Universidade de Aveiro Departamento de Biologia

Ano 2016

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Caracterização molecular de comunidades microbianas na aquacultura

Molecular characterization of microbial communities in aquaculture

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Molecular characterization of microbial communities in aquaculture

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Dr. Newton C.M. Gomes, Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) e dos Professores Doutores Victor Quintino e Ana Maria Rodrigues, Professores do Departamento de Biologia da Universidade de Aveiro.

Este trabalho foi financiado pelo Centro de Estudos do Ambiente e do Mar e pelo PROMAR através do projeto AQUASAFE (31-03-05-FEP-0016).

Patricia Martins recebeu apoio financeiro da Fundação para a Ciência e Tecnologia (FCT) e do Fundo Social Europeu (FSE) no âmbito do III Quadro Comunitário de Apoio sobre a forma de Bolsa Individual de Doutoramento com referência SFRH/BD/73889/2010.

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agradecimentos

Primeiramente gostaria de agradecer ao meu orientador, o Dr. Newton Gomes. Obrigada pela disponibilidade que sempre teve, obrigada pela confiança, obrigada por todo o apoio e um obrigada especial por todo o conhecimento que me foi passando ao longo destes quatro anos.

Ao Dr. Victor Quintino e à Dra. Ana Rodrigues pela sua co-orientação. Obrigada pelo apoio.

Ao Dr. Daniel Cleary e ao Dr. Ricardo Calado pela sua disponibilidade e auxílio, foram fundamentais para a concretização deste trabalho.

Aos colegas do LEMAM, sem eles não teria tanta piada. Obrigada pelas conversas sobre os mais variados assuntos, desde temas relacionados com biologia molecular como outros não tão importantes mas não menos interessantes. Realmente trabalhar num ambiente tão agradável propicia um interesse ainda maior pelo trabalho.

Á minha amiga Alexandra, pela compreensão relativa aos meus atrasos constantes por motivos de trabalho.

Aos meus amigos, à Marta, ao Fernando, ao Renato, à Luísa e ao Fábio por apesar de irem cobrando a minha ausência, terem compreendido a minha constante falta de disponibilidade. Obrigada por estarem presentes.

Aos meus pais e irmãs pelo apoio incondicional. Obrigada por acreditarem em mim.

Em especial ao meu marido e filho. Obrigada por estarem presentes na minha vida, convosco tudo é mais fácil.

Aos restantes amigos que me apoiaram nos momentos mais difíceis.

A todos, muito obrigada.

palavras-chave

Aquacultura, Comunidades Bacterianas, Doenças, Deteção de Espécies Patogénicas, Técnicas de Biologia Molecular.

resumo

Os microrganismos têm um papel fundamental no processo de produção de peixes, bem como na manutenção da saúde dos mesmos nos sistemas de aquacultura. Contudo, existe ainda falta de informação acerca da composição e diversidade bacteriana destes sistemas.

O objetivo principal deste estudo é adquirir conhecimento fundamental acerca das communidades bacterianas de dois sistemas distintos de aquacultura (sistema intensivo e semi-intensivo) recorrendo a técnicas de biologia molecular. Adicionalmente, avaliou-se também a utilização de técnicas de biologia molecular para detetar potenciais bactérias patogénicas e desenvolvemos uma nova abordagem molecular para deteção rápida de uma espécie bacteriana patogénica de peixes (*Photobacterium damselae*) em sistemas de aquacultura.

Na aquacultura intensiva estudou-se a composição das comunidades bacterianas presentes num sistema de aquacultura em recirculação (RAS) com produção de pregado (*Scophthalmus maximus*) e linguado (*Solea solea*). As técnicas de biologia molecular utilizadas, eletroforese em gel de gradiente desnaturante (DGGE) e pirosequenciação de fragmentos do gene 16S, revelaram diferenças entre as comunidades bacterianas dos dois sistemas de produção, sugerindo que as espécies produzidas promovem um efeito acentuado nas comunidades bacterianas presentes na água. Várias espécies potencialmente patogénicas foram identificadas, contudo nenhum peixe demonstrou sinais de doença durante o estudo. Neste estudo, a pirosequenciação mostrou ser uma técnica sem precedente para estudar as comunidades bacterianas e revelar potenciais espécies patogénicas.

Na segunda fase deste estudo, investigámos padrões sazonais na composição do bacterioplâncton e na sua função num sistema de aquacultura semi-intensivo de produção de robalo (*Dicentrarchus labrax*) localizado na Ria de Aveiro. Os resultados indicaram que as alterações nas comunidades do bacterioplâncton foram provocadas pelas variações sazonais dos fatores abióticos da água do estuário. Além disso, a utilização de ferramentas bioinformáticas para prever o conteúdo funcional do metagenoma, como o PICRUST, permitiu-nos conhecer melhor a qualidade microbiológica deste sistema de aquacultura.

Tendo em conta o impacto dos surtos de doenças no crescimento do sector da aquacultura, desenvolvemos

uma nova abordagem utilizando métodos moleculares para uma rápida deteção de subespécies de *Photobacterium damselae* responsávies por enormes perdas económicas em sistemas de aquacultura nos países mediterrâneos da Europa.

Foram desenvolvidos *primers* específicos utilizando o gene *toxR* para quantificar a espécie *P. damselae* e detetar as suas subespécies (*P. damselae piscicida and P. damselae damselae*) utilizando a técnica de PCR em tempo real (RT-PCR) e PCR – eletroforese em gel de gradiente desnaturante (PCR-DGGE), respetivamente. Esta abordagem mostrou elevada especificidade para a quantificação e deteção das subespécies de *P. damselae* em amostras de água provenientes de um estuário e de um sistema de aquacultura.

keywords

Aquaculture, Bacterial communities, Diseases, Pathogen Detection, Molecular Techniques, *Photobacterium damselae*.

abstract

Microorganisms play key roles in fish production and in the maintenance of fish health in aquaculture systems. However, there is still a lack of fundamental knowledge regarding the bacterial composition and diversity of these systems.

The general goal of this study is to gain fundamental knowledge on the bacterial communities of different aquaculture systems using molecular techniques. Furthermore, we evaluate the use of molecular methodologies to detect potential fish pathogens and develop a new molecular approach for early detection of a fish pathogen (*Photobacterium damselae*) in aquaculture systems.

In the intensive aquaculture, we assessed the composition of bacterial communities inhabiting recirculating aquaculture systems (RAS) of turbot (*Scophthalmus maximus*) and sole (*Solea solea*). Both molecular approaches, DGGE profiles and barcoded-pyrosequencing of 16S rRNA gene fragments, revealed differences between the bacterial communities of both RAS, suggesting that the fish species being cultured promoted a strong effect on water bacterial communities. Several potential pathogenic species were detected, however despite the presence of these pathogens, no symptomatic fish were observed during the study. In this study barcoded pyrosequencing showed to be an unprecedented technique for assessing bacterial communities and reveal potential pathogens.

In the second phase of this study, we investigated seasonal patterns of bacterioplankton (free-living and particle-associated bacteria) composition and their putative function in a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system located at Ria de Aveiro. The results indicated that shifts in the bacterioplankton communities are driven by seasonal changes of abiotic characteristics of the estuarine water. Furthermore, the use of bioinformatics tools such as PICRUSt to predict metagenome functional content allowed us a better understanding of microbial quality in this aquaculture system.

Given the impact of disease outbreaks in the development of aquaculture sector, we developed a new molecular approach for fast detection of *Photobacterium damselae* subspecies, responsible for major economic losses in the aquaculture systems in Mediterranean countries of Europe. Specific primers targeting the *toxR* gene were designed for overall quantification of *P. damselae* species and specific detection of its subspecies (*P. damselae piscicida and P. damselae damselae*) based on real time PCR (RT-PCR) and PCR denaturing gradient gel electrophoresis (PCR-DGGE), respectively.

This approach showed good specificity for quantification and detection of *P. damselae* subspecies in estuarine and aquaculture water samples. The molecular approach developed here can contribute for a more effective long-term management of fish health in aquaculture systems.

List of Publications

This thesis includes results which have already been published in the journals listed below:

Martins, P., Cleary, D.F.R., Pires, A.C.C., Rodrigues, A.M., Quintino, V., Calado, R. and Gomes, N.C.M., 2013. Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for *Scophthalmus maximus* and *Solea senegalensis*. PLoS ONE. 8: e80847. DOI: 10.1371/journal.pone.0080847

Martins, P., Navarro, R.V.V., Coelho, F.J.R.C. and Gomes, N.C.M., 2015. Development of a molecular methodology for fast detection of *Photobacterium damselae* subspecies in water samples. Aquaculture. 435: 137-142. DOI:10.1016/j.aquaculture.2014.09.028

CONTENTS

List of Figures

List of Figures given as supplementary material

List of Tables

Chapter 1. Introduction

Aquaculture overview

The growth of the human population in the last decades has led to a substantial increase in the demand for fish, and subsequently a decrease in natural stocks. According to the Food and Agriculture Organization (FAO, 2014) world per capita food fish supply increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012. As a response to this growing demand, aquaculture has become one of the fastest growths of food production industry. From 1980 to 2012 aquaculture production increased at an average rate of 8.6 percent per year. In 12 years (from 2000 to 2012) world food fish aquaculture production more than doubled, increasing from 32.4 million tonnes to 66.6 million tonnes (FAO, 2014). In Portugal, from 2011 to 2012 the aquaculture production increased 12.3 percent reaching a production of 10,317 tons (INE, 2013). China is, by far, the largest producer with 43.5 million tonnes of food fish and 13.5 million tonnes of aquatic algae.

In 1988, FAO (1988) defined aquaculture as "the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants. Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc..".

Aquaculture systems can be classified according to the technology or the production system used. Frequently, these systems are classified as "extensive", "semi-intensive" and "intensive" based on the production per unit volume (m3) or per unit area (m2) farmed. Extensive aquaculture involves production systems with low density of organisms (stock density of less than 1 ton per hectare per year), minimal input of artificial nutrition (depends mostly on natural food), low level of technology and very low investment. The semi-intensive aquaculture involves production systems with lower densities (stock density of 1-5 kg/m^3) and the animal nutrition depends mostly on natural food but is complemented with an input of artificial nutrition. Basically, this system is an intermediate production system between the extensive and the intensive aquaculture. Intensive aquaculture involves production systems with high density of organisms (stock density of 12-18 kg m^3), high input of artificial nutrition (addition of complete diets), the use of more technology and subsequently much higher investment costs (FAO, 1988).

Aquaculture systems can also be classified depending on other characteristics, such as: where the production system is located (within the sea, in a tidal zone or on land), the type

of environment where the animals are cultivated (freshwater, marine and brackish water) or the life stage of the species produced (eggs, fry, juvenile or on-growing), among others.

The main benefits of aquaculture are an increase of global production of food for human consumption, less impacts on wild stocks, new business opportunities, the creation of employment mainly in rural areas, increase of national exports and decrease of imports by local production (FAO, 1988; Koldewey et al., 2010).

Worldwide, more than 600 species are cultured using different production systems of varying input intensities, technological sophistication and type of water used (FAO, 2014). There is a wide variety of species cultured in aquaculture systems, such as molluscs, crustaceans, amphibians and reptiles, aquatic invertebrates and marine and freshwater algae. According to the latest information, FAO estimates that in 2012 aquaculture produced over 44 million tonnes of finfish (87 499 US$million), 6 million tonnes of crustaceans ($30 million), 15 million tonnes of molluscs ($15 million) and 0.865 million tonnes of other species ($3 million) (Table 1.1) (FAO, 2014).

Table 1.1. World production of farmed species groups from inland and marine aquacultures in 2012.

	Inland aquaculture (Million tonnes)	**Mariculture** (Million tonnes)	**Quantity subtotal** (Million tonnes)	(Percentage by volume)	**Value subtotal** (US$ million)	(Percentage by value)
Finfish	38.599	5.552	44.151	66.3	87 499	63.5
Crustaceans	2.530	3.917	6.447	9.7	30 864	22.4
Molluscs	0.287	14.884	15.171	22.8	15857	11.5
Other species	0.530	0.335	0.865	1.3	3512	2.5
Total	**41.946**	**24.687**	**66.633**	**100**	**137 732**	**100**

Microbial communities in aquaculture

Bacteria, micro algae and protozoans have major roles in aquatic ecosystems. These microorganisms are involved in several processes in aquaculture systems productivity, nutrient cycling, nutrition of the cultured animals, water quality, disease control and environmental impact of the effluent.

In aquaculture systems the organic matter resulting from unconsumed food and fish metabolites is recycled by a diverse array of microbes. The bacterial communities in

aquaculture systems include two main groups of bacteria: autotrophic (mostly nitrifying bacteria) and heterotrophic. Autotrophic and heterotrophic bacteria play an important role in oxidation of ammonia to nitrate and degradation of organic matter. Both, autotrophic and heterotrophic bacteria have a complementary relationship. Heterotrophic bacteria produce carbon dioxide as an end product, which provide a carbon source for autotrophic bacteria, which in turn create biomass that will be later be consumed by the heterotrophic bacteria.

One of the major metabolic wastes produced from aquatic animals is ammonia which is toxic to aquatic animals. The high production densities of fishes in these systems lead to a high accumulation of ammonia–nitrogen in the water as a result of fish excretion products (feces, urine and unconsumed food). Furthermore, heterotrophic bacteria mineralize the organic matter of unconsumed food and of organism excretions, increasing even more ammonia concentrations in water. Nitrogen-containing organic molecules are decomposed into ammonia by heterotrophic bacteria, with ammonia subsequently being converted into nitrite, and nitrite into nitrate, by nitrifying bacteria. It is well known that nitrifying bacteria activity is influenced by several factors such as temperature, salinity, dissolved oxygen, sunlight, degree of water exchange, transparency of water tank or depth of the tank (Stenstrom et al., 1980; Antoniou et al., 1990; Rysgaard et al., 1999). Thus, the type of aquaculture has a strong influence on bacterial activity. For instance, in a semi-intensive aquaculture system located in an estuarine environment, the degree of water exchange is high (output of water rich in organic material and an input of water from the open ocean) and subsequently the low activity of nitrifying bacteria is observed when compared to a recirculating aquaculture systems (RAS).

Most of the studies addressing the bacterial communities in intensive aquaculture systems are mainly related to bacterial populations involved in the nitrogen (N) cycle (Sugita et al., 2005; Schreier et al., 2010), while in the semi-intensive the majority of studies focus on physical and chemical parameters to assess water quality (Tucker et al., 1993; Guerrero-galván et al., 1998). However, there is a lack of information about the overall diversity and composition of bacterial communities in the water of the aquaculture systems during fish production.

The in-depth analysis of microbial communities in aquaculture systems provides quantitative and qualitative outputs with potential to unravel a comprehensive picture of the "normal" aquaculture microbiome and their role during fish growth and health. Furthermore, this

information can improve our ability to understand and control the microbial quality of water and decrease the risk of disease outbreaks.

Diseases in aquaculture with fish production

Disease outbreaks are one of the main constrains to the development of the aquaculture sector. In aquaculture systems, fish are in permanent contact with microbial communities and fish metabolites, a feature that can affect their health and growth. Diseases in aquaculture are caused by viruses, bacteria, fungi or protistan and metazoan parasites, however bacterial pathogens probably cause more disease problems than all others combined (Meyer, 1991). Many of the bacteria inhabiting the aquaculture that are capable of causing disease are saprophytic in nature only becoming pathogenic when fishes are physiologically unbalanced. Usually diseases occur as the result of a disruption of the normal environment in which animals are being reared. Some conditions such as crowding, temperature fluctuations, inadequate dissolved oxygen, excessive handling, or toxic substances may be propitious for the development of diseases in the aquaculture system (Meyer, 1991; Moriarty, 1997). "Stress" is considered to be an important predisposing factor in most bacterial diseases of fish.

The increased intensification of aquaculture practices implies higher density of individuals, which requires great use and management of inputs, greater waste products and increase potential for the spread of pathogens.

The main diseases in aquaculture systems are vibriosis (causative agent - *Vibrio*), photobacteriosis (causative agent - *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*)), furunculosis (causative agent - *Aeromonas salmonicida* subsp. *salmonicida*), flexibacteriosis (causative agent - *Tenacibaculum maritimum* (formerly, *Cytophaga marina*, *Flexibacter marinus* and *F. maritimus*)), pseudomonadiasis "Winter disease" (causative agent - *Pseudomonas anguilliseptica* (among other *Pseudomonas* species)), bacterial kidney disease (BKD) (causative agent - *Renibacterium salmoninarum*), streptococcosis (causative agent - *Streptococcus parauberis*) and mycobacteriosis (causative agent - *Mycobacterium marinum* (among other *Mycobacterium* species)) (Table 1.2) (Toranzo et al., 2005). Nowadays, diseases considered as typical of fresh water aquaculture, such as furunculosis (*A. salmonicida*), bacterial kidney disease (BKD) (*R. salmoninarum*) and some types of streptococcosis, are a major problem also in marine aquaculture systems.

Table 1.2 - Aetiological agents of the main bacterial fish diseases affecting aquaculture systems.

Causative agent	Disease	Main host fish
Vibrio (Listonella) anguillarum	Vibriosis	Salmon, turbot, sea bass, striped bass, eel, ayu, cod, and red sea bream
Mycobacterium marinum	Mycobacteriosis	Seabass, turbot, Atlantic salmon
Photobacterium damselae	Photobacteriosis	Seabream, seabass, sole, striped bass, yellowtail
Pseudomonas anguilliseptica	Pseudomonadiasis "Winter disease"	Seabream, eel, turbot, ayu
Aeromonas salmonicida	Furunculosis	salmon, trout, goldfish, koi and a variety of other fish species
Tenacibaculum maritimum	Flexibacteriosis	Turbot, salmon, sole, seabass, gilthead seabream, red seabream, flounder
Renibacterium salmoninarum	Bacterial kidney disease (BKD)	Salmon
Streptococcus parauberis	Streptococcosis	Turbot
Flavobacterium psychrophilum	Coldwater disease	Salmon, carp, eel, tench, perch, ayu

Among these diseases, vibriosis and photobacteriosis are the diseases that cause major economic losses in the aquaculture systems with production of gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax)* and sole (*Solea senegalensis* and *Solea solea*) in the Mediterranean countries of Europe (Toranzo et al., 1991; Magariños et al., 1996; Romalde et al., 1999; Zorrilla et al., 1999).

Vibriosis is caused by *Listonella (Vibrio) anguillarum* in a wide variety of warm and cold water fish species: Pacific and Atlantic salmon (*Oncorhynchus spp*. and *Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), seabass (*D. labrax*), seabream (*S. aurata*), striped bass (*Morone saxatilis*), cod (*Gadus morhua*), Japanese and

European eel (*Anguilla japonica and Anguilla anguilla*), and ayu (*Plecoglossus altivelis*) (Toranzo et al., 1993; Toranzo et al., 2005).

Photobacteriosis, also known as pasteurellosis or pseudotuberculosis is caused by *P. damselae piscicida* (Fig. 1.1). The species *P. damselae* comprises two subspecies, *P. damselae* subsp. *damselae* and *P. damselae* subsp. *piscicida*, both described as emergent fish pathogens in aquaculture systems. Each subspecies cause a different disease.

P. damselae subsp. *piscicida* is the causative agent of photobacteriosis disease and was first detected in natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) from Chesapeake Bay (Maryland, USA) (Snieszko et al., 1964). Nowadays *P. damselae* subsp. *piscicida* infects a variety of marine fish species (Toranzo et al., 1991; Pujalte et al., 2003; Pedersen et al., 2009).

P. damselae damselae is responsible to infect several aquatic animals (fishes, crustaceans, dolphins and molluscs, among others) (Hanlon et al., 1984; Buck et al., 1991; Fouz et al., 1992; Song et al., 1993) and is frequently associated with epizootic outbreaks in cultured fish species (turbot, rainbow trout and sea bream, among others) (Vera et al., 1991; Fouz et al., 1992; Pedersen et al., 2009). Only the subspecies *P. damselae damselae* is able to infect humans (Shin et al., 1996; Fraser et al., 1997), causing necrotizing fasciitis. According with Yamane et al. (2004) this subspecies is more aggressive and causes higher mortality rate in humans than *Vibrio vulnificus*.

Figure 1.1 - Hemorrhaged areas on a tongue sole (*Cynoglossus semilaevis*) fish infected with *P. damselae* subsp. *piscicida* (in Austin and Austin, 2007).

Despite both *P. damselae* subspecies be considered emergent fish pathogens, *P. damselae piscicida* causes higher mortality rates in aquaculture systems. Thus, it is crucial to identify and distinguish *P. damselae* subspecies. Given the high phenotypic similarities between the subspecies, classical microbiological and biochemical methods are the most commonly used strategies to identify and distinguish them. However, due to the limitations of the conventional microbiological techniques (laborious, time-consuming and lack of sensitivity), a variety of DNA-based protocols have also been developed (Zhao et al., 1989; Osorio et al., 1999; Kvitt et al., 2002; Zappulli et al., 2005; Amagliani et al., 2009).

Although previous molecular techniques showed the ability to differentiate between the two *P. damselae* subspecies, most of these studies are based on the 16S rRNA gene (Zappulli et al., 2005; Amagliani et al., 2009), which is not considered a suitable phylogenetic marker to distinguish between *P. damselae piscicida* and *P. damselae damselae* (Osorio et al., 1999; Osorio et al., 2000). Thus, it is of crucial importance to develop a fast, specific and sensitive tool to detect and distinguish the two *P. damselae* subspecies.

The spread of infectious diseases in aquaculture systems has a serious impact on this sector. Therefore, the early detection of pathogens is crucial for an effectiveness disease control.

The use of molecular methods in aquaculture systems

Until the last decade, microbial identification required the isolation of pure cultures. The traditional culture dependent methods were used to access the microbial diversity in all types of environments, however, only approximately 1% of bacteria on Earth can be cultivated *in vitro* (Amann et al., 1995). Thus, the use of culture dependent methodologies underestimates dramatically the diversity of bacterial communities. Some advances were made with these techniques, mainly in the identification of particular pathogenic bacteria in clinical microbiology. However, the use of these techniques are difficult or are not adequate for studying the bacterial communities in natural environments. For instance, the role of bacteria in the decomposition of organic matter and nutrient cycling was not fully understood until recently, with the development of new molecular methodologies.

Within the past few decades, molecular methods have been developed and used as alternative to the culture dependent methods. The development of molecular techniques allows to

overcome the limitations of the traditional microbiological methods, providing a rapid, sensitive and high resolution description of microbial communities (Dahllöf et al., 2000). Several molecular techniques were developed, such as polymerase chain reaction (PCR), real-time PCR (RT-PCR), loop mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), PCR - denaturing gradient gel electrophoresis (PCR-DGGE), single-strand conformation polymorphism analysis (SSCP), terminal restriction fragment length polymorphism (T-RFLP) and barcoded pyrosequencing. Despite PCR, RT-PCR, LAMP or NASBA have already been widely accepted for fast detection of pathogens in aquaculture systems (Saulnier et al., 2009; García-González et al., 2011), the use of these technologies depends on the selection of a range of pathogens to consider in the assay and, therefore, new or unexpected pathogens will not be detected. Alternatively, molecular fingerprint analyses of microbial communities (e.g. PCR-DGGE, SSCP and T-RFLP) allow us to profile complex microbial communities in a range of environments (Muyzer et al., 1993; Acinas et al., 1997; Schwieger et al., 1998). Overall, these community fingerprint techniques are cost-effective and allow a fast characterization of different microbial assemblages in multiple samples, and can easily be used to monitor microbial community structure in fish farms (De Schryver et al., 2008). The major constrain of these techniques is the lack of information about the identity of the microbial population. As a result, the use of high throughput sequencing technologies can be especially interesting for monitoring the bacterial communities and detect bacterial pathogens in the aquaculture systems. This technique can provide an in depth compositional analysis of complex microbial communities and an accurate characterization of microbial diversity, with an unprecedented level of resolution (Roesch et al., 2007; Gomes et al., 2010; Pires et al., 2012). However, the use of this technique requires specialized personnel and high-performance computing resources. Meanwhile, several studies (Cleary et al., 2012; Pires et al., 2012), showed that a combined DGGE and barcoded 16S rRNA pyrosequencing approach can decrease the costs associated with the analysis of several samples and allows a fast and reliable overall profile of microbial communities prior to pyrosequencing. However, high-throughput sequencing technologies are now widely used in scientific research and, given the rapid reduction in their operating costs, it is likely that they will soon be routinely used to screen for pathogens and compare bacterial communities in aquaculture systems.

Objectives and thesis outline

The general objective of this work was to gain fundamental knowledge on the bacterial communities of different aquaculture systems. The in-depth analysis of microbial communities provides quantitative and qualitative outputs that may allow us to obtain a comprehensive definition of the "normal" aquaculture microbiome. In turn this information can improve our ability to understand and control the microbial quality of the water and decrease the risk of disease outbreaks. Furthermore, knowledge on bacterial pathogens of the aquaculture systems can contribute to the development of new methodologies for a fast detection of specific pathogens.

A more detailed description of each chapter is outlined below:

Chapter 1 – Introduction

In this chapter we present a brief introduction addressing the importance of the aquaculture systems as a food fish supply, the role of bacterial communities and the development of diseases in these systems, and the importance of the use of molecular techniques in the study of bacterial communities.

Chapter 2 – Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for *Scophthalmus maximus* and *Solea senegalensis*

In this chapter we assessed the composition of bacterial communities inhabiting recirculating aquaculture systems (RAS) of turbot (*Scophthalmus maximus*) and sole (*Solea solea*) using a combined DGGE and barcoded 16S rRNA pyrosequencing approach. Additionally, we also evaluated the use of barcoded pyrosequencing to detect and determine pathogen loads in RAS. All production system of twin RAS operating in parallel (one for each fish species) was sampled: water supply pipeline (Sup), fish production tanks (Pro), sedimentation filter (Sed), biofilter tank (Bio), and protein skimmer (Ozo; also used as an ozone reaction chamber).
This chapter was published in the journal PlosOne with the following reference: DOI: 10.1371/journal.pone.0080847

Chapter 3 – Seasonal patterns of bacterioplankton in a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system

In this chapter we study the seasonal patterns in the bacterioplankton composition and function of a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system (Ria de Aveiro, Portugal). To assess the bacterioplankton composition we used a barcoded pyrosequencing analysis while their putative function was assessed using a predicted metagenome analysis using the bioinformatics tool PICRUSt. The detection of potential pathogens was also evaluated as well as their seasonal dynamics.

