

**INFLUENCE OF MARSH FLORA ON DENITRIFICATION
RATES AND THE ABUNDANCE AND COMMUNITY
STRUCTURE OF DENITRIFYING BACTERIA**

ANA MARGARIDA PINTO HENRIQUE MACHADO

**Dissertação de Mestrado em Ciências do Mar – Recursos
Marinhos**

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AND THE ABUNDANCE AND COMMUNITY STRUCTURE OF
DENITRIFYING BACTERIA

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Orientador – Professor Doutor Adriano A.
Bordalo e Sá

Categoria – Professor Associado com
Agregação

Afiliação – Instituto de Ciências Biomédicas de
Abel Salazar da Universidade do Porto.

Co-orientador – Doutora Catarina Pinto
Magalhães

Categoria – Pos-Doc Investigadora

Afiliação – Centro Interdisciplinar de
Investigação Marinha e Ambiental

“The finish line is a good place to start”

Ao Rui e à Dharma
Aos meus Pais

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Abstract

Temperate salt marshes are typical estuarine ecosystems and are among the most productive environments on Earth, harboring diverse communities implicated in multiple ecosystem functions including microorganisms. Owing to their location, estuaries also receive multiple pollutants from the drainage basin and the coast, such as metals. The influence of salt marsh plants (*Halimione portucaloides*) and the level of sediment metal contamination on the distribution and activity of microbial communities, including those associated to the N-cycle were investigated in two Portuguese estuarine systems with different degrees of metal contamination: Cavado (41.5228 N; 8.7846 W) and Sado estuaries. In Sado, two salt marshes were investigated: Lisnave (38.4879 N; 8.7912 W) and Comporta (38.4425 N; 8.8312 W). Moreover, denitrification in eutrophic coastal and estuarine systems influences the nitrogen budget and may result in increased fluxes of nitrous oxide (N₂O), a potent greenhouse gas that also contributes to the destruction of the ozone layer. The presence of plants in salt-marshes may influence physically and biochemically denitrification, since sediment characteristics and organic carbon availability may be affected. PCR rDNA-DGGE approach and direct microscopic counts of DAPI-stained cells were applied to study the biodiversity and abundance of prokaryotic communities in colonized (rhizosediments) and un-colonized sediments. Sediment characteristics and metal concentrations (Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn) were concomitantly evaluated to identify possible environmental constraints on spatial and temporal microbial dynamics. Denitrification and nitrous oxide (N₂O) rates were measured in sediment slurries using the acetylene technique. The diversity of genotypes of nitrate (*narG*), nitrite (*nirS* and *nirK*) and N₂O reductase (*nosZ*) genes were evaluated by DGGE. Abundance and phylogeny of *nirS* and *nirK* genes, considered the key enzymes in the denitrification, were also studied. Redundancy analysis (RDA) revealed that Lisnave salt marsh microbial community was usually associated to a higher degree of metal contamination, especially the metal Pb. In clear contrast, Cavado estuary microbial assemblage composition was associated to low metal concentrations but higher organic matter content. Comporta salt marsh bacterial community clustered in a separate branch, and was associated to higher levels of different metals, namely Ni, Cr and Zn. Additionally, the microbial community structure of Lisnave and Cavado showed a seasonal pattern, clustering in the summer. Moreover, microbial abundance correlated negatively with metal concentrations, being higher in Cavado, generally yielding higher counts in the rhizosediments. Denitrification potential varied between 0.41 and 26 nmol N₂ g wet sed⁻¹ h⁻¹, presenting a strong temporal variation, with higher rates during summer and fall. On the other hand, rhizosediments N₂O production rates were higher than in un-

