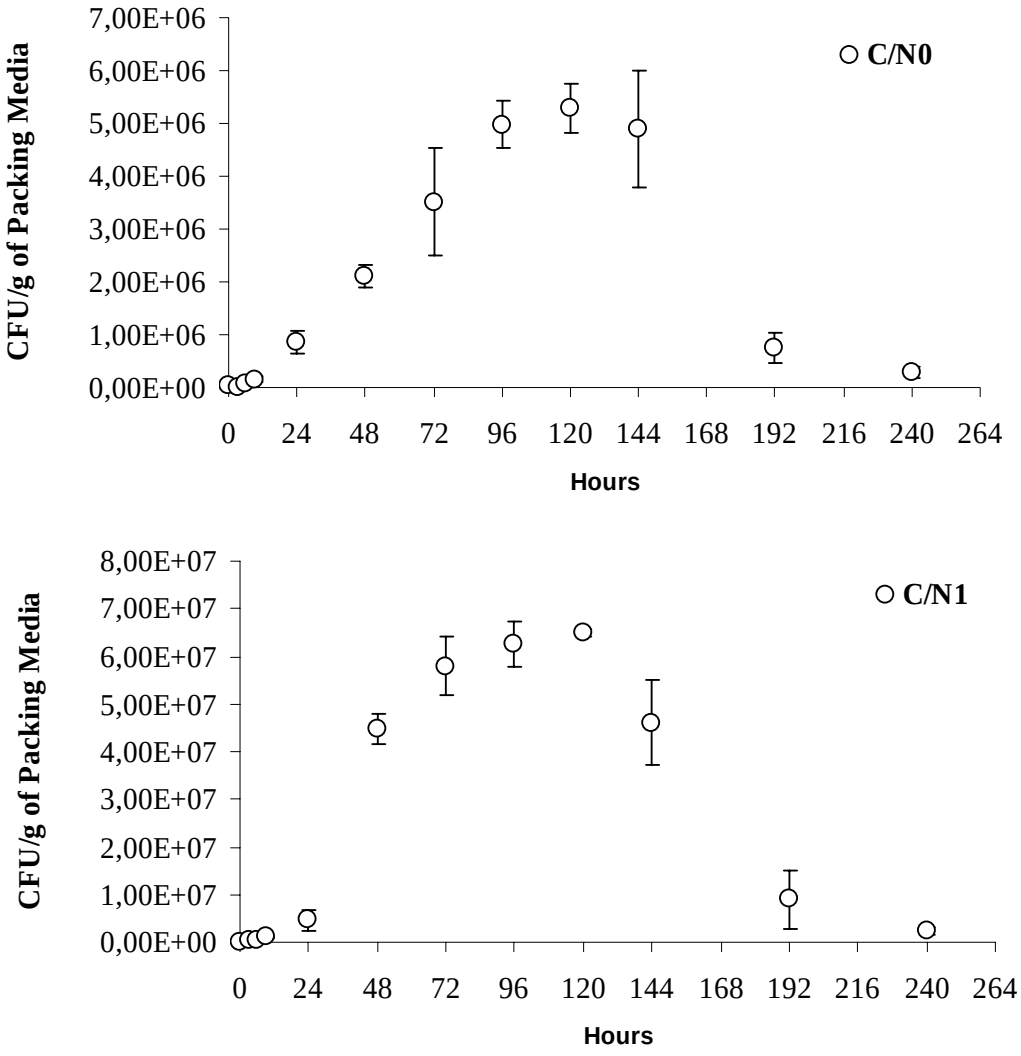

Figure 7.5: Biofilm bacteria growth curves in batch for C/N ratio enrichment of 0 and 1.

In this batch experiment, the maximum bacterial abundance reached was higher than abundances evaluated both in the pilot scale biofilters used for C/N ratio experiments (Chapter 4) that ranged between 10^4 and 10^6 CFU/g of packing media, and for the 0.7 m^3 biofilter used for the community characterization (Chapter 5), where the cultivable bacteria were 10^6 CFU/g of packing media. This strongly suggests that the water flow

significantly contributes to reduce the biofilm biomass and that biofilm growth model should take into account the sharing stress, that varies with the biofilter clogging and

