RECIRCULATING ACQUACULTURE SYSTEM (RAS) BIOFILTERS: FOCUSING ON BACTERIAL COMMUNITIES COMPLEXITY AND ACTIVITY

Tesi di Dottorato
Dott. Filippo INTERDONATO

Il Coordinatore
Prof. Emilio De Domenico

Il Tutore
Prof. Maria De Francesco

I Cotutori
Dott. Jean Paul Blancheton

Dott. Luigi Michaud

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Riassunto

La presente ricerca è stata incentrata sulla comprensione delle relazioni che intercorrono tra le comunità batteriche, instaurate all'interno dei filtri biologici di un sistema di acquacoltura ricircolato, e l'influenza esercitata dalle forzanti del sistema stesso.

Il lavoro è stato suddiviso in 3 obiettivi principali, volti a studiare:

- la composizione della frazione batterica metabolicamente attiva tramite l'allestimento di librerie di cloni su cDNA (RNA) e l'applicazione di tecniche di *fingerprinting* (quali la T-RFLP);

- l'effetto del carbonio organico particellato sulla nitrificazione e sulle comunità batteriche in diverse tipologie di filtri biologici;

- l'effetto di un moderato aumento del potenziale di ossido-riduzione (ORP), tramite l'insufflazione d'ozono, nei confronti dell'attività e della struttura della comunità batterica.

Le librerie di cloni su cDNA hanno permesso di suddividere la comunità attiva in 48 filotipi, corrispondenti ad altrettante specie. I gruppi batterici sono stati rappresentati principalmente da Gammaproteobatteri (59,7%), seguiti da Alfaproteobatteri (11,5%) e Bacteroidetes (7,9%). Molti dei cloni analizzati, soprattutto tra i Gammaproteobatteri, appartenevano a specie potenzialmente patogene per i pesci, anche se questi ultimi si trovavano in un eccellente stato sanitario durante il periodo della ricerca. Tale dato potrebbe suggerire l'esistenza di un effetto protettivo della flora batterica autoctona presente in tali impianti contro patogeni opportunisti, comunque sempre presenti in questo tipo di sistemi.

I risultati emersi hanno messo in evidenza una relazione negativa tra l'efficienza di nitrificazione ed il rapporto C/N (inteso come rapporto fra Carbonio organico particellato ed Azoto inorganico disciolto), con una sensibile diminuzione della nitrificazione quando il rapporto C/N passa da 0 a 4. L'aumentare di tale rapporto ha portato ad un drastico aumento dell'abbondanza batterica (coltivabile e totale) sia sul supporto filtrante che nell'acqua in uscita dai filtri. Ciò suggerisce come l'aumento del carbonio organico porti alla predominanza di batteri eterotrofi su quelli autotrofi, responsabili della nitrificazione, con il conseguente drastico decremento dell'efficienza di filtrazione.

I dati riguardanti gli effetti causati dall'ozonizzazione su campioni d'acqua e supporto filtrante all'interno del filtro biologico hanno evidenziato come l'ossidazione delle macromolecole organiche, ad opera dell'ozono, abbia conseguenze sull'intero comparto microbico. Infatti, l'utilizzo di diverse metodiche, molecolari e chimiche, ha confermato che sia la composizione e la struttura della comunità batterica (citometria a flusso e librerie di cloni) sia la sua attività, intesa come efficienza di filtrazione (analisi chimiche sui nutrienti) e attività metabolica (attività eso-enzimatica), mostrino differenze rispetto al filtro biologico di controllo, non sottoposto ad ozonizzazione.

In conclusione, i risultati ottenuti forniscono un importante contributo alle conoscenze attuali su dinamiche e relazioni che intercorrono tra i differenti comparti in sistemi complessi come gli impianti d'acquacoltura ricircolati, soprattutto per quanto concerne la corretta gestione dei filtri biologici in relazione ai parametri del sistema.

Abstract

This research has been carried out in order to better understand the relationships between bacterial communities, established within the biofilter of a recirculating aquaculture system, and the influence exerted by forcing factors on the system itself. The work was divided into three main objectives that were aimed at studying:

- the composition of the metabolically active bacterial fraction through the construction of cDNA clone libraries and the application of fingerprinting techniques (e.g., the T-RFLP);

- the effect of particulate organic carbon on both the nitrification process and the microbial communities in different types of biological filters;

- the effect of a moderate increase in the oxidation-reduction potential (ORP), through the injection of ozone, towards the activity and structure of the bacterial communities.

The cDNA clone libraries allowed subdividing the active community in 48 phylotypes, each corresponding to a species. The *Gammaproteobacteria* (59.7%) were predominant, followed by *Alphaproteobacteria* (11.5%) and *Bacteroidetes* (7.9%). Most clones, especially among the *Gammaproteobacteria*, belonged to species that are potentially pathogenic to fish, even these latter were in an excellent health state during the experimentation period. This might suggest the existence of a shelter effect by the autochthonous bacterial flora against opportunistic pathogens, which are always present in such systems.

Results showed a negative relationship between the nitrification efficiency and C/N ratio (defined as the ratio of particulate organic carbon and dissolved inorganic nitrogen), with a significant decrease in nitrification when the C/N ratio increased from 0 to 4. The increase of such ratio led to a dramatic increase in bacterial abundance (viable and total counts) on both the packing media and the water outlet. This suggests that the increase in organic carbon could allow to the predominance of heterotrophic bacteria on those autotrophic, which are responsible for the nitrification process, with the consequent drastic decrease in the filtration efficiency.

Data regarding the effects caused by the ozonation process on water and packing media samples showed that the oxidation of organic macromolecules, by ozone, has

consequences on the whole microbial compartment. In fact, based on results from the filtration efficiency (chemical analysis on nutrients) and metabolic activity (exo-enzymatic activities) determinations, the use of various methods (both molecular and chemical) confirmed that both the composition and structure of the bacterial community (as it was determined by the application of flow cytometry and clone libraries) in addition to bacterial activity, were different in untreated and ozonated biological filters.

In conclusion, results provide an important contribution to the current knowledge on the dynamics and relationships between the different compartments in complex systems such as recirculated aquaculture systems, mainly giving indications about the proper management of the filters in relation to the parameters that characterize the system itself.

CHAPTER 1

Introduction and problem statement

Recent decades have witnessed a rapid growth and development of aquaculture systems for the intensive rearing of fish (van Rijn, 1996). This was in response to the increasingly high worldwide per capita demand (16.7 kg) showing a steady upward trend for the coming decade and where aquaculture provides 47% (FAO, 2009). In order to alleviate the pressure of fishing on marine stocks, it is necessary that the production (especially fish) should be accelerated through aquaculture (Tal *et al.,* 2009). This production increasing, in addition to be economically viable, also takes into account the impact that it has on resources (environment, water availability, location on land, etc.) (Schneider *et al.,* 2007; Zohar *et al.,* 2005).

Among the many existing aquaculture systems, the RAS (Recirculating Aquaculture System) seems to overcome these limitations and can provide a form of sustainable farming for both marine and freshwater species (Schreier *et al.,* 2010). Efficient RAS management allows: the effective control and treatment of waste (soluble and particulate) coming from the system; minimal inputs of water if not to make up for losses due to evaporation (Tal *et al.,* 2009; Zohar *et al.,* 2005; Michaud, 2007); provides the ability to monitor the parameters associated with the rearing environment during the life cycle of farmed fish, maximizing production yield; reduces the occurrence of infections caused by pathogenic bacteria or parasites (Michaud, 2007).

The treatment of wastewater within a RAS is carried out by several steps of filtration, which are mainly divided into mechanical and biological filtration: the former uses physical agents (oxygen, temperature, ozone, UV, pH and salinity) for the removal of waste substances in the water outlet from the rearing tanks and for its disinfection; the latter uses biological oxidation and redox reactions thanks to micro-organisms. Just the microbial compartment plays a key role in wastewater treatment: in fact, the importance and influence of the bacterial communities are comparable to those of fish in terms of biomass, processes related to their activity (Michaud, 2007) and oxygen consumption (Blancheton, 2000).

Also, to get a proper management of a RAS is necessary to study and deeply understand all the mechanisms of both filtration approaches. In fact, while the mechanical processes can be monitored and managed, biological filtration systems, based on the interaction of microbial communities among themselves and with their environment, are not easily controlled. For this purpose, studies conducted in recent years by using molecular methods, have allowed not just describing the microbial diversity, but they also provided data on bacterial activity to a greater understanding of community interactions (Schreier *et al.,* 2010). Therefore, the expansion of knowledge of metabolic activity, inside the bacterial community, turns out to be of primary importance for the determination of the relations intra-and inter-specific.

Moreover, as suggested by previous studies (Michaud *et al.,* 2009), a good management of rearing environmental determines the proper maintenance of the physical and chemical parameters of water recirculated systems. Considering the large number of variables that exist in the RAS filtration, a deepening of studies about different biotic and abiotic parameters is necessary, in order to improve the filtration and farming quality, maximizing profits.

CHAPTER 2

State of the Art

2.1.   Aquaculture

World aquaculture has had a significant growth over the past 50 years. From a production below 1 million tons in the '50s, it has gone in 2006 to 51.7 million tons *(Fig. 2.1).* This means that it continues to grow faster than any other field in the production of food of animal origin. Although the supply of fish products from fishing is in a stalemate, the demand for fish and fish products continues to grow. Consumption has more than doubled since 1973, resulting in consequential growth of aquaculture production. In fact, its contribution in the supply of fish has increased significantly, reaching the historical record of 47% in 2006, compared to 6% in 1970. This trend is projected to continue and will reach a rate of 60% in 2020 (FAO, 2009).
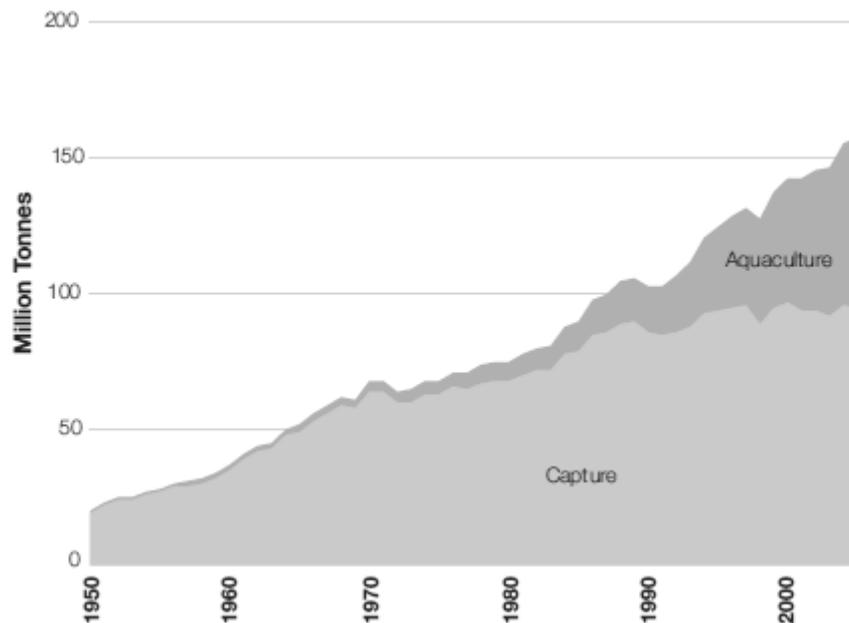
Fig. 2.1: Global fisheries and aquaculture production, 1950-2005 (from Allsopp *et al.,* 2008)

The expected production increase that aquaculture should reach in the next decade does not seem to will be followed by fishing. In fact, high rates of production until now

supported by fishing, have inevitably led to a depletion of wild marine stocks. Overall, about 80% of worldwide marine stocks, for which ones information are available, are reported as fully exploited or overexploited (FAO, 2009). This fact shows how the absence of an effective and precautionary management of fisheries, with the concomitant modernization of fishing fleets (more powerful engines, more efficient fishing gears, tools for the location of fish stocks, etc.), has led to reaching the maximum recruiting potential in world's oceans.

The rapid development of farms with *intensive* or *hyper-intensive* production, both *on-shore* and *off-shore*, in response to the growing demand for fish products, has often led to solutions that tolerate very high production rates, but that produced as result a very high environmental impact. In fact, they produce large quantities of wastewater containing high concentrations of suspended solids [SS] (waste of fish metabolic cycle and not eaten food) and micronutrients, such as ammonia nitrogen and phosphates, capable of establishing local pollution in the water due to increased consumption of biological and chemical oxygen demand (BOD-*Biological Oxygen Demand* / COD-*Chemical Oxygen Demand*), as well as an increase in water turbidity and they can often create anaerobic conditions (Michaud, 2007) and eutrophication in the bottom of the sea.

The systems consist in *off-shore* modular cages placed directly into the sea; in the different modules the fish are fed until they reach commercial size and weight. However, although farming in cages show excellent cost-benefit ratios, is far from being ecological problem-free. In *off-shore* salmon farms, for example, waste products affect the aquatic environment nearby the various modules and this is pronounced if the areas in which they are installed are not adequate (too close to the coast, closed bays, etc.), often leading to anoxia. In extreme cases the large number of farmed fish can generate sufficient quantities of waste to cause the collapse of the minimum levels of oxygen necessary to the life of the aquatic ecosystem, with the result of possible suffocation of the benthic biocoenoses and the same stock bred. Even an efficient and careful management, however, causes an impact that can usually be found in a significant reduction of biodiversity around the cages (Allsopp *et al.,* 2008).

In intensive farming systems on-shore hydraulic organization can be classified into "*open*" and "*recycled*" (closed) (van Rijn, 1996). Both consist of tanks of different shape and material, placed on land and connected to the water resources using a pumping systems.

"*Open*" farms are usually designed for the production of freshwater, brackish and seawater species (van Rijn, 1996). The quantity and quality of available water represent the main factors that affect the productivity of systems. In fact, they significantly exploit the available water resources as they require of large volumes of water for their supply. Also, this dependency limits their design on the territory to the proximity of a exploitable water body.

On the other hand, the high biomass that is reached in the rearing tanks (20-40 kg/m^3) leads to the formation of highly concentrated wastewater in terms of particulate and dissolved organic matter (POM and DOM), ammonia nitrogen and phosphates (Michaud, 2007).

In these systems, in order to limit the impact on the aquatic ecosystem, different solutions have been adopted: they consist in the removal of the bottom deposits of the fish tanks by recovery mechanical actions and waste water phytoremediation in suitable reservoirs. Although, these are quite effective, they are not decisive, producing themselves waste products (active sludge). Therefore, the "*classics*" farming systems remain linked to the processing of waste, through their storage and sent to wastewater purification stations, which complete the process of clarification up to include the wastewater between the limits provided by law. So, it is evident as the future expansion of fish production, through aquaculture, depends significantly on the ability of farmers to combine the best conditions for the marketing of fish with the reduction of the risk and impact that these activities produce interacting with the environment (Tal *et al.,* 2009).

2.2. Recirculating Aquaculture Systems (RAS)

Recirculating Aquaculture Systems (RAS) are one of the future platforms that offer a *sustainable* method for the intensive rearing of marine and freshwater fish. The possibility thanks to these systems to handle, store and treat waste products accumulated during the growth of farmed fish represent a key factor for the development of environmentally friendly management of aquaculture production systems (Piedrahita, 2003; van Rijn, 1996). RAS has been developed as an alternative to traditional aquaculture systems. They adopt a closed farming system (or recycled) that allows the reuse of farm water thanks to a series of filtrations, thus limiting not only the removal

from natural water resources, but also the impact on the environment reducing the volume of waste generated treated in the same system (Buono, 2005; Lahav *et al.,* 2009).

These systems offer several advantages compared to traditional technologies: the possibility to be placed near the fish markets, high product quality, shorter production cycles due to high food conversion factors and a constant monitoring of the farm environment in order to improve rearing conditions (Singh *et al.,* 1999). However, one of the biggest problems that recirculating aquaculture companies meet is linked to the high initial investment required for the design and construction of plants; it is also recovered relatively quickly thanks to the high productions obtained (Buono, 2005).

As mentioned previously, the treatment of wastewater is carried out directly into the system by providing their treatment and reuse. In a RAS, in fact, several filtration processes are used and managed in order to make the water usable again for farming; they consist of mechanical, chemical and biological treatments.

Also, a RAS can be made more efficient by adding accessory components to the system as: unit for the administration of ozone, for the wastewater disinfection and organic waste removal; degassing unit, for carbon dioxide removing; monitoring and control systems (Michaud, 2007) (*Fig. 2.2*).

Fine & Dissolved Solids Removal

Foam fractionation

Aeration or Oxygenation

Air stone diffuser
Packed column
Down-flow contactor
Low head oxygenator
U-tube

Fish Culture Tank
Round, Octagonal, Rectangular or D-ended

Carbon Dioxide Removal

Air stone diffuser
Packed column

Disinfection

Ultraviolet light
Ozone contact

Waste Solids Removal

Sedimentation
Swirl separators
Screen filters
Bead filters
Double drain

Biological Filtration (Nitrification)

Fluidized bed filters
Mixed bed filters
Trickling filters
Rotating bio contactor

Fig. 2.2: Units required for the process and some typical components used in a recirculating aquaculture production system (from Losordo *et al.,* 1998).

- With the appellation suspended solids (SS) it is identified all the particulate matter that settles on the bottom of the fish tank and cannot be removed easily in conventional sedimentation basins. If not removed can significantly limit the amount of fish that can be reared into the system and can cause irritation to the gills of fish (Losordo *et al.,* 1998). Furthermore, the concentration of suspended solids in the water body coming out from a rearing tank is often very high, causing a significant decrease in water quality. In fact, the particles in suspension, as well as result in increased turbidity in the wastewater, are mainly composed of particulate organic matter (POM) which can quickly led to putrescence and collapse of the dissolved oxygen content. The suspended solids are primarily removed by mechanical filtration: the two types most commonly used filters are the "*drum*" and "*sand*" filters (Losordo *et al.,* 1998). They allow to drastically reduce the amount of particulate matter in the wastewater, using mechanical processes that allow the separation from the water, resulting in the accumulation and removal of waste substances.

- The ultraviolet irradiation (UV) and/or ozonation can be an effective solution for the treatment and sometimes recirculated water disinfection, before entering into fish rearing tanks (Summerfelt *et al.,* 2009). Without an internal disinfection process, in fact, obligated or opportunistic pathogens may accumulate on farms that treat and reuse water, causing the spread of diseases and the death of farmed stocks, as a result of the proliferation of pathogens from their hosts to the entire system (Brazil, 1996; Bullock *et al.,* 1997; Summerfelt *et al.,* 1997; Christensen *et al.,* 2000; Krumins *et al.,* 2001a, b; Summerfelt, 2003; Sharrer *et al.,* 2005; Summerfelt *et al.,* 2004; Sharrer and Summerfelt, 2007). In addition, the ability of *biofilms* (*see 2.2.2.*) to act as potential "microbial shelters" further suggests the application of decontamination of sea water for aquaculture uses, in order to limit the entrance of microorganisms potentially harmful in the circulating water and/or on surfaces of the system (Wietz *et al.,* 2009). To this end, in recirculated aquaculture systems it has been shown that the ozonation represents a functional approach (Wietz *et al.,* 2009) and the use UV irradiation inactivates microorganisms (Farkas *et al.,* 1986; Zhu *et al.,* 2002; Sharrer *et al.,* 2005) restricting the entry of pathogenic species.

- The nitrogen compounds (ammonia, nitrites and nitrates) are considered the main contaminants in the waste water produced as waste in aquaculture (Qin *et al.,* 2005). Ammonia is the main waste produced by the metabolism of fish. Acute exposure to high concentrations, causing brachial hyperventilation, hyperexcitability, loss of balance while swimming, convulsions and even death (Smart, 1978; Thurston *et al.,* 1981). Instead, chronic exposure to lower concentrations of ammonia cause tissue damage, decreased reproductive capacity, decreased growth, increased susceptibility to disease (Thurston *et al.,* 1984; Thurston *et al.*, 1986) and even death (Randall and Wright, 1987). In order to reduce and/or eliminate harmful waste products resulting from the metabolism of fish, different configurations are used in biological filters, which are adapted to the requirements of different farmed fish species (Schreier *et al.,* 2010).

2.3.   The biological filtration

2.3.1.   The biological filter

One of the key points in the architecture of a RAS is the biological filtration through the use of biological filters (also known as bio-filters). They usually consist of a cylindrical bioreactor containing substrates of different materials (*Media*), designed to have maximum contact surface in order to promote growth of the bacterial community through the production of biofilms (Avnimelech, 2006; Gutierrez-Wing and Malone, 2006).

Independently of the type of system (sea water or freshwater, small aquaria or large production systems), the biofilter integrates aerobic and anaerobic microbial processes for the elimination of waste products of nitrogen in the form of ammonia excreted by fish, and carbon from the feed not consumed and the fecal matter (Schreier *et al.,* 2010) (*Table 2.1; Fig.2.3*).

Primary activities associated with RAS biofiltration units and participating microorganisms.

Process	Reaction	Microorganism	
		Freshwater	Marine
Nitrification			
Ammonium oxidation	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$	*Nitrosomonas oligotropha*	*Nitrosomonas* sp. *Nitrosomonas cryotolerans* *Nitrosomonas europaea* *Nitrosomonas cinnybus/nitrosa* *Nitrosococcus mobilis*
Nitrite oxidation	$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$	*Nitrospira* spp. *Nitrospira marina* *Nitrospira moscoviensis*	*Nitrospira marina* *Nitrospira moscoviensis*
Denitrification			
Autotrophic (sulfide-dependent)	$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow$ $SO_4^{2-} + 0.8N_2(g) + 0.8H_2O$		*Thiomicrosporia denitrificans* *Thiothrix disciformis* *Rhodobacter litoralis* *Hydrogenophaga* sp.
Heterotrophic	$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow$ $10HCO_3^- + 4N_2(g) + 4H_2O$	*Pseudomonas* sp. *Comamonas* sp.	*Pseudomonas fluorescens* *Pseudomonas stutzeri* *Pseudomonas* sp. *Paracoccus denitrificans*
Dissimilatory nitrate reduction to ammonia (DNRA)	$NO_3^- + 2H^+ + 4H_2 \rightarrow NH_4^+ + 3H_2O$		Various *Proteobacteria* and *Firmicutes*
Anaerobic ammonium oxidation (Anammox)	$NH_4^+ + NO_2^- \rightarrow N_2(g) + 2H_2O$		*Planctomycetes* spp. *Brocadia* sp.
Sulfate reduction	$SO_4^{2-} + CH_3COO^- + 3H^+ \rightarrow$ $HS^- + 2HCO_3^- + 3H^+$		*Desulfovibrio* sp., *Dethiosulfovibrio* sp., *Fusibacter* sp., *Bacteroides* sp.
Sulfide oxidation	$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$		*Thiomicrospira* sp.
Methanogenesis	$4H_2 + H^+ + HCO_3^- \rightarrow CH_4(g) + 3H_2O$		Methanogenic Archaea [Mirzoyan and Gross, unpublished]

Table 2.1: Main bacterial reactions associated with a biological filter (modified from Schreier *et al.,* 2010)
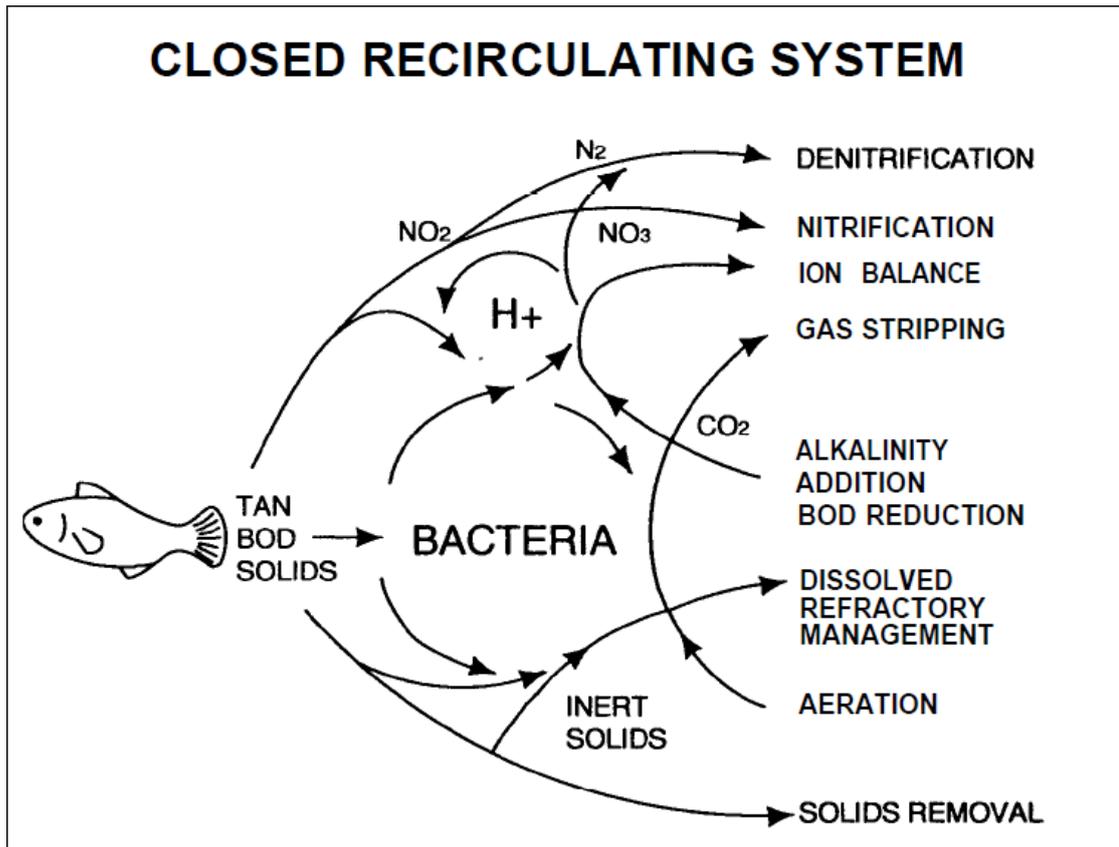
Fig 2.3: Diagram of the fate of waste products from fish and their effect on bacterial and chemical interactions in a recirculated system (Masser *et al.,* 1992).

They are traditionally dimensioned according to the amount of substrate (m^3) that are able to contain, or the total area (m^2/m^3) provided by the substrate used (Drennan *et al.,* 2005). Today there are on the market a wide variety of substrates: rocks, shells, sand, expanded clay or plastic are the materials mainly used to support bacterial films (Malone and Pfeiffer, 2006).

The choice of a proper biofilter influence the investment and operating costs in a RAS, water quality and of course the efficiency of water treatment (Summerfelt, 2006). A perfect model would remove all the ammonia present in the effluent, not produce nitrite, support dense populations of nitrifying bacteria, requiring for its realization inexpensive materials and low maintenance. Unfortunately, no biofilter has all these characteristics, but each type has its own advantages and disadvantages (Rusten *et al.*, 2006; Michaud, 2007).

There are many different types of biological filters: trickling filters, Rotating Biological Contactors (RBC), granular substrate biofilters, submerged fixed substrate biofilters (Static Bed), mobile substrate biofilters (Moving Bed), etc. In particular, the Static Bed filters support excellent volumes of water with good purification rates, but

require significant maintenance (frequent cleaning due to occlusion, periodic washing of the internal material, etc.), while the Moving Bed filters need very little maintenance, but they need more time for the organization of bacterial community.

2.3.2. *The bacterial biofilm*

The water of a RAS includes the presence of large populations consisting of bacteria, protozoa and micrometazoa (Michaud, 2007). Some of these organisms are involved in the degradation of particulate organic matter present in the waste within the system (Franco-Nava *et al.,* 2004), others in the degradation of dissolved waste substances in water, including dissolved organic compounds, ammonia, nitrites and nitrates (Sharrer *et al.,* 2005; Itoi *et al.,* 2006).

Microorganisms can be found freely floating in the water flow in planktonic phase or, viceversa, living in complex aggregations, characterized by the presence of a protective and adhesive matrix: the biofilm (Léonard, 2000; Michaud *et al.,* 2009). However, because the microbial activity is mainly associated with the contact with surfaces (Costerton *et al.,* 1995; Davey and O'Toole, 2000; O'Toole *et al.,* 2005), the majority of bacteria resident in the aquatic environment is thus organized as biofilms, which adhere easily to any solid support, that are organic or inorganic, in contact with water (Lewandowski *et al.,* 1993; MacDonald and Brözel, 2000; Watnik and Kolter, 2000; Characklis and Marshall, 1990; Costerton, 1999; Møller *et al.,* 1998). Defined by Zhu and Chen (2001b) as "*viscoelastic layer of microorganisms*", it represents a water-substrate interface, site of active metabolic exchange (Characklis and Marshall, 1990) and plays a key role both in nature and in technological processes (Michaud, 2007).

Usually, the structure of the bacterial biofilm consists of complex cellular aggregations immersed in a protective self-produced matrix, composed of extracellular polymeric substances: this structure prevents that other microorganisms can adhere on it, limiting the competition for essential substances (Davey and O'Toole, 2000; Hall-Stoodley *et al.,* 2004). In addition, the spatial heterogeneity involves a significant impact on the behavior and overall functionality of the biofilm (Xavier *et al.,* 2004).

The formation of a biofilm in a cyclic process occurs divided into three stages: adsorption of molecules essential for the contact of bacteria, colonization of the pioneer bacterial groups, reproduction and detachment (Characklis, 1981; Costerton, 1999). In

fact, the planktonic bacterial cells, after conditioning of the surface of the medium by organic molecules and minerals, initially adhere in a reversible way; at this stage occurs the inhibition of synthesis and the subsequent loss of the flagellum, which would destabilize the structure of biofilms, and simultaneously the increase in the production of exopolysaccharides (EPS), which play a protective (increasing of the resistance to antibiotics, disinfectants and detergents) and mechanical role (adhesion to the substrate) (Watnik and Kolter, 2000; Michaud, 2007). After, the growing occurs resulting in cell division, which gradually leads to the enlargement of the structure until it reaches a stage of *mature biofilms* in which there is an efficient inter-cellular communication (Quorum Sensing). Finally, a portion of the mature biofilm is detached, which again will release free planktonic bacteria that can colonize a new free surface (*Fig.2.4*) (Costerton, 1999; Ghigo, 2006).
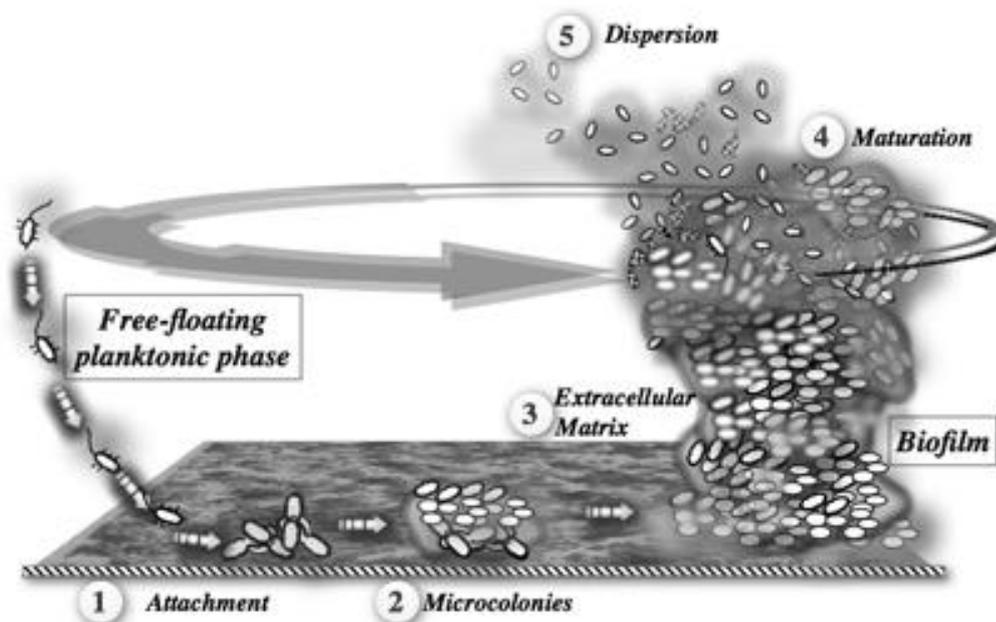
Fig 2.4: Essential steps in the formation of bacterial biofilm (from Ghigo, 2006).