colonized sediments. Moreover, cluster analysis of DGGE profiles showed differences in the composition of denitrifier assemblages. In general, rhizosediments showed greater diversity than un-colonized sediments. Samples were primarily clustered by sampling sites, and within them, by season. Rates of potential denitrification and N₂O accumulation were not directly related to the degree of metal contamination among the different marshes. However, the diversity of genes implicated on this processes was found to be significantly correlated ($p < 0.05$) to the concentration of metals. While the diversity *narG* was negatively affected by almost all metals, *nirS*, *nirK* and *nosZ* diversity were positively related to metals that function as micronutrients (e.g. Cu, Fe). These findings suggest that increased metal concentrations affect negatively the abundance of prokaryotic microorganisms and that salt marsh plants may have a pivotal role in shaping the microbial community structure. Moreover, denitrifier communities in rhizosediment can have an important contribution to the greenhouse effect through N₂O emissions. Since salt-marshes can colonize large areas in temperate estuaries, the dynamic of denitrification pathway in these sediments should not be disregarded in the recovery and mitigation strategies in those systems.

Resumo

Os sapais são típicos ecossistemas estuarinos temperados que se encontram entre os ambientes mais produtivos do planeta, abrigando diversas comunidades implicadas em múltiplas funções ecossistémicas, microrganismos incluídos. Em virtude da sua localização, os estuários recebem inúmeros poluentes originários da bacia hidrográfica e do mar. A influência das plantas de sapal (*Halimione portucaloides*) assim como nível de contaminação por metais dos sedimentos sobre a distribuição e actividade das comunidades microbianas, incluindo aquelas associadas ao ciclo de azoto, foram investigadas em dois sistemas estuarinos portugueses com diferentes graus de contaminação por metais: Cávado (41.5228° N; 8.7846° W) e Sado. No estuário do Sado, dois locais foram estudados: Lisnave (38.4879° N; 8.7912° W) e Comporta (38.4425° N; 8.8312° W). A desnitrificação em sistemas costeiros e estuarinos eutrofizados pode influenciar o balanço de azoto e conduzir ao aumento de fluxos de óxido nítrico (N_2O), um potente gás estufa que também contribui para a destruição da camada de ozono. A presença de plantas de sapal pode influenciar física e bioquimicamente a desnitrificação, uma vez que as características do sedimento e disponibilidade de carbono orgânico podem ser afectadas. A abundância e biodiversidade das comunidades procarióticas em rizosedimentos e sedimentos não colonizados foi estudada através de análise de genes de 16S rDNA e por reacção em cadeia de polimerase (PCR), electroforese em gradiente desnaturante (DGGE) e contagem directa de células com coloração DAPI em epifluorescência. As características do sedimento e concentrações de metais (Cd, Cr, Cu, Fe, Pb, Mn, Ni e Zn) foram, de igual modo, avaliadas para identificar possíveis influências ambientais sobre a dinâmica espaço-temporal microbiana. As taxas potenciais de desnitrificação e óxido nítrico (N_2O) foram medidas em “slurries” de sedimentos utilizando a técnica do acetileno. A diversidade dos genes nitrato (*narG*), nitrito (*nirS* e *nirK*) e óxido nítrico (*nosZ*) redutases foram avaliados por DGGE. A abundância e filogenia dos genes *nirS* e genes *nirK*, que codificam enzimas-chave da desnitrificação, foram também estudadas. A análise de redundância (RDA) revelou que a comunidade microbiana do sapal da Lisnave se encontrava associada a um maior grau de contaminação por metais, especialmente Pb. Em claro contraste, a composição microbiana do sapal do estuário do Cávado foi associada a menores concentrações de metais, mas fortemente condicionada pela maior disponibilidade de matéria orgânica. Por outro lado, a comunidade bacteriana do sapal da Comporta foi agrupada num ramo separado, associado a níveis mais elevados de metais como Ni, Cr e Zn. A estrutura da comunidade microbiana presente nos sapais da Lisnave e Cávado mostrou um padrão sazonal, sendo mais semelhantes, entre si, no verão. Além disso, a abundância