Chapter 4 – Development of a molecular methodology for fast detection of *Photobacterium damselae* subspecies in water samples

In this chapter, we developed a molecular approach for fast detection of *Photobacterium damselae* subspecies in the water of aquaculture systems. *P. damselae*-specific primers targeting the *toxR* gene were designed for quantification of *P. damselae* species and detection of its subspecies (*piscicida* and *damselae*). First, to detect and quantify *P. damselae* species we used real time PCR (RT-PCR) and then to distinguish its subspecies we used a nested PCR-DGGE approach. Importantly, the developed methodology was tested against environmental samples (estuary and aquaculture water samples) and the results validated using a state of the art barcoded pyrosequencing approach.
This chapter was published in the journal Aquaculture with the following reference: DOI:10.1016/j.aquaculture.2014.09.028

Chapter 5 – Conclusions

In this chapter, we summarize the main conclusions of the work.

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Chapter 2. Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for *Scophthalmus maximus* and *Solea senegalensis*

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PLoS ONE. 8: e80847

Abstract

The present study combined a DGGE and barcoded 16S rRNA pyrosequencing approach to assess bacterial composition in the water of a recirculating aquaculture system (RAS) with a shallow raceway system (SRS) for turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*). Barcoded pyrosequencing results were also used to determine the potential pathogen load in the RAS studied. Samples were collected from the water supply pipeline (Sup), fish production tanks (Pro), sedimentation filter (Sed), biofilter tank (Bio), and protein skimmer (Ozo; also used as an ozone reaction chamber) of twin RAS operating in parallel (one for each fish species).

Our results revealed pronounced differences in bacterial community composition between turbot and sole RAS, suggesting that in the systems studied there is a strong species-specific effect on water bacterial communities. *Proteobacteria* was the most abundant phylum in the water supply and all RAS compartments. Other important taxonomic groups included the phylum *Bacteriodetes.* The saltwater supplied displayed a markedly lower richness and appeared to have very little influence on bacterial composition. The following potentially pathogenic species were detected: *Photobacterium damselae* in turbot (all compartments), *Tenacibaculum discolor* in turbot and sole (all compartments), *Tenacibaculum soleae* in turbot (all compartments) and sole (Pro, Sed and Bio), and *Serratia marcescens* in turbot (Sup, Sed, Bio and Ozo) and sole (only Sed) RAS. Despite the presence of these pathogens, no symptomatic fish were observed. Although we were able to identify potential pathogens, this approach should be employed with caution when monitoring aquaculture systems, as the required phylogenetic resolution for reliable identification of pathogens may not always be possible to achieve when employing 16S rRNA gene fragments.

Keywords

Bacteria, aquaculture, RAS, DGGE, pyrosequencing, pathogen detection, turbot and sole

Introduction

The growing worldwide demand for fish has prompted research towards intensive aquaculture. Innovative technologies, such as recirculating aquaculture systems (RAS) and

shallow raceway systems (SRS) (Øiestad, 1999), have been developed to improve the efficiency and sustainability of intensive aquaculture practices. RAS basically consists of the recycling and re-using of production water thus reducing adverse environmental impacts associated with water usage and release of nutrient-rich effluents (Blancheton, 2000; Borges et al., 2008). SRS represent an improvement of common raceways, as they have an optimized hydrodynamic performance due to their low water level and plug-flow pattern thus enabling producers to employ higher fish stocking densities (Øiestad, 1999). The organic matter resulting from unconsumed food and fish metabolites is recycled in RAS by a diverse array of microbes. Nitrogen-containing organic molecules are decomposed into ammonia by heterotrophic bacteria, with ammonia subsequently being converted into nitrite, and nitrite into nitrate, by nitrifying bacteria in biological filters. Given the key role played by these microorganisms in the operation of RAS, it is no surprise that most studies addressing bacterial communities in these production systems have mainly focused on biological filters (Sugita et al., 2005; Schreier et al., 2010). However, there is a lack of information on the overall diversity and composition of bacterial communities in the different components of these intensive aquaculture systems. An in-depth analysis of these microbial communities will provide quantitative and qualitative outputs that should allow us to obtain a comprehensive definition of the 'standard' microbiome of a RAS. In turn this information can improve our ability to understand and control the microbial quality of production systems and reduce the risks associated with disease outbreak.

Traditionally, conventional microbiological techniques, such as culture-based approaches, serology and histology, have been used for pathogen detection in aquaculture. However, these techniques are often laborious and time-consuming. The development of molecular tools has allowed researchers to overcome these limitations [e.g. polymerase chain reaction (PCR) and real-time PCR (RT-PCR)] (Saulnier et al., 2009; García-González et al., 2011). However, the application of these technologies depends on the selection of a range of pathogens to be targeted by the assay and, therefore, new or unexpected pathogens will not be detected. Alternatively, molecular fingerprint analyses of microbial communities [e.g. PCR - denaturing gradient gel electrophoresis (PCR-DGGE)] enable us to profile complex microbial communities in a range of environments (Muyzer et al., 1993; Acinas et al., 1997; Schwieger and Tebbe, 1998). Overall, these community fingerprinting techniques are cost-effective, allow a fast characterization of different microbial assemblages in multiple

samples and can easily be used to monitor microbial community structure in fish farms (De Schryver et al., 2008). However, although this approach can provide important information on the structural diversity of microbes at different taxonomic levels (group specific PCR-DGGE for different kingdom, phylum, class, order, family, genus and species) (Gomes et al., 2010), no information concerning the identity of microbial populations is provided.

In order to overcome these constraints, it is now possible to employ high throughput sequencing technologies (e.g. pyrosequencing) to achieve an in depth compositional analysis of complex microbial communities with an unprecedented level of resolution (Roesch et al., 2007; Gomes et al., 2010; Pires et al., 2012). Additionally, these technologies can be especially interesting for monitoring fish disease in aquaculture systems. However, high throughput sequencing technologies demand specialized personnel and high-performance computing resources, and thus may not be readily available for most fish producers. Alternatively, DGGE can be used as a rapid proxy for the determination of compositional variation among different samples and/or experimental treatments. In this way, researchers can have a rapid overall characterization of the microbial communities being studied through DGGE and, at the same time, select the best strategy for sequencing analysis (Cleary et al., 2012; Pires et al., 2012). Here, for the first time, a DGGE - barcoded pyrosequencing approach was applied to explore the diversity of bacterial communities and detect potential fish pathogens in an intensive aquaculture operating RAS (with a SRS) for the production of *Scophthalmus maximus* (turbot) and *Solea senegalensis* (sole). The results obtained are critically discussed to highlight the advantages and limitations of this approach for the detection and characterization of bacterial pathogen assemblages in RAS.

Materials and methods

Study site

The present study was carried out in a turbot (*S. maximus*) and sole (*S. senegalensis*) super-intensive fish farm located in northern Portugal, which employed RAS technology combined with SRS. Briefly, saltwater was pumped from a well and was strongly aerated and sand-filtered before entering each RAS; the water was recycled as it passed from the production tanks to the sedimentation tank where mechanical filtration was performed. The water then

flowed to the biofilter tank for biological filtration and subsequently to the protein skimmer, which was also used as an ozone reaction chamber (Fig. 2.1).

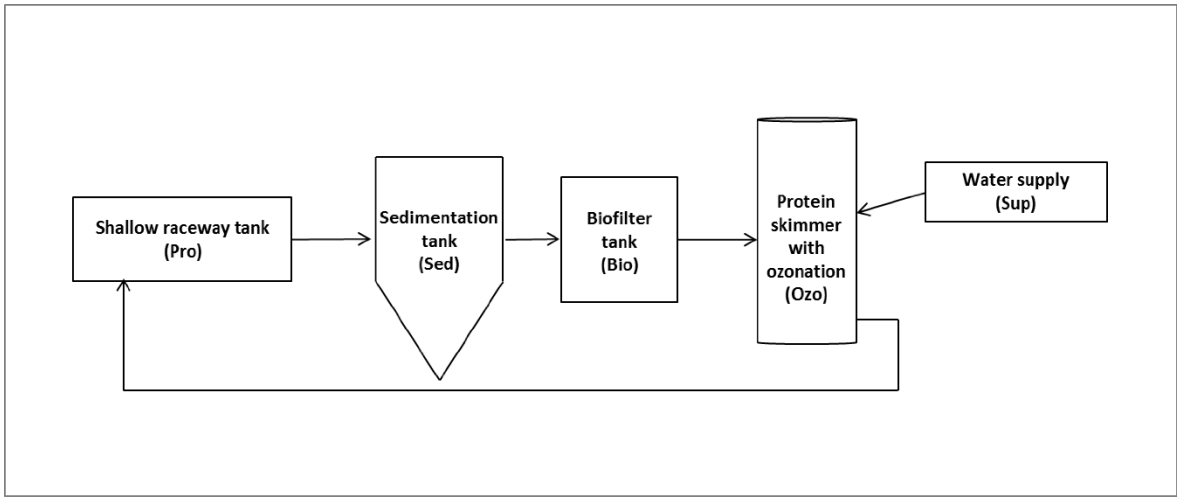

Figure 2.1 - Schematic representation of the sampling compartments in RAS. Water circuit of the recirculating aquaculture system studied (not to scale).

Sampling and DNA extraction

In RAS the external environmental parameters, such as temperature and natural photoperiod, have no influence on the system. Therefore, RAS display little to no seasonal variability in water parameters, allowing us to have a representative sample from the system by sampling at a single time point. Four sampling compartments for each species (*S. maximus* and *S. senegalensis*) were sampled: 1) shallow raceway tank (Pro) (containing fishes ~380 days old, weighing approximately 200 g to 300 g and densities with about 15 Kg/m^2), 2) sedimentation tank (Sed), 3) biofilter tank (Bio) and 4) ozone tank (acting both as protein skimmer and ozone reaction chamber) (Ozo). We also sampled the water supply pipeline (Sup), which was the same for both RAS systems. The turbot and sole RAS were fully independent.

Water samples (three replicates) were collected at different parts of the tank in each RAS compartment studied. Ammonia (NH_3), nitrites (NO_2^-), nitrates (NO_3^-), bromine residuals (BR_2), sulfates (SO_4^{2-}) and phosphates (PO_4^{3-}) were determined following the 8507, 8016 and 8155 methods described in the Hach Spectrophotometer (DR 2800) standard analytical procedures and according to EPA Method 300.1 and 351.2. Total organic carbon analysis (TOC) in the water was performed according to the European Norm 1484. Conventional

physical-chemical parameters (temperature, pH, dissolved oxygen (DO), suspended particles and salinity) were also recorded.

For bacterial community analysis, water samples (250 ml) were filtered through 0.2 μm pore polycarbonate membranes (Poretics, Livermore, CA, USA) and total community (TC) DNA extraction was performed directly on the filter using an E.Z.N.A Soil DNA Extraction kit (Omega Bio-Tek, USA) following the manufacturer's instructions.

PCR-DGGE bacterial community fingerprinting

In this study DGGE fingerprinting was used as a rapid tool to determine compositional variation among bacterial communities in different samples prior to barcoded pyrosequencing (Cleary et al., 2012). After statistical analysis of DGGE profiles, we applied a more-in-depth compositional barcoded pyrosequencing analysis of composite samples. Amplified 16S rRNA gene fragments suitable for bacterial DGGE fingerprints of total microbial community DNA samples were obtained using a nested approach following Gomes et al. (2008). The V6-V8 regions of bacterial 16S rDNA fragments were amplified using the primers set 27F and 1494R (Weisburg et al., 1991; Gomes et al., 2001) and 968GC - 1378R (Nübel et al., 1996), for the first and second PCR, respectively. The PCRs were conducted in a Professional Thermocycler (Biometra). Positive and negative controls were run for each PCR.

DGGE was performed on a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Samples were loaded onto 8% (w/v) polyacrylamide gels in 1x Tris-acetate-EDTA (TAE) with the denaturing gradient ranging from 40% to 58% (100% denaturant contains 7 M urea and 40% formamide) and performed at 58 ºC at 160 V during 16 hours. Gels were silver-stained according to Byun et al. (2009), except for the stop solution that in our case was replaced by a Na_2CO_3 solution. The image was acquired using the Epson perfection V700 Photo Scanner.

Barcoded pyrosequencing

A barcoded pyrosequencing approach was used for in-depth compositional analyses of bacterial communities in turbot and sole RAS intensive cultures. Prior to pyrosequencing, TC-DNA of all three replicates of each sampling compartment was combined, forming one DNA library for each stage of the production system. This procedure was performed for the

water supply and water of both fish species (*S. maximus* and *S. senegalensis*). The V3-V4 region of bacterial 16S rRNA gene amplicons were amplified using barcoded fusion primer V3 Forward (5´ -ACTCCTACGGGAGGCAG-3') and V4 Reverse (5´ -TACNVRRGTHTCTAATYC-3') with the Roche 454 titanium sequencing adapters. Pyrosequencing libraries were generated by the 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd., West Sussex, United Kingdom) (Wang and Qian, 2009; Cleary et al., 2013). PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA) was used to quantify the PCR product, and was pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to the manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA).

Data analyses

Analysis of the DGGE banding profile was performed with the software package BioNumerics v6.6 (Applied Maths, Belgium). Band standardization was carried out automatically by the program, but was always confirmed visually with changes made when necessary. Subsequently, the program constructed a matrix that incorporated the position and intensity of each band. Briefly, the matrix containing both band position and intensity were processed in a spreadsheet and transformed into relative abundance. The relative abundance matrix was uploaded to R, $\log_{10}$ (x+1) transformed and a distance matrix constructed with the Bray Curtis similarity coefficient using the vegdist() function in the vegan package (Oksanen, 2008) in R (version 2.11.1; http://www.r-project.org/). Variation in composition was visualised using principal coordinates analysis (PCO) with the cmdscale() function in R. Differences in the bacterial composition of RAS and water supply were tested using the adonis() function in vegan.

Pyrosequencing libraries were first analysed using QIIME (Quantitative Insights Into Microbial Ecology) (http://qiime.sourceforge.net/). First, data was filtered using the split_libraries.py script, which removed forward primers, barcodes and reverse primers. Sequences shorter than 200 base pairs were also removed. Operational taxonomic units (OTUs) were selected (97% similarity) using the pick_otus.py script with the 'usearch_ref' method and the most recent Greengenes release (Greengenes 12_10; http://qiime.wordpress.com/2012/10/16/greengenes-12_10-is-released/). Chimeras were

identified and removed using de novo and reference based chimera checking based on a reference fasta file from the Greengenes 12_10 release. Representative sequences were selected using the pick_rep_set.py script with the 'most_abundant' method and taxonomic identity was determined using the assign_taxonomy.py script with the Ribosomal Database Project (RDP) method (Wang et al., 2007). We used the make_otu_table.py script in QIIME to produce an OTU by sample table containing the abundance and taxonomic assignment of all OTUs. Unique OTUs were identified by assigning them to arbitrary numbers. This table was uploaded to R and non-bacteria, chloroplasts and mitochondria were removed from the analysis. Rarefaction curves were made for each sampling compartment using a self-written function in R (Gomes et al., 2010). Variation in OTU composition was visualised using principal coordinates analysis following the same method used for DGGE band data. Variation in the relative abundance of the most abundant bacterial taxa (two phyla, eight classes and ten orders) was assessed using bar plot graphs. The relative abundance was calculated considering the total of reads for each taxonomic level. In addition to this, OTUs taxonomically classified into genera known to be fish pathogens (Toranzo et al., 1993; Vigneulle and Laurencin, 1995; Imsland et al., 2003; Toranzo et al., 2005) (Data S2.1) were selected and their phylogeny investigated. BLAST search (http://www.ncbi.nlm.nih.gov/) was used to obtain the closest relatives of selected OTUs (pathogens and abundant taxa, i.e., where the number of sequences > 400). These sequences were also aligned using ClustalW and a phylogenetic tree was constructed using the neighbour-joining method in Mega 5.1 (http://www.megasoftware.net/). The evolutionary distances were computed using the Maximum Composite Likelihood method with a gamma distribution (four categories) and 500 bootstraps.

The DNA sequences generated in this study were submitted to the NCBI SRA: Accession number SRP026529.

Results and discussion

Bacterial community profiles

In this study, temperature, salinity and pH were similar in both RAS systems (Table 1.1). However, ammonia, nitrites and nitrates were present in lower concentrations in water samples collected from the RAS employed for sole production. The sole RAS also showed

the highest values for solid particles and TOC. In line with these analyses, the water of different fish cultures also showed distinct microbial communities. The PCO ordination analysis of bacterial DGGE profiles (Fig. 2.2) showed clear separation of three main groups in the ordination representing the water supply and the RAS compartments for turbot and sole production (Fig. 2.2A).

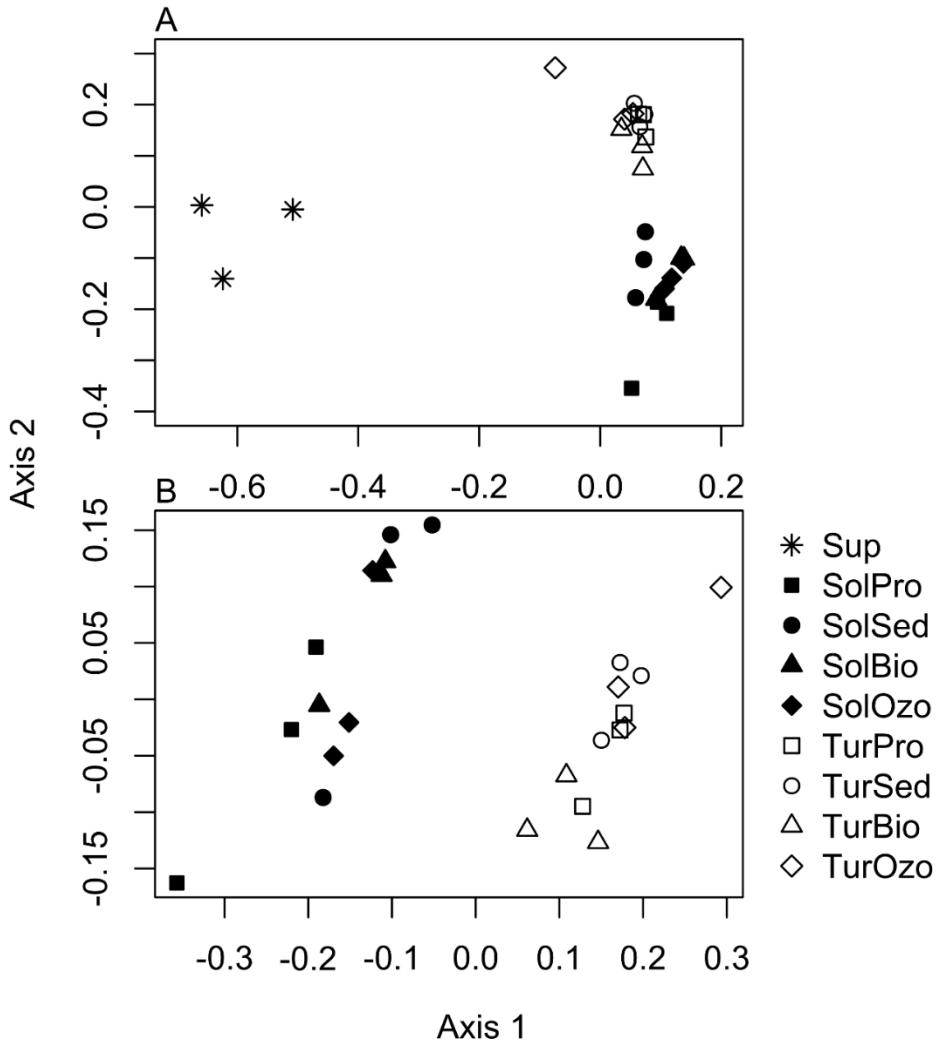

Figure 2.2. Ordination diagrams (PCO) of the bacterial community based on DGGE profile. A) in all sampling compartments (water supply (Sup), turbot production tank (TurPro), turbot sedimentation tank (TurSed), turbot biofilter tank (TurBio), turbot ozone tank (TurOzo), sole production tank (SolPro), sole sedimentation tank (SolSed), sole biofilter tank (SolBio), sole ozone tank (SolOzo)); B) only in turbot and sole sampling compartments (without water supply).

The differences in bacterial composition were highly significant ($F_{8,18} = 6.94$, $P < 0.001$, $R^2 = 0.755$) among groups. The primary axis of variation of the PCO in Fig 2.2A revealed that bacterial communities from the water supplied to the RAS and the samples collected from the compartments of the RAS clearly differed. In order to gain a better insight on how the bacterial communities differed between the two fish RAS and among the different compartments of each RAS, a new PCO was performed (Fig. 2.2B) excluding the samples from water supply. The second PCO (Fig. 2.2B) confirmed the differences between the two fish RAS, as illustrated by the primary axis of variation, with a significant difference in composition among groups being recorded ($F_{7,16} = 7.48$, $P < 0.001$, $R^2 = 0.766$). Previous studies have shown that gut fish microbes may colonize the biofilm of marine aquaculture systems and that their microbial composition will depend on the fish species being cultured (Sugita et al., 2005; Lahav et al., 2009). In fact, fish faeces and unconsumed feed are important parameters controlled in RAS. Large amount of organic materials form suspended particles, which support the growth of heterotrophic bacteria. This adversely affects nitrifiers and increases concentrations of ammonia, nitrites and nitrates and may trigger the growth of pathogenic microorganisms (Liltved and Cripps, 1999; Michaud et al., 2006; Hess-Erga et al., 2008). However, in contrast to current knowledge (Welch and Bartlett, 1998; Zhu and Chen, 2001), neither solid particulates nor TOC were associated with increased values of dissolved nitrogen in turbot RAS (Data S2.2). Sole RAS compartments showed the highest concentration of solid particulates and the lowest levels of dissolved nitrogen (Table 2.1). The PCO did not show pronounced separation between bacterial communities from different RAS compartments with the exception of the sedimentation compartment of the turbot RAS (Data S2.3).

Table 2.1. Mean values and standard deviation of temperature, pH, dissolved oxygen (DO), salinity, ammonia, nitrites, nitrates, bromine residuals, suspended particles, sulfates, phosphates and total organic carbon (TOC) in the aquaculture system [water supply (Sup), turbot production tank (TurPro), turbot sedimentation tank (TurSed), turbot biofilter tank (TurBio), turbot ozone tank (TurOzo), sole production tank (SolPro), sole sedimentation tank (SolSed), sole biofilter tank (SolBio), sole ozone tank (SolOzo)]. The sign < indicates values below detection limit.

	Temperature (Cº)	**pH**	**DO (mg /L)**	**Salinity**	**Ammonia (mg /L)**	**Nitrites (mg /L)**	**Nitrates (mg /L)**	**Bromine residuals (mg /L)**	**S. Particules (mg /L)**	**Sulfates (mg /L)**	**Phosphates (mg /L)**	**TOC (mg /L)**
Sup	17.60 ± 0.08	7.40 ± 0.02	6.87 ± 0.09	24 ± 0	1.47 ± 0.05	0.80 ± 0.14	<10	0.06 ± 0.01	4.80 ± 1.23	1782.00 ± 13.64	<10	2.10 ± 0.26
SolPro	18.40 ± 0.00	6.77 ± 0.08	13.37 ± 2.30	24 ± 0	1.03 ± 0.12	0.48 ± 0.06	32.67 ± 9.29	0.16 ± 0.01	7.60 ± 1.78	1766.33 ± 9.24	<10	8.00 ± 0.00
SolSed	18.60 ± 0.10	6.60 ± 0.07	10.93 ± 0.06	24 ± 0	1.40 ± 0.17	0.53 ± 0.04	36.00 ± 1.73	0.06 ± 0.01	185.27 ± 33.69	1787.33 ± 4.04	<10	6.67 ± 1.53
SolBio	18.43 ± 0.06	6.97 ± 0.01	6.73 ± 0.51	24 ± 0	0.73 ± 0.06	0.30 ± 0.00	36.00 ± 2.00	0.07 ± 0.01	20.80 ± 2.31	1745.67 ± 50.08	<10	9.67 ± 1.53
SolOzo	18.50 ± 0.00	7.18 ± 0.19	8.90 ± 0.00	24 ± 0	0.60 ± 0.17	0.27 ± 0.06	26.00 ± 10.82	0.13 ± 0.03	10.00 ± 3.94	1777.67 ± 12.66	<10	6.00 ± 0.00
TurPro	18.77 ± 0.06	6.76 ± 0.04	16.60 ± 2.43	24 ± 0	2.33 ± 0.12	1.67 ± 0.72	82.33 ± 2.08	0.06 ± 0.01	6.73 ± 1.36	1760.00 ± 39.89	<10	7.33 ± 0.58
TurSed	18.73 ± 0.06	6.69 ± 0.01	11.33 ± 0.15	24 ± 0	3.43 ± 0.49	1.23 ± 0.35	85.00 ± 15.62	0.13 ± 0.02	6.60 ± 1.60	1708.00 ± 129.32	<10	7.00 ± 1.00
TurBio	19.00 ± 0.00	6.88 ± 0.04	7.33 ± 0.06	24 ± 0	1.60 ± 0.17)	1.57 ± 0.15	86.00 ± 10.54	0.11 ± 0.02	10.20 ± 2.95	1720.33 ± 22.37	<10	8.00 ± 1.00
TurOzo	18.80 ± 0.00	6.98 ± 0.01	8.73 ± 0.06	24 ± 0	1.73 ± 0.21	2.00 ± 0.65	57.33 ± 11.72	0.15 ± 0.06	5.33 ± 2.14	1567.00 ± 208.32	<10	7.67 ± 0.58

Overall assessment of bacterial composition in RAS

Barcoded pyrosequencing analysis yielded 5553, 24214 and 22786 sequence reads for water supply, turbot and sole RAS compartments, respectively. In terms of bacterial community diversity, the water in the turbot RAS showed the highest bacterial richness (Fig. 2.3). Controlling for sample size (n = 4300 individual sequences), OTU richness varied from 562.58 ± 7.58 OTUs in the shallow raceway tank to 527.65 ± 8.40 OTUs in the biofilter tank of turbot RAS. OTU richness of the sole RAS varied from 504.45 ± 7.70 OTUs in the biofilter tank to 445.03 ± 5.34 OTUs in the sedimentation tank. The water supply used in the RAS had the lowest richness, 33.24 ± 0.82 OTUs. Although the ozone compartment in sole RAS exhibited relatively low richness, the ozone compartment in turbot had similar values to those obtained in other compartments.