2.3.3. The bacterial depuration

In recirculating aquaculture systems, bacteria can be divided into two main groups:

Almost all organic matter is represented of compounds as carbohydrates, aminoacids, peptides and lipids; it is derived from uneaten feeds/diets, dead bodies and

excreta of fish and is mineralized for their metabolism by *Heterotrophic Bacteria* (HB), both on filter materials and in rearing water. On the contrary, *Autotrophic Bacteria* (AB) use CO_2 as a carbon source and take energy from the oxidation of inorganic nitrogen compounds, sulfur or iron. In the process of mineralization proteinous nitrogen is decomposed to ammonia (NH_4^+) by both proteases and deaminases produced by bacteria. Moreover, ammonia is also excreted directly by fish (Sharrer *et al.,* 2005; Sugita *et al.*, 2005).

For their operation, the filters take advantage of the biological pathway of a heterogeneous group of chemo-litho-autotrophic bacteria strictly aerobic, not phylogenetically linked: the *Nitrifying Bacteria* (Aoi *et al.*, 2000; Michaud, 2007). This process, called "Nitrification", consists in the conversion of ammonia, as mentioned earlier extremely toxic to fish, into nitrite and immediately after nitrate, much less toxic (Schuster and Stelz, 1998); this mechanism, therefore, allows to purify the inlet water, charged of ammonia, returning reusable water for the rearing process and reducing the water requirement of the system.

The nitrification process for the wastewater biological treatment can be carried out by two bacterial fractions: the fixed fraction (that is adherent to the biofilm) and the suspended fraction (i.e. freely floating). The main rate-limiting factor in a nitrifying biofilm can be either TAN (Total Ammonia Nitrogen) or DO (Dissolved Oxygen) concentration assuming other nutrients are supplied at adequate levels for biofilm growth (Zhu and Chen, 2002). In fact, the maximum nitrification efficiency is achieved with a oxygen saturation around 80% and no reactions are possible for concentrations of dissolved oxygen below 2 mg/l (Michaud, 2007). In addition, the nitrification rate in the biofilm can be interpreted as the balance between the demand for substrate, due to the growth of bacterial biomass, and the availability of the substrate, determined by diffusion processes (Rasmussen and Lewandowski, 1998).

The nitrification is divided in two distinct phases.

In the first one ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (AOB), classified in two phylogenetic groups: the first is *Nitrococcus*, belonging to the β subclass of proteobacteria, and it is represented by two described marine species (Koops and Pommerening-Röser, 2001); the second group, belonging to the γ subclass of proteobacteria, is represented by clusters *Nitrosospira* and *Nitrosomonas* (Michaud, 2007).

The oxidation of ammonia is described by the following reaction:

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O + 84\ kcal\ mol^{-1}$$
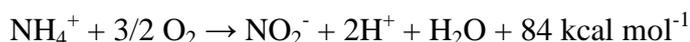
Ammonia is first oxidized to hydroxylamine, a reaction intermediate, and then to nitrite. This process involves two enzymes: ammonia monooxygenase (AMO) and hydroxylamine oxido-reductase (HAO) (Tsang and Sukuki, 1982; Bock *et al.,* 1991). The hydroxylamine is the first product of ammonia aerobic oxidation, but also the reduction produces nitrite in the anammox process (van de Graaf *et al.,* 1996).

In the second phase, nitrite is oxidized to nitrate by a distinguished group of microorganisms, the *nitrite-oxidizing bacteria* (NOB), which are classified into four groups (Egli, 2003). The main group, which belongs to the α subclass of proteobacteria, is represented by a single genus, *Nitrobacter*, itself subdivided into four species, two of which, *N. mobilis* and *N. gracilis*, are marine and belong respectively to the β and γ subclasses of proteobacteria (Koops and Pommerening-Röser, 2001). Another genus is *Nitrospira* that includes two species, *N. marina* and *N. mascoviensis* (Ehrich *et al.,* 1995) which are part of a phylum belonging to the δ subclass of proteobacteria (Michaud, 2007).

The oxidation that converts the nitrites to nitrates follows the following reaction:

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

In this case the enzyme complex involved is made of the nitrite oxidoreductase (NOR), the cytochromes a1 and c1, a quinine and a NADH dehydrogenase (Bock *et al.,* 1986; Bock *et al.*, 1990).

If the mechanisms of the nitrification process have already been extensively described (van Rijn, 1996; Aoi *et al.,* 2000; Koops and Pommerening-Röser, 2001; Egli, 2003; Tal *et al.,* 2003; Sharrer *et al.,* 2005; Michaud, 2007), the importance of a more detailed investigation of the heterotrophic bacterial flora has been recognized only recently (Michaud *et al.,* 2006, 2009). These bacteria are an important factor in terms of oxygen consumption, production of by-products of metabolism after cell lysis, onset of disease in farmed fish and also they actively compete for oxygen and space with the autotrophic bacteria, significantly inhibiting the nitrification (Zhu and Chen, 2001a; Léonard *et al.,* 2002; Michaud *et al.,* 2006). In fact, within a biological filter

heterotrophic bacteria, having a more rapid growth, dominate the outer layers of the biofilm matrix, directly taking the dissolved oxygen in the water, that to the detriment of the autotrophic bacteria, which are slow growing and are located in the deeper layers where the oxygen diffuses in a limited way (Lewandowski *et al.,* 1993; Zhu and Chen, 2002). This competition crucially influence the efficiency of the biological filter in terms of rate of ammonia oxidation and seems to be linked to the rate of organic carbon available for the heterotrophic fraction (Zhu and Chen, 2001b; Michaud *et al.,* 2006).

2.3.4. *Impact on the nitrification process*

Nitrification in a biological filter involves physical, chemical and biological agents, which are governed by a series of abiotic parameters (Chen *et al.*, 2006). Several studies have been conducted on parameters such as temperature (Zhu and Chen, 2002; Urakawa *et al.*, 2008), organic matter (Michaud *et al.*, 2009), dissolved oxygen (DO), pH (Chen *et al.*, 2006) and suspended particulate matter (Reeders and Bij de Vaate, 1991). Other factors, however, have been still little studied.

Some investigations on the transfer of nutrients within the biofilm seem to show that this process is directly related to the turbulence of the water flow with a considerable impact on nitrification (Chen *et al.*, 2006). Instead, little is known about the effects that the thrust of the water flow causes on the microbial community, because of the hydrodynamic conditions that are subjected the media filters. For example, in the case of mobile subunits (Moving Bed), water motion causes shock and friction, causing the thinning (Rusten *et al.*, 2006) and the probable detachment of biofilm portions.

The efficiency of ozone (O_3) in the processes of disinfection and water purification in aquaculture facilities or drinking water has been largely demonstrated in several studies (Krumins *et al.,* 2001a-b; Camel and Bermond, 1998; Rueter and Johnson, 1995; Summerfelt, 2003; Summerfelt *et al.,* 2009). Few others, however, have demonstrated the efficacy that ozone may have to get higher production rates for the rearing of some species: rotifers (Suantika *et al.,* 2001), *Artemia salina* (Wietze *et al.,* 2009), lobsters (Ritar *et al.,* 2006).

Wietz *et al.* (2009) have also demonstrated that the ozonation of rearing water involves direct effects on the development and growth of bacterial biofilm. By changing the oxidation-reduction potential (ORP) up to 290-320mV by treatment with ozone, a

stabilization of the internal structure and the acceleration of the cycle of bacterial biofilm formation were observed. Chang *et al.* (2004) define the redox potential (ORP) as the electric potential required to transfer electrons from one compound to another, to be used as indicative value of the oxidation state of a liquid. There are still few studies on the role that variations of compounds concentration that determine the ORP (such as organic carbon, NH_3, NO_2^- e NO_3^-) possess in causing changes in the bacterial community and in the biofilms development. Thus, it is clear that a deeper understanding of the effects of environmental variables on the biofiltration process can allow to better manage the biological filtration of recirculated systems, with a consequent increase in overall productivity of the farming system.

Several authors (Zhu and Chen, 1999; Zhu and Chen, 2001a-b; Michaud *et al.,* 2006) showed that the nitrification process, understood as the removal of TAN, is inhibited by increasing concentrations of particulate organic matter (POM). This effect seems to promote, as mentioned earlier, the growth of the heterotrophic component to the detriment of the nitrifying one (Léonard *et al.,* 2002). Moreover, the increase of POM, rather than DOM, composed mainly of humic substances (Léonard *et al.,* 2002), seems to be the real control factor of heterotrophic bacterial growth and biological filtration efficiency, increasing not only the number of bacteria but also their physiological activity (Michaud, 2007).

In particular, Michaud *et al.* (2006) have highlighted the effect that the accumulation of particulate organic matter causes on the efficiency of filtration of bacterial communities associated to biofilms of fixed bed biological filters (Static Bed). The authors have shown that within this type of biological filter, the increase of POM leads to a rapid decrease in the capacity of nitrification by the autotrophic bacteria, this is probably due to a different arrangement of the different layers that make up the thickness of the biofilm.

In fact, at high C/N ratio, fast-growing heterotrophic bacteria were found in the outer layers and they may represent an effective barrier against the diffusion of oxygen and ammonia to the deeper layers, where slow growing autotrophic nitrifying bacteria are probably pushed (Michaud *et al.,* 2006). In addition, because of their high rate of growth and reproduction, heterotrophic populations produce significant amounts of bacterial biomass with direct consequences on the operation of the filter: clogging and reduced nitrification capacity (Michaud *et al.,* 2006). In view of this, it is necessary to extend the study on the dynamics of nitrification in relation to the concentration of

organic matter by studying other mechanisms of biological filtration. This is very important to understand and control the evolution of a biological filter in order to be able to optimize the functionality.

2.4.   Aim of the work

The aim of present Ph.D. thesis was to deepen study the microbial community structure, dynamics and activities of recirculating aquaculture systems biological filters. Such new information, coupled with the already available literature, will contribute to allow reaching the possibility to really manage and control the microbial community in a RAS.

From previous works some experimental questions remain unanswered. The work has been divided in four objectives that will be treated in four separated chapters:

Objective 1 (Chapter 3). Michaud and colleagues (2009) reported the phylogenetic characterization of a RAS biofilter microbial community via the cloning and sequencing of packing media DNA. However, from that work any information could be obtained concerning the metabolically active fraction. Thus, the first objective of present Thesis was the study of the metabolically active fraction of the microbial community of a RAS biofilter via the extraction and cloning of the RNA.

Objective 2 (Chapter 4). If the impact of the C/N ratio (organic carbon/inorganic nitrogen) on the biofiler nitrification efficiency and on the bacterial abundances have been investigated by various authors (Zhou and Chen, 2001b; Michaud *et al*., 2006), to the best of our knowledge, little information is available on the impact of C/N ratio on the structure of the biofilter microbial communities. The second objective of present Thesis was to study if and how the increasing C/N ratio influenced two typologies of labscale biofilter communities (Mineral Static Bed and Plastic Moving Bed).

Objective 3 (Chapter 5). Various studies exist on the use of ozone as disinfectant in aquaculture but, to the best of our knowledge, no information are available on the impact of Redox potential (modified via ozone injection) on the biofilter nitrification efficiency and on the associated microbial communities. This experiment was carried out both at laboratory and pilot scale.

Objective 4 (Chapter 6). Several authors reported that inside aquaculture facilities obligate and/or opportunistic pathogens have been found, even if reared fish

resulted in a perfect sanitary status. In particular, *Vibrio* sp. related bacteria are widespread pathogens that cause many troubles in aquaculture industry. The aim of this last objective was to test the possibility to couple the ARISA fingerprinting approach (used for the two previous objectives) to the ITS (Internal Transcribed Spacer) sequencing, to rapidly identify Vibrios in biofilter.

CHAPTER 3

Diversity of the metabolically active bacterial fraction in the biological filter of a Recirculating Aquaculture System

3.1.   Introduction

A recirculating aquaculture system (RAS) is a complex environment whose microbiology is influenced by the rearing management protocols, the fish associated microflora and the makeup water parameters (Schreier *et al.,* 2010). In a RAS maintaining the fish health is directly dependent not only on good rearing water parameters (dissolved oxygen levels, solid waste and CO_2 removal and efficient nitrification), but also on the effective management of the microbial populations colonizing the system itself (Michaud *et al.,* 2009).

Significant differences in microbial communities composition were found between biofilter packing media and the rearing water (Michaud *et al.,* 2009), even if the biofilter has been recognized as the main bacteria supplier of a RAS in terms of abundance (Léonard *et al.,* 2000; 2002). Both communities represent unique and complex microenvironments where different actors (bacteria, virus, protozoa and micrometazoa) are tightly linked (Sharrer *et al.,* 2005; Wietz *et al.,* 2009). In particular, biofilter biofilms develop via the segregation of individual community members into distinct layers, according to their nutritional requirement (Schreier *et al.,* 2010).

Wietz *et al.* (2009), by using the Fluorescence In Situ Hybridization (FISH) approach for the study of biofilm formation in a marine RAS, observed that the predominant group was represented by *Gammaproteobacteria* followed by *Alpha-* and *Betaproteobacteria*, *Cytophaga-Flavobacteria-Bacteroidetes* (CFB) group and *Planctomycetes*. On another hand, Michaud *et al.* (2009), by using a clone library approach, reported that the large part of the microflora in a mature marine RAS was dominated by *Alpha-* and *Gammaproteobacteria* followed by CFB. Finally, Itoi *et al.* (2007), making libraries from freshwater aquarium filters, demonstrated that *Nitrospira* sp. was restricted to the outside layers.

It is well known that the RAS microbial flora includes both chemoautotrophic (e.g. nitrifiers) and heterotrophic bacteria, that actively consume oxygen and organic matter. Such bacterial communities harbor species that are obligate or facultative pathogens and that may cause disease in fish (Michaud *et al*., 2009). However, the main part of the heterotrophic bacterial compartment is constituted by "neutral microbes" that can contribute to maintaining a good microbial water quality by occupying niches and preventing proliferation of harmful species (Attramadal, 2011). Microbiological studies on aquaculture were generally addressed to the effects of diseases caused by fish pathogens and the use of antibiotics as treatment. However, an in-depth knowledge of the total heterotrophic microflora of the rearing system is necessary to detect the presence and accumulation of potential fish pathogens depending on the rearing conditions (Michaud *et al.,* 2006). Moreover, such investigation could provide information about the presence ∕ absence of potential biocontrol bacteria in the systems (Smith and Davey 1993; Austin *et al.,* 1995; Gram *et al.,* 1999).

To date, even if the new generation sequencing approaches (e.g. pyrosequencing) are rising more and more, the most widely used approach to examine bacterial diversity is based on 16S rRNA gene clone libraries construction (Cottrell and Kirchman, 2000). However, the cloning approach is unable to describing the whole diversity of a study site/environment (due to DNA extraction, PCR biases and so on), but also the study of the DNA gives any information on the phylogenetic affiliation of the physiologically active fractions within a bacterial community (Pedrós-Alió, 2006).

In this context, the aim of the present objective was to analyze the metabolically active fraction of the microbial community of a RAS biofilter via the extraction and cloning of the RNA.

3.2.   Material and Methods

3.2.1.   *Experimental RAS description*

The experimental RAS used for this work was located at the IFREMER research station of Palavas les Flots (France). It was equipped with three self-cleaning fish tanks (1 m^3) with particle separators. A simplified diagram of the system is shown in *Fig. 3.1*

and *Table 3.1*. The reared fish were sea bass (*Dicentrarchus labrax*) with an average weight of 80 ± 3 g.
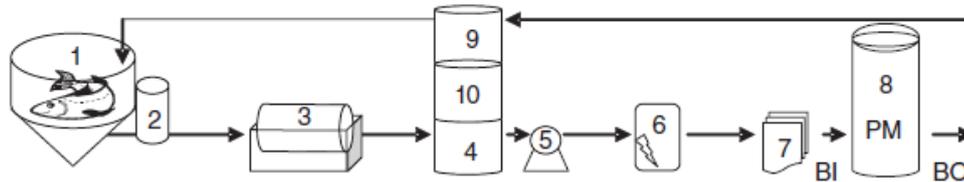
Fig 3.1: Simplified diagram of the Recirculating Aquaculture System and the Sampling Points.
System components (described in Table 1): 1) Fish tank; 2) Particle trap; 3) Mechanical filter; 4) Pump tank; 5) Pump; 6) UV lamp ; 7) Heat exchanger; 8) Biological filter; 9) CO_2 stripping; 10) Surplus Storage tank.
Points: BI) Biofilter Inlet; BO) Biofilter Outlet; PM) Biofilter Packing Media.

Table 3.1: Description of the marine RAS used through this study.

Number in Fig.3.1	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	$6{\sim}9m^3$/h
6	UV lamp	Bacteria control	$20{\times}10^{-3}J/cm^2$
7	Heat exchanger	Thermoregulation	$18\pm1°C$
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®

3.2.2. *Collection and preliminary treatment of samples*

Water samples were collected by using sterile polycarbonate 2 l bottles at the biofilter inlet (BI) and at the biofilter outlet (BO) (*Fig. 3.1*) as described in Michaud *et al.* (2009). In addition, subunits of the biofilter packing medium (PM) were sampled using a sterile and RNAse free beaker and treated with a detachment buffer as previously described by Michaud *et al.* (2009) in order to harvest cells.

For DNA and RNA extraction 1000 ml of water from BI and BO and 500 ml of PM detachment buffer, were concentrated on sterile 47 mm diameter, 0.22 μm pore size membranes (Millipore) and stored at -80°C until processing.

3.2.3. Bacterial enumeration

Samples for direct enumeration of free living and attached bacteria were fixed in 2% (vol/vol) formalin and stored at -20°C until processing. In order to overcome interference with particles, formalin-fixed samples were 50 fold diluted with sterile phosphate buffer saline (130 mM NaCl, 10 mM Na_2HPO_4 and 10 mM NaH_2PO_4, pH 7.4) and pre-filtered on 3 μm membranes (Nucleopore, Millipore) (Michaud *et al.*, 2006). Samples aliquots (0.5 ml) were filtered (<5 mm Hg) on 25 mm diameter, 0.22 μm pore size black polycarbonate filter, and stained with DAPI (4',6– diamidino–2–phenilindole) (Porter and Feig, 1980). Cells were visualized by epifluorescence microscope (Axioplan, Zeiss).

3.2.4. Nucleic acids extraction and RT-PCR

Genomic DNA and total RNA extraction were carried out by using RNA/DNA mini 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-transcribed by using the universal primer 1492R (5′-GGTTACCTTGTTACGACTT-3′). Quality of DNA and RNA samples was examined by agarose electrophoresis and concentrations were determined by Nanodrop[®] ND-1000.

3.2.5. Preliminary fingerprinting of the bacterial communities

A Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu *et al.*, 1997; Moeseneder *et al.*, 1999; Dunbar *et al.*, 2001) approach was used to preliminarily estimate the genetic diversity of bacterial communities for both DNA and cDNA.

The extracted DNA and cDNA 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 labelled 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. 300 ng 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 Genelab Laboratory (Italy).

Only profiles with a cumulative peak height ≥5,000 fluorescence unit 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:

$$Ap= ni/N \ x100$$

in which *ni* 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). Each sample was run in triplicate.

3.2.6. *Biofilter community cDNA cloning*

Amplification of 16S cDNA was carried out by using PCR as previously described by Michaud *et al.* (2009) and performed with an ABI 9600 thermocycler (PE; Applied Biosystems) by using the forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). The PCR was performed in a 50 µl (total volume) mixture containing 1× Q solution (Qiagen), 10× Qiagen reaction buffer, 1 µM of each primer, 10 µM dNTPs (Fermentas), 50 ng of cDNA template and 2.0 U of *Taq* Polymerase (Qiagen). The PCR program was as follows: 3 min at 95°C followed by 30 cycles for 1 min at 94°C, 1 min at 50°C, 2 min at 72°C and a final extension step of 10 min at 72°C.

The 16S cDNA fragments were cloned into the pGEM Easy Vector System (Promega) according to the manufacturer's instructions. The resulting ligation products were used to transform Escherichia coli ElectroMAX DH10B cells (Invitrogen). Two hundred and fifty white colonies were randomly picked, and the cells were directly used in a PCR reaction with standard primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') under the conditions described above. Amplified inserts of expected size (approx. 1200 bp) were identified by gel electrophoresis. Sequencing was performed by an outsourcing service (Macrogen Korea). Sequence analyses were performed by using the basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) and FASTA (Pearson, 1990). Sequence data were checked using the CHECK_CHIMERA program to determine the presence of any hybrid sequences (Maidak *et al.*, 2001). All sequences with similarity ≥97% were considered to represent one phylogenetic group or phylotype. Sequences were further aligned using the program Clustal W (Thompson *et al.*, 1994) to the most similar orthologous sequences retrieved from database. 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 tree was constructed using the MEGA 5 (Molecular Evolutionary Genetics Analysis) software (Kumar *et al.*, 1993). The robustness of the inferred trees was evaluated by 500 bootstrap resamplings.

3.2.7. *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 150 µl of the same cells suspension (in detaching buffer), used for DAPI counts (before formalin fixation), and were kept at room temperature ($25 \pm 1°C$). 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 RT. 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.

3.2.8. *Data analysis*

For statistical analyses clones were considered to define phylotypes at 97% of similarity, while for the T-RFLP analysis it was assumed that the number of peaks represented the species number (phylotype/genotype richness) and that the band

intensity peak height represented the relative abundance of each bacterial species (Danovaro *et al.,* 2006).

Cluster analysis and diversity indices were calculated by using Primer 6 software, version 6β R6 (Copyright 2004, PRIMER-E Ltd). In particular the Shannon-Wiener (H') and the Simpson Reciprocal (1/D) indices were computed.

To perform rarefaction analysis, total number of obtained clones compared with the number of clones representing each unique phylotype was used to produce the rarefaction curves. Coverage values were calculated to determine how efficient our clone library described the complexity of a theoretical community such as 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.

3.3.   Results

The bacterial abundances at the sampling time ranged from $1.05x10^6 \pm 1.68x10^5$ to $1.15x10^6 \pm 1.40x10^5$ cells ml^{-1} (MD±SD; mean ± standard deviation) at the inlet and the outlet of the biofilter respectively. The estimated total abundance of the biofilm colonizing the biofilter, were found to be $3.69x10^7 \pm 7.78x10^6$ cells g^{-1} of packing media.

In order to preliminarily investigate the structure of the bacterial community in both the rearing water (before and after the biological filter) and associated to the biofilter packing media, a T-RFLP approach has been carried out.
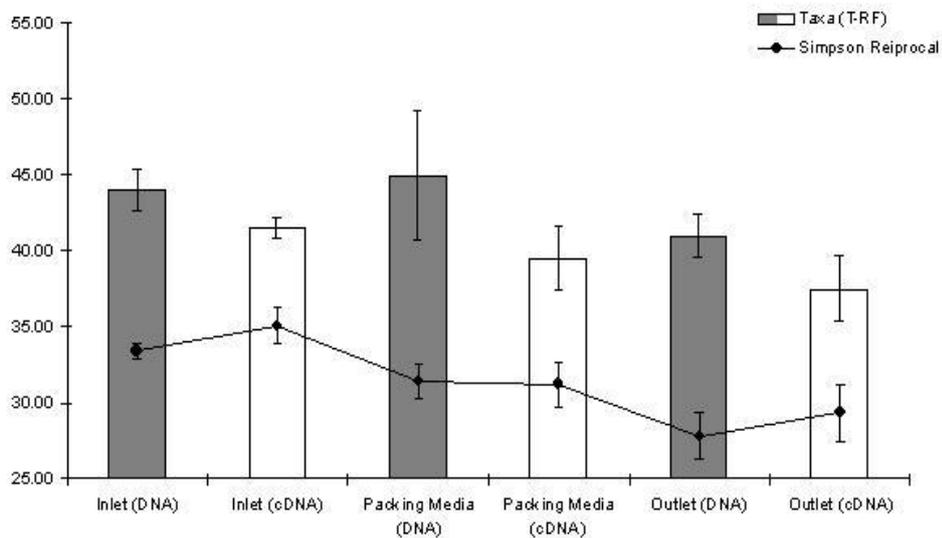
Fig. 3.2: Simpson reciprocal index (dots) compared to taxa (bars), expressed as T-RF.

The T-RF richness resulted comprised between 45 ± 4.24 to 37.5 ± 2.12 in the Biofilter packing Media (DNA) and in the Outlet (cDNA) (*Fig. 3.2*).

Results suggest that the T-RF richness decreased slightly from the biofilter Inlet to the Outlet, passing through the biofilter. In all the three samples the richness was higher for DNA than for the cDNA. If the Shannon diversity index did not significantly vary among all the samples (average H'= 3.27 ± 0.07) the dominance reciprocal Simpson Index decreased from 35.1 ± 1.2 at the Inlet (cDNA) to 29.3 ± 1.9 at the Outlet (cDNA).

The cluster analysis computed on the samples results highlighted that Inlet and Outlet samples were strictly correlated each other, while there was a strong difference between the rearing water samples and the packing media ones (*Fig. 3.3*).
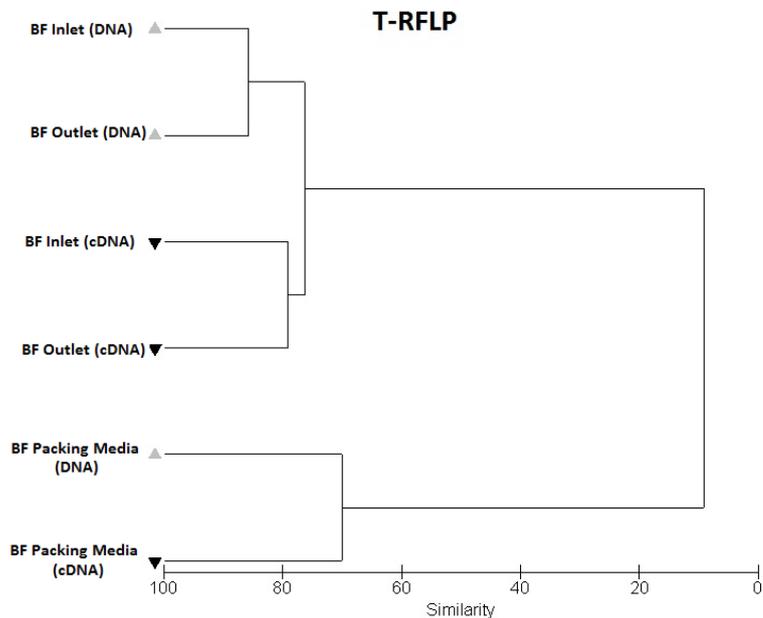
Fig. 3.3: T-RFLP cluster between cDNA and DNA samples

A total of 226 clones and 48 phylotypes (*Table 3.2; Fig. 3.4* and *3.5*) were obtained and sequenced (Coverage of 92%).

The 59.73% of screened clones belonged to the *Gammaproteobacteria*, mainly represented by the genus *Vibrio* (53 clones out of 135) (*Table 3.2; Fig. 3.4* and *3.5*).

Alpha-, Beta- and *Deltaproteobacteria* represented the 11.50, the 1.77 and the 0.88, respectively (*Fig. 3.4*). Among the remaining phyla the CFB represented the 7.96 % of the total clones.

RNA

Gammaproteobacteria
59.73%

Betaproteobacteria
1.77%

Deltaproteobacteria
0.88%

Alphaproteobacteria
11.50%

CFB
7.96%

Nitrospirae
5.31%

Unidentified
2.65%

Firmicutes
2.65%

Planctomycetes
3.98%

Acidobacteria
0.88%

Actinobacteria
0.44%

Verrucomicrobia
2.21%
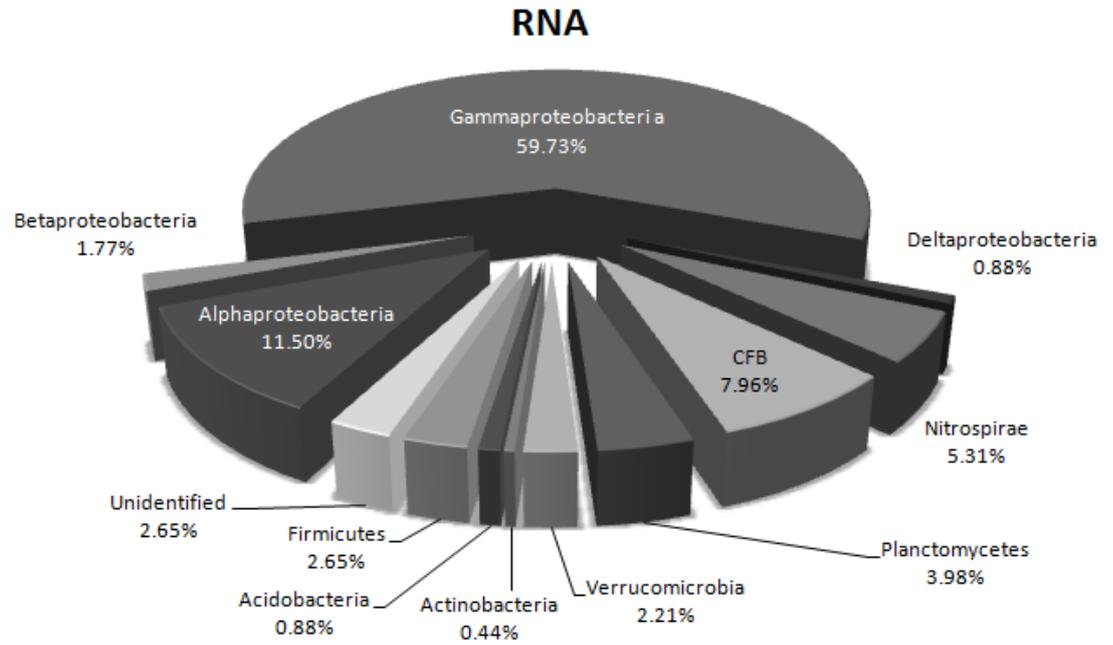
Fig. 3.4: Percentage presence of phylotypes described in *table 3.2*.