microbiana correlacionou-se negativamente com as concentrações de metais, sendo mais elevada no Cávado, onde os sedimentos colonizados apresentaram maior abundância microbiana. O potencial de desnitrificação variou entre 0,41 e 26 nmol g N₂ sed⁻¹ h⁻¹, apresentando uma forte variação temporal, com taxas de desnitrificação superiores durante o verão e outono. Por outro lado, as taxas de produção de N₂O foram maiores no sedimento colonizado do que em sedimentos não colonizados. A análise dos perfis de DGGE revelou importantes diferenças na composição das comunidades desnitrificantes. Em geral, os sedimentos colonizados apresentaram maior diversidade do que os não colonizados. As amostras foram primeiramente agrupados por local de amostragem e, dentro destes, por estação do ano. As taxas de desnitrificação e acumulação potencial de N₂O dos diferentes sapais não se mostraram directamente relacionadas com o grau de contaminação por metais. No entanto, a diversidade dos genes implicados no processo de desnitrificação correlacionou-se significativamente ($p < 0.05$) com a concentração de metais. Enquanto a diversidade do nitrato reductase (*narG*) foi negativamente afectada por quase todos os metais, os genes *nirS*, *nirK* e *nosZ* foram correlacionados positivamente com metais que funcionam como micronutrientes, como o Cu e o Fe. Estes resultados sugerem que as concentrações de metais afectam negativamente a abundância de procariontes e que as plantas de sapal desempenham um papel não negligenciável na formação da estrutura da comunidade microbiana. Além disso, as comunidades desnitrificantes podem ter uma importante contribuição para o efeito de estufa através das emissões de N₂O. Assim, e como os sapais podem colonizar grandes áreas em estuários de clima temperado, a dinâmica associada à desnitrificação nos sedimentos deve ser tida em conta na elaboração de estratégias de recuperação e mitigação nesses sistemas.

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Chapter 1

General Introduction

Microbial populations are ubiquitous of all environments but less than of 1% of all existent bacterial species have been described (Colwell and Hawksworth 1991), and according to the Systematics agenda 2000 (1994), the majority of the remaining 4×10^5 to 3×10^6 bacterial species are unknown. Microbes play an important role in all biological structures of the environment, so the biodiversity of microbial communities always has been an object of great interest (e.g. Crump *et al.* 1999, Abreu *et al.* 2001, Bouvier and del Giorgio 2002, Bernan and Francis 2006). For many decades, microbiologists applied standard physiological and biochemical approaches to assess microbial biodiversity of natural ecosystems that only dealt with cultivated microorganisms, leading to an underestimation of the actual diversity and abundance (e.g. Barnes *et al.* 1994, Woese 1994). Indeed, more than 99% of microorganisms are not cultivated by routine techniques (Amann *et al.* 1995). The application of molecular techniques to ecological studies, such as analysis of 16S ribosomal RNA genes (rDNA), Polymerase Chain Reaction and DNA probing, unveiled the presence of a huge diversity of microorganisms, previously undetected (e.g. Pace *et al.* 1986, Liesack and Stackebrandt 1992). Actually, fingerprinting methods like PCR rDNA-DGGE approach have been routine use to analyze simultaneously multiple samples of microbial community in different and diverse ecosystems (e.g. Abreu *et al.* 2001, Magalhães *et al.* 2005, Wu *et al.* 2006, Ferrari and Hollibaugh 1999, Zhao *et al.* 2008).

1.1. Nitrogen cycle

The element nitrogen (N) is an essential component of proteins and nucleic acids, two macromolecules constituent of all living beings. Nevertheless, the majority of other biological materials contain nitrogen as well. It was estimated that plants and animals in soils and waters of the planet together contain about 1.5×10^{10} tons of N, being the nitrogen cycle responsible for processing approximately a fifth of this amount per year (Postgate 1987).