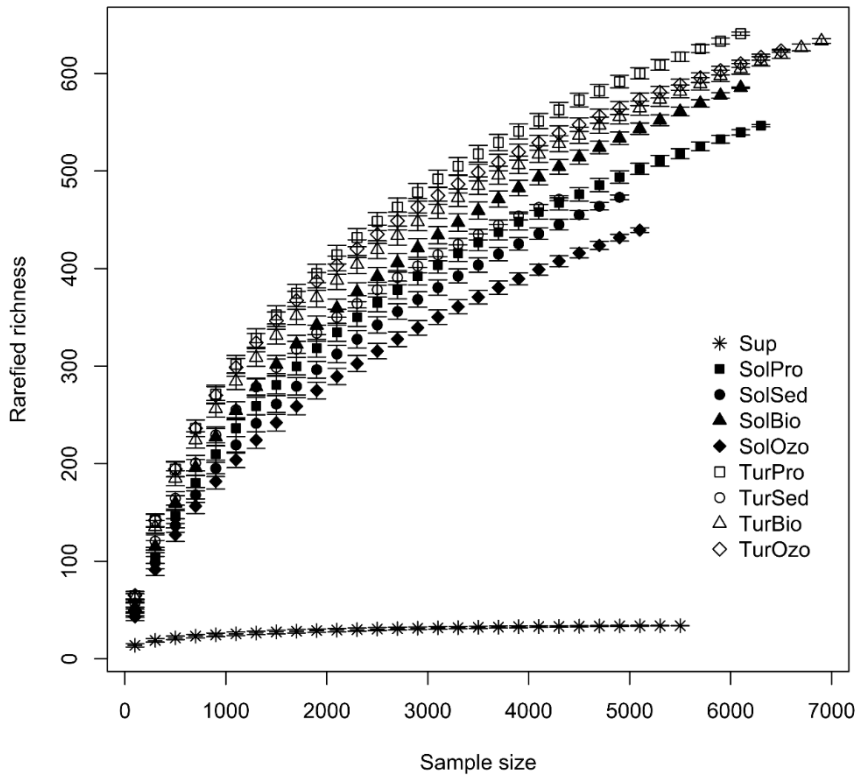

Figure 2.3. Bacterial richness. Rarefied OTU richness in all sampling compartments (water supply (Sup), turbot production tank (TurPro), turbot sedimentation tank (TurSed), turbot biofilter tank (TurBio), turbot ozone tank (TurOzo), sole production tank (SolPro), sole sedimentation tank (SolSed), sole biofilter tank (SolBio), sole ozone tank (SolOzo)).

In line with the PCO of DGGE profiles (Fig. 2.2), the ordination of barcoded-pyrosequencing data (OTU composition) showed that the water supply had the most distinct microbial community (Fig. 2.4A). The PCO comprising only turbot and sole production systems also showed clear differences between the two RAS (Fig. 2.4C) with a range of abundant OTUs (> 400 sequences) specific to each system (Fig. 2.4C and D). Only few bacterial OTUs were found in the water supply and RAS. These results indicate that bacterial populations entering the system through the water supply are probably out-competed by those already established in turbot and sole RAS.

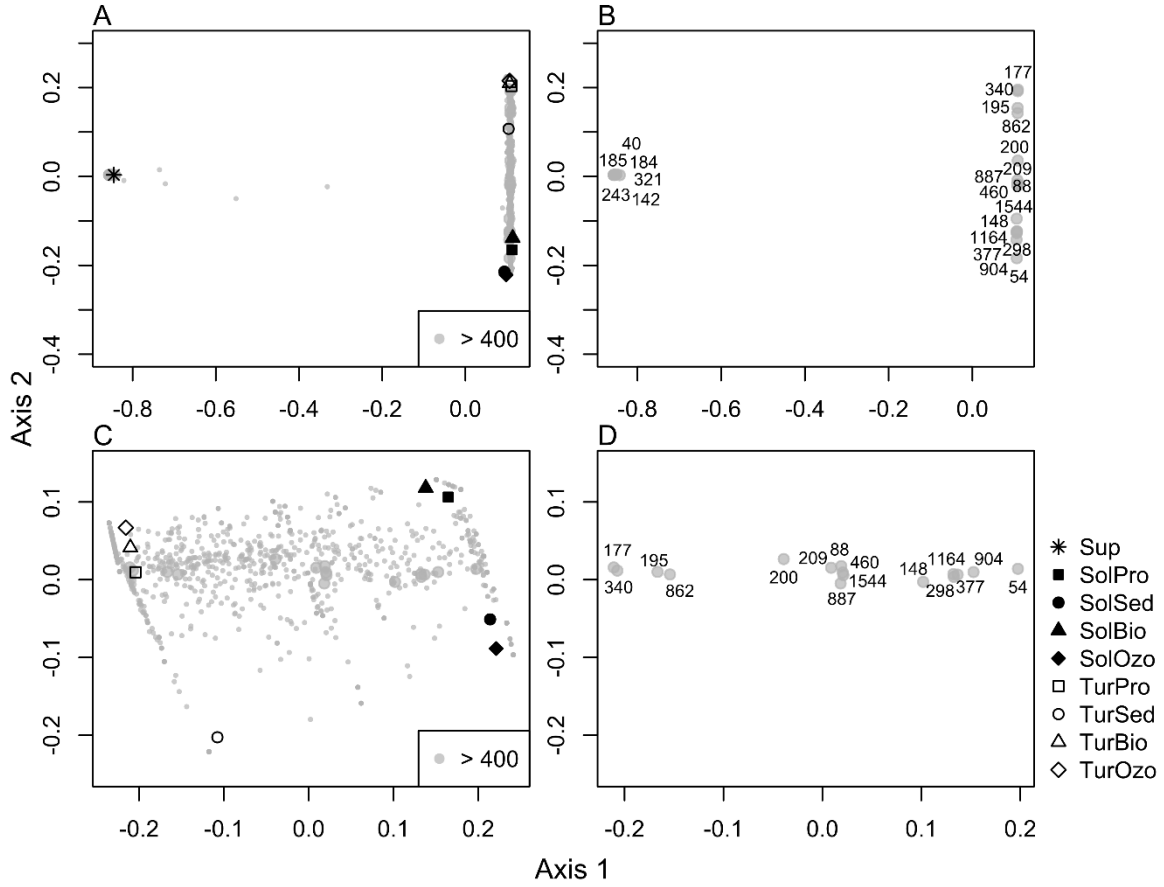

Figure 2.4. Ordination diagrams (PCO) of the bacterial community based on barcoded pyrosequencing data. (A) in all sampling compartments; (B) the most abundant OTUs associated to all sampling points; (C) only in turbot and the sole sampling compartments (without water supply); (D) the most abundant OTUs associated to turbot and the sole sampling compartments.

The overall taxonomic analyses grouped bacterial sequences into twenty-four phyla, forty-two classes and sixty-one orders. At the phylum level, about 8% of OTUs remained unclassified. Fig. 2.5 shows the relative abundance of the most dominant bacterial groups (≥ 400 reads).

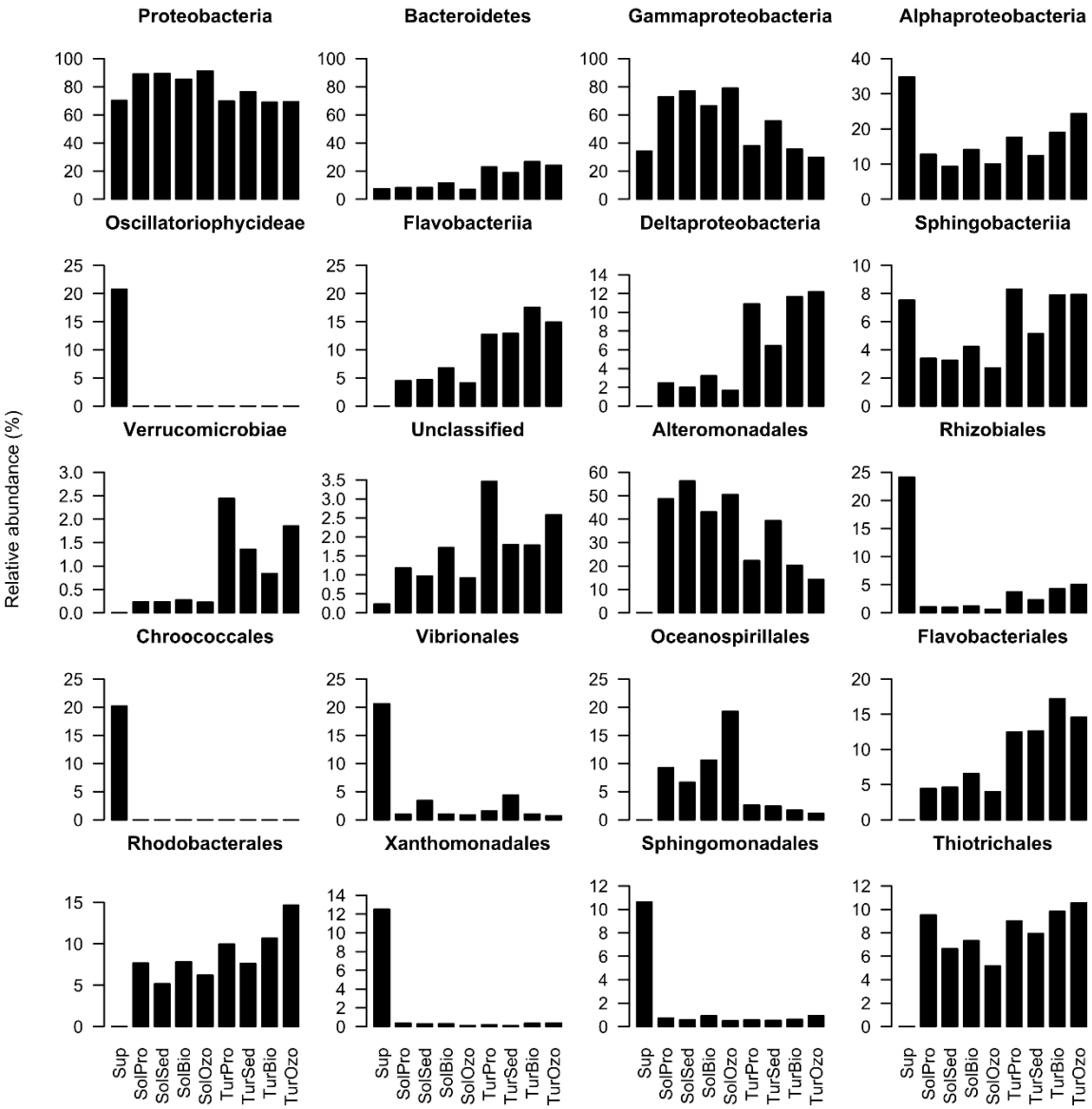

Figure 2.5. Relative abundance of the most dominant bacterial groups (2 phyla, 8 classes and 10 orders) in each sampling compartments (water supply (Sup), turbot production tank (TurPro), turbot sedimentation tank (TurSed), turbot biofilter tank (TurBio), turbot ozone tank (TurOzo), sole production tank (SolPro), sole sedimentation tank (SolSed), sole biofilter tank (SolBio), sole ozone tank (SolOzo)). Groups are present from the most abundant to the least abundant.

In agreement with the PCO analysis, the water supply had the most distinct composition, showing the highest abundance values for *Oscillatoriophycideae* (20.7%), *Rhizobiales* (24.1%), *Chroococcales* (20.2%), *Vibrionales* (20.6%), *Xanthomonadales* (12.5%) and *Sphingomonadales* (10.6%) (Fig. 2.5). *Proteobacteria* was the most abundant phylum in all fish sampling compartments and displayed a slight dominance in sole RAS. In general, its relative abundance ranged between 70% and 90%. This phylum is widely dispersed in marine environments and plays an important role in the processes of nutrient cycling and mineralization of organic compounds (Kirchman, 2002; Kersters, 2006). *Bacteroidetes* was the second most abundant phylum with a relative abundance ranging from 7% to 11% in sole RAS and 18% to 26% in turbot RAS compartments. The *Bacteroidetes* (previously *Cytophaga-Flexibacter-Bacteroides*) are dominant members of marine heterotrophic bacterioplankton and are frequently found colonizing macroscopic organic matter particles (marine snow) (Woebken et al., 2007). Further differences were also observed at lower taxonomic levels between turbot and sole RAS compartments (e.g., *Gammaproteobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Oceanospirillales* and *Verrucomicrobiae*) (Fig. 2.5).

Composition analysis of dominant OTUs

The dominance analysis revealed six OTUs found in the water supply: 40 (unknown *Chroococcales*), 142 (unknown *Sphingobacteriales*), 184 (*Sphingomonas*), 185 (*Stenotrophomonas*), 243 (*Aliivibrio*) and 321 (*Phyllobacterium*) (Fig.2.4 and Table 2.2). However, none of these OTUs was dominant in the RAS compartments.

The most abundant OTUs in turbot RAS compartments were assigned to *Kordia* (OTU 195), *Polaribacter* (OTU 862), unknown *Hyphomicrobiaceae* (OTU 177) and unknown *PB19* (OTU 340) group (Fig. 2.4 and Table 2.2). Previous studies on *Kordia* spp. have shown that members of this genus can exhibit algicidal activity and produce extracellular proteases responsible for the cell lysis of diatom species (Paul and Pohnert, 2011). *Kordia* spp. was also previously detected in biofilter media employed in RAS for the culture of goldfish *Carassius auratus* (Itoi et al., 2006). Gómez-Consarnau et al. (2007) showed that some *Polaribacter* species have the ability to produce specific bacterial compounds (namely rhodopsins) that induce growth when associated with light (photoheterotrophy). OTU 177

was classified as an unknown member of the family *Hyphomicrobiaceae* and was closely related with a partial sequence of a 16S rRNA gene retrieved from marine bacterioplankton communities after environmental disturbance (Sjöstedt et al., 2012) (Fig. 2.6). Species in this family have been recognized as important methylotrophic denitrifiers in fluidized bed reactors and activated sludge (Liessens, 1993; Osaka et al., 2006). The most dominant OTU in the turbot RAS was OTU 340 (1749 reads). This OTU was classified as unknown PB19, and according to Blast (http://www.ncbi.nlm.nih.gov/) was related to an uncultured bacterium [GenBank accession number (acc.) EU283402] isolated from activated sludge produced by an aerated submerged membrane bioreactor for domestic wastewater treatment (Du et al., 2008) (Fig. 2.6).

OTUs 54, 377, 904 and 1164 were the most abundant in the sole RAS compartments, and were classified as *Pseudoalteromonas* spp. (*Alteromonadales*), except for OTU 54 that was identified as an unclassified *Oceanospirillales* (Fig.2.4 and Table 2.2). According to Wawrik et al. (2012) in a study of assimilatory nitrate utilization by bacteria on the West Florida Shelf, *Alteromonadales* and *Oceanospirillales* orders were identified as relevant marine heterotrophic bacteria involved in the uptake of dissolved inorganic nitrogen (DIN). Therefore, their higher abundance in sole RAS compartments may be in part responsible for the lower DIN values observed in this system when compared to the turbot RAS (Table 2.1).

Table 2.2. Taxonomic affiliation of the most abundant OTUs (>400 reads) in water supply, turbot and sole RAS-SRS, and their closest relatives (using Blast) with the respective accession number, sequence identity (Sq ident) and source. Reads indicates the number of sequences obtained for each OTU in water supply (Sup), turbot (Tur) and sole (Sol).

OTU	Reads			Class	Order	Family	Genus	Acession	Sq ident	Source
	Tur	Sol	Sup							
40	0	0	1079	*Oscillatoriophycideae*	*Chroococcales*	*Xenococcaceae*	Unclassified	EU780251	99	disease affected *Turbinaria mesenterina* colony
54	10	681	0	*Gammaproteobacteria*	*Oceanospirillales*	*Oleiphilaceae*	Unclassified	JN092240	100	gut *Nephrops norvegicus*
88	281	361	0	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Olleya*	JN175350	100	seawater particulates, water temperature 5ºC
142	0	0	418	*Sphingobacteriia*	*Sphingobacteriales*	Unclassified	Unclassified	FJ178015	95	*Austrocochlea concamerata*
148	196	559	0	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*	Unclassified	KC756863	100	*Paralichthys olivaceus*
177	466	9	0	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	Unclassified	FR647917	99	seawater
184	0	1	564	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingomonas*	JQ229600	100	Crater Cirque Lake
185	0	0	679	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Stenotrophomonas*	EU438980	100	paper mill pulps containing recycled fibres
195	756	97	0	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Kordia*	FJ015036	100	turbot larval rearing unit, tank surface
200	1405	1060	0	*Gammaproteobacteria*	*Thiotrichales*	*Thiotrichaceae*	*Leucothrix*	GU451651	100	macroalgal surface
209	347	398	0	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Phaeobacter*	NR_043888	100	*Phaeobacter arcticus* DSM 23566
243	7	8	998	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Aliivibrio*	AY292946	100	*Aliivibrio fischeri*
298	314	1481	0	*Gammaproteobacteria*	*Oceanospirillales*	*Oleiphilaceae*	Unclassified	EF215752	99	inert artificial surfaces submerged in marine water
321	7	0	1003	*Alphaproteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Phyllobacterium*	JQ316262	100	soil from Fazenda Nova Vida
340	1749	28	0	*Deltaproteobacteria*	PB19	Unclassified	Unclassified	EU283402	95	*Phaeobacter arcticus* DSM 23566
377	1060	4681	0	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	HQ401050	100	biofilm from surface of coral reef
460	1526	1926	0	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*	*Thalassomonas*	HM237288	98	*Thalassomonas* sp. M-M1
862	528	87	0	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Polaribacter*	EU586892	100	aquaculture seawater
887	362	421	0	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*	*Thalassomonas*	HM237288	100	*Thalassomonas* sp. M-M1
904	64	418	0	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	FJ154991	99	ocean water
1164	143	687	0	*Gammaproteobacteria*	*Alteromonadales*	*Pseudoalteromonadaceae*	*Pseudoalteromonas*	AB257337	100	*Pseudoalteromonas mariniglutinosa*
1544	297	378	0	*Gammaproteobacteria*	*Alteromonadales*	*Colwelliaceae*	*Thalassomonas*	HM237288	98	*Thalassomonas* sp. M-M1

Members of the order *Oceanospirillales* are also often involved in symbiotic interactions with marine animals; however, their putative functional role in fish is yet to be determined (Jensen et al., 2010). In line with the higher-taxon analysis (Fig. 2.5), the most abundant OTUs (OTUs 1164, 377 and 904) detected in sole RAS were related to *Pseudoalteromonas mariniglutinosa* (acc. AB257337) (Fig.2.6). A member of the *Alteromonadales* order previously isolated from an organically enriched sediment below fish farms (Wada et al., 2008). Recently, Aranda et al., (2012) showed that *Pseudoalteromonas* sp. strains (related to *P. mariniglutinosa*) could be used as *Vibrio*-biocontrol agents, as they were able to produce a putatively novel class of bacteriostatic compounds.

The dominant OTUs 88 (*Olleya*), 148 (unclassified *Alteromonadales*), 200 (*Leucothrix*), 209 (*Phaeobacter*), 298 (unclassified *Oceanospirillales*), 460 (*Thalassomonas*), 887 (*Thalassomonas*) and 1544 (*Thalassomonas*) were abundant in both RAS (Fig. 4D). OTU 88 was assigned to the genus *Olleya* and was closely related to *Olleya marilimosa* (Fig. 2.6) isolated from a Danish turbot farm (Porsby et al., 2008). This bacterium produces an exopolysaccharide in liquid media which may contribute to the capture, sinking and mineralization of organic substances in natural marine environments (Kirchman, 2002; Nichols et al., 2005) OTUs 148 and 298 were closely related with *Colwellia aestuarii* (acc. KC756863) and an unknown member of the *Oceanospirillales* order (acc. EF215752), respectively (Fig. 2.6). These taxa comprise marine bacterial guilds often associated with nitrate reduction (Jung et al., 2006; Wawrik et al., 2012). OTU 200 was classified as *Leucothrix* and was closely related to an epibiont bacteria (acc. GU451651). The epibiont *Leucothrix mucor* is the most studied member of this genus and is known to cause fouling diseases in prawns, although it is not considered to be a true pathogen (Gutiérrez-Salazar et al., 2011).

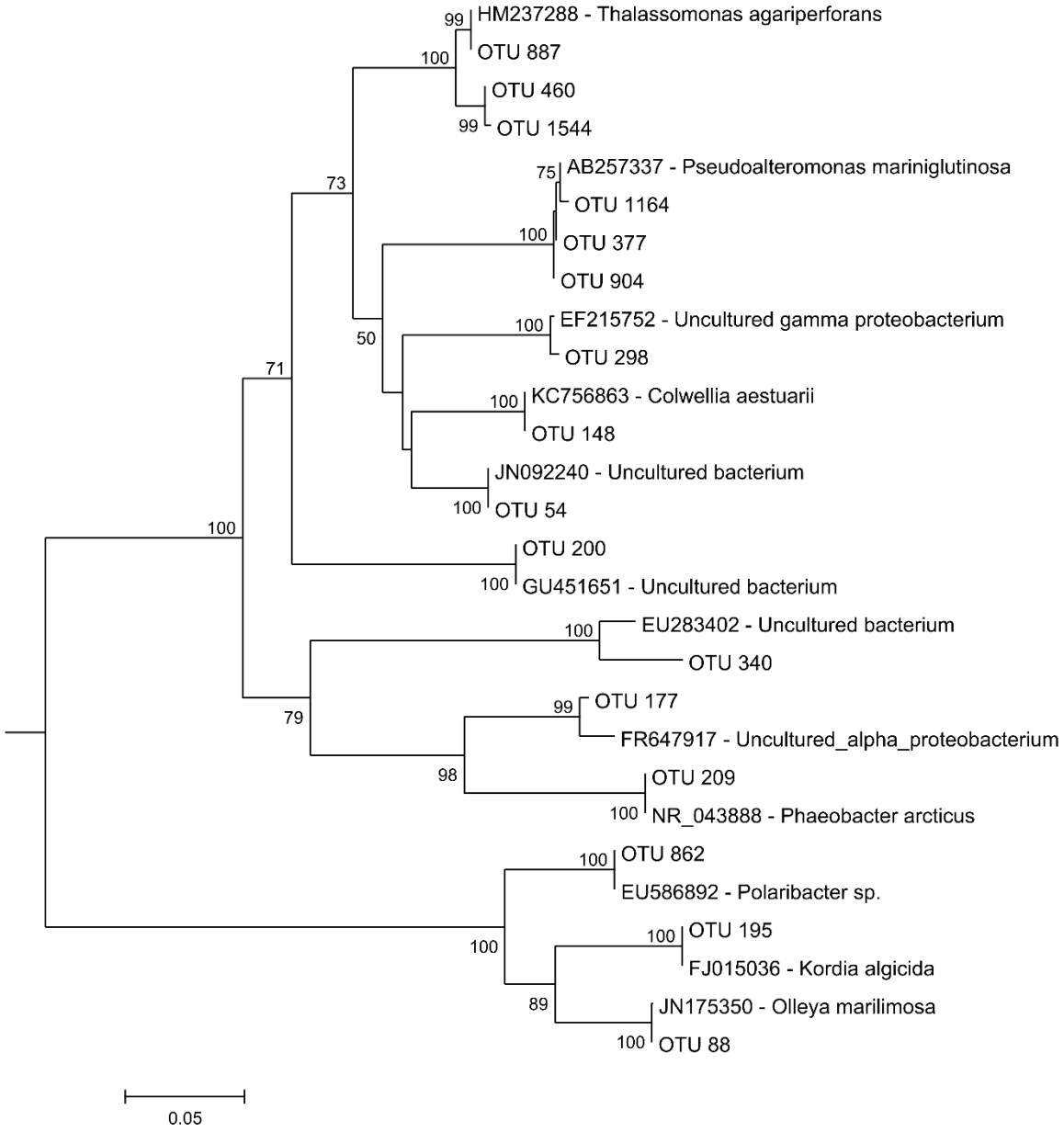

Figure 2.6. Neighbour-joining phylogenetic tree (16S rRNA gene sequences) of the most dominant OTUs in turbot and sole RAS and their closest relatives (accession number-species). Bootstrap values generated from 500 replicates. Values lower than 50% were omitted.

Interestingly, our analysis detected OTUs phylogenetically related to taxa comprising bacterial species with potential activity against several aquaculture pathogens. OTUs 460, 887 and 1544 were closely related with *Thalassomonas agariperforans* previously isolated from marine sand (Park et al., 2011). Recently, Torres et al. (2003) showed that a member of this genus (*Thalassomonas* sp. PP2-459) isolated from a bivalve hatchery was capable of

degrading N-acylhomoserine lactone (AHL) signal molecules (quorum quenching). The degradation of this molecule may affect the quorum sensing system of potential pathogens. Quorum sensing is a mechanism that allows bacteria to coordinate the expression of certain genes (including virulence genes) in response to the presence of small signal molecules such as AHL (Waters and Bassler, 2005). Recent studies showed that the use of bacterial strains with quorum quenching activity might be a useful strategy to biocontrol aquaculture pathogens (Defoirdt et al., 2004; Defoirdt et al., 2011). We also detected an abundant OTU (209) closely related to *Phaeobacter arcticus*. Recent studies showed that members of the genus *Phaeobacter* can also have antagonistic activity against known aquaculture pathogens (Prado et al., 2009; Prol et al., 2009; D'Alvise et al., 2012).

Phylogenetic analyses of potential fish pathogens

In order to understand the distribution of potential fish pathogens in the aquaculture system studied, a list of the most frequent bacterial pathogens responsible for fish diseases in aquacultures located in Europe was compiled (Toranzo et al., 1993; Vigneulle and Laurencin, 1995; Imsland et al., 2003; Toranzo et al., 2005) (Data S2.1). The 16S rRNA gene sequences of these bacteria were then phylogenetically compared with related OTUs (same genera) detected in this study (potential fish pathogens) and their closest GenBank relatives (blastn tool - http://blast.ncbi.nlm.nih.gov/) (Fig. 2.7). In addition to this, the spatial distribution and the relative abundance of potential pathogens in RAS (turbot and sole) were investigated (Table 2.3). OTUs 257 and 467 were closely related to an unknown *Photobacterium* sp. and *P. damselae*, respectively. *P. damselae* subsp. *piscicida* is one of the most common pathogens associated with sole aquaculture (Zorrilla et al., 1999; Romalde, 2002). However, in our study, *P. damselae* (OTU 467) was only detected in the turbot RAS compartments, and was more abundant in production tanks and the sedimentation filter. In contrast, we detected OTUs closely related to *T. discolor* (OTU 107) and *T. soleae* (OTU 350) in nearly all turbot and sole RAS compartments. *T. soleae* and *T. discolor* are known fish pathogens responsible for tenacibaculosis disease and were first isolated from diseased sole (*S. senegalensis* and *S. solea*) and turbot (*S. maximus*) in Spain (Piñeiro-Vidal et al., 2008; Piñeiro-Vidal et al., 2008). Eight OTUs were classified (OTUs 12, 219, 428, 503, 847, 865, 1127 and 1135) as members of the genus *Vibrio* (data not shown). OTUs 12, 1135, 1127 and 428 were closely related with 16S rRNA gene sequences of *V. ichthyoenteri, V.*

parahaemolyticus, *V. gallaecicus* and *V. xuii*, respectively (Fig. 2.7). All these *Vibrio* species were isolated from marine aquaculture environments. However, only *V. ichthyoenteri* and *V. parahaemolyticus* are potentially pathogenic, as they are often associated with diseased animals (Zorrilla et al., 2003; Gauger et al., 2006). These species were detected in nearly all RAS compartments. Our analysis also detected an OTU with strong homology to *S. marcescens* (OTU 17). This species is known to be an opportunistic pathogen previously detected in brackish and freshwaters and is a causative disease agent in natural population of white perch (Baya et al., 1992). This potential pathogen was also isolated from diseased tilapia fish (*Oreochromis niloticus*) in Malaysia (Chang et al., 2012).