Finally, computed diversity index showed that the community was moderately dominated, as suggested by Simpson Reciprocal (1/D) with a value of 15.66 out of 48 max. In the same time, Shannon index (H) revealed that the community was highly diverse (3.3 out of 3.9 maximum).

Table 3.2: Phylotypes from cDNA sequencing

Next relative by GenBank alignment (AN*, organism)	Representative clone/isolate	Sim† (%)	N° of clones
Alphaproteobacteria			
Roseobacter sp. (AB257326)	RAS cDNA 47	97	5
Roseovarius aestuarii (EU156066)	RAS cDNA 48	98	4
Prosthecomicrobium sp. ATCC 27833 (GQ221767)	RAS cDNA 6	83	1
Sinorhizobium sp. enrichment culture clone Van49 (HQ222284)	RAS cDNA 22	93	1
Mesorhizobium sp. BA151 (EU748911)	RAS cDNA 24	93-96	3
Hyphomicrobium sp. Ellin112 (AF408954)	RAS cDNA 25	92	1
Alpha proteobacterium C32 (AB302373)	RAS cDNA 14	88-98	10
Iodide-oxidizing bacterium Mie-8 (AB159210)	RAS cDNA 47	92	1
Betaproteobacteria			
Nitrosomonas sp. (AJ296605)	RAS cDNA 2	94-96	4
Gammaproteobacteria			
Alkalispirillum sp. ACO5 (FJ976681)	RAS cDNA 45	92	1
Colwelliaceae bacterium AK17-004 (AB376285)	RAS cDNA 4	95	1
Coxiella burnetii (D89792)	RAS cDNA 11	93-95	4
Cronobacter turicensis (EF059863)	RAS cDNA 23	92	1
Ectothiorhodospira imhoffii (AM902494)	RAS cDNA 29	91-93	10
Glaciecola sp. #105 (AB238790)	RAS cDNA 28	94-99	5
Kangiella sp. K17-13 (FJ449700)	RAS cDNA 42	94	1
Pseudomonas stutzeri (AJ270453)	RAS cDNA 49	98	14
Vibrio alginolyticus (AB497062)	RAS cDNA 40	97-99	2
Vibrio brasiliensis (HM584042)	RAS cDNA 31	97	1
Vibrio campbellii (NR_029222)	RAS cDNA 36	97	1
Vibrio gigantis (JF836190)	RAS cDNA 32	93-100	4
Vibrio mediterranei (HM031977)	RAS cDNA 27	94-99	3
Vibrio lentus (AY292927)	RAS cDNA 37	98	1
Vibrio rotiferianus (JF907572)	RAS cDNA 41	100	1
Vibrio shilonii (JN039144)	RAS cDNA 34	99	2
Vibrio splendidus (JF836193)	RAS cDNA 43	95-99	3
Vibrio vulnificus CMCP6 (AE016795)	RAS cDNA 39	96	1
Vibrio sp. JZ08ML73 (HM117132)	RAS cDNA 30	93-100	34
Gamma proteobacterium N2yML2 (EF629832)	RAS cDNA 15	92-100	33
Marine gamma proteobacterium HTCC2089 (AY386332)	RAS cDNA 19	88-95	2
Uncultured bacterium clone 15 (JN157650)	RAS cDNA 10	88-100	10
Deltaproteobacteria			
Nannocystis exedens (DQ491074)	RAS cDNA 18	93	2
Nitrospirae			
Nitrospira marina (HQ686084)	RAS cDNA 3	90-99	12
CFB			
Algibacter sp. J13-26 (GU949539)	RAS cDNA 9	98	1
Bacteroidetes bacterium ArSB (AB539997)	RAS cDNA 16	87-94	6
Flexibacteraceae bacterium DG1232 (DQ486479)	RAS cDNA 5	83-98	2
Flavobacteriaceae bacterium scsio713 (JN116829)	RAS cDNA 8	90-97	4
Mucilaginibacter sp. KOPRI 25889 (HQ825037)	RAS cDNA 20	87	1
Maribacter sp. YA2 (JF751049)	RAS cDNA 26	94	1
Reichenbachiella sp. YMK (AB540004)	RAS cDNA 38	91-98	2
Winogradskyella thalassocola (GU799438)	RAS cDNA 44	88	1

Table 3.2: *(continued)*

Next relative by GenBank alignment (AN*, organism)	Representative clone/isolate	Sim† (%)	N° of clones
Planctomycetes			
Pirellula sp. (X81943)	RAS cDNA 50	96	4
Planctomycete A-2 (AM056027)	RAS cDNA 7	89-97	5
Verrucomicrobia			
Verrucomicrobium sp. KLE1210 (GQ262724)	RAS cDNA 17	93-96	5
Actinobacteria			
Actinobacterium CN30_LM100 (FN554443)	RAS cDNA 21	96	1
Acidobacteria			
Uncultured *Acidobacterium* sp. clone VERDEA88 (FJ902657)	RAS cDNA 1	92-93	2
Firmicutes			
Uncultured marine bacterium (GU326794)	RAS cDNA 33	95-98	6
Uncultured bacterium			
Uncultured bacterium clone DSJB94 (DQ499222)	RAS cDNA 13	85-99	6

*AN, accession number.

†Sim, similarity with the next relative.
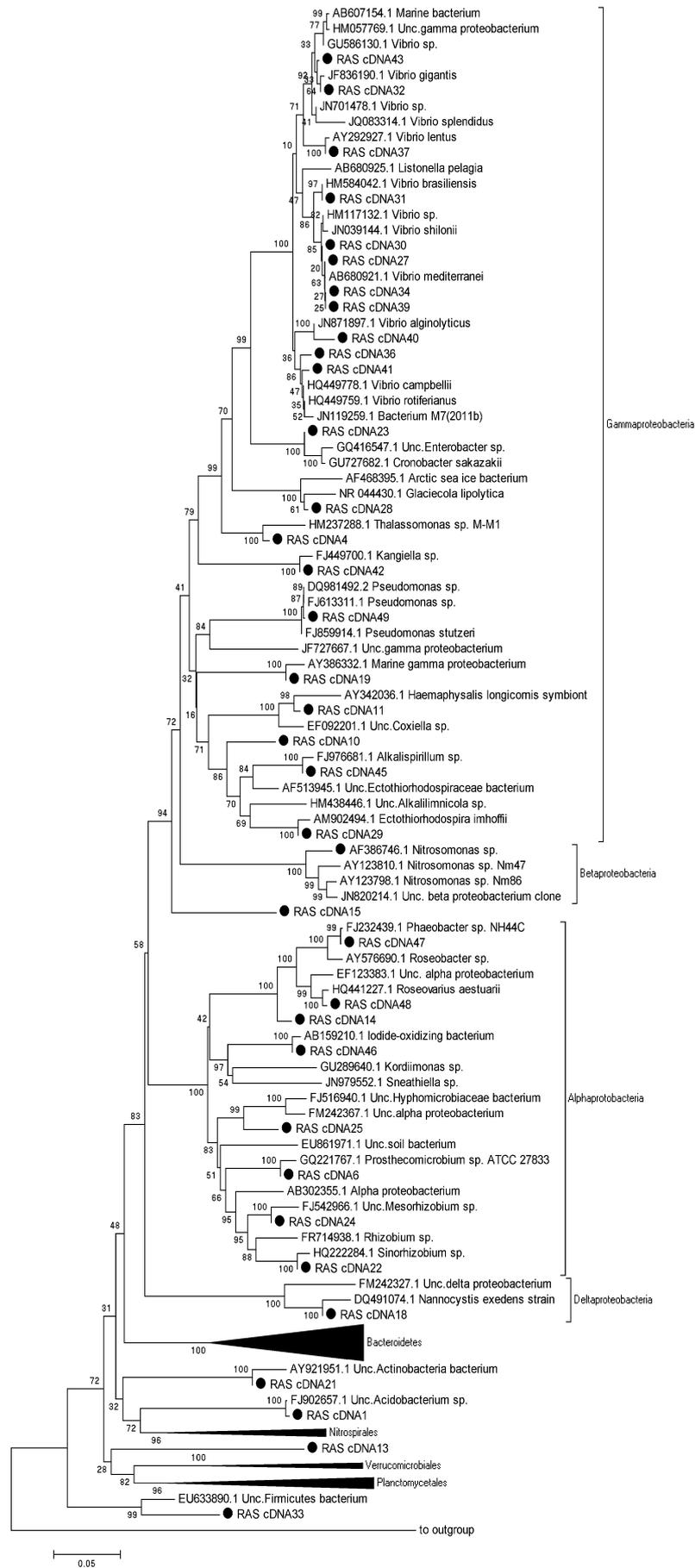
Fig. 3.5: Rooted phylogenetic tree showing affiliation of clones to closest-related sequences from cloned members of different bacterial clusters. Representative clones of different phylotypes are indicated with black dots. Sequence of Uncultured Crenarchaeote clone pSL17 (U63339) was used as outgroup, as described by Gentile and colleagues (2006).
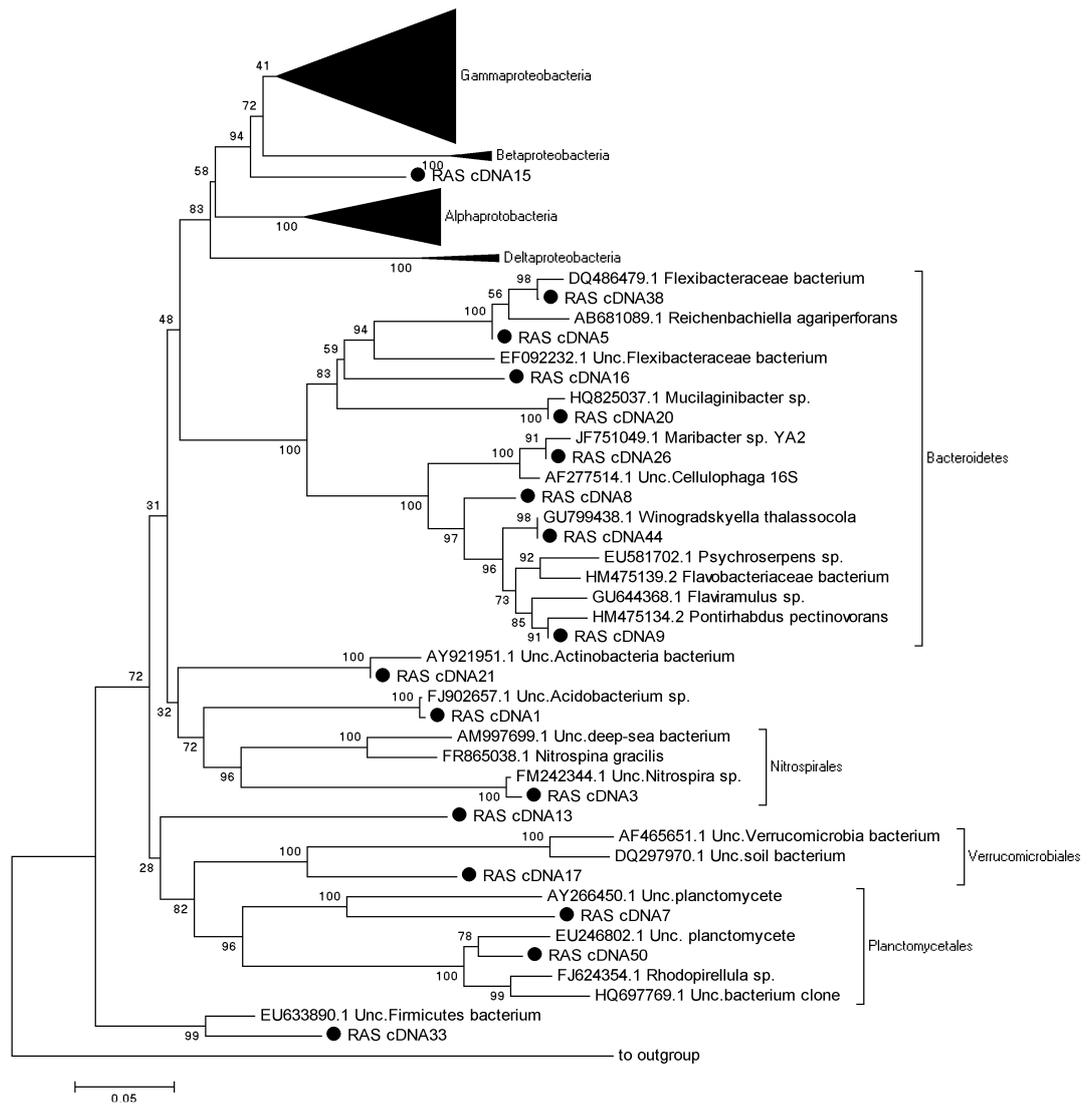
Fig. 3.5: *(continued).*

Plate counts about community-level physiological profiles (CLPP) did not show a real variation in bacterial community substrate degradation. A little increase was detectable from inlet water flow within the biofilter (Packing Media), but no variations were observed from Packing Media to outlet water flow (*Fig. 3.6*).
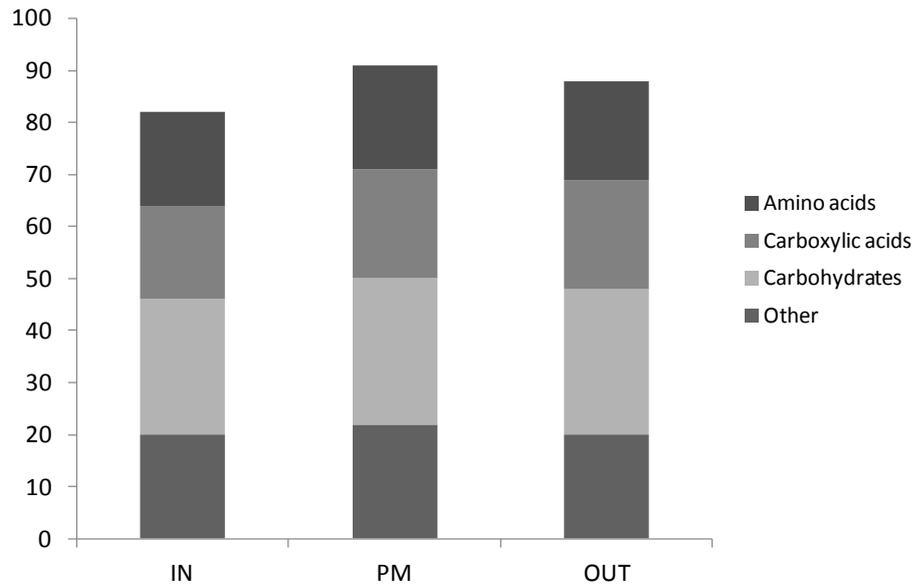
Fig. 3.6: CLPP analysis described number of carbon sources oxidized in both water and filtration media. IN, Inlet; PM, Packing Media; OUT, Outlet.

3.4.   Discussion

Metabolically active microbial fractions in RAS biofilters have not been investigated intensively. To our knowledge, there is no previous study that has retrieved 16S rDNA sequences from a pilot-scale RAS. For this reason, a better comprehension of the actors that determine the global activity of the microbial community into the biofilm, represent an key aspect in RAS ecology.

In this study a total of 226 clones were sequenced and 48 phylotypes were identify. Microbial communities was analyzed by 16S rDNA and 16S rRNA clones libraries creation and compared with each other in order to reveal the differences (if any) between total and metabolically active bacteria.

Around a quarter of whole sequences belonged to the *Vibrio* group (*Gammaproteobacteria*). These bacteria are generally widespread in the coastal and estuarine environments but also in/on aquatic animals, notably invertebrates, often associated with animal and human diseases (Farmer *et al.,* 2005; Austin and Austin, 2007). In particular, *V. alginolyticus*, a potentially fish pathogen (Austin and Austin, 2007; Austin 2009), and *V. vulnificus*, causing disease in both aquatic animals (Austin and Austin, 2007) and humans (Mouzopoulos *et al.,* 2008), were found. In particular, the latter is an important fish pathogen present in aquaculture systems (Hoffmann *et al.,* 2010).

The *Gammaproteobacteria* group was also represented, among others, by *Coxiella brunetii*, a gram-negative intracellular obligate bacterium, causative agent of the zoonosis Q fever (Voth and Heinzen, 2007) and *Pseudomonas stutzeri*, also detected in DNA samples, a gram-negative denitrifying bacterium in semiaerobic conditions, with oxygen as terminal electron acceptor (Lalucat *et al.,* 2006).

The second most abundant phylotype was that of the *Alphaproteobacteria* (11.50%). The principal species found are reported below.

Roseobacter spp., a genus common in cDNA and DNA sequences in this study; these organisms are gram-negative living in aquatic habitat with anaerobic oxygen requirements and they seem to be very important as probiotic organisms in environment like biological filters. Moreover, a genus belonging to the *Roseobacter* clade, *Roseovarius* sp., was also found. It was,represented by the novel species *R. aestuarii*, a gram-negative bacteria isolated from a Yellow Sea tidal flat located on the coast of Korea, growing with 7% NaCl (Yoon *et al.,* 2008).

Mesorhizobium spp., a genus of nitrogen-fixing organisms that lives in symbiosis with plants, present in marine habitats (Gage, 2004; Krick *et al.,* 2007).

Additional bacteria involved into the nitrification process were also detected in the biological filter, as described below.

Nitrosomonas spp., a genus of ammonia-oxidizing *Betaproteobacteria* (AOB), important players in wastewater treatment plants, where they get rid of excess ammonia by converting it to nitrite. Such species is slow growing, sensitive to environmental changes and, although ubiquitous in most aquatic environments, usually present only in low numbers and with low activity during adverse growth conditions (Painter, 1986).

Nitrospira marina has been isolated from ocean water, freshwater, aquarium water, deltaic sediment, deep-sea sediments, soils, and an iron pipe of a heating system (Daims *et al.,* 2001). *N. marina* is part of the nitrification process and it is now considered as the primary nitrite-oxidizing bacterium (NOB) in aquariums, as well as in wastewater treatment systems and other reactors (Hovanec *et al.,* 1998). However, high concentration of ammonia and acid pH inhibits *Nitrospira* nitrifying activity.

Furthermore, *Planctomycetes*, a phylum abundant in terrestrial and marine habitats, were also found. Such organisms are known to derive energy from the anaerobic oxidation of ammonia (Anammox bacteria) (Glöckner *et al.,* 2003).

Finally, members of *Bacteroidetes*, *Deltaproteobacteria*, *Verrucomicrobia* and *Acidobacteria* groups were also well represented.

Therefore, despite during start-up period all pioneers colonizing bacteria come from the same water body (Michaud, 2007), during biofilter activity bacterial community change and specializes in function of the ecologic niche that occupy and environmental forcing agents.

The presence of all the actors of Nitrogen cycle into detected active microbial fraction underline the importance of this metabolic pathway in biofilter performance.

Nitrosomonas spp. and *Nitrospira marina*, as seen before, are directly involved into nitrification process. It is common to all RAS and it is critical for maintaining sub-lethal ammonia concentrations between 0.1 and <3 mg L^{-1} (NH_3-N) depending on parameters including species, pH, and salinity (Stickney, 2000) with the ionized form, $NH4^+$, being relatively harmless (Stickney, 2000). Ammonia oxidation is associated with *Nitrosomonas* sp. in marine and freshwater aquaculture biofilters (Shreier *et al.,* 2010). Nitrite and ammonia oxidation occur together in nitrification biofilters and *Nitrospira sp.* is the dominant nitrite oxidizer. The alphaproteobacterial *Nitrobacter* sp. was not detected in RAS presumably because of its low substrate affinity compared to *Nitrospira* sp. (Urakawa *et al.,* 2008).

Several 16S rRNA gene phylotypes related to denitrifying heterotrophs including *Pseudomonas* sp. were found in anaerobic nitrate-stimulated enrichments derived from marine RAS nitrification communities, which likely contributed to nitrate-reducing activities (Borges *et al.,* 2008; Tal *et al.,* 2003). *Pseudomonas* spp., in particular the denitrifying bacteria *P. stutzeri* was found in the present work, appear to be the most abundant *Gammaproteobacteria* in marine RAS nitrification filters, by 16S rDNA clone libraries and enrichments (Borges *et al.,* 2008; Léonard *et al.,* 2000; Michaud *et al.,* 2009; Shreier *et al.,* 2010). However, present results about active microbial community composition demonstrate that *Pseudomonas* group was underrepresented compared to others Gamma, e.g. *Vibrio* sp., probably because denitrification process is promoted under anaerobic conditions, in dedicated compartments or deep inside biofilm layers (Lalucat *et al.,* 2006; van Rijn *et al.,* 2006; Morrison *et al.,* 2008), suggesting a limited activity in deeply anaerobic niches into biofilm.

In addition to denitrifyers, RNA sequences have provided evidence for the presence of *Planctomycetes*. This group is related to Anammox loop in marine RAS biofilters (Lahav *et al.,* 2009; Tal *et al.,* 2009). For RAS, the anammox pathway is important in removing ammonia, although it cannot replace denitrification because of

its sensitivity to organic acids and very slow doubling times (>11 days) (Paredes *et al.,* 2007).

As seen before, active fraction of the bacterial community, described through to RNA affiliations, is mainly represented by *Gammaproteobacteria*. In particular, a variety of bacteria potentially pathogenic for reared fish, belonging to the genus *Vibrio*, were identified in this study, despite the optimal sanitary status of the system. Due to relatively little dilution with make-up water and to the large organic loading rates placed upon these system, pathogens can accumulate to much higher concentrations within recirculating systems than in single-pass systems (Sharrer *et al.,* 2005). Microorganisms are carried into the recirculating system through the make-up water supply, stocked eggs or fish, building air exchange, fish feed, equipment used in and about the system, and staff/visitor contact with the system (Sharrer *et al.,* 2005). From these three-dimensional structures, bacterial cells could be detached by the water flux action and injected in the rearing system causing recurring disease in stressed fish or could lead to the presence of infected asymptomatic fish being consumed. Due to the high resistance of biofilms to antibiotics (Kimberly, 2004) it seems difficult to avoid the presence of such potential pathogens in RAS. The non expression of their virulence in such systems, without the use of antibiotics, could be ascribed to good system management and to the good physiological status of reared fish.

Alternatively, the presence of bacteria previously described as probiotics or producers of inhibitory compounds, as members of the *Roseobacter* clade (Gatesoupe, 1991, 1999; Irianto and Austin, 2002; Bruhn *et al*., 2005), suggests that the indigenous microbiota could control pathogenic organism development in fish rearing systems. In particular, *Roseobacter* strains have been found to be very active against vibrios (Hjelm *et al*., 2004).

In conclusion, considering both rearing water and biofilter packing medium, data obtained on the bacterial diversity of a marine RAS underline that good management of the rearing environment would benefit the maintenance of favorable water physicochemical parameters in these systems. High amounts of organic matter 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). Solid waste removal (fish faeces and potential unconsumed feed) is an important parameter to be controlled in recirculating aquaculture systems.

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 due to the increasing ineffectiveness of the currently legally available antibiotics. The ability to maintain pathogen-free facilities is a difficult task, however 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. It is well accepted that all microbial populations can be involved in maintaining an effective and stable rearing environment (*shelter effect*), probably through the release of chemical substances that have a bactericidal or bacteriostatic effect on other microorganisms or which outcompete for chemicals and available energy (Verschuere *et al.,* 2000).

One of the key aspects for improving the reliability and the sustainability of such RAS is having the ability to ''manage'' these bacterial populations in order to exploit their potentiality and use them as biocontrol agents (Verschuere *et al.,* 2000; Léonard *et al.,* 2000, 2002).

The analysis of the community structure (cluster analysis and T-RFLP) showed that it is not possible to describe a real difference between water community at the inlet and outlet of the biofilter. However, it also revealed that the biofilter RW community and the PM community are different, confirming what previously described by Michaud *et al.* (2009).

These results suggest that free-living bacteria, members of the rearing water community, are not influenced in structure and composition by Packing Media ones, probably because the exchange of bacterial cells between the water and the biofilm and *vice versa* can involve only superficial biofilm layers, while deeper ones remain stable and protected from the flow action (Michaud *et al.,* 2009). Rather, it could be argued that the RW bacterial community structure and composition are more sensitive to both the make-up water renewal rate (whose community is certainly influenced by the catchment environmental conditions) and the UV disinfection (Michaud *et al.,* 2009).

CHAPTER 4

Effect of C/N ratio on microbial communities structure associated to laboratory scale biological filters

4.1. Introduction

The aquaculture industry is actually challenging with major changes, growing from small-scale homestead-level activities to large-scale commercial farming, exceeding landings from capture fisheries in many areas (Gutierrez-Wing and Malone, 2006).

The need to increase aquaculture production is driving the industry toward more intensive practices. However, it has been estimated that 85% of phosphorus, 80–88% of carbon, 52–95% of nitrogen and 60% feed input in the rearing water will end up as particulate matter, dissolved chemicals, or gases (Gutierrez-Wing and Malone, 2006).

In Recirculating Aquaculture Systems (RAS) one of the key water treatment steps is represented by the biological filtration, as the primary means of production system water renovation for reuse within the system (Guerdat *et al.,* 2011).

Number of different types of biological filters can be used in a RAS and they all have their advantages and disadvantages. Therefore, proper selection and sizing of biofilters are critical to both the technical and economic success of a RAS (Malone and Pfeiffer, 2006). Classical biofiltration process is normally a single-stage where removal of organic matter and ammonia nitrogen is carried out simultaneously and where the nitrification is inhibited depending on the C/N ratio of influent (Michaud *et al.,* 2006; Pfeiffer and Wills, 2011; Zhu and Chen, 2001b).

Conventionally, biological filters adopt submerged granular static media (mineral or plastic) as the support for microbial biofilms that also provides the depth filtration action (Mendoza-Espinosa and Stephenson, 1999). However, recent development in biofilters has lead towards the use of moving bed biofilm reactors (MBBR) because of their commercial success in large scale wastewater treatment plants and relatively low costs to manufacture (Pfeiffer and Wills, 2011).

Typically, the capacity of a biological filter results limited by its nitrification ability and by various environmental factors (Michaud *et al.,* 2006; Pfeiffer and Wills, 2011). Biofilter nitrification efficiency is influenced by the organic load (often expressed as C/N ratio), the dissolved oxygen (DO) concentration of the rearing water, the Total Ammonia Nitrogen (TAN) concentration within the reactor, the water temperature, pH, alkalinity and salinity, and, obviously the biofilter management (Michaud *et al.,* 2006; Pfeiffer and Wills, 2011; Zhu and Chen, 2001b). Moreover, biofilter nitrification rates are influenced by the internal hydrodynamics which vary with the media used, the water flow and aeration rates, and the filling fraction of media in the reactor (Rusten *et al.,* 2006).

It has been reported that the organic matter has a significant negative effect on the nitrification efficiency (Michaud *et al.,* 2006; Zhu and Chen, 2001b). In fact, when dissolved and/or particulate organic carbon concentrations (DOC and POC, respectively) increased, the nitrification process is reduced due to competition between autotrophic and heterotrophic bacteria (Zhu and Chen, 2001b; Léonard *et al.,* 2002; Ling and Chen, 2005; Michaud *et al.,* 2006). Thus, the physical sharing of media surface, in addition to the competition for nutrients and oxygen, result in a stratified biofilm structure (Nogueira *et al.,* 2002). In this matrix the fast growing heterotrophic bacteria occupy the outer layer of the stratified biofilm where the substrate and oxygen concentration and detachment rates are higher, while the slower growing autotrophic nitrifying bacteria occupy the inner layer of the biofilm (Ohashi *et al.,* 1995; Satoh *et al.,* 2000; Nogueira *et al.,* 2002; Michaud *et al.,* 2006).

The C/N (organic carbon / inorganic nitrogen) ratio concept has often been used as a link between the availability and competition for organic carbon and ammonium (Hu *et al.,* 2009). At high C/N ratios the heterotrophic bacteria reduce the diffusion of nitrogenous substrate and dissolved oxygen (DO) to the autotrophic nitrifying bacteria, thus negatively affecting the rate of nitrification (Nogueira *et al.,* 2002; Chen *et al.,* 2006). Reduction in TAN removal rates as high as 70% have been reported at C/N ratios above 1.0 (Zhu and Chen, 2001b; Ling and Chen, 2005) for dissolved carbon, while a reduction of 73% has been reported at C/N ratio of 2 for particulate carbon (Michaud *et al.,* 2006).

It should be pointed out that to date, to the best of our knowledge, the effect of organic carbon on the biofilter has been studied with respect either to total reactor performance or to microbial spatial and quantitative distribution (FDZ-Polanco *et al.,*

2000; Michaud *et al.,* 2006; Ohashi *et al.,* 1995), but hardly to the community shift (Hu *et al.,* 2009).

In this context, the aim of present experiment was to determine the adaptation of the microbial community structure, associated to biofilm in pilot-scale biofilters, at the increasing of C/N ratio, expressed as organic carbon/inorganic nitrogen.

4.2. Material and Methods

4.2.1. *Experimental system*

Two identical laboratory scale biofilter units (A and B) were used for this work receiving the same influent water constituted from heated (25 °C ± 2), sand-filtered, and UV disinfected seawater (salinity 37 ‰ ± 1). The pH (7.5 ± 0.5) was maintained constant by adding 10 M NaOH, when necessary.

Each unit (*Fig 4.1*) was constituted by 4 biofilters (volume of 9.5 l). 2 of them (replicates) were operated as submerged reactors (defined as Static Bed, SB) and filled with a mineral packing media (cooked clay with a high specific surface usable by bacteria of more than 600 m^2/m^3, Biogrog). The other 2 biofilters (replicates) were operated as moving bed reactors (defined as Moving Bed, MB) with a plastic packing media (Acui-T, specific surface of 800 m^2/m^3). For each unit one 1 m^3 tank was used to store the nutrient enrichments (inorganic N and organic C, as described below) and a 300 l tank was used as a buffer for make up water (40 µm prefiltered seawater). The raw seawater was pumped into each biofilter at a constant flow rate of 2 l min^{-1} (controlled with one flow rate meters per biofilter) and the enrichment was pumped at a constant flow rate of 0.2 l min^{-1}.