The nitrogen cycle consists of multiple redox reactions of nitrogen compounds performed in different ways, primarily mediated by bacteria, archaea and some specialized fungi. The nitrogen plays a central role in biogeochemical cycles, ultimately controlling the primary

production in aquatic systems. Human activity, particularly the anthropogenic nitrogen enrichment affects the nitrogen cycle, being implicated in the eutrophication and degradation of coastal marine systems. Some gaseous nitrogen products, such as nitrous oxide (N_2O) and nitric oxide (NO), mainly produced from denitrification and nitrification, are associated with severe impact on our atmosphere contributing to the greenhouse effect causing the destruction of the ozone layer and, therefore, are potentially involved in controlling the climate of the Earth (Schlesinger 1997, Zehr and Ward 2002).

The nitrogen compounds can be found in the environment in various forms, which can be described in terms of their chemical structure, oxidation state and phase solid - liquid - gas. The many oxidation states of nitrogen and the resulting large number of nitrogen species give rise to many redox reactions that transform one species to another. The complexity of the nitrogen cycle is shown in Figure 1, where one can notice that reactions such as oxidation / reduction are implied in the nitrogen transformation, whose oxidation state varies between nitrate (NO_3^- , +5), the most oxidized and ammonia (NH_4^+ , -3), in addition to existing compounds in the intermediate states. These microbiological transformations includes: (i) reduction of nitrate (NO_3^-) and nitrite (NO_2^-) to nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2) (denitrification), (ii) conversion of ammonia to nitrogen organic by assimilative process, (iii) production of NH_4^+ from the decomposition of organic nitrogen (ammonification), (iv) oxidation of NH_4^+ to NO_2^- and NO_3^- (nitrification), (v) reduction of N_2 to NH_4^+ and organic nitrogen (nitrogen fixation), (vi) reduction of NO_3^- to NH_4^+ (dissimilar reduce nitrate to ammonia), (vii) the oxidation of NH_4^+ to N_2 with NO_2^- and NO_3^- as electron acceptor (anaerobic ammonia oxidation). These biochemical conversions can be energetically favorable (e.g. nitrification and denitrification) or energy-demanding (e.g. nitrogen fixation), and are fundamental processes in microbial biosynthesis and bioenergetics (Madigan *et al.* 2003).