Some selected OTUs in this study were closely related to potential new fish pathogens. For example, OTU 127 was close related to *P. putida.* This species is known to be an opportunistic human pathogen, although a previous report suggests that *P. putida* may also be a disease causative agent in rainbow trout aquaculture (Altinok et al., 2006). OTUs 599 and 881 were closely related to *Mycobacterium conceptionense* and *Streptococcus infantarius*, respectively. These species can be found colonizing human and environmental samples, but there is no previous report on their occurrence in aquaculture systems. However, it is important to note that the genera *Mycobacterium* and *Streptococcus* comprise several members able to cause mycobacteriosis and streptococcosis diseases among both wild and captive fishes worldwide (Chinabut, 1999; Salati, 2011).

Despite the presence of pathogens, no diseased fish were registered during the study period. The composition and relative abundance analysis of the potential bacterial pathogens in turbot and sole RAS (Table 2.3) indicated that OTUs closely related to fish pathogens were present at a low abundance (low infective concentration) in both RAS. Fish density and infectious dose have been considered as key factors to control fish mortality (Holm et al., 1990; Agnew and Barnes, 2007).

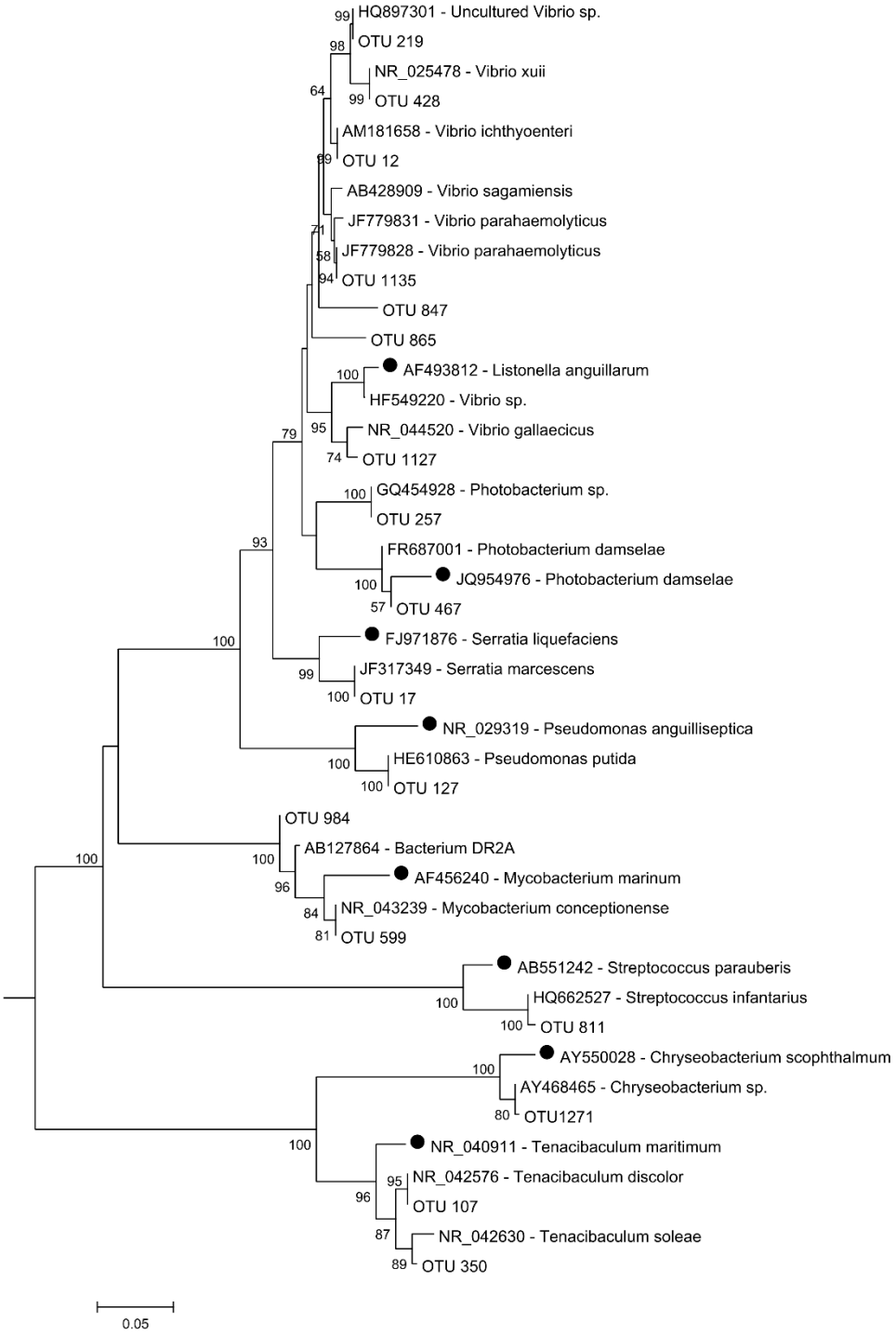

Figure 2.7. Neighbour-joining phylogenetic tree (16S rRNA gene sequences) of OTUs related potential fish pathogens and their closest relatives (with accession number). Black circles indicate the most frequent pathogens described in literature. Bootstrap values generated from 500 replicates. Values lower than 50% were omitted.

Table 2.3. Values of relative abundance (%) of potential fish pathogens detected in water supply (Sup), turbot production tank (TurPro), turbot sedimentation tank (TurSed), turbot biofilter tank (TurBio), turbot ozone tank (TurOzo), sole production tank (SolPro), sole sedimentation tank (SolSed), sole biofilter tank (SolBio), sole ozone tank (SolOzo).

Potential fish pathogen	OTU	Sup	SolPro	SolSed	SolBio	SolOzo	TurPro	TurSed	TurBio	TurOzo
Chryseobacterium scophthalmum	1271	ND	ND	ND	0.016	ND	ND	ND	ND	ND
Mycobacterium conceptionense	599	ND	ND	ND	0.016	ND	ND	0.023	ND	0.015
Photobacterium damselae	467	ND	ND	ND	ND	ND	0.064	0.069	0.014	0.030
Pseudomonas putida	127	0.018	0.079	0.039	0.065	0.114	0.081	0.092	0.141	0.227
Serratia marcescens	17	0.990	ND	0.039	ND	ND	ND	0.046	0.042	0.030
Streptococcus infantarius	811	ND	ND	ND	ND	ND	ND	0.023	ND	ND
Tenacibaculum discolor	107	ND	0.189	0.138	0.114	0.152	0.307	0.253	0.212	0.242
Tenacibaculum soleae	350	ND	0.031	0.039	0.033	ND	0.081	0.069	0.014	0.015
Vibrio ichthyoenteri	12	ND	0.252	0.454	0.147	0.133	0.275	1.034	0.198	0.167
Vibrio parahaemolyticus	1135	ND	0.031	0.020	0.016	0.038	0.032	0.184	0.014	ND

ND – Not detected

Possibly, the high abundance of naturally occurring antagonistic strains detected in this study (e.g., bacterial populations closely related to known antagonistic strains belonging to the genus *Thalassomonas* and *Phaeobacter*; see above) may have contributed to suppress the development of potential fish pathogens.

Critical evaluation of barcoded pyrosequencing of 16S rRNA gene fragments for fish disease detection

The results obtained in this study were critically evaluated to underline the advantages and disadvantages of using barcoded pyrosequencing of 16S rRNA gene fragments for monitoring potential fish pathogens in aquaculture systems. The 16S rRNA gene is widely used in phylogenetic studies and is an important marker for molecular diagnostics and molecular ecology studies. However, this gene may fail to provide a sufficient phylogenetic resolution or a correct classification of some bacterial pathogens. For example, the 16S rRNA gene fragments used in this study, are unable to resolve differences between two different *P. damselae* subspecies, namely, *damselae* and *piscicida* (Osorio and Klose, 2000). These subspecies may, however, cause very different infections in a variety of fish species (Romalde, 2002; Toranzo et al., 2005). In addition to this, *P. damselae* subsp. *piscicida* is more infectious. This bacterium is the causative agent of pasteurellosis, one of the most common diseases in marine fish farms in the Iberian Peninsula (Fouz et al., 1992; Zorrilla et al., 1999). The same problem may be observed at the species level for other bacteria. The 16S rRNA gene phylogeny provides an accurate classification of *Vibrio* species at family and genus level, but due to the high similarity between distinct species, it fails to provide an accurate identification at species level (Thompson et al., 2005). This was the case for *V. ichthyoenteri* (OTU 12), *V. parahaemolyticus* (OTU 847 and 1135), *V. gallaecicus* (OTU 1127) and *V. xuii* (OTU 428) detected in this study. Thompson et al. (2004), showed that *V. ichthyoenteri* and *V. scophthalmi* and *V. nereis* and *V. xuii* had 99% 16S rRNA sequence similarity but only shared 90% *recA* gene sequence similarity. According to Osorio and Klose (2000), *V. parahaemolyticus* and *V. alginolyticus* sequences were 99.8% similar using the 16S rRNA marker gene while using partial *toxR* gene showed only 61.7% similarity. Beaz-Hidalgo (2012) also showed that the *recA* gene is a better genetic marker to discriminate *V. gallaecicus* than the 16S rRNA gene. Several other studies reported that the

degree of resolution obtained with the 16S rRNA gene is not sufficiently robust for phylogenetic analysis of some known bacterial fish pathogens (Ruimy et al., 1994; Yamamoto and Harayama, 1995; Venkateswaran et al., 1998; Suzuki et al., 2001; Dauga, 2002; Mignard and Flandrois, 2006).

Another additional problem is that none of the next generation sequencing technologies developed until now can provide long sequence reads of gene fragments (pyrosequencing provides the longest fragment size ≤ 600 bp). Very often long stretch nucleotide sequences or complete nucleotide sequences of the 16S rRNA gene (~1,500 bp) are necessary for clear differentiation of closely related species. Other phylogenetic markers can be used, however, while the 16S rRNA gene sequence database (The Ribosomal Database Project) has currently over 2,765,278 sequences, other genetic databases are substantially smaller and incomplete (lacking representative sequences for all taxa and limited numbers of sampled ecotypes) (http://rdp.cme.msu.edu/; Accessed 30 July 2013). Therefore, this problem will hamper a fast identification of a range of bacterial species or the establishment of relationships between the sequence retrieved and their closest relative in the GenBank database. Currently, this approach allows us to associate 16S rRNA gene sequences with other sequences in the database previously detected in a specific environment or case of study (e.g., ecotypes and case studies about emergent causative agents of disease outbreaks).

Conclusion

In this study we applied a combined DGGE and pyrosequencing approach to assess the structural variation and composition of bacterial communities in RAS for turbot and sole production. The DGGE approach revealed significant structural differences between bacterial communities from turbot and sole RAS. This result suggests a strong fish species-specific effect on bacterial communities of both RAS studied. The pyrosequencing approach provided fundamental information about the bacterial composition and pathogen load in different RAS compartments. However, despite detecting potential fish pathogens in sole and turbot RAS, no symptomatic fish were observed during this study. Our goal in the future should be to identify the triggering mechanism(s) causing fish infection and disease progress in aquaculture facilities. The use of a high throughput sequencing approach using the 16S rRNA gene allowed us an unprecedented means to detect pathogens in the aquaculture systems studied. However, while the use of the 16S rRNA gene is commonly recognized to

be a suitable option when studying microbial composition, it may not be optimal for detecting some bacterial groups, including potential fish pathogens. Therefore, data obtained needs to be carefully examined and critically evaluated in terms of the level of resolution provided by the 16S RNA gene. Other conventional molecular tools may be used in combination with this technology to ensure the correct identification of some specific bacteria [e.g. Real Time PCR, Fluorescence In Situ Hybridization (FISH) and Isothermal DNA amplification], as well as the use of a different phylogenetic marker.

High-throughput sequencing technologies are now widely used in scientific research and, given the rapid reduction in their operating costs, it is likely that they will soon be routinely used to screen for pathogens and compare bacterial communities in aquaculture systems.

Acknowledgements

Patricia Martins benefited from a PhD grant (SFRH/BD/73889/2010) provided by the Portuguese FCT (Fundação para a Ciência e Tecnologia). This study has been carried out with the financial support of project AQUASAFE - Development of new technologies to anticipate and diagnose disease outbreaks in aquaculture (PROMAR 31-03-05-FEP-0016) (PROMAR, a Portuguese instrument for the sectors of fisheries and aquaculture funded by the European Fisheries Fund). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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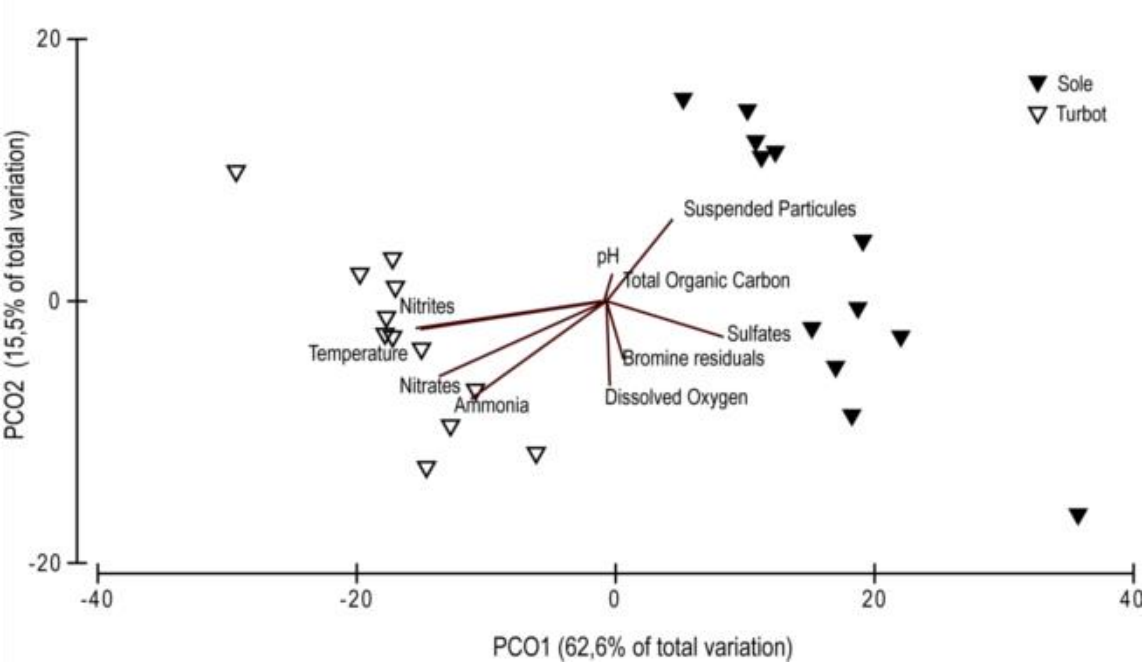
Supplementary material

Data S2.1 Most frequent bacterial pathogens in aquaculture systems of turbot and sole production described in literature and the pathogen species identified in this study.

Fish pathogen species	**Common fish pathogens (literature)**		**Putative fish pathogens (this study)**	
	Turbot	**Sole**	**Turbot**	**Sole**
Aeromonas salmonicida	X			
Chryseobacterium scophthalmum	X			
Mycobacterium conceptionense			X	X
Mycobacterium marinum	X			
Photobacterium damselae	X	X	X	
Pseudomonas anguilliseptica	X	X		
Pseudomonas putida			X	X
Serratia liquefaciens	X			
Serratia marcescens			X	X
Streptococcus infantarius			X	
Streptococcus parauberis	X			
Tenacibaculum discolor			X	X
Tenacibaculum maritimum	X	X		
Tenacibaculum soleae			X	X
Vibrio anguillarum	X	X	X	X
Vibrio gallaecicus			X	
Vibrio ichthyoenteri			X	X
Vibrio parahaemolyticus			X	X
Vibrio sagamiensis			X	X
Vibrio xuii			X	X

Data S2.2. Principal Coordinates Analysis of the RAS bacterial communities. Water parameters (ammonia, nitrites, nitrates, bromine residuals, sulfates, total organic carbon, temperature, pH, dissolved oxygen, suspended particles and salinity) are represented by vectors.



Data S2.3. ANOSIM, pairwise test comparing among sampling compartments (global R=0.62).

		R statistic								
		Sup	**SolPro**	**SolSed**	**SolBio**	**SolOzo**	**TurPro**	**TurSed**	**TurBio**	**TurOzo**
R statistic	**Sup**	-								
	SolPro	1	-							
	SolSed	1	0.222	-						
	SolBio	1	0.407	0.111	-					
	SolOzo	1	0.37	0.074	0.63	-				
	TurPro	1	1	1	1	1	-			
	TurSed	1	1	1	1	1	0.481	-		
	TurBio	1	1	1	1	1	0.148	0.593	-	
	TurOzo	1	1	1	0.96	1	0.481	0.556	0.444	-

Chapter 3. Seasonal patterns of bacterioplankton in a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system

Abstract

In the present study, in depth 16S rRNA gene pyrosequencing and PICRUSt functional prediction analyses were used to study seasonal variation in the structure and function of bacterioplankton of a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system (Ria de Aveiro, Portugal). Water samples were collected randomly in the estuary near the entrance of the aquaculture (E) and in the aquaculture tanks (T) at six different sampling events over the year 2012: (01) January (winter), (03) March (beginning of spring), (05) May (end of spring), (07) July (summer), (09) September (beginning autumn) and (11) November (end of autumn). Our results revealed that seasonal variations of bacterioplankton composition were related to changes of environmental factors. In general, we observed a dominance of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* phyla in all samples analysed. No apparent differences were found between bacterial communities from estuarine and aquaculture water during all sampling events, although an in depth composition data analysis indicated a slight increase in the abundance of some bacterial groups. For example, actinobacterial populations were more abundant in aquaculture tanks in mid-summer and autumn. Our results also showed seasonal variation of potential fish pathogens (e.g., *Tenacibaculum discolor*, *Serratia marcescens* and *Streptococcus agalactiae*). In agreement with these results, the PICRUSt prediction indicated an enrichment of the KEGG subcategory infectious diseases at the beginning of autumn (September). Interestingly, a disease outbreak and fish mortality was also observed during the first weeks of October (direct field observation). In this study, we showed that shifts in the bacterioplankton communities are driven by seasonal changes of abiotic characteristics of the estuarine water. The use of 16S rRNA gene pyrosequencing and PICRUSt analyses improved our understanding of bacterioplankton temporal dynamics and potential function in a semi-intensive aquaculture, providing relevant information for fish health and production.

Keywords

Bacteria, DGGE, pyrosequencing, pathogen detection, seasonal variability, semi-intensive aquaculture, predictive metagenomics

Introduction

The increasing human population has, over the past decades, led to a substantial increase in the demand for fish. In response to this growing demand, aquaculture has become a strategic sector for food production and economic development in several countries around the globe. From 1980 to 2012, aquaculture production increased at an average rate of 8.6% per year. From 2000 to 2012, aquaculture production more than doubled, increasing from 32.4 million tonnes to 66.6 million tonnes (FAO, 2014). However, aquaculture systems may have high mortality rates due to fish diseases. This is assumed to happen due to disruption of water physical and chemical properties during fish production. Factors such as temperature, pH, dissolved oxygen, salinity, nutrient input and phytoplankton biomass play key roles in the maintenance of fish health and fish production (Swann, 1990; Ashley, 2007). However, fluctuations in theses parameters are common in semi-intensive aquaculture systems located in estuaries, where the production environment is strongly influenced by seasonal environmental variation. Several parameters such as nutrient concentrations, fall which affects salinity, water temperature and different levels of sunlight exposure along the year represent key drivers of a range of biological interactions, including fish-microbe interactions. For example, elevated nutrient concentrations (organic and inorganic) or temperature can act as a stressor to fish health by increasing susceptibility to disease (Meyer, 1970; Snieszko, 1974; Walters and Plumb, 1980; Evans et al., 2002). Glibert et al. (2002) reported a case of a massive fish kill in Kuwait Bay involving the species *Streptococcus agalactiae* that was apparently driven by a variety of stressful environmental conditions (high temperature, calm conditions, elevated nutrient concentrations and depressed oxygen concentrations). However, studies are lacking about the effects of seasonal environmental dynamics on bacterioplankton communities in semi-intensive aquaculture systems and their potential effects on fish health.

Molecular techniques have been routinely used to characterize complex microbial communities in various types of environments (Muyzer et al., 1993; Acinas et al., 1997; Gomes et al., 2008), and have been used in the last years as tools for pathogens detection in aquaculture systems (Ji et al., 2004; Saulnier et al., 2009; García-González et al., 2011; Martins et al., 2015). Currently, high throughput sequencing technologies (e.g., pyrosequencing and Illumina) provide an in depth sequencing analysis of complex microbial communities and can provide an accurate characterization of microbial diversity, with an

unprecedented level of resolution (Roesch et al., 2007; Gomes et al., 2010; Pires et al., 2012). A previous study successfully used a pyrosequencing-based 16S ribosomal RNA (rRNA) gene sequencing approach to detect potential pathogens in water samples of a recirculating aquaculture system (Martins et al., 2013). Currently, massive 16S rRNA sequence data have also been used for *in silico* prediction of functional profiles of microbial communities. Langille et al. (2013) described a bioinformatic tool, PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states), which predicts metagenomic functional content of microbial communities using marker gene data and a database of reference genomes. This algorithm can predict diverse features of complex microbial communities such as metabolism and function of gene content of uncultured microorganisms. Such information can provide a fast prediction of microbial traits in a range of environments. In this study, an in depth 16S rRNA gene pyrosequencing approach and PICRUSt functional prediction analysis were used to investigate seasonal patterns of bacterioplankton (free-living and particle-associated bacteria) composition of a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system. Our specific goals were to (1) test to what extent variation in bacterioplankton composition and putative function can be explained by environmental conditions in aquaculture tanks and estuary at different sampling times, (2) compare the dynamic of higher taxa abundance during the year, (3) identify dominant (> 900 reads) bacterial operational taxonomic units (OTUs) and (4) investigate the seasonal occurrence of potential fish pathogens.

Materials and methods

Sampling and DNA extraction

This study was carried out in an aquaculture producing European seabass (*Dicentrarchus labrax*) and located in the north-west coast of Portugal (Aveiro, Portugal). The aquaculture operates under semi-intensive management, receiving natural water from the Ria de Aveiro estuary. Water samples were collected randomly (three replicates) in the estuary (E) near the entrance of the aquaculture and in three aquaculture tanks (T) during six sampling events in 2012: (01) January (winter), (03) March (beginning of spring), (05) May (end of spring), (07) July (summer), (09) September (beginning autumn) and (11) November (end of autumn). Seasons were defined based on the Portuguese Institute for Sea and Atmosphere

(IPMA) (http://www.ipma.pt/) that consider: December, January and February as winter; March, April and May as spring; June, July and August as summer; September, October and November as autumn. The sampled tanks had approximately the same fish densities. Fish juveniles (11 months old) were placed in the tanks in August 2011. Water samples (250ml) were filtered through 0.2 µm pore polycarbonate membranes (Poretics, Livermore, CA, USA) and DNA extraction was performed directly on the filter using an E.Z.N.A. Soil DNA Extraction kit (Omega Bio-Tek, USA) following the manufacturer's instructions.

Environmental parameters

Water aliquots were filtered (Whatman GF/C glass-fibre filter) and analysed for dissolved inorganic nutrients (DIN and DIP) and sulfates in all sampling times. Ammonium (NH_4), nitrites (NO_2^-), nitrates (NO_3^-), sulfates (SO_4^{2-}), and phosphates (PO_4^{3-}) were determined following the 8507, 8016 and 8155 methods described in the Hach Spectrophotometer (DR 2800) standard analytical procedures and according to EPA Method 300.1 and 351.2. Total organic carbon (TOC) analysis in the water was performed according to the European Norm 1484. The following physical and chemical parameters were measured *in situ* in surface water (about 60 cm deep) in each sampling time using field sensors/probes: temperature, pH, dissolved oxygen (DO), and salinity.

Bacterial community analysis

In this study, bacterial DGGE fingerprinting was used as a rapid proxy of compositional variation among sample replicates and sampling events (Cleary et al., 2012). In general, this analysis showed that the replicate samples of each compartment (estuary and fish tanks 1, 2 and 3) were similar to one another (Data S3.1). Therefore, for in depth sequencing analysis, DNA replicate samples from estuary and fish tanks 1, 2 and 3 were combined into one composite sample for each compartment, reducing the number of samples to 4 per sampling event (24 samples in total). In a second step, a pyrosequencing approach was used for compositional analyses of bacterial communities. 16S rRNA gene amplicons were obtained using bacterial specific primers 27F and 1494R after a denaturation step at 94ºC for 5 min, 25 thermal cycles of 1 min at 94ºC, 1 min at 56ºC and 2 min at 68ºC were carried out followed by an extension step at 68ºC for 10 min. The amplicons from the first PCR were used as template for amplification of the V3-V4 region [V3 forward primer 5'-

ACTCCTACGGGAGGCAG-3' (Yu et al., 2005) and V4 reverse degenerate primer 5'-TACNVRRGTHTCTAATYC-3' (Vaz-Moreira et al., 2011) using barcoded fusion primers with the Roche-454 Titanium sequencing adapters.

Pyrosequencing libraries were first analysed using QIIME (Quantitative Insights Into Microbial Ecology) (http://qiime.sourceforge.net/). First, data were filtered using the split_libraries.py script, which removed forward primers, barcodes and reverse primers. Sequences shorter than 218 base pairs were also removed. Operational taxonomic units (OTUs) were selected using UPARSE (Edgar, 2013) with the usearch7 script. Chimeras were identified and removed using the UCHIME algorithm (Edgar et al., 2011). Quality filtering was performed using the -fastq_filter command with fastq_trunclen 250 -fastq_maxee 0.5 -fastq_truncqual 15 arguments. The dereplication and abundance sort was performed using the commands -derep_fulllength and -sortbysize, respectively. After performing the OTU clustering using the -cluster_otus command, an additional chimera check was applied using the -uchime_ref command with the gold.fa database (http://drive5.com/uchime/gold.fa). Representative sequences were selected using the pick_rep_set.py script with the 'most_abundant' method and OTUs were assigned to taxa using the assign_taxonomy.py script with the Ribosomal Database Project (RDP) method (Wang et al., 2007). The Greengenes 13_8 release (ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz) was used for the RDP classifier. We used the make_otu_table.py script in QIIME to produce an OTU by sample table containing the abundance and taxonomic assignment of all OTUs. This table was uploaded into R and non-bacteria, chloroplasts and mitochondria were removed prior to analysis. Data was log10 (x+1) transformed and a distance matrix was constructed using the Bray Curtis similarity coefficient with the vegdist() function in the vegan package (Oksanen, 2008) in R (version 2.11.1; http://www.r-project.org/). Variation in OTU composition was visualised using principal coordinates analysis (PCO) with the cmdscale() function in R. Differences in the bacterial composition of samples collected in the estuary and in the aquaculture tanks during each sampling event were tested using the adonis() function in vegan.

