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Dinâmica das comunidades procariotas estuarinas e o ciclo do Azoto

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Maria Rovisco Correia Gonçalves Monteiro

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Dynamic of estuarine prokaryotic communities and the Nitrogen cycle

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Catarina Maria Pinto Mora Pinto de Magalhães, Investigadora do CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental, e co-orientação do Doutor Newton Carlos Marcial Gomes, Investigador Principal do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho ao meu pai,
pela sua enorme força e rectidão de carácter
que fazem parte de mim.

o júri

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Palavras-chave

Estuário, Salinidade, Sedimentos, Bacteria, Archaea, Archaea oxidadoras de amónia,16s rRNA, *amoA*, Nitrificação

Resumo

Estuários são ecossistemas aquáticos altamente dinâmicos, possuindo grandes gradientes físicos e químicos, como é o caso da salinidade, influenciando as comunidades microbianas em termos de diversidade e abundância. A análise das respostas e adaptações destas comunidades às flutuações ambientais torna-se essencial para a compreensão dos ciclos biogeoquímicos que regulam estes ecossistemas, que tem vindo nos últimos anos a sofrer pressões ambientais devido à crescente atividade antropogénica. Neste estudo, investigámos a dinâmica da diversidade de Archaea e Bacteria ao longo de um gradiente de salinidade no estuário do Rio Douro (NW, Portugal). As amostras foram recolhidas em quatro locais cobrindo um gradiente de salinidade que variou entre 4.9 - 21.7 ppt. A aplicação da técnica de electroforese em gel com gradiente desnaturante (DGGE), revelou uma variação na diversidade de Bacteria e Archaea ao longo do gradiente salino. A diversidade das comunidades de Archaea com a capacidade de oxidar a amónia (AOA) foi também avaliada através da análise de diversidade do gene funcional *amoA*. Paralelamente, foram avaliados os fluxos líquidos dos compostos de azoto inorgânico (NH_4^+, NO_3^-, NO_2^-) bem como as taxas de nitrificação através da utilização do método do acetileno e da análise isotópica de ^{15}N. Os resultados mostraram que apesar de ter ocorrido um aumento da diversidade das AOA com a diminuição da salinidade, as maiores magnitudes das taxas de nitrificação foram registadas nos locais com salinidades intermédias, onde se registou maior disponibilidade de NH_4^+. Este estudo permitiu-nos obter importantes conhecimentos sobre o efeito da salinidade na estrutura das comunidades procariotas estuarinas bem como na dinâmica de processos chave do ciclo do azoto.

Keywords

Estuary, Salinity, Sediments, Bacteria, Archaea, Ammonia Oxidizing Archaea,16s rRNA, *amoA*, Nitrification

Abstract

Estuaries are highly dynamic aquatic systems, having steep physical and chemical gradients, such as salinity, influencing microbial communities in terms of their abundance and diversity. The analysis of microbial responses and adaptations to those environmental fluctuations became essential to understand the biogeochemical cycles that regulate these ecosystems, which have been undergoing progressive anthropogenic pressures. In this study, we investigated the dynamics of Archaea and Bacteria diversity along the salinity gradient of the Douro River estuary (NW Portugal). Samples were collected at four locations covering the salinity gradient, ranging from 4.9 - 21.7 ppt. The application of denaturing gradient gel electrophoresis (DGGE) showed a variation of Bacteria and Archaea diversity along the salinity gradient. The diversity of ammonia oxidizing Archaea (AOA) was also assessed by the analysis of *amoA* diversity. Simultaneously, were measured net fluxes of inorganic nitrogen (NH_4^+, NO_3^-, NO_2^-) and nitrification rates by using acetylene and ^{15}N isotope analysis. The results showed that although there was an increase in the diversity of AOA with the decrease of salinity, the highest magnitudes of nitrification rates were registered at intermediary saline sites, where there was a higher availability of NH_4^+. This study revealed important insights on the effect of salinity on estuarine prokaryotic diversity structure as well on the dynamics of key processes of the nitrogen cycle.

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ABREVIATIONS

AOA Ammonia Oxidizing Archaea

AOB Ammonia Oxidizing Bacteria

amoA Ammonia monooxygenase, subunit A

AOM Ammonia Oxidizing Microorganism

ATU Allythiourea

DAPI 4',6-diamidino-2-phenylindole

DGGE Denaturing Gradient Gel Electrophoresis

HCA Hierarchical Cluster Analysis

IRMS Isotope ratio-mass spectrometry

MDS Multidimensional Data Scaling

OM Organic matter

PCA Principal Component Analysis

PTIO 2 Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide

RDA Redundancy Analysis

TCC Total Cell Counts

LIST OF PAPERS

The elaboration of this dissertation benefited from the following submitted scientific paper and additional conference presentation with a relevant contribution:

Magalhães C. and Monteiro M. (2013).The history of aerobic ammonia oxidizers: from the first discoveries to today. Submitted to *Archives of Microbiology*

Monteiro, M., Santoro A., Gomes N.C.M. and Magalhães C. Shifts in sediment prokaryotic community structure and function along an estuarine gradient and its role on nitrification process. First EMBO Conference on Aquatic Microbial Ecology: SAME 13, Stresa, Italy, 8-13 September 2013.

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CHAPTER 1 – INTRODUCTION

“In nature, nothing is created, nothing is lost, everything changes”
Antoine Lavoisier

1.1– ENVIRONMENTAL MICROBIOLOGY OVERVIEW

When the word “ecology” appeared for the first time, in 1866 by the ecologist Ernst Haeckel, the idea of Earth functioning only through the application of only physical and chemical laws became outdated, and the activity of living beings started to be linked with the surrounded environment. This was an important step in the history of science because since then it is common knowledge that living beings are permanently exchanging fluxes of energy and chemical elements with the environment. Most of these fluxes act in a cyclic way, called the biogeochemical cycles, describing the movement of crucial substances for life through the ecosystem, such as: Carbon, Oxygen, Nitrogen, Phosphorous and Sulfur. Making the analogy, they can be compared to a recycling system of Earth matter, being most of the times supported by microbial activity.

Prokaryotes (Bacteria and Archaea) are the most ubiquitous and simple forms of life. Yet, they catalyze unique and indispensable transformations in nature, representing a large portion of life genetic diversity, occupying from the most extreme to the most mesophilic environments (Whitman et al., 1998). Even so, although being so diverse, they are poorly known and the main reason is because of their invisible size for our eyes, being only recognized in the XVII century by the invention of the microscope by Leeuwenhoek, “the father of Microbiology”.

Because in the earliest times, microscope technology was very rudimentary, only simple descriptions of microbial morphologies could be done (Neff, 1956; Ford, 1981). At that time the development of microbiology as a science was dependent on two main steps: the improvement of microscopic technologies and the development of laboratory methodologies, like culture methods. Soon, the knowledge about microbial diversity increased with the emergence of methodologies for cultivating microbes. Martinus Beijerick pioneered those practices, starting to isolate many aquatic and soil microorganisms into enrichment cultures (Beijerick, 1901). At the same time, Sergei Winogradsky (1890) ascertained the autotrophic mode of life to Bacteria, being the first to propose the term *chemiolitotrophy*, the oxidation of inorganic compounds for energy purposes (Winogradsky, 1890).

With the development of pure-culture techniques, microbial organisms started to be studied as individual types and characterized with some accuracy (mainly by nutritional criteria). Although those techniques greatly improved our knowledge, they were found to be very limited for the understanding of the natural and complex microbial assemblages. In 1969, Whitakker reorganized organisms into the “Five Kingdoms” of life: animals, plants, fungi, protists (“protozoa”), and monera (Bacteria) (Whitakker, 1969). The distinction between them was mainly based on nutrition requirements and form of life (uni or multicelular), which were not proper for the establishment of taxonomic and evolutionary relationships (Pace, 1997).

The relationship of organisms in phylogenetic terms was only possible after the discovery of DNA structure (Watson and Crick, 1953) and the genetic code (Nirenberg et al., 1966). Those discoveries triggered the emergence of new molecular tools (for example the development of sequence-based methods - Sanger sequencing), which quickly became of great importance in Science. Appreciating ecology and evolution, Woese and Fox (1977) built a phylogenetic tree which could relate all organisms and established the history of life. They designated three domains (or *urkingdoms*) of Life: Eucarya (eukaryotes), Bacteria and Archaea (or *ArchaeBacteria*, how it was initially called) (Figure 1). To build this tree several 16S/18S rRNA sequences from a diversity of living beings were aligned and their nucleotide sequences compared (Woese and Fox, 1977). The nucleotide differences were used as a measurement for evolutionary distance between the different domains, and since only the change in the nucleotide sequence was considered, similar sequences were representative of closer organisms. This interpretation considered this tree as a rough map of the evolution of the living beings (Pace, 1997).

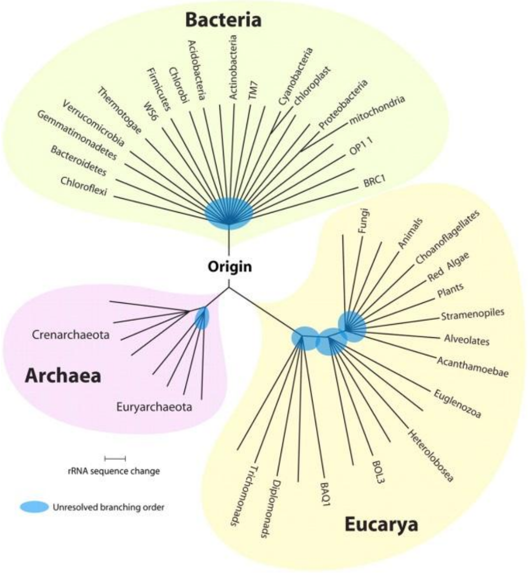

Bacteria
Chloroflexi
Bacteroidetes
Gemmatimonadetes
Verrucomicrobia
Thermotogae
WS6
Firmicutes
Chlorobi
Acidobacteria
Actinobacteria
TM7
Cyanobacteria
chloroplast
Proteobacteria
mitochondria
OP11
BRC1
Origin
Crenarchaeota
Archaea
Euryarchaeota
rRNA sequence change
Unresolved branching order
Fungi
Animals
Choanoflagellates
Red Algae
Plants
Stramenopiles
Alveolates
Acanthamoebae
Euglenozoa
Heterolobosea
BOL3
BAQ1
Diplomonads
Trichomonads
Eucarya

Figure 1 - The molecular tree of life. The diagram compiles the results of many rRNA sequence comparisons (Pace, 2009).

Still, the knowledge of microorganisms was very dependent on their culture and if we consider that in an environmental sample more than 99% of organisms seen microscopically are not cultivated by routine techniques (Table 1) (Amman et al., 1995), those traditional methods seriously constrained the view of natural microbial diversity.

The introduction of culture-independent methods to study microbial communities allowed to start doing representative surveys of microbial diversity in the environment and scientists became aware of how rudimentary their knowledge was about natural microbial world. Those innovative technologies were first introduced in environmental ecology by Pace and colleagues, in 1984, by analyzing ribosomal genes - 16S rDNA - recovered from nucleic acids extraction directly from the environment. Those genes are suitable for genetic comparisons because of their ubiquitous distribution, high conservation, and by the possession of some hyper-variable zones capable to distinguish different microorganisms (Stahl et al., 1984). Besides, 16S rRNA makes part of protein synthesis machinery, having an essential function conserved across all prokaryotes and is not involved in horizontal gene transference, making it the most commonly used phylogenetic marker until now (DeLong and Pace, 2001). This approach has led to the discovery of novel microbial *taxa,* suggesting that most of life forms inhabiting Earth are still undiscovered (DeLong and Pace, 2001).

Table 1 - Culturability determined as a percentage of cultivable Bacteria in comparison with total cell counts (Amman et al., 1995).

Habitats	Culturability (%)*
Seawater	0.001 – 0.1
Freshwater	0.25
Mesotrophic lake	0.1 – 1
Unpolluted estuarine waters	0.1 – 3
Activated sludge	1 – 15
Sediments	0.25
Soil	0.3

* Culturable Bacteria are measured as CFU (colony forming units).

The molecular-phylogenetic perspective becomes an important view to organize microbial diversity. However, not only the phylogeny but also the information about the metabolic community patterns could be retrieved by genome sequencing. In fact, the improvement of molecular tools able us to target microorganisms, not only by their phylogenetic genes, but also by their functional genes (Pace, 1997). Thus, molecular biology also provided a framework from which we can look to the metabolic machineries within a microbial community, connecting the microbial composition of a community with their function in the ecosystem. Indeed, after scientists start to get results from environment data, there was an enormous improvement in the knowledge of environmental microbiology. Not only the nucleic acid data, but also the information about functional genes that are being expressed and the proteins itself, which therefore are being produced, have the potential to provide the genotypic and phenotypic traits of the community members linking their physiology to their function and evolution (Willms and Bond, 2009).

The sequencing revolution, and more recently with the implementation of next-generation sequencing methods in microbial ecology studies (Mardis, 2008), doors were opened to the vast information contained in the molecular sequences. Millions of sequences are being compared and introduced in web databases every day, unraveling the microbial world. Starting with 16S rRNA to the sequencing of the whole community genome, microbiology has getting unprecedented insights about the genetic and physiological dynamics of complex microbial assemblages (Wilmes and Bond, 2009).

1.2 - BACTERIA PHYSIOLOGY AND DIVERSITY

Although the twelve major Bacterial divisions identified until 2001 (DeLong and Pace, 2001) still remains equal, most of the recent cultivation-independent molecular surveys have revealed that Bacterial domain consists of much more divisions (Rappé and Giovannoni, 2003). This approach led to the discovery of many novel microbial *taxa* and contrarily to what was expected, these newly recognized microbes are not minor players in the environment. They often represent major *taxa* in earth ecosystems (DeLong and Pace, 2001), suggesting that the phenotypic and physiologic properties of many of the most abundant microbes inhabiting Earth remain to be determined.

It is estimated that the number of prokaryotes and their biomass (calculated in respect to the total amount of their cellular carbon) on Earth are between 4–6 x 10^{30} cells and 350–550 Pg of carbon (1 Pg = 10^{15} g), respectively (Whitman et al., 1998) (Table 2). Besides their widespread distribution, they can be found in relative low species numbers in some environments as the atmosphere, glacial ice, and acidic stream waters, while in other environments like soils, microbial mats, marine water, soil subsurfaces, human body and sewage, they can be found in very high numbers (Whitman et al., 1998; Fierer and Lennon, 2011).

Table 2 - Number and biomass of prokaryotes in the world (1 Pg = 1015g) (Whitman et al., 1998).

Environment	No of prokaryotic cell, x 10^{28}	Pg of C in Prokaryotes
Aquatic habitats	12	2.2
Ocean subsurface	355	303
Soil	26	26
Terrestrial subsurface	25 – 250	22 - 215
Total	415 – 640	353 - 546

Their high taxonomic diversity and abundances is mainly justified by their underlying metabolic diversity. Some are autotrophic having a photosynthetic metabolism, while others use inorganic compounds like H_2S, Fe^{2+} or NH^{3+} and "alternate" electron acceptors (e.g. NO_3^-, Fe^{3+}, Mn^{4+}, $SO4^{3-}$, or CO_2 instead of O_2) to produce energy. Others, have a heterotrophic metabolism or are able to switch their metabolism (called mixotrophs) enabling them to survive under rapid environmental changes. Their capacity to establish symbiotic interactions with other organisms (ex: plants, corals, animals) also gave them better chances of survival (Marinelli and Waldbusser, 2005). However, despite this metabolic variety, it remains unclear if such specialization, alone, can explain such coexistence around the world. Other Bacterial feature is their fast generation time and easy capacity of mutation (Kassen and Rainey, 2004). Slowly growing populations may have higher generation times and fewer mutational events comparing to rapidly growing populations. Transposing to the environment, the highest cellular productivity is found in the open ocean, so mutational events are more likely to occur in marine population then in others populations (Whitman et al., 1998). In addition, their capacity of lateral gene transference achieved by transformation (the uptake of extracellular DNA), conjugation (the swapping of plasmids between cells) or transduction (virus-mediated movement of genes among cells) is a good weapon to gain, for example, many resistance factors, introducing novel traits into distantly related taxa and opening doors for new species interactions (Boucher et al., 2003). Another interesting Bacterial feature is their dormancy strategy. When an environment becomes not favorable for some Bacteria, they can enter into a reversible state of reduced metabolic activity, allowing them to persist in the unfavorable conditions until a better one appears (Jones and Lennon, 2010).

All those features announced here help us to understand the Bacterial way of life, how did they evolved and survived to ecological changes and even to environmental disasters that occurred on Earth since the formation of Life. However, we believe that this is just the beginning, not only in respect to the fewer number of microorganism described but to the type of environments that are still unexplored and for sure harbor an unique Bacterial world.

1.3 - ARCHAEA PHYSIOLOGY AND DIVERSITY

Archaea were described for the first time by Woese in 1977, and although prokaryotic in cellular ultra-structure, they are evolutionarily distant from Bacteria.

They have a small size, with coccus or bacillus shape, holding one or more flagella. Like Bacteria their nucleus is not organized and their DNA exists in a single loop (plasmid). However they do have histones that compact DNA into structures that resembles the nucleosomes of Eukaryotes (Reeve et al., 1997). The translation process shows similarities to both eukaryote and Bacterial translation: besides using Shine-Delgarno sequences for the initiation of translation the initiation factors are homologous to the eukaryote ones (Baumann et al., 1995). Another interesting feature is their cell wall structure, distinct from Bacteria and eukaryotes, composed by a polysaccharide quite similar to peptidoglican – pseudopeptedoglican – which is formed by repetitive and alternate units of N-acetilglicosamid and N-acetiltalosaminuronic (instead of N-acetilmuramic of Bacterial peptidoglican). Instead of β-1,3glicosidic links, the skeleton of pseudopeptidolglican is assured by β-1,4 links. Their membrane is predominantly constituted by isoprenoid glycerol diethers or diglycerol tetraethers, which was another feature, used by Woese,for the establishment of this domain (Woese et al., 1990). While hydrogen-based energy metabolism is very common within this domain, other metabolic pathways were already identified, many of them with unique enzymatic systems; for example the enzymatic complex of the first step of ammonia oxidation (Vajrala et al., 2013).

During many years, Archaea were known as extremophiles, being always associated to the most extreme environments. However, with the progressive investigations, rapidly it was demonstrated that these organism are worldwide distributed across different ecosystems including the ocean open waters (DeLong, 2005; Santoro et al., 2010), sediments (Abreu et al., 2001; Magalhães et al., 2009), solid gas hydrates, tidal flat sediments, freshwater lakes, soil, plant roots, peatlands, petroleum-contaminated aquifers and even in the human mouth and gut (Robertson et al., 2005). In fact, the discovery of this widespread diversity in such mesophilic habitats is one of the particularly striking findings of culture-independent surveys, revealing a high prevalence of Archaea organisms in some environments. In addition, a specific symbiotic association with marine sponges indicates that those microorganisms have radiated into associations with metazoan hosts (Preston et al., 1996). All these investigations strongly suggest that the newly detected Archaea are active, dynamic, and likely to have marked impacts and interactions with surrounding habitats and biota (DeLong and Pace, 2001).

In the early phylogenetic tree, the Archaea domain fell into two distinct groups— the Euryarchaeota and the Crenarchaeota, - based on cultured organisms information in the late 70s (using less than twenty 16S rRNA sequences). Crenarchaeotes included hyperthermophiles organisms whereas Euryarchaeotes, included the methanogens, halophiles and termophiles (Woese et al., 1990). Later, using a higher number of rRNA sequences retrieved from natural samples, a new phylogenetic tree was produced. Besides the two-branch form has not changed, new groups appeared and older ones were replaced or disappeared (Robertson et al., 2005). Very recently an idea for restructuring Archaeal tree was proposed by considering a third Archaeal phylum, the Thaumarchaeota (Brochier-Armanet et al., 2008, 2011). Such decision was based on the analysis of ribosomal protein markers of the *Cenarchaeum symbiosum* genome (Brochier-Armanet et al., 2008). Higher analytical resolution of ribosomal proteins indicated a new phylogenetic position for *C. symbiosum* and its mesophilic Crenarchaeota relatives, in a robust branch located before the separation between Euryarchaeota and Crenarchaeota (Brochier-Armanet et al., 2008).

The rate of Archaeal sequences submission to public sequence databases had increased dramatically in the recent years. Those advances have largely been fueled by the advance of sequencing techniques which, along with Bacteria, have permitted the detection of Archaea in worldwide environments without cultivation. Indeed, sequencing of 16S rRNA genes from environmental samples has revolutionized scientists perception about microbial systematics and diversity, revealing how scarce is the knowledge about prokaryotic diversity. We know that their existence is driven by environmental conditions, so, integrating the information from environmental surveys with the biological data, we might start to get a perception of how easily microbes tolerate different kinds of environmental changes and in what manner did that contributed to microbial function, evolution and their survival.

1.4 - NITROGEN BIOGEOCHEMICAL CYCLE

The growth of all organisms depends on the availability of different resources, including mineral nutrients. The availability of those nutrients in nature is dependent of a multitude of reactions, mediated by a metabolically diverse range of autotrophic and heterotrophic organisms. In fact, microbial communities support the biogeochemical cycles and drive the nutrient cycles making the planet habitable for larger organisms. In this section, It will be specifically described the main pathways of nitrogen biogeochemical cycle and characterize the microbial communities involved in nitrogen (N) biogeochemistry.

Nitrogen is a very abundant element on Earth, however 94% of it is found on the Lithosphere (mainly on rocks in the mantle), and only 0.001 % is found in the Biosphere. Inside the Biosphere, only 0.04% is available for living beings (Rosswal, 1981) (Table 3). The low availability of N contradicts its high requirement by living organisms, once it is an essential component of their proteins and nucleic acids. Moreover, N is typically the nutrient that controls primary production in most temperate estuarine zones and other marine coastal ecosystems, being commonly implicated in the eutrophication and consequently degradation of coastal marine systems due to anthropogenic nitrogen enrichment (Galloway, 1998).

Table 3 - Nitrogen distribution on Earth (Rosswall, 1981).

	Amount of Nitrogen (Tg)	Proportion %
Lithosphere	574 x 10^8	93.8
Atmosphere	38x 10^8	6.2
Hydrosphere	0,23 x 10^8	0,04
Biosphere	0,009 x 10^8	0,001

Nitrogen cycle can be decomposed in several pathways of oxidation and reduction reactions (Figure 2). N fixation, is the process by which N is converted into ammonia (NH_3), being then able for organisms to use it and assimilate it. This step was first discovered by Beijerinck (1925) and is carried out by a limited group of organisms (*Azotobacter*, *Azospirillum*, *Rhizobium* and *Bradyrhizobium),* which holds a special enzyme, *nitrogenase,* that catalyzes the conversion of atmospheric nitrogen (N_2) into NH_3 (Postgate, 1982).

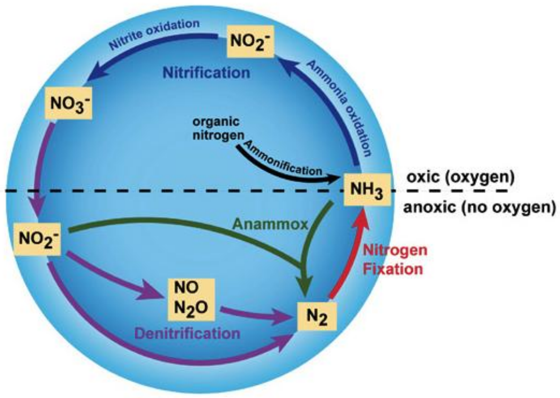


Figure 2 - Nitrogen Biogeochemical cycle (Bernhard, 2010).