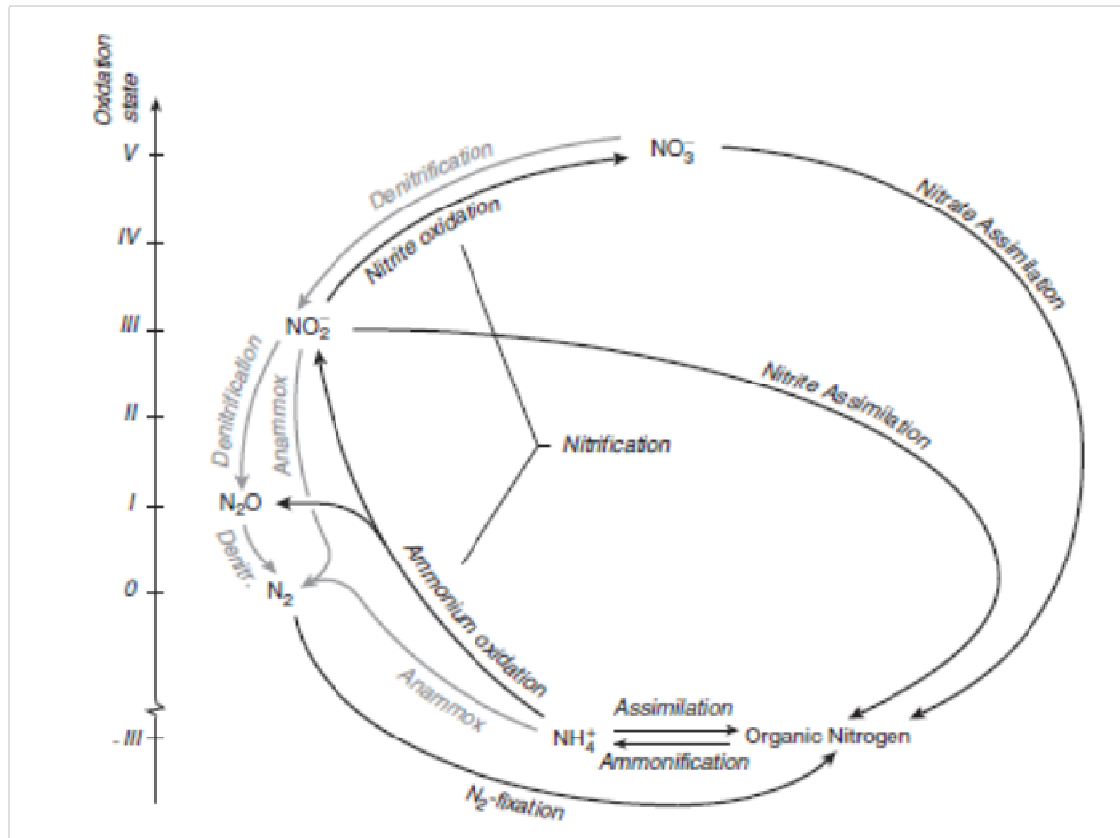


Figure 1: Schematic of the key process involved in the nitrogen cycle (Gruber 2008). The various chemical forms of nitrogen are plotted versus their oxidation state. Processes shown in grey occur in anoxic environments only.

The complex nitrogen cycle has then some key reactions that are briefly described in a summarized form:

Nitrogen fixation - Most microorganisms can assimilate N in various forms, yet they cannot generally use the N_2 directly. Although 79% of the atmosphere of the Earth is composed of molecular nitrogen, the major reservoir of nitrogen is unavailable directly to animals and plants. The biological nitrogen fixation is the process of conversion of N_2 into NH_4^+ and organic nitrogen, with the addition of three electrons per atom. It involves breaking a triple bond ($\text{N} \equiv \text{N}$), whose very high activation energy requires large amounts of cellular energy. The ability to fix nitrogen is found only in some prokaryotes and apparently arose relatively early in bacteria. This specialized group possesses the key enzyme in the process, nitrogenase, and includes anaerobic bacteria and photosynthetic cyanobacteria (Postgate 1987, Ward 1992, Herbert 1999).

The magnitude of N_2 fixation has been a topic of intense research and discussion in the last two decades, in particular the extent to which the fixed nitrogen budget is actually in balance is still controversial. In temperate coastal systems, the N fixation is considered to have a smaller contribution to N budgets than in the open ocean (Seitzinger 1988). However in some temperate coastal systems, high rates of N fixation can be found, but their contribution to the annual budget may be modest (Nixon 1981, Joye and Paerl 1993). The N fixation revealed to be unimportant in systems with high nitrogen availability in the water column and sediments such coastal marine environments, where extensive meadows of rooted macrophytes are present.

Ammonification - All living matter contains nitrogenous macromolecules such as nucleic acids, proteins, polyamines sugars and low molecular weight compounds, which become available after cellular death for decomposing organisms (putrefaction) or are excreted into the surrounding environment. Ammonification is the process by which primary amines are deaminated during decomposition of organic compounds, the transformation of organic nitrogen to NH_4^+ . Most of this process is done by heterotrophic bacteria, which use the oxidation of organic carbon to CO_2 as a source of energy, but release the organic nitrogen as NH_4^+ (Ward 1992, Herbert 1999). A large percentage of the NH_4^+ produced during mineralization (40 to 60%) of organic N in sediments can also be lost from the ecosystems as N_2 . Essentially, the NH_4^+ produced in the sediment is nitrified and subsequently denitrified (Seitzinger 1990).