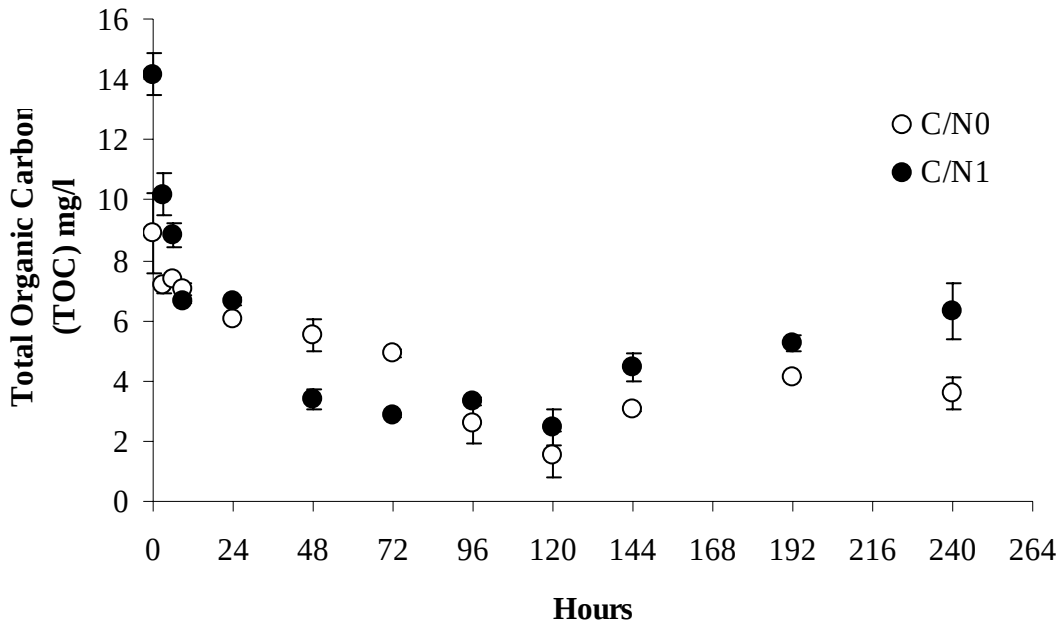

Figure 7.6: Evolution over time of Total Organic Carbon in batches.

subsequent backwash procedures.

Biofilm bacteria grazing. Another aspect to be considered in the bacterial dynamic control is the loss of biomass caused by grazing.

Grazing of biofilms is poorly understood, but an often published opinion is that the biofilm phenotype provides protection against predation (Huws *et al.,* 2005). Pederson (cited in Huws *et al.,* 2005), stated that the effects of protozoan grazing on the biofilm community are probably the most important factors controlling biofilm composition in aquatic ecosystems.

Biological processes such as protozoan grazing can result in the continual detachment of biofilm bacteria adding as a controlling or limiting factor to biofilm accumulation (Block *et al.,* 1997). Several authors have also pointed out the importance of predator/prey relationships to control biofilm communities (Hahn and Höfle, 1999, 2001).

Bomo *et al.* (2003) indicated that biological wastewater filters are dynamic systems and that biological factors (i.e., predation by the indigenous protozoa) can be of major importance in these systems. Stevik *et al.* (1998, 1999) found an increase in bacterial removal efficiency in the upper zones of biological filters, possibly as a result of protozoan predation.

The grazing activity on planktonic bacteria inside the biofilter can be considered quite negligible due to the low retention time of water in the biological filter (some minutes). On the contrary, grazing should be taken into account for modeling the growth and decay of the bacteria attached to the packing medium.

A preliminary direct microscopic observation was carried out in order to describe the main taxonomic groups of animals associated to the biofilm.

For this purpose, packing medium subunits were collected in triplicate from the biofilter, and washed three times with the detaching buffer (Chapter 3), by a gentle manual shaking. An aliquot of the obtained suspension was fixed with Lugol solution and stored at 4°C in the dark, until processing. Fixed samples were analyzed by using the Utermöhl technique (Carrias *et al.*, 1996). A volume of 100 ml of sample was settled at least for 24 h in a plankton chamber at 4°C in the dark and then visualized under a inverted microscope (Zeiss) at 250X in order to enumerate the grazers organisms.

The results are plotted in Figure 7.7. They revealed that nematodes represented the 24 % of observed "grazers" followed by anellidae (20 %) and ciliates (16.6 %).