The characteristics of the biofilters are shown in *Table 4.1*.
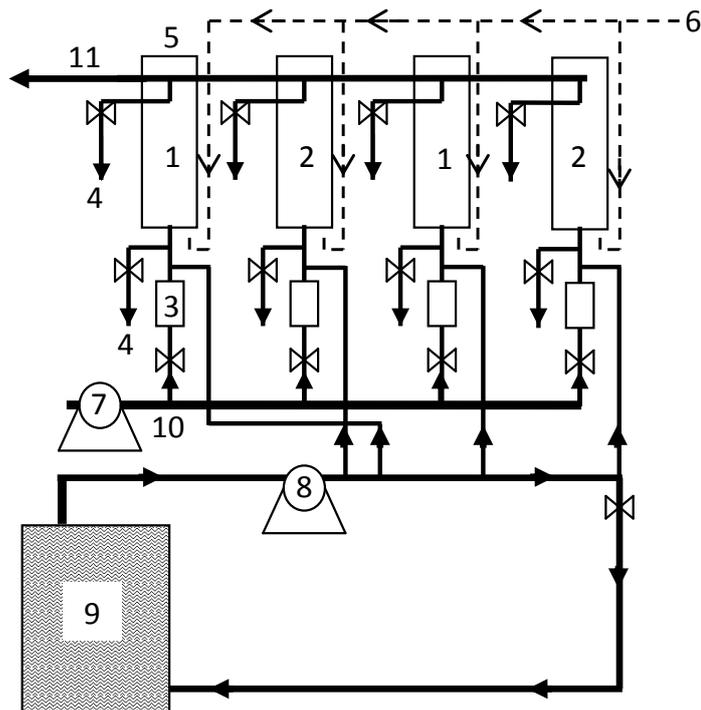
Fig. 4.1: Pilot scale biofilter unit scheme. 1) Static Bed filters (x 2, Biogrog packing media); 2) Moving Bed filters (x 2, Acui-T media); 3) Flow metres (x 4); 4) sampling ports for waters analyses (x 2 for each filter); 5) sampling port for packing media; 6) Air line; 7) circulation pump; 8) peristaltic pump for nutrients enrichments; 9) enrichment reservoir; 10) water inlet; 11) water outlet.

Table 4.1: Pilot scale biofilters specifications.

	Symbol	Biofilter
Diameter	$\varnothing$	11 cm
Cross-sectional area	$\pi \times r^2$	95 cm²
Height	h	85 cm
Volume	S x h	8075 cm^3
Flow rate	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

4.2.2. *Experimental procedures*

Inlet water was enriched with Particulate Organic Matter (POM) and ammonium chloride (see below). Theoretical Carbon/Nitrogen levels were fixed as 0, 0.5 and 2 for unit A and 0, 0.8 and 4 for unit B. Each C/N ratio step, set up in duplicate (two filters for MB and two for SB), was run for at least four weeks to allow the formation of a

Steady state biofilm. A daily routine was carried out for chemical and physical parameters measurement (controlling parameters) all the experiment period.

At the end of each C/N ratio step, samples were collected for chemical and microbiological analyses (see below), and the sequent step was set up and run again for 4 more weeks.

4.2.3.   Enrichment mixture

The input of ammonium chloride (Sigma) was set to achieve an ammonia concentration of 1 mg l^{-1} at the inlet of each biofilter. This concentration was kept constant for all the experiment and the C/N ratio was obtained through the change of the only carbon source concentration.

The organic carbon used for the experiment was composed by fish feces and unconsumed feed collected from particle separators at the outlet of various experimental seabass rearing systems at the IFREMER Research Station (Palavas les Flots, France). Such mixtures (94% ± 0.1 dry matter) were pooled and sterilized. Then it was freeze dried and finally grinded in a fine homogenous powder as described in Michaud *et al.* (2006). The resulting powder was chemically analyzed with and auto-analyzer Carlo Erba Instruments 1500 CHN (Verardo *et al.,* 1990) for the determination of average carbon content (42% ± 0.8 of Total Organic Carbon, data not shown), and stored in the dark at -20°C during all the experiment duration.

4.2.4.   Sampling

Bacterial communities associated with few packing media subunits were detached following the procedure described in Michaud *et al*. (2006). Briefly, packing media subunits were placed in a detachment buffer (0.1% of sodium pyrophosphate in a phosphate buffer saline: 130mM NaCl, 10mM Na_2HPO_4, and 10mM NaH_2PO_4 at a pH of 7.4), manually scraped with a sterile blade or brush and then placed in an ultrasonic bath for 10min at 20 kHz, in an ice bath. This mixture was collected, divided in various aliquots and used for microbiological analyses.

All media and solution used were sterilized by autoclaving at 121°C, 1 atm for 20 min.

4.2.5. Bacterial enumeration

Bacterial concentrations were expressed as CFU or cells per gram of wet packing media (Léonard *et al.,* 2000) because the surface area of the mineral packing material was not well defined. All data are expressed as mean plus standard deviation (M±SD).

***Culturable fraction*.** Cultivable heterotrophic bacteria associated with the packing media were enumerated by Colony Forming Units (CFU ml^{-1}) counts on Marine Agar 2216 (MA, Difco) as described in Léonard *et al.* (2001) and Michaud *et al.* (2006). Plates were set up in duplicate for each dilution. Only plates having between 20 and 200 colonies were considered.

***Total counts*.** The bacterial cell counting was performed through DAPI staining, as described in the precedent chapter (section 3.2.3.).

4.2.6. Fluorescence In Situ Hybridization (FISH)

Fluorescence In Situ Hybridization (FISH) with rRNA-targeted oligonucleotide probes was carried out according to Aman *et al.* (1995), MacDonald and Brözel (2000) and Nogueira *et al.* (2002). Cells fixation was carried out with paraformaldehyde in phosphate-buffered saline (final concentration of 4%, w/v) and incubated at 4°C for 12 h. Triplicate samples were concentrated from fixed detachment solution on white polycarbonate filters (Isopore, diameter, 25 mm; pore size, 0.22 mm). FISH was performed by using CY-3 labelled probes (MWG, M-Medical, Italy) listed in *Table 4.2*.

Hybridization procedures were carried out as described by Glöckner *et al.* (1999). Hybridized cells were counter-stained with 4',6'-diamidino-2-phenylindole (DAPI) (1 μg ml^{-1}) for 10 min and washed in PBS and distilled water (Amann *et al.,* 1995).

Cells were observed using an Axioplan epifluorescence microscope (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.

4.2.7. Genomic DNA extraction

Membranes were pretreated with 150 µl of a 5 mg ml^{-1} lysozyme solution for 10 min and minced filters were put 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.

4.2.8. Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Standard ARISA protocol. In order to analyze the bacterial community structure in all samples by automated rRNA intergenic spacer analysis (Fisher and Triplett, 1999), extracted DNA was amplified in duplicates using the bacteria-specific primers ITSReub (5'-GCCAAGGCATCCACC-3') and ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') (Cardinale *et al.,* 2004) with the latter being labeled with the phosphoramidite dye FAM. Normalized DNA quantities of 25 ng per reaction were used to perform PCR reactions as described by Ramette (2009). PCR was carried out in an Eppendorf MasterCycler (Eppendorf, Hamburg, Germany) with an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 90 s, with a final extension at 72°C for 5 min. The resulting amplified fragments were purified with Sephadex G-50 Superfine (Sigma Aldrich, Munich, Germany) and identified by capillary electrophoresis on an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems) as follows.

A standardized amount of DNA (100 ng of DNA, as determined spectrophotometrically) was added to a separation mix containing 0.5 µl of internal size standard Map Marker 1000 ROX (50–1000 bp) (BioVentures, Inc., Washington, DC), 0.5 µl of tracking dye (BioVentures) and 14 µl of deionized Hi-Di formamide (Applied Biosystems, Foster City, CA). The preparation was denatured 3 min at 95°C and kept on ice at least 5 min before being further processed by the sequencer as described by Ramette (2009).

The obtained ARISA profiles were analyzed using the GeneMapper Software v 3.7 (Applied Biosystems, Carlsbad, CA, USA) with minimum peak heights of 50 fluorescence units for all dyes.

Binning. From GeneMapper output tables, conversions to sample-by-fragment tables and subsequently to sample-by-binned-OTU tables were performed by using custom R with automatic and interactive binner scripts. The algorithm rearranges the data and calculates the relative fluorescence intensity (RFI) of each peak by dividing individual peak areas by the total peak area for the respective sample. All peaks with RFI values of 0.09% were not included in further analysis since they consisted of background peaks. To include the maximum number of peaks while excluding background fluorescence, only fragments above a threshold of 50 fluorescence units and ranging between 100 and 1000 bp were taken into consideration (Ramette, 2009).

The window size (WS) was estimated empirically for each fingerprinting pipeline by running appropriate controls at different times, for instance. Binning has a significant effect on the obtained sample similarities and must thus be accounted for to avoid falsely describing ecological differences that would be only due to technical variability (Hewson and Fuhrman, 2006). A shifting window size binning strategy was used since it offers the possibility to optimally align electrophoretic profiles and to deal with different window starting positions (Hewson and Fuhrman, 2006). The binning frame that offers the highest similarity among samples is identified out of all binning frames starting at a given position. The distance between two consecutive binning frames is defined as the shift (Sh) value that was 0.1 bp. The interactive script reported the best frame among all calculated as the one that maximizes sample similarities. Another script allows for an automatic calculation of a series of WS values (e.g., 0.5, 1, 2, 3, 4, and 5 bp) for a given Sh value (e.g., 0.1 bp). To obtain the best binning strategy for a data set without a priori knowing the ideal WS value it was used the automatic binner script (Ramette, 2009). A compromise between high resolution (low WS) and high similarity among samples (high WS) was found based on the output of the scanning mode script. The interactive and automatic binning algorithms were implemented in the free, R programming language (The R Foundation for Statistical Computing).

4.2.9.   *Physiological diversity of bacterial communities*

CLPPs were determined as described in the section 3.2.7.

4.2.10. *Statistical analyses and diversity indices calculation*

Results of bacterial abundances were analyzed using analysis of variance (ANOVA). Comparison between groups for a significant difference of mean or rank values was performed after normality and variance tests.

Statistical calculations were performed with SigmaStat software for Windows, version 2.0 (Copyright 1992-1995 Jandel Corporation).

ARISA peaks profiles were analyzed after *ad hoc* data pretreatment, the analyses of resemblances among communities (Bray Curtis) were computed by using Primer 6 software, version 6β R6 (Copyright 2004, PRIMER-E Ltd). Genotype richness was expressed as the total number of peaks within each gel lane or electropherogram.

The ARISA data reflecting relative OTU abundance, FISH counting and CLPP profiles, were separately used to calculate Bray–Curtis pairwise distances between samples which were further visualized in a lower dimensional space by applying non-metric multidimensional scaling (nMDS) and cluster analysis. nMDS is a nonmetric procedure that is robust to outliers and preserves the rank orders of the relative distances among points in the higher dimensional data cloud as well as possible in a smaller number of dimensions (Lear *et al.,* 2008).

Analysis of similarities (ANOSIM) was carried out in order to assess statistical differences between bacterial community structures in multivariate data sets. ANOSIM was used to compare Static Bed vs Moving bed. ANOSIM result produces a sample statistic, R, which represents the degree of separation between test groups (Clarke, 1993). A value close to 1 indicates the community composition is totally different, whereas a value of 0 indicates no difference.

In order to evaluate structural diversity between samples the Shannon-Wiener diversity index (H') and the reciprocal Simpson dominance index (1/D) were computed. The former takes into account the number of species present and their relative importance within the assemblage, the latter by computing the probability that two taxa chosen at random will be from the same OTU gives a strong weighting to the dominants taxa. Fnally, the evenness (Pielou index, J), which reflects the relative importance of each taxon within the entire assemblage, were calculated (Danovaro *et al.,* 2006). For these calculations, it was assumed that the number of peaks represented the species number (phylotype/genotype richness) and that the band intensity peak height

represented the relative abundance of each bacterial species. These calculations were performed using Primer 6 software, version 6β R6 (Copyright 2004, PRIMER-E Ltd).

4.3.   Results and Discission

4.3.1   Bacterial Abundances

Static Bed (SB). The viable counts in the Static Bed filters ranged from $2.09 \times 10^4 \pm 8.43 \times 10^3$ CFU ml^{-1} (MD±SD; mean ± standard deviation) for C/N 0 to a maximum of $8.23 \times 10^5 \pm 1.24 \times 10^4$ CFU ml^{-1} (MD±SD) for C/N 4. The total bacterial counts were $2.53 \times 10^6 \pm 4.23 \times 10^5$ cells ml^{-1} (MD±SD) for C/N 0 and $1.43 \times 10^7 \pm 5.73 \times 10^5$ cells ml^{-1} (MD±SD) at C/N 4 (*Table 4.3*).

Moreover, ANOVA results revealed that there was a statistically significant difference (*P*<0.001) for viable counts. The subsequent Pairwise Multiple Comparison (PMC) procedure (Tukey Test) pointed out that there was not a statistical significant difference between C/N 0 and 0.5 as well as between C/N 2 and 4. On the other hand, total count were statistically different (ANOVA, *P*<0.001) and the subsequent PMC analysis confirmed this difference for all C/N ratios pairwise excepted between C/N 0 and 0.5 (P< 0.05). Finally, there was a linear relationship between total counts and viable counts with an R^2 of 0.983 (*P* = 0.00275) (not shown).

Moving Bed (MB). In the Moving Bed biofilters the viable counts ranged from $3.01 \times 10^4 \pm 8.94 \times 10^3$ CFU ml^{-1} (MD±SD) at C/N 0.5 to $7.99 \pm 1.77 \times 10^4$ (MD±SD) at C/N 0.8, while total counts ranged from $2.68 \times 10^6 \pm 1.00 \times 10^5$ (MD±SD) at C/N 0 to $3.83 \times 10^6 \pm 1.04 \times 10^5$ (MD±SD) at C/N 2 (*Table 4.3*). Also in this case ANOVA results revealed that there was a statistically significant difference (*P*<0.001) both among viable and total counts. However, the subsequent PMC procedure (Tukey Test) revealed that there was not a statistical significant difference among C/N 0, 0.5 and 0.8 as well as between C/N 2 and 4 (*P* >0.050) for both viable and total counts. Linear correlation showed a good R^2 (0.932, *P* = 0.021) between total counts and viable counts (not shown).

SB vs MB. In the two biofilter configurations the bacterial abundances at C/N 0 and 0.5 resulted quite similar without significant differences (Tukey Test, > 0.05), while on the contrary at the three other ratios tested the abundances, both viable and total

counts, resulted significantly higher in the Static Bed filter than in the Moving one (Table 4.3). Finally, the cultivability resulted similar at C/N 0 and 0.5 between the two biofilter configurations, but significantly higher for Static than Moving bed at the other C/N ratios (not shown).

Table 4.3: Bacterial abundances (St.Dev.: Standard Deviation) and EUB –stained cells.

Static Bed	CFU ml^{-1}		Cells ml^{-1}		CFU/Total	EUB 338
	Mean	St. Dev.	Mean	St. Dev.	Counts (%)	(% of DAPI)
C/N 0	2.09×10^4	8.43×10^3	2.53×10^6	4.23×10^5	0.83	49.5 ± 7
C/N 0.5	7.30×10^4	2.45×10^4	3.25×10^6	3.33×10^4	2.25	55.4 ± 9
C/N 0.8	3.80×10^5	1.09×10^5	5.78×10^6	6.22×10^5	6.59	50.2 ± 5
C/N 2	6.81×10^5	1.30×10^5	1.30×10^7	2.19×10^5	5.25	59.9 ± 3
C/N 4	8.23×10^5	1.24×10^5	1.43×10^7	5.73×10^5	5.75	79.9 ± 8
Moving Bed	CFU ml^{-1}		Cells ml^{-1}		CFU/Total	EUB 338
	Mean	St. Dev.	Mean	St. Dev.	Counts (%)	(% of DAPI)
C/N 0	3.65×10^4	4.62×10^3	2.68×10^6	1.00×10^5	1.36	70.3 ± 9
C/N 0.5	3.01×10^4	8.94×10^3	2.94×10^6	3.07×10^5	1.02	64.4 ± 9
C/N 0.8	5.02×10^4	1.17×10^3	2.93×10^6	1.00×10^5	1.71	68.1 ± 6
C/N 2	7.99×10^4	1.77×10^4	3.83×10^6	1.04×10^5	2.09	70.1 ± 5
C/N 4	7.94×10^4	3.54×10^3	3.62×10^6	1.19×10^5	2.22	86.5 ± 10

4.3.2   FISH

Static Bed (SB). The percentage of DAPI-stained cells that were enumerated by the EUB 338 probe mix ranged from $49.5 \pm 7\%$ for C/N0 and $79.9 \pm 8\%$ for C/N 4 (ANOVA *P*<0.001) (*Table 4.3*). FISH probes, reported as percentage of EUB stained cells, for the main phylogenetic groups revealed that for C/N 0 and 0.5 the *Gammaproteobacteria* were predominat (25 and 28.1%, respectively) (*Fig 4.2a*), followed by *Alphaproteobacteria* (21 and 22%, respectively) and HGC (11 and 15.6%, respectively). For the higher C/N ratio, namely 2 and 4, HGC became dominant (21.1 and 25.1%, respectively). CFB became dominant, together with HGC only at C/N 4. LGC (namely *Firmicutes*) remain stable, ranging from 7% at C/N 0 to 10.1% at C/N 2.
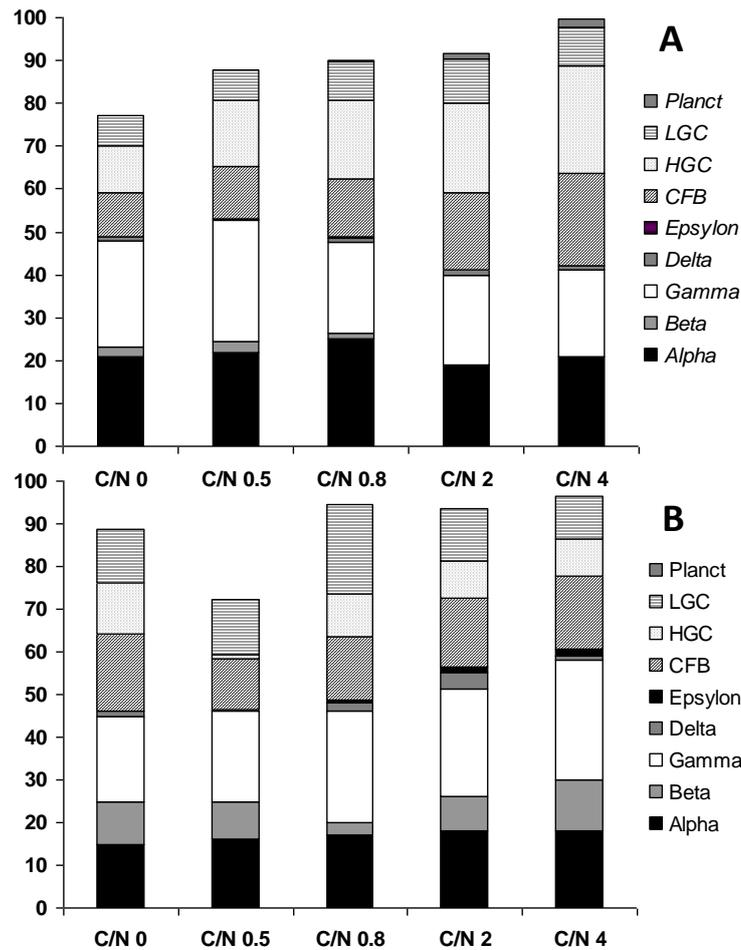
Fig. 4.2: Bacterial community composition by FISH analysis at increasing C/N ratios.

Finally, *Planctomycetes* were detected only at higher C/N ratios (from 0.8 to 4), ranging from 0.2 to 2.1% (*Fig. 4.2a*). Bray-Curtis similarities resulted on average 86.7 ± 5% with the maximum value (93.6%) between C/N 2 and C/N 4. The subsequent SIMPER analysis pointed out that *Gamma-* and *Alphaproteobacteria* contributed for 27.3 and 26.3%, respectively, to the group similarity.

Moving Bed (MB). The percentage of DAPI-stained cells detected with EUB 338 probe mix ranged from 64.4 ± 9% for C/N 0.5 and 86.5 ± 10% for C/N 4 (*Table 4.3*). In *Fig. 4.2b* results of FISH probes for main phylogenetic groups for Moving Bed filters are reported (percentage of EUB stained cells). In this case *Gammaproteobacteria* were predominat within the community at each C/N ratio (ranging from 20% at C/N 0 to 28.1% at C/N 4). *Alphaproteobacteria* and CFB remained stable at each C/N ratio (16.8 ± 1.3% and 15.6 ± 2.3% average, respectively). LGC were maximal at C/N 0.8 (21 %) and HGC decreased from C/N 0 to C/N 4 (ranging from 12% to 8.7%). Bray Curtis similarities resulted on average 86.1 ± 3.9%. Maximal similarities were computed

between C/N 2 and 4 (92.8%). Also in this case, *Gamma-* and *Alphaproteobacteria* contributed for 28.6 and 20.9%, respectively, to the similarity of MB group (SIMPER analysis).

SB vs MB. The nMDS computed on the whole FISH data-set (Static and Moving Bed together) revealed that the two biofilter configurations were different in their community structure (*Fig 4.3a*). In both case it can be possible to see three different situations: one corresponding to very low or null added carbon, the second corresponding to an intermediate carbon supply and the last corresponding to the maximum carbon addiction (namely C/N 2 and 4). On the basis of the Bray-Curtis similarities ANOSIM was computed by using the "Biofilter Configuration" as factor. This technique calculates a Global R-value, which usually falls between 0 and 1, indicating some degree of discrimination between treatments. In our case the analysis showed a clear global difference among the two biofilter configurations (R= 0.78 p < 0.01). Finally, SIMPER analysis pointed out that between SB and MB the main contribution to their average dissimilarity (21.5%), was due to HGC, *Betaproteobacteria* and LGC (cumulative contribution 60.6%).
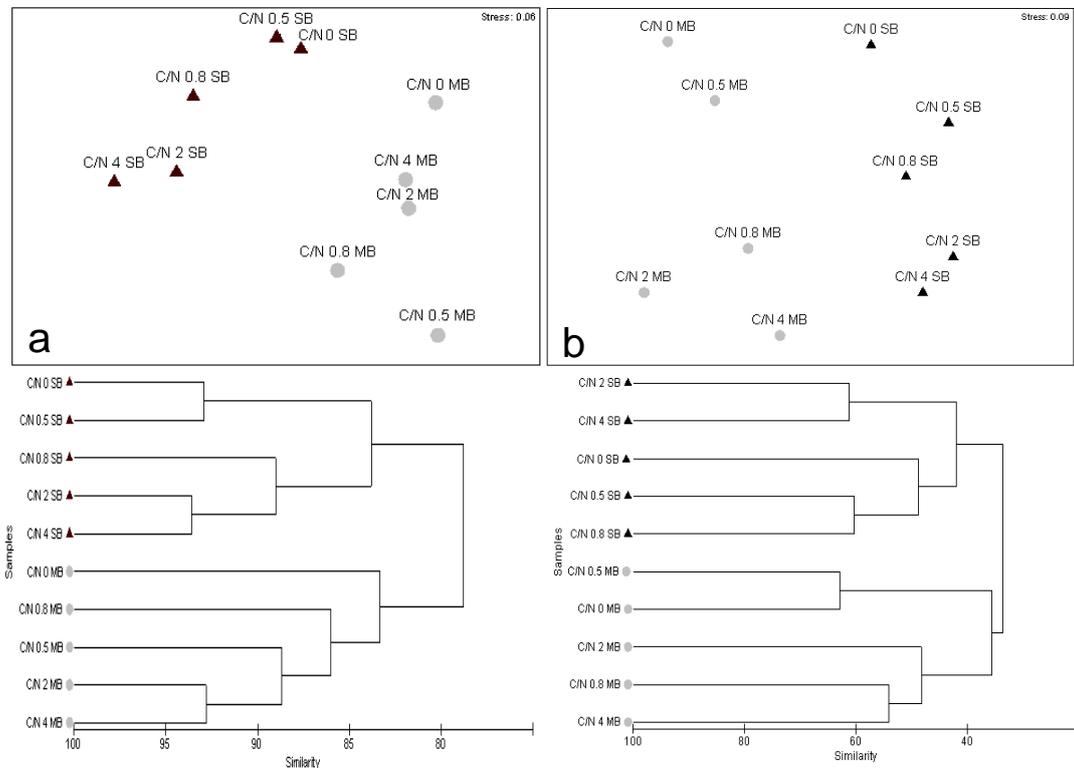
Fig 4.3: Community structure in SB *vs* MB: a) FISH results; b) ARISA results.

4.3.3   Communities fingerprinting

Static Bed (SB). The richness of ARISA fragments ranged from 73 to 54 at C/N 0 and 4, respectively. Only 12 fragments were common to all the C/N ratios, while 29 fragments were found exclusively at C/N 0. Diversity indices computed revealed that at the two lowest C/N ratios the communities were the most diverse and also the most dominated. From C/N 0.8 to 4 the reciprocal of Simpson index falls down.

Moving Bed (MB). For Moving Bed biofilters the richness ranged from 70 to 51 genotypes at C/N 0 and 4, respectively. 10 fragments were common to all the C/N ratios. Only 19 fragments were exclusively from C/N 0. In this case the, Diversity Indices revealed that at C/N ratios from 0 to 0.8 the diversity was maximal.

SB vs MB. If the shift of the communities structures seems to follow a similar pattern in both the Static and Moving Bed biofilters, the Pairwise comparison of the ARISA profiles at each tested C/N ratio revealed only an average $42.5 \pm 5.5\%$ of similarity between the two communities. In fact, as observed for the FISH analysis, the biofilter community structure resulted different (*Fig. 4.3*). The ANOSIM, computed by using the "Biofilter Configuration" as factor, confirm a global difference among the two biofilter configurations (R= 0.678 $p < 0.01$).

All diversity indices described showed a decrement from C/N 0 to C/N 4 in both Static and Moving filters (*Fig. 4.4*).
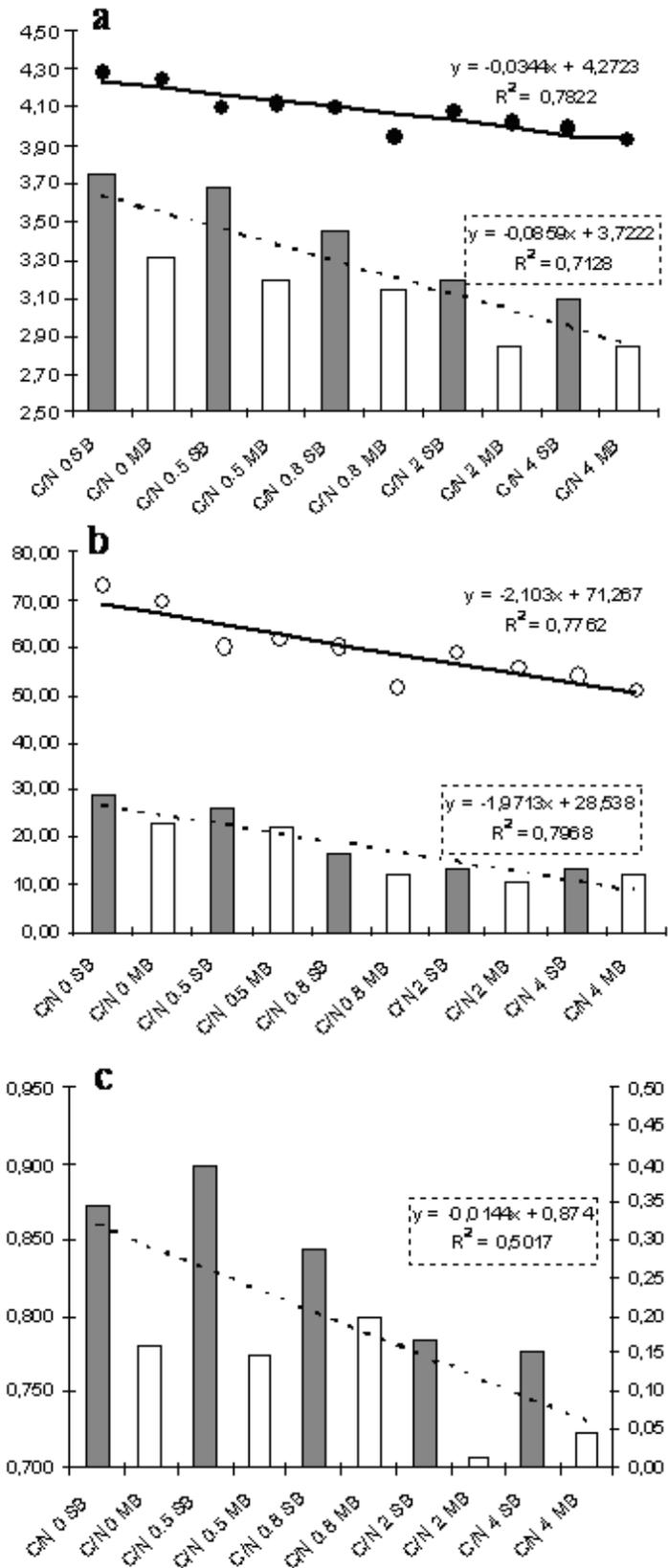
Fig. 4.4 : Diversity indices: a) Shannon (bars) and Shannon max (dots); b) Simpson reciprocal (bars) and Simpson max (dots); c) Pielou Evenness (bars)

4.3.4   *Physiological diversity of bacterial communities*

Community level physiological profiles (CLPP) indicated an interesting trend in the biofilter microbial community structures over different C/N ratios, in both SB and MB.

Total number of oxidized carbon sources grew up from low C/N ratios to higher ones in both systems, although communities appeared to have two different metabolic patterns. In fact, SB and MB were 68 and 60 at C/N 0 respectively, to reach 81 and 88 at C/N 4, highlighting a more important variation of MB associated community rather than SB one, following organic matter increasing (*Fig. 4.5*).