Nitrification – Nitrification represents the oxidative part of N cycle completing the redox cycle of nitrogen from most reduce to most oxidized form. The oxidation of ammonium to nitrate is a process that involves two-steps: in the first step, mediated by ammonium oxidizing bacteria (e.g., *Nitrosomonas*) and archaea, ammonium is oxidized to nitrite that subsequently is oxidized to nitrate, in the second step mediated by nitrite oxidizing bacteria (e.g., *Nitrobacter*) and archaea. The nitrification process is a strictly prokaryotic process undertaken by a specialized group of chemo-autotrophic aerobic microorganisms (Postgate 1987, Ward 1992, Herbert 1999). Nitrification tends to be inhibited by light, which can have important implications for the upper ocean nitrogen cycle. Normally, although timings are different, the two steps are closely linked, so no significant accumulation of nitrite in the environment occurs. Nitrification is a source of nitrate to denitrifying bacteria playing an essential role in the N cycle of coastal sediments. The coupling of this obligate aerobic process (nitrification) with an aerobic process (denitrification) promotes the loss of nitrogen to the atmosphere as nitrous oxide and dinitrogen (Seitzinger 1988). Nevertheless, the degree of coupling between these two

processes is variable according to inherent environmental characteristics of each system, and is still subject of much discussion.

Denitrification - Microbiological process involving a series of four reductions, by which heterotrophic bacteria oxidize organic matter using nitrate as electron acceptor. The end product is nitrogen gas - molecular nitrogen (N_2) or nitrous oxide (N_2O) (Ward 1992, Zumft 1997, Herbert 1999). Each step is carried out by a specific enzyme and nitrite reductase is closely coupled with subsequent enzyme in the reduction sequence to nitric oxide and nitrous oxide, since neither of these gases nor nitrite is accumulate in the environment in large amounts. Denitrification is ubiquitous in aquatic systems. Coastal sediments present an ideal environment for denitrification given that they concentrate organic matter from the water column, which upon decomposition releases NH_4^+ to nitrification, and subsequently NO_3^- would support denitrification (Seitzinger 1988). Anthropogenic N-enrichment (e.g. agriculture) can be an additional source of NO_3^- for denitrification (Nowicki *et al.* 1999). Denitrification is an important process for the effective removal of nitrogen from aquatic systems as dinitrogen gas, reducing the amount of N transported downstream and to the ocean (Nixon 1981) (for further details see below).

Dissimilatory Nitrate Reduction to Ammonium (DNRA) - A second mechanism of nitrate reduction, also called nitrate ammonification involves heterotrophic bacteria, predominantly fermentative, with the ability to reduce nitrate to ammonia (Koike and Hattori 1978, Herbert 1999). In contrast to denitrification where N is lost from the ecosystem, DNRA retains the nitrogen fixed in the system. This process is quite important in organically rich environments and low nitrate concentrations (Rysgaard *et al.* 1996, Bonin *et al.* 1998, Master *et al.* 2005).

Anaerobic ammonia oxidation (anammox) - Denitrification has been described as the only important process of removing the existing pool of nitrogen in natural environments. Recently, however, it was found that ammonia can be oxidized anaerobically by chemo-autotrophic bacteria in sediments in the presence of nitrate or nitrite (Mulder *et al.* 1995, van de Graaf *et al.* 1995). This process, first uncovered in wastewater bioreactors, has been demonstrated to occur in marine environments only very recently (e.g. Kuypers *et al.* 2003, Dalsgaard *et al.* 2003). Although, its quantitative significance is not yet known on a global scale, studies showed that this alternative can contribute significantly for the benthic production of N_2 (Thamdrup and Dalsgaard 2002). On the other hand, in surface sediments and in the presence of oxygen, oxidation can occur in organic N and NH_4^+ by manganese oxide (MnO_2) with formation of N_2 (Luther *et al.* 1997). From a geochemical

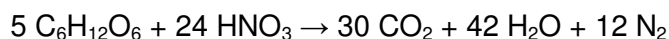
perspective, denitrification and anammox have the same implication; they both lead to a loss of fixed nitrogen from the ocean, albeit with a somewhat different stoichiometry.