The next step, Nitrification, can be divided in two pathways: the first where NH_4^+ (ammonium) is oxidized into nitrite (NO_2^-) and the second where NO_2^- is subsequently oxidized into nitrate (NO_3^-). Those two steps are carried out by two different types of microorganisms: the ammonia – oxidizers, which involve Bacteria and Archaea representatives (Konneke et al., 2005, Treusch et al., 2005) and nitrite-oxidizing Bacteria, which include the Bacteria genus like *Nitrospira*, *Nitrobacter*, *Nitrococcus*, and *Nitrospina* (Burrell et al., 1998).

Until recently, the ammonia oxidation step has been described as an aerobic process; however the discovery of an alternative way of ammonia oxidation under anoxic conditions, called Anammox (Van de Graaf et al., 1995), changed previous assumptions. Anammox is a process mediated by bacterial conversion of NH_4^+ and NO_2^- into N_2, with CO_2/bicarbonate as the carbon source (Jetten et al., 2009). This is an alternate pathway to denitrification for nitrogen loss to the atmosphere as N_2 gas (Jetten et al., 2009). While denitrification, the process of nitrate (NO_3^-) reduction to produce molecular nitrogen (N_2), is primarily performed by heterotrophic Bacteria, anammox is an autotrophic process carried out by Bacteria belonging to Planctomycetes phylum (*Brocadia anammoxidans)* (Kuenen, 2008). In an overall vision it can constitute a shortcut in N cycle, where nitrification and denitrification are linked at the level of $NO_2,^-$ without going through NO_3^- (Zehr and Ward, 2002).

During denitrification, several compounds are being produced, such as nitrous oxide (N_2O), a strong greenhouse gas that reacts with ozone accelerating climate change. This process is mainly mediated by Bacteria, although there are evidences that some eukaryotes can also do it (Risgaard-Petersen et al., 2006). This step has a large human and ecological contribution, acting like a two-edged knife: in one hand it is essential for removing the NO_3^- from the ecosystem returning it to the atmosphere, being particularly useful to remove it from wastewater effluents, reducing the chances for undesirable consequences like algae blooms. On the other hand, it can be harmful, mainly for agricultural activities, once the loss of NO_3^- in fertilizers is detrimental and costly.

The anthropogenic influence on the N cycle is greater than that on any other biogeochemical cycle. It began with the industrial revolution in the 90s, with the production of nitrogenous fertilizers by the industrial process known as the Haber–Bosch process. The massive acceleration of the N cycle triggered a massive production and agricultural usage of artificial N fertilizers, enabling humankind to increase food production, supporting the population increase at a global scale over the past century. As a consequence, total N loading to the global landmass has nearly doubled since the pre-industrial era, from about 111 Tg yr^{-1} to 223 Tg yr^{-1}, all due to human activities (Frink et al., 1999), having necessarily negative consequences for the environment ranging from eutrophication of terrestrial and aquatic systems to stratospheric ozone loss (Gruber and Gallaway, 2008).

1.4.1 – Ammonia-oxidizing step of nitrification pathway

In 1890, with the beginning of environmental microbiology, Sergei Winogradsky identified for the first time, nitrifying Bacteria, isolating ammonia oxidizers from soil and showing to scientific knowledge that this process was mediated by Bacteria. At that time he proposed for the first time the chemoautotrophic concept, as the mode of life of this Bacteria group (Winogradsky, 1890). Several Bacteria started to be isolated including *Nitrosomonas europaea,* a very well-known ammonia oxidizing Bacteria, collected from Swiss and French soils (Omeliansky, 1899; Winogradsky, 1904) which became the most studied ammonia-oxidizing specie. It was the first and most important Bacterial model organism for ammonia oxidation studies being its physiology, biochemistry and phylogeny very well characterized (Clark and Schmidt, 1967; Ritchie and Nicholas, 1974; Abeliovich and Vonsahk, 1992; McTavish et al., 1993; Beaumont et al., 2002).

In 1933, with the advances on molecular culture-independent tools, Head and collaborators started to use those approaches to study nitrifying communities phylogeny based on 16S rRNA analysis (Head et al., 1993). With the development of specific primers to amplify environmental ammonia oxidizing Bacteria (AOB) sequences, their distribution, phylogeny and diversity started to be reported. The first reports described AOB in water samples from an estuarine system (Stehr et al., 1995), from Lake Bonney located in Antarctica and from Santa Monica Basin (Voytek and Ward, 1995) and then in a wide range environments (Bano and Hollibaugh, 2000; Burrell et al., 2001; Kowalchuk and Stephen, 2001; Hollibaugh and Ducklow, 2002; Freitag and Prosser, 2003; Bock and Wagner, 2006). Those studies have brought many data, which triggered even more the study of these new organisms function within the N cycle (Zehr and Ward, 2002).

At this stage the necessity of combining biogeochemical studies with functional molecular markers, by integrating disciplines and methodologies became a priority to fully understand the ecosystem-level importance of the different species responsible for ammonia oxidation. The increasing interest in linking nitrification with the diversity of microorganisms encouraged the use of probes based on enzymes or genes directly involved in ammonia oxidation (Gieseke et al., 2001; Francis et al., 2003; O'Mullan and Ward, 2005; Bock and Wagner, 2006). All AOM (ammonia-oxidizing microorganisms) possesses a crucial enzyme – AMO, ammonia monooxigenase – which is responsible for the conversion of NH_4^+ into hydroxylamine (NH_2OH), an intermediate metabolite between ammonia oxidation and NO_2^- production (Hollocher et al., 1981). The gene is composed by three subunities (A, B and C), being the first subunit, AMO-A, sequenced in 1993 by McTavish and detected in environmental samples by Sinigalliano et al. (1995).

AOB quantification techniques emerged with the first attempts of direct quantification performed by fluorescence *in situ* hybridization (FISH) (Schramm, 2003). FISH results revealed that, unexpectedly, AOB numbers represented only a very small fraction (0.1 – 2%) of the total microscopic Bacterial counts (Schramm, 2003; Urakawa et al., 2006). Subsequently, real-time PCR technique proved to be a highly sensitive method for enumerating the relatively low AOB numbers in natural environments, becoming a widely used method to analyze AOB *amoA* gene copy numbers in different locations (Harms et al., 2003; Limpiyakorn et al., 2005). However, these quantification studies reinforced the still unanswered question of how a rare Bacterial group could be responsible for such a worldwide, and critical biogeochemical process as nitrification.

Just ten years after the detection of Bacterial *amoA* in environmental samples, the gene was again found but in a different prokaryotic domain. From a 1.2 Gb large-insert environmental fosmid library of soil samples, a 43kb genomic fragment was isolated with affiliation to group 1.1b of Crenarchaeota phylum within, Archaea domain, encoding two proteins related to subunits of ammonia monooxygenases (Treusch et al., 2005) (Figure 3). In parallel, these *amoA-like genes* were also identified in Sargasso Sea metagenomic database (Venter et al., 2004), suggesting that mesophilic terrestrial and marine Crenarchaeota might be capable of ammonia oxidation. Those studies were followed by the isolation of the first ammonia oxidizing MG-1 Archaea, from a seawater aquarium, denominated as *Nitrosopumilus maritimus* (Könneke et al., 2005).

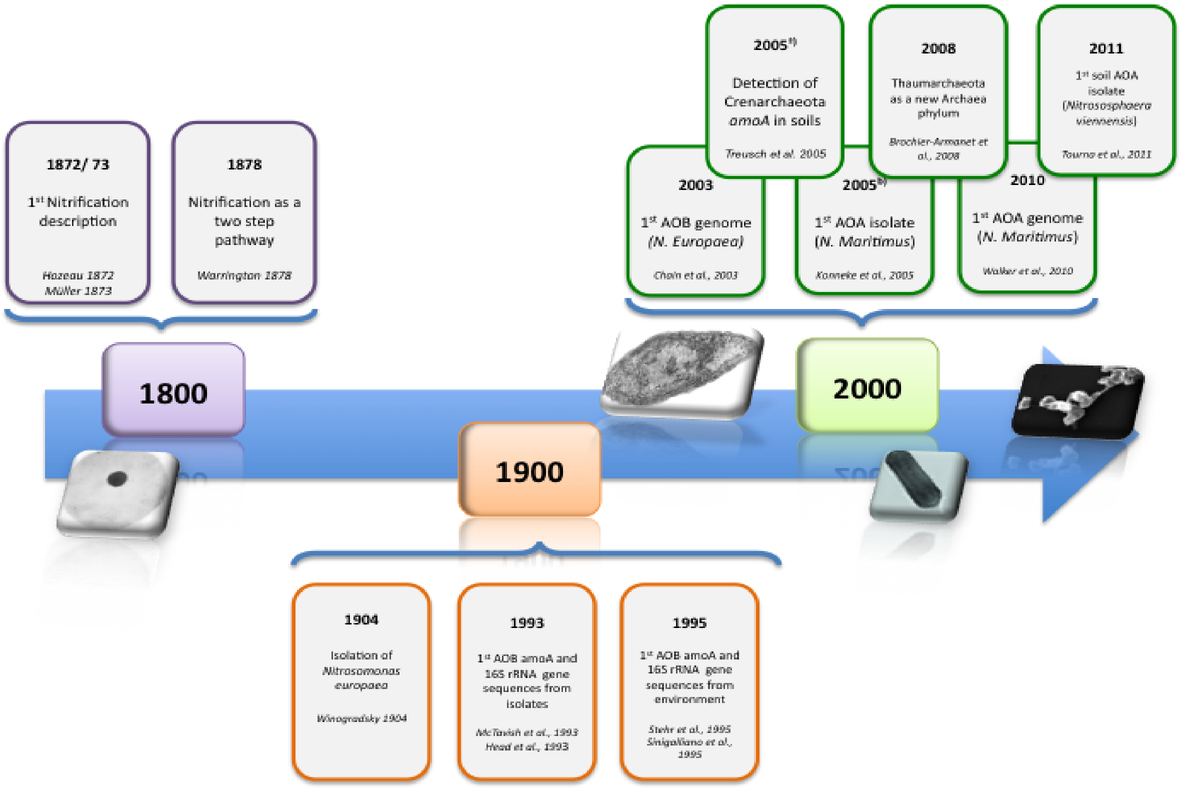

1872/ 73
1st Nitrification description
Hazeau 1872
Müller 1873
1878
Nitrification as a two step pathway
Warrington 1878
1800
1900
2000
1904
Isolation of Nitrosomonas europaea
Winogradsky 1904
1993
1st AOB amoA and 16S rRNA gene sequences from isolates
McTavish et al., 1993
Head et al., 1993
1995
1st AOB amoA and 16S rRNA gene sequences from environment
Stehr et al., 1995
Sinigalliano et al., 1995
2003
1st AOB genome (N. Europaea)
Chain et al., 2003
2005a)
Detection of Crenarchaeota amoA in soils
Treusch et al. 2005
2005b)
1st AOA isolate (N. Maritimus)
Konneke et al., 2005
2008
Thaumarchaeota as a new Archaea phylum
Brochier-Armanet et al., 2008
2010
1st AOA genome (N. Maritimus)
Walker et al., 2010
2011
1st soil AOA isolate (Nitrososphaera viennensis)
Tourna et al., 2011

Figure 3 - Historical time line with the important discoveries on nitrification. It highlights the discoveries made on aerobic ammonia oxidation and its respective microorganisms (Magalhães and Monteiro submitted to Archives of Microbiology).

This discovery sparked again the attention of scientific community for the biogeochemical N cycle, once the role of Archaea in this cycle could complete the scenery of nitrification step. The boom of studies triggered again the introduction of many *amoA* sequences in world databases revealing a surprisingly differentiation of those sequences between organism from different environments (Biller et al., 2012; Stahl and de la Torre, 2012) (Figure 4). In addition to *amoA* sequences, the sequence of new genomes obtained by enrichment cultures from agricultural soils, estuaries, sediments or even high temperature habitats or low pH ones defined new ammonia oxidizing Archaea (AOA) candidates: *"Nitrosoarchaeum koreensis", "Nitrosopumilus salaria", "Nitrosoarchaeum limnia,* and *Cenarchaeum symbiosum", "Nitrososphaera gargensis", "Nitrosocaldus yellowstonii", "Nitrosotalea devanaterra"* , among many others, expanding more our knowledge about this group. Very recently, was isolated the first representative of soil-inhabiting ammonia oxidizing Archaea, "Candidatus *Nitrososphaera viennensis* EN76", being widely distributed in soils (Tourna et al., 2011).

Those discoveries enhance the knowledge about the distribution of those new ammonia oxidizers. In fact they are spread among many different habitats just like natural and managed soils, marine and estuarine water sediments, wastewater treatment plant bioreactors, Antarctica soils, hot springs and in many other environments (Francis et al., 2005; Beman and Francis, 2006; Leininger et al., 2006; Park et al., 2006; Dang et al., 2008; Magalhães et al., 2009). The wide distribution of AOA raised questions about their relative contributions to the nitrification pathway in natural ecosystems (Hallam et al., 2006a; Leininger et al., 2006; Wuchter et al., 2006; Caffrey et al., 2007b; Mosier and Francis, 2008; Nicol et al., 2008; Santoro et al., 2008); Are AOA the main contributors to nitrification and not AOB? What environmental parameters drive their distribution and speciation? What controls the prevalence of one group over the other? In fact, higher numbers of AOA have been observed in many systems, with a general prevalence of AOA *amoA* copy numbers over *betaproteoBacteria* - AOB (Leininger et al., 2006; Wuchter et al., 2006; Nicol et al., 2008). However, in other particular systems, like agricultural soils, coastal and estuarine sediments, there were evidences of AOB *amoA* dominance (Mosier and Francis, 2008; Santoro et al., 2008; Jia and Conrad, 2009; Magalhães et al., 2009). Those findings are based on the quantification of *amoA*, but those abundances do not provide information about the relative contribution of AOA and AOB for ammonia oxidation, since the gene might not be expressed or its transcript or enzyme might be inactivated. The potential use of selective AOA and/or AOB inhibitors to distinguish AOA *vs* AOB activities has been shown, for example the use of inhibitors (PTIO, ATU (allythiourea) or Sulfathiazole) whose action might differentiate Archaeal from Bacterial nitrification (Martens-Habbena and Stahl, 2010; Santoro et al., 2010; Yan et al., 2012). However, the efficiency of those inhibitors in natural complex samples is questionable, since they were mainly tested in pure cultures and are still being developed.

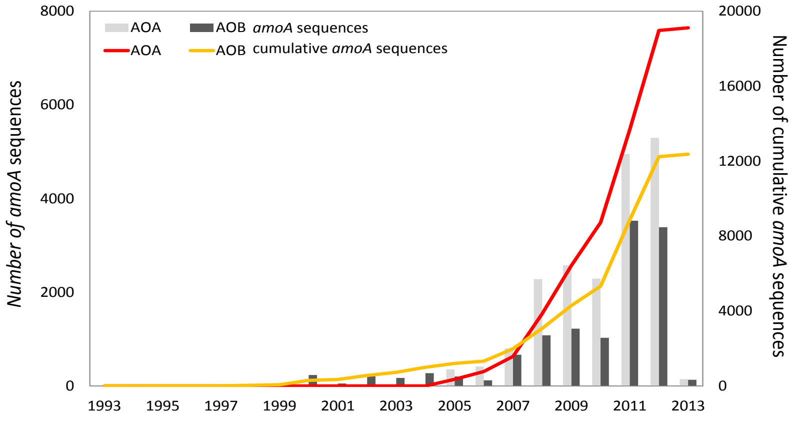


Figure 4 - Evolution of the number of Bacteria and Archaeal *amoA* sequences that have been submitted to the National Center for Biotechnology Information (NCBI) database. Topics used for the advance search were: ammoniaoxidizing Archaea or ammoniaoxidizing Bacteria (*all fields*), *amoA* (*gene name*) and Archaea or Bacteria (*organism*). (Magalhães and Monteiro, submitted to Archives of Microbiology).

1.4.2 - Ammonia oxidizing organism phylogeny and metabolism

Based on 16 S rRNA gene sequences, ammonia-oxidizing Bacteria (AOB) are divided into two monophyletic lineages: the first lineage belongs to the betaproteobacteria including the genus *Nitrosomonas* and *Nitrosospira*, whereas the second lineage is affiliated with the gammaproteobacteria contains species like *Nitrosococcus oceani* and *Nitrosococcus halophilus*. (Head et al., 1993; Teske et al., 1994; Purkhold et al., 2000). The main pitfall of the 16S rRNA gene as a molecular marker is that it is not necessarily related to the physiology of the target organisms (Kowalchuk and Stephen, 2001; Calvó et al., 2004), making the use of functional markers a better alternative for ecological studies. In all known AOB, the genes encoding the enzyme AMO belong to an operon with the structure *amo* CAB (Stein et al. 2000; Chain et al. 2003; Klotz et al. 2006). Two to three copies of the operon are present in the genomes of beta-AOB (Stein et al. 2000; Chain et al. 2003), whereas a single copy has been reported for gamma-AOB (Klotz et al., 2006). This enzyme (as it was mentioned before) catalyzes the oxidation of ammonia into hydroxylamine, which is subsequently oxidized by hydroxylamine oxidoreductase (*hao*) into NO_2^-.

As mention above, in the last few years the evolutionary relationships between Archaeal species was restructured by considering a third Archaeal phylum, the Thaumarchaeota (Brochier-Armanet et al., 2008). The presence of a unique membrane lipid, called Crenarchaeol in all Thaumarchaeota representatives involved in ammonia oxidation pathway, also helped for this restructuration, being also a good biomarker for AOA identification (Zhang et al., 2006; de la Torre et al., 2008; Schouten et al., 2008; Pitcher et al., 2009; Pester et al., 2011; Damsté et al., 2012). After the isolation of the first ammonia oxidizing Archaea and its genome sequenced (Konneke, et al., 2005; Walker et al., 2010), some differences, comparing with its counterparts ammonia oxidizing Bacteria, stand out, mainly in regard to its ammonia oxidation biochemistry, which was confirmed by the dissimilarity of ammonia monooxygenase (AMO) sequences and structure (Walker et al., 2010). While Bacterial ammonia oxidation passes through a first step of ammonia oxidation into hydroxylamine (NH_2OH), catalyzed by AMO enzyme, and then a reoxidation into NO_2^- by HAO (hydroxylamine oxireductase) complex, AOA have an AMO enzyme with higher Cu-requirements and an absence of genes coding for HAO complex. Thus, an alternative pathway was proposed for ammonium oxidation involving the catalysis of ammonia to nitroxyl hydride (HNO), instead of NH_2OH, which is then oxidized into NO_2^- (Walker et al., 2010). It was, however, discussed that AOA might have a similar metabolic pathway to AOB but catalyzed by different, and still unidentified, enzymes (Schleper and Nicol, 2010; Hatzenpichler, 2012). Just recently, it was demonstrated that the ammonia oxidation metabolism of AOB also occurs in *N. maritimus* (Vajrala et al., 2013). This last study represents a major contribution to the AOA metabolic pathway by identifying hydroxylamine (NH_2OH), as the immediate product of AMO in *N. maritimus*, and suggesting similar ammonia oxidation mechanisms between AOA and AOB. Although, the lack of HAO gene in AOA still suggests a distinct enzyme complex for NH_2OH oxidation. Another difference is the absence of cytochrome c proteins and the presence of many copper-containing proteins in AOA. This suggests a different electron transport mechanism from the highly iron dependent AOB, which could also explain the higher ratio of AOA/AOB in the marine environments with generally high copper concentrations than iron (Hatzenpichler, 2012 and references therein). Besides N_2O is being produced by the denitrifying pathway, it is also a product of Bacteria and Archaea ammonia oxidation, however the enzymatic machinery of N_2O production in AOA is still under debate. Despite the lack of evidence of hydroxylamine oxireductase gene in Archaeal genome, it was found in the genome of *N. maritimus,* as well on environmental samples genomes, evidences of nirK genes (Bartossek et al., 2010, Walker et al. 2010) providing a potential theory of N_2O production by nitrifier-denitrification pathway (Jung et al., 2011; Santoro et al., 2011; Loescher et al., 2012). In another hand, the recent reports that show the production of hydroxylamine as an AMO product, together with some acetylene inhibition experiments, strongly suggested that N_2O is a product of ammonia oxidation and not from NO_2^- reduction (Vajrala et al., 2013). With these last discoveries, AOA have been proposed to be potential contributors for the upper ocean N_2O emissions (Santoro et al., 2011).

1.4.3 - Environmental factors that differentiate AOA from AOB distribution

Environmental factors are main forces that dictate AOA and AOB population dynamics along their worldwide distribution, acting like a web on AOA and AOB niche distribution and functionality. Although a single factor cannot discriminate AOA from AOB distribution, for our better and easier understanding most of the studies tend to isolate just one factor and analyze its influence on ammonia-oxidizing communities. For example, one of the main factors that were found to shape the relative distribution of AOA versus AOB is the substrate concentration. Most of AOA are linked to oligotrophic environments with low concentration of ammonia. In fact, it was demonstrated that *N. maritimus* SCM1 has an ammonia monooxigenase enzyme with high affinity to low thresholds for ammonia concentrations (Martens-Habbena et al., 2009; Martens-Habbena and Stahl, 2010). A preference for low NH_3 was also found to be true for other AOA species (Hatzenpichler et al., 2008; Lehtovirta-Morley et al., 2011). However, the soil isolate, *N. viennensis* showed a higher tolerance for ammonia concentrations compared with *N. maritimus* SCM1

(Tourna et al., 2011), however they are still low when compared to AOB ammonia tolerance (Koops et al., 2006). It is also well accepted that AOA are the major contributors to ammonia oxidation in low pH terrestrial systems (Zhang et al., 2011; He et al., 2012), along with the low ammonia concentrations that characterize those soils (He et al., 2012). Compared to AOB, AOA are more transcriptionally active in low pH soils showing to have phylotypes that are specifically adapted to low-pH conditions (Gubry-Rangin et al., 2011). Although the temperature effect is not very well studied on the selection of the two groups of ammonia oxidizers (AOA, AOB), there are evidences of Archaeal *amoA* genes in environments with temperatures up to 97°C (Wang et al., 2009; Dodsworth et al., 2011). Oxygen may be also a key factor in shaping AOA and AOB populations, with an AOA domination in reduced O_2 habitats, suggesting a tolerance for low O_2 environments (Lam et al., 2009; Martens-Habbena and Stahl, 2010). Studies performed within a salinity gradient ecosystems, also identified salinity as a potential environmental regulator of AOA and AOB habitat selectivity, with higher salinities favoring numerical dominance of AOB over AOA in coastal and estuarine sediments (Santoro et al., 2008; Magalhães et al., 2009).

Although the increasing interest in evaluating ecological variables that shape the dynamics of natural AOA and AOB populations, more work must be done. Plus, most of those studies are based on correlations between environmental sample and the environmental factor, not taking in account the representativeness of the organisms in the sample and in the environment. In addition, in natural ecosystems a single factor is not operating alone, thus not one, but a web of environmental factors may dictate AOA and AOB distribution. A good news is that combined study approaches are starting to appear making use of genome analyses, controlled experiments (microcosms) and environmentally cultivated representatives in order to search for the combination of key environmental drivers and environmental speciation of AOA and AOB (Prosser and Nicol, 2012).