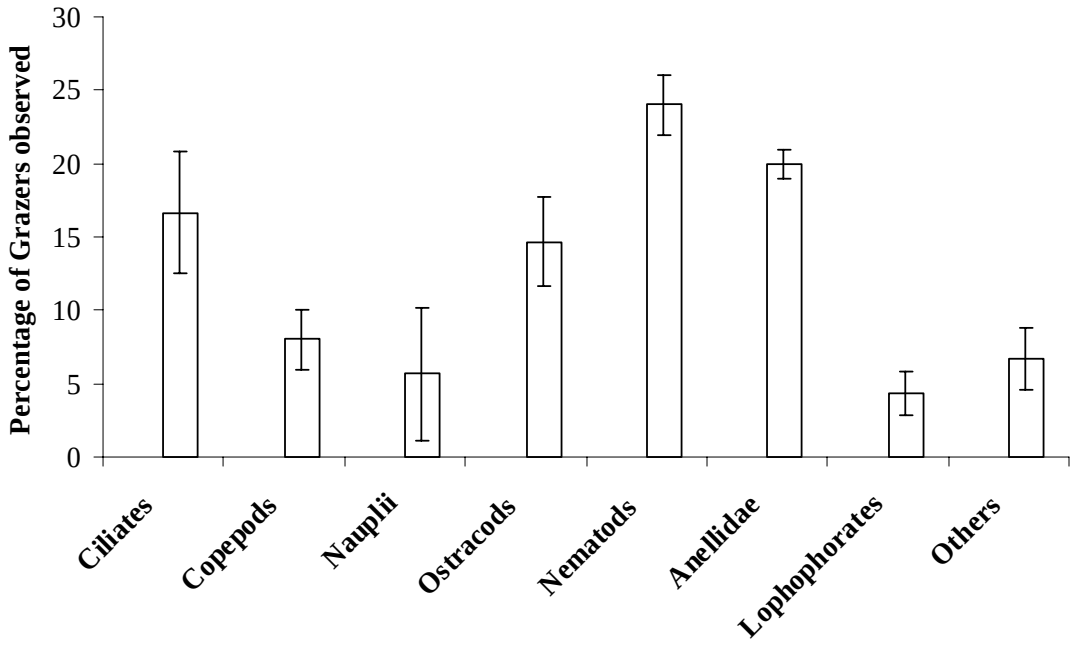

Figure 7.7: Percentage of grazers associated to the biofilter packing media.

Protists and metazoa are known to be important consumers of bacteria in wastewater purification systems, such as activated sludge plants, rotating biological contactors or percolating filters (Fried *et al.*, 2000). It has been suggested that nematodes play an

important role in wastewater bio-oxidation by their grazing activity on the bacterial community, and that they play a fundamental role on the biofilm development: due to their motility, they perform a mechanical function in the biofilm that enhances oxygen diffusion (Salvadò *et al*., 2004). Ciliates have been indicated as the most common bacteria predators in activated sludge (Ratsak *et al.*, 1996; Cybis and Horan, 1997). To our knowledge any information is available on the presence of Anellidae in biological filters.

Chung and Strom (1991) found a remarkable similarity in the microfauna in trickling filters and rotating biological contactors (RBC) filters compared with activated-sludge systems. The same authors, however, found more flagellates, ciliates, rotifers and nematodes in the RBC than the trickling filters.

The influence of these grazers was tentatively evaluated in a batch experiment. Two series of two batches were made as described in the previous paragraph. The four batches were supplied with 4 mg/l of NH_4 and with the same amount of the same particulate organic carbon used for the previously described batch experiments. One series was run as a control and the second was aliquotated with a solution containing 100 mg/l (w/v) final concentration each of two grazer inhibitors: cychloheximide, that inhibits the 80S ribosomal operation (De Lorenzo *et al.*, 2001; Vazquez-Domınguez *et al.*, 2005) and colchicine (Taylor and Pace, 1987), that inhibits microtubules polymerization. Batches were monitored for nutrient evolution (NH_4, NO_2 and NO_3) and bacterial abundance until bacterial biofilms reached the plateau.

Results did not show a difference for the nutrient evolutions between treated and control batches. In particular, the nitrate production was constant and almost identical in the four batches, suggesting that the grazing did not affect nitrifiers, which could profit of a sort of protection by the external heterotrophic layer (Figure 7.8). This is confirmed by Griffiths (1989 cited in Lee and Oleszkiewicz, 2003) who did not find any grazing effect on nitrifiers. He explained this observation by the tendency of autotrophs to form large agglomerates of bacteria, making them more resistant to grazing by protozoa than single bacterial cells.