1.2. Denitrification

Being by excellence the process of removing nitrogen from the aquatic environment and the main focus of this thesis, should analyze it in some detail.

Denitrification is known for more than a century as the main mechanism of conversion of combined nitrogen, the form available to the eukaryotes in molecular nitrogen gas, thus completing the nitrogen cycle. Recently, denitrification has received increased attention for being the main source of NO and N₂O gases of fundamental importance to atmospheric ozone depletion and global warming (Ye *et al.* 1994).

The denitrifying bacteria use NO₃⁻ as electron acceptor in anaerobic oxidation of organic matter releasing gaseous N₂ through the following reaction:



that produces 570 kcal / mole (Delwiche 1970).

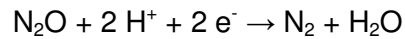
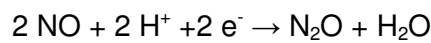
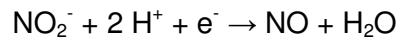
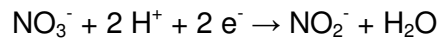
Denitrification, in the aquatic environment, occurs when oxygen begins to be depleted throughout the water column or sediments (below the level of penetration of oxygen) as a result of induction of an aerobic facultative bacteria enzyme system that can only use nitrogen oxides when the oxygen level is strongly reduced or absent.

The capacity of performing denitrification is widespread among bacteria and is distributed across various taxonomic subclasses. The majority of currently characterized denitrifiers fall within the *Proteobacteria* group (Zumft 1997). Denitrification has been described also in some archaea and fungi, however the ecological significance of the process in these organisms still needs to be characterized.

Because denitrifying bacteria are facultative anaerobes, with few exceptions they can also use oxygen as terminal electron acceptor when this gas is present in sufficient concentrations. However, when oxygen becomes limiting, the ability to use nitrate as

terminal oxidant allows denitrifying bacteria continue respiration using an alternative electron acceptor (Zumft 1997, Shapleigh 2001).

The N-oxide reduction pathway during denitrification has been well worked out and involves the sequential reduction of nitrate to nitrite, followed by nitric oxide, nitrous oxide and finally to nitrogen gas in a process that develops in several steps:



All steps within this metabolic pathway are catalyzed by complex multisite metalloenzymes with characteristic spectroscopic and structural features (Cole 1978), (Figure 2).

In all bacteria, the enzymes of denitrification receive e^- from the respiratory chain system that is part of the cytoplasmatic membrane. In the first step of denitrification, the two electron reduction of nitrate to nitrite is catalyzed by nitrate reductase (Nar). Four types of nitrate reductase have been described so far: a eukaryotic assimilative nitrate reductase and three bacterial enzymes: a cytoplasmic enzyme, an enzyme associated with the respiratory membrane and a dissimilated periplasmic enzyme (Einsle and Kroneck 2004). The direct electron donor used by the nitrate reductase is quinone membrane (Zumft 1997, Shapleigh 2001, Einsle and Kroneck 2004). In brief, the quinone is oxidized towards the periplasmic surface of the membrane, with the release of H^+ to the periplasm but transfer of e^- across the membrane to the active site, which is located on a globular domain that protrudes into the cytoplasm. That transfer of e^- through Nar, together with H^+ release and uptake at the two sides of the membrane, generates a H^+ -motive force across the membrane. The location of the site of NO_3^- reduction on the cytoplasmic side of the membrane requires a transport system for NO_3^- , that is believed to be provided by NarK proteins. One of these proteins catalyses NO_3^- symport with one or more H^+ , allowing the initiation of respiration. In the steady state the NO_3^- import would be in exchange for NO_2^- export to the periplasm.