1.5 – SCOPE OF THIS STUDY AND GOALS

Estuaries are the interfaces between freshwater and marine environments being extremely dynamic zones. The mixing of freshwater and saltwater along with the geomorphology, winds, tidal heights and anthropogenic impacts creates physical-chemical gradients that are accompanied by shifts in the resident microbial communities, turning these ecosystems a place of great interest in microbiological and ecological studies.

In coastal systems, nitrification is often coupled to denitrification (Jenkins and Kemp, 1984; Sebilo et al., 2006), returning N to the atmosphere. Once microorganisms catalyze those processes, it is important to identify the main environmental factors that regulate their diversity, distribution, and activity. Much of nitrification research has been addressed to open ocean or terrestrial systems (Leininger et al., 2006; Park et al., 2006; Santoro et al., 2010), however in such dynamic systems like estuaries, Archaeal and Bacterial ammonia oxidizers face several environmental stresses, which may be reflected in a variable pattern of abundance, diversity and activity. Salinity has been addressed as an important factor regulating Bacteria and Archaea community dynamics; however we must be aware that in natural environment it is very difficult to isolate the influence of just one environmental parameter. Thus, along with salinity, nitrogen, pH, oxygen, temperature, organic matter, among many other parameters, have been correlated with shifts in microbial communities, including the nitrifier communities (Del Giorgio and Bouvier, 2002; Crump et al., 2004; Hewson and Fuhrman, 2004; Bernhard et al., 2005, 2010a, 2010b; Bernhard, 2010).

In some estuarine locations characterized by high salinity gradients, beta-ProteoBacteria AOB were found to be more abundant than AOA (Santoro et al., 2008; Magalhães et al., 2009) contradicting previous reports indicating that AOA are always more abundant than AOB (Prosser and Nicol 2008). Thus, based on these observations, and taking advantage of the gradient of salinity of the Douro estuary, the main goal of this study was to investigate the regulatory role of salinity on Archaea, Bacteria and Archaea ammonia oxidizing communities diversity and on some biogeochemical processes mediated by those microbial communities. This was addressed by evaluating the benthic microbial diversity, by means of fingerprinting techniques, and several N biogeochemical processes and environmental parameters in different stations located along the salinity gradient of the Douro estuary.

This study contributed to increase our knowledge on the effect of salinity changes in the structure of estuarine prokaryotic communities and gave us insights about the role of Archaea communities in ammonia oxidation step of nitrification conditioned by a salinity gradient.

CHAPTER 2 - MATERIAL AND METHODS

“Fortunate are those who now start”

Martinus W. Beijerinck

In this chapter It will be described the sampling area in the Douro River estuary followed by an explanation of how the field samples were collected and treated, emphasizing the approaches used in order to pursue the study goals. For a better and clear reading, the methodology was divided between chemical, with special emphasis on the isotope paring and inorganic nutrient analyses, and biological analysis, with a deeper description of the methodologies used for microbial diversity evaluation.

2.1 - DOURO RIVER ESTUARY CHARACTERIZATION

Douro river begins in Spain, at *Sierra de Urbión* peaks, having a length of about 930 km and a watershed extension of about 98000 km^2, draining 17% of Iberia Peninsula (Bordalo and Vieira, 2005; Magalhães et al., 2005a) and making of it one of the largest rivers of the Iberian Peninsula. Over its course 15 dams were constructed for irrigation and electrical power generation purposes; the last one, Crestuma Dam, works as an electrical power generator, being located 21.6 km from the mouth of the river, defining the upstream limit of the Douro estuary.

The estuary has a total area of 7.5 km^2 and a watercourse that extends for 21.6 km draining in the Atlantic Ocean between Porto and Gaia cities. Because of the high pressure from anthropogenic activities along the river course and the high proximity of those two highly populated cities, the lower estuary is considered to be polluted (Bordalo and Vieira, 2005).

This system has been liable for several monitoring programs, being very well described along the years (Vieira and Bordalo, 2000; Magalhães et al., 2002, 2005a, 2005b and 2008; Bordalo and Vieira, 2005; Azevedo et al., 2008). The average estuary depth is about 8.2 m, tides are semi-diurnal with an average of tidal range of 2.8 m at the mouth and 2.6 m at the head, being described as a mesotidal estuary (Vieira and Bordalo, 2000). At the lower part of the estuary, the water temperatures can range from 7 °C, during winter season, to 22 °C, during summer season and the salinity between 0 to 35 ppt (Magalhães et al., 2002). It also possesses an extensive intertidal flat representing 9% of the total area of the estuary and 37 % of its lower part being mainly composed by sand (0.60 km^2) and a small muddy marsh (0.05 km^2) (Magalhães et al., 2002). Freshwater inflow have an average of 488 m^3 s^{-1}, with a highly marked seasonal pattern (Magalhães et al., 2002). The water residence time ranges between 0.3 and 16.5 days, being considered a very stratified river and a salt-wedge estuary: an estuary in which a wedge of salt water is established under low river flows (Vieira and Bordalo, 2000).

In spite of being mostly an heterotrophic estuary, some benthic intertidal areas are autotrophic, with an important role in removing inorganic nutrients from the water column, being also a source of oxygen (for the water column) and organic matter (for the trophic chains)

(Magalhães et al., 2003, 2005b). The input of NO_3^- and silicates to the estuary is mainly done by the river; in fact in the past years studies reported an increase of NO_3^- concentration in the upstream reservoir which is leading to higher loads of this nutrient into the estuary, affecting its productivity (Magalhães et al., 2005a, 2008). Compared to other Portuguese estuaries, these concentrations are higher but still below the threshold established by the European NO_3^- directive (Azevedo et al., 2007). In the case of other nutrients like phosphates and NH_4^+, other sources than the river flow are considered, mainly because NH_4^+ highest values coincide with the high numbers of fecal coliform Bacteria which were detected in the most urbanized zones, probably being sourced by wastewater discharges (Bordalo, 2003; Magalhães et al., 2003, 2008; Azevedo et al., 2008). Primary production dynamics showed to have a large interannual variability in intertidal sediments of the estuary but a decreasing trend in water column from the upper to the lower estuary has been described (Azevedo et al., 2006). Moreover, if we compare phytoplankton biomass concentration between Douro and other Portuguese rivers, it is lower, which may be explained by the short water residence time in this system. In what Bacterial distribution is respected, higher abundance in water column has been described at intermediate salinity (Bordalo and Vieira, 2005).

In terms of the river sanitary conditions, it was frequently described high number of fecal coliforms in the water column, especially in the most urbanized area of the estuary, with values higher than the maximum determined by European legislation (Bordalo, 2003; Bordalo et al., 2006).

Once the primary forcing agent for water circulation in the Douro estuary is the freshwater inflow, periodical monitoring studies should be done in order to assess changes in water quality and river “health”. With the increase of urban areas surrounding the river, new inputs of nutrients and other substances, either contaminants or not, might affect the estuary ecosystem and all the biogeochemical cycles that are supporting it. A cause of concern are the NO_3^- loads into Douro estuary mainly due to anthropogenic sources, which certainly affect the primary producers of the ecosystem, promoting phytoplankton and algae blooms endangering the health of such important aquatic environment. In addition, the progressive decrease in freshwater flow into the estuary, due to water diversion for agriculture within the watershed and climate change, has led to alter the estuarine salinity regime (Vieira and Bordalo, 2000).

2.2 – SAMPLING PROCEDURES

Once Douro river has a marked gradient of salinity during low tide, we chose four stations along that gradient, in the middle of July (Figure 5). Within each station, were collected sediment samples from 3 points (10-20 steps between each) in order to cover intra-site variability. Salinity and water temperature were measured *in situ*. At each station, sediments were homogenized, stored in sterile plastic bags and transported to the laboratory in the dark inside refrigerated ice chests. In addition, at one sub-site from each station, three undisturbed cores were collected to perform isotopic nitrification measurements. Subsamples of the homogenized sediment were immediately processed for the analysis of total organic matter, inorganic N fluxes and nitrification rates by acetylene inhibition technique, and later frozen and stored at –70°C until DNA extraction. Sediments were also characterized in terms of their grain size as well as in terms of total nitrogen and carbon content in the samples stored at -20ºC. Estuarine water was also collected adjacent to each sampling site for nutrient analysis. In the laboratory, water collected was filtered by a 0.45 µm membrane, and used to measure NH_4^+, NO_2^- and NO_3^- concentrations as well as TOC, (total organic carbon) and TN (total nitrogen) concentrations, according to the methodologies described below.

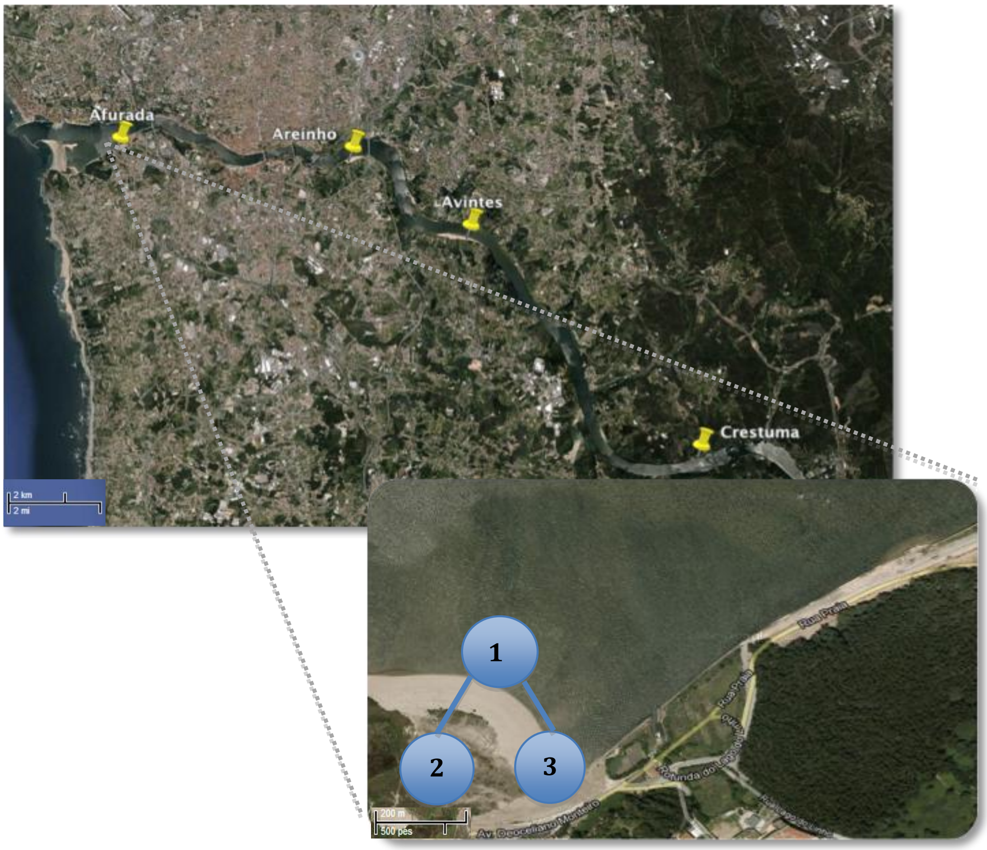


Figure 4 - Sampling stations location along river Douro estuary.

2.3 - SLURRIES INCUBATION

In order to assess nitrification rates and nutrient fluxes, sediment slurries incubations were performed (in triplicate) for the three sites of each sampling station (Figure 6). Ten mL of sediment were weighted and introduced into a 50 mL serum bottle, to which it was added 20 mL of pre-filtered estuarine water from each station. Nitrification rates in the slurries were measured by using the acetylene (C_2H_2) inhibition technique (Sørensen,1978). The nutrient fluxes were measured according to referenced methodology (Magalhães et al., 2002; 2003).

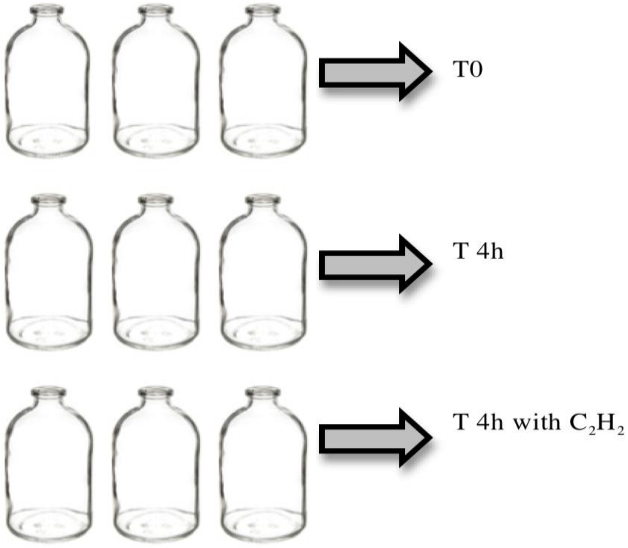


Figure 5 - Slurry treatment scheme (applied for each site).

2.3.1 - Acetylene Inhibition Technique

Nitrification rates in slurries were measured using the acetylene inhibition technique according to Sørensen (1978). This compound is an inhibitor of *amo* enzyme both in Bacteria and Archaea (Offre et al., 2009). Slurries were prepared by adding 20 ml of filtered estuarine water from each local into 50 mL serum bottles with 10 ml of homogenized sediment previously weighed (around 8 g). Samples were run in triplicate with and without 8 mL of C_2H_2. All samples were incubated in the dark for 4 h at constant temperature and stirring (70 rpm). At time zero (T0) and 4 h (T1), slurries were mixed and the overlying water collected, centrifuged, filtered (by 0.45 µm membrane filters) and stored at -20ºC in 5 mL tubes for later nutrient analysis. Nitrification rates were calculated by the difference between NH_4^+ concentration measured in treatments with and without C_2H_2. The NH_4^+ concentrations were quantified according to Grasshoff et al. (1983) as described below.

2.3.2 - Isotopic Analyses of ^{15}N

There are two isotopic forms of atomic nitrogen in nature: ^{14}N (more abundant- 99 %) and ^{15}N (less abundant- 0.370 %). It was stated that natural abundance of these stable isotope ratios present on NO_3^- and N_2O can be used to understand the cycle of these species by integrating information from both the sources and sinks (Santoro et al., 2010). The method to measure the uptake of ^{15}N has already been described by many authors (Dugdale and Goering, 1967; Santoro et al., 2010) where basically, the $^{15}N/^{14}N$ ratio present in atomic composition of NO_2^- plus NO_3^- produced by the nitrification pathway gives a measurement of this process.

In our experiments, isotopic nitrification measurements were performed using three undisturbed cores collected at one of the sub-sites at all stations. Two of the cores were supplied with labeled $^{15}NH_4^+$ (at half concentration of the natural one) and the ^{15}N present on the $^{15}NH_4^+$ supplied was measured in the forms of $^{15}N\text{-}NO_2^-$ and $^{15}N\text{-}NO_3^-$ produced at the end of incubation, throughout nitrification process (Sigman et al., 2001, Santoro et al., 2010). These compounds were measured in the filtered (0.2 µm) overlying water collected after 2 h of incubation. These samples were then incubated with a pure culture of denitrifying Bacteria lacking N_2O *reductase (Pseudomonas chlororaphis*)*,* being transformed into N_2O. Then, all the labeled $^{15}N_2O$ formed during this transformation was measured by mass spectrometer according to the atomic masses of correspondents to $^{15}N/^{14}N$ ratios of N_2O (Sigman et al., 2001; Casciotti et al., 2002).

Following mass spectrometry, and using data of the $^{15}N_2O$, it was calculated the atomic percentage of ^{15}N contained in the NO_2^- and NO_3^- product pool. Calculations of the representativeness of the $^{15}NH_4^+$ added in our sample were also performed. The nitrification rates were assumed as being the ^{15}N flux between the $^{15}NH_4^+$ supplied and the NOx pool produced ($^{15}N\text{-}NO_2^-$ and $^{15}N\text{-}NO_3^-$). The atomic percentage of ^{15}N measured in $NO_2^- + NO_3^-$ pool when compared to the atomic percentage of ^{15}N contained in the initial $^{15}NH_4^+$ gives the quantity of ^{15}N that was transferred along the incubation from NH_4^+ to NO_3^- by the nitrification pathway (Dugdale and Goering, 1967; Santoro et al., 2010). In order to calculate the NH_4^+ transformation rate, a modified equation of Dugdale and Goering was used (Dugdale and Goering, 1967).

The equation:

$$VNO_3^- = p14/N1$$

Where,

VNO_3^- - growth rate of NO_3^-

p14 – the rate of transport of nitrate

N1 – the concentration of nitrogen in the particulate nitrogen fraction

was modified to:

$$VNH_4^+ = pNO_3^- /N1$$

Where,

VNH_4^+ - fluxes of transportation rate of ^{15}N- NH_4^+

pNO_3^- - slope of the time course ^{15}N-NO_3^- in atomic percentage

N1 – atomic percentage of ^{15}N in NO_3^- (the product pool) - atomic percentage of ^{15}N in NH_4^+ (substrate pool).

Atomic percentage = $^{15}N/(^{15}N + ^{14}N)$.

Nitrification rates were calculated by the fluxes of ^{15}N from initial $^{15}NH_4^+$ to ^{15}N-NO_2^- and ^{15}N-NO_3^- product pool, measured after 2 hours of incubation.

2.4- CHEMICAL ANALYSES

2.4.1 – Inorganic Nitrogen quantification

Elements that play a key role on nitrification process (NH_4^+, NO_2^- and NO_3^-) present in the filtered water from the four sampling locations and in the selected slurries treatments were analyzed by spectrophotometry. Concentrations of NO_2^- and NH_4^+ were evaluated using methods described in Grasshoff et al. (1983). Nitrates were assayed using an adaptation of the spongy cadmium reduction technique (Jones, 1984), with the NO_2^- value subtracted from the total. The precision of all determinations was in the range of 0.1 to 8%, depending on the particular nutrient concentration. All samples were run in triplicate and a standard curve for each nutrient was made. A detailed description of the methodologies used is given below.

The method used for NO_2^- quantification is based in a reaction between NO_2^- and an aromatic amine (sulfanilamid) producing a nitrogenised compound which reacts with a second aromatic amine (N-(1-naftil)-etilenodiamine) producing a pink complex. The color intensity of that compound is proportional to the NO_2^-concentration. After this reaction samples were read in a spectrophotometer at a λ of 540 nm.

A dilution of 10x was performed and for the calculation of NO_2^- concentration the following formula was applied:

$$NO_2^- = D \times (A_a - A_b) \times FD$$

Where:

NO_2^- - Nitrite concentration

D - Slope of the line pattern

A_a – Absorbance value of the sample

A_b – Absorbance value of the solution without nitrites

FD – Dilution factor of the sample

For NO_3^- quantification, a chemical reduction was performed. This reduction was triggered by mixing the sample (diluted 10x) with spongy cadmium (Cd^{2+}) (reductor agent) in the presence of a buffer, ammonium chloride, (an alkaline solution that complexes the oxidized Cd^{2+}). Nitrate was reduced to NO_2^- and from this step the methodology was the same as previously described for NO_2^- quantification.

For the calculation of NO_3^- concentration the following equation was applied:

$$NO_3^- = ((D \times (A_a - A_b) - NO_2^-) \times C \times FD$$

Where:

NO_3^- - Nitrate concentration

D – Slope of the line pattern

A_a – Absorbance value of the sample

A_b – Absorbance value of the solution without nitrates

NO_2^- - Nitrite concentration

C – 1.2 dilution factor for ammonium chloride solution

FD – Dilution factor of the sample

For NH_4^+ quantification the method used is based on the fact that this nutrient, when present in a more or less alkaline solution, reacts with hipochloride forming the compound monochloromine which, in turn, in the presence of a catalyzer (nitroprussiate), phenol and hipochloride (that exceeded) produces a colorful blue complex. The reaction takes at least 6 hours, in the dark, at room temperature until it become complete. At a pH higher than 9.6 the ions Mg^{2+} and Ca^{2+} can precipitate. According to Koroleff (1983) those ions can be dissolved in solution by the use of citrate. In our methodology, an alternative of this method was performed and instead of citrate, a magnesium solution was added in order to cause precipitation of magnesium hydroxide, present in saline water. This step avoids possible interferences by particles in suspension (Grasshoff et al., 1983).

After this reaction samples were read in a spectrophotometer at a λ of 630 nm. A dilution of 2x was applied and for the calculation of NH_4^+ concentration the following formula was applied:

$$NH_4^+ + NH_3 = D \times (A_a - A_b) \times FD$$

Where:

NH_4^+ - Ammonium and ammonia concentration

D - Slope of the line pattern

A_a – Absorbance value of the sample

A_b – Absorbance value of the solution without ammonia

FD – Dilution factor of the sample

2.4.2 – Water and sediments total dissolved carbon and nitrogen analysis and total organic matter in sediments

Estuarine water determination of total dissolved carbon (TDC) and total dissolved nitrogen (TDN) was performed using a Shimadzu Instruments TOC-VCSN analyzer coupled to a total nitrogen measuring unit (TNM-1, Shimadzu), according to the methodologies previous described (Magalhães et al. 2008). TDC was measured by high temperature catalytic oxidation followed by nondispersive infrared detection of CO_2. TDN was thermally decomposed in a combustion tube and the resulting nitric oxide detected by chemiluminescence. Three to five replicates were performed per sample for TDC and TDN.

For sediment analyzes of total carbon and nitrogen, the sediments, from each sub site, were weighted (2 mg) and them analyzed by IRMS (isotope ratio-mass spectrometry), linked to an elementar analyser Flash 2000 (Bahlmann et al., 2010). Samples for sediment organic matter content were processed by drying to constant weight at 60°C, weighted, followed by ignition at 500°C for 4h and reweighting (Magalhães et al. 2002). Sediment granulometry was performed by sieving (10min) the sediment previously dried and weighted (100g). Each sediment fraction was recovered according to its size (< 0.063, > 0.063, > 0.125, > 0.25, > 0.5, > 1 and > 2 mm) and reweighted (Magalhães et al., 2002).

2.5 - MICROBIAL COMMUNITY ANALYSIS

2.5.1 – Total Cell Counts

The total number of prokaryotic cells in the sediment samples were estimated by epifluorescence microscope by a direct count of DAPI (4',6-diamidino-2-phenylindole) stained cells (Pernthaler et al., 2001; Llobet-Brossa, et al., 1998). Sediment samples (0.5 g of fresh sediment), from each of the three sub-sites from the four stations, were fixed in 2% formaldehyde. This was followed by two washing steps with PBS 1x. In the last washing step a solution of PBS 1x and Ethanol 80% (1:1) was used, and fixed sample was storage at -20 °C, for latter analysis. An aliquot of 5 µL of each sample was mixed with 10 mL of PBS 1x. Cells were collected on the surface of 0.2 µm pore-size polycarbonate membranes by vacuum filtration, stained with DAPI (2 µL/mL), and mounted in Citifluor immersion oil solution (Citifluor Ltd, London, UK). Cells were visualized with an epifluorescence microscope with a mercury bulb and filter sets 31000 (Chroma) (Clearly et al., 2012a). Finally, cells were counted with 1000 magnification in 30 randomly selected optical fields. The following formula was used to estimate total DAPI counts of prokaryotic cells in each sample:

$$\mathrm{NTB} = \frac{(\text{cell nº/field x D x F}) / V,}{w}$$

Where:

NTB – Number of total Bacteria (cells per gram)
Cell nº/ field – Average of cells numbers per optical field
D – Dilution
F – Dilution factor
V – Volume of the sample (l)
w – Weigh of the sediment (g)

2.5.2 - DNA extraction

Total DNA was extracted from a total of 12 sediment samples, with two replicates of the three sub-sites of the four stations located along the salinity gradient of Douro estuary. A total of 0.5 to 1 g wet weight of homogenized sediment was used to extract total DNA by a PowerSoil DNA isolation kit (MoBio Laboratories Inc., Solana Beach, CA). The two extraction replicates were used to prepare a composed sample. The reproducibility of the amount of the DNA extracted was already tested (CV= 14%) (Magalhães et al., 2011). The quality of the DNA extracted was certified by running 5 µL of the total DNA in an agarose gel stained with sybersafe stain.