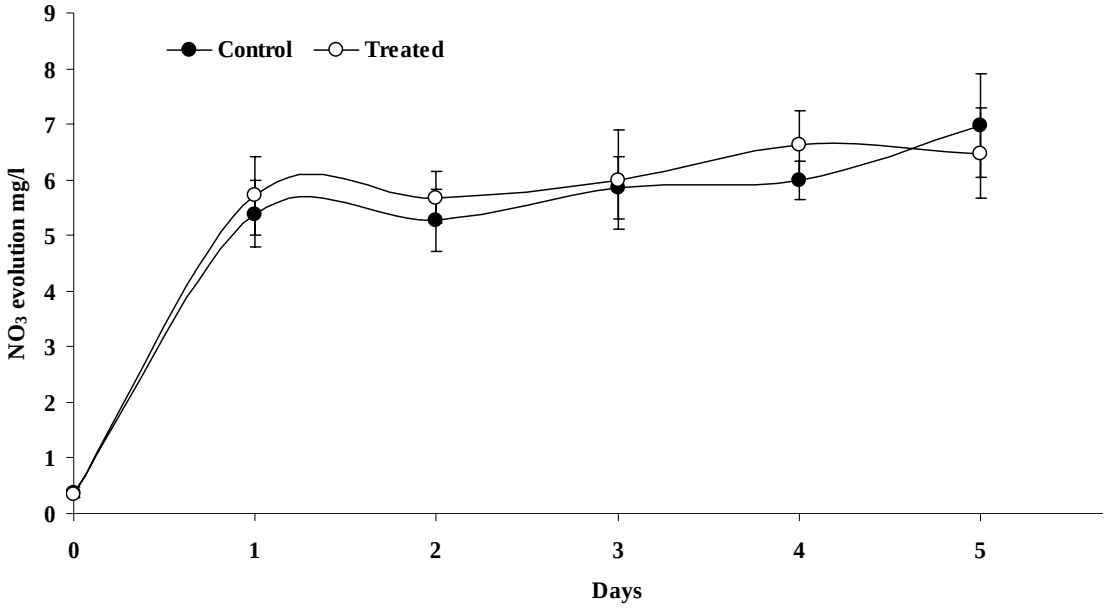

Figure 7.8: NO_3 evolutions over time in grazing inhibition experiments ("Treated" supplemented with eukaryotes inhibitors).

The bacterial growth in the two "Controls" batches reached on average $6.67x10^7 \pm 4.77x10^6$ CFU/g of PM, while in the "Treated" ones the biofilm abundance reached $9.27x10^7 \pm 6.86x10^6$ CFU/g of PM (Figure 7.9). By calculating the difference between the growth rate for the biofilm in the "Controls" batches (μ = 2.13 day^{-1}) and in the

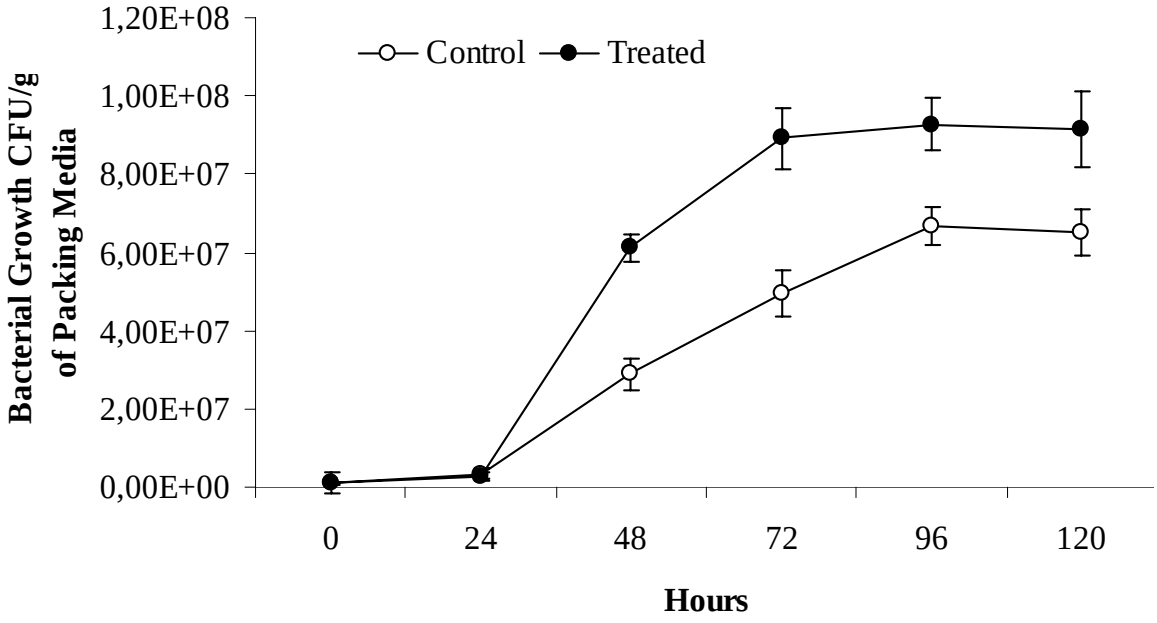

Figure 7.8: Growth curves for grazing inhibition experiments ("Treated" supplemented with eukaryotes inhibitors).

"Treated" ($\mu = 3.18\ day^{-1}$) we obtained a theoretical grazing rate of $1.06\ day^{-1}$.

It must be pointed out that because metabolic inhibitors do not equally affect all members of the targeted communities, quantitative interpretations of such experiments are strictly limited. However, this was a preliminary investigation on the effect of the grazing on the dynamics of the microbial communities in these systems.