MDS and cluster analysis confirmed this discrepancy, showing a sensible difference between SB and MB communities. In particular, MB data plotted in MDS appear to shift more over different C/N ratio than SB ones (*Fig. 4.6; 4.7*).
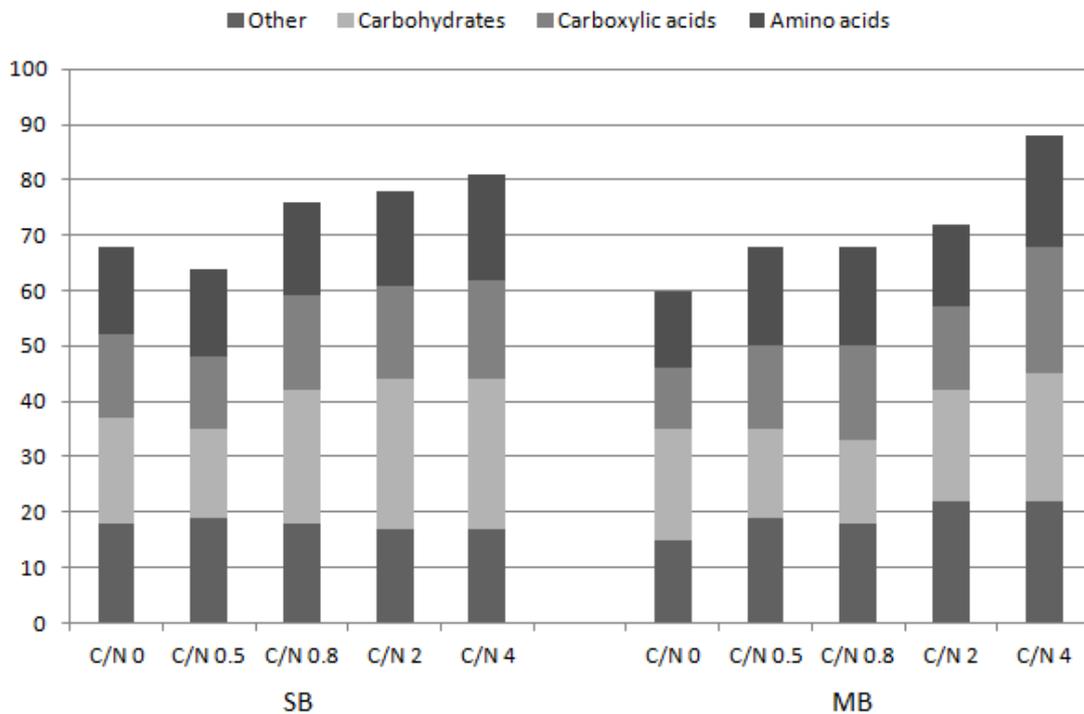
Fig. 4.5: Number of carbon sources oxidized in both biological filters at the different C/N ratio enrichments. SB, Static Bed; MB, Moving Bed.
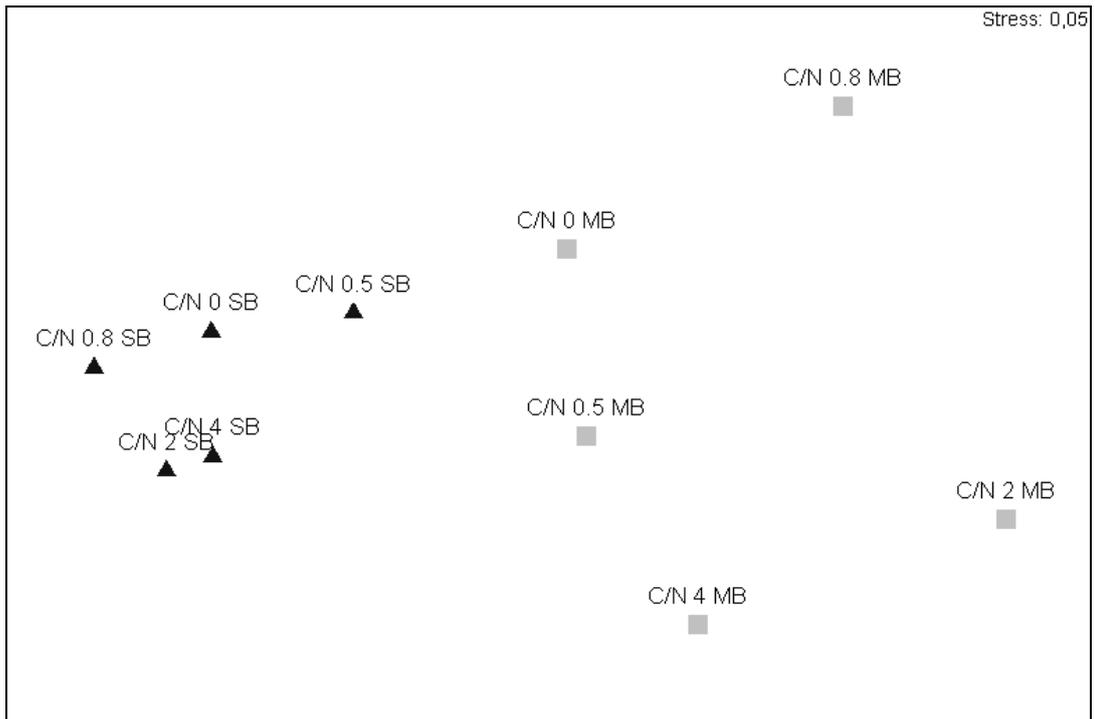
Fig 4.6: MDS showing similarity between carbon source utilization profiles between both biological filters at the different C/N ratio enrichments. SB, Static Bed; MB, Moving Bed.
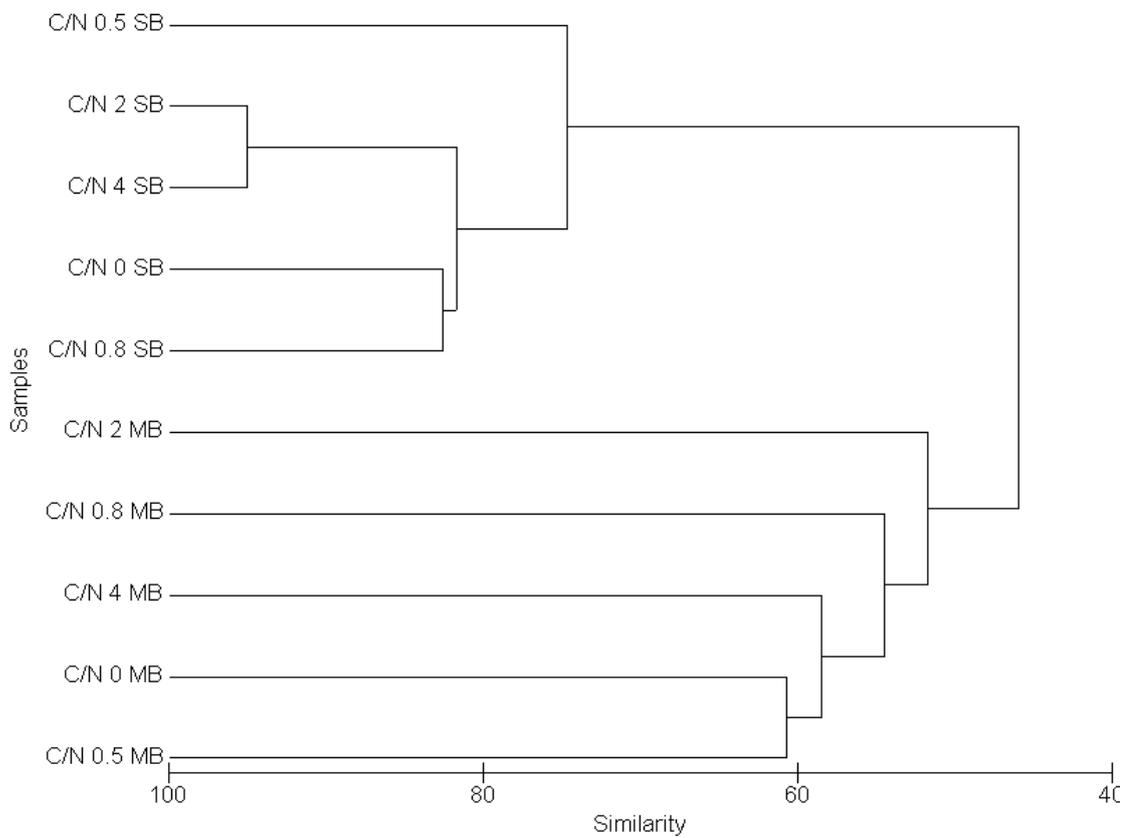
Fig 4.7: Cluster plot showing similarity in both biological filters types. SB, Static Bed; MB, Moving Bed.

Static Bed and Moving Bed produced quite different diversity patterns, calculated through Shannon index, at increasing organic matter concentrations.

Shannon index values for SB revealed an overall growth of bacterial community diversity from C/N 0 to 0.8; from this point onwards diversity was stabilized until high organic matter concentration (C/N 4) (*Fig. 4.8*).

Diversity in MB showed a different trend compared to SB. For low organic carbon concentration (C/N 0 to 0.8) Shannon index decreased gradually, it became constant at C/N 2 and grew up at C/N 4 (*Fig. 4.8*).

■ Static Bed   ■ Moving Bed
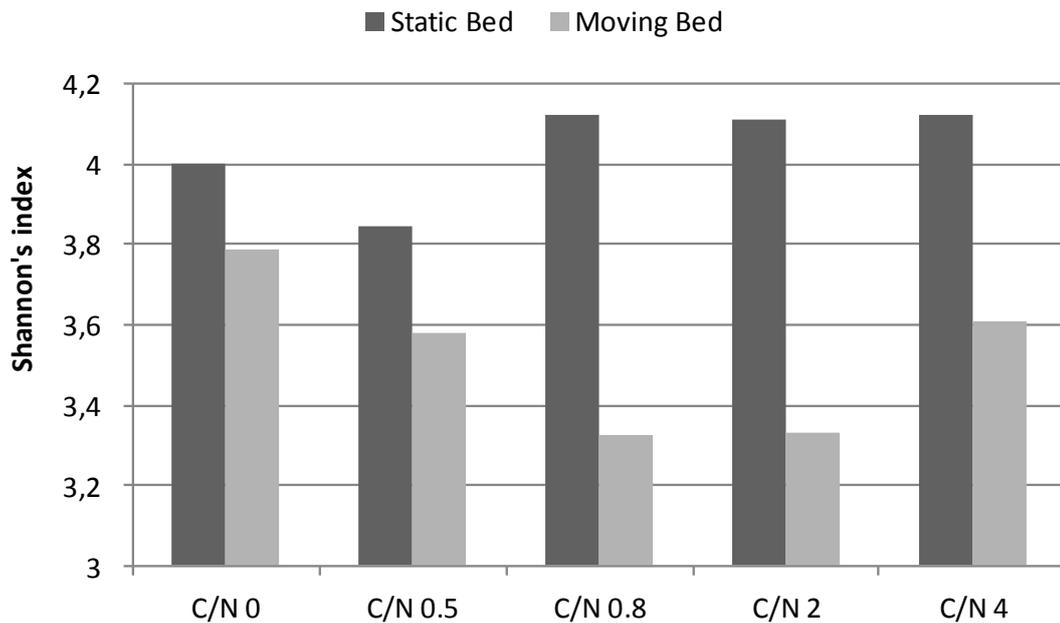
Fig 4.8: Shannon Diversity Index of carbon substrate use by the bacterial community in Static Bed and Moving Bed.

4.4.   Conclusions

Biofilms play an important role in all aquatic ecosystems due to their pivotal position at the interface between physical–chemical and biotic components. Because they are complex communities that include representatives of several taxonomic kingdoms, biofilms integrate responses to chemical stressors from heterotrophic organisms (mostly bacteria and fungi) to autotrophic organisms (algae, cyanobacteria, chemiolithoautotrophic bacteria). Different types of microorganisms evolve jointly within biofilms, which are complex communities of interacting microorganisms (Fechner *et al.,* 2010).

Changes in biofilm community structure can be assessed using fingerprinting techniques as denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-RFLP) and, more recently, automated ribosomal intergenic spacer analysis (ARISA) (Fechner *et al.,* 2010; Lear *et al.,* 2008). This latter provides a high-throughput molecular method with which to screen large numbers of environmental samples for differences in microbial community structure giving reproducible descriptions of the community diversity and composition to be attained with a high level of taxonomic resolution (Jones *et al.,* 2007).

In this study, CLPP, FISH and ARISA were used in order to provide broad and fast snapshots of the microbial diversity of the complex communities of aquaculture biofilter biofilm for assessing the effect of different C/N ratio on two biofilter typology.

Results showed that the Particulate Organic Matter present in the rearing water influenced not only the biofilter efficiency (Michaud *et al.,* 2006) but also the community structure. The increasing of the C/N ratio resulted, both for static and moving, in a shift of the bacterial community structure in term of reduction of taxa richness and diversity indices (Shannon, Simpson reciprocal and Evenness). This effect seems to be more marked in the Static Bed than in the Moving ones, probably due to the self-cleaning properties of these latter. There was any relation between the C/N ratio and the dissimilarity among the two filters typology. The microbial community colonizing Static Mineral and Plastic Moving bed media are quite different (57% dissimilarity on average) due to the different nature of the medium and on the different hydraulic regime. In any case, both communities were forced to adapt to the increasing of C/N ratio, probably by the selection of bacteria able to cope with big amounts of carbonaceous substances. This is in line with previous studied (Michaud *et al.,* 2006) in with the increasing of C/N ratio caused an increasing of the presence of so called "presumptive vibrios". In a biofilter, especially in those using mineral packing media, a multitude of microhabitats is present, providing a diverse range of niches and resulting in patchy distributions and community variation at different spatial scales.

Such results underline that a biofilter bacterial community is influenced by system conditions and operation and that one way to manage such microbiota is the management of the water chemistry.

Finally, even it should be pointed out that fingerprinting techniques cannot be used to assess the diversity of a sample, ARISA and FISH combined with multivariate analysis is an efficient tool to identify succession patterns or dynamics. From this

perspective, performing ARISA using a bioanalyzer is a powerful analytical means to obtain ultrafast fingerprints of a large number of samples and is therefore an efficient technique to compare fingerprints and thus reveal succession patterns (Fechner *et al.,* 2010; Lear *et al.,* 2008). In fact bacterial communities are thought to be highly responsive indicators of changing environmental conditions as a consequence of their rapid life cycle.

CHAPTER 5

Effect of redox potential on microbial community structure, diversity and activity on both laboratory and pilot-scale biological filters

5.1.   Introduction

Ozone (O_3) is a clear blue colored gas that is formed when an oxygen molecule (O_2) is forced to bond with a third oxygen atom (O). The third atom is only loosely bound to the molecule, making ozone highly unstable. This property makes ozone an excellent oxidizing agent and ideal for use in water treatment (Schröeder, 2010).

Ozone is widely used for drinking water processing and oxidation of sewage and industrial wastewaters (Katzenelson and Biedermann, 1976; von Gunten, 2003). Since several decades, it is increasingly being used in aquaculture as a strong oxidant for disinfection water treatment, and bacteria control and improvement of water quality (Summerfelt and Hochheimer, 1997; Forneris *et al.,* 2003; Buchan *et al.,* 2005;). It destroys viruses, bacteria, fungi and protozoa, giving it broad applications in the aquaculture industry (Colberg and Lingg, 1978; Danald *et al.,* 1979; Schneider *et al.,* 1990; Buchan *et al.,* 2005; Liltved *et al.,* 2006).

One way commonly used to measure residual ozone concentrations in seawater is to utilize a probe or electrode that measures oxidation-reduction potential, commonly referred to as ORP (Buchan *et al.,* 2005; Metzger, 2009).

ORP is not a measurement specifically for ozone but rather all oxidizing agents, including other disinfectants (Metzger, 2009). In fact, the disinfection mechanism also involve molecular reactions independent of ORP, in which case ORP is not equivalent to disinfection potential (Tango and Gagnon, 2003) and it varies as a function of the standard potential, relative ion concentration, temperature, and the number of electrons transferred (Banhidi, 1995; Tango and Gagnon, 2003; Summerfelt *et al.,* 2009). Despite this, it appears to be an excellent method to continuously detect ozone addition and thus ensure the desired treatment objective (Tango and Gagnon, 2003).

It was seen that continuous exposures to the utilization of UV disinfection and ozonation treatments could steer the microbial community in variation in structure and composition (Schreier *et al.,* 2010). However, these forcing agents play significant roles in perturbing these communities (Wietz *et al.*, 2009) but appear to have little effect in deep layers of filter biofilms (Urakawa *et al.*, 2008; Michaud *et al.*, 2009).

To date, few knowledge exist about the real impact that ozonation can cause on microbial community organization and composition. Thus, such more investigation could provide very useful information about the right way to manage ozone in aquaculture process, optimizing microbial environment and, at the same time, promoting fish production.

As seen in Chapter 4, the management and the control of all components of a RAS represent a key aspect in the production process. For this reason this experiment had as objective to deeper understand about the effects on microbial community structure, diversity and activity of a crucial environmental parameter as Oxidation-Reduction Potential (ORP) on both laboratory and pilot-scale biological filters.

5.2. Material and Methods

5.2.1. Experimental Design

The system at the IFREMER research station of Palavas les Flots (France) was adapted to that previously described in section 4.2.1.

Experiments were performed into two systems named "**EcoMicro**" and "**Lagunage**" that were settled as follows: the first consisted of 12 *laboratory-scale* biofilters and the latter of 4 *pilot-scale* biofilters supplied with seawater having the same quality described before (see 4.2.1.).

The characteristics of the biofilters are shown in *Table 5.1*.

Table 5.1: Pilot scale biofilter specifications.

	Symbol	EcoMicro	Lagunage
Diameter	$\varnothing$	16 cm	50.5 cm
Cross-sectional area	$\pi \times r^2$	200 cm²	2000 cm²
Height	h	120 cm	150 cm
Volume	$S \times h$	24 dm^3	250 dm^3
Flow rate	$Q=V/t$	0.24 m^3 h^{-1}	3 m^3 h^{-1}
Water retention time	$t=V/Q$	6 min	5 min
Water velocity	$v=Q/S$	12 m h^{-1}	15 m h^{-1}

In each system, the biofilters were divided into two main Units: "**A**" and "**B**" for EcoMicro and "**Tribord**" and "**Babord**" for Lagunage.

In EcoMicro each Unit was composed of three independent filters pairs (replicates), operating in parallel. In order, they were: moving plastic substrate (Moving Plastic Bed - MPB), plastic fixed substrate (Static Plastic Bed - SPB) and mineral fixed bed (Static Mineral Bed - SMB) *(Fig 5.1a)*; Lagunage Units were constituted of two couple of Moving Plastic Bed biofilters (MPB) each. (*Fig. 5.2a*)

The columns were made of semi-transparent PVC and contained Biogrog support for SMB filters and a Acui-T support for SPB and MPB filters. The latter, moreover, were provided at the base of an air insufflator which supplied a steady stream (10 l min^{-1}) in order to maintain the oxygen at the highest level and ensure the continuous mixing of the plastic substrates.

In EcoMicro a tank of 500 l was associated to each couple, which served as reserve of water and where the compounds that were added to simulate the presence of fish (ammonium chloride, NH_4Cl and carbon source) were mixed (see 5.3.2.). As shown in *Fig 5.1b*, the inlet flow to each column came from the tank, passed through each biofilter and finally arrived to the same tank in order to recreate the conditions present in a RAS.

Lagunage, instead, represented a real RAS (pilot-scale) (*Fig 5.2a*). Units were provided of 3 fish tanks, 1 m^3 each, which provided the biofilters of ammonia and organic matter. As shown in *Fig 5.2b* the inlet flow to each column came from fish tanks, passed trough mechanical and physical filtration units and, after filtration, the outlet flow went directly to the fish tanks, closing the system.

In both cases, each couple of biofilters was associated with a protein skimmer (AQUAVIE PS700 and AQUAVIE PS5000 for EcoMicro and Lagunage, respectively), thanks to which organic material in excess was extracted. Also, in order to compensate

for losses due to evaporation, all biofilters in both experiments were supplied with seawater at the same quality of the system ($0.2 \, l \, min^{-1}$).

Each column was provided of two sampling taps on the top and at the bottom for inlet and outlet water, respectively.

At first the system was subjected to an initial start-up phase (1 month), required to reach the optimal working conditions. Subsequently, the EcoMicro and Lagunage experiments were started according to the scheme shown in *Tables 5.2* and *5.3*.

In both cases, there were reproduced two different Oxidation Reduction Potential (ORP) values for the whole duration of the experiment. In the Units A and Tribord, the ORP was modified to reach 320 mV, thanks to the addition of 100 mg h^{-1} and 4 g h^{-1} (maximum rate) of ozone (O_3), respectively. This latter was provided by an ozonator (Certizon C100, Sander for EcoMicro and BMT 802N, BMT Messtechnik, Berlin for Lagunage) which was connected to each protein skimmer. In the Units B and Babord, the ORP was naturally selected between 260 and 280 mV.

Table 5.2: EcoMicro experimental desing: **Biof** = Type of biological filter; **ORP** = Oxidation Reduction potential; **BioFilters** = Biofilter identification code; **MPB** = Moving Plastic Bed; **SPB** = Static Plastic Bed; **SMB** = Static Mineral Bed

Units	Biof.	ORP	BioFilters	C/N Condition
Unit A Marine water	*MPB*	Ozonated	BF1	C/N 0.8
	MPB	Ozonated	BF2	C/N 0.8
	SPB	Ozonated	BF3	C/N 0.8
	SPB	Ozonated	BF4	C/N 0.8
	SMB	Ozonated	BF5	C/N 0.8
	SMB	Ozonated	BF6	C/N 0.8
Unit B Marine water	*MPB*	Natural	BF7	C/N 0.8
	MPB	Natural	BF8	C/N 0.8
	SPB	Natural	BF9	C/N 0.8
	SPB	Natural	BF10	C/N 0.8
	SMB	Natural	BF11	C/N 0.8
	SMB	Natural	BF12	C/N 0.8
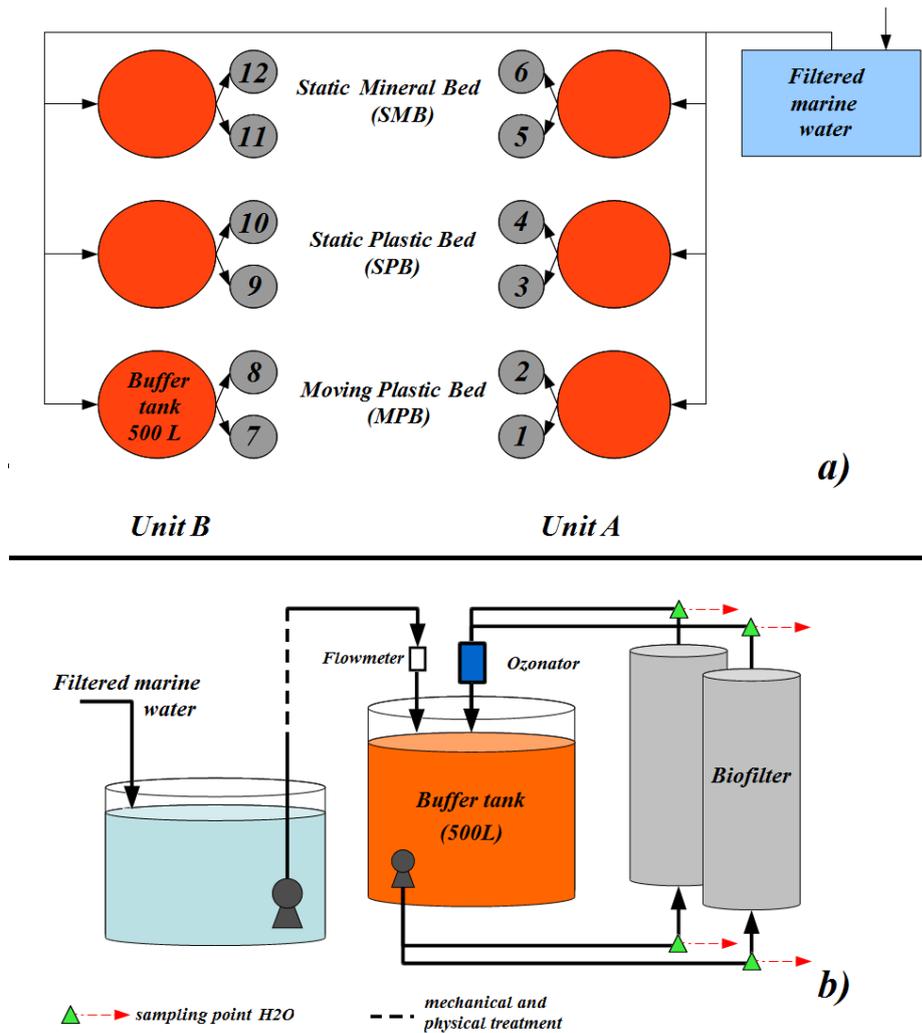
Fig 5.1: General configuration of EcoMicro system *a)* and detail of each pair of biofilters *b)*.
Note: the presence of the ozonator in figure *b)* is referred only to the Unit A.

Table 5.3: Lagunage experimental desing: **Biof** = Type of biological filter; **ORP** = Oxidation Reduction potential; **BioFilters** = Biofilter identification code; **MPB** = Moving Plastic Bed.

Units	Biof.	ORP	BioFilters
Tribord Marine water	*MPB*	Ozonated	BF-TA
	MPB	Ozonated	BF-TB
Babord Marine water	*MPB*	Natural	BF-BA
	MPB	Natural	BF-BB
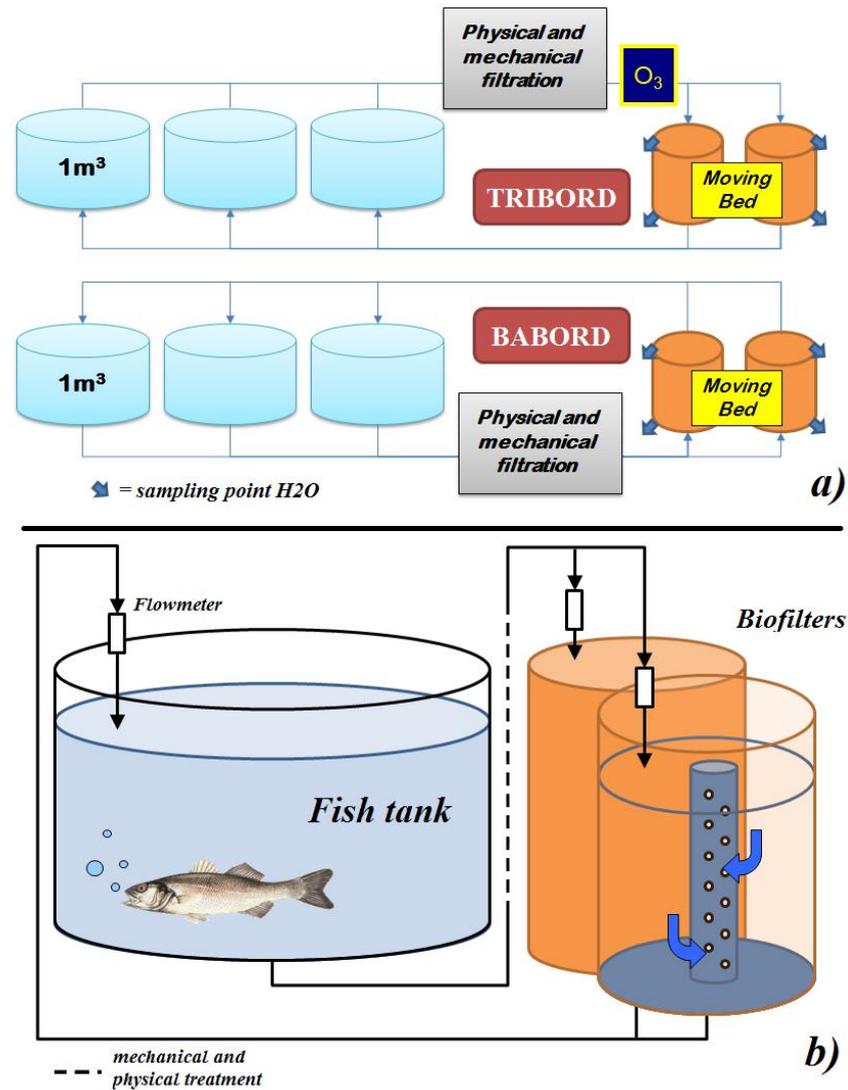
Fig 5.2: General configuration of Lagunage system *a)* and detail of biofilters *b)*.

5.2.2. *Source of carbon and nitrogen*

In EcoMicro, the filters were enriched at C/N ratio corresponding to a biomass typically reared in a real system (C/N 0.8) and tested for a period of four weeks (*Table 5.2*).

The carbon used for the enrichment consists of very fine granular food (diameter 150-300 µm) that is routinely used in the same research station (Marin Start AL-0, Le Gouessant, France). Before each application it was sterilized at 121°C for 20 min by autoclaving, then dried in an oven at a temperature of 105°C.

In order to estimate the percentage content of carbon in the food, TOC analysis was carried out (Schimadzu TOC-5000 Analyzer) in triplicates on serial dilutions of food dissolved in MilliQ sterile water using an Ultraturrax homogenizer (Raw sample, 1:50, 1:100, 1:500 and 1:1000 g l^{-1}), (*Table 5.4*).

Table 5.4: Carbon content of the food used as carbon source.

	Units of Measurement	Sample 1	Sample 2	Sample 3
Total Dry Matter (DM) (105°C)	**%**	**94.1**	**94**	**94.1**
Total Organic Carbon	**g C / kg of DM**	**422.3**	**430.6**	**414.5**

The percentage content of carbon was on average 10% of dry weight and, thus, it was possible to choose the required amount of POM to be mixed with nitrogen to select the target C/N ratio. The ammonia input consisted of ammonium chloride (NH_4Cl) (Sigma) and its concentration was kept constant throughout the experiment (4 mg l^{-1}).

The rates of NH_4Cl and food as source of carbon were weighed, mixed and placed uniformly on automatic tape feeders, by which the mixture was added continuously for 24 h, after which the operation was repeated.

As described before, for Lagunage the source of carbon and nitrogen was naturally provided by the rearing of fish in the tanks linked to the system.

5.2.3. *Chemical and biochemical analyses*

For each pair of biofilters a multiparametric probe (Ponsel Odeon Classic, EMS) continuously acquired physical measurements (every 10 min): temperature, oxygen, ORP and pH. After four weeks of enrichment it was assumed that the multi-specific nitrifying biofilms had reached an equilibrium state (*Steady state*), adapting to the environmental conditions.

Because EcoMicro was a laboratory scale system, so provided with a small total volume of water, the analysis on the biofilter efficiency was carried out on each couple of columns using a closed-loop system filter (*Batch configuration*) in order to determine the reaction kinetics of nitrification by collecting nutrient samples for ammonia, nitrite, nitrate and phosphate. In each buffer tank, a volume of seawater (300 l) with a NH_4Cl concentration of 10 mg l^{-1} was placed, sampled manually at regular intervals (every 45 min, for a total period of 270 min) and analyzed as described below.

For Lagunage, as pilot scale system provided of a large total volume of water, nutrient samples were collected manually for ammonia, nitrite, nitrate and phosphate and analyzed with the same method of EcoMicro nutrient samples (see below).

To express nitrification efficiency in EcoMicro was calculated the ammonia degradation kinetics velocity using the following equation:

$$V = \frac{V_{max}\,[NH_4]}{[NH_4] + K_m}$$

where V is the reaction velocity and Vmax is the maximum reaction rate, $[NH_4]$ is the concentration of ammonia and Km is the ammonia concentration at Vmax/2.

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

$$TAN\ removal\ rate = [TAN]in - [TAN]out \times Q/V$$

where [TAN]in and [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.

5.2.3.1. Nutrient analyses

N-NO$_3^-$, N-NO$_2^-$, N-NH$_4^+$, P-PO$_4^{3-}$ concentrations were estimated using standard spectrophotometric techniques. All spectrophotometric measurements were performed using a Varian Cary 50 Probe using cuvettes of 40 or 10 mm and converting the adsorbances into micromoles per liter with appropriate factors derived from calibration curves developed for each chemical species.

Ammonia was determined as indophenol blue, formed by reaction of ammonia with phenol dissolved in alkaline buffered solution and with the presence of an oxidant, the hypochlorite and a catalyst, the sodium ferrocyanide. For the development of color,

24 h were expected and the spectrophotometer measurements were performed at a wavelength of 630 nm (Aminot and Chaussepied, 1983).