Environmental parameters, namely ammonium (NH_4), nitrites (NO_2^-), nitrates (NO_3^-), and phosphates (PO_4^{3-}), total organic carbon (TOC), temperature, pH, dissolved oxygen (DO), and salinity were fit in the PCO ordinations using the envfit function in vegan. Variation in

the relative abundance of the most abundant bacterial taxa (two phyla, five classes and five orders) was assessed using barplot graphs. The relative abundance was calculated considering the total reads for each taxonomic level. Rarefaction curves were made for each sample using a self-written function in R (Gomes et al., 2010). Rarefied OTU richness was selected for a standard sampling size of 3000 sequences and a barplot graph was constructed. In addition to this, OTUs taxonomically classified into genera known to be fish pathogens (Toranzo et al., 2005; Austin and Austin, 2007; Martins et al., 2013) (Data S3.2) were selected and their phylogeny investigated. BLAST search tool (http://www.ncbi.nlm.nih.gov/) was used to obtain the closest relatives of dominant OTUs (> 900 reads) and potential pathogens. These sequences were also aligned using ClustalW and a phylogenetic tree was constructed using the neighbour-joining method in Mega 5.1 (http://www.megasoftware.net/). The evolutionary distances were computed using the Maximum Composite Likelihood method with a gamma distribution (four categories) and 500 bootstraps. For visualization we used the iTOL webserver (Letunic and Bork, 2007).
The DNA sequences generated in this study were submitted to the NCBI SRA: Accession number SRP044694.

Predictive metagenome analysis

The PICRUSt algorithm (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict metagenome functional content using the 16S rRNA dataset of the aquaculture water samples over time. This analysis was based on the Kyoto encyclopedia of genes and genomes (KEGG) classification (Kanehisa and Goto, 2000). The predict_metagenomes.py script was used to create the final metagenome functional predictions and the normalized OTU table was created using the normalize_by_copy_number.py script. To collapse the PICRUSt predictions into individual pathways, we used the categorize_by_function.py script. Additionally the Nearest Sequenced Taxon Index (NSTI) was calculated to quantify the availability of nearby genome representatives for each sample (Langille et al., 2013). We used the linear discriminant analysis (LDA) effect size (LEfSe) method to test for functional differences among sampling events. The results are presented hierarchically using a cladogram with category, subcategory and individual pathway designations.

To test for significant variation in KO composition of the most abundant KO (> 250000 genes), an analysis of deviance was performed using the glm() function in R. The GLM model was used to test for significant variation among sampling times using the anova() function in R with the F-test.

The relative abundance of selected pathways for the KEGG subcategory infectious disease was assessed using stacked bar graphs. The relative abundance was calculated considering the total genes for each sample.

Results

Water properties/ physical and chemical parameters

Temporal variation of physical and chemical parameters measured in the estuary and aquaculture tanks during the sampling events is shown in Table 3.1. Slight differences were found in values of temperature, salinity and pH, and on the concentrations of TOC, pH, phosphate and nitrite between estuary samples and aquaculture samples for each sampling time.

In general, TOC and dissolved oxygen concentrations showed similar pattern over the course of the study, with the highest values during summer (July). The pH values showed a variation, ranging between 7.4 in winter (January) and 8.1 in summer (July). Salinity did not vary much over the sampling (remained near 35), except at the end of spring (May) when it decreased to 30. Temperature ranged between 11.4 ºC at the end of autumn (November) and 25.5 ºC during summer (July).

The relation between bacterioplankton communities and the physical and chemical parameters is shown in Fig. 3.1A. Communities sampled in winter and in the beginning of spring (January and March) are mainly associated with high nitrate concentrations (envfit for 1^{st} and 2^{nd} axes: P=0.002). In contrast, community samples collected in summer and at the beginning of autumn (July and September) are associated with higher temperatures (envfit for 1^{st} and 2^{nd} axes: P=0.001), higher TOC (envfit for 1^{st} and 2^{nd} axes: P=0.024) and higher pH (envfit for 1^{st} and 2^{nd} axes: P=0.006). There were no significant associations between dissolved oxygen, salinity, nitrite, phosphate, ammonium and the ordination of the 1^{st} and 2^{nd} axes.

Significant differences were found among sampling events for all physical and chemical parameters (Temperature: $F_{5,18} = 384.09$, $R^2 = 0.99$, $P = 0.001$; pH: $F_{5,18} = 6.24$, $R^2 = 0.63$, $P = 0.003$; Dissolved oxygen: $F_{5,18} = 32.05$, $R^2 = 0.90$, $P = 0.001$; Salinity: $F_{5,18} = 66.82$, $R^2 = 0.95$, $P = 0.001$; TOC: $F_{5,18} = 10.87$, $R^2 = 0.75$, $P = 0.001$; Nitrate: $F_{5,18} = 241.79$, $R^2 = 0.98$, $P = 0.001$; Nitrite: $F_{5,18} = 4.70$, $R^2 = 0.57$, $P = 0.004$; Silica: $F_{5,18} = 8.55$, $R^2 = 0.70$, $P = 0.002$; Sulfate: $F_{5,18} = 6.53$, $R^2 = 0.64$, $P = 0.001$; $P = 0.109$; Ammonium: $F_{5,18} = 5.05$, $R^2 = 0.58$, $P = 0.009$), with the exception of phosphate ($F_{5,18} = 2.05$, $R^2 = 0.36$).

Table 3.1 - Mean values and standard deviation of temperature, pH, dissolved oxygen (DO), salinity, total organic carbon (TOC), nitrate, nitrite, sulfate, phosphate and ammonium in each sampling time for the estuary and the aquaculture tanks. E01 (Estuary January, Winter), T01 (Aquaculture Tank January, Winter); E03 (Estuary March, Beginning of spring), T03 (Aquaculture Tank March, Beginning of spring); E05 (Estuary May, End of spring), T05 (Aquaculture Tank May, End of spring); E07 (Estuary July, Summer), T07 (Aquaculture Tank July, Summer); E09 (Estuary September, Beginning of autumn), T09 (Aquaculture Tank September, Beginning of autumn); E11 (Estuary November, End of autumn), T11 (Aquaculture Tank November, End of autumn).

	E01	T01	E03	T03	E05	T05	E07	T07	E09	T09	E11	T11
Temperature (ºC)	12.00 ± 0.00	12.73 ± 0.70	13.2 ± 0.20	13.96 ± 0.85	22.6 ± 0.10	22.56 ± 0.70	25.47 ± 0.60	24.84 ± 0.71	20.37 ± 0.15	20.17 ± 0.22	11.43 ± 0.06	11.73 ± 0.25
pH	7.44 ± 0.00	7.72 ± 0.10	7.42 ± 0.11	7.55 ± 0.10	7.81 ± 0.08	7.99 ± 0.11	7.84 ± 0.03	8.30 ± 0.160	8.15 ± 0.00	7.78 ± 0.07	8.01 ± 0.03	7.49 ± 0.06
Oxygen (mg/L)	9.40 ± 0.00	8.77 ± 0.93	10.2 ± 0.62	9.43 ± 0.67	8.27 ± 0.40	8.09 ± 0.51	15.93 ± 3.76	14.33 ± 4.33	5.27 ± 0.92	4.56 ± 0.79	6.03 ± 0.40	5.91 ± 0.71
Salinity	35.00 ± 0.00	35.00 ± 0.00	33.67 ± 0.58	35.00 ± 0.00	30.00 ± 0.00	30.56 ± 0.53	36.00 ± 0.00	35.33 ± 0.50	35.67 ± 0.58	34.78 ± 0.44	33.33 ± 0.58	34.00 ± 0.87
TOC (mg/L)	1.70 ± 0.00	1.63 ± 0.12	1.97 ± 0.06	2.38 ± 0.34	2.83 ± 0.21	3.04 ± 0.52	14.00 ± 8.49	7.44 ± 4.81	1.37 ± 0.15	3.29 ± 1.34	2.00 ± 0.79	3.80 ± 1.16
Nitrate (µmol/L)	128.49 ± 1.51	115.13 ± 6.58	123.26 ± 9.90	118.61 ± 4.91	122.39 ± 6.92	145.93 ± 5.99	5.12 ± 3.64	2.62 ± 2.55	0.87 ± 0.31	2.20 ± 0.34	11.62 ± 0.76	7.07 ± 1.96
Nitrite (µmol/L)	1.13 ± 0.42	0.68 ± 0.35	1.13 ± 0.42	0.68 ± 0.21	0.66 ± 0.06	0.90 ± 0.34	0.60 ± 0.12	0.14 ± 0.55	0.22 ± 0.10	0.87 ± 0.24	1.45 ± 0.00	1.86 ± 0.38
Sulfate (mg/L)	2581.22 ± 174.19	2652.3 ± 40.48	2676.24 ± 139.71	3027.26 ± 211.72	2773.48 ± 32.20	2908.66 ± 123.42	2856.35 ± 439.88	3063.84 ± 360.90	2507.18 ± 705.75	2531.12 ± 497.12	2436.46 ± 403.43	2377.53 ± 683.59
Phosphate (µmol/L)	0.68 ± 0.04	0.85 ± 1.72	0.51 ± 0.07	0.49 ± 0.18	0.20 ± 0.04	0.16 ± 0.13	0.00 ± 0.00	1.48 ± 0.58	0.00 ± 0.00	1.63 ± 0.78	1.12 ± 0.04	1.26 ± 0.34
Ammonium (µmol/L)	4.01 ± 0.23	3.28 ± 0.84	14.13 ± 4.05	8.46 ± 4.41	7.50 ± 0.10	24.03 ± 7.57	5.04 ± 0.54	7.06 ± 3.89	1.18 ± 0.40	11.05 ± 1.41	13.08 ± 0.68	26.92 ± 2.90

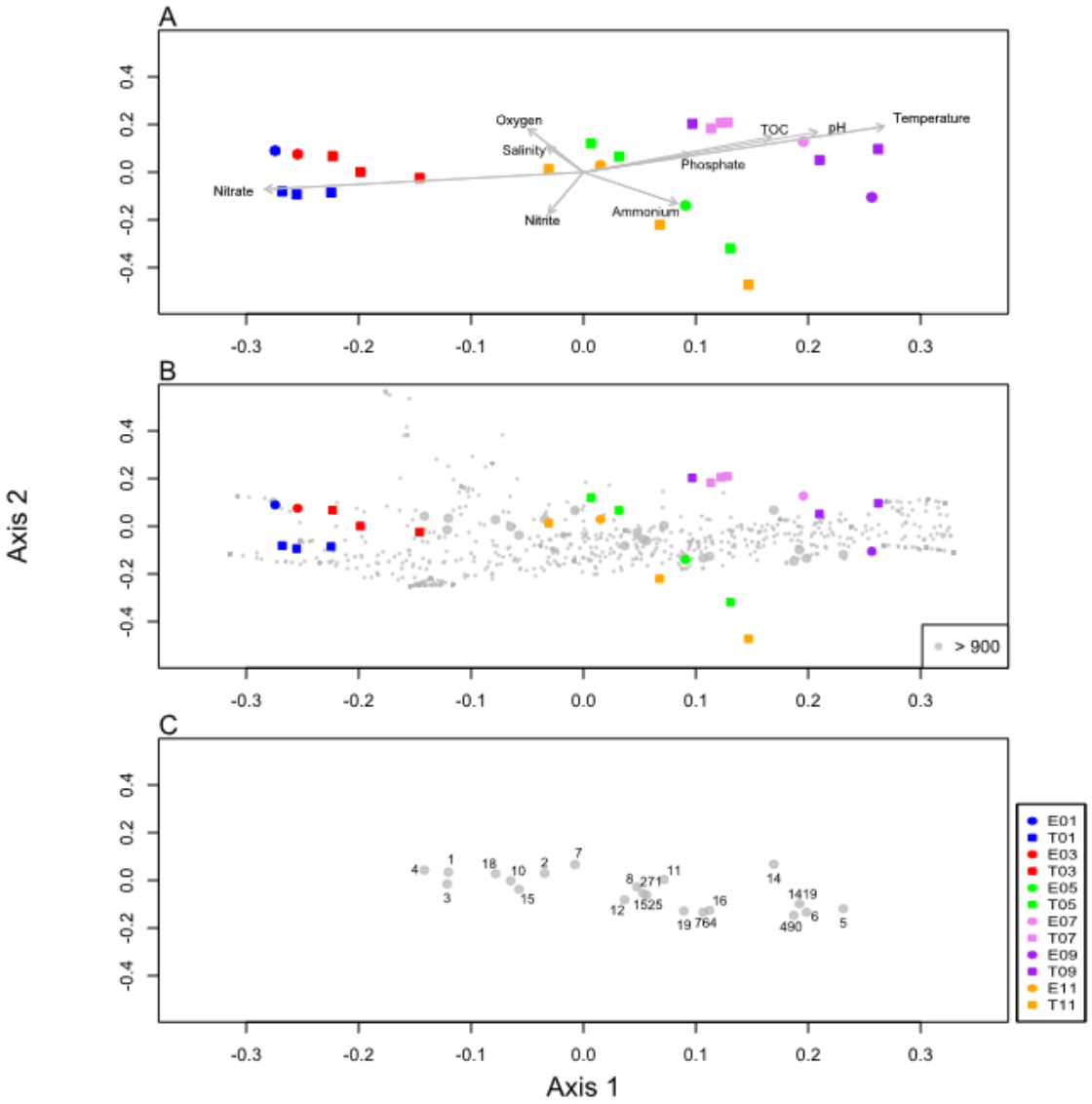


Figure 3.1. Ordination diagrams (PCO) of the bacterial community based on barcoded pyrosequencing data and the physical and chemical parameters (A), operational taxonomic unit (OTU) distribution (B) and the most abundant OTUs associated to the sampling points (C). Winter: E01 – Estuary January and T01 – Aquaculture Tank January; Beginning of spring: E03 – Estuary March and T03 – Aquaculture Tank March; End of spring: E05 – Estuary May and T05 – Aquaculture Tank May; Summer: E07 – Estuary July and T07 – Aquaculture Tank July; Beginning of autumn: E09 – Estuary September and T09 – Aquaculture Tank September; End of autumn: E011 – Estuary November and T011 – Aquaculture Tank November.

Bacterioplankton composition

Barcoded-pyrosequencing analysis yielded 131,398 sequence reads that were assigned to 1,685 OTUs. The PCO ordination analysis of bacterioplankton communities (OTU composition) (Fig. 3.1A) showed a clear separation between samples collected in winter and beginning of spring (January and March) and samples collected in summer and at the beginning autumn (July and September). Samples collected in May and November (end of spring and end of autumn) were grouped together. In general, the primary axis of variation of the PCO follows the succession of the bacterial communities in line with seasonal variation. There was a highly significant difference in bacterial composition among sampling events ($F_{5,18}$=2.50, P<0.001, R^2=0.41), however no apparent differences were found between samples collected in the aquaculture tanks and samples collected in the estuary in each sampling event.

Controlling for sample size (*n* = 3000 individual sequences), OTU richness varied from 370.42 ± 7.35 OTUs in the water from the estuary collected in winter (January) to 69.77 ± 1.65 OTUs in the water from the estuary collected at the beginning of autumn (September). OTU richness of the tanks varied from 199.61 ± 0.57 OTUs in the water from the tanks collected in summer to 94.71 ± 1.91 OTUs in the water from the tanks collected at the end of autumn. In January, March and November (winter, beginning of spring and end of autumn, respectively), bacterial richness was higher in samples collected in the estuary than in the tank and in May, July and September (end of spring, summer and beginning of autumn, respectively), the opposite was found (Fig. 3.2).

The overall taxonomic analyses grouped bacterial sequences into 37 phyla, 75 classes and 103 orders. At the phylum level, about 1.3% of OTUs remained unclassified. Fig. 3.3 shows the relative abundance of the most dominant bacterial groups (> 900 reads). *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were the most dominant phyla in all sampling times. Relative abundance of the phyla *Proteobacteria* and *Bacteroidetes* did not vary substantially between water samples from the estuary and water samples from the aquaculture tanks. Furthermore, no apparent differences were found in the relative abundances of these phyla over time. The relative abundance of the phylum *Actinobacteria* ranged between 0.4% and 13.1% and was higher in the aquaculture tanks in July, September and November. Interestingly, our results showed that all members of the phylum *Actinobacteria* detected were classified into the order *Actinomycetales.*

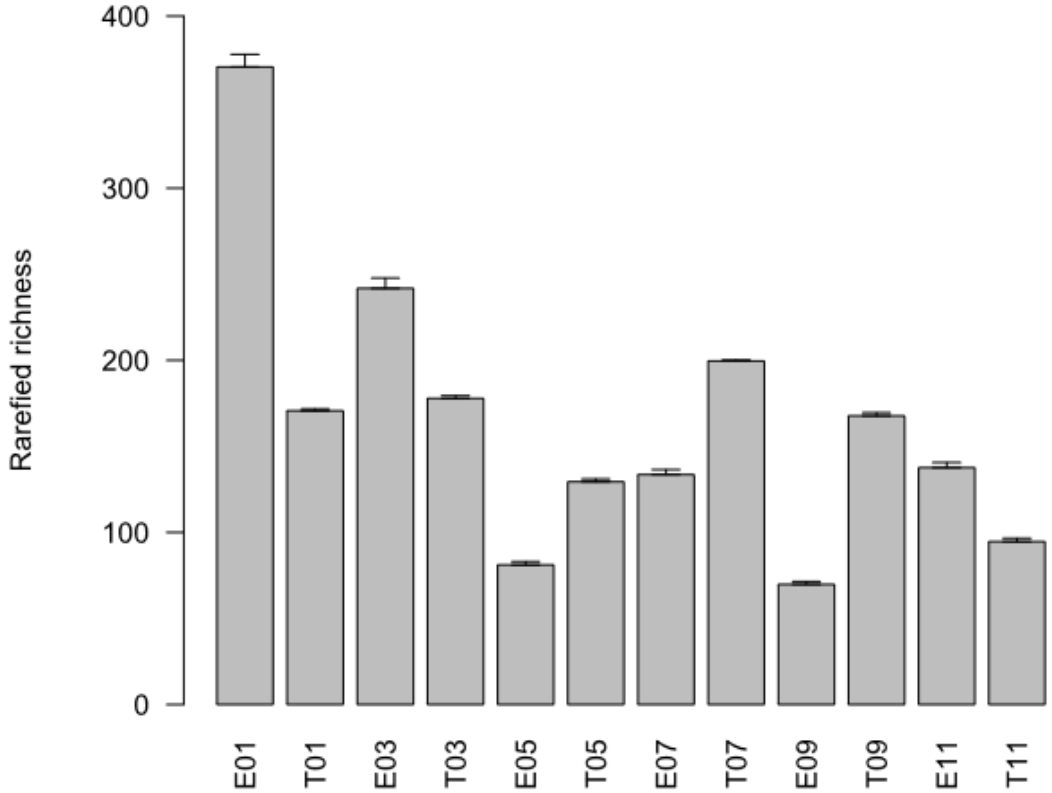


Figure 3.2. Barplot of rarefied OTU richness controlled for 3000 sequences. E01 (Estuary January, Winter), T01 (Aquaculture Tank January, Winter); E03 (Estuary March, Beginning of spring), T03 (Aquaculture Tank March, Beginning of spring); E05 (Estuary May, End of spring), T05 (Aquaculture Tank May, End of spring); E07 (Estuary July, Summer), T07 (Aquaculture Tank July, Summer); E09 (Estuary September, Beginning of autumn), T09 (Aquaculture Tank September, Beginning of autumn); E11 (Estuary November, End of autumn), T11 (Aquaculture Tank November, End of autumn)

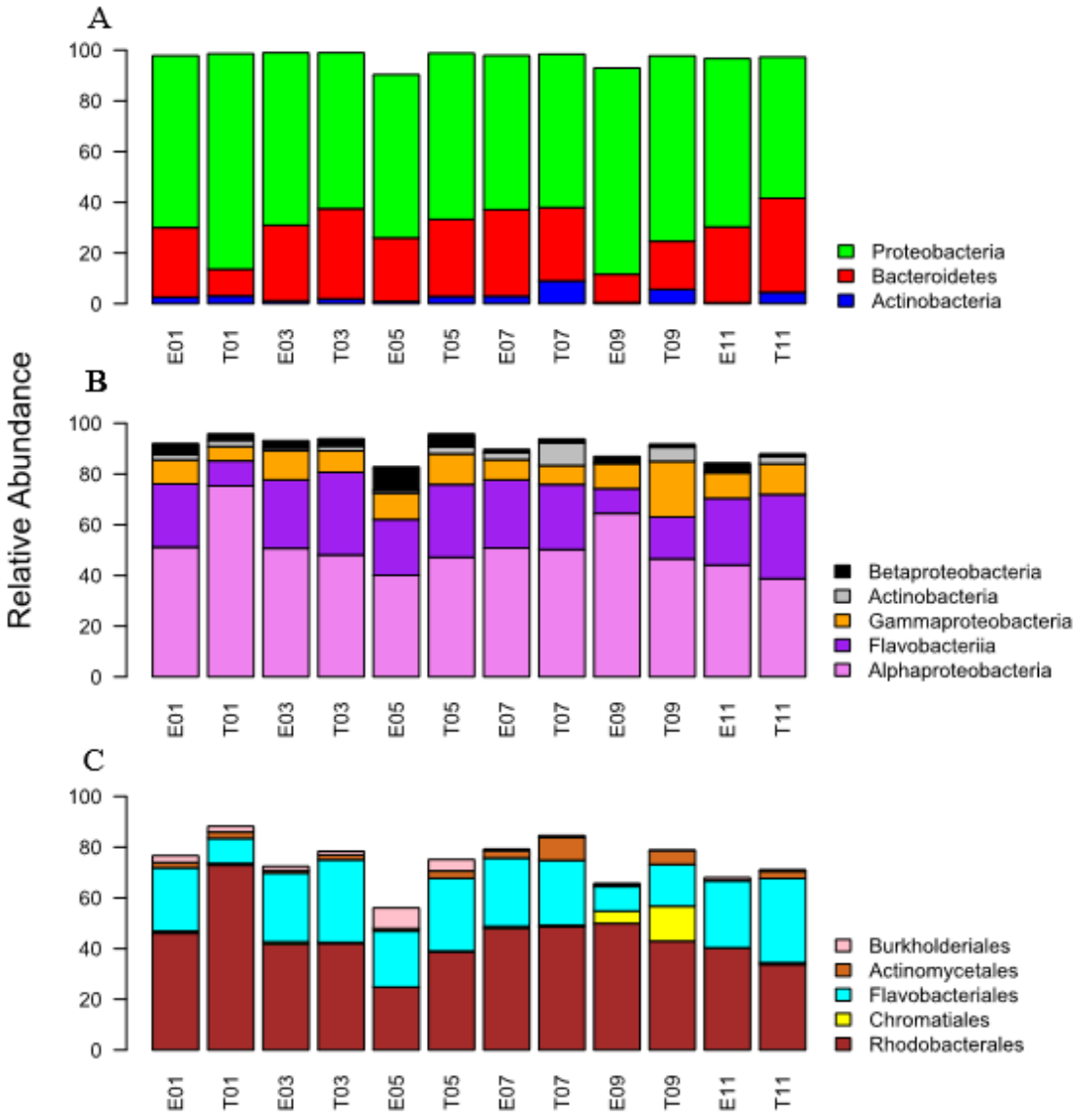


Figure 3.3. Phylum- (A), class- (B) and order-level (C) bacterioplankton community composition in the estuarine and aquaculture tanks in each sampling event. Relative abundance of the most 3 dominant phyla, 5 dominant classes and 5 dominant orders. E01 (Estuary January, Winter), T01 (Aquaculture Tank January, Winter); E03 (Estuary March, Beginning of spring), T03 (Aquaculture Tank March, Beginning of spring); E05 (Estuary May, End of spring), T05 (Aquaculture Tank May, End of spring); E07 (Estuary July, Summer), T07 (Aquaculture Tank July, Summer); E09 (Estuary September, Beginning of autumn), T09 (Aquaculture Tank September, Beginning of autumn); E11 (Estuary November, End of autumn), T11 (Aquaculture Tank November, End of autumn).

A total of 21 dominant OTUs (> 900 reads) were detected (Fig. 3.1B). The PCO ordination of bacterial OTUs (Fig. 3.1B and Fig. 3.1C) indicates that the majority of the dominant OTUs were associated with water samples collected after the beginning of spring. OTU 1 was the most dominant bacterium (17,053 reads) and was found predominantly in samples collected in winter, spring and autumn and was classified as belonging to the genus *Octadecabacter*. OTU 4 (*Loktanella*) was also dominant, but more abundant in January (Table 2). Both OTUs belonged to the family *Rhodobacteraceae*. According to the Blast search tool, the closest relative of OTU 1 was *Planktomarina temperate* (Table 3.2), isolated from the Wadden Sea, near Neuharlingersiel (Germany) (Voget et al., 2014).

The abundance analysis revealed that only OTU 2 (16,453 reads) was dominant in all seasons (winter, spring, summer and autumn). This OTU was classified as an unknown member of the family *Cryomorphaceae.* OTUs 3, 5 and 1519 were classified as unknown members of the family *Rhodobacteraceae,* however OTU 3 was dominant at the beginning of spring (March) and OTU 5 and 1519 were dominant during summer and the beginning of autumn (July and September). According to Blast OTU 5 (5915 reads) was closely related to *Phaeobacter* sp.. At the beginning of autumn (September), OTU 6 showed clear dominance and was identified as an unknown *Chromatiales*. OTU 7 was assigned to an unknown member of the *Oceanospirillales* order and was dominant at the end of spring (May). OTU 19 was dominant during summer (July) and was identified as an unknown *Flavobacteriales*. OTU 1525 (10,639 reads) was dominant during spring, summer and autumn (March, May, July, September and November) and was assigned to *Octadecabacter.*

The abundance analysis revealed several dominant OTUs, which were abundant during all seasons (OTUs 8, 10, 11, 12, 14, 15, 16, 18, 271, 490 and 764). OTUs 8 and 10 were classified as *Sediminicola* and unknown *Alteromonadales,* respectively. OTUs 11 and 764 were assigned to "*Candidatus Aquiluna rubra*" while OTUs 12 and 16 were assigned to the family *Flavobacteriaceae* and OTU 15 was assigned to the order *Flavobacteriales.* OTUs 14 and 18 were classified as *Pseudomonas* and unknown *Alphaproteobacteria*, respectively. OTUs 271 and 490, were both assigned to the family *Rhodobacteraceae* and classified as *Octadecabacter* and unknown *Rhodobacteraceae*, respectively. All these OTUs were assigned to either the *Flavobacteriaceae* family (*Flavobacteriales*), *Rhodobacteraceae* family (*Rhodobacterales*) or *Microbacteriaceae* family (*Actinomycetales*).