2.5.3 – PCR amplification of 16S rRNA and *amoA* gene

Bacteria and Archaea 16S rRNA genes were amplified using a nested PCR approach suitable for DGGE fingerprinting (Pires et al., 2012). For Bacteria, the initial amplification was carried out using the general Bacteria set of primers 1512R/27F at 10 µM each (Heuer et al., 1997). PCR was run with 1 µL of template DNA in 25 µL reaction volume using the following conditions: 5 min of denaturation at 94 °C, 25 thermal cycles of 45 s at 94°C, 45 s at 56 °C, and 1min and 30 s at 72 °C. A final extension step at 72 °C for 10 min was performed to finish the reaction. A portion of the PCR product obtained (1 µl) was used in a secondary amplification using the set of primers 1378R/ 984F (Heuer et al., 1997), where a GC clamp with the sequence: 5'- CGCCCGGGGCGCGCCC CGGGCGGGGCGGGGGCACGGGGGG-3' was added to the forward primer (Nübel et al., 1996). PCR was run in 25 µL reaction volume using the following conditions: 4 min of denaturation at 94 °C, 25 thermal cycles of 1 min at 95 °C, 1 min at 53 °C, and 1 min and 30 s at 72 °C. A final extension step at 72 °C for 10 min was also performed. Negative control reactions mixtures containing no template were included in all amplifications performed.

For Archaea, 16S rRNA was amplified using the set of primers ARC344f-mod /Arch958R-mod at 10 µM (Pires et al., 2012) in the first amplification, and the set of primers 524F/ Arch958R-mod at 10 µM (with the GC clamp attached to the reverse primer) in the second one (Pires et al., 2012). The nested PCR methodology was similar to the described for Bacteria with changes on volume of DNA loaded in the second PCR reaction (2µL) and on the PCR cycles: 5 min denaturation at 94°C, 30 thermal cycles of 1 min at 94 °C, 1min at 56 °C, and 1min at 72 °C, with a final extension step at 72 °C for 7 min for the first amplification. For the second amplification a similar program was run with changes in the number of thermal cycles which increased to 35 and on the annealing temperature which decreased to 50 ºC.

For Archaeal ammonia oxidizing (AOA) diversity analysis a single PCR was performed. The amplification was done using the set of primers Cren *amoA* 23F/ Cren *amoA* 616R (Tourna et al., 2008), specific for the amplification of the gene *amoA*. PCR was run with 2 μL of template DNA in 25 μL reaction volume using the following conditions: 4 min denaturation at 95 °C, 30 thermal cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C were carried out. A final extension step at 72 °C for 5min was performed to finish the reaction (Yamamoto et al., 2010).

2.5.4 - DGGE

DGGE was performed using a Dcode system (BioRad). All runs were performed in 7 L of TAE 1x buffer at 60 °C and a constant voltage of 70 V for 16 h. For Bacterial and Archaeal 16S rRNA runs was used a denaturant gradient of 40 % - 58 %. For Bacteria 5 μL of each PCR product was loaded with 4 μL of loading buffer, while for Archaea 9 μL of each PCR product was loaded with 4 μL of loading buffer, with the exception of samples A1, A2 and A3 (from site Afurada) where 15 μL of PCR product were loaded. For AOA PCR products was used a denaturant gradient of 25 % - 45 %. Five μL of each PCR product was loaded with 4 μL of loading buffer, with the exception of samples A1, A2 and A3 (from site Afurada) where 10 μL of PCR product were loaded. All DGGE gels were stained using silver nitrate protocol (Heuer et al., 2001). Gels were scanned using a Molecular Image FX apparatus (Bio-Rad, Hercules, CA).

2.6 - STATISTICAL ANALYSIS

Data analysis was performed at the 95 % confidence level ($p < 0.05$). Environmental data were tested for normality using the Kolmogorov-Smirnov test, and for homoscedasticity using Levene's test (Zar, 1999). Nitrification data were *log* transformed in order to follow the previous assumptions. One-way ANOVA was performed (Zar, 1999) in order to test the significance of each variable measured. Correlations were performed between the main environmental parameters and biological data measured in all sampling sites using Statistica software (version 11). Principal component analysis (PCA) was performed in PRIMER 6 software package (version 6.1.11) (Clarke and Gorley, 2006) to ordinate the samples collected based on the values of environmental data. Environmental data used in the multivariate analysis was log $(X + 1)$ transformed and normalized prior to the analysis, in order to have comparable scales. A resemble similarity matrix was created using Euclidean distances and then examined using an hierarchical cluster analysis (HCA). The clusters generated were tested for differences using a similarity profile permutation test (SIMPROF test).

For biological data, the digitalized DGGE gels were analyzed with the software package GelCompar (version 4.0; Applied Maths). Briefly, both band position, number and intensity were processed in a spreadsheet. The data matrix of the gels was transformed into a binary matrix according to the absence/presence of the bands over the gel profile, and a distance matrix was constructed using the Bray-Curtis coefficient index by PRIMER 6 software (version 6.1.11). For all gels it was performed an HCA and a method of nonmetric multidimensional scaling (MDS) based on Bray–Curtis similarities. Samples in MDS plots were grouped together according to their similarity. In the two-dimensional plots, the relationships between samples (i.e. goodness of fit) were determined by a stress coefficient: if its value is < 0.1 that indicates a good portrayal of data with no real prospect of misleading interpretation (Clarke and Warwick, 1994). The ANOSIM test (Clarke and Warwick, 1994) was used to test the differences between the groups previously identified as being correspondent to each sampling site. R statistic values are an absolute measure of how well the groups are separate, ranging between 0 (indistinguishable) and 1 (well separated). In order to establish relationships between environmental factors and diversity of Bacterial, Archaeal and AOA communities, a redundancy analysis (RDA) was performed as a method of samples ordination using Canoco Software. For RDA Bacteria, Archaea and AOA richness values were log $(x+1)$ transformed, and the environmental variables were normalized (i.e. adjusted for a mean of 0 and SD of 1). A Monte Carlo permutation test was used to assess the statistical significance of the relationships. In the RDA ordination diagram, the angle and length of the arrow relative to a given axis reveals the extent of correlation between the variable and the canonical axis (environmental gradient) (ter Braak and Smilauer, 2002).

CHAPTER 3 - RESULTS

“The deepest sin against the human mind is to believe things without evidence”

Thomas Huxley

3.1. – SAMPLING SITES CHARACTERIZATION

3.1.1 – Chemical parameters

Along the four sampling stations (Figure 5), as the salinity decreases (21.7 – 4.9), the temperature increases, ranging between 18 °C and 24 °C (Table 4). Estuarine water total dissolved carbon (TDC) and total dissolved nitrogen (TDN) were found to be higher in most saline waters (Afurada) (Table 4). Estuarine water concentrations of NO_3^-, NO_2^-, and NH_4^+ varied according to the study sites: Afurada, showed higher concentrations of NH_4^+ (3.39 µM), but lower concentrations of NO_3^- (19.77 µM) which tended to increase in more upstream stations (Avintes and Crestuma) (Table 4).

Contents of total carbon and nitrogen in the sediments ranged between 19 – 25 % and 7 – 8 % respectively (Table 4), not being significantly different between the different sampling sites (one-way ANOVA, $p > 0.05$, $n = 12$). The percentage of total organic matter fluctuated according to the sites, being the lowest and highest values observed in Areínho (0.46 %) and in Avintes (1.08 %), respectively (Table 4).

Table 4 - Water column and sediments chemical characteristics in the Douro estuary sampling sites. Data are presented as mean values and standard deviations (±sd) for the three sub-sites of each station.

	Water								Sediment			
Sites	TDC (mg/l)	TDN (mg/l)	C:N	NH_4^+ (µM)	NO_2^- (µM)	NO_3^- (µM)	Salinity (ppt)	Temperature (ºC)	%C	%N	C:N	Organic matter %
Afurada	27.74 (0.10)	1.52 (0.02)	18.30	3.39 (1.1)	0.59 (0.03)	19.77 (1.4)	21.70	18.00	24.8 (4.5)	8.5 (3.9)	4.9 (1.3)	1.03 (0.1)
Areinho	24.04 (0.35)	1.19 (0.01)	20.29	2.55 (0.2)	0.69 (0.02)	42.61 (0.3)	9.30	21.80	19.3 (4.3)	7.2 (1.1)	2.43 (0.3)	0.46 (0.01)
Avintes	21.25 (0.10)	1.48 (0.04)	14.37	2.86 (0.9)	0.73 (0.02)	78.23 (3.9)	6.70	22.30	19.5 (1.6)	7.7 (0.4)	2.58 (0.1)	1.08 (0.03)
Crestuma	22.56 (0.35)	1.19 (0.02)	19.02	2.51 (0.1)	0.78 (0.02)	61.47 (0.7)	4.90	24.70	19.3 (1.7)	7.9 (2.3)	2.37 (1.1)	0.78 (0.2)

3.1.2 – Sediment grain size

Sediment grain sizes were found to be heterogeneous between the different locations (Figure 7). Areinho sediments have higher content of gravel (56-65%) and low content of fine sand, slime and clay (0.1%). On the other hand, Afurada showed lower percentages of gravel (20-25%) but higher coarse and fine sands (3.6%) and the highest of slime and clay (2.5%). Avintes and Crestuma displayed a high content of gravel (40%) and also relative higher contents of fine sands (6-9%) but very low contents of slime and clay (Figure 7).

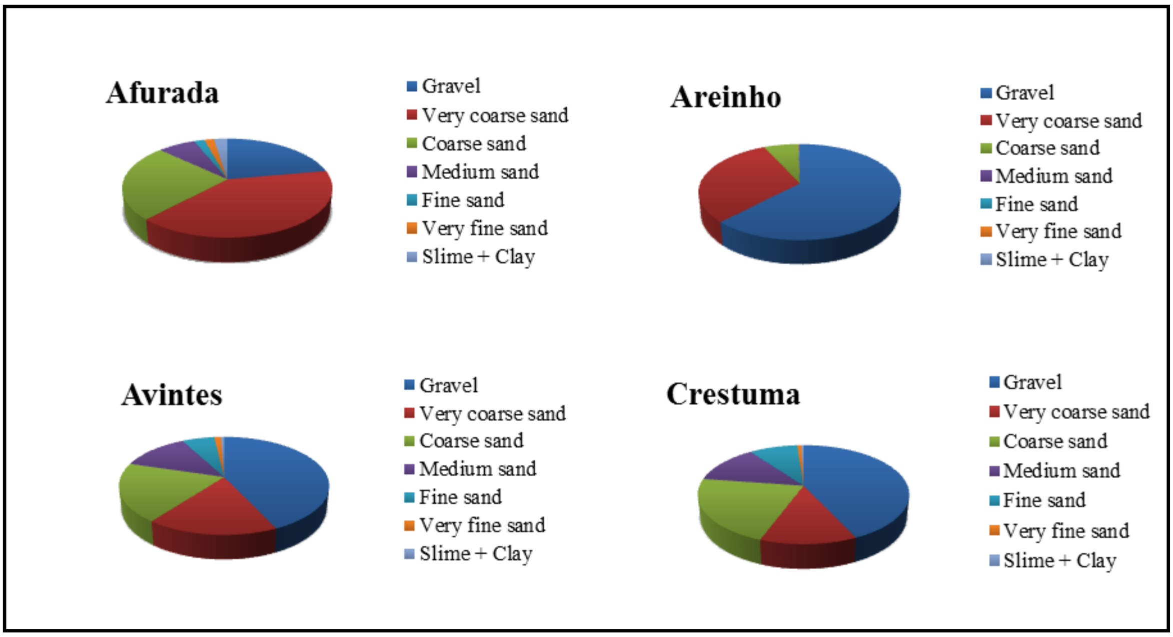


Figure 6 - Distribution of sediment grain sizes at each sampling site. The values are means of the results obtained from the sediments collected at the three sites per station sampled.

3.2 – NITROGEN BIOGEOCHEMICAL PROCESSES

3.2.1 - Inorganic Nitrogen Fluxes

Concentrations of NH_4^+ in the interstitial water of the incubation step ranged between 64 µM and 9 µM in Avintes and Crestuma, respectively (table 5). On the other hand, NO_3^- concentrations tend to be higher in the interstitial water, with the exception of the Afurada station, where NO_3^- values equal NH_4^+ ones (Table 5). Interstitial water concentrations of NO_3^- were positively correlated with temperature and with its concentration in water column ($r = 0.84$, $p = 0.001$, $r =$

0.89, $p < 0.001$, respectively for n =12) and negatively correlated with salinity (r= -0.83, p = 0.001, n = 12). Low concentrations of NO_2^- were observed in all sites, and showing irregular fluxes between the different stations (Table 5, Figure 8b).

Table 5 - Concentrations (means ± SD) of NH_4^+, NO_2^- and NO_3^- in the interstitial water along the different sampling sites of Douro river estuary.

Sampling Site	**Sub site**	**NH_4^+ (µM)** (± sd)	**NO_2^- (µM)** (± sd)	**NO_3^- (µM)** (± sd)
Afurada (A)	A1	26.12 (3.01)	1.61 (0.23)	31.06 (7.66)
	A2	38.24 (3.61)	1.56 (0.27)	27.00 (6.07)
	A3	26.82 (2.59)	1.86 (0.08)	22.75 (2.82)
Areinho (B)	B1	33.68 (0.9)	5.07 (0.77)	70.69 (22.16)
	B2	62.61 (0.64)	1.15 (0.10)	40.54 (2.29)
	B3	37.80 (0.63)	1.35 (0.08)	47.61 (4.76)
Avintes (C)	C1	47.71 (0.70)	0.93 (0.14)	108.17 (37.83)
	C2	64.86 (0.51)	3.24 (0.49)	103.25 (6.44)
	C3	47.53 (0.67)	0.67 (0.06)	121.38 (3.71)
Crestuma (D)	D1	13.87 (0.82)	0.96 (0.13)	151.69 (5.01)
	D2	11.36 (0.50)	0.79 (0.28)	122.97 (8.74)
	D3	9.76 (0.58)	0.65 (0.05)	91.57 (3.42)

A general efflux of NH_4^+ was observed, ranging from 10 (± 14) (Crestuma) to 325 (±49) nmol cm^{-2} h^{-1} (Afurada) (Figure 8c). While net fluxes of NH_4^+ did not significantly vary between Afurada, Areinho Avintes and Crestuma (one way ANOVA, p = 0.07; n = 12) they tend to decrease in the less saline stations reaching the lowest values in Crestuma. Results revealed that net fluxes of this nutrient were inversely related with NO_3^- net fluxes (r = - 0.65; p = 0.02; n = 12), NO_3^- interstitial water concentration (r = - 0.64; p = 0.024; n = 12) and temperature (r = -0.61; p = 0.03; n=12), being positively correlated to NH_4^+ interstitial water concentration (r = 0.58; p = 0.045; n = 12). In what NO_3^- net fluxes is respected, a distinct pattern was observed (Figure 8a). In this case, a general net uptake of NO_3^- from overlying water was registered, being those results significantly different in Crestuma where effluxes of this nutrient (mean values for the station of 21.02 (±29) nmol cm^{-2} h^{-1}) were registered (one way ANOVA, p = 0.001, n = 12). These fluxes were positively correlated with temperature and negatively correlated with NH_4^+ fluxes and its concentration in the interstitial water (r = 0.63, p = 0.028; r = - 0.65, p = 0.020; r = - 0.79, p = 0.003, respectively, n = 12). Net fluxes of NO_2^- measured were always on the threshold between its uptake and intake (Figure 8b), being in agreement with its concentrations observed in the interstitial water (Table 5). However, mean values showed that in Crestuma occurred general effluxes of NO_2^- (15 (±8) nmol cm^{-2} $h^{-1})^-$, contrasting significantly with Areinho and Avintes (one way ANOVA, p = 0.01, n = 12) where NO_2^- influxes were observed. These results were positively correlated with the net NO_3^- fluxes (r= 0.67, p = 0.015, n = 12) and negatively related with NH_4^+ interstitial water concentration (r = -0.65, p = 0.023, n = 12).

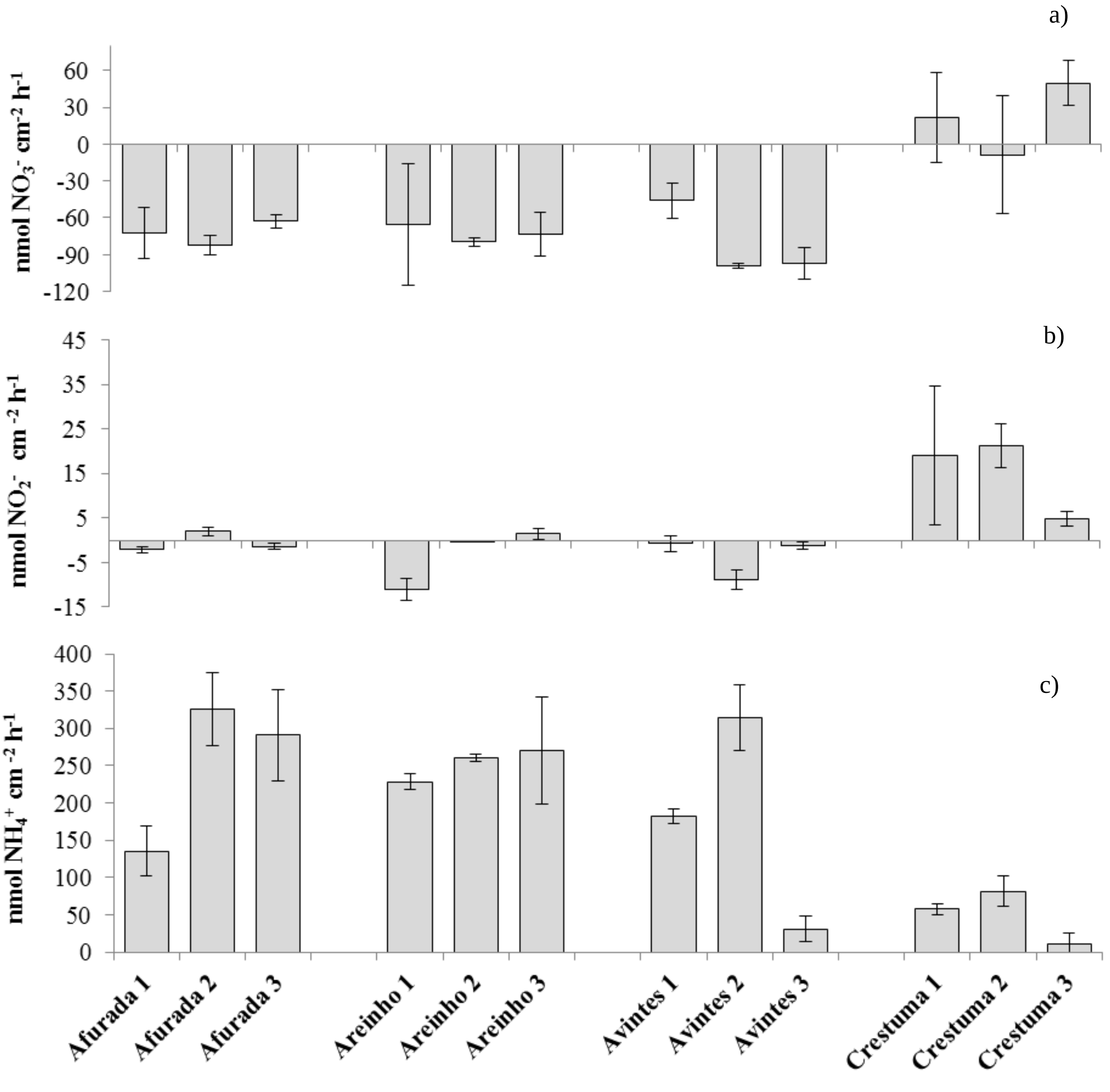


Figure 7 - Net NO_3^- (a), NO_2^- (b) and NH_4^+ (c) fluxes (nmol cm^{-2} h^{-1}). Positive values represent release of the nutrient to the water column and negative values represent uptake of the nutrient by the sediments.

3.2.2. Acetylene Inhibition Technique

Results of nitrification rates measured by the acetylene inhibition technique are presented in Figure 9. Areinho, the sampling site with an intermediate salinity (9.3), showed the highest nitrification rates (mean values 190 (±38) nmol NH_4^+ cm^{-2} h^{-1}), followed by Avintes (6.7 of salinity and 120 (±58) nmol NH_4^+ cm^{-2} h^{-1}), Crestuma (4.9 of salinity and nitrification rates values of 113 (±19) nmol NH_4^+ cm^{-2} h^{-1}) and Afurada (21.7 of salinity and nitrification rates values of 108 (±51) nmol NH_4^+ cm^{-2} h^{-1}). Beside this pattern, no significant differences were achieved for those rates between the different sites (one way ANOVA, $p > 0.05$, n =12).

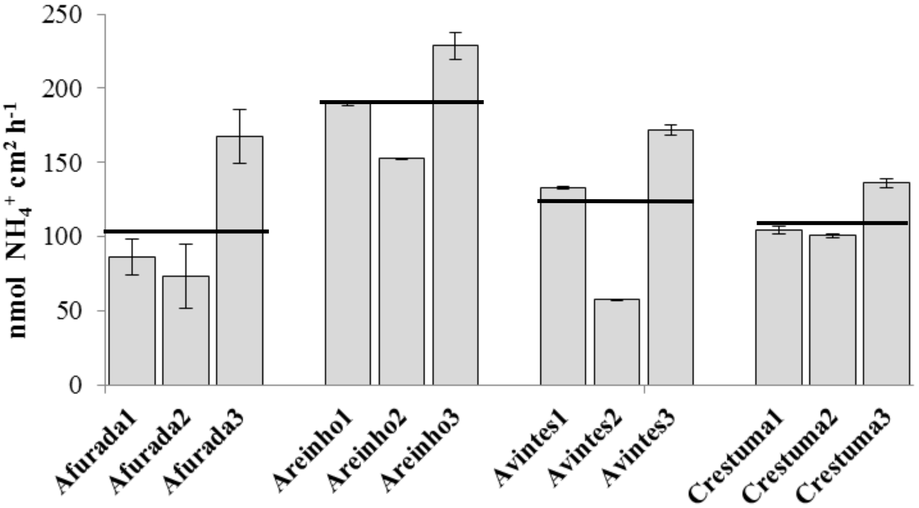
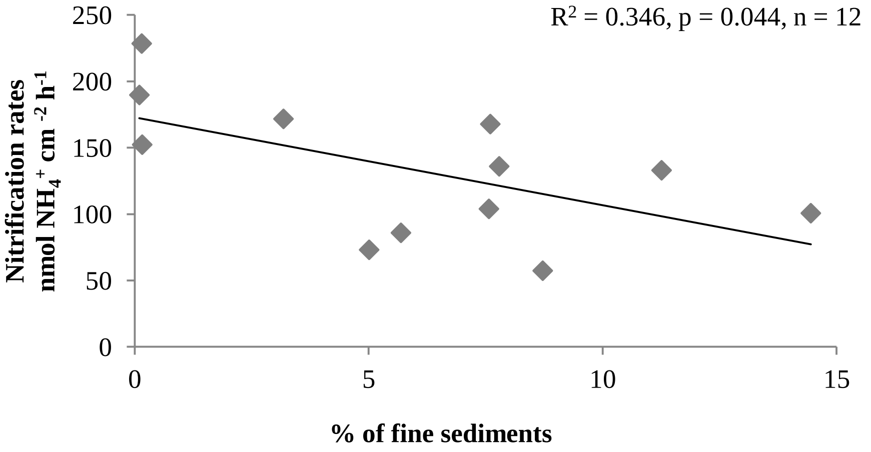


Figure 9 – Nitrification rates (nmol NH_4^+ cm^{-2} h^{-1}) and standard deviations measured in the three subsites of each sampling station along the salinity gradient of Douro estuary, using the acetylene inhibition technique. The bars show the mean values of nitrification rates at each station.