Finally, in Table 7.3 some values of the parameters that characterize the main processes governing the dynamics of biofilm bacteria are reported.

Table 7.3: Values of some parameters for the biofilm bacterial model.

Parameter	Symbol	Values	Units - Comments	Reference
Growth yield of heterotrophic bacteria	$Y_{x/s\ (H)}$	0.63 (a) 0.60 (b) 0.92 (b)	g of bacterial heterotrophic C produced/ g of DOC consumed	(a) Billen *et al.*, 1988 cited in Léonard, 2000 (b) Horn and Hempel, 1997 (c) Tsuno *et al.*, 2002
Growth yield of autotrophic bacteria	$Y_{x/s\ (A)}$	0.24 (a) 0.021 (b) 0.062 (c)	g of bacterial autotrophic C produced/ g of NH4 consumed	(a) McBride and Tanner, 2000 (b) Horn and Hempel, 1997 (c) Tsuno *et al.*, 2002
Specific fixing rate	k_{fix}	20 – 40	h^{-1} for water velocity around 1 cm s^{-1}	Léonard, 2000
Specific shearing rate	k_{shear}	0.75	h^{-1}	Léonard, 2000
Specific mortality rate	k_{mor}	0.264 (a)	day^{-1} at 24°C	Billen et al., 1988 cited in Léonard, 2000
Specific Autotroph lysis rate	$k_{lysis\ aut}$	0.05 (c)	day^{-1}	McBride and Tanner, 2000
Specific Heterotroph lysis rate	$k_{lysis\ H}$	0.02 (c)	day^{-1}	McBride and Tanner, 2000
Grazing rate	k_g	1.06	day^{-1}	This study
Heterotrophic bacteria growth rate	μ_H	2.27 0.904	day^{-1} for C/N 1 at 25°C day^{-1} for C/N 0 at 25°C	This study This study
Autotrophic bacteria growth rate	μ_A	0.20 (*) 0.50 (**)	day^{-1} *17.5°C, day^{-1} **20.1°C	McBride and Tanner, 2000
Maximum growth rate for heterotrophs	μ_{maxH}	5.5 (e)	day^{-1} (mean of several values)	Horn and Hempel, 1997
Maximum growth rate for autotrophs	μ_{maxA}	0.14 (e)	day^{-1} (mean of several values)	Horn and Hempel, 1997

7.3. Conclusions and perspectives

In an intensive recirculating fish production facility, the role of heterotrophic bacteria is a key element to be taken into consideration for the routine management of the system. The possibility to make prediction on their dynamics and their interaction with the system is vital to the well development of RAS.

The construction of the model, as well as all other models, is limited by the difficulty to up-scale results obtained at the lab-scale level, with the real system. However, this kind of artificial environment, presents important advantages compared to a natural

systems, as, for example, the stability of some physico-chemical parameters (temperature, pH, ORP) that can be computed as constant.

The aim of present work was to put together available data and new findings, in order to upgrade a conceptual model of the heterotrophic bacterial compartment and to settle the basis for the future development of the mathematical model describing this compartment of recirculating aquaculture systems.

However, more information are necessary in order to achieve a better description of the bacterial compartment before the mathematical description of the model. In particular it could be pointed that researches are needed on: 1) the biodegradable fraction of the POC in the rearing water; 2) the biofilm microscopic structure and dynamics, by employing microelectrodes and confocal microscopy, in order to get more precise information on the interactions among the biological, physical and chemical components of this complex system; 3) the biofilm composition and structure evolution over an entire rearing cycle; and finally, 4) the bacterial community composition and structure of the different layers of the biofilter.

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CHAPTER 8

General conclusions and perspectives

The environment of a RAS supports large populations of bacteria, protozoa, and micrometazoa, that can metabolize the high amounts of dissolved and particulate organic carbon of the recirculating water.

If the nitrifying bacteria and the nitrification process have been widely studied, the importance of deeper investigation on the heterotrophic bacterial flora has been recognized only recently.

Nowadays, the ideas that such bacterial communities play a key role in the stability and the quality of the recirculating water and that the good management of “friendly” bacteria is vital for the viability of recirculating systems is more and more accepted.

The present Thesis was aimed at investigating the heterotrophic bacterial communities of a marine recirculating aquaculture system.

This thesis work focused on four main objectives: 1) a study on the influence of organic carbon on the heterotrophic bacterial populations in relation to the efficiency of the biological filter, 2) a molecular characterization of the heterotrophic bacteria inhabiting the rearing water and the biofilter associated biofilm, 3) an investigation on the stability of the bacterial communities and, 4) the description of a conceptual model of the heterotrophic bacterial compartment of a RAS, focusing on the biological filter.