Table 3.2. Taxonomic affiliation of the most abundant OTUs (>900 reads) detected in this study and their closest relatives (using Blast) with the respective accession number, sequence identity (Sq ident) and source. Sum of reads indicates the number of sequences obtained for each OTU.

OTU	Sum of reads	Class	Order	Family	Genus	Acession	Sq ident	Source
1	17053	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter	CP003984	100	Planktomarina temperata strain RCA23
2	16453	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Unclassified	JF488529	100	Gulf of Maine (HNA bacterioplankton fraction)
3	4712	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified	GU061023	100	Yellow Sea intertidal beach
4	5044	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Loktanella	KC160936	99	Antarctic sea sediment
5	5915	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified	AB498882	99	Biofilm on control surface-winter sample
6	2423	Gammaproteobacteria	Chromatiales	Unclassified	Unclassified	JQ269275	100	Biofilm on control surface-winter sample
7	2080	Gammaproteobacteria	Oceanospirillales	Unclassified	Unclassified	JF488585	99	Gulf of Maine (HNA bacterioplankton fraction)
8	2025	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Sediminicola	AB496663	99	Seawater samples
10	2154	Gammaproteobacteria	Alteromonadales	HTCC2188	HTCC	GU061024	100	Yellow Sea intertidal beach
11	2148	Actinobacteria	Actinomycetales	Microbacteriaceae	Candidatus Aquiluna	NR125489	99	Freshwater, Canal Roanne a Digoin
12	2290	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Unclassified	JF488593	100	Gulf of Maine (HNA bacterioplankton fraction)
14	1314	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	JN609540	100	Intestines of a juvenile Salmo salar
15	1181	Flavobacteriia	Flavobacteriales	Unclassified	Unclassified	AB498909	89	Gulf of Maine (HNA bacterioplankton fraction)
16	1335	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Unclassified	JF488532	99	Gulf of Maine (HNA bacterioplankton fraction)
18	1131	Alphaproteobacteria	Unclassified	Unclassified	Unclassified	JF488476	100	Gulf of Maine (HNA bacterioplankton fraction)
19	1804	Flavobacteriia	Flavobacteriales	Unclassified	Unclassified	JF488529	89	Gulf of Maine (HNA bacterioplankton fraction)
271	1545	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter	EU600595	100	Uncultured Rhodobacteraceae bacterium
490	1706	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified	EU167485	99	Seawater
764	1256	Actinobacteria	Actinomycetales	Microbacteriaceae	Candidatus Aquiluna	AM999985	99	Freshwater, artificial pond
1419	3438	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified	HQ675338	98	HOT Station ALOHA, subtropical ocean gyre
1525	10639	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter	EF215743	100	Uncultured Rhodobacterales bacterium

Phylogenetic analyses of potential fish pathogens

In order to understand the seasonal dynamics of potential fish pathogens, we used a list of the most frequent bacterial pathogens responsible for fish diseases in aquacultures located in Europe (modified from our previous study, Data S3.2). The 16S rRNA gene sequences of these bacteria were then phylogenetically compared with related OTUs (same genera) detected in this study (potential fish pathogens) and their closest GenBank relatives (blastn tool) (Fig. 3.4). In addition to this, the seasonal dynamics (relative abundance) of detected genera, which are known to comprise potential fish pathogens, was also investigated (Fig. 3.4).

OTUs 127 and 1366 were closely related to *Photobacterium* sp. and *Photobacterium aestuarii*, respectively. Both OTUs were only detected in the aquaculture tanks during the end of spring, however OTU 127 had higher relative abundance. Our analysis also detected OTUs similar to *Vibrio ichthyoenteri* (OTUs 246 and 924). Curiously, the genus *Vibrio* was only detected during the beginning of autumn (September) (Fig. 3.4).

OTU 951 was closely related to *Tenacibaculum discolor,* a known fish pathogen. This OTU was not very abundant and was only detected in the aquaculture tanks during summer. OTU 95 was closely related with *Serratia marcescens*. Potential fish pathogens related to the genus *Serratia* were only detected in samples collected after the beginning of spring (March) and their relative abundance gradually decreased in samples collected after summer (July). Overall, the relative abundance of *Serratia* was always higher in samples collected in the estuary than in samples collected in tanks, except at the beginning of autumn (Fig. 3.4).

OTUs 14, 1252, 1309, and 197 were closely related to an unknown *Pseudomonas* spp. and *Pseudomonas psychrophila*, respectively. The genus *Pseudomonas* was detected in all seasons with high relative abundances during the end of spring, summer and autumn. OTU 14 had the highest relative abundances. Four OTUs were classified as members of the genus *Streptococcus* (OTUs 65, 116, 445 and 1356). OTUs 65, 116, 445 and 1356 were related to 16S rRNA gene sequences of *Streptococcus thermophilus*, *Streptococcus agalactiae* and an unknown *Streptococcus* sp., respectively (Fig. 3.4). The genus *Streptococcus* was detected in the aquaculture tanks in all seasons. OTUs 65, 116 and 445 had the highest relative abundances, in the aquaculture tank during the end of autumn and in the estuary samples during the beginning of autumn and the end of spring, respectively (Fig. 3.4).

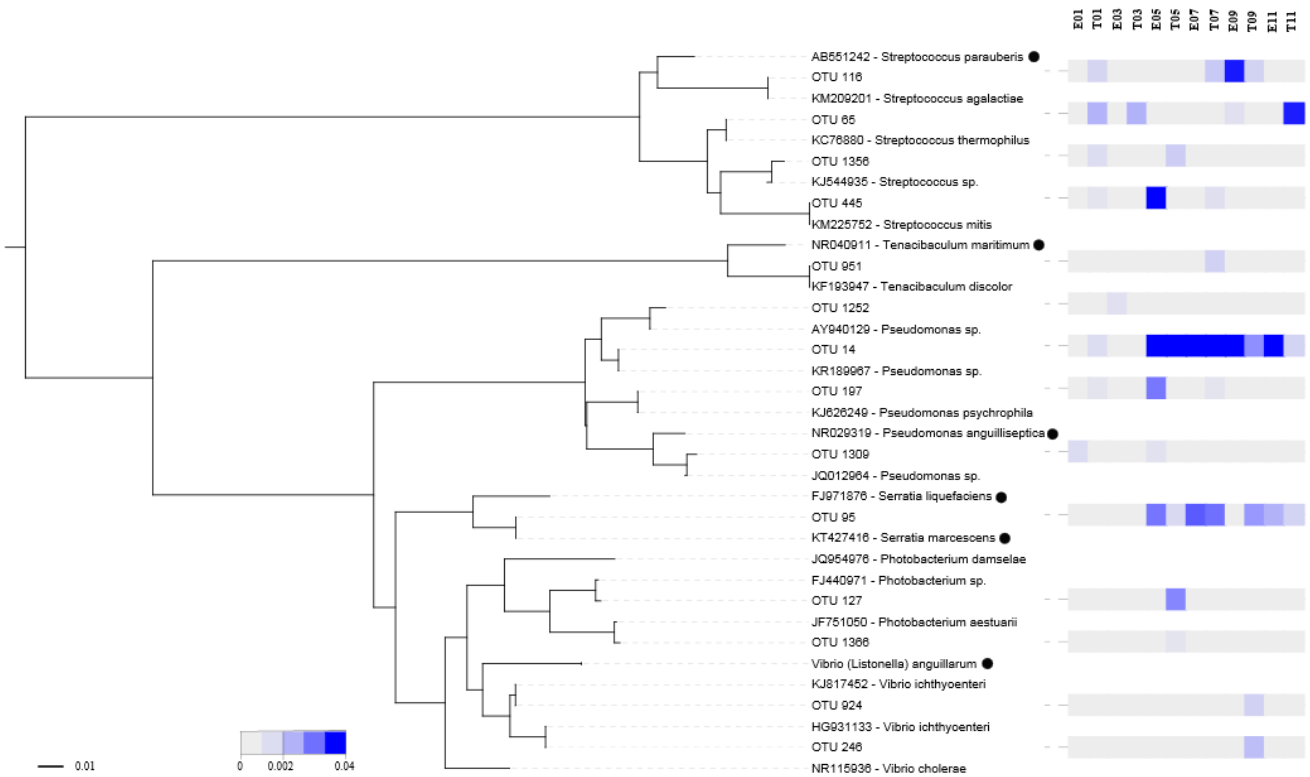


Figure 3.4. Phylogenetic tree (16S rRNA gene sequences) generated by the Mega 5.1 software and drawn using the online iTOL software package of the OTUs related to potential fish pathogens, their closest relatives (with accession number) and the relative abundance of each OTU in all sampling points. Black triangles indicate the known or emerging fish pathogens and black circles the most frequent pathogens described in literature. E01 (Estuary January, Winter), T01 (Aquaculture Tank January, Winter); E03 (Estuary March, Beginning of spring), T03 (Aquaculture Tank March, Beginning of spring); E05 (Estuary May, End of spring), T05 (Aquaculture Tank May, End of spring); E07 (Estuary July, Summer), T07 (Aquaculture Tank July, Summer); E09 (Estuary September, Beginning of autumn), T09 (Aquaculture Tank September, Beginning of autumn); E11 (Estuary November, End of autumn), T11 (Aquaculture Tank November, End of autumn).

Predictive metagenome analysis

The PICRUSt software package was used to predict metagenome functional content from the 16S rRNA gene data set. Mean (and standard deviation) of Nearest Sequenced Taxon Index (NSTI) values for the sampled environments varied between 0.115 and 0.133 in the water samples from the estuary and between 0.085 and 0.117 in water samples from the tanks. Based on the observed NSTI values, our *in silico* metagenomic analysis should yield reliable predictions (Langille et al., 2013).

A total of 15 dominant KO (> 250000 genes) were detected. The majority of the dominant KO belonged to "ABC transporters" pathway (K02026, K02025, K02023 and K02057: "Saccharide, polyol, and lipid transporters"; K02032, K02033, K02034 and K02035: "Peptide and nickel transporters") and three of these KOs differed significantly among sampling events (K02032: $F_{23,11} = 3.08$, $P = 0.03$, K02033: $F_{23,11} = 2.96$, $P = 0.038$, K02034: $F_{23,11} = 2.95$, $P = 0.038$ and K02035: $F_{23,11} = 3.20$, $P = 0.029$). These KOs are all related to the "peptides/nickel transport system" involved in the "ATP-binding cassette (ABC) transporters" pathway. Using LEfSe we found significant differences in several categories and pathways among sampling events (Fig. 3.5). These included an enrichment in: "environmental information processing" in winter (January), "metabolism" at the beginning of spring (March) and "genetic information processing" in summer (July). At the subcategory level, there was significant enrichment in the "xenobiotics biodegradation and metabolism" during winter (January), "Immune system diseases" during summer (July) and "infectious diseases", "cell growth and death", "cellular processes and signaling" at the beginning of autumn (September). Metagenome functional content of samples collected at the end of autumn (November) did not show significant enrichment of any specific subcategory.

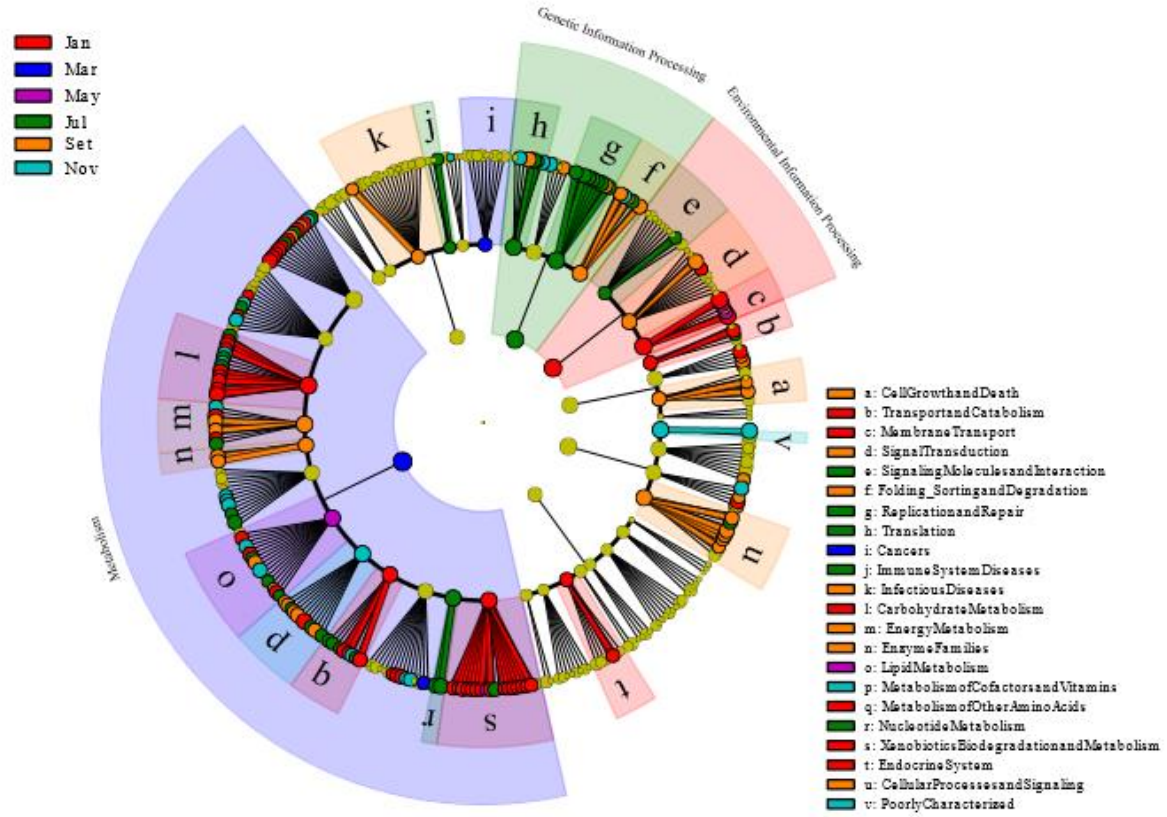


Figure 3.5. Cladogram created using LEfSe with the significant differences in gene counts of the KEGG PATHWAY hierarchy between the bacterial communities of the sampling events: Jan – January, Winter; Mar – March, Beginning of spring; May – May, End of spring; Jul – July, Summer; Sep – September, Beginning of autumn; Nov – November, End of autumn.

In this study, we paid special attention to the KEGG category for "infectious diseases". This enabled us to investigate the occurrence of a disease outbreak in the aquaculture system during October (Fig. 3.6). We observed a high relative abundance of KOs in the subcategory "infectious diseases" during the beginning of autumn (September), some weeks prior to the occurrence of a disease outbreak in October (direct field observation). The relative abundance analysis of selected functional individual pathways of the subcategory infectious diseases is shown in Fig. 3.6. The pathway "*Vibrio cholerae* infection" was enriched in samples collected in the aquaculture tanks during the beginning of autumn (September). During summer, we also observed an increase in estuary samples but not in the aquaculture tanks; subsequently, at the beginning of autumn the abundance of "*Vibrio cholerae* infection" declined in the estuary sample and increased in the aquaculture tanks (reaching its greatest abundance). At the end of autumn there was a decline in both aquaculture and estuary samples.

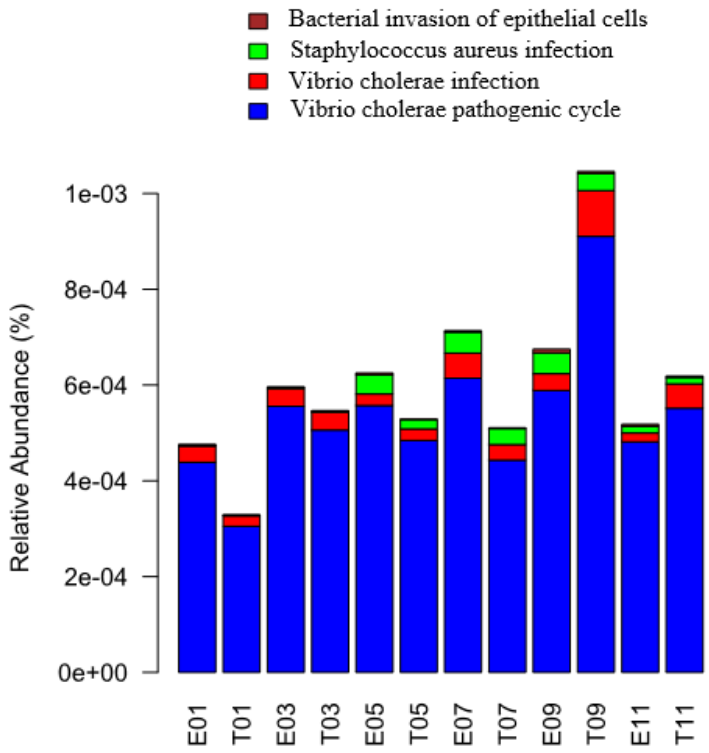


Figure 3.6. Relative abundance of selected pathways for KEGG subcategory "infectious diseases" in each sampling event for the estuary and the aquaculture tank. E01 (Estuary January, Winter), T01 (Aquaculture Tank January, Winter); E03 (Estuary March, Beginning of spring), T03 (Aquaculture Tank March, Beginning of spring); E05 (Estuary May, End of spring), T05 (Aquaculture Tank May, End of spring); E07 (Estuary July, Summer), T07 (Aquaculture Tank July, Summer); E09 (Estuary September, Beginning of autumn), T09 (Aquaculture Tank September, Beginning of autumn); E11 (Estuary November, End of autumn), T11 (Aquaculture Tank November, End of autumn).

Discussion

Seasonal changes of bacterioplankton communities in estuarine and aquaculture water

The PCO analysis showed seasonal variations of bacterioplankton communities, which were in turn related to changes of environmental factors (Data S3.3). While the community cluster formed during the winter and beginning of spring (January and March) showed correlation with significant increase of nitrate concentration, the cluster formed during summer and beginning autumn (July and September) was associated with higher TOC (14.00 mg L^{-1}) , higher temperature (25.47 ºC) and pH (8.30). Samples collected in November (end of autumn) tended to share more similarities with samples from May (end of spring), forming a cluster in the center of the PCO with intermediary values for environmental parameters. These results are in agreement with previous studies indicating that Ria de Aveiro estuary has a seasonal variation of nutrients (Lopes et al., 2007; Rodrigues et al., 2012), nitrate with higher concentrations in winter and spring and lower concentrations in summer (varied between 118.00 and 6.00 µmol L^{-1}) following the precipitation trend. The water temperature showed a seasonal variation with a maximum in the summer (23.50 ºC) and the minimum in the winter (11.20 ºC) (Lopes et al., 2007).

Interestingly, the PCO analyses did not show pronounced separation between bacterial communities from estuary and aquaculture samples, although the relative abundance analysis of the most dominant bacterial groups indicated a slight increase in the abundance of the *Actinomycetales* in aquaculture tanks in mid-summer and autumn. Members of the *Actinobacteria* phylum are often uncultured and, therefore, are more frequently detected using molecular techniques (Rheims et al., 1999). The ecological function of *Actinobacteria* in marine or estuarine waters is not very clear, but they may be key players in the maintenance of fish health. Several studies reported the use of marine *Actinobacteria* as a potential probiotic in aquaculture systems (You et al., 2005; Das et al., 2006; You et al., 2007; Das et al., 2008; Das et al., 2010). For example, You et al. (2007) suggest that marine *Actinomycetes* have an antagonistic activity against members of the genus *Vibrio*. The overall community composition analysis (estuarine and aquaculture water) showed that *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were the most abundant phyla during all sampling events. *Proteobacteria* are widely dispersed in marine environments and play an important role in nutrient cycling and mineralization of organic compounds (Kirchman,

2002; Kersters, 2006) while *Bacteroidetes* (previously *Cytophaga-Flexibacter-Bacteroides*) include dominant marine heterotrophic bacterioplankton and are frequently found colonizing macroscopic organic matter particles (marine snow) (Woebken et al., 2007).

Seasonal dynamics of dominant bacterial OTUs

An in depth analysis of seasonal variation of dominant bacterial OTUs showed that two of the most dominant OTUs, OTU 1 (*Octadecabacter*) and OTU 4 (*Loktanella*), assigned to the *Roseobacter* clade-affiliated (RCA) group (family *Rhodobacteraceae*, class *Alphaproteobacteria*), were more abundant during winter, spring and autumn. This group is an important component of the bacterioplankton in marine environments and probably one of the most extensively studied groups of marine bacteria (Brinkhoff et al., 2008). Previous studies have shown that several members of the RCA group can exhibit antibacterial activity or have an antagonistic effect on pathogenic bacteria, and can be used for disease control in aquaculture systems (Brinkhoff et al., 2004; Bruhn et al., 2005; Planas et al., 2006).

OTU 2 was dominant in all seasons (winter, spring, summer and autumn) and was classified as unknown *Cryomorphaceae.* The family *Cryomorphaceae* belongs to the phylum *Bacteroidetes* and most of its genera are of marine origin. Several studies reported that members of the family *Cryomorphaceae* cannot utilize carbohydrates and require complex organic compounds for growth (Bowman et al., 2003; Lau et al., 2005). Bacteria belonging to this family were previously isolated from an aquaculture system of abalone (*Haliotis discus*) in Southern Korea (Lee et al., 2010). According to Lau (2006), the high carotenoid content in these bacteria can lead to an eventual reduction in the oxidative stress caused by white spot syndrome virus (WSSV) infection, and thus has potential probiotic use against this virus in aquaculture systems.

OTU 3 was most abundant at the beginning of spring (March) and OTUs 5 and 1419 were most abundant during summer and at the beginning of autumn (July and September); they were classified as unknown members of the family *Rhodobacteraceae*. OTU 7 was identified as an unknown *Oceanospirillales* and was most abundant at the end of spring (May), while OTU 19 was most abundant during summer (July) and was identified as an unknown member of the order *Flavobacteriales.* Members of the order *Oceanospirillales* are often involved in symbiotic interactions with marine animals; however, their putative functional role in fish is yet to be determined (Jensen et al., 2010).

OTU 1525 (10,639 reads) was assigned to the genus *Octadecabacter* and was abundant during spring, summer and autumn (March, May, July, September and November). Dominant OTUs which were abundant during all seasons were assigned to the groups *Flavobacteriaceae* (*Flavobacteriales*), *Rhodobacteraceae* (*Rhodobacterales*) and *Microbacteriaceae* families (*Actinomycetales*). For example, OTUs 271 and 490, were classified as *Octadecabacter* and unknown *Rhodobacteraceae*, respectively. The phylogeny of the *Roseobacter* group is considered problematic due to the assignment of genera names to more than one monophyletic lineage (e.g., *Roseobacter* and *Ruegeria*). However, an overview of the marine *Roseobacter* lineage indicated three robust superlineages (more than 10 non-redundant members): the *Otadecabacter-Ruegeria* group, the *Sulfitobacter-Staleya-Oceanibulbus* group, and the *Silicibacter-Ruegeria* group (Buchan et al., 2005). Members of the *Roseobacter* group are known for their ability to produce acylated homoserine lactones (AHL) and secondary metabolites (Brinkhoff et al., 2004; Martens et al., 2007). Some *Phaeobacter* and *Ruegeria* strains produce tropodithietic acid (TDA) that is able to inhibit the growth of *V. anguillarum* (Ruiz-Ponte et al., 1999; Bruhn et al., 2005; Porsby et al., 2008). In fact, several studies have evaluated the use of members of the *Roseobacter* group as potential probiotics in the aquaculture systems (Hjelm et al., 2004; Planas et al., 2006).
OTUs 14 and 8 were classified as *Pseudomonas* and *Sediminicola*, respectively. *Sediminicola luteus* is the most studied member of this genus firstly described by Khan et al. (2006) and isolated from marine sediment from the Sea of Japan. OTU 14 was closely related to *Pseudomonas* sp.. *Pseudomonas* are common inhabitants of the aquatic environment and compose the natural flora of European seabass (*Dicentrarchus labrax*) (Taliadourou et al., 2003; Castro et al., 2006). Although the majority of members of the genus *Pseudomonas* are considered opportunistic pathogens, several studies reported that some members of this genus have an antagonistic activity against fish pathogens (Chythanya et al., 2002; Korkea-aho et al., 2011).

Seasonal dynamics of potential fish pathogens

The phylogenetic analysis of OTUs related to known aquaculture pathogens showed seasonal trends, occurrence and relative abundance, of potential fish pathogens in the semi-intensive aquaculture studied. OTUs 246 and 924 were detected in aquaculture tanks during the beginning of autumn and showed strong homology with *Vibrio ichthyoenteri*. This

species is a potential pathogen previously reported as the main cause of mortalities in fish aquaculture (Alvarez et al., 1998; Zhang and Austin, 2000; Zorrilla et al., 2003). OTU 95 also showed strong homology to *Serratia marcescens*. This bacterium is an opportunistic fish pathogen that was previously detected in brackish and freshwaters and is a causative disease agent in natural populations of white perch (Baya et al., 1992). This species was detected during the end of spring, summer and autumn, and was relatively abundant at the end of spring and summer however declined in abundance after summer (July). OTUs classified as members of the genus *Pseudomonas* were detected in estuarine and aquaculture water in all seasons, with a strong dominance of OTU 14 in the end of spring until the end of autumn. However, none of these OTUs showed homology with known fish pathogens. Several members of the genus *Pseudomonas* are considered opportunistic pathogens, although previous reports indicate that they are part of the natural flora of European seabass (*Dicentrarchus labrax*) (Taliadourou et al., 2003; Castro et al., 2006).

In this study, four dominant OTUs (65, 116, 445 and 1356) classified as members of the *Streptococcus* genus alternated their abundance during the seasons. *Streptococcus* comprises several members capable of causing streptococcosis in both wild and captive fishes worldwide (Salati, 2011). However, the phylogenetic analysis of dominant OTUs related to *Streptococcus*, showed that only OTU 116 was phylogenetically close related to a potential fish pathogen (*Streptococcus agalactiae*). This OTU showed an increased dominance in estuarine water in September (beginning of autumn). Interestingly, disease outbreak and fish mortality was observed during the first weeks of October (direct field observation). A recent study published by Evans et al. (2015) provided evidences that *S. agalactiae* strains from different geographic origin can cause fish mortality and can have different pathogenic capacities. *Streptococcus agalactiae* was previously isolated from Nile tilapia (*Oreochromis niloticus*) cultured in net cages in Lake Sentani in Indonesia (Anshary et al., 2014). Another potential fish pathogen, *Tenacibaculum discolor* (OTU 951), was only detected in the aquaculture tanks during summer. This species is a known fish pathogen responsible for tenacibaculosis disease and was firstly isolated from diseased sole (*S. senegalensis*) and turbot (*S. maximus*) in Spain (Piñeiro-Vidal et al., 2008).