Nitrification rates at the different estuarine stations were significantly correlated with the grain size of each sampling site, having a positive correlation with the percentage of gravel and a negative one with the content of fine sands in the sediment composition ($r = 0.632$, $p = 0.027$ and $r = -0.588$, $p = 0.044$ respectively, n = 12). These results agree with the fact that gravel allows more air circulation, triggering aerobic biogeochemical processes as the case of nitrification (Figure 10). No significant correlations were detected between nitrification rates and salinity, temperature or with any inorganic nutrient net fluxes measured ($p > 0.05$, n = 12).



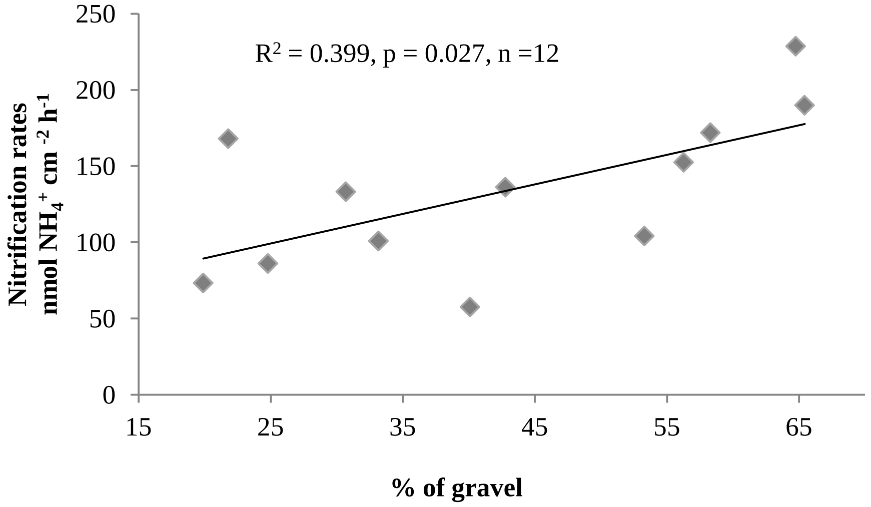


Figure 9 - Linear regression between sediment grain sizes fractions and nitrification rates measured at the different stations using the acetylene inhibition technique.

3.2.3. - ^{15}N Isotope Technique

Nitrification measurements by using the isotope analysis showed clearly higher rates at intermediate salinities (Avintes – 7430 (±2473) nmol NH_4^+ cm^{-2} h^{-1} and Areinho – 6356 (±586) nmol NH_4^+ cm^{-2} h^{-1}). Figure 11 represents the amount of NH_4^+ used by ammonia oxidizing microorganism to produce NO_2^- and NO_3^-, during nitrification process, measured by isotopic analysis. Values for the relative rates of nitrification between stations were in agreement with the results obtained with the C_2H_2 technique, showing higher rates at intermediate salinities (Figure 11), however the magnitude of the rates were found to be very different, mainly due to the different methodologies used during the sediment incubation process. At higher salinities (Afurada) and at the less saline station (Crestuma), the $^{15}NH_4^+$ intake was lower (1333 (±41) and 2233 (±447) nmol NH_4^+ cm^{-2} $h^{-1,}$ respectively) (Figure 11). Significant differences were obtained between the nitrification rates measured in Afurada and the intermediary saline site Avintes (one-way ANOVA, $p < 0.05$, n = 8).

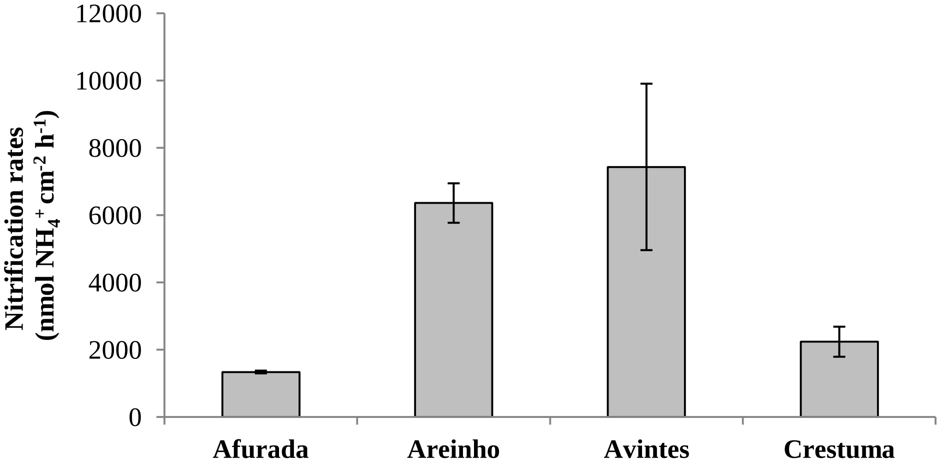


Figure 10 - Nitrification rates (nmol NH_4^+ cm^{-2} h^{-1}) measured along the salinity gradient of Douro's estuary by ^{15}N isotope method.

3.3 – SITE-SPECIFIC VARIATION OF THE ENVIRONMENTAL AND BIOGEOCHEMICAL PARAMETERS

A two-dimensional principal components analysis (PCA) was applied to the environmental and biogeochemical variables measured at the different sites, which included salinity, OM, C:N ratios in the estuarine water and sediments, net N fluxes, temperature, water column and interstitial water NH_4^+ and NO_3^- concentrations, sediment grain size and nitrification rates.

The PCA analysis showed that sub-samples of each sampling station group with each other and distance themselves from the others locations (Figure 12). PC1 was mainly related to salinity (negatively), interstitial water NO_3^- concentrations (positively), NO_3^- and NH_4^+ net fluxes (negatively) and temperature (positively), explaining 38% of the variance. Meanwhile PC2 (explaining 27.6% of the variance) was related to C:N of the water column, % of gravel (negatively), % of fine sediments (positively) and organic matter (positively) (Figure 12).

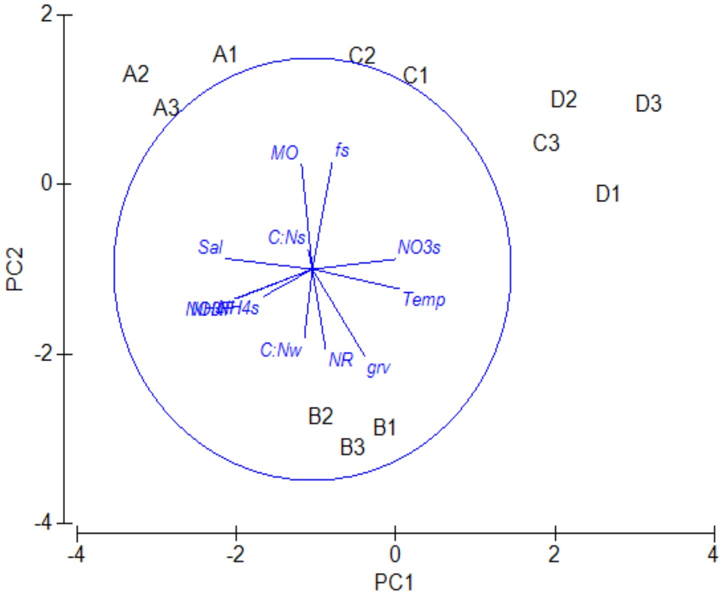


Figure 12 – Two dimensional principal component analysis (PCA) ordination based on the values of environmental and biogeochemical data (salinity, OM, C:N ratios in sediments and water, NH_4^+ and NO_3^- fluxes, temperature, NH_4^+ and NO_3^- interstitial water concentrations, grain size and nitrification rates).

The hierarchical cluster analysis (HCA) of the same input data used for the PCA resulted in four clusters separated at the Eucledian distance level of 3.73 (Simprof permutation test, $p < 0.05$) (Figure 13). Each cluster includes samples from the sub-sites of each independent sampling station, showing that the main environmental parameters of each station differ between them, contributing for a good ordination of the locations along the salinity gradient of Douro estuary. Samples from Afurada site (A1, A2, A3), the most saline one, stand out first, being characterized by their higher saline values in the water column, high NH_4^+ concentrations both on water column an interstitial water and higher C:N ratios in the sediments along with higher percentages of silt and clay. The second cluster, which includes the three sub-sampling sites from Areinho (B1, B2, B3), has the highest nitrification rates measured and the highest C:N ratios in the water. Moreover, sediments from Areinho station showed higher percentage of gravel comparing to other sites and lower contents on organic matter. Avintes and Crestuma split at the Eucledian distance level of 5.49, and presented the highest concentrations of NO_3^- in both water and sediments being the ones more influenced by freshwater.

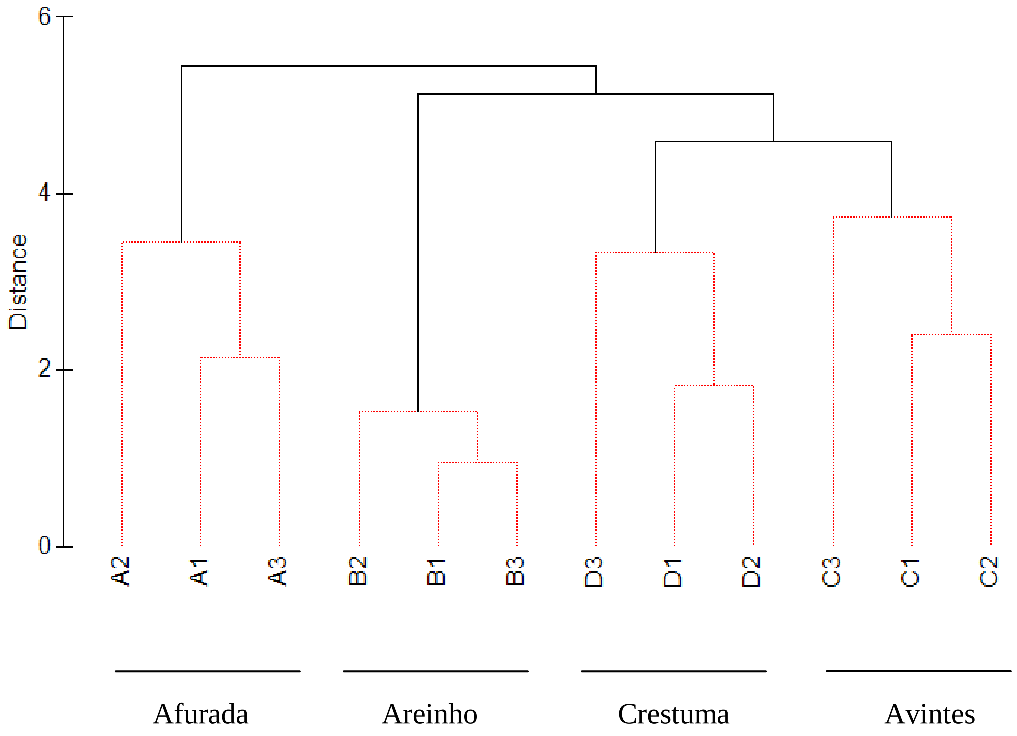


Figure 13 – Dendogram generated from hierarchical analysis based on Euclidean distances calculated for log (X+1) transformed environmental and biogeochemical data and using Simprof test to verify differences between clusters generated. Red branches grouped the most similar samples, according to Simprof test.

3.4 – SEDIMENT MICROBIAL COMMUNITY ANALYSIS

3.4.1 – Sediment total cell counts

The number of prokaryotic cells, varied in average between 9.82 x 10^7 (Crestuma) and 1.94 x 10^8 (Areinho), without being significantly different along the different sampling stations (one way ANOVA, $p > 0.05$, $n = 12$) (Figure 14). Values of TCC were positively correlated with NH_4^+ fluxes and with NH_4^+ interstitial water concentration ($r = 0.63$, $p = 0.027$; $r = 0.71$, $p = 0.009$, respectively, $n = 12$).

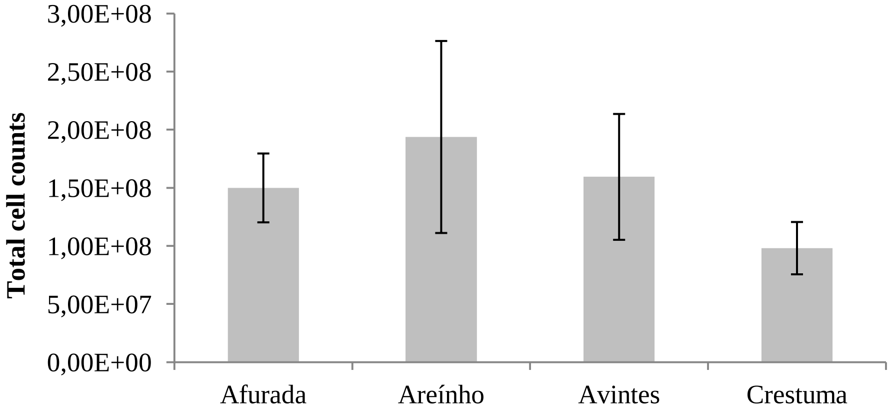


Figure 13 - Mean and standard deviations of total cell counts (TCC) stained by DAPI at the different sampling stations along the salinity gradient of the Douro estuary.

3.4.2 – Bacterial diversity

The DGGE profiles of Bacterial 16S rRNA gene fragments revealed clearly higher similarity between samples from the same site than between different sampling sites (Figure 15).

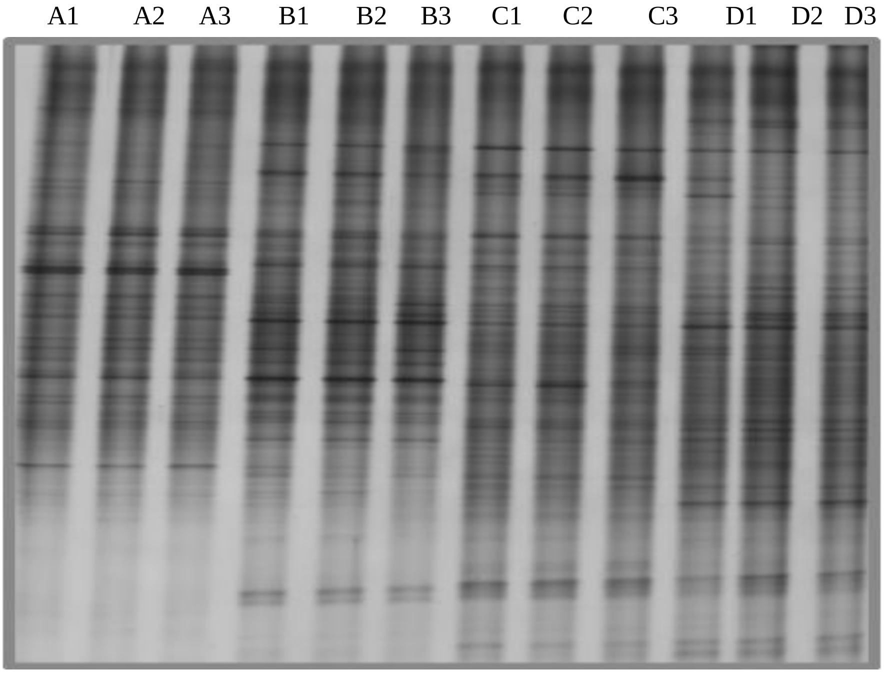


Figure 14 - Image of the DGGE gel containing 16S rRNA gene fragments amplified for Bacteria. Beginning from the left to the right the codes refers to: Afurada (A1, A2 and A3), Areinho (B1, B2 and B3), Avintes (C1, C2 and C3) and Crestuma (D1, D2 and D3).

The HCA analysis (Figure 16) performed, based on the computation of distance values from the absence/presence DGGE matrix, revealed that samples within each sampling site were more similar to each other than between samples from the different stations. Additionally, ANOSIM analysis, revealed a global R value equal to 1 ($p < 0,01$), indicating significantly different Bacteria communities diversity between the different stations located along the salinity gradient.

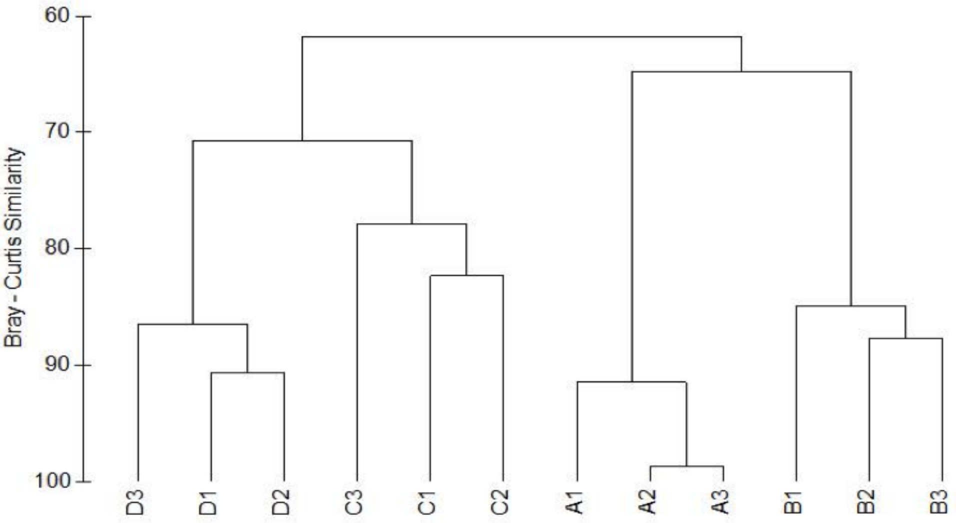


Figure 16 - Bacteria hierarchical cluster analysis based on group average linking of Bray – Curtis similarity, using the presence or absence of DGGE bands, from each sampling site sediment, as input variables. The coding letters refers to: Afurada (A1, A2 and A3), Areinho (B1, B2 and B3), Avintes (C1, C2 and C3) and Crestuma (D1, D2 and D3).

Despite the high similarity between samples (60% of similarity), four clusters were formed sharing 75% of similarity. While the examination of the R value for each pairwise comparison between the stations was 1, ($R = 1$, for all pairwise comparisons), indicative of a complete separation of the groups generated, the test was not statistically significant ($p = 0.1$). This is a result of having a low number of replicates: while R calculations are not affected by the number of replicates between the groups, the statistical significance is affected. Since we only have 3 replicates within each group, the test can only do 10 permutations, being unable to have a significance level better than 0.1. This was true for the further ANOSIM tests performed.

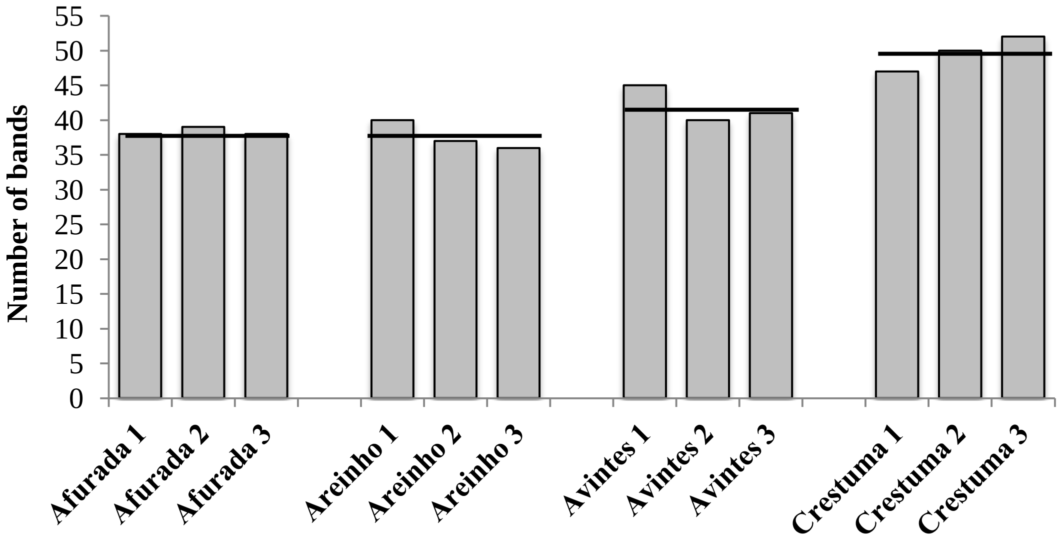


Figure 16 - Bacteria richness calculated as the number of bands in the DGGE profile at the three sediment sub-sample sites of each station. The bars indicate mean values for each station.

Based on the number of bands retrieved from the DGGE profile, and assuming that one band is relative to one phylotype, community richness was calculated, and results revealed a tendency for bacterial richness increase with the decrease of salinity (Figure 17). Bacterial richness was observed in Crestuma was significantly higher when compared to Avintes, Areinho and Afurada (one-way ANOVA, $p = 0.0004$, $n = 12$). No significant differences were observed between Afurada, Areinho and Avintes (one way ANOVA, Tuckey test, $p > 0.05$, $n = 12$).

3.4.3 – Archaea Diversity

Archaea DGGE profile showed a clear differentiation in the band pattern as we get closer to the mouth of the estuary (A1, A2 and A3). Another feature is the appearance of unique bands in specific stations, which may indicate specificity of some Archaea groups to the environmental characteristics, intrinsic to each station along the Douro estuary. However, there are also some bands that appear across all sampling sites (Figure 18)

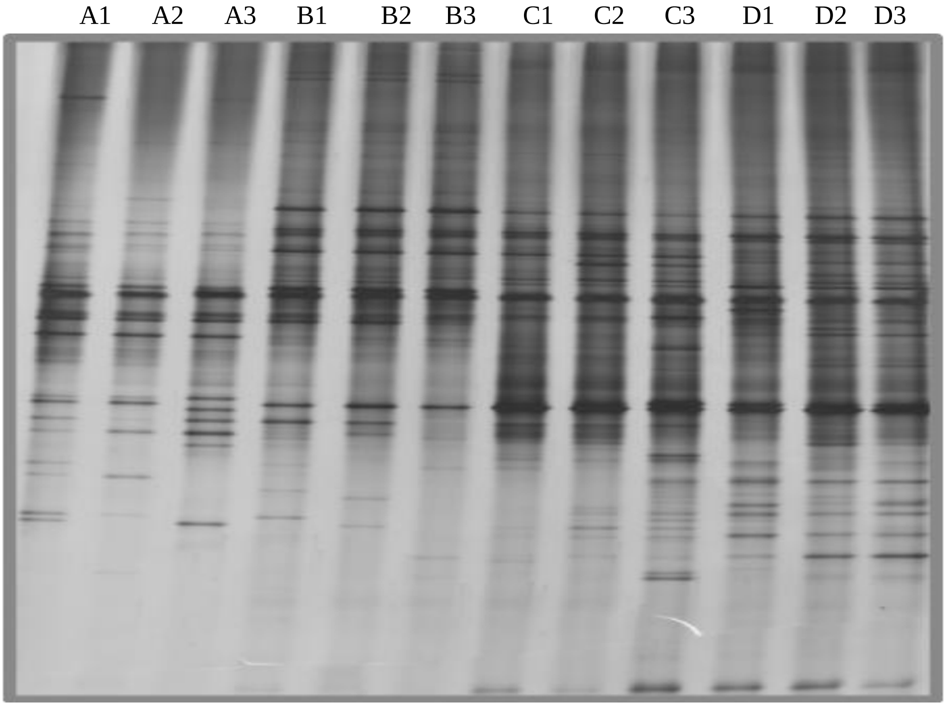


Figure 17 - Image of the DGGE gel containing 16S rRNA gene fragments amplified for Archaea. Beginning from the left to the right the codes refers to sediments from: Afurada (A1, A2 and A3), Areinho (B1, B2 and B3), Avintes (C1, C2 and C3) and Crestuma (D1, D2 and D3).