The concentration of _nitrites_ was determined by reaction with solfanilamide in hydrochloric acid followed by a second reaction with N-(1-naphthyl) ethylenediamine hydrochloride. The absorbance of the salt of diazonium salt thus formed was read spectrophotometrically at 543 nm after color development (Bendschneider and Robinson, 1952).

Nitrates were analyzed such as, but by reduction to nitrite on cadmium-copper column at pH 8.0 and subsequent determination as nitrites. The absorbance read, then focused on the total amount of nitric and nitrous salts present in the sample and the concentration of dissolved nitrate was obtained by subtraction of the result for nitrites (Wood _et al._, 1967).

The concentration of _phosphates_ was determined spectrophotometrically by reaction with ammonium molybdate and potassium tartrate under acidic conditions. The resulting phosphomolybdic acid was reduced by ascorbic acid and then with the formation of molybdenum blue, which was read at 885 nm (Murphy and Riley, 1962).

Total Organic Carbon (TOC) and Total Suspended Solids (TSS) were performed as described in Michaud _et al._ (2006). Ammonia, nitrite and nitrate were analyzed with a Technicon[®] Autoanalyser II as described by Treguer and La Corre (1974). Total Organic Carbon (TOC) was analyzed with a Shimadzu TOC5000 analyzer and Total Suspended Solids (TSS) were determined by filtering a known volume of water on a pre-weighted and pre-combusted (450°C for 3-4 h) glass fiber filter (Whatman GF/C, Springfield Mill, UK). Filters were dried at 65°C for two days and weighted again.

5.2.3.2. Biological Oxygen Demand (BOD$_5$)

Measurement of Biological Oxygen Demand in 5 days (BOD$_5$) was performed by Winkler method, in order to control the amount of dissolved oxygen needed by aerobic biological organisms in both inlet and outlet biofilter water flow of Ozonated and Natural sides.

5.2.4. Microbiological analyses

Packing media samples were conditioned as described in the section 4.2.4. for quali/quantitative analyses in order to determine changes in the microbial community associated with the biofilm. In particular, for bacterial enumeration flow cytometry was applied as reported in the section 5.2.4.1. below. To assess the community physiological and biochemical features, the CLPP was carried out as reported in the section 3.2.7., while ectoenzymatic activities were investigated as described in the section 5.2.4.2. below. Finally, the community structure and composition were analyzed by ARISA (section 4.2.8.) and clone library construction (see below section 5.2.4.3.).

5.2.4.1. Flow cytometric assessment of bacterial cell abundance

The total prokaryotic abundance was estimated by using the Flow Cytometer A-50 (Apogee Flow System, Hertfordshire, England) equipped with a solid-state laser set at 20 mV and tuned to an excitation wave length of 488 nm. The light scattering signals (forward scatter, LS1 and side scatter, LS2) and the green fluorescence FL2 channel (530/30 band passing filter) were acquired for microbial cell detection. Samples were stained with SYBR Green I (1:10000 dilution; Molecular Probes, Eugene, Oregon, USA) and incubated for 15 min in the dark at room temperature. The data were analyzed using the Apogee Histogram Software 2.05 (Apogee Flow System). The total number of prokaryotic cells was detected by their signatures in a plot of the side scatter (LS2) vs. the green fluorescence (FL2). Samples were run at low flow rates to keep the 27 number of events below 1000 event/sec in order to reduce the probability that two or more particles could pass together through the electronic systems, being considered as a single larger particle (Amalfitano and Fazi, 2008).

5.2.4.2. Ectoenzymatic activities (EEA)

The study of bacterial metabolic activity was performed by analyzing bacterial extracellular enzymatic activity (EEA). 14 units of Biogrog mineral substrate and 20 units of Acui-T plastic support were sampled at the same time of sampling and analyzed the same day at the laboratory IRSA-CNR in Rome.

The enzymatic activities were tested through the fluorimetric method (Hoppe 1993) using the organic substrates (Sigma-Aldrich), MUF-β-D-glucopyranoside, MUF-phosphate, MUF-oleate, L-Leucine-4-methylcoumarin-7-starch, to measure the enzymatic activity of beta-glucosidase, alkaline phosphatase, and lipase dell'aminopeptidase, respectively.

The intact biofilm grown on plastic supports (Acui-T) and mineral one (Biogrog) was subjected to the measurement of enzyme activities by modifying the method used by Smucker *et al.* (2009) for lotic ecosystems biofilms.

For both samples, a volume of substrate equivalent to 3 ml of plastic substrates and 10 ml of mineral substrates were submerged in sterile seawater, in order to preparing three replicates for each sample, and considering two negative controls (blanks). These latter were prepared by boiling the samples for 1 h at 100°C.

The enzymatic activities were tested by choosing saturating concentrations (300μM for MUF-β-D-glucopyranoside, MUF-phosphate, L-Leucine-4-methylcoumarin-7-starch and 500μM for MUF-oleate, final concentration) found in the literature (Smucker *et al.,* 2009). Samples and blanks were incubated for 40 min at 20°C in a shaker (80 oscillations per min).

After incubation, an aliquot (200 μl) was placed in a plate and the fluorescence emission was measured in the spectrofluorometer (Victor X3 - PerkinElmer). To measure the fluorescence a wavelength of excitation (365 nm for MUF and 380 nm for AMC) and one of emission (446 for MUF and 440 nm for AMC) were used. The intensity of fluorescence emitted by the sample was converted into concentration of AMC or MUF released by using the calibration line.

Each fluorimetric method for the standardization needs of a calibration curve for known concentrations of substrates. Stock solutions of MUF and AMC for the construction of the standard line were prepared by dissolving the pure compounds (MUF-4-Metilumbelliferone and AMC-7-Amino4metilcumarine) in metilcellosolve. The solutions thus prepared can be stored in sterile tubes, in the dark at -20°C, without any alteration.

The extracellular enzyme activity (EEA) was calculated considering the slope of the calibration line and the volume occupied by the plastic supports (Acui-T) and for mineral ones (Biogrog). Depending on the volume occupied the surface of the biofilm (600 m^2/m^3 for Acui-T and 800 m^2/m^3 for Biogrog) was calculated.

5.2.4.3. Biofilter community DNA cloning

Biofilter community structure and composition was investigated as described before in the section 3.2.6., with the exception that cloning was performed on DNA than cDNA.

5.3. Results and discussion

Results for the two analyzed systems, laboratory scale EcoMicro and Lagunage, are reported into separate sections.

5.3.1. *Laboratory scale experiment "EcoMicro"*

5.3.1.1 Chemical and biochemical analyses

The TOC analysis (*Fig. 5.3*) showed that the differences between the outlet and inlet in ozonated biofilters assumed a high positive value (0.797 ± 0.1 mg l^{-1}) in MPB, whereas it went to negative values in SPB and SMB (-0.160 ± 0.1 and -0.042 ± 0.1 mg l^{-1}, respectively). On the contrary, natural biofilters showed positive values in MPB and SPB (0.052 ± 0.1 and 0.208 ± 0.1 mg l^{-1} respectively), and a high negative value in SMB (-0.628 ± 0.1 mg l^{-1}).
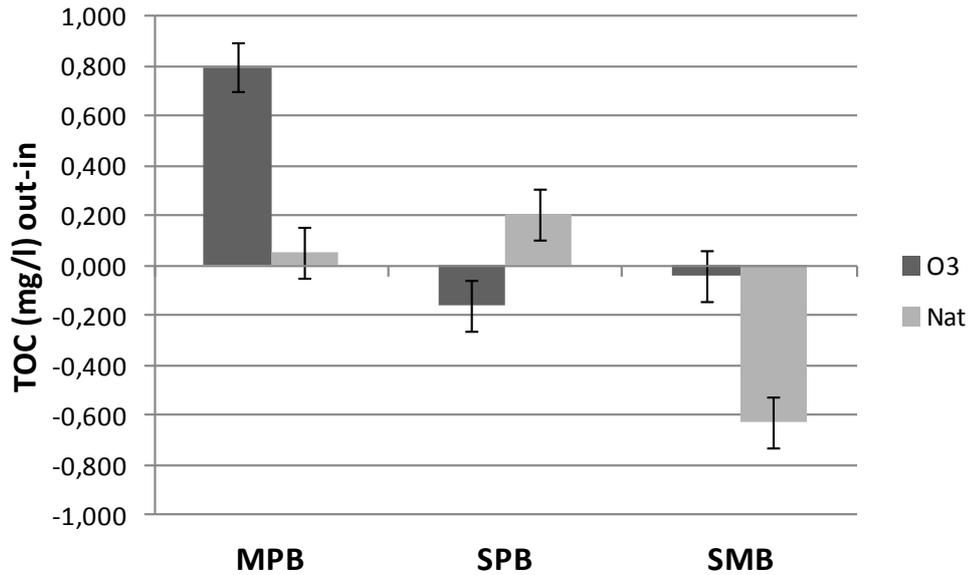
Fig 5.3: TOC differences between outlet and inlet in EcoMicro system. MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

Outlet and inlet Δ-TSS (*Fig. 5.4*) underlined a sensible increasing between ozonated plastic media biofilters (-0.40 ± 0.1 and -1.10 ± 0.1 mg l^{-1}) and ozonated mineral media one (1.90 ± 0.1 mg l^{-1}). Conversely, in natural biofilters there was not a real variation in the different biofilter typologies (2.30 ± 0.1, 1.90 ± 0.1 and 2.93 ± 0.1 mg l^{-1} in MPB, SPB and SMB, respectively).

These values were comparable with those found by Wolters and colleagues (2009) and seemed to underline a positive effect of ozonation on the removal of Suspended Solid (SS). In fact, all ozone treated filters presented a lower concentration of SS than natural ones. In agreement with this, Summerfelt *et al.* (1997) demonstrated that adding ozone to the recirculating system resulted in an overall improvement in water quality due to more complete oxidation of total suspended particulate solids, producing a 35% reduction of TSS in freshwater recirculating aquaculture system. Moreover, the improved solid removal was probably due to oxidation producing precipitation of dissolved organic molecules and microflocculation of colloidal organic solids, as first described by Maier (1984).
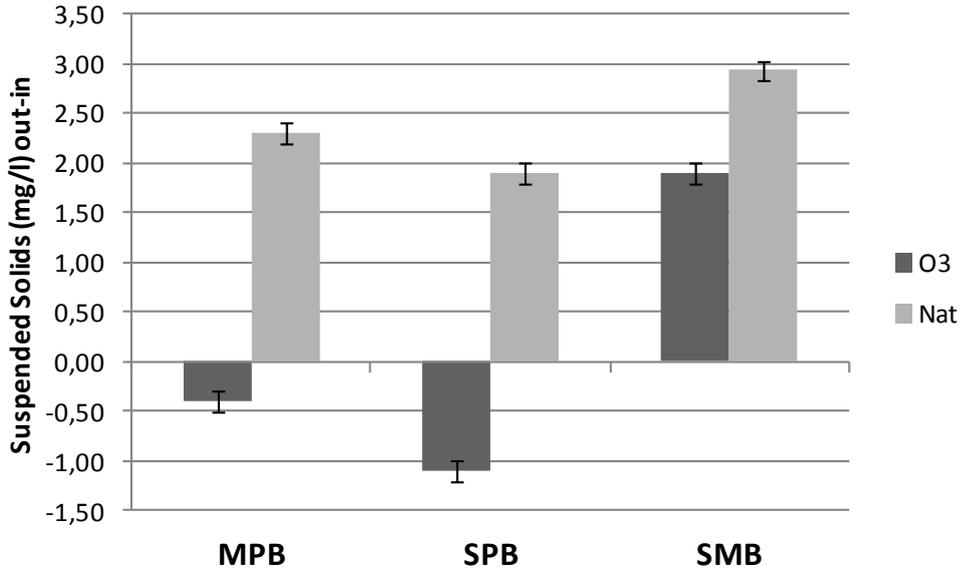
Fig 5.4: Differences between outlet and inlet TSS in the EcoMicro system. MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

In the EcoMicro system the nitrification efficiency was determined through nutrient sampling in the system in a *Batch configuration* (see 5.2.3.). As shown in the *Fig. 5.5*, natural biofilters had always Vmax/2 values higher than ozonated biofilters, ranging from 0.041 ± 0.03 in SPB to 0.063 ± 0.03 in MPB, and from 0.026 ± 0.02 in SPB to 0.043 ± 0.02 in MPB, respectively.
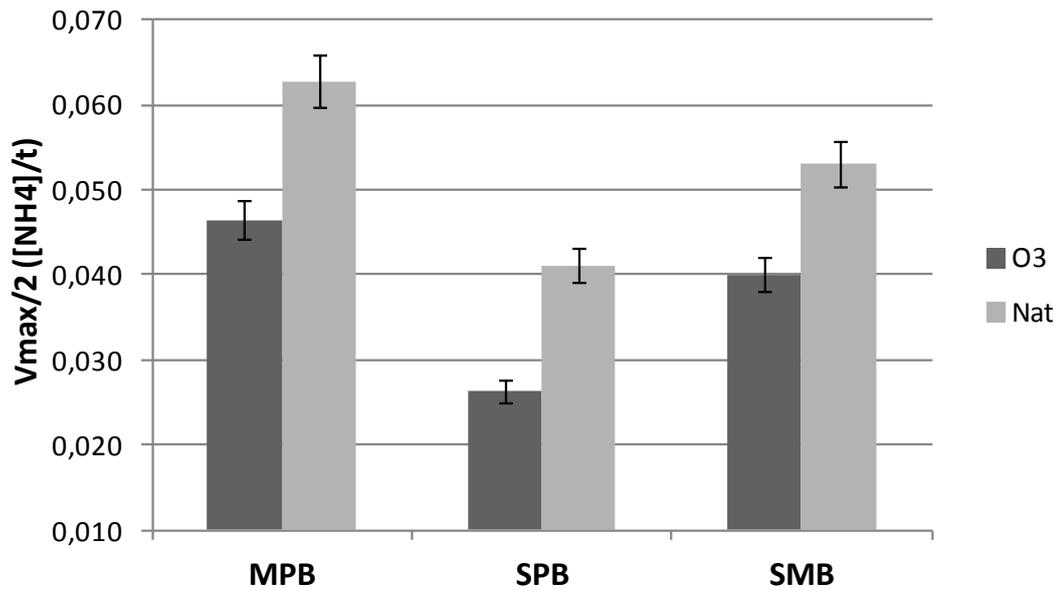
Fig 5.5: Vmax/2 in both ozonated and natural biofilters. MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

As shown in *Fig. 5.6*, Km data seemed to confirm this aspect. In fact, such constant was always higher in natural biofilters (8.015 ± 0.401 and 8.195 ± 0.410 for MPB and SPB), than in ozonated ones (7.240 ± 0.362 and 6.996 ± 0.350 for MPB and SPB, respectively). Exception was SMB filters where Km values in ozonated and natural biofilters were similar (8.543 ± 0.427 and 8.494 ± 0.425, respectively).
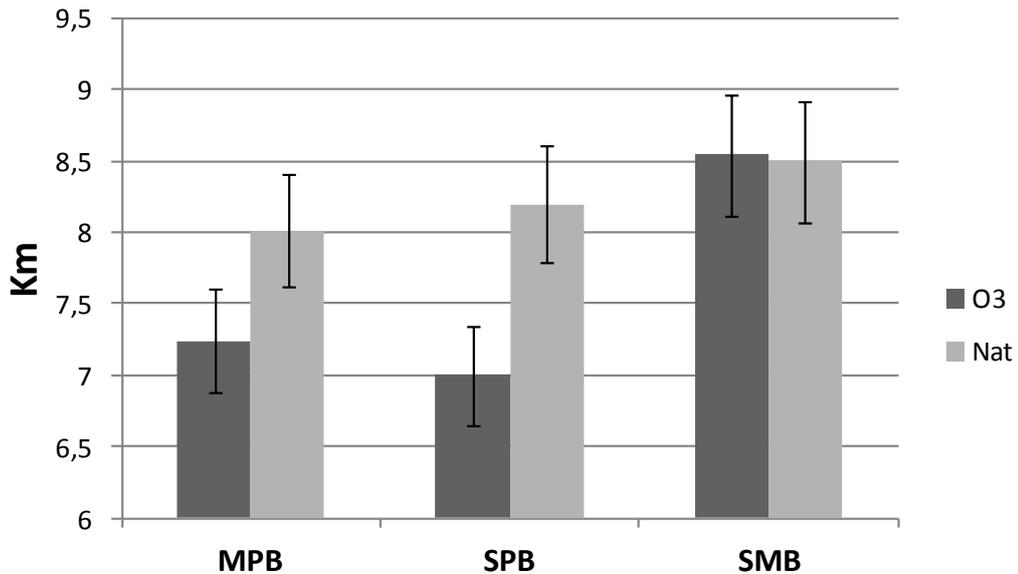
Fig 5.6: Km values in ozonated and natural biofilters. MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

The reaction kinetics of nitrification was expressed by plotting the values of ammonia [$N\text{-}NH_4$] versus time for each biofilter: in each case the ammonia degradation was higher in natural biofilters than in ozonated ones (*Fig 5.7*).

Data on the degradation kinetics allowed to calculate the ammonia oxidation velocity (*Fig 5.8*), at increasing substrate concentration, for the different biofilters. Results confirmed that all natural biofilters velocities were higher than ozonated ones (MPB natural: 54.77 - 498.71 and MPB ozonated: 63.86– 376.64; SPB natural: 104.22– 321.93 and SPB ozonated: 69.51 - 220.10; SMB natural: 66.26 – 411.25 and SMB ozonated: 43.73 - 311.71).

In order to calculate Km, Lineweaver-Burk graphics were plotted as shown in *Fig 5.9*
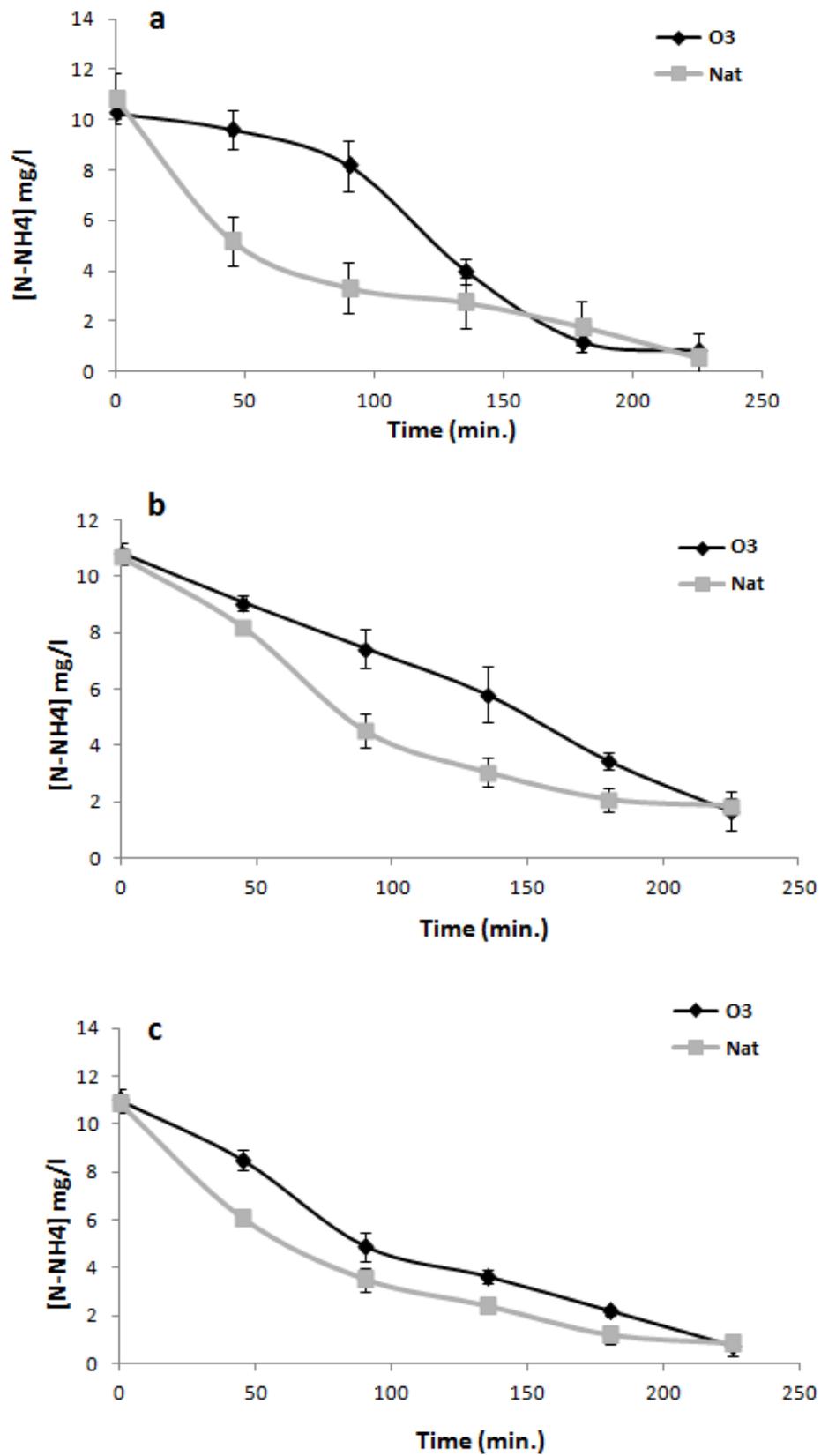
Fig 5.7*:* Comparison between the different biofilter types for ammonia degradation. a) MPB, Moving Plastic Bed; b) SPB, Static Plastic Bed; c) SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.
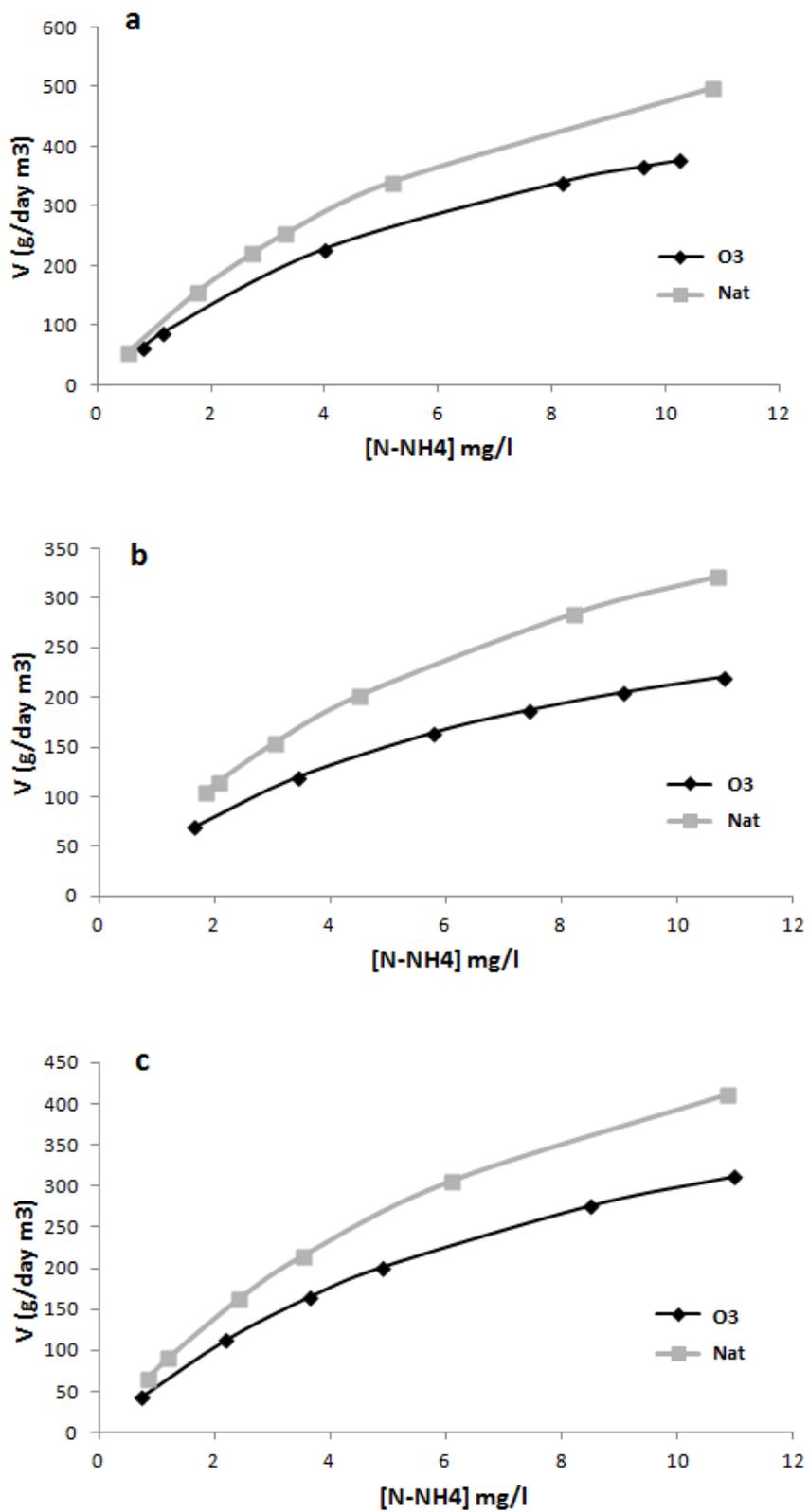
Fig 5.8*: Comparison between the different biofilter types for ammonia oxidation velocity.
a) MPB, Moving Plastic Bed; b) SPB, Static Plastic Bed; c) SMB, Static Mineral Bed; O3,
Ozonated samples; Nat, Natural samples.
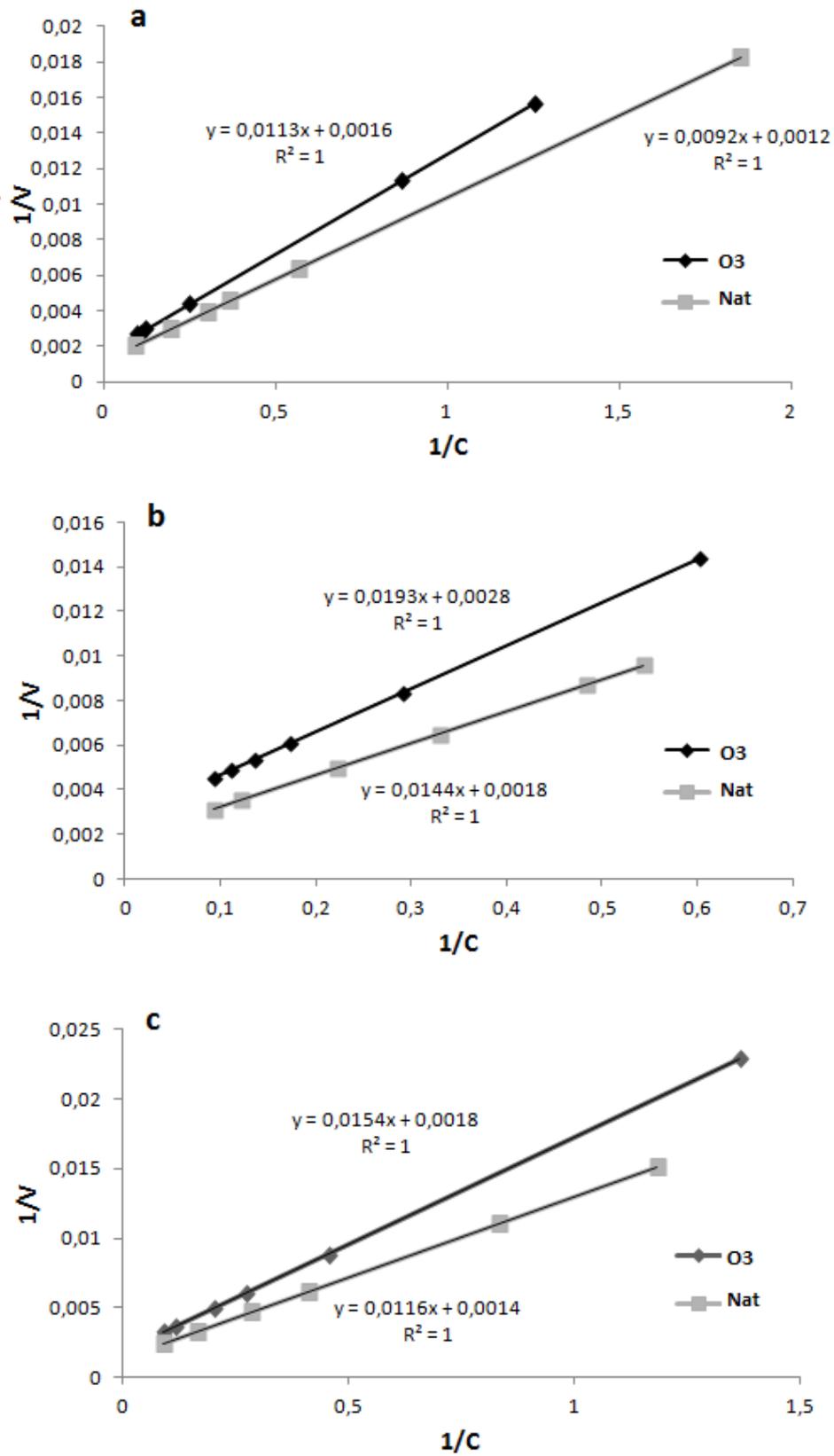
Fig 5.9: Lineweaver-Burk analysis in each biofilter type. a) MPB, Moving Plastic Bed; b) SPB, Static Plastic Bed; c) SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

5.3.1.2 BOD$_5$

There were not very significant differences in BOD$_5$ values between ozonated and natural biofilters, but total averages showed that natural biofilters utilized more oxygen than ozonated ones (1.44 ± 0.30 and 0.98 ± 0.32 mg l^{-1} respectively) (*Fig. 5.10*). Although the difference is small, this could suggest a subtraction of organic matter (degradation and/or microflocculation) (Buchan *et al.,* 2005; Metzger, 2009; Summerfelt *et al.,* 1997) by ozone treatment, resulting in a less oxygen demand in these filters.
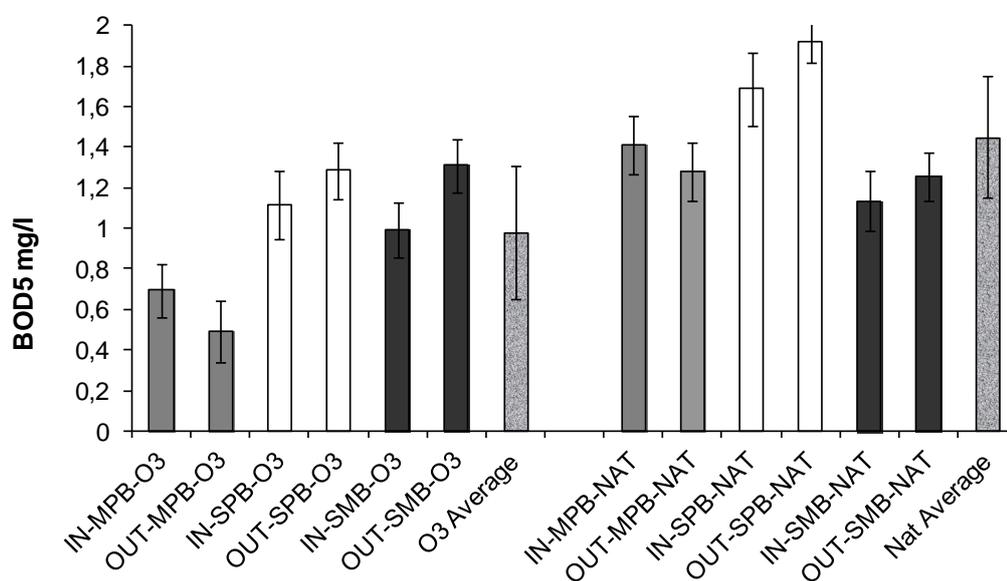
Fig 5.10: BOD$_5$ trend in the EcoMicro system. IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

5.3.1.3 Microbiological analyses

Microbial abundances

High bacterial abundance was detected by flow cytometry in SPB filters (both natural and ozonated), followed by MPB and SMB biofilters (*Fig. 5.11*). In particular, the highest ($1.83 \times 10^8 \pm 9.17 \cdot 10^6$ cells ml^{-1}) and lowest ($2.26 \cdot 10^7 \pm 1.13 \cdot 10^6$ cells ml^{-1}) values were found in the ozonated SPB biofilter and in the natural SMB biofilter, respectively. This finding suggests that plastic media could better support bacterial colonization than mineral substrates (SPB and MPB *versus* SMB).