In the first part of the work it was put on evidence how the high amount of particulate carbon available in the system, deriving from the normal metabolism of reared fish and from uningested feed, can negatively affect the nitrification process and can increase the bacterial abundance, favoring the presence of vibrios, most of them potentially pathogens. It was pointed out that the carbon concentration, expressed as C/N ratio, regulated not only the bacterial abundance, but also the community structure in a biofilter.

The heterotrophic communities were then investigated, in order to assess their viable and total abundance both and their phylogenetic affiliation.

Bacterial abundances vary between 10^3 CFU/ml at the outlet of the UV reactor 10^5 CFU/ml at the outlet of the biological filter for cultivable bacteria, and 10^5 cells/ml at

the outlet of the UV reactor and 10^6 cells/ml at the outlet of the mechanical filter for total abundance. Moreover, by comparing data about viable and total abundance, the percentage of cultivability ranged between 0.33 % the outlet of the UV reactor and 27.31 % the outlet of the biological filter. Abundances of bacteria associated to the biological filter packing media were ranged from 10^6 CFU/g for cultivable bacteria to 10^7 cells/g for total ones, with a cultivability of 20.9 %. Such high cultivability, compared with values normally reported in literature for seawater, suggests a good physiological status of the bacterial populations, probably due to large amounts of available organic carbon sources and to a good oxygenation of the system.

The relative abundance of the different phyla identified was comparable between the rearing water community and the biofilter biofilm. The RAS heterotrophic community can be described as a typical marine one with important allochthonous influences. Some bacterial isolates and clones were closely related to potential fish pathogens (e.g. *Vibrio harveyi, Vibrio ichthyoenteri, Enterococcus sanguinicola*), while many others belonged to species described as probiotics (*Roseobacter Clade*). Finally, the most frequent species in the biofilter biofilms was *P. stutzeri*, a well described denitrifying bacterium. This finding and the subsequent positive search for the presence of anammox bacteria in the biofilter, can explain the loss of ammonia in the mass balance calculation for biofilters.

In the third part, the stability of rearing water and biofilter communities was tested against the introduction of a commercial biological activator, made up of a confidential bacterial mixture. If the rearing water communities were only scarcely affected and the biofilter biofilm evolved in a divergent way of the control one. However, this change in community structure, by the increasing of the dominance of some taxa, did not affect the biofilter performances. Such changes cannot be unambiguously linked to the addition of the biological activator, as we do not have data on the potential natural divergence of bacterial community in biofilters. This point is a very critical one, and outlines the necessity to better design further experiments including replication at the biofilm level.

Finally, considering fixed and free status of the bacterial cells in the biofilm and rearing water respectively, the main related dynamic processes (growth, attachment, shearing and grazing) were analyzed in order to contribute to the development of a

conceptual model that can allow the construction of a mathematical one in a future work.

The results of this work highlight that a fundamental step for the future development of RAS is the reduction and management of particulate organic matter, in order to reduce the rearing water degradation, enhancing its reuse, and to control the bacterial communities proliferation. The effective control of the microbial ecology by controlling the system operation could be applied to engineering systems with direct monitoring them using molecular techniques (Aoi *et al.*, 2000). The application of modern methods for the study of microbial communities makes possible to correlate microbial systematic and dynamics with aquaculture management.

The biofilter is a complex environment, with plenty of microniches where a large variety of microrganisms can develop and proliferate. Future works have to take into account such heterogeneity and diversity, getting over the classical "operational" distinction between nitrifiers bacteria and the "others" bacteria. The simple distinction between autotrophic nitrifiers and heterotrophic bacteria is inadequate to describe the micro world inhabiting a RAS, where nitrifiers, anammox, denitrifiers, heterotrophs, cyanobacteria, archaea, protozoa, live, interact and contribute to the stability of this artificial environment.

Many aspect of the microbial ecology of a RAS remain to be described, as the comprehension of roles and interactions between such different groups.

Results from this thesis put on evidence a high diversity among heterotrophic bacteria inhabiting a marine RAS. In our opinion, further analyses are necessary in order to assess the ecophysiology of isolated strains belonging to the main bacterial groups in the biofilter. Such additional information, based on the similarity of the physiologic features examined, could allow to consider inside the bacterial model not only a single state variable of "heterotrophic bacteria", but to integrate also more state variables corresponding to different functional groups.

Moreover, further researches are still necessary in order to investigate the "buffer" effect of autochthonous bacteria of the system, as well as their so-called "shelter" effect against potential pathogens. Keeping facilities pathogen-free is an impossible task, but reducing levels of pathogens to below infective levels, by promoting the presence and

the activity of probiotics bacteria, should decrease the chance of fish becoming clinically infected.