Predictive metagenome analysis

The PICRUSt metagenomic prediction indicated that the most enriched KOs belong to "ABC transporters" pathway. ABC transporters form one of the largest known protein families, and are widespread in bacteria, archaea, and eukaryotes. They couple the energy released from ATP hydrolysis to transport a wide variety of substrates such as ions, sugars, lipids, sterols, peptides, proteins, and drugs into or out of cells and organelles (http://www.genome.jp/) (Davidson and Chen, 2004). Bacterial ABC transporters are associated with the ability to survive in widely diverse environments. Linton and Higgins (1998), in a study addressing the ATP-binding cassette (ABC) proteins in *Escherichia coli*, suggested that the high number of ABC transporters in this species may reflect its versatility and ability to adapt to changing environments. We also found differences among seasons in three of the most enriched KOs. They were all related to peptides/nickel transport system involved in the ATP-binding cassette (ABC) transporters pathway. Peptide transporters are responsible for adjusting the expression of specific genes to environmental conditions (Detmers et al., 2001). Nickel plays a significant role in several cellular processes and is an essential component of several metalloenzymes involved in energy and nitrogen metabolism (Mulrooney and Hausinger, 2003). In the aquaculture system, the consumed feed is partially transformed in fish biomass and in part excreted as ammonia in the water. Ammonia is subsequently converted into nitrite and nitrite into nitrate by nitrifying bacteria. The nitrification process plays a key role in controlling the concentration of ammonia in the water. It is well known that nitrifying bacterial activity is influenced by several factors such as temperature, salinity, dissolved oxygen, sunlight, transparency of water tank or depth of the tank (Stenstrom and Poduska, 1980; Antoniou et al., 1990; Rysgaard et al., 1999). As this study was carried out in an aquaculture located in an estuarine system, the significant differences observed between sampling times in the KOs related to the peptides/nickel transport system could be related to seasonal variation and consequently by fluctuations in the hydrodynamic conditions that naturally occurs in the estuarine system.

The predicted metagenome analysis for the infectious diseases subcategory showed high relative abundances of KOs during the beginning of autumn (September). This data is in line with the seasonal analysis of potential fish pathogens, which also showed an increased relative abundance of potential fish pathogens in September. Interestingly, these results preceded the disease outbreak and fish death during subsequent weeks (direct field

observation). An in depth analysis of the subcategory showed an increase in the abundance of the pathway "*Vibrio cholerae* infection" and "*Vibrio cholerae* pathogenic cycle" during the beginning of autumn (September).

As mentioned in Martins et al. (2013), the 16S rRNA gene is widely used in phylogenetic analysis and is an important marker for molecular diagnostics and molecular ecology studies. However, this gene fails to provide an accurate identification at the species level for some bacteria. Several studies reported that the degree of resolution obtained with the 16S rRNA gene is not sufficiently robust for the phylogenetic analysis of *Vibrio* species (Thompson et al., 2005). Figure 4 shows that *Vibrio cholerae* is closely related to *Photobacterium* and *Vibrio* species. Furthermore, several studies already reported that some fish bacterial pathogens have the same virulence factors (Sudheesh et al., 2012). For instance, the gene for type III secretion system (T3SS2 gene), involved in the pathogenicity of the bacterium, highly conserved with other T3SS2 genes reported in *V. parahaemolyticus*, *V. cholera*, and *V. mimicus* (Caburlotto et al., 2009; Okada et al., 2010). Type III secretion systems are important virulence factors in several bacterial pathogens of animals and plants (Hueck, 1998). Burr et al. (2005) suggested that the type III secretion system is required for virulence in *A. salmonicida*, i.e, is required to establish systemic infection. Thus, *Vibrio cholerae* infection in the KEGG classification can be used in fact as a general indicator for emergence of potential fish pathogens.

Conclusion

In this study, we used an in depth sequencing approach and bioinformatic tools to analyze seasonal dynamics of bacterioplankton communities and their putative function in an open semi-intensive aquaculture system. Our results revealed that variations in bacterioplankton composition and putative function in aquaculture and estuarine water can be related to seasonal variations of estuarine environmental parameters. In addition, the results also indicated few differences between structural and functional patterns of bacterioplankton communities in aquaculture and estuarine water during all sampling events. However, to confirm these results we are currently analyzing more estuarine water samples. The in depth bacterioplankton composition analysis allowed us to detect seasonal changes of potential fish pathogens (e.g., *T. discolor, S. marcescens* and *S. agalactiae*). In line with these results, the predicted metagenome analysis for the subcategory "infectious diseases", showed a high

relative abundance of related KOs during the beginning of autumn (September). Interestingly, these results preceded a disease outbreak and fish death during subsequent weeks. In this study, the use of 16S rRNA gene pyrosequencing and PICRUSt analyses improved our understanding of bacterioplankton temporal dynamics and potential function in a semi-intensive aquaculture, providing relevant information for fish health and production. Similar studies in the future can contribute to a better understanding of the 'standard' microbiome of fish aquacultures and help to improve management practices, fish health, quality and the safety of aquaculture systems.

Acknowledgements

Patricia Martins benefited from a PhD grant (SFRH/BD/73889/2010) provided by the Portuguese FCT (Fundação para a Ciência e Tecnologia). This study has been carried out with the financial support of project AQUASAFE - Development of new technologies to anticipate and diagnose disease outbreaks in aquaculture (PROMAR 31-03-05-FEP-0016) (PROMAR, a Portuguese instrument for the sectors of fisheries and aquaculture funded by the European Fisheries Fund). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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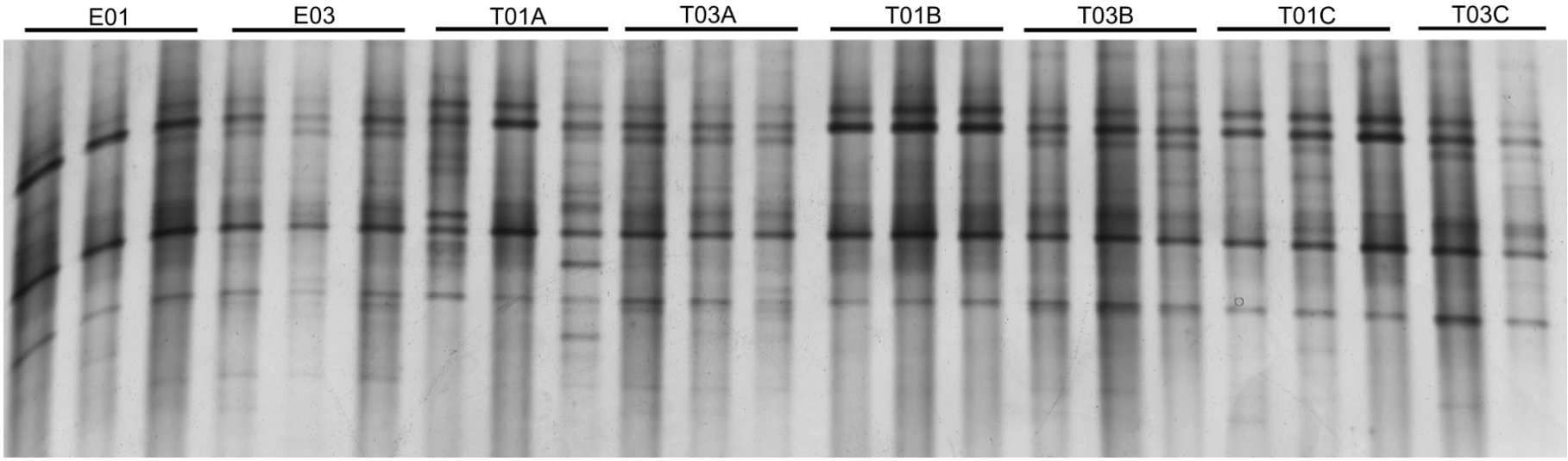
Supplementary material

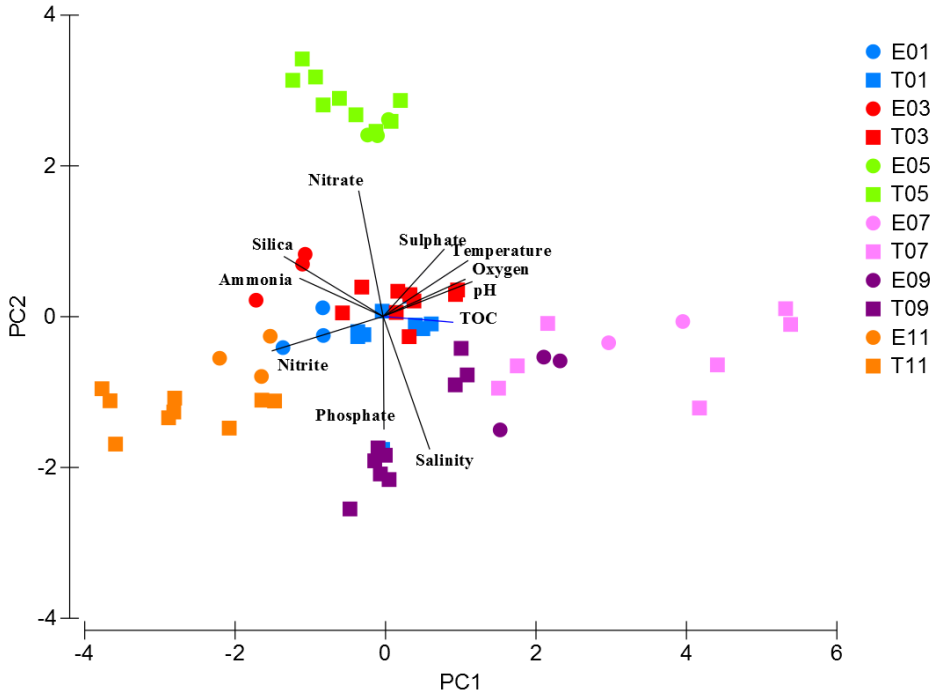
Data S3.1. Representative DGGE gel showing the similarity among replicates in samples collected during winter (January) and the beginning of spring (March). E01 - Estuary January, E03 - Estuary March, T01A – Aquaculture Tank January replicate A, T03B – Aquaculture Tank March replicate A, T01B – Aquaculture Tank January replicate B, T03B – Aquaculture Tank March replicate B, T01C – Aquaculture Tank January replicate C, T03C – Aquaculture Tank March replicate C.



Data S3.2. Most frequent bacterial pathogens in aquaculture systems production described in literature.

Fish pathogen species	Fish hosts (literature)
Aeromonas salmonicida	Salmonids, turbot
Mycobacterium marinum	Seabass, turbot, Atlantic salmon
Photobacterium damselae	Seabream, seabass, sole, striped bass, yellowtail
Pseudomonas anguilliseptica	Seabream, eel, turbot, ayu
Serratia marcescens	Salmonids,
Streptococcus iniae	Yellowtail, flounder, seabass, barramundi
Tenacibaculum maritimum	Turbot, salmonids, sole, seabass, gilthead seabream, red Seabream, flounder
Vibrio anguillarum	Salmonids, turbot, seabass, striped bass, eel, ayu, cod, red seabream

Data S3.3. Principal component analysis of the environmental parameters. E01 (Estuary January, Winter), T01 (Aquaculture Tank January, Winter); E03 (Estuary March, Beginning of spring), T03 (Aquaculture Tank March, Beginning of spring); E05 (Estuary May, End of spring), T05 (Aquaculture Tank May, End of spring); E07 (Estuary July, Summer), T07 (Aquaculture Tank July, Summer); E09 (Estuary September, Beginning of autumn), T09 (Aquaculture Tank September, Beginning of autumn); E11 (Estuary November, End of autumn), T11 (Aquaculture Tank November, End of autumn).



Chapter 4. Development of a molecular methodology for fast detection of *Photobacterium damselae* subspecies in water samples

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Aquaculture. 435: 137-14

Abstract

Photobacterium damselae subspecies *damselae* and *piscicida* are known fish pathogens responsible for disease outbreaks in several cultured fish species. Fast detection of these pathogens is important for management and control of disease outbreaks. In this study, we developed a molecular approach for quantification of *P. damselae* species and detection of its subspecies (*piscicida* and *damselae*) based on real time PCR (RT-PCR) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE) of *toxR* gene fragments. In addition, the efficacy of this molecular methodology was tested against water samples from a natural environment (Ria de Aveiro estuary, Aveiro, Portugal) and from an intensive recirculating aquaculture system (RAS). In the first phase of this study we developed a RT-PCR targeting the *toxR* gene suitable for detection and quantification of *P. damselae* species. After the first RT-PCR trial, a nested PCR-DGGE was developed to determine the presence of *P. damselae* subspecies in the positive samples. Confirmation of the identity of different subspecies was obtained by Sanger DNA sequencing and phylogenetic analysis of *toxR* gene fragments obtained from excised DGGE bands. Our RT-PCR detected between 1797.85 ± 376.15 (ca 0.71 fg) and 2976.68 ± 1253.63 (ca 1.76 fg) gene copy numbers of *P. damselae toxR* genes in the aquaculture samples. The DGGE analysis of these samples detected two equally abundant bands near the DGGE reference position for *P. damselae* subsp. *piscicida.* The sequence and phylogeny analyses of excised bands revealed the presence of two populations with distinct *toxR* gene sequences suggesting a close phylogenetic relationship with *P. damselae* subsp. *piscicida*. Contrary to aquaculture samples, no RT-PCR signal was obtained for DNA extracted from estuarine water samples.

Here we provide evidences that the molecular methodology developed in this study can be used for overall quantification of *P. damselae* and subsequent detection of its subspecies in natural ecosystems and aquaculture systems.

Keywords

Aquaculture; pathogen detection; barcoded pyrosequencing; bacteria; disease

Introduction

Aquaculture plays an increasingly important role in food production worldwide. However, disease outbreaks have direct effects on fish production, causing severe economic losses in the aquaculture sector. Members of the species *Photobacterium damselae* are frequently associated with disease outbreaks, and have been described as emergent fish pathogens in aquaculture systems. Currently, the species *Photobacterium damselae* comprises two subspecies, *P. damselae* subsp. *damselae* and *P. damselae piscicida.* These two species are able to cause different diseases. *P. damselae damselae* is a pathogen able to infect several aquatic animals (fishes, crustaceans, dolphins and molluscs, among others) (Hanlon et al., 1984; Buck et al., 1991; Fouz et al., 1992; Song et al., 1993) that is frequently associated with epizootic outbreaks in cultured fish species (turbot, rainbow trout and sea bream, among others) (Vera et al.1991; Fouz et al., 1992; Pedersen et al., 2009). Usually, fishes infected with this pathogen present hemorrhaged area (eyes, mouth, and jaws) and skin ulcerative lesions (Fouz et al., 1992). Recently, Labella et al. (2011) suggested *P. damselae damselae* as an emergent fish pathogen affecting new cultured marine fish species in Southern Spain. This subspecies has been also reported as a human pathogen, in most of the cases by exposure to wounds inflicted during handling diseased fishes (Clarridge and Zighelboim-Daum, 1985; Perez-Tirse et al., 1993). Only the subspecies *P. damselae damselae* is able to infect humans (Shin et al., 1996; Fraser et al., 1997). This pathogen causes necrotizing fasciitis in humans, reported as more aggressive and with higher mortality rate than that caused by *Vibrio vulnificus* (Yamane et al., 2004). The potential pathogenicity of these bacteria to humans should be considered of zoonotic interest (Pedersen et al., 2009; Rivas et al., 2013). *P. damselae piscicida* is the causative agent of photobacteriosis, also known as pasteurellosis or pseudotuberculosis, one of the most important fish diseases in aquaculture systems. *P. damselae piscicida* is a halophilic bacterium that causes septicemia in infected fishes and in extreme cases can lead to the formation of whitish tubercles in several internal organs (Romalde, 2002). This subspecies is more aggressive, causing higher mortality rates in aquacultures.

In general, *P. damselae* subspecies *piscicida* and *damselae* have distinct differences in their motility, gas production from glucose, nitrate reduction, hemolysin production, and optimal temperature for growth (Fouz et al., 1992; Magariños et al., 1996). Due to the phenotypic differences, classical microbiological and biochemical methods are the most commonly used

strategies to identify and distinguish *P. damselae piscicida* and *P. damselae damselae.* However, these techniques are often laborious, time-consuming and lack sensitivity. In the last years, despite the high homology in the DNA sequence of both subspecies (Gauthier et al., 1995; Osorio et al., 1999), several studies developed protocols using molecular techniques to overcome the limitations of the conventional microbiological techniques and to allow the detection of *P. damselae* (Zhao and Aoki, 1989; Osorio et al., 1999; Kvitt et al., 2002) and distinguish the two subspecies (Zappulli et al., 2005; Amagliani et al., 2009). Previous studies showed that different genotypes of *P. damselae* subspecies *piscicida* and *damselae* can have different phenotypic characteristics and pathogenicity potential (Hawke et al., 2003; Takahashi et al., 2008). Therefore, the evaluation of the occurrence of distinct populations within a subspecies can be important for proper assessment of each case and effective disease control.

Recently, well-established techniques to assess microbial diversity, such as PCR - denaturing gradient gel electrophoresis (PCR-DGGE) and high throughput sequencing approaches have been successfully used as a tool for pathogens detection (Ji et al., 2004; Petersen et al., 2007; Martins et al., 2013). Although previous molecular studies showed the ability to differentiate between the two *P. damselae* subspecies, none of these methodologies were tested directly against DNA samples extracted from environmental or aquaculture water samples. Furthermore, most of these studies are based on the 16S rRNA gene, which is not considered a suitable phylogenetic marker to distinguish *P. damselae piscicida* and *P. damselae damselae*. Several authors have reported that 16S rRNA gene does not provide a sufficient phylogenetic resolution at the species level for *Vibrio* or *Photobacterium* species (Osorio and Klose, 2000; Thompson et al., 2005). According to Osorio C. (1999, 2000) the subspecies *P. damselae damselae* and *P. damselae piscicida* have 100% homology between sequences of 16S rRNA gene and only 91% homology between sequences of *toxR* gene. In this study our goal was to develop a molecular methodology based on *toxR* gene for quantification of *P. damselae* and rapid detection of its subspecies. First, to detect and quantify *P. damselae* species a real time PCR (RT-PCR) was developed using *P. damselae*-specific primers targeting the *toxR* gene. Then, a nested PCR-DGGE approach based on *toxR* gene analysis of *P. damselae* subspecies *damselae* and *piscicida* was developed. Importantly, the methodology presented in this study was tested against environmental

samples (estuary and aquaculture water samples) and the results validated using a state of the art barcoded pyrosequencing approach.

Material and methods

Sampling and DNA extraction

Water samples (three replicates containing 250 ml of water each) were collected at the Ria de Aveiro estuary (Aveiro, Portugal – coordinate 40°38'58.9"N/8°39'51.1"W) (this study) in July 2012 and from biological filters in a recirculation aquaculture system (RAS) for sole (SolBio1, SolBio2, SolBio3) and turbot (TurBio1, TurBio2, TurBio3) in July 2012 (Martins et al., 2013). The samples were filtered, stored in ice during 24 hours and processed for DNA extraction in accordance with the procedure described by Martins et al. (2013). Briefly, estuarine and aquaculture samples were filtered through 0.2 µm pore polycarbonate membranes (Poretics, Livermore, CA, USA) and the total community (TC) DNA extraction was performed directly on the filter using an E.Z.N.A Soil DNA Extraction kit (Omega Bio-Tek, USA) following the manufacturer's instructions.

Barcoded pyrosequencing

A barcoded pyrosequencing approach was used for in-depth bacterial composition analyses of estuary and aquaculture samples as previously described in Martins et al. (2013), but using the most recent Greengenes release (Greengenes 13_8; ftp://greengenes.microbio.me/greengenes_release/gg_13_5/gg_13_8_otus.tar.gz). Briefly, prior to pyrosequencing, aliquots of all three replicates were combined forming one DNA library for each treatment (estuary, turbot and sole). Pyrosequencing libraries were analysed using QIIME (Quantitative Insights Into Microbial Ecology) (http://qiime.sourceforge.net/). Operational taxonomic units (OTUs) were selected (97% similarity) using the pick_otus.py script, representative sequences were selected using the pick_rep_set.py script and the taxonomic identity was determined using the assign_taxonomy.py script with the Ribosomal Database Project (RDP) method (Martins et al., 2013). The OTU table containing the abundance and taxonomic assignment of all OTUs was produced using the make_otu_table.py script.

The DNA sequences generated in this study were submitted to the NCBI SRA: Accession number SRX658559.

Primer design and validation.

Initially two primer sets were designed, the first pair of primers (ToxRn1-F and ToxRn1-R) was suitable for RT-PCR and for conventional PCR and the second pair of primers (ToxRn2-(GC)-F and ToxRn2-R) was designed to target the PCR fragments generated with the primers ToxRn1-F and ToxRn1-R for a nested PCR approach suitable for DGGE fingerprinting of *P. damselae* subspecies. Briefly, nucleotide sequences of partial *toxR* gene from *P. damselae* were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/), 16 belonging to *P. damselae damselae* and 5 belonging to *P. damselae piscicida*. Sequence alignment was performed in Mega software (Molecular Evolutionary Genetics Analysis) using the ClustalW algorithm. On the basis of the alignment two sets of primers were designed (for a nested PCR approach). The designed primers were optimized with the program Oligo (version 4.0; National Biosciences Inc.). In order to obtain PCR amplicons suitable for DGGE fingerprinting, a GC clamp was added to the primer ToxRn2F to prevent complete melting of double-stranded DNA during DGGE. The primer sets, locations in the gene and product lengths are presented in Table 4.1. The oligonucleotides were synthetized by IBA GmbH Nucleic Acids Product Supply (Göttingen, Germany).

A preliminary *in silico* analysis of the primers designed in this study was performed using BLAST reference database in order to evaluate the specificity of the primers. Primers designed were tested against all *toxR* gene sequences available in the GenBank (GenBank, National Centre for Biotechnology Information, Rockville Pike Bethesda, USA). Degenerate bases were added in the primer pair for the first PCR (ToxRn1-F and ToxRn1-R) (Table 1) to allow a perfect primer match with target *toxR* genes. No degenerate bases were added in the DGGE primers. However, all DGGE primers displayed a perfect match with at least the first 14 nucleotides in direction 3`-5`. Such modifications could result in multiple bands for each DGGE band genotype and should be avoided. The optimal annealing temperature were tested in a temperature gradient ranging from 58ºC and 63°C, using as template DNA of *P. damselae damselae* (DSMZ nº 7482) and *P. damselae piscicida* (DSMZ nº 22834). Visualization of PCR products on agarose gel showed a single band of the expected size

(~323bp fragment in the first PCR and ~300bp fragment in the second nested PCR), without unspecific amplification at 63ºC.

Table 4.1. PCR primers used in the study, with the position according to the *toxR* of *Photobacterium damselae subspecies damselae GenBank* acc. no. AF170886.

Primer name	Sequence (5' - 3')	Location in the gene	Product lengths (bp)
ToxRn1-F	GGCTATTRCAKCAACSGAACACG	162	323
ToxRn1-R	TTTGGYGTTACRACTTGCACCCCT	485	
ToxRn2-(GC)-F[a]	CAGCAACGGAACACGCAGAAGA	170	300
ToxRn2-R	TGCACCCCTTTAACCGAAAAGAGT	470	

[a] Primer ToxRn2-(GC)-F had a GC clamp attached to the 5' end used for DGGE analyses; GC clamp sequence: 5'–CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG -3' (Nübel et al., 1996).

Real-time PCR quantification

Quantification of the gene *toxR* was carried out with StepOne real time PCR system (Apllied Biosystems) using standard curves constructed from 10-fold serial dilutions. The RT-PCR master mix (20 µL) contained 1 µL of template DNA, 2x Luminaris Color HiGreen High ROX qPCR Master Mix (Thermo Scientific, Waltham, Massachusetts, USA) and 0.3 µM of ToxRn1-F and ToxRn1-R primers. The amplification protocol involved an initial incubation step at 50°C for 2 min, followed by 10 min of incubation at 90°C and then 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Fluorescence signal intensity was measured during a 80°C step for 10 s to dissociate the primer dimers. Product specificity was confirmed by melting curve analysis. The melt curve data were obtained starting at 60°C and increasing by 0.3°C every 15 seconds to a maximum of 95°C. During the melting stage a plate read was obtained at every 0.3°C increment. Based on pyrosequencing data analysis estuarine samples negative for *P. Damselae* were used as negative control for evaluation of primer specificity under real environmental conditions (high bacterial diversity and the presence of PCR

inhibitors). The standard curves were constructed using gene fragments with ca. 323 bp previously obtained by PCR amplification (ToxRn1-F and ToxRn1-R primers) of *toxR* gene from pure DNA extracts of *P. damselae piscicida* DSMZ nº 22834. After amplification the DNA was quantified with the Quant-iT dsDNA high sensitivity assay kit (Invitrogen, Eugene, Oregon, USA) and the Qubit fluorometer (Invitrogen, Carlsbad, California, USA). The gene copy number in the initial standard was calculated from the DNA content, the length of the fragment and the average weight of a base pair (650 Da). The standard ranges were created by producing a ten times dilution series from 10^9 to 10^1 target gene copies per µL. All the quantifications were performed in triplicate.

Nested PCR-DGGE

Here a nested PCR-DGGE approach was used to detect and distinguish *P. damselae* subspecies from environmental (estuary and aquaculture) samples. For the first PCR reaction mix (25µl) we used 1 µl of template DNA, 2µM of each primer (ToxRn1-F and ToxRn1-R), 4% (vol/vol) of acetamide and 12.5µl of DreamTaq PCR Master Mix (2x) containing Dream Taq DNA polymerase, optimized Dream Taq buffer, MgCl2 and dNTPs according to the manufacturer's instructions (Fermentas, Vilnius, Lithuania). After 5min denaturation at 94ºC, 30 thermal cycles of 45s at 94ºC, 45s at 63ºC and 1min30s at 72ºC, the PCR ended with a final extension at 72ºC for 10 min. The amplification products were checked on an electrophoresis in 1% agarose gels. In the second PCR reaction mix (25µl) 1 µl of template DNA obtained from the first PCR was added in the PCR mix containing 2µM of each primer, 4% (vol/vol) of acetamide and 12.5µl of DreamTaq PCR Master Mix (2x) containing Dream Taq DNA polymerase, optimized Dream Taq buffer, $MgCl_2$ and dNTPs according to the manufacturer's instructions. (Fermentas, Vilnius, Lithuania). The temperature profile was 10 min of initial denaturation at 95ºC, 25 thermal cycles of denaturation (45s at 94ºC), annealing (45s at 63ºC) and extension (1 min at 72ºC) and a final extension of 10 min at 72ºC. The PCRs were conducted in a Professional Thermocycler (Biometra). Positive and negative controls were run for every PCR, and the amplification products were checked on an electrophoresis in 1% agarose gels.