Further, bacterial abundance seemed to be favored by static conditions rather than moving ones, probably due to the mixing of the media units in the latter biofilter. In fact, the continuously exposition of media surface to oxidants present into ozonated water of MB could favour the degradation and destabilization of biofilm. Instead, SB organization seemed to produce a sort of biofilm protection in internal space, among media.
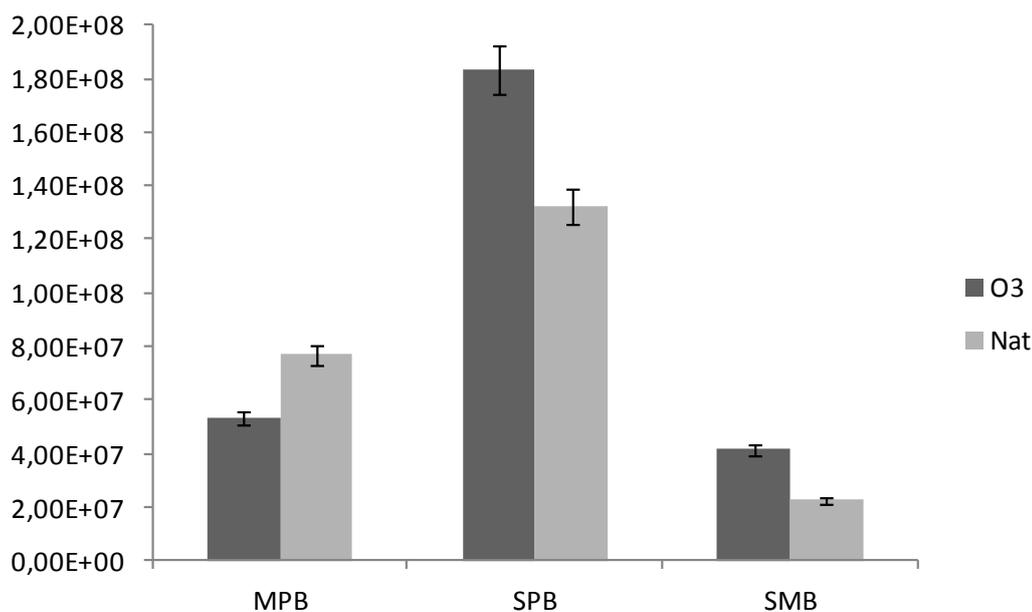
Fig 5.11: Flow cytometry in PM of EcoMicro system. MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

Community Level Physiological Profile (CLPP)

CLPP analysis revealed that globally PM bacteria community in ozonated filters utilized a higher number of carbon sources than natural ones (*Fig. 5.12*).

MDS analysis showed that ozone application produced a shift between both ozonated and natural bacterial communities, resulting in a separation of PM samples in two mainly groups with a percentage similarity of 40-60% (*Fig. 5.13*). MPB represented the only exception. In fact, it was separated from the other samples, but showed anyway a close similarity percentage (40%) with natural sample than ozonated ones (<40%).

This suggests that ozonation affected marginally MPB community, probably due to the continuously mixing and cleaning that occurs in the filter, resulting in a destabilization of biofilm structure and stability.
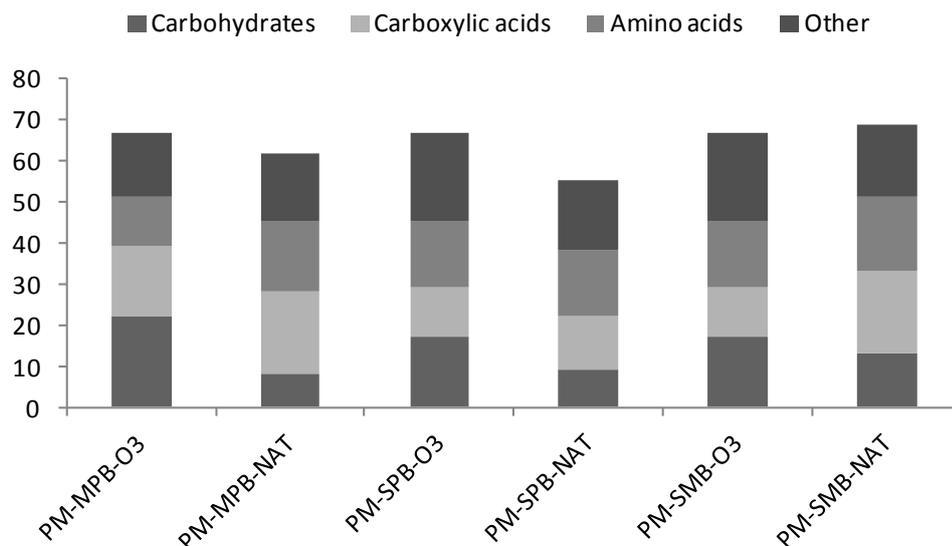
Fig 5.12*:* CLPP analysis described number of carbon sources oxidized in both water and filtration media. IN, Inlet; PM, Packing Media; OUT, Outlet.
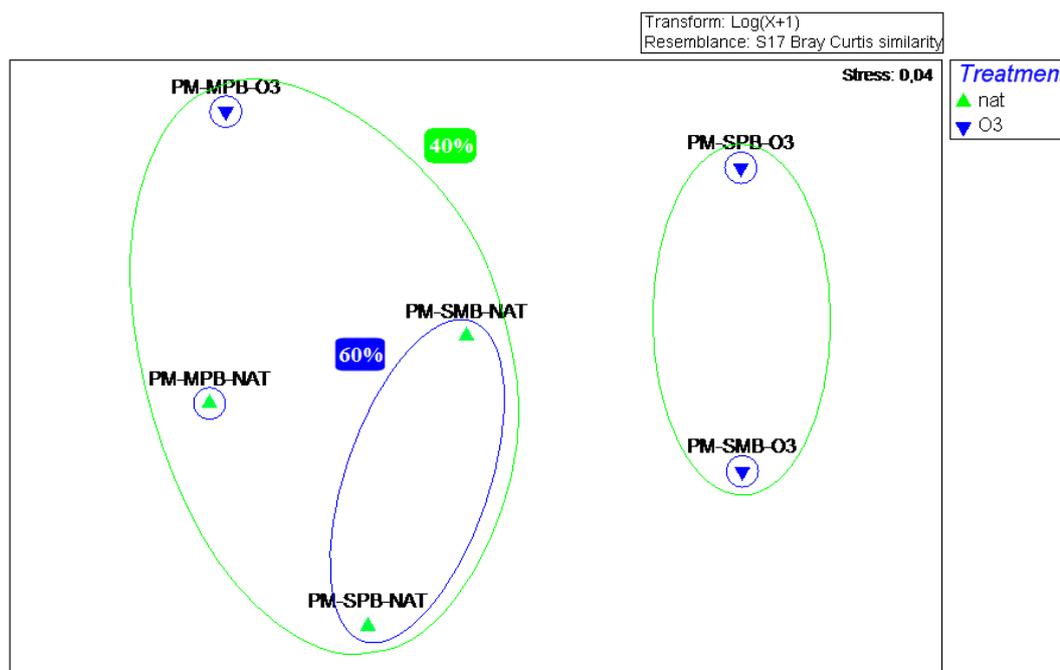
Fig 5.13*:* MDS computed on CLPP results. IN, Inlet; PM, Packing Media; OUT, Outlet.

Ectoenzymatic activities (EEAs)

The high-molecular weight organic compounds that are largely present in aquaculture systems must be hydrolyzed prior to the bacterial uptake throughout enzymatic activities. Extracellular enzyme activity (EEA) determinations were carried out for water and PM samples by taking into consideration 4 different enzymes (alkaline

phosphatase, β-D-1,4 glucosidase, leucine aminopeptidase and lipase), as described in the materials and methods section.

EEAs in water samples. The EEAs determined in water samples are reported in *Fig. 5.14*. Overall, the microbial community seemed to be more efficient in the utilization of proteinaceous compounds, as indicated by the high leucine aminopeptidase activity, followed by carbohydrates (β-D-1,4 glucosidase activity) and phosphorylated molecules (alkaline phosphatase activity). Conversely, any lipase activity was found in the water (both inlet and outlet) of the EcoMicro system.

With few exceptions, all detected activities were generally higher in natural than in ozonated biofilters, suggesting that ozone treatment probably inactivated (partially or totally, as in the case of β-D-1,4 glucosidase) the enzymes analyzed in the present study. In addition, in each biofilter type no significative differences were generally observed between the inlet and outlet water.

In detail, the alkaline phosphatase activity resulted always higher in natural biofilters than in ozonated ones, both in inlet and outlet water, ranging from 24.97 ± 15.21 to 9.11 ± 0.44 µmol l^{-1} h^{-1}, and from 16.52 ± 1.20 to 2.16 ± 0.64 µmol l^{-1} h^{-1}, respectively. The β-D-1,4 glucosidase activity was found only in outlet samples of natural biofilters and ranged from 1112.79 ± 402.32 µmol l^{-1} h^{-1} in MPB to 402.43 ± 124.50 µmol l^{-1} h^{-1} in SPB, respectively. No activity was determined in the remaining samples. Finally, the leucine aminopeptidase activity was generally higher in natural biofilters, ranging from 6802.78 ± 222.49 µmol l^{-1} h^{-1} at the outlet of MPB filter. Exception was the SPB filter as the activity in ozonated biofilters was higher (9178.53 ± 140.01 µmol l^{-1} h^{-1} – inlet -) than in natural ones (5020.01 ± 209.68 µmol l^{-1} h^{-1}), representing the highest activity.

Alkaline phosphatase

μmol l-1 h-1

45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0

IN-MPB-O3, OUT-MPB-O3, IN-MPB-NAT, OUT-MPB-NAT, IN-SPB-O3, OUT-SPB-O3, IN-SPB-NAT, OUT-SPB-NAT, IN-SMB-O3, OUT-SMB-O3, IN-SMB-NAT, OUT-SMB-NAT

β-D-1,4 glucosidase

μmol l-1 h-1

1600,0
1400,0
1200,0
1000,0
800,0
600,0
400,0
200,0
0,0

IN-MPB-O3, OUT-MPB-O3, IN-MPB-NAT, OUT-MPB-NAT, IN-SPB-O3, OUT-SPB-O3, IN-SPB-NAT, OUT-SPB-NAT, IN-SMB-O3, OUT-SMB-O3, IN-SMB-NAT, OUT-SMB-NAT

Leucine aminopeptidase

μmol l-1 h-1

10000,0
9000,0
8000,0
7000,0
6000,0
5000,0
4000,0
3000,0
2000,0
1000,0
0,0

IN-MPB-O3, OUT-MPB-O3, IN-MPB-NAT, OUT-MPB-NAT, IN-SPB-O3, OUT-SPB-O3, IN-SPB-NAT, OUT-SPB-NAT, IN-SMB-O3, OUT-SMB-O3, IN-SMB-NAT, OUT-SMB-NAT

Fig 5.14*: Extracellular enzyme activity (EEA) in the inlet (IN) and outlet (OUT) water of the EcoMicro system. IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

EEAs in the packing media. The EEAs determined in PM samples are reported in *Fig. 5.15*. As it was observed in water samples, the microbial community inside the biofilters (PM) seemed to be more efficient in the utilization of proteinaceous compounds, as indicated by the high leucine aminopeptidase activity, followed by carbohydrates (β-D-1,4 glucosidase activity), phosphorylated molecules (alkaline phosphatase activity) and lipids (lipase activity). All detected activities were generally comparable between natural and ozonated biofilters.

In detail, the alkaline phosphatase activity was comparable in natural and ozonated biofilters, both in MPB (42.80 ± 7.32 and 43.56 ± 11.00 µmol l^{-1} h^{-1}, respectively) and SMB (1.67 ± 0.86 and 1.80 ± 0.96 µmol l^{-1} h^{-1}, respectively) biofilters. Only in SPB the activity resulted higher in natural biofilters than in ozonated ones (41.49 ± 4.51 and 7.38 ± 0.71 µmol l^{-1} h^{-}1, respectively). The β-D-1,4 glucosidase activity was higher in MPB, in both ozonated and natural ones (53.19 ± 2.27 and 51.42 ± 4.56 µmol l^{-1} h^{-1}, respectively), than in the other biofilter types (SPB 7.38 ± 0.71 µmol l^{-1} h^{-1} – ozonated, and 41.49 ± 4.51 µmol l^{-1} h^{-1} – natural; SMB 1.67 ± 0.86 µmol l^{-1} h^{-1} ozonated and 1.80 ± 0.96 µmol l^{-1} h^{-1} natural). The leucine aminopeptidase activity in PM was higher in ozonated MPB biofilter (1454.56 ± 229.13 µmol l^{-1} h^{-1}) than in the natural one (864.71 ± 76.82 µmol l^{-1} h^{-1}). In both ozonated and natural SPB filters the activity was comparable (552.77 ± 33.73 and 648.74 ± 209.04 µmol l^{-1} h^{-1}, respectively) and almost no activity was found in SMB filters. Finally, the lipase activity was found in all natural biofilters (between 10.84 ± 1.62 and 1.63 ± 7.63 µmol l^{-1} h^{-1}), whereas no activity was found for those ozonated, with the exception of SPB (7.17 ± 2.05 µmol l^{-1} h^{-1}).

Overall, results suggest that ozone treatment had probably higher influence on water than on the biofilter packing media. In addition, alkaline phosphatase (and lipase) activity was higher in PM samples than in water. Conversely, β-D-1,4 glucosidase and leucine aminopeptidase activities were higher in water samples.
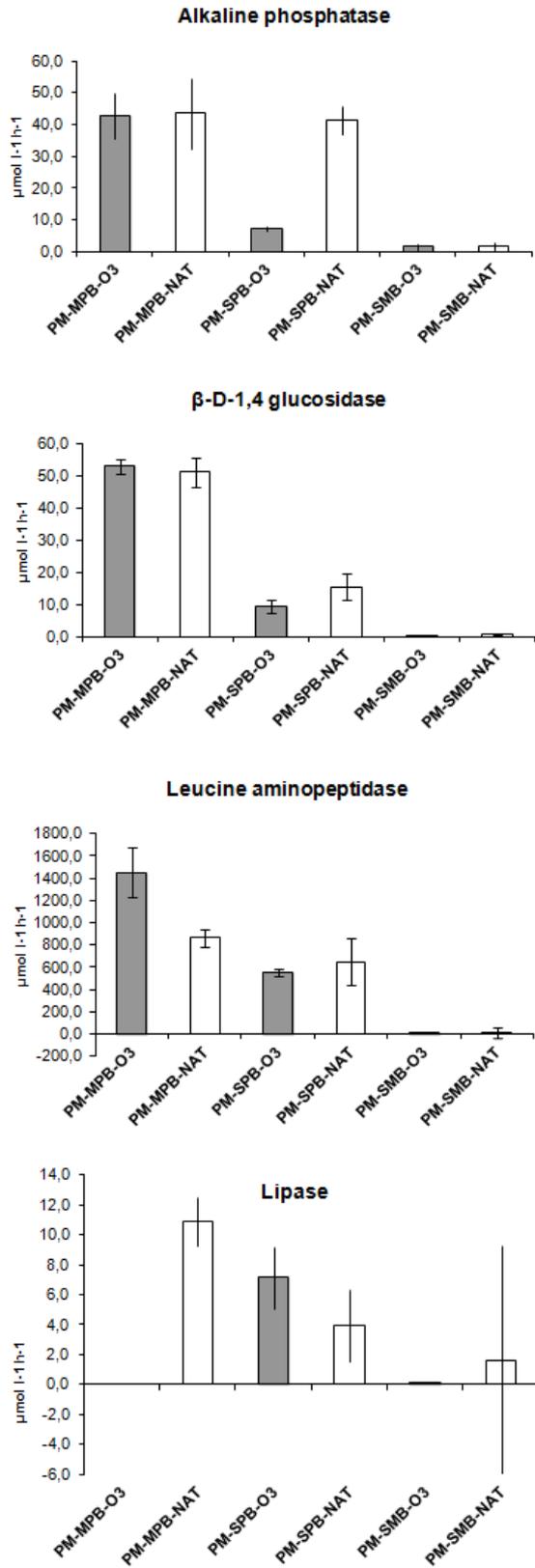
Fig 5.15: Extracellular enzyme activities (EEAs) in the PM of the EcoMicro system (note the different scale). IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

ARISA

The application of ARISA to samples from EcoMicro showed that there was a difference between communities inhabiting water and PM (*Fig. 5.16*). In fact, MDS analysis showed that water samples grouped in couple, with respect to the system (i.e. MPB, SPB or SMB) and treatment (i.e. ozonated and natural units) types, indicating that the composition of the bacterial community in water was really influenced by ozone treatment. On the contrary, PM community seem to have not greatly influenced free-living water community in the whole system, showing a similarity percentage of 60% between inlet and outlet of each filter type, excepting for ozoned MPB that had similarity of 80%.

PM samples appeared to be separated in two groups with a percentage similarity of 40%: 1) natural and ozonated MPB biofilters and 2) ozonated SPB and SMB, revealing an effect of ozone on PM community. The only exception was represented by ozonated MPB, which was closer to natural PM samples. This suggest a less impact of ozone on MPB community, probably due to the characteristics of biofilm in this kind of biofilter: in fact, the continuously mixing and rubbing between media units, due to the injection of air, could cause a destabilization of biofilm structure and stability. Therefore, ozone action maybe have more consequences on a more structured and stable biofilm, like in static configuration filters, than in a moving one.
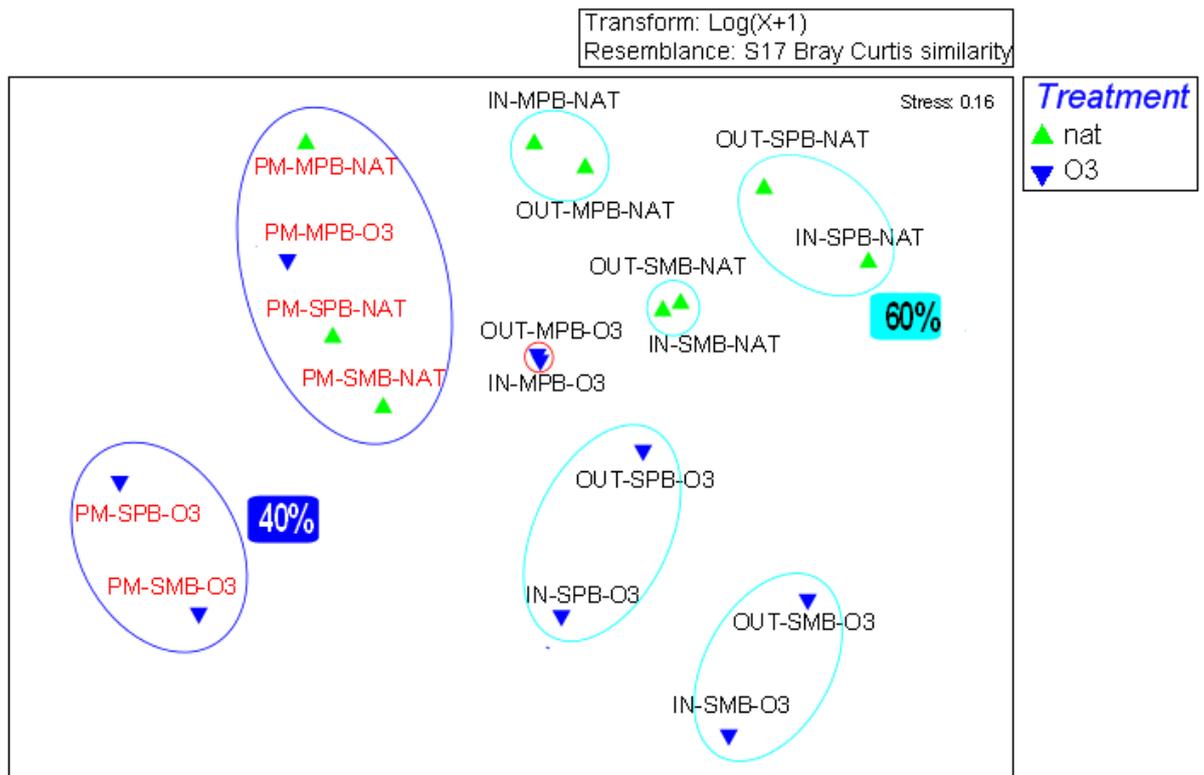
Fig 5.16: MDS computed from ARISA carried out on water and PM samples and respective similarity percentage from the EcoMicro system. IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

Computed indices on both inlet and outlet water showed that ozone treatments resulted in a shift of the bacterial community structure in term of reduction of taxa richness and diversity indices (Shannon, Simpson reciprocal and Pielou-Evenness) in natural biofilters compared to ozonated ones (*Fig. 5.17*). A possible explication is that a little laboratory system, like EcoMicro, allows a massive accumulation of total oxidants in water flow, causing a general bacteriostatic effect that could homogenise and uniform free-living bacterial community. However, this effect seems to be less marked in the Static Mineral Bed than in the others, probably due to a sort protective effect linked to packing media nature (expanded clay *vs* plastic in MPB and SPB).

Conversely, PM samples showed that ozone impacted deeply on ozonated samples than natural ones (*Fig. 5.18*). Probably, the microbial community colonizing packing media, being more structured and organized in the biofilm matrix, is more susceptible to oxidation by ozone by-products than free-living one.

In any case, results underlined that both ozonated communities (water and PM) were forced to adapt to the increased ORP regime, probably by the selection of bacteria able to cope with high oxidant conditions.
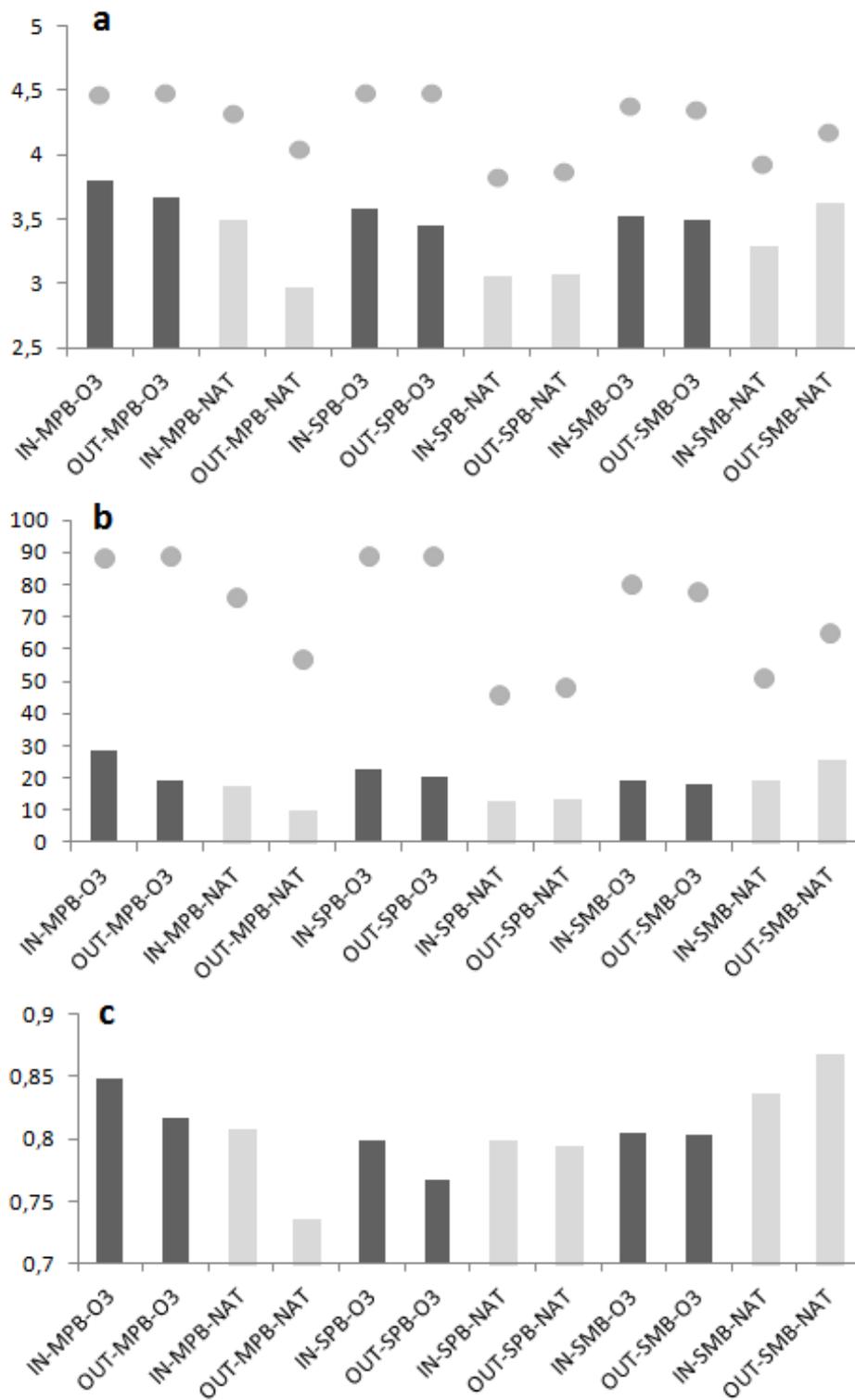
Fig 5.17: ARISA diversity indices in water samples: a) Shannon; b) Simpson reciprocal; c) Pielou Evenness. IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.
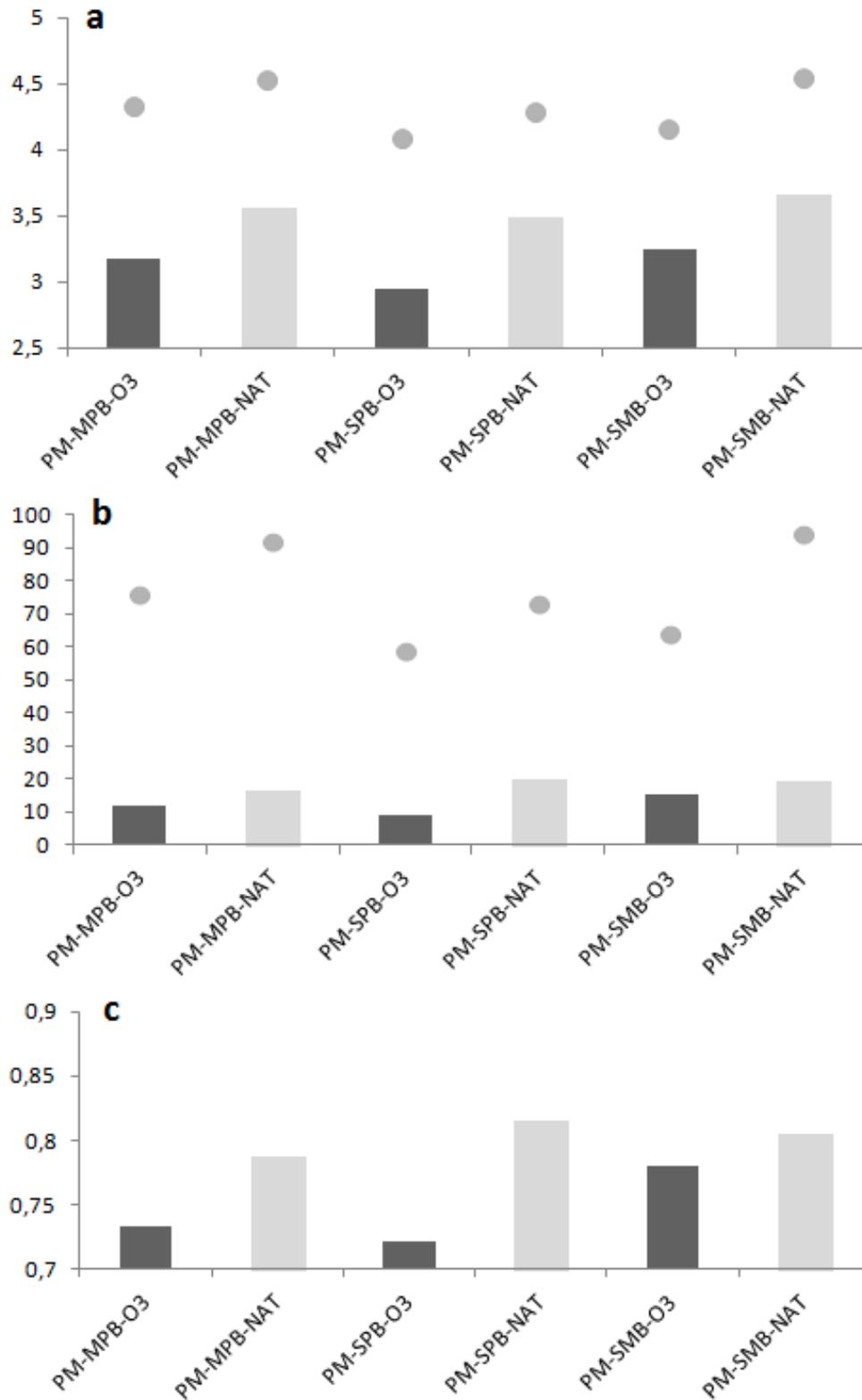
Fig 5.18*:* ARISA diversity indices in PM samples: a) Shannon; b) Simpson reciprocal; c) Pielou Evenness. PM, Packing Media; MPB, Moving Plastic Bed; SPB, Static Plastic Bed; SMB, Static Mineral Bed; O3, Ozonated samples; Nat, Natural samples.