These information are necessary in order to create a global model, enabling to predict the functioning of the bacterial compartment and consequently the water quality in the rearing system. Because “microbes and their activities bridge the macroscopic and the microscopic worlds” (J. Wimpenny), the comprehension of microbes role in RAS is vital to the management and the development of such promising systems.

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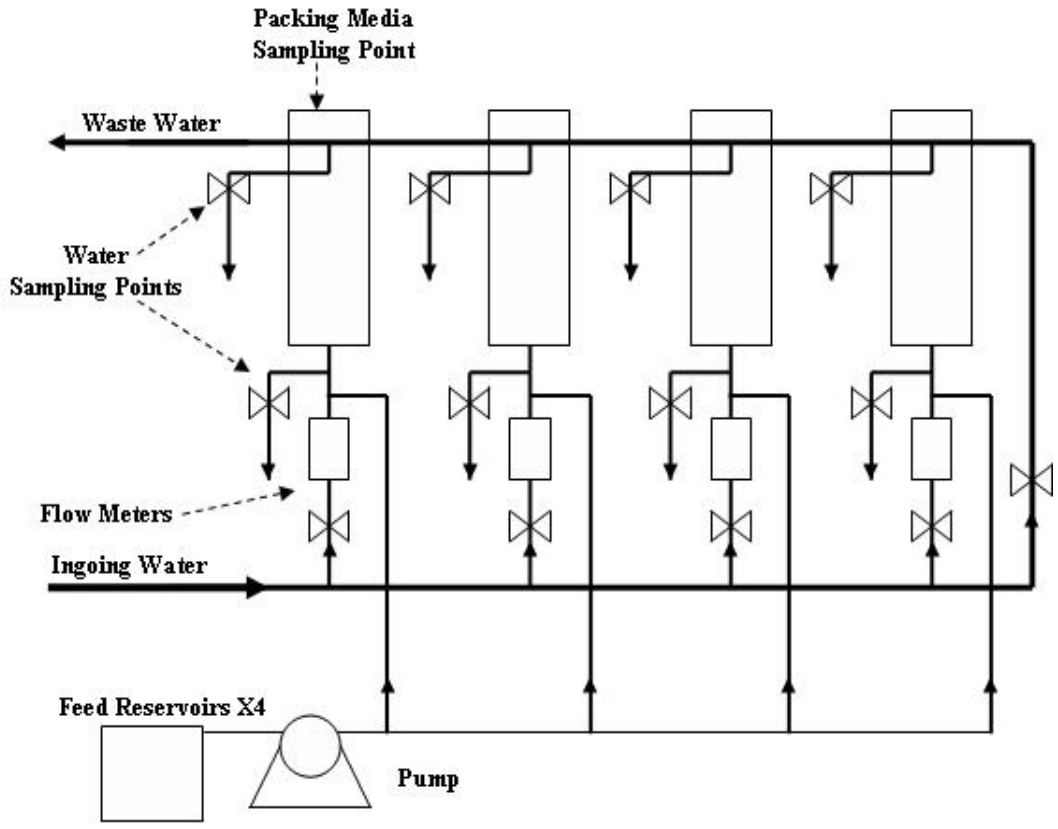
ANNEX

Annex 1: Pilot scale biological filters used for experiments in Chapter 4.



Annex 2: Batches cultures used in Chapter 8.

Annex 3: Bacterial activator, used in Chapter 5.

AQUACEET LB/M

MICRO-ORGANISMS-BASED LIQUID BIOLOGICAL ACTIVATOR TO PURIFY WATER AND REDUCE SHRIMPS HATCHERIES/ NURSERIES SLUDGE

ADVANTAGES

- **Biodegradation** (decomposition) : solid organic material, faeces… in aquaculture. Strongly **reduce** sludge and stress rates.
- **Biodegradation :** fongicides and hydrocarbons. Increase shrimps larva and juveniles survival rates.
- Contributes to **eliminate pathogenic** micro-organisms : improve sanitary conditions.

USES

- Permanent **depollution** of shrimps hatcheries and juveniles breeding water.

USE INSTRUCTIONS

- First **reactivate** AQUACEET LB/M for **48 hours**, maintaining temperature at **30°C** with a with an immersion heater at 30°C and an aquarium type aerator-bubble making device for oxygenation. This operation prepares the **weekly dose** to use just before spreading.
- For a 1m³ tank, spread 1 ml of AQUACEET LB/M diluted 100 times for one week at the most.

Note : This dose can be doubled or tripled depending on pollution. AQUACEET LB/M is to be used with AQUACEET LN.

CHARACTERISTICS

- Liquid with a slight sulphur smell.
- Turpide suspension, dark olive green.
- pH = 7,4.
- Contains selected micro-organisms (Class 1, Norm X42040).