As it was observed in the bacterial profiles, samples from the same sampling site tended to group in the same cluster, presenting the highest similarity values of banding pattern (Figure 19). Samples from the three sub-sites of Afurada grouped with each other demonstrating that Archaea communities are very similar within the same station. This site is the one who first stands out from the dendogram, suggesting a clear differentiation between Archaea communities from Afurada and the ones located upstream, with less than 40% of similarity. The same pattern occurs in Areinho, however this site starts to be more related with Avintes and Crestuma, showed by the higher similarity with these stations. The similarity pattern between the two most upstream sites was very high, enough to group samples in the same cluster at a similarity level of 60%. This indicates that Archaea diversity in Avintes and Crestuma stations might be regulated by environmental parameters that are quite similar between those two sites, selecting the same type of communities, turning the DGGE bands profiles similar.

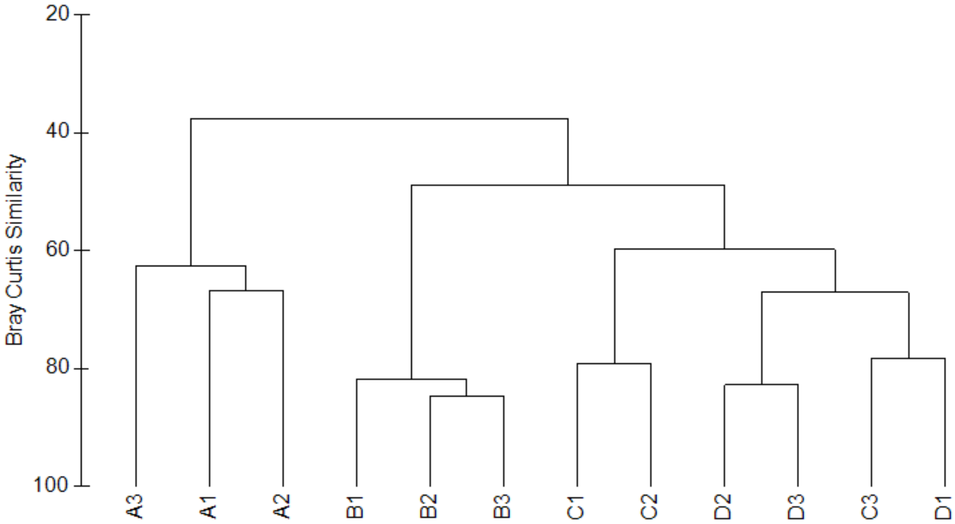


Figure 19 - Archaea hierarchical cluster analysis based on group average linking of Bray – Curtis similarity, using the presence or absence of DGGE bands, from each sampling site sediment, as input variables. The code refers to: Afurada (A1, A2 and A3), Areinho (B1, B2 and B3), Avintes (C1, C2 and C3) and Crestuma (D1, D2 and D3).

ANOSIM analyses showed that the ordination of the samples in relation to their sampling sites differs significantly with a global R = 0.957 ($p < 0.01$). Pairwise comparisons showed R values equal to 1 with the exception of the comparison between Avintes and Crestuma, R = 0.778, p = 0.1. These results are in accordance with the HCA where samples from Avintes (C3) were mixed with the ones from Crestuma (D1), as previously mentioned.

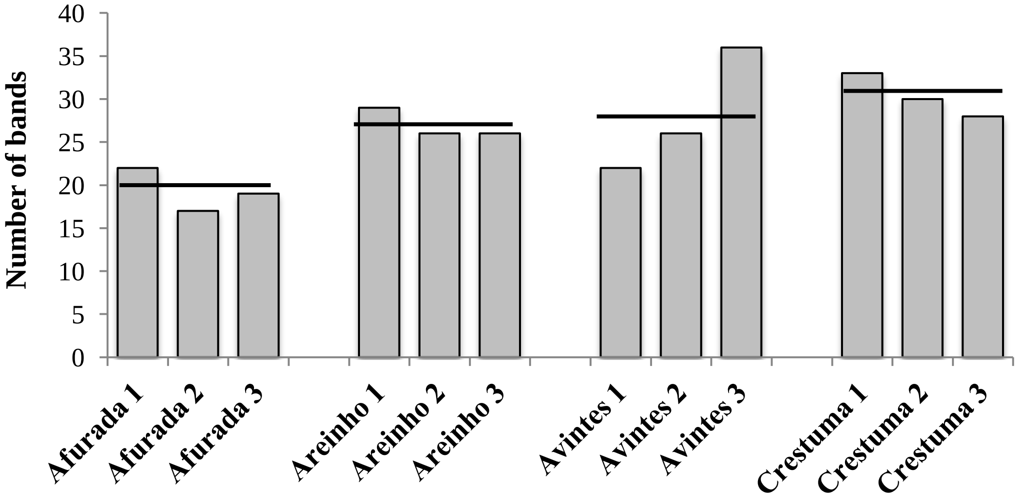


Figure 19 - Archaea richness at each sampling site sediment calculated as the number of bands in each DGGE profile at the three sub-sample sites of each station. The bars indicate mean values for each station.

Results showed that Archaea richness tended to decrease with the proximity of the estuary mouth, however there were no significant differences along the sampling sites (one way ANOVA, p = 0.05, n = 12). Despite these results, the p test is on the threshold limit. Performing the Tuckey test there are significant differences between Crestuma and Afurada (p = 0.045, n = 12).

3.4.4 – Archaeal Ammonia Oxidizers Diversity

The diversity of ammonia oxidizing Archaea (AOA) *amoA* along the salinity gradient of Douro estuary is showed in the DGGE gel displayed above (Figure 21). In agreement with to what was found for Bacteria and Archaea, AOA DGGE profile showed the occurrence of unique bands in different stations and a clear differentiation on the AOA profiles between the most extreme stations in terms of the salinity gradient (Figure 21).

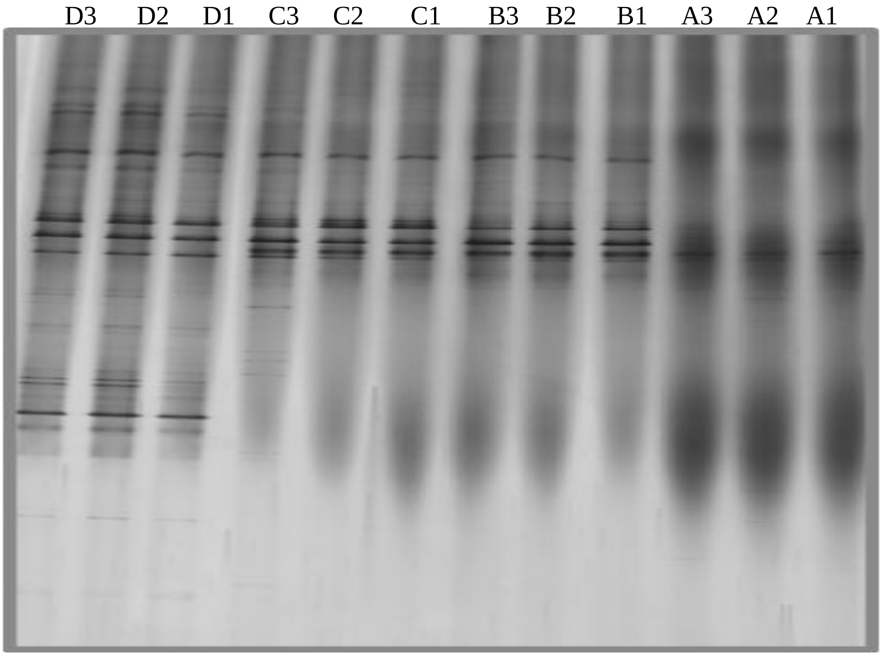


Figure 21 - Image of the DGGE gel containing *amoA* fragments amplified from Archaea. In the gel, beginning from the right to the left, the codes refers to Afurada (first three bands at the right side – A1, A2 and A3), Areinho (B1, B2 and B3), Avintes (C1, C2 and C3) and Crestuma (last three bands of the left side – D1, D2 and D3).

The low PCR product recovered in the Afurada samples (Figure 22) led to a higher load of amplicons, which in turns promoted an higher input of PCR non-specific compounds, being a possible cause of the appearance of the darkest background in those DGGE profiles (Figure 21).

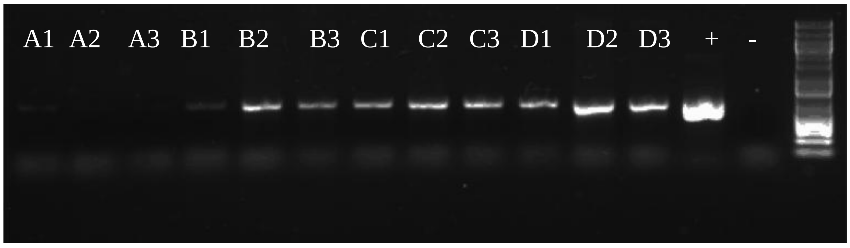


Figure 21 - Agarose gel with Archaeal *amoA* amplification. Five µL were loaded into an agarose gel (2%) stained with gelRad and marked with 1Kb ladder.

The HCA of ammonia oxidizing Archaea, based on the presence/absence of DGGE bands gel profile, is shown in Figure 23.

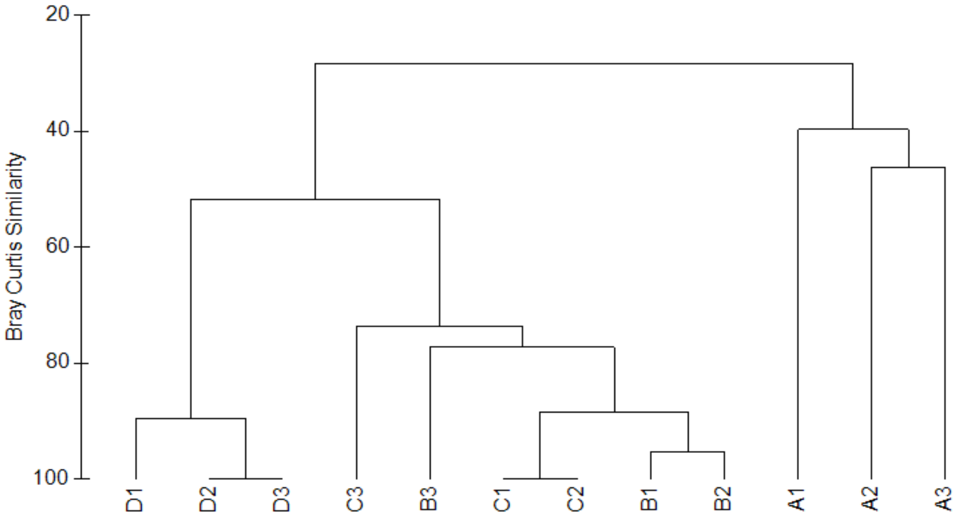


Figure 23 – Archaea *amoA* gene hierarchical cluster analysis based on group average linking of Bray–Curtis similarity, using the presence or absence of DGGE bands, from each sediment sampling site, as input variables. The code refers to: Afurada (A1, A2 and A3), Areinho (B1, B2 and B3), Avintes (C1, C2 and C3) and Crestuma (D1, D2 and D3).

HCA generated three different clusters, where samples from Areinho and Avintes were mixed with each other (Figure 23). The DGGE patterns of those samples were very similar, which may be explained by similarities of some the environmental conditions.

The samples ordination into their sampling locations, was significant by ANOSIM analysis with a global R = 0.684 and a significance p < 0.01. While pairwise comparisons showed R values equal to 1 between Afurada and Crestuma or between the last station and Avintes and Areinho the low R value obtained for Avintes and Areinho (R = 0.37, p = 0.1), revealed a very similar diversity pattern of *amoA* diversity between those two stations.

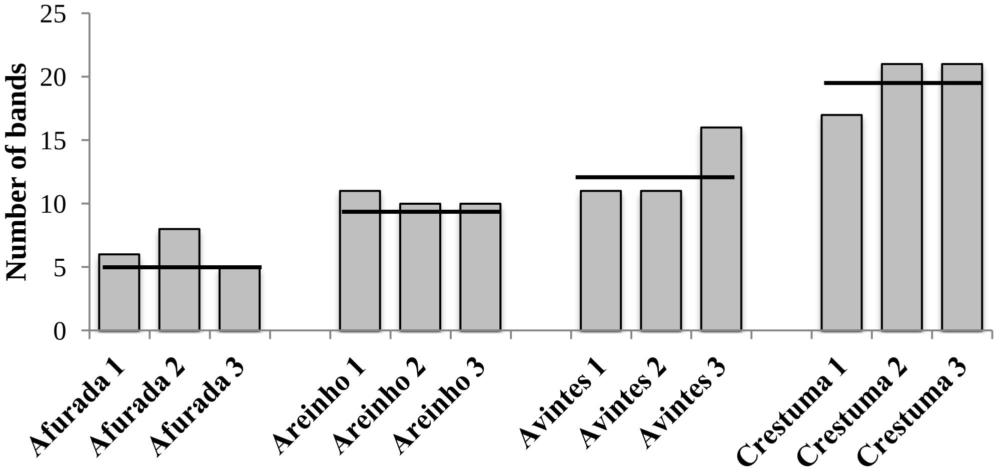


Figure 23 - Archaeal ammonia oxidizing amoA richness at each sampling site calculated as the number of bands in each DGGE profile at the three sub-sample sites of each station. The bars indicate mean values for each station.

AOA *amoA* richness (figure 24) was found to decrease significantly with the proximity to the estuary mouth (one-way ANOVA, p = 0.0002, n = 12). The main differences were between Crestuma and all downstream stations and between Afurada and Avintes. No significant differences were found between Areinho and Avintes or Areinho and Afurada (Tuckey test, p > 0.05, n =12).

3.5 – MICROBIAL DIVERSITY VS ENVIRONMENTAL DRIVERS

Relationships between Bacteria, Archaea and AOA assemblage composition and the environmental variables and the biogeochemical processes measured were established by plotting those parameters on MDS (Multidimensional Data Scaling) ordination diagrams (Figures 26, 27 and 28). In agreement with cluster analysis (Figures 16, 19 and 23), nonmetric MDS of the different groups of microorganisms analyzed (using the presence or absence of DGGE bands from all sample profiles as input variables) identified differences in the distribution of these prokaryotes assemblages among different stations located within the salinity gradient of the Douro estuary (Figures 26, 27 and 28).

Salinity was found to have a negative effect on the richness of all microbial groups analyzed (Figures 25a and 25b and 25c). In what Bacteria is respected, a logarithmic relationship was established between their richness and salinity (R^{2} = = 0.5, $p < 0.05$, n =12) (Figure 25a) and for Archaea and AOA richness a linear significant and negative relationship was established with salinity ($R^2 = 0.59$, $R^2 = 0.63$; n = 12; $p < 0.05$; respectively for Archaea and AOA) (Figures 25 b and c respectively). These results suggested salinity as an important factor that contributed to microbial richness variability.

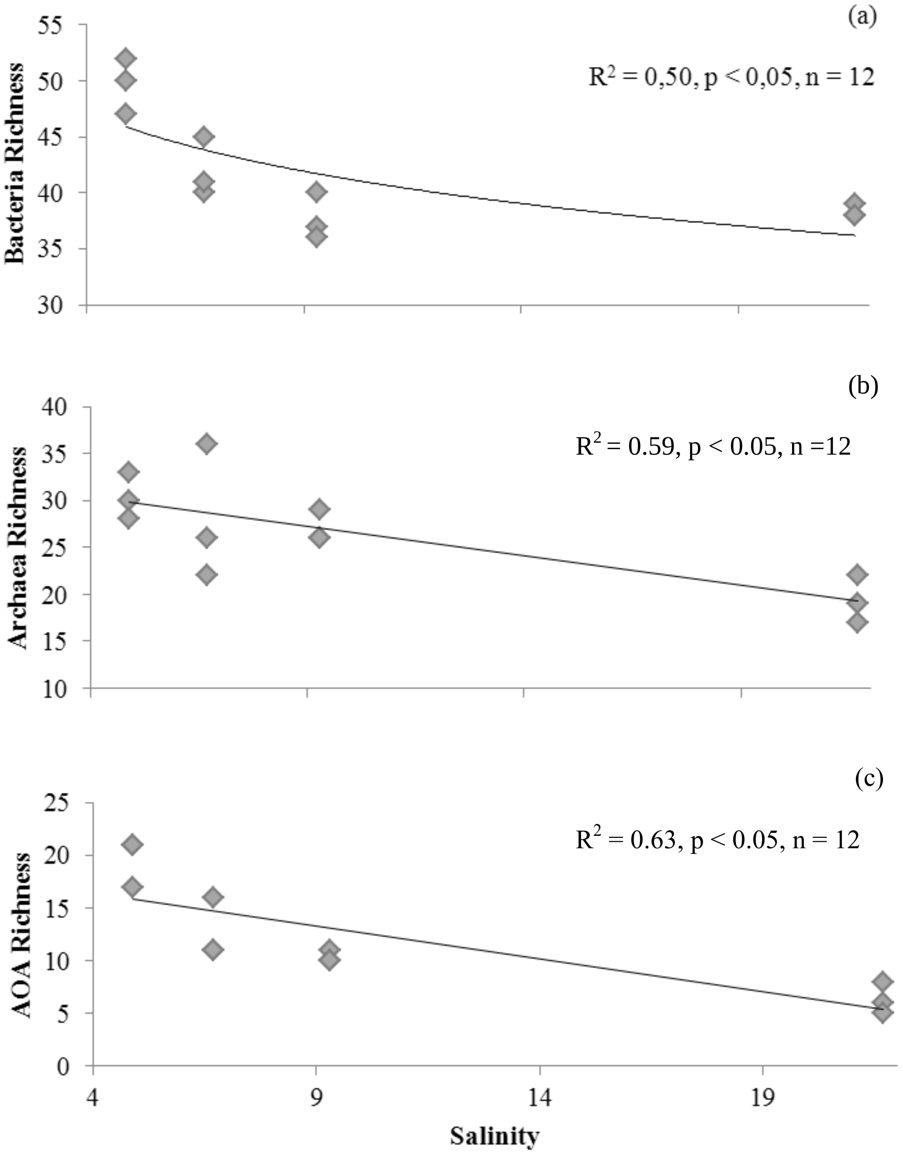


Figure 24 - Relationship between salinity and Bacteria (a), Archaea (b) and Archaea ammonia oxidizing (c) richness.

Temperature, higher concentrations of NO_3^- in the interstitial water, its net fluxes and the percentage of fine sands also correlated positively for the Bacterial richness (r =0.74, r= 0.70, r =0.86 and r = 0.68 respectively, $p < 0.05$, n = 12), whereas NH_4^+ interstitial water concentrations was negatively correlated (r = -0.62, $p < 0.05$, n =12) (Figure 26).

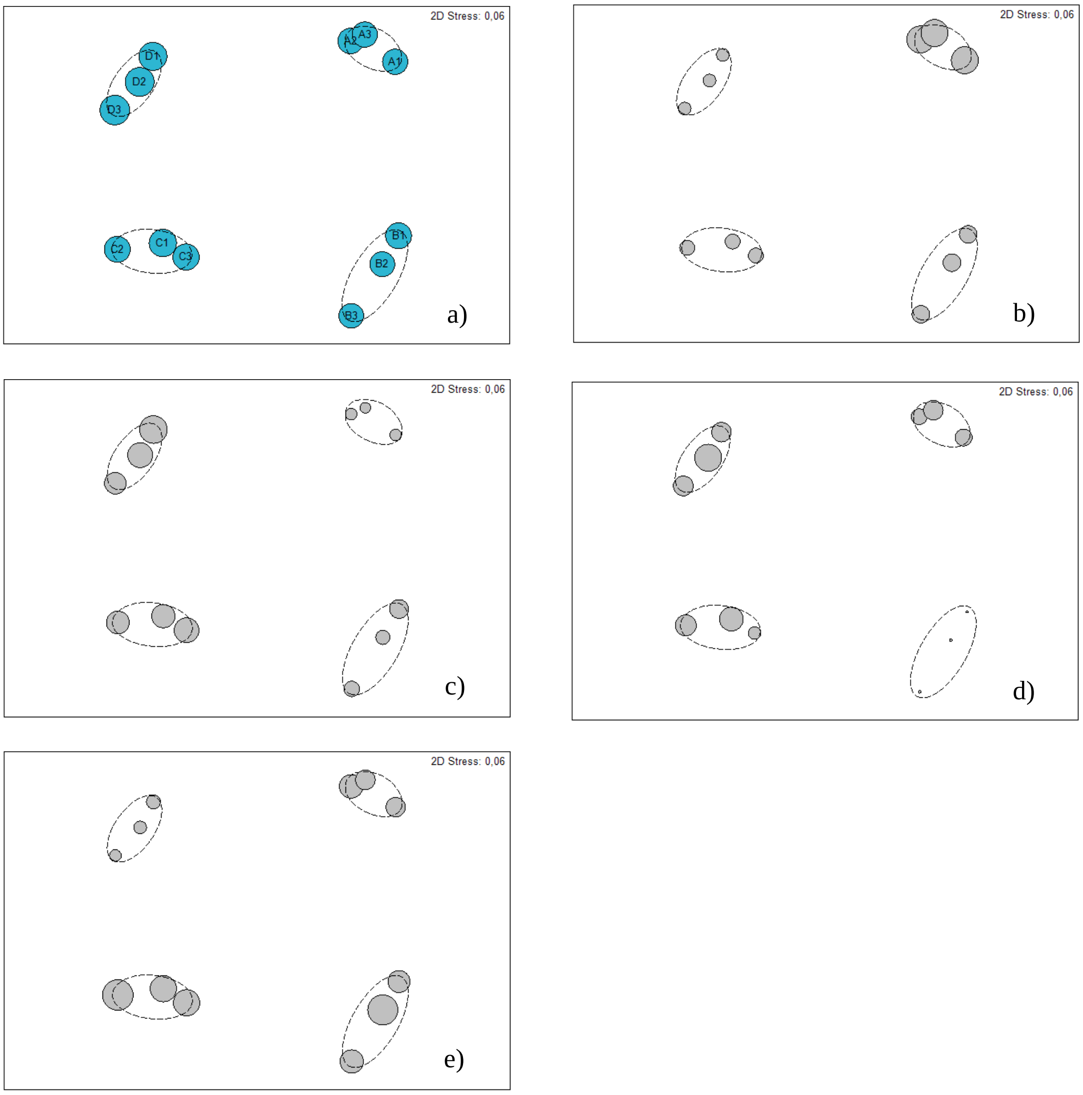


Figure 25 - Non-metric multidimensional scaling (MDS) ordination of the sites using single linkage of Bray–Curtis similarities using the presence or absence of Bacteria DGGE bands as input variables. Stress value = 0.06 for all panels. Large ovals indicate discrete groups of samples referred in the text, which were grouped within 75% of similarity. a) through e) are the values of environmental variables for each sample, being represented as circles which diameters are scaled linearly to the magnitude of the value: richness of the sample in each site (a); salinity (b); NO_3^- concentrations in the interstitial water (c); percentage of fine sediments (d) and NH_4^+ in the interstitial water (e).