5.4.1. Pilot scale experiment "Lagunage"

5.4.1.1. Chemical and biochemical analyses

Ozonated and natural biofilters in the Lagunage system resulted highly different about TOC (expressed as the difference between outlet and inlet values) that was 0.734 ± 0.0367 and 0.127 ± 0.0063 mg l^{-1}, respectively.

Nitrification performance in Lagunage was expressed as Total Ammonial Nitrogen (TAN) removal rate (see 5.3.3.). Confirming observations made for the EcoMicro biofilters, results showed that the untreated system (natural) was slightly more efficient than ozonated system in ammonia removal (761.36 ± 7.61 and 738.78 ± 7.38 g/m^3 day, respectively).

5.4.1.2. BOD$_5$

BOD$_5$ in Lagunage system revealed a trend comparable with the EcoMicro one. In fact, as shown in the *Fig. 5.19*, no significant differences were found in the oxygen consumption of ozonated and natural biofilters of Lagunage system ($1,32 \pm 0,076$ and $1,39 \pm 0,16$ mg l^{-1}, respectively). However, also in this case natural biofilters utilized more oxygen than ozonated ones, highlighting an ozone effect on oxygen consumption, as before suggested for EcoMicro system (see 5.3.1.2.).
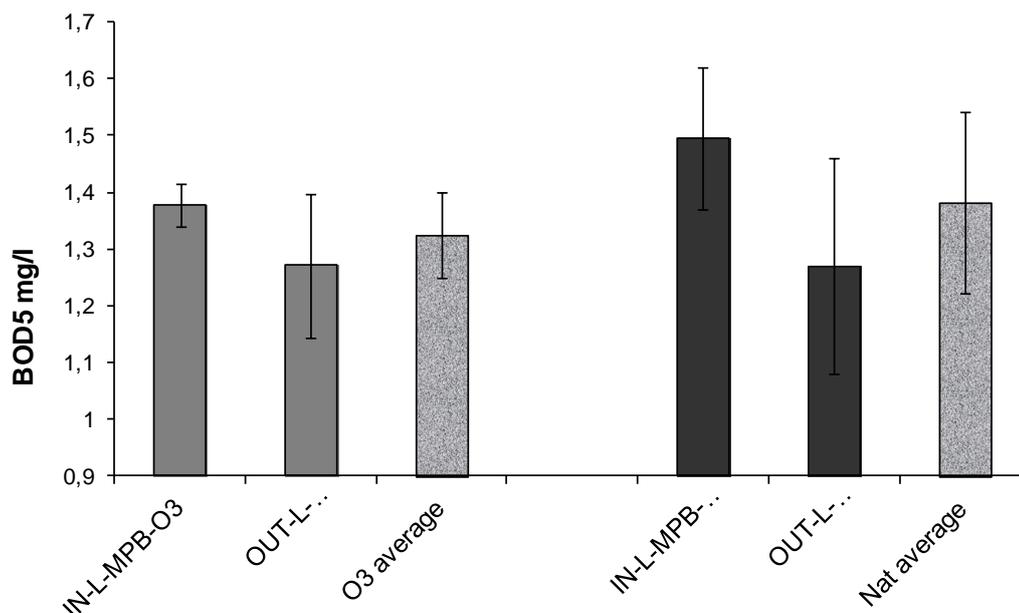
Fig 5.19: BOD$_5$ trend in the Lagunage system. IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; O3, Ozonated samples; Nat, Natural samples.

5.4.1.3. Microbial analyses

Microbial abundance

Confirming what reported before for EcoMicro system, flow cytometry showed a higher number of bacteria in natural biofilters than in ozonated ones being them 1.59 x 10^8 ± 7.93 x 10^6 and 2.03 x 10^8 ± 1.02 x 10^7 cells ml^{-1}, respectively.

Community Level Physiological Profile (CLPP)

CLPP did not highlighted real differences between both ozonated and natural units, with 76 and 74 carbon sources utilized, respectively. Moreover, in both cases communities preferred carboxylic acids over other substrates: 21 for ozonated filter and 23 for natural one.

Ectoenzymatic activities (EEAs)

The activity of 4 different enzymes (alkaline phosphatase, β-D-1,4 glucosidase, leucine aminopeptidase and lipase) were determined in water and PM of the Lagunage system, as described in the materials and methods section.

EEAs in water samples. The EEAs determined in the water of the Lagunage system are reported in *Fig. 5.20*. Overall, the microbial community seemed to be more

efficient in the utilization of proteinaceous and lipidic compounds, as indicated by the high leucine aminopeptidase and lipase activities.

The alkaline phosphatase activity was higher in ozonated biofilters than in natural ones, ranging from 31.00 ± 1.68 to 9.01 ± 0.64 µmol l^{-1} h^{-1} in the outlet water of ozonated and natural biofilters, respectively.

The β-D-1,4 glucosidase activity results underlined a tendency to a higher enzymatic activity in ozonated biofilters than in natural ones: the maximum was 17.28 ± 7.33 µmol l^{-1} h^{-1} in inlet water of ozonated biofilters and the minimum was 2.47 ± 1.26 µmol l^{-1} h^{-1} in outlet water of natural ones.

The leucine aminopeptidase showed a higher activity in ozonated biofilters: they went from a maximum of 1681.04 ± 48.59 µmol l^{-1} h^{-1} to a minimum of 434.30 ± 26.12 µmol l^{-1} h^{-1} in the outlet water of ozonated and natural biofilters, respectively.

The lipase activity was found only in the inlet water of both biofilters, being 875.63 ± 25.22 and 424.31 ± 16.42 µmol l^{-1} h^{-1} in ozonated and natural biofilters, respectively.

EEAs in the Packing Media. The EEAs determined in PM samples are reported in *Fig. 5.20*. As reported for the water samples, the microbial community seemed to be more efficient in the utilization of proteinaceous and lipidic compounds. The alkaline phosphatase and β-D-1,4 glucosidase activities were higher in natural biofilters than in ozonated ones: 103.55 ± 4.43 (O3) and 51.92 ± 14.96 (nat.) µmol l^{-1} h^{-1} for the former; 82.07 ± 9.88 (O3) and 41.08 ± 9.31 (nat.) µmol l^{-1} h^{-1} for the latter. The leucine aminopeptidase activity was comparable in both ozonated and natural biofilters (944.23 ± 46.05 and 1023.64 ± 59.51 µmol l^{-1} h^{-1}, respectively). Finally, no evident lipase activity was found in the Lagunage system.

Moreover, in the Lagunage system the alkaline phosphatase and β-D-1,4 glucosidase activities were higher in the PM, leucine aminopeptidase activity was resulted of the same order of magnitude in water and PM and, finally, lipase activity was the less represented ad itt was found only in the ozonated and natural SPB biofilters.
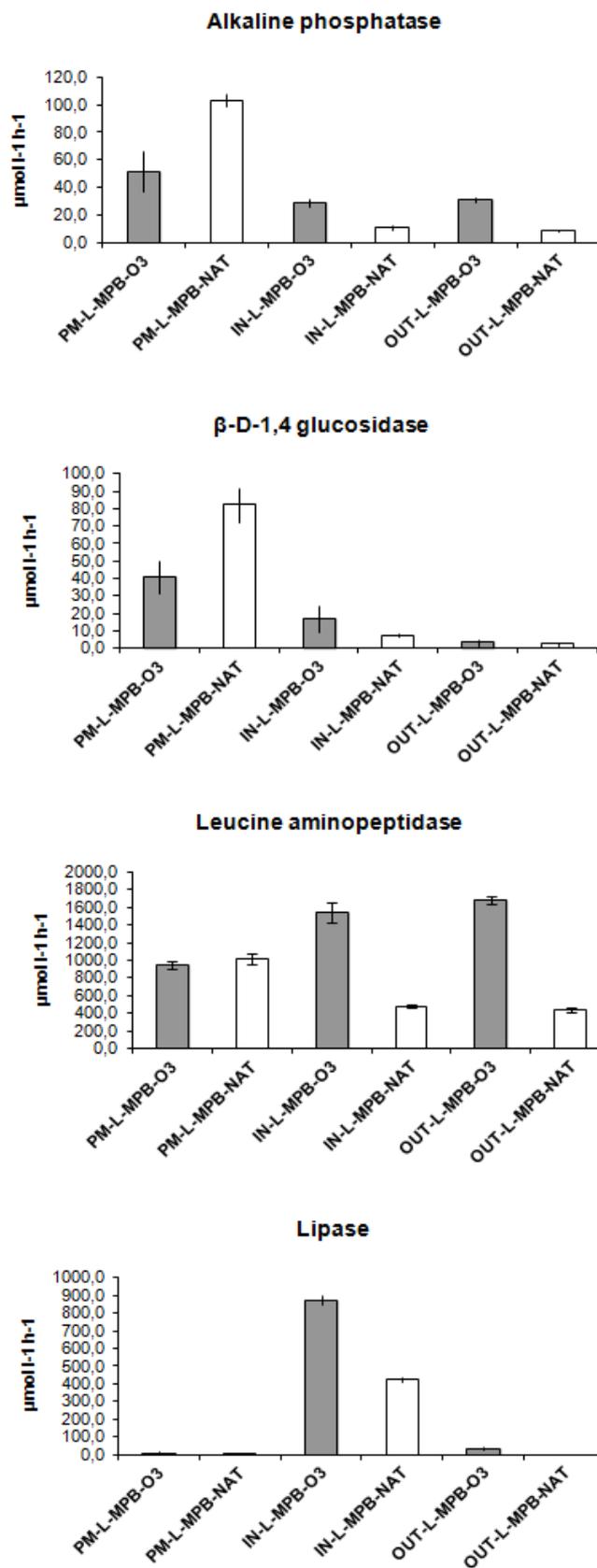
Fig 5.20: Extracellular enzyme activities (EEAs) in water and PM of the Lagunage system (note the different scale). IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; O3, Ozonated samples; Nat, Natural samples.

ARISA

ARISA in Lagunage demonstrated that there was a difference between communities inhabiting water and PM (*Fig. 5.21*). As observed for EcoMicro, MDS analysis on ARISA data showed that in the Lagunage system water samples from ozonated and natural biofilters were very similar and thus water samples grouped in couple, in relation to both filtration configuration (i.e. MPB, SPB or SMB) and treatment (i.e. ozonated and natural units) types, confirming a really influence of ozone treatment on water bacterial community.

Moreover, free-living water community was slightly affected by PM one, showing a similarity percentage of 60 between inlet and outlet of each filter type.

MDS also highlighted that PM biofilters appeared to be quite different from water ones, as they grouped apart, suggesting a greatly impact of ozone on community adherent to PM (*Fig. 5.21*).
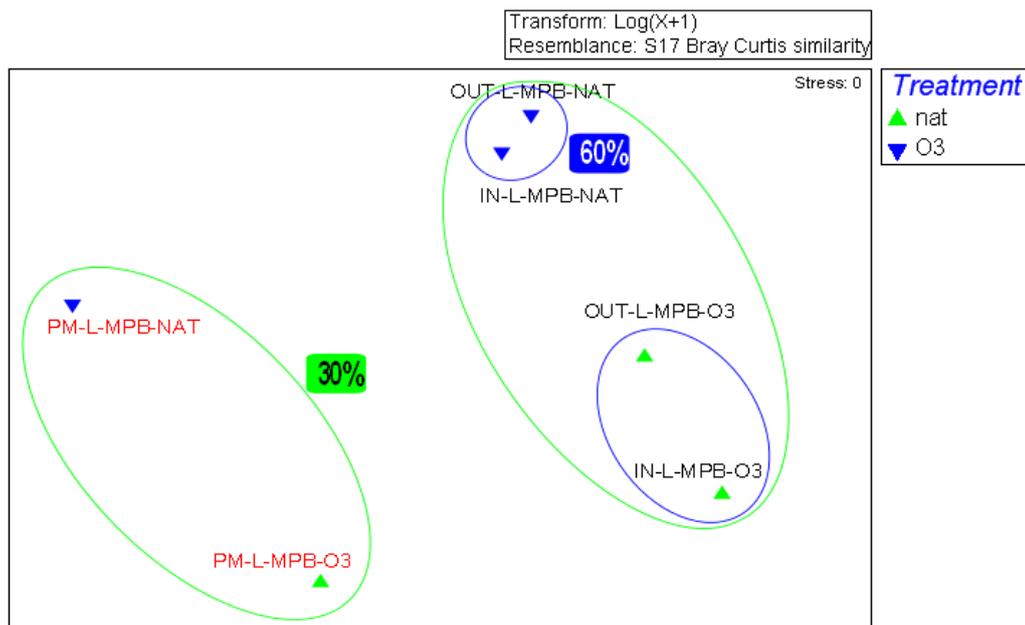
Fig 5.21: MDS computed from ARISA carried out on water and PM samples and respective similarity percentage from the Lagunage system. IN, Inlet; OUT, Outlet; MPB, Moving Plastic Bed; O3, Ozonated samples; Nat, Natural samples.

Shannon, Simpson reciprocal and Pielou-Evenness indices in Lagunage were computed on inlet water, PM and outlet water, revealing a different trend compared to EcoMicro analysis.

As shown in *Fig 5.22*, ozonation clearly impacted on inlet water and PM bacterial communities, but this effect seem to be opposite in outgoing water community. System size, concerning important dilution and recirculation period, could result in a decrease

of ORP, thus producing a reduction of oxidation in water. At the same time, own fish bacterial community could make an alteration of water community composition. These aspect could justified the trend inversion from EcoMicro system.

On the other side, higher ORP levels could determine a partial detachment of biofilm in PM, also promoting by the self-cleaning properties of MPB filters, and a consequence increasing of bacterial community in the outlet water.
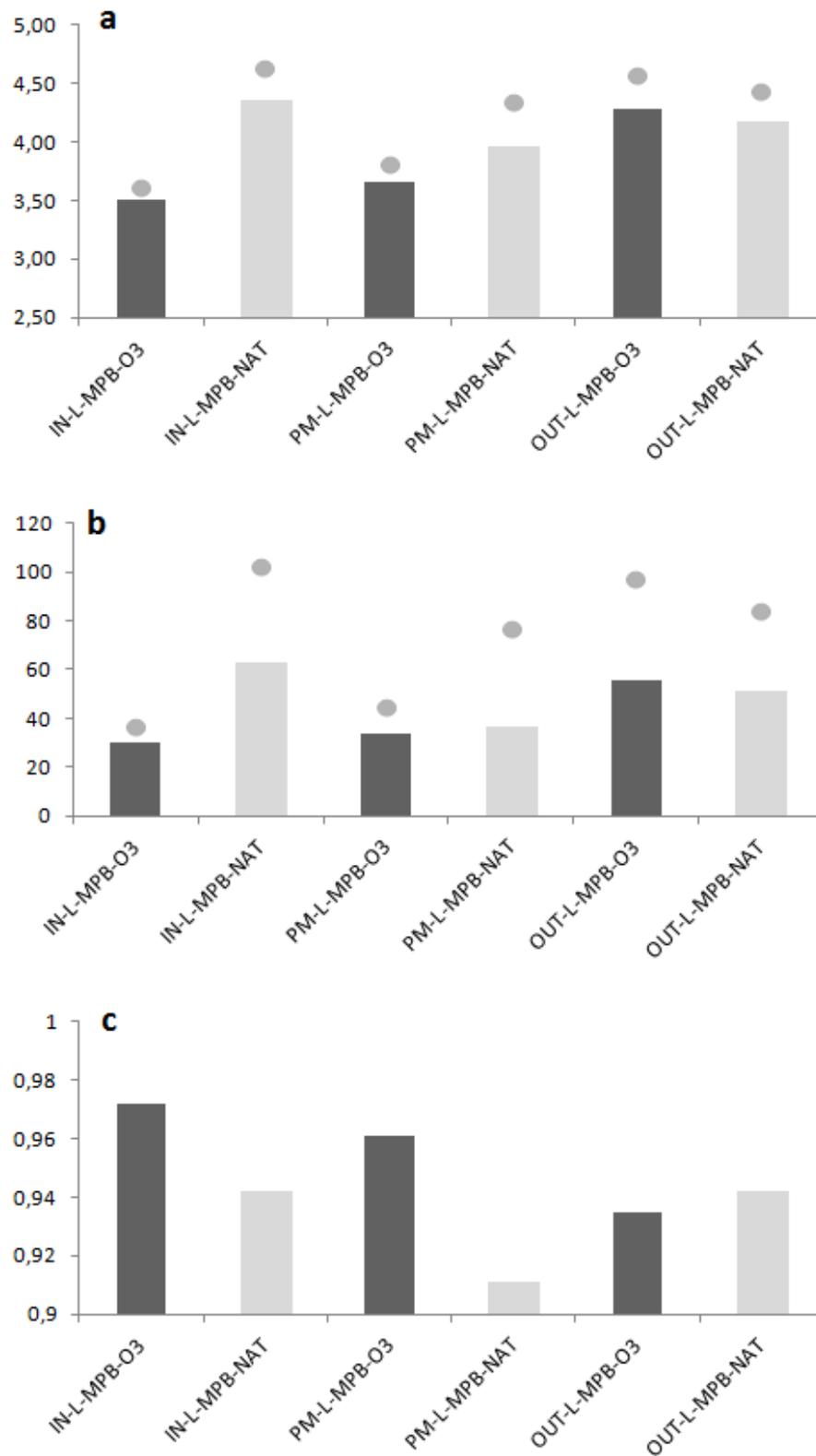
Fig 5.22: ARISA diversity indices in both water and PM samples: a) Shannon; b) Simpson reciprocal; c) Pielou Evenness. IN, Inlet; OUT, Outlet; PM, Packing Media; L, Lagunage; MPB, Moving Plastic Bed; O3, Ozonated samples; Nat, Natural samples.

In this study clone libraries were performed in order to investigate and compared the phylogenetic diversity of 16S rDNA clones associated to MPB filters in a pilote system, in order to reveal the precences of differences (if any) between ORP modified and natural.

Lagunage was chosen as closer and more representative system of industrial large scale ones, with the purpose of bring the comprehension of biofilter microflora as much as possible to a realistic level.

An amount of 116 clones were sequenced and 40 phylotypes were identify in ozonated unit (Tribord) (*Fig. 5.23*) versus 138 clones and 43 phylotypes in natural unit (Babord) (*Fig. 5.24*).

Clone in the both units were mainly represented by *Alphaproteobacteria* with a total percentage of 36.21 and 32.61 respectively.

In ozonated unit the second most abundant phylotype was that of the *Gammaproteobacteria* (26.72 %) followed by *Flavobacteria* (namely CFB) at just 15.52 %, confirming results of Michaud *et al.* (2009).

Conversely, natural side show that both *Gammaproteobacteria* and *Flavobacteria* (namely CFB) were at very similar percentage (23.19 % and 22.46 %, respectively).

Even though, *Flavobacteria* are a group of ubiquitous commensal bacteria and opportunistic pathogens, was founded that they could grow very slowly in some aquatic environments (Simek *et al.,* 2001). This aspect could suggest that ozone treatment had an effect on physiology of CFB, reducing their growth rate.
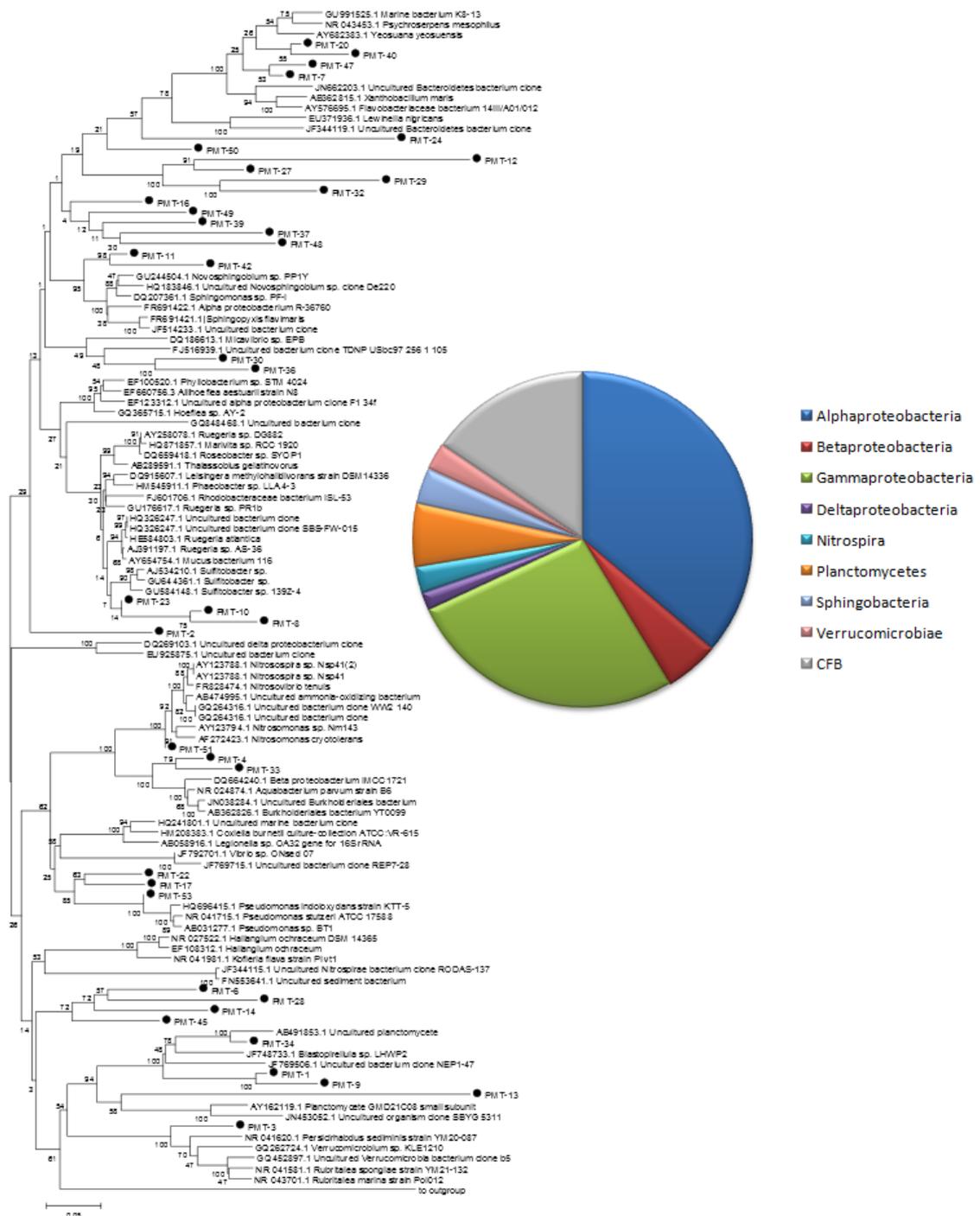
Fig 5.23: Rooted phylogenetic tree of ozonated system (PMT) showing affiliation of clones to closest-related sequences from cloned members of different bacterial clusters. Representative clones of different phylotypes are indicated with black dots. Sequence of Uncultured Crenarchaeote clone pSL17 (U63339) was used as outgroup, as described by Gentile and colleagues (2006).
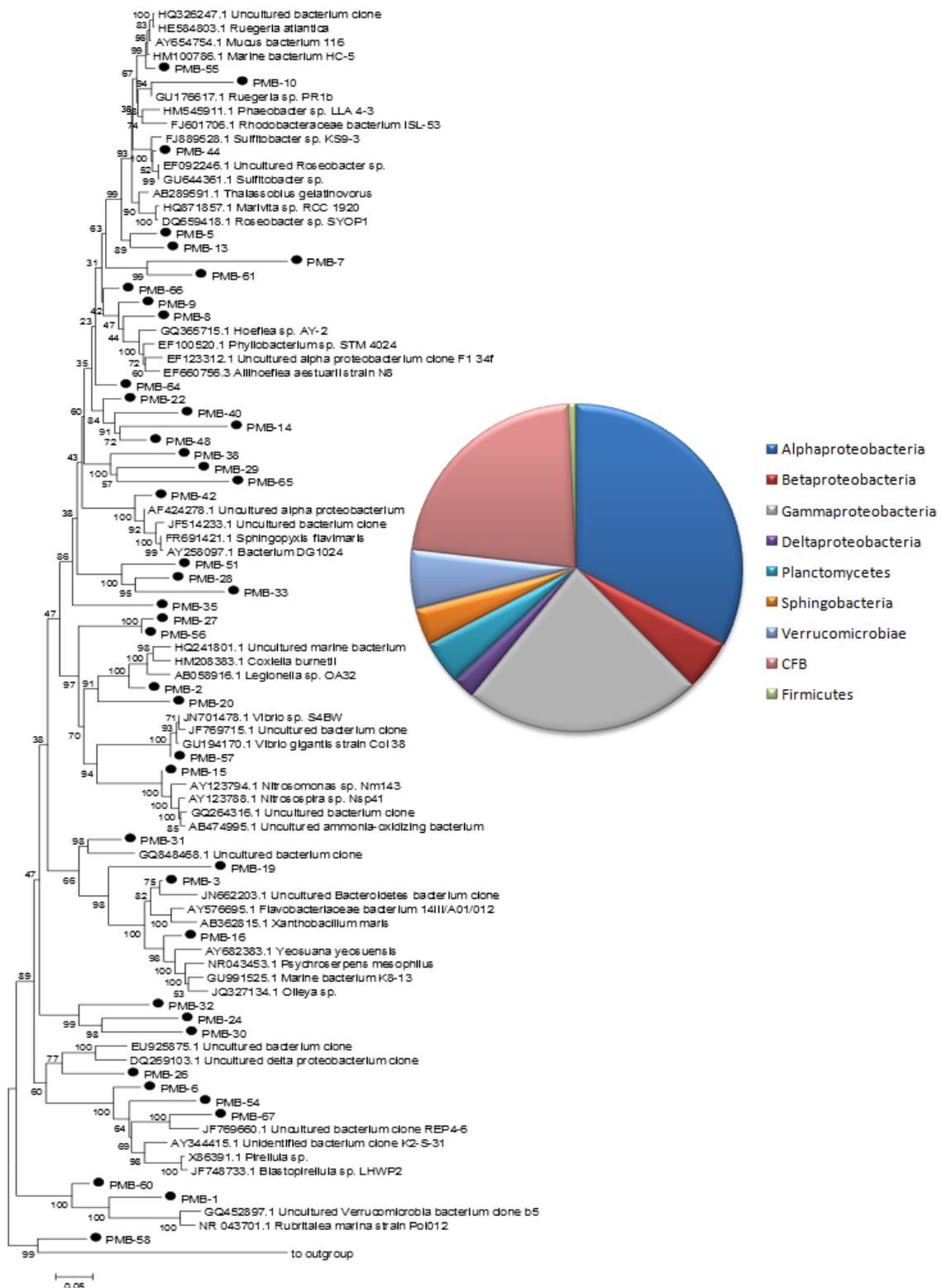
Fig 5.24: Rooted phylogenetic tree of natural system (PMB) showing affiliation of clones to closest-related sequences from cloned members of different bacterial clusters. Representative clones of different phylotypes are indicated with black dots. Sequence of Uncultured Crenarchaeote clone pSL17 (U63339) was used as outgroup, as described by Gentile and colleagues (2006).

CHAPTER 6

General discussion and conclusion

RAS technologies efficiency to production of marine species has been largely demonstrated (Manthe *et al.,* 1985, 1988; Davis and Arnold, 1998) but the commercial production has been limited by a number of factors (Mozes *et al.,* 2003). Lisac and Muir (2000) report a production cost 19% lower for offshore facilities compared with land based extensive operations.

Seawater recirculating systems play an important role in the production of healthy, properly sized fingerlings (Fielder and Allan, 1997). They are very adapt and compatible with the complex reproduction processes of commercial marine species and in rearing of fingerlings and marine ornamentals (Howerton, 2001; Palmtag and Holt, 2001), also diminishing the impact of waste processing costs. Reliable supply of fingerlings is a bottleneck for the commercial production of marine species, as sea bass, sea bream, flatfish and cobia among others (Watanabe *et al.,* 1998; Schwarz *et al.,* 2004). The higher market prices of marine fishes make this an attractive niche for recirculating systems.

Biofiltration represent the key point in RAS dedicated to marine species and hatchery. This expanding niche will demand operations under oligotrophic regimens (Malone and de los Reyes, 1997) which are rarely demanded by freshwater applications. The biofilter efficiency is evaluated according to his nitrification capacity, wich is proportional to the TAN (or nitrite-N) concentration: TAN must to remain below 1.0 mg/l (Zhu and Chen, 2001b).

The reduction of particulate and dissolved organic concentrations in recirculating systems represent one of the main goals in aquaculture: feed quality and assimilation (Losordo *et al.,* 1998; Leonard *et al.,* 2002), mechanical filtration of waste solids (Guerdat *et al.,* 2011), low maintenance biofiltration procedures (Gao *et al.,* 2011) are important fields for the reduction of the overall amount of POM and ultimately DOC in a recirculating system. Particulate organic matter (POM) in culture systems is comprised of uneaten feed particles, feces, and dead bacteria. Accumulation of POM in biofilters has been shown to significantly reduce the TAN removal rates in recirculating systems (Guerdat *et al.,* 2011; Léonard *et al.,* 2002; Michaud *et al.,* 2006). In the same

time, incorporating particle filtration immediately prior to the biofilters will reduce the available POM and increase TAN removal rates.

Increase of organic carbon concentration also promote heterotrophical metabolism and the consequent accumulation of potential pathogen bacteria into biofilter environment. Maintain the health of RAS represent a real challenge: use of antibiotics and chemicals in the water remain strictly regulated by law, but at the same time, the microflora living in aquaculture systems became more and more resistant to antibiotic.

However, 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 (Michaud *et al.,* 2009).

Although, in this work cloning on active microbial fraction in RAS shown that community is largely composed by *Vibrio sp.* group, outbreak of diseases did not occur. This seem to confirm the capacity of microbial populations in biofilters to maintain an effective and a stable rearing environment (shelter effect) (Michaud *et al.,* 2009), by competition for space, oxygen and energy sources and chemicals release in water and/or biofilm matrix (Verschuere *et al.,* 2000).

While antibiotic therapy is often used to treat pathogenic microorganisms, enhanced resistance and its consequences (Smith, 2008) have necessitated incorporating UV and ozone treatments to safeguard against infection (Shreier *et al.,* 2010).

In particular ozonation has been used in several aquaculture systems to disinfect or sterilize water supplies or discharge systems and it is also applied to control bacterial proliferation in closed recirculation systems (Suantika *et al.,* 2001).

Ozonation, as shown in the present work, reserve a deep impact on bacterial community in different biofilter types, modifying her composition, structure and metabolism. Also, nitrification process was also inhibited by ozone treatment respect to control, showing less enzymatic reaction velocity.

In conclusion, the characterization of RAS biofilter communities trough molecular culture-independent methods, principally relied on 16S rRNA gene analyses, have proved to be a very important tool for understanding microbial processes involved in managing and maintaining efficient and stable wastewater treatment and water quality control. Moreover, further studies on RAS system parameters (i.e. C/N ratio) and management procedures (i.e. ozonation) must to be performed in order to improve the production rate and the system health.

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