SAFETY AND ENVIRONMENT

- Preparation **non subject to toxicity labelling** according to the European directive n° : 88-379.
- **No danger** for fishes, shrimps, young shrimps and aquatic flora when instructions for use are respected.
- Keep in closed drums **between 5°C and 40°C**.
- Maximum storage in original packaging : **2 years** and 2 to 3 months once the packaging is opened.
- Avoid skin and eye contact.
- Consult the data safety sheet.

FOR PROFESSIONAL USE ONLY
NOTA : All above data are communicated from our laboratory as information only.
They do not commit us as far as injuries and damages following a wrong use of the product are concerned.

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TITRE : Microbial communities of recirculating aquaculture facilities: interaction between heterotrophic and autotrophic bacteria and the system itself

RESUME : Les systèmes d'aquaculture en circuit recirculé (Recirculating Aquaculture Systems, RAS) peuvent être considérés comme une alternative à la technologie de l'aquaculture en milieu ouvert et en bassins (en consommant moins d'eau pour un même rendement de production) ou peuvent être intégrés dans une chaîne de production avec des systèmes d'enclos en filet.
L'intérêt des RAS est dû à leurs avantages intrinsèques comme la réduction des besoins en surface et en eau, le haut niveau de contrôle environnemental permettant une croissance optimale à tous moment de l'année et la possibilité de produire à proximité des marchés. De plus, le haut niveau de contrôle améliore la gestion des risques lié aux infections bactériennes et parasitaires ou aux invasions de prédateurs. Finalement, la production de déchets (particulaires et dissous) peut être contrôlée et minimisée, contribuant ainsi à rendre ces systèmes écologiquement durables.
Cependant, il n'existe que peu de connaissance sur l'écologie microbienne des RAS, et il est fondamental d'acquérir des informations sur la dynamique des populations de procaryotes, sur l'abondance relative des pathogènes *versus* celle des pro biotiques et sur les interactions entre les bactéries hétérotrophes et nitrifiantes.
Cette thèse a pour objectif général d'étudier les communautés bactériennes hétérotrophes d'un système d'aquaculture marine recirculé et plus particulièrement celles associées au filtre biologique qui constitue une composante centrale du système. Elle a été ciblée sur 4 objectifs principaux : (i) l'influence du carbone organique particulaire sur les populations bactériennes hétérotrophes en relation avec l'efficacité du filtre biologique, (ii) une caractérisation moléculaire des bactéries hétérotrophes de l'eau recirculée et du biofilm associé au filtre biologique, (iii) la stabilité de la communauté bactérienne hétérotrophe en réponse à l'utilisation d'un bioactivateur, (iv) la description d'un modèle conceptuel du compartiment bactérien hétérotrophe d'un RAS avec une attention spéciale sur le filtre biologique.

MOTS-CLES : Aquaculture en circuit recirculé - écologie microbienne - filtre biologique - carbone organique particulaire - caractérisation moléculaire - bioactivateur - modèle conceptuel

SUMMARY: Recirculating Aquaculture Systems (RAS) may be considered an alternative to pond aquaculture technology (by consuming lower amounts of water producing similar yields of crop) or can be integrated in a sort of "production chain" with net pen systems.
The interest in RAS is due to their intrinsic advantages like the reduction of land and water requirements, the high degree of environmental control allowing year-round growth at optimum rates and the possibility to produce in close proximity to markets. Moreover, the high level of control improves risk management linked with bacterial and parasitic infections and predator pests. Finally, waste production (both particulate and soluble) can be controlled and minimized contributing to make these systems ecologically sustainable.
Nowadays, there are a few knowledge on the microbial ecology within Recirculating Aquaculture Systems, and a better understanding on the microbial populations will be helpful to optimize the growing protocols and the welfare of reared animals. In particular, it is vital to acquire information on the dynamic nature of the prokaryotic populations, the relative abundance of pathogens versus probionts and the interaction between heterotrophic and nitrifying bacteria.
The present Thesis was aimed at investigating the heterotrophic bacterial communities of a marine recirculating aquaculture system. It was focused on four main objectives: 1) a study on the influence of organic carbon on the heterotrophic bacterial populations in relation to the efficiency of the biological filter, 2) a molecular characterization of the heterotrophic bacteria inhabiting the rearing water and the biofilter associated biofilm, 3) an investigation on the stability of the bacterial communities and, 4) the description of a conceptual model of the heterotrophic bacterial compartment of a RAS, focusing on the biological filter.

KEYWORDS: Recirculating Aquaculture System – microbial ecology – biological filter – particular organic carbon – molecular characterization – biological activator – conceptual model

DISCIPLINE : Ecologie, biodiversité et evolution

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