DGGE analysis

The DGGE technique allows the separation of DNA fragments (PCR amplicons) of identical length according to their base-pair sequences. This separation is based on the alteration of the electrophoretic mobility of the fragments as they migrate through an increasing gradient of DNA denaturants. Therefore, different DNA sequences have different melting temperature, which influences the migration of the sequence in the gel (Lerman et al., 1984). Here we used this approach to differentiate *toxR* gene fragments amplified from environmental samples belonging to different *P. damselae* subspecies.

DGGE was performed on a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Samples were loaded onto 8% (w/v) polyacrylamide gels in 1x Tris-acetate-EDTA (TAE) with the denaturing gradient ranging from 40% to 58% (100% denaturant contains 7 M urea and 40% formamide) and performed at 58 ºC at 160 V during 16 hours. Gels were silver-stained according to Byun et al. (2009), except for the stop solution that in our case was replaced by a Na_2CO_3 (0.75%) solution. The image was acquired using the Epson perfection V700 Photo Scanner. The position of the bands on the DGGE allows us to identify different subspecies by comparing their migration pattern with known reference strains for *P. damselae damselae* and *P. damselae piscicida*.

Selected DGGE bands were excised using a sterile scalpel blade and eluted in 30µl of Tris-EDTA (TE) buffer overnight at 4ºC. After that, this solution was used as template (4µl) for a re-amplification using the original primer set as described above (second PCR). The re-amplified product and the original sample were run in the DGGE to confirm the presence and the position of the band in the gel (one band at the expected position). Sequencing analysis was performed by a certified laboratory (GATC Biotech, Germany). Band sequences were compared with sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/) by using the BLASTN program. The DNA sequences obtained from DGGE bands were then submitted to NCBI's GenBank database under accession no. KM224457 and KM224458.

A phylogenetic tree for *toxR* gene sequences obtained from DGGE bands and sequences from NCBI's GenBank database was constructed using the Mega 5.1 (http://www.megasoftware.net/) software. The evolutionary distances were computed using the Maximum Composite Likelihood method with a gamma distribution (four categories) and 1000 bootstraps.

Results and discussion

In the last two decades efforts have been made to develop rapid and specific protocols enabling fish farmers to early detect fish pathogens and prevent potential disease outbreaks (Romalde et al., 1999; del Cerro et al., 2002; Zappulli et al., 2005). Although these approaches proved to be appropriate for positive identification of the subspecies of *P. damselae*, none of them were able to perform direct simultaneous detection of both subspecies in water samples from natural or artificial systems, which limit their application in the process of disease outbreak control.

Barcoded pyrosequencing data analysis

The bacterial communities of water samples from Ria de Aveiro estuary and a RAS were thorough characterized by barcoded pyrosequencing (Data S4.1) priory evaluation of the newly developed RT-PCR and PCR-DGGE methodologies for *P. damselae* analyses. The pyrosequencing data analysis showed a total of 173 OTUs for the estuarine samples and 458 OTUs (SolBio) and 582 OTUs (TurBio) for the aquaculture samples, indicating that both environments were colonized by a diverse range of bacterial species. The most abundant OTUs in the aquaculture sample were assigned to *Pseudoalteromonas* (OTU 7) and *Thalassomonas* (OTU 497). OTUs 3, 5 and 310 were the most abundant in the estuarine sample, and were classified as an unknown member of the family *Rhodobacteraceae* (Data S4.2). The majority of the dominant OTUs in the estuarine sample did not appear in the aquaculture samples, and vice versa. Concerning the fish pathogen *P. damselae,* the pyrosequencing analysis could only detect sequence reads related to this species in the TurBio samples (Accession number SRP026529) (Martins et al., 2013). The pyrosequencing data provided valuable base line information about the bacterial community composition of environmental water samples used to evaluate the molecular methodology developed in this study.

Real-time PCR analysis

The ToxRn1-F and ToxRn1-R primer set developed here, proved to be successful to detect and quantify *P. damselae* species with RT-PCR (Fig. 4.1). The standard curves were linear (R^2=0.98) over the range of 10^6 to 10^1 target gene copies per µL and the overall amplification efficiency was 90%. The detection limit, corresponding to the smallest serial dilution with

detectable levels of PCR amplification, was about 1×10^3 target molecules per µL (0.40 fg). Interestingly, in contrast to the pyrosequencing results, our RT-PCR approach for *P. damselae toxR* gene was able to detect the species *P. damselae* in both TurBio and SolBio samples, indicating the presence of *P. damselae* species in all RAS samples studied. The number of *toxR* gene copies present in the aquaculture samples was 2976.68 ± 1253.63 (ca 1.76 fg) for TurBio and 1797.85 ± 376.15 (ca 0.71 fg) for SolBio. In agreement with the pyrosequencing results, RT-PCR analysis did not detect *P. damselae* species in the estuarine samples. The low abundance of *P. damselae* detected here is in agreement with our previous study where we showed that a healthy RAS comprises a diverse range of bacterial fish pathogens with very low relative abundance when compared with the overall RAS microbial community (Martins et al., 2013).

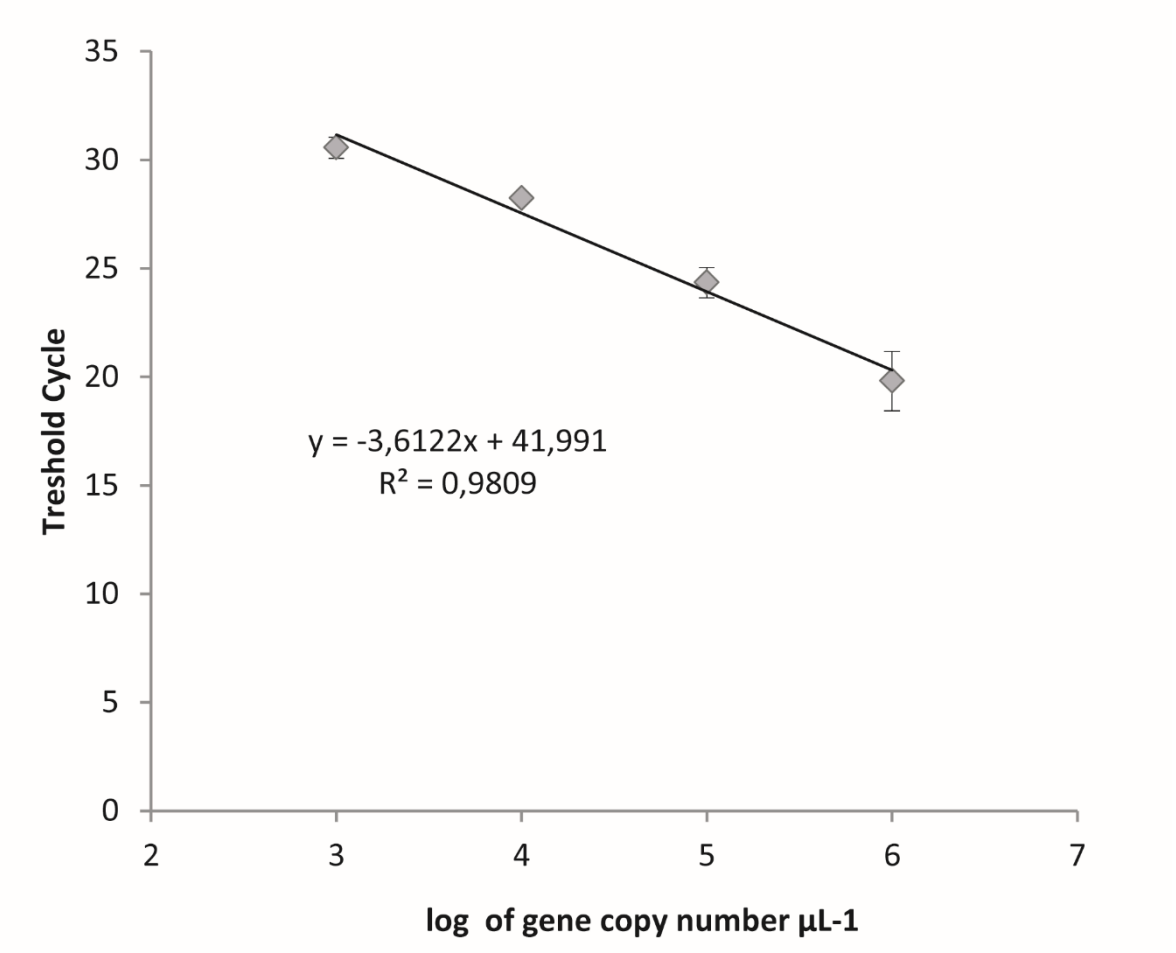

Figure 4.1. Standard curve for target *toxR* gene. The symbols represent means±SD of triplicates of PCR amplifications. The line is the trend line of linear regression.

DGGE analysis of *toxR* gene fragments and sequence analysis

Here we used a DGGE method to differentiate PCR amplicons obtained from *P. damselae toxR* gene (nested PCR) for fast detection of different subspecies of *P. damselae damselae* and *P. damselae piscicida* (detect different *P. damselae toxR* genotypes).

The DGGE loaded with PCR product (*P. damselae toxR* gene nested PCR) obtained from reference strains of *P. damselae damselae* and *P. damselae piscicida* showed one main single band for each subspecies at different positions (Fig. 4.2). The PCR products of the reference strains *P. damselae damselae* and *P. damselae piscicida* were always used in the DGGE as a reference for their band position (DGGE band type).

The specificity of the nested PCR-DGGE to detect *P. damselae* subspecies was evaluated with estuarine and RAS samples (Fig. 4.2). In agreement with the RT-PCR analysis, while no PCR product was detected for estuarine samples, all aquaculture samples were positive for *P. damselae* (data not shown). The DGGE analysis of these amplicons showed two bands, one besides the reference strain *P. damselae piscicida* (DGGE band 4) and other slightly above (DGGE band 3) (Fig. 4.2). The bands detected were excised from the DGGE gel and sequenced for further analysis. These results suggest that the PCR-DGGE approach for *P. damselae* subspecies developed in this study was able to detect two different *P. damselae toxR* genotypes in TurBio and SolBio aquaculture samples.

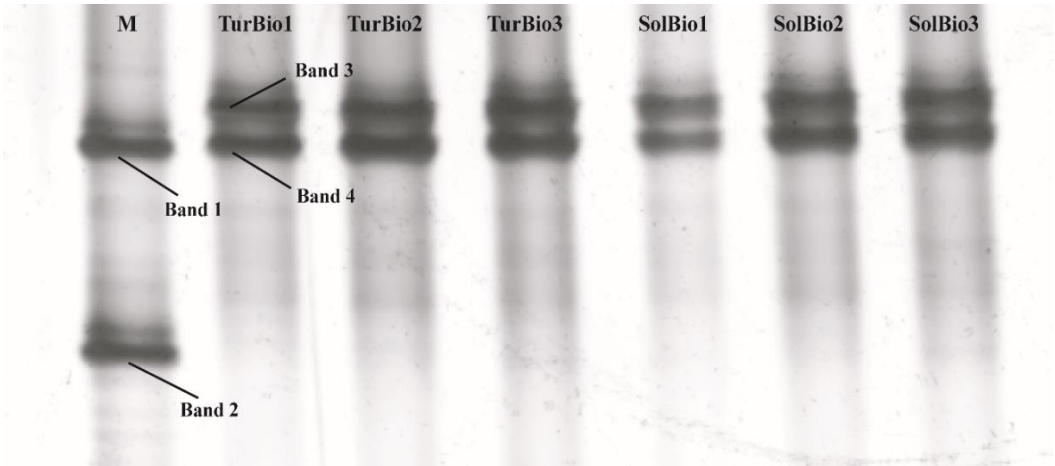

Figure 4.2. Denaturing gradient gel electrophoresis (DGGE) fingerprint of the aquaculture samples (TurBio - turbot RAS biofilter tank and SolBio - sole RAS biofilter tank). M: DGGE marker constructed using both reference strains (Band 1 - *P. damselae piscicida* – GenBank acc. no. AB364559 and Band 2 - *P. damselae damselae* - *GenBank* acc. no. AB364543). Band 3 and Band 4: identified as *P. damselae piscicida*.

The BLASTN algorithm was used to assess the similarities of *toxR* gene sequences obtained from excised DGGE bands with sequences available in the GenBank database. This analysis showed that sequences from both DGGE bands had a 99% similarity to *P. damselae piscicida* strain NCIMB 2058 [GenBank accession number (acc.) AB364559]. This bacterium is a reference strain from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland) isolated from yellowtail (*Seriola queradiata*) which was identified as causative agent of pseudotuberculosis in Japan. Strain NCIMB 2058 was first identified as *Pasteurella piscicida*, but later based on morphological, biochemical and phylogenetic analyses was renamed *Photobacterium damselae* subsp. *piscicida* (Gauthier et al., 1995). The gene *toxR* that encodes for the transmembrane transcription regulator, was first described in *Vibrio cholera* (Miller et al., 1987) and later reported in other bacteria (Welch and Bartlett, 1998; Osorio and Klose, 2000). Although this gene appears to be well conserved among *Vibrio* species, it presents highly divergent regions that could be useful for identification of this species (Kim et al., 1999; Osorio and Klose, 2000; Conejero and Hedreyda, 2003). Several studies indicate *toxR* gene as an ancestral gene of the *Vibrionaceae* family (Osorio and Klose, 2000; Conejero and Hedreyda, 2003). Osorio and Klose (2000) showed that this gene could also be useful for the distinction of *Photobacterium* species. According to Osorio and Klose (2000), *P. damselae damselae* and *P. damselae piscicida* sequences were 100% similar using the 16S rRNA marker gene while partial *toxR* gene fragments showed only 91% similarity.

The phylogenetic tree of *toxR* gene sequences detected in RAS samples and their closest relatives is presented in Fig. 4.3. The high bootstrap values obtained in each branch validate the phylogenetic clusters and point out the high resolution power of this gene to distinguish the *P. damselae* subspecies.

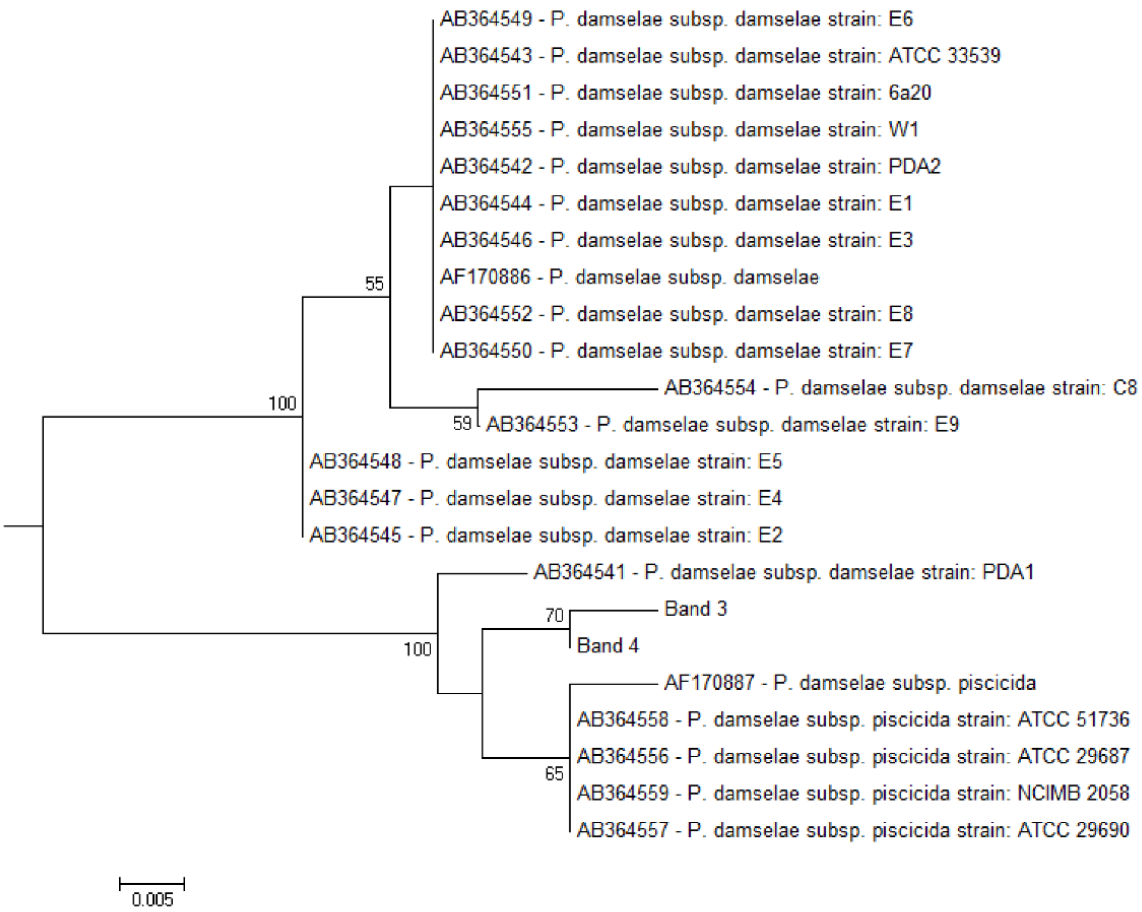

Figure 4.3. Maximum Likelihood phylogenetic tree (16S rRNA gene sequences) containing all *toxR* sequences from *P. damselae* available at GenBank database (with their accession number) and *toxR* sequences from the excised DGGE bands (band 3 and band 4). Bootstrap values generated from 1,000 replicates. Values lower than 50% were omitted.

Overall, the *toxR* gene sequences formed two monophyletic clusters, one containing subspecies *P. damselae damselae* and other containing mainly subspecies *P. damselae piscicida*, with exception of *P. damselae damselae* PDA-1. Curiously, the strain *P. damselae damselae* PDA1 was clustered apart in the main *P. damselae piscicida* branch. This result suggests that *toxR* gene of different members of *P. damselae damselae* may also form a phylogenetic cluster with *P. damselae piscicida.* However, up to now, only *P. damselae damselae* PDA1 isolated from a clinical case (fatal necrotizing fasciitis in human) (Yamane et al., 2004) showed a *toxR* gene phylogenetically related to *P. damselae piscicida* apart from

the main *P. damselae damselae* branch. This trend has to be confirmed in the future after sequencing *toxR* genes of more isolates.

Conclusion

The molecular methodology developed in this study showed good specificity for *P. damselae* quantification (RT-PCR) and subspecies detection (PCR-DGGE) in estuarine and aquaculture samples. Quantification of *P. damselae* can be important to improve our knowledge about the pathogen growth dynamic and infection. For example in this study the RT-PCR provided evidences that *P. damselae* was present in low abundance in all aquaculture samples analyzed, however, no disease symptoms or fish death were observed. In our previous study we also showed that despite the presence of a range potential fish pathogens, no symptomatic fish were observed during the study period (Martins et al., 2013). Thus, it is important to know the basal levels of the pathogen, i.e, levels wherein the presence of the pathogen does not affect the health of the fishes. This provides fundamental information to monitoring fish disease in aquaculture systems, and can also contribute to develop the best strategy to control aquaculture pathogens and the risks associated with disease outbreak. However, after the first signs of aquaculture infection with *P. damselae* and/or when an outbreak is already occurring, an important concern is to detect the pathogen responsible for the disease and not only pathogen quantification. In this case, our PCR-DGGE approach can provide information on the presence of *P. damselae* subspecies and their inter-subspecies variability based on their *toxR* genotypes. This approach can be important to monitor the incidence rate of a specific *P. damselae* subspecies and detect fish infection with multiple subspecies genotypes. Therefore, the information obtained with the molecular methodology developed in this study can contribute for a more effective long-term management of fish health and possible improvements in the epidemiological predictions.

Acknowledgements

This study has been carried out with the financial support of project AQUASAFE - Development of new technologies to anticipate and diagnose disease outbreaks in

aquaculture (PROMAR 31-03-05-FEP-0016) (PROMAR, a Portuguese instrument for the sectors of fisheries and aquaculture funded by the European Fisheries Fund). Patricia Martins and Francisco Coelho benefited from a PhD grant (SFRH/BD/73889/2010 and SFRH/BD/46322/2008, respectively) provided by the Portuguese FCT (Fundação para a Ciência e Tecnologia). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Supplementary material

Data S4.1. Taxonomic affiliation of the most abundant OTUs (>300 reads) in estuarine (Estuar) and aquaculture samples [turbot biofilter tank (TurBio), sole biofilter tank (SolBio)]. Reads indicates the number of sequences obtained for each OTU in estuarine sample (Estuar), turbot biofilter tank (TurBio), sole biofilter tank (SolBio).

OTU	Reads			Class	Order	Family	Genus
	TurBio	SolBio	Estuar				
3	0	0	774	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter
5	0	0	1086	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified
7	272	1137	0	Gammaproteobacteria	Vibrionales	Pseudoalteromonadaceae	Pseudoalteromonas
9	448	298	0	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Leucothrix
12	10	3	272	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
13	637	11	0	Deltaproteobacteria	PB19	Unclassified	Unclassified
18	72	328	0	Gammaproteobacteria	Oceanospirillales	Oleiphilaceae	Unclassified
21	0	0	376	Sphingobacteriia	Sphingobacteriales	Unclassified	Unclassified
33	308	39	0	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Kordia
37	0	0	266	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Unclassified
48	93	135	0	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Olleya
55	0	0	584	Flavobacteriia	Flavobacteriales	Cryomorphaceae	Unclassified
72	0	0	215	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified
174	2	0	291	Flavobacteriia	Flavobacteriales	Unclassified	Unclassified
272	185	26	14	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Polaribacter
278	0	0	202	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified
310	0	1	735	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified
320	139	102	0	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Leucothrix
337	73	142	0	Gammaproteobacteria	Alteromonadales	Colwelliaceae	Unclassified
444	0	0	303	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Unclassified
497	470	620	0	Gammaproteobacteria	Alteromonadales	Colwelliaceae	Thalassomonas
502	0	0	268	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Unclassified
721	0	0	232	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Octadecabacter

Chapter 5. Conclusions

Conclusions

The success of fish production in aquaculture systems depends on the maintenance of a healthy system. Microbial communities play important roles in several processes in these systems. Thus, knowledge on the 'standard' microbiome of aquaculture systems can improve our ability to understand and control the microbial quality of production systems and reduce the risks associated with disease outbreaks. The present study provides new insights and important information on the microbiology of two different aquaculture systems (semi-intensive and intensive) in Portugal.

The main conclusions of this study are synthesized below.

Chapter 2 - Molecular Analysis of Bacterial Communities and Detection of Potential Pathogens in a Recirculating Aquaculture System for *Scophthalmus maximus* and *Solea senegalensis*

The molecular analysis of bacterial communities of two different fish production in a recirculating aquaculture system showed clear differences between the two fish RAS and among the different compartments of each RAS. These results suggest a strong fish species specific effect on water bacterial communities.

We were able to detect some potential fish pathogens (*Serratia marcescens, Photobacterium damselae, Tenacibaculum discolor* and *Tenacibaculum soleae*) in sole and turbot cultures, however no symptomatic fish were observed during the study. The low relative abundance (low infective concentration) of these potential pathogens and the high abundance of populations close related to bacterial degraders of AHL (quorum quenching activity) found in this study could explain the absence of diseased animals.

Our results show that a combined DGGE and barcoded pyrosequencing approach provides a cost effective means for characterizing bacterial communities in RAS. Furthermore, the use of a high throughput sequencing approach using the 16S rRNA gene allowed us an unprecedented means to detect pathogens in the aquaculture systems studied.

Here, we provided fundamental information about the bacterial composition and pathogen load in a recirculating aquaculture system using molecular techniques.

Chapter 3 - Seasonal patterns of bacterioplankton in a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system

The seasonal patterns of bacterioplankton composition and putative function of a semi-intensive European seabass (*Dicentrarchus labrax*) aquaculture system were assessed using molecular techniques such as barcoded pyrosequencing and the bioinformatic tool PICRUSt. Our results revealed that seasonal variations in the bacterioplankton composition seems to be related to changes in the estuarine environmental parameters.

Using barcoded pyrosequencing we also detected seasonal changes in some potential fish pathogens. The predicted metagenome analysis showed high relative abundance of KOs in the subcategory "infectious diseases" during the beginning of autumn (September). Curiously, these results preceded a disease outbreak during the subsequent weeks (field observation). Overall, the use of bioinformatic tools such as PICRUSt to predict metagenome functional content contributed to a better understanding of microbial function in the aquaculture system.

This study provided fundamental knowledge on the temporal dynamics of bacterial communities present in a semi-intensive aquaculture system, which can help to improve management practices, fish health, quality and the safety of aquaculture fish production.

Chapter 4 - Development of a molecular methodology for fast detection of *Photobacterium damselae* subspecies in water samples

The molecular methodology developed in this study for fast detection of *Photobacterium damselae* consists of two phases. In the first phase we developed a real time PCR (RT-PCR) targeting the *toxR* gene for quantification and detection of *P. damselae* species, and in the second phase a nested PCR-DGGE was developed to determine the presence of different *P. damselae* subspecies in the positive samples. This methodology was evaluated directly against DNA samples extracted from environmental or aquaculture water samples.

P. damselae-specific primers targeting the *toxR* gene were developed to detect and quantify *P. damselae* species using a real time PCR (RT-PCR). Our RT-PCR detected between 1797.85 ± 376.15 (ca 0.71 fg) and 2976.68 ± 1253.63 (ca 1.76 fg) gene copy numbers of *P. damselae toxR* genes in the aquaculture samples. The detection limit, corresponding to the

smallest serial dilution with detectable levels of PCR amplification, was about 1×10^3 target molecules per μL (0.40 fg).

A nested PCR-denaturing gradient gel electrophoresis (PCR-DGGE) was used to distinguish the subspecies *P. damselae damselae* and *P. damselae piscicida.* The positions of the bands on the DGGE allowed us to identify different subspecies by comparing their migration pattern with known reference strains for *P. damselae damselae* and *P. damselae piscicida*.

The developed methodology proved to be a successful tool to detect *P. damselae* subspecies in estuarine and aquaculture water samples. The high sensitivity and specificity of this methodology to detect *P. damselae* can contribute for an early detection of these pathogens allowing an important management and control of disease outbreaks in the aquaculture systems.

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