Besides salinity, differences in NO_3^- concentration between each site may also have an influence on Archaeal diversity ($r= 0.69$, $p < 0.05$, $n = 12$) (Figure 27c). Indeed Crestuma and Avintes displayed similar high concentrations of this nutrient being the ones with higher similarities in terms of Archaea diversity. However, the availability of NO_3^- in the water column co-vary with salinity ($r = -0.83$, $n = 12$, $p < 0.05$) and a strong co-variation between the salinity was also achieved for temperature, which can also influence Archaea richness ($r = 0.76$, $p < 0.05$, $n = 12$). Interstitial water concentrations of NH_4^+, contrasting to Bacteria were not significantly related to the diversity of the Archaea domain.

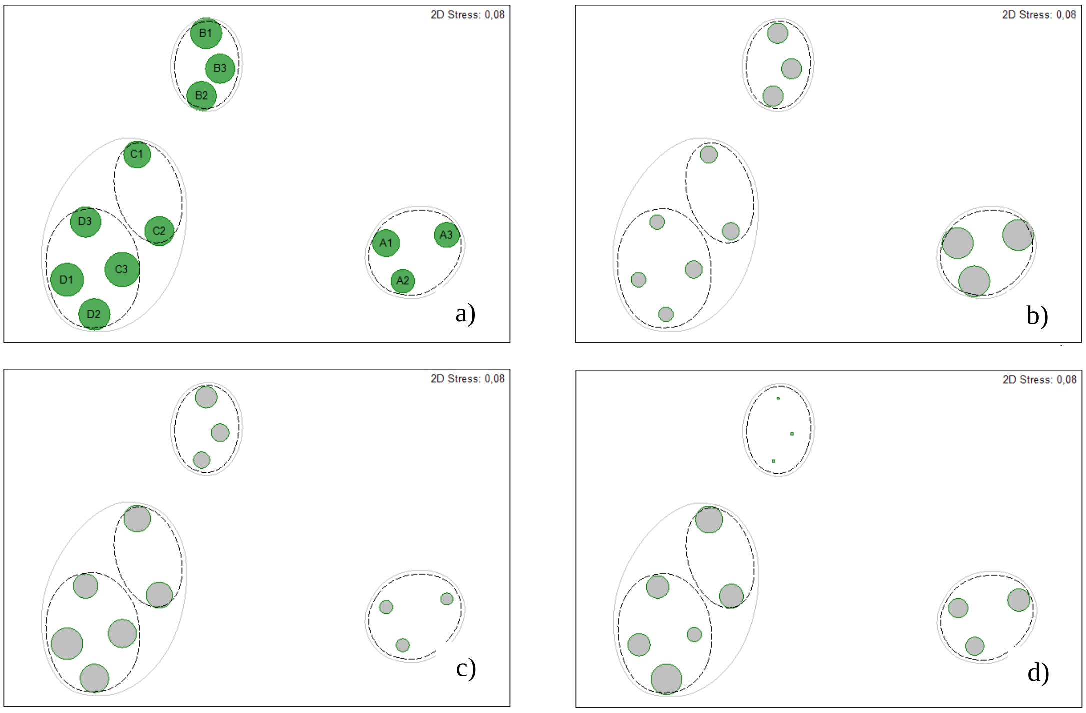


Figure 26 - Non-metric multidimensional scaling (MDS) ordination of the sites using single linkage of Bray-Curtis similarities using the presence or absence of Archaea DGGE bands as inputs variables. Stress values = 0.08 for all plots. Large ovals indicate discrete groups of samples referred in the text which were grouped within 50% and 60% of similarity. a) through d) are the values of environmental variables for each sample represented as circles of diameter scaled linearly to the magnitude of the value: richness of the samples in each site (a); salinity (b); NO_3^- present in the sediment slurries in the beginning of incubation (c) and percentage of fine sediments (d).

In respect to the spatial differences of *amoA* richness, Crestuma was the station which presented the higher richness values of this gene. It was also overlapped by the highest NO_2^- and NO_3^- effluxes (Figure 28e), being those positively correlated with Archaea ammonia-oxidizing richness ($r = 0.63$ and 0.73 respectively, $p < 0.05$, $n = 12$). In addition, NH_4^+ effluxes were correlated negatively with Archaea ammonia-oxidizing richness ($r = -0.77$, $p < 0.05$, $n = 12$) (Figure 28f). In opposition, the high saline site, Afurada, was characterized by the highest net NH_4^+ effluxes and by higher interstitial water concentration of this nutrient, presenting the lowest richness values. Samples from this site appear much dispersed between themselves, which might be due to the low defined band pattern in the gel leading to a more difficult interpretation during the gel bands analysis.

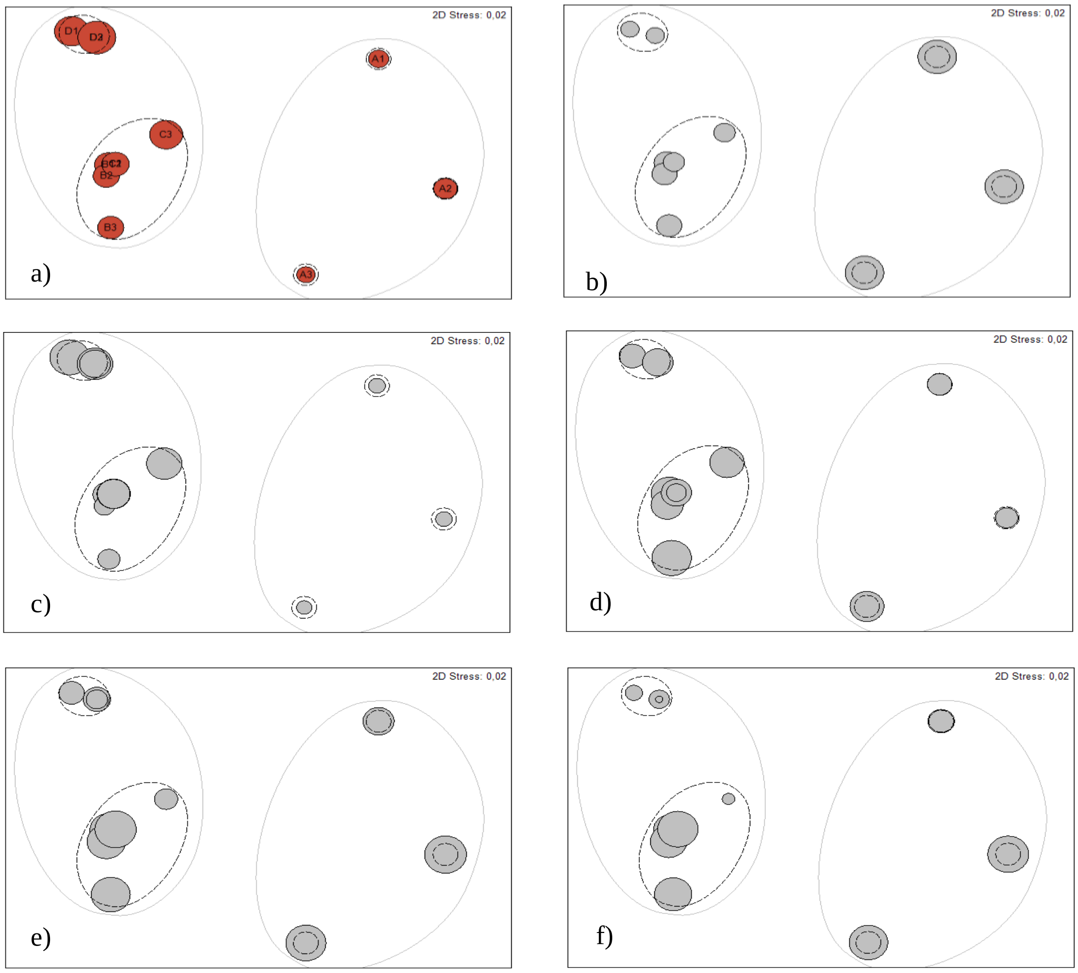


Figure 27 - Non-metric multidimensional scaling (MDS) ordination of the sites using single linkage of Bray- Curtis similarities and the presence or absence of AOA DGGE bands as inputs variables. Stress values = 0.02 for all plots. Large ovals indicate discrete groups of samples referred in the text which were grouped within 40% and 60% of similarity. a) through f) are the values of environmental variables for each sample represented as circles of diameter scaled linearly to the magnitude of the value: richness of the sample in each site (a); salinity (b); NO_3^- present in the sediment (c); nitrification rates (d); NO_3^- effluxes (e) and NH_4^+ effluxes (f) (in order to not having negative values of the fluxes, that are relative to nutrient influxes, a constant was added to all negative data).

Nitrification rates were not correlated with AOA richness ($p > 0.05$, n =12), however at the sites where nitrification rates were higher (Areinho and Avintes), and where the interstitial water concentration of NH_4^+ was also higher, the *amoA* diversity was very similar (Figure 28d and f).

Correlations between environmental variables and richness of all prokaryotic groups analyzed were also examined using redundancy analysis (RDA) (Figure 29).

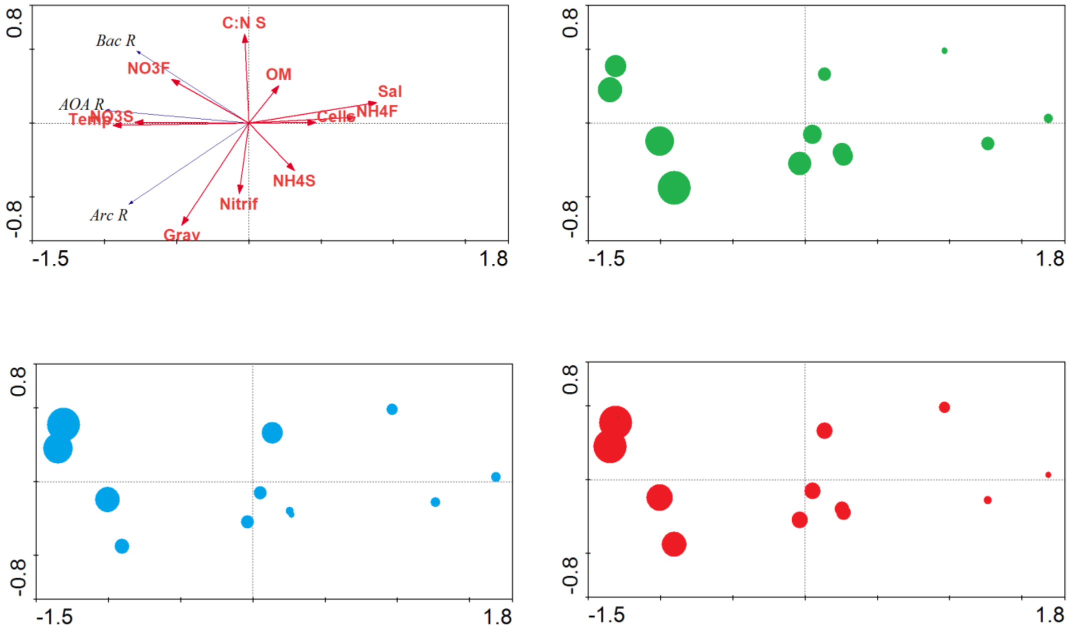


Figure 28 - Redundancy analysis ordination (RDA) plot for the environmental variables and richness of the different group of microorganisms analyzed. The environmental variables analyzed were the following: fluxes of NO_3^- and NH_4^+, NO_3^- and NH_4^+ interstitial water concentrations, C:N in sediments, water temperature and salinity, TCC, and percentage of gravel in sediments. In terms of richness green bubbles refer to Archaea, blue bubbles refer to Bacteria and red bubbles refer to ammonia oxidizing Archaea.

The first gradient (RDA 1, horizontal) explained 90.8% of the total richness variability and was well correlated with the environmental data, suggesting that the data set is governed by a single dominant gradient represented by RDA 1 (horizontal). The RDA projection of the environmental variables revealed that the RDA 1 axis is negatively correlated with temperature, net NO_3^- fluxes and NO_3^- concentrations in the interstitial water, percentage of gravel in the sediments, nitrification and C:N ratio in the sediments. In the other hand, RDA 1 is positively correlated with organic matter, salinity, total cell counts, net NH_4^+ fluxes and NH_4^+ concentrations in the interstitial water (Figure 29). The correlation matrix generated by RDA analysis confirmed that relationships of all measured environmental variables with the second axis (RDA 2, vertical) were rather weak. This analysis was resolved by Monte-Carlo test of F-ratios.

The position of the individual richness data showed that higher diversity (richness) of Bacteria, Archaea and AOA are favored by the decrease of salinity (Figure 29). However, in terms of total cell accounts of prokaryotic organisms, the numbers seems to be higher at higher salinities and where high magnitudes of net NH_4^+ fluxes, and high NH_4^+ concentrations in the interstitial water were registered (still the results of this parameter were not statistical significant along the sampling stations). Besides salinity, the richness of all prokaryotic communities was also influenced by temperature. Bacteria and Archaea richness was also influenced by NO_3^- concentration in the interstitial water and the diversity of Archaea communities by the percentage of gravel in the sediments (Figure 29).

Variability in AOA richness was strongly influenced by net NO_3^- fluxes, being more diverse in the site where an efflux of this nutrient was registered, and lower NH_4^+ fluxes and interstitial water concentration were registered as well (Figure 29). Thus, with a similar pattern as salinity, NH_4^+ effluxes by the sediments have a negative influence on the diversity of this group of organisms.

CHAPTER 4 - DISCUSSION

“The secret of genius is to carry the spirit of the child into old age, which means never losing your enthusiasm.”
Aldous Huxley

Estuaries are the interfaces between land and coastal sea, through which pass all material leached from land into the rivers, as well as anthropogenic discharges, being highly dynamic, productive and impacted systems (Nixon et al., 1986; Bricker et al., 2008). Such dynamism is reflected in constant changes of nutrient concentrations, tidal currents, and salinity (Bernhard et al., 2005), which is causing stress in the biotic organisms, including the microbial communities, that inhabit those transitional environments. Among the abiotic factors, salinity has been shown to affect the distribution patterns, diversity and activity of microbial communities (Crump et al., 2004; Hewson and Furhman, 2004; Herlemann et al., 2011; Campbell and Kirchman, 2013), inducing mortality (Painchaud et al., 1995) or adaptation, once many communities are either adapted to life in saltwater or freshwater conditions (Bordalo et al., 1993; Painchaud et al., 1995; Herlemann et al., 2011). Furthermore, changes in salinity also affect chemical constituents. In fact, as nutrients pass along the salinity gradient, they are exposed to a number of physical, chemical and biological processes such as adsorption, precipitation, dissolution, flocculation, regeneration, biological uptake, among others (Eyre and Balls, 1999). Therefore, the different physical and chemical properties of the estuaries are affected and have an effect by/in the local microbial activity.

4.1- DYNAMICS OF NITROGEN PROCESSES

Nutrients, especially nitrogen (N), strongly influence the productivity and environmental quality of estuaries. Once N biogeochemical processes influence the inorganic nitrogen budget in estuaries, it is very important to understand the role of environmental factors controlling those processes (Ogilvie et al., 1997; Eyre and Balls 1999; Rysgaard et al., 1999). Specifically, nitrification is a microbial mediated process that plays a central role in the global cycling of N having a great economic importance in agriculture and wastewater treatment. It ensures the conversion of NH_4^+ (derived from organic N during decomposition and mineralization processes) into the oxidized and more soluble form of NO_3^-, providing the substrate for denitrification, which has the ability to return N back to the atmosphere. Ammonia can be nitrified in the sediments and in the water column, being this process in nature controlled by a range of environmental variables such as: temperature, salinity, dissolved oxygen (DO) concentrations, NH_4^+ availability, light and sulfide concentrations (Caffrey et al., 2007b and references therein). The transformation of N into its oxidation states is a key process to ensure productivity in the biosphere, being highly dependent on the activities of a diverse assemblage of microorganisms (Bernhard et al., 2010a).

Our results showed that along the Douro estuary there is a split between Crestuma, the more upstream site, and the more downstream sites, in terms of N biogeochemistry. Crestuma is much more influenced by freshwater, and once the estuary has been undergoing from freshwater NO_3^- inputs (Magalhães et al., 2005a), this site showed higher concentrations of this nutrient, both in water column and interstitial water. In fact, along the sampling sites water column NO_3^- and NO_2^- concentrations were inversely related with salinity, previously described as being part of dilution mechanisms rather than an *in situ* processes of regeneration (Magalhães et al., 2005a). Contrasting to NO_3^-, water column NH_4^+ concentrations were positively related with the salinity. In agreement, interstitial water NH_4^+ concentrations on freshwater site (Crestuma) were low increasing in the mid-estuarine sites (Areinho and Avintes) and in Afurada. These results suggested the occurrence of NH_4^+ sources and/or internal inputs in the lower and middle Douro estuary. In fact, the human-induced contamination in these areas of the Douro estuary has been described (Azevedo et al., 2008; Magalhães et al., 2008), and it was previously found that they may enhance respiration, contributing to the heterotrophy situation observed all year round in the pelagic metabolism of the lower and middle stretches of the estuary (Azevedo et al., 2006). It was also reported that large mineralization potential sustained by a microbial community can be triggered by the input of particulate and dissolved organic material introduced trough river course or wave tidal pumping (Franke et al., 2006). In the mid-estuarine sites (Areinho and Avintes) and Afurada, where higher interstitial water NH_4^+ concentrations were measured, it was also detected higher numbers of total cell accounts, which may be related with the high organic carbon (C) and N input in these areas of the estuary (Bolatek and Graça, 1996; Magalhães et al., 2008).

General positive net NH_4^+ fluxes were registered between the sediment and water column interface, along the salinity gradient of the Douro estuary. However, rates tend to be lower in the most upstream site (Crestuma). This trend is in agreement with previous studies performed on the influence of salinity in the desorption/adsorption processes of NH_4^+ in the sediments, by describing a stimulation of NH_4^+ desorption in more saline environments (Boatman and Murray, 1982; Gardner et al., 1991). Fluxes of NH_4^+ are also dependent on the type of sediment, more precisely, on the fraction of fine particles, with higher effluxes typically occurring in silty or sludgy silt sediments, which is related to higher organic matter content on these type of sediments (Bolatek and Graça, 1996). Moreover, salinity can affect nitrifier and denitrifier communitiy diversity and activity which may alter the efficiency by which N is transformed (Caffrey et al., 2003; Bernhard et al., 2007; Magalhães et al., 2005a, 2007, 2009; Santoro et al., 2008). Net fluxes of NH_4^+ are also highly influenced by the presence/absence of light (Magalhães et al., 2002; 2005b). Magalhães et al., (2005b) showed distinct inorganic nutrient dynamics between the water column and sediments according to the different phase of the day (daylight or dark). Higher percentages of organic matter in the sediments might also stimulate H_2S production which inhibits nitrification (Joye and Hollibaugh, 1995; Magalhães et al., 2002). In addition, higher effluxes of NH_4^+ could also be supported by dissimilatory NO_3^- reduction to NH_4^+ (DNRA) (Magalhães et al., 2005b). In the other hand, this release of NH_4^+ to the system, can potentiate estuarine primary productivity influencing the structure of communities of higher trophic levels (Kemp et al., 2005), which in turn can increase nitrogen assimilation and decrease O_2 concentrations, which may again enhance the dissimilatory reduction of NO_3^-

Higher denitrification rates were related with the increase of NO_3^- concentrations, which enhanced the importance of this nutrient as a key regulator of denitrification (Magalhães et al., 2005a). However, in our case, the site that presented higher NO_3^- concentrations in interstitial water (Crestuma) also showed a release of this nutrient to the water column, suggesting that denitrification and other processes involved in NO_3^- uptake were saturated in terms of subtract availability, and that can explain why NO_3^- tend to accumulate within the sediments, at this station. An opposite scenario was observed in the three downstream stations, where a general net influx of NO_3^- was measured. Differences in the net NO_3^- fluxes along the gradient of salinity may reflect differences on the activity of the sediment microbial communities (Magalhães et al. 2005a). In fact, a general uptake of NO_3^- into sediments has also been described in previous studies performed in the Douro estuary, and attributed to microbial NO_3^- removal instead of primary producers assimilation (Magalhães et al. 2002; Magalhães et al., 2005b). Concentrations of NO_2^- in coastal ecosystems tend to be low, since it is rarely accumulated during the processes of its formation, acting as an intermediate of nitrification, denitrification and DNRA. Indeed, NO_2^- concentrations along Douro River estuary were low which is in agreement with previous measurements in similar locations (Magalhães et al., 2002, 2005a, 2005b). The general influx of NO_3^- and net NH_4^+ effluxes observed along the salinity gradient could be attributed to denitrification (Magalhães et al., 2005b) and/or DNRA (Magalhães et al., 2005b; Giblin et al., 2010), once at those sites NH_4^+ is being released, with the exception of Crestuma, where occurred a small efflux of NO_2^- and NO_3^-. At the Crestuma station, the positive correlation between net NO_3^- and NO_2^- fluxes might indicate that at this site higher rates of nitrification might be occurring. Still, those assumptions are not in agreement with our nitrification results and beside being in much lower intensity, NH_4^+ is being released to the interstitial water instead of being consumed (as it was expected to occur with nitrification). Thus low effluxes of NH_4^+ and their low concentrations in interstitial water might be a product of lower N-mineralization processes.

The general NH_4^+ efflux to the water column observed in our flux measurements may be indicative of a reduced nitrification processes, which is related to estuaries with high nutrient loads (Sloth et al., 1995; Burford & Longmore, 2001; Caffrey et al., 2007a) or it can suggest a link between nitrification rates and DNRA (Magalhães et al., 2005b). In our study, the consistent results both from acetylene inhibition method and isotopic measurements show higher magnitudes of nitrification in the stations located at intermediary salinities (Areinho and Avintes). Interestingly, previous studies also showed higher nitrification rates at intermediary salinities both in natural conditions and controlled experiments (Jones and Hood, 1980; Somville, 1984; Pakulski et al., 1995, Meyer et al., 2001; Magalhães et al., 2005a, Bernhard et al., 2007). Although, when this process is analyzed *in situ*, many factors can influence this process, for example, the inputs of NH_4^+ or its *in situ* regeneration (Pakulski et al., 1995). The stations which revealed higher nitrification rates were the ones where higher concentrations of NH_4^+ in the interstitial water were registered, thus this factor could indeed induce nitrifier communities to enhance their activity (Butturini et al., 2000, Magalhães et al., 2005a; 2007). Another explanation is that salinity effect can produce physiological changes in AOB and AOA populations or shifts in their community composition and/or abundance that resulted in higher activity rates (Magalhães et al., 2005a, 2009; Bock and Wagner, 2006; Bernhard et al., 2007; Santoro et al., 2008). Indeed, there are studies, which refer a high capability of nitrifiers to adapt to the salinity that is prevailing in the environment (Caffrey et al., 2003; Smith and Caffrey, 2009). In agreement to our results, Magalhães et al., (2005a) showed optimum benthic nitrification rates at intermediary salinities (15 psu). In addition, other culture experiments with estuarine isolates showed higher nitrification rates at salinities between 5-10 (Jones and Hood, 1980) or even between 0- 20 psu (MacFarlene and Herbert, 1984), with a subsequent reduction of the activity at higher salinities. Those results might reflect an intrinsic characteristic of ammonia oxidizer physiology to better adapt to those salinities (Bernhard et al., 2007).

Furthermore, our results showed lower nitrification rates at the highest saline site (Afurada), where higher NH_4^+ effluxes were observed, which can be a result of an ion exchange increase that would reduce the NH_4^+ residence time within the sediment, being less accessible for nitrifiers (Gardner et al., 1991; Rysgaard et al., 1999). Thus, at this site NH_4^+ would diffuse out of the sediment before nitrification could take place. Salinity and NH_4^+ availability together were previously related with nitrification (Magalhães et al., 2005a) however, being our study performed in a natural system where many variables are acting, the individually effect of those two parameters might be masked. The only environmental parameter that had significant correlation with nitrification rates was the grain size of the sediments. Indeed, sediments with higher grain sizes allow more oxygen diffusion within the sediments, which can enhance nitrification process (Henriksen et al., 1981; Henriksen and Kemp, 1986; Rysgaard et al., 1994). In fact, the oxygen availability is referred as an important variable that relates both Archaeal and Bacterial nitrifier communities in the environment (Caffrey et al., 2007b, Erguder et al., 2009; Hatzenpichler, 2012). Moreover, the type of sediments can also influence the processes through specific adsorptive properties; different diffusive coefficients or even the occurrence of aerobic and/or anaerobic microsites influencing the prevalence of the different processes involved in the recycling of N (Roswall, 1981; Sloth et al., 1995).

While the two methodologies used to estimate nitrifications rates within the salinity gradient of Douro estuary gave us similar relative results between stations, the absolute rates were found to be very discrepant. It must be emphasized, however, that ^{15}N measurements were done in undisturbed cores and with an initial addition of $^{15}NH_4^+$, which could induce higher magnitudes of nitrifier activity. Moreover ^{15}N methodology is a much more sensitive approach (Dugdale and Goering, 1967; Santoro et al., 2010). Besides acetylene had a great inhibitory effect on nitrification (Sahrawat et al., 1987), it has been shown that in some cases the usually low concentrations used are not sufficient to total inhibit nitrification (Garrido et al., 2000). Moreover, it was found that this compound did not inhibit heterotrophic nitrification (Schimel et al., 1984).

4.2 – MICROBIAL COMMUNITIES ALONG AN ESTUARINE SALINITY GRADIENT

4.2.1 Bacteria and Archaea diversity

Shifts in estuarine microbial communities are regulated by the ability of the community to overcome various environmental stresses (Bernhard et al., 2005). Among them, salinity and temperature appear to be the most important parameters in distinguishing aquatic communities over large spatial scales since many of the environmental factors studied co-vary with those two parameters (for example, dissolved oxygen, nutrients and several biogeochemical fluxes) (Fortunato et al., 2012). In fact, salinity has been suggested to be a major determinant of microbial community composition (Wu et al., 2006; Jeffries et al., 2012), exceeding the influence of temperature or pH (Lozupone and Knight, 2007), affecting Bacterial abundance and activity as well chemical constituents which may, in turn, affect changes in microbial communities (Prieur et al., 1987; Eyre et al., 1999; Revilla et al., 2000; Bernhard et al., 2005; Fortunato and Crump, 2011; Herlemann et al., 2011).

In agreement, our results from DGGE analysis of Bacterial 16S rRNA gene fragments revealed that the structure of Bacterial communities differed significantly between all sampling stations and that those differences were significantly related with the salinity gradient of the Douro estuary. In fact, a decrease of Bacteria richness was demonstrated to occur with the increase of salinity in more brackish waters (Benlloch et al., 2002; Fortunato et al., 2012; Jeffries et al., 2012 and references therein). However, Afurada and Areinho did not showed significant differences in

Bacteria richness, besides presenting differences in their communities. This suggested that communities from Afurada may be well adapted to high salinities whether communities from Areinho, being in a site influenced by shifts of salinity, have Bacterial communities with faster capabilities of adaptation to those shifts or are probably composed by communities from either freshwater and marine ones (Herlemann et al., 2011; Fortunato et al., 2012). In fact, a recent study using a metagenomic approach showed the occurrence of a shift in genes content of sediment microbial communities along the salinity gradient, announcing the acquisition, by microbial communities, of genes responsible for the adaption of those higher salinities (Jeffries et al., 2012). Thus, it seems that the salinity gradient is accompanied by an adjustment of the diversity and function of microbial communities, which may reflect different controls of estuarine biogeochemistry.

Along with salinity, the temperature increased due to the mix between saline and freshwaters which also had an influence on Bacterial communities. Our results are supported by other studies showing that Bacterial diversity in a given habitat is largely influences by temperature (Furhman et al., 2008), which also enhances Bacterial productivity (Shiah and Ducklow, 1994). Inorganic N concentration as NH_4^+ and NO_3^- also had great influence on Bacterial richness (Magalhães et al., 2007, 2008). While our results did not show any positive relation between NH_4^+ availability and Bacterial richness, higher interstitial water concentrations of NH_4^+ was observed at stations where higher TCC were detected, suggesting an influence of NH_4^+ in microbial abundance. Actually, the inputs of inorganic nutrients into the estuary from wastewater discharges could influence microbial biomass, being the NH_4^+ a source for bacterial assimilation (Revilla et al., 2000). In fact, NH_4^+, was mentioned to be preferred assimilated by phytoplankton and heterotrophic bacteria, once NO_3^- demands a higher energetic cost to be transformed into amino acids (Kirchman et al., 1992, Jorgensen et al., 1994). Ammonium is also a source of energy for nitrifying Bacteria and Archaea (Bernhard and Bollman, 2010a). On the other hand, the station where lower NH_4^+ concentrations were measured (Crestuma), presented higher Bacterial richness values and lower total cell numbers. Once NH_4^+ is limited in this site, and NO_3^- very abundant, those nutrient changes might induce differences in the structure of bacteria populations, once NO_3^- are less required but still might be used (Kirchman et al., 1992, Middelburg and Nieuwenhuize, 2000). In fact, nutrient concentrations along with temperature and salinity act as limiting factors for heterotrophic Bacteria as reported (Pomeroy and Wiebe, 2001). In this study we showed that the availability of nutrients in the estuary is influenced by salinity, and thus changes in the bioavailability of the nutrients is related to the gradient of salinity within the system.

From this study, there is no phylogenenic information about the Bacterial communities that inhabit the different stations along the salinity gradient, not being possible to ensure if there is a mix between riverine, marine and native estuarine phylotypes as it was demonstrated to occur in other studies (Crump et al., 1999; Herlemann et al., 2011). However, our Bacterial DGGE profiles revealed that certain bands consistently appear across all sites, which may be related to the fact that microorganisms tend to adapted to the shifts of the environmental parameters that characterized an estuarine system (Crump et al., 1999). In contrast, other DGGE bands appear only at specific locations, which can be translated in specific adaptations to some environmental constrains, that characterized each specific station along the estuarine gradient. In fact, previous studies reported differences between freshwater and marine microbial communities mainly because of the salinity effects and the required adaptations for the ability to cope with osmotic stress (Bernhard et al., 2005; Jeffries et al., 2012). Another parameter we must have into account is the relative low water residence time characteristic of the Douro estuary, which was reported to prevent the formation of adapted estuarine assemblages (Troussellier et al., 2002; Crump et al., 2007). In turn, it could be suggested that part of the genetic diversity may be a result of the interface position of the estuary where freshwater and marine Bacterial communities are mixed (Troussellier et al., 2002). Nevertheless, when considering ecological diversity and community structure, it is believed that species diversity is an important feature to maintain a certain degree of stability within the community (Leibold et al., 2004).

Only after the use of culture-independent molecular techniques, involving the amplification of 16S rRNA genes was shown that Archaea are not exclusively from extreme habitats but have an ubiquitous distribution, in significant numbers, in environments such as soils, marine plankton, sediments, mangrove and deep subsurface (DeLong, 1998; DeLong and Pace, 2001; Schleper et al., 2005; Pires et al., 2012). Our results showed a clear shift in Archaea 16S rRNA DGGE profiles between the different stations, demonstrating that salinity affected the structure of the community, as it was described in other studies (Oueriaghili et al., 2013). In fact, in agreement to what was observed for Bacteria, changes in Archaea community structure was observed along the salinity gradient, while no significant differences were registered for Archaea richness. Furthermore, as salinity increased, there was a disappearance of many phylotypes while others appeared specifically in the sites more downstream. This pattern was also shown in a salinity gradient in soils, where no differences in richness were achieved but sequencing analysis revealed a clear shift of Archaeal communities (Walsh et al., 2005). Beside we do not know what organisms our bands are related to, in a study performed by Abreu et al., (2001) in the Douro estuary, the presence of Euryarchaeotes, characterized by halophyles organism (ex: *Halobacterium* sp.) has been described. This previous study, also reported Archaea phylotypes from temperate estuarine sediments and deep-sea sediments, indicating an ability of those organisms to survive in a wide range of environments (Abreu et al., 2001). Beside the dissimilarities of the Archaea communities between sites, in Crestuma and Avintes the richness patterns are more similar, being those sites mostly characterized by lower salinities, higher temperatures and higher NO_3^- concentrations. In fact, it was found that Archaea can drive N cycle processes, being able to reduce NO_3^- by assimilatory or respiratory pathways, possessing also and more frequently dissimilatory NO_3^- reduction pathways, showed by the presence of genes encoding putative NO_3^- transporters, NO_3^- reductases and NO_2^- reductases in both crenarchaeota and euryarchaeota phylotypes (Cabello et al., 2004, and references therein).

Thus, salinity, temperature and inorganic nutrients concentrations also have a great influence in the dynamics of Archaeal communities like it was observed in previous studies (Berdjeb and Pollet, 2013). The presence of bands in same position through the DGGE profiles generated for the different stations might suggest that some phylotypes lead better with the environmental gradients of the estuary, having an important function in the system. On the other hand, there is again the possibility of in the middle estuarine stations, part of this high diversity be consequence mix of estuarine, riverine and coastal ocean assemblages, just like it was previous observed in Bacterial diversity studies (Abreu et al., 2001; Crump and Hobbie, 2005; Vieira et al., 2007; Singh et al., 2010). Another information we can retrieve from our DGGE analysis is that, in both Bacteria and Archaea profiles, bands located in the same position became gradually weak along the salinity gradient. This might be due to a gradually substitution of some phylotypes by others along the estuarine mixing gradient, indicating that some Archaea or Bacteria phylotypes might be replaced by others more adapted to those conditions (Crump et al., 2004). In other words, it seems there is a spatial succession of prokaryotic communities, along the estuarine gradient.

The methodology used to ascertain prokaryotic community was based in a fingerprinting method which has an advantage to be cheap, faster to analyse and to obtain results (Cleary et al., 2012b). However, it cares a critic analysis of the results. According to this method, microbial assemblages are generally dominated by a few taxa (3 to 35 bands), although many more are likely to be present but in lower abundance (Casamayor et al., 2002). Once it relays on a PCR amplification, being a technique that may introduce different biases, may alter the natural abundance of sequences, being also influenced by variations in operon copy numbers, it can produce a misleading interpretation of the results (Hewson and Furhman, 2004). Furthermore, DGGE, also has the problem of the presence of heteroduplexes (Muyzer et al., 1998), different sequences that might stop at the same position in a gradient (Casamayor et al., 2002). In contrast, the same species may be represented by multiple bands (Cleary et al., 2012b), inducing again a wrong interpretation of the results. Another problem arises from nucleic acid extraction procedures, once microbial cells may exhibit different degrees of resistance to cell breakage, decreasing extraction efficiency (Casamayor et al., 2002). While the calculated richness in this study is helpful to understand the relative changes along the estuary, it does not necessarily correspond exactly to the actual Bacterial-cell richness. Furthermore, species less abundant might not be amplified, narrowing the range of target microbial groups and underestimating the results (Cleary et al., 2012b; Hewson and Furhman, 2004). However, since we are comparing the community structure along the estuarine gradient, this methodology suits well our goals to understand the shift of community profiles as environmental parameters change. Thus, the results of richness are not absolute and do not represent the community diversity, but provide a valuable tool for monitoring the structure and dynamics of microbial populations, at a first level, over the influence of environmental changes (Gafan et al., 2005; Smalla et al., 2007).

4.2.2 – Archaeal ammonia oxidizing diversity

Until recently, our knowledge about the diversity of aerobic ammonia oxidizers was restricted to Bacteria domain. With the enrolment of Archaea in this process there was a shift in the way of looking to the nitrification process (Francis et al., 2007; Schleper and Nicol, 2010). Many investigations have addressed the relationships between environmental variables and nitrification process, with the distribution of ammonia oxidizing populations (Bernhard et al., 2007; Magalhães et al., 2007, 2009; Mosier and Francis, 2008; Santoro et al., 2008). The knowledge of the N transformations in estuarine sediments, characterized by steep physicochemical gradients, and their nitrifier communities, is essential to understand the nitrogen budgets (Santoro et al., 2008). Among many parameters, salinity has been shown to affect species composition of nitrifying communities (Magalhães et al., 2005a, 2009; Sahan and Muyzer, 2008; Santoro et al., 2008; Bernhard and Bollmann, 2010a). Moreover, a study performed with Archaeal *amoA* sequences retrieved from aquatic systems revealed that large part of its variability could be explained by this parameter (Biller et al., 2012).

Our findings revealed a great influence of salinity on the diversity of Archaea *amoA*, which is in agreement with what was previously reported by Sahan and Muyzer (2008), which identified salinity and temperature as key variables that contributed to the diversity and distribution of both ammonia oxidizing Archaea (AOA) and ammonia oxidizing Bacteria (AOB). In our study, the diversity of AOA *amoA* decreased with the increase of salinity contrasting to the positive effect of temperature, besides that covaried with salinity. Some studies relating salinity with AOA *amoA* showed a lower abundance in higher salinities (Mosier and Francis, 2005; Santoro et al., 2008;

Magalhães et al., 2009). In this study, *amoA* diversity was found to be very low in the high saline sites, but still the presence of those bands may indicated the existence of some high tolerant AOA ecotypes that were selected at higher salinities. Moreover, a lower recovery of PCR product, obtained in samples from the more saline sites, might indicate a lower abundance of these organisms in the most saline site of the estuary. Still, Archaeal *amoA* were first detected in the Sargasso Sea with mean salinities of 36.6 psu (Venter et al., 2004), which reflects an adaptation by some phylotypes of this phylum to high salinities. In the other hand, a study performed by Francis et al. (2005) discovered that Archaeal *amoA* sequences from low saline sites (0.5 psu) clustered together in distinct cluster, indicating the possibility of the existence of unique low-salinity AOA types. From the AOA *amoA* DGGE profiles we can see the presence of some bands across all profiles, despite many of them disappear as salinity decreases. Thus, it is likely that some AOA ecotypes are tolerant to the wide range of salinity conditions, whereas others are well adapted to unique environmental conditions (Erguder et al., 2009). In a previous study Magalhães et al. (2009), reported higher abundance of AOB *amoA* comparing to AOA *amoA* in more saline sites of Douro estuary, a result shared by Santoro et al., (2008), in a subterranean estuarine system. Thus, these studies revealed that salinity was a key environmental parameter driving AOB abundances, which outnumbered AOA in more saline stations (Santoro et al., 2008¸ Magalhães et al., 2009; Wankel et al., 2011). Also, other study showed that AOA were more abundant than AOB only in the low saline sites (Mosier and Francis, 2008). Besides salinity, also temperature had a great influence on the distribution of Archaeal *amoA*, as it was previous described in other studies (Sahan and Muyzer, 2008). Still, in the Douro estuary this parameter is co-related to salinity as previous mentioned. Biller et al. (2012), using *amoA* gene sequences form genomic databases, concluded that temperature would explain 9.7% of the *amoA* sequence variation, however it could decrease when compared to the influence of habitat type.

Although, our results did not show any correlation between the *amoA* diversity and nitrification rates, we saw a higher similarity pattern between AOA *amoA* diversity from Areinho and Avintes on the DGGE profile and further analysis described. In fact, those intermediary saline sites were mostly characterized by high NH_4^+ interstitial water concentrations, higher nitrification rates and low C:N ratios; environmental variables that can be affect those communities. Still the major richness of the AOA *amoA* was achieved in the most upstream station (Crestuma), with lower NH_4^+ concentration. In fact, the majority of the studies indicate that Archaeal *amoA* are more adapted to low ammonium-containing environments (Di et al., 2009; Martens-Habbena et al., 2009 Martens-Habbena and Stahl, 2010; Santoro et al., 2010), where nitrification processes occurs with NH_4^+ released through mineralization (Verhamme et al., 2011). In agreement to these previous findings in our study we showed that Archaeal *amoA* diversity was higher in the site with less NH_4^+

availability and lower NH_4^+ effluxes. Contrary, in the more saline station (Afurada), where higher NH_4^+ effluxes and interstitial water concentrations were registered, AOA *amoA* diversity clearly decreases. However, higher effluxes of NH_4^+ were achieved in intermediary saline sites, as well. Very recently, Sintes et al. (2012) suggested the presence of two different ecotypes of Archaeal ammonia oxidizers adapted to medium and low NH_4^+ concentration. This could indicate that in the Douro estuary the differential shifts in NH_4^+ concentration imposed an adaptation of AOA communities, which is reflected in a differential pattern of *amoA* diversity. A previous study performed in the Douro estuary, suggested a connection between the phylogenetic composition of the AOB assemblage and the physiological ability to tolerate high NH_4^+ concentrations (Magalhães et al., 2005a). Actually, it has been shown different growth responses to NH_4^+ concentration by AOA and AOB organisms (Park et al., 2006), which may indicate a differential distribution in their ecological niches (Verhamme et al., 2011), which might influence nitrification rates.

In our study, the lower PCR *amoA* product recovered and the lower AOA diversity observed at the more saline site, plus the lack of relation with NH_4^+ availability or nitrification rates, suggested that AOB might have a greater contribution to nitrification processes in the more saline sites of Douro River estuary. These results are in agreement with previous studies (Magalhães et al, 2009) by the fact that we show a clear decrease of AOA diversity with the increase of salinity. Indeed, salinity and NH_4^+ concentrations might act as stress factors to AOA communities, being overcame by AOB communities, more adapted to those site conditions. Thus, along estuarine environmental gradient, AOA diversity pattern may be controlled by a variation of selective pressures on the function of the AMO enzyme complex (Biller et al., 2012).

CHAPTER 5 - CONCLUSIONS AND NEW PERSPECTIVES

“Even though discreet, life in form of Bacteria and its many communities changed the surface and atmosphere of the planet Earth.”
Lynn Margulis

The progressive increases of N loading and reduction of freshwater discharge into estuaries have led to worldwide serious ecological problems in coastal ecosystems. Indeed, water diversion from agricultural activities within the watershed and the consequences of climate change has led to a progressive decrease in freshwater flow, altering the salinity regime of the estuaries. Because benthic microbes play an important role in estuarine biogeochemical transformations, it is important to investigate how salinity may influence microbial diversity and activity as well as the biogeochemical processes mediated by those organisms, within systems with a high range of salinity regimes.

This study represented a first comparative approach to analyse the microbial communitiy diversity along the salinity gradient of the Douro estuary. Our combined chemical, physical, biogeochemical and biological findings add new knowledge about the dynamics of the microbial communities and biogeochemistry of the Douro estuary, and also corroborate the findings of previous studies performed in the same estuarine system.

Our findings suggest that, the saltwater intrusion into the Douro estuary, allowed the creation of a gradient of salinity along the estuary disturbing NH_4^+ behaviour by enhancing its effluxes and promoting a dilution of NO_3^-, with a clear impact on the diversity of benthic prokaryotes. Thus, along the estuary, besides the gradient of salinity, there is also a steep chemical gradient, reflecting differences in the nutrients availability which in turn has an influence on the microbial communities, which are believe to be key drivers of estuarine biogeochemistry. Moreover, the mix of saline cold waters with freshwaters creates a gradient of temperature that also has been found to influence estuarine microbial community composition.

Nitrification process was detected in the four different stations located along the salinity gradient of the Douro estuary. However, both acetylene inhibition technique and ^{15}N isotopic analysis registered higher rates of this process at the intermediary salinities. Interestingly, this is in agreement to what was previously observed in other investigations performed in the Douro estuary or in other coastal systems. The higher nitrification rates were registered at sites where higher NH_4^+ concentrations were measure in the interstitial water, which possibly induced nitrifier communities to enhance their activity. Our findings also indicated a role of the granulometric properties of the sediments in controlling the magnitudes of nitrification. Indeed, higher percentage of gravel within the sediments was significantly related to higher nitrification rates, which in turn, could allow more oxygen diffusion within the sediments, favouring aerobic processes like nitrification.

Comparative DGGE profiles of Bacterial and Archaeal 16S rRNA gene fragments, between the different stations, clearly indicated a shift in the diversity of those two groups of prokaryotes along the estuarine salinity gradient. Moreover, our study identified the important role of salinity and temperature in controlling those spatial changes on microbial diversity. The chemical estuarine gradient established due to the mix of saline and freshwater masses, which in turns influence the nutrient availability for organisms, could also contribute to the shift of the estuarine microbial community structure observed. Indeed the diverse nutritional requirements that characterized prokaryotes and the high plasticity for adaptation to environmental changes, also may explain the diversity patterns seen along the Douro estuary.

The diversity of ammonia oxidizing Archaea (AOA) composition along the salinity gradient of Douro estuary suggested that AOA are physiologically influenced by the increase of salinity, with a deep decrease of the diversity in the most saline station. Moreover, besides the differences on *amoA* diversity along the different sampling sites, at the intermediary salinity sites, the communities were found to be very similar. Those sites were the ones that displayed higher concentrations of NH_4^+ in the interstitial water, a variable that might select the AOA communities of those sites, with an impact on the magnitudes of nitrification. The higher richness of the AOA *amoA* gene were found in the more upstream station (Crestuma) characterized by low salinities and less NH_4^+ availability, which is in accordance with the fact that AOA are referenced to have low NH_4^+ requirements.

We believe that the data generated from this study represent an important background to help us understand the dynamics of microbial communities along a salinity gradient, contributing to the knowledge of the estuarine biogeochemical cycles. However, future research must be focused in characterizing the phylogeny of both Bacterial and Archaeal communities in order to understand if a mix of freshwater and marine microbial communities is occurring or if there are specific phylotypes that are actually selected by the imposed gradient of salinity. Additionally, the diversity of AOA should be extended to their Bacteria counterparts (AOB), in order to improve our understanding of how salinity may control the relative abundance and diversity of the two groups of microorganisms involved in the nitrification process (AOA and AOB). Also, quantitative studies of the abundance of *amoA* gene from both domains (AOA, AOB) will be essential to relate the magnitude of the nitrifications rates, with the representativeness of each group of ammonia oxidizer microorganisms at each environment. Finally, because many environmental variables are correlated within an estuary, it would be very useful to set up controlled experiments in order to isolate the regulatory effect of salinity. Such approach would enable to characterize in detail the role of salinity in driving shifts of relative abundance and diversity of the active AOA and AOB and consequently its independent contribution for the nitrification activity.

CHAPTER 6 – REFERENCES

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