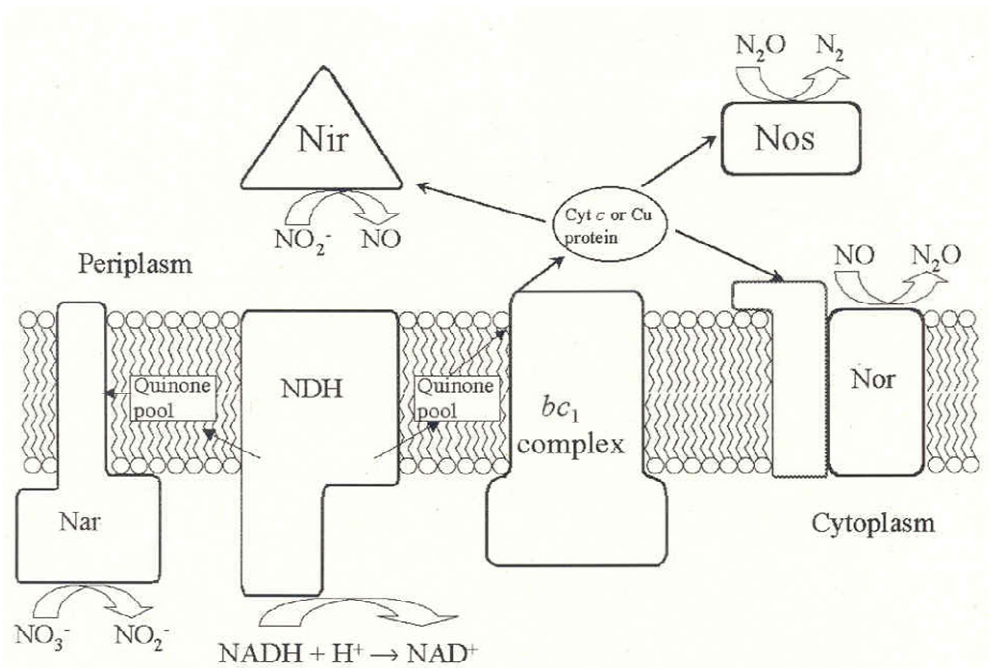


Figure 2: Basic layout of the reductases involved in denitrification (Shapleigh 2001) (Nar - nitrate reductase, Nir - nitrite reductase; In - N_2O reductase, Nor - NO reductase).

The reduction of nitrite is particularly important because distinguishes denitrifiers from other bacteria that use NO_3^- metabolism without being able to reduce NO_2^- to gas (Shapleigh 2006). The nitrite reductase (Nir) catalyzes the one electron reduction of nitrite to nitric oxide. There are two structurally different but functionally and physiologically equivalent forms of nitrite reductases, the Cu-nitrite reductase and cytochrome cd1. Both are water-soluble proteins located in the periplasm and they have never been found to coexist in the same denitrifying organism (Coyne *et al.* 1989). The cytochrome has also the ability to reduce molecular oxygen to water (Ye *et al.* 1994, Zumft 1997, Shapleigh 2001, Einsle and Kroneck 2004).

The reduction of NO to N_2O occurs at a binuclear center. The enzyme that catalyzes this process, the nitric oxide reductase (Nor) is an integral membrane protein (Zumft 1997, Shapleigh 2001). Two NO molecules are reduced at each time, with heme groups in common with the NO_2^- reductase, involved in the transfer of electrons (Ye *et al.* 1994, Einsle and Kroneck 2004). The NO generated must be restricted to low concentrations because of its potential toxicity, but nonetheless it is a definite free intermediate of denitrification. The activity of this enzyme is strictly dependent of copper.

The final step of denitrification pathway, the reduction of nitrous oxide to molecular nitrogen is catalyzed by N₂O-reductase (Nos), another periplasmic enzyme. It is assumed that the immediate e⁻ donor proteins are common to NO₂⁻ reductase. N₂O-reductase is a Cu-enzyme. This step can be blocked, so the end product of denitrification is not necessarily the molecular nitrogen. The acetylene (C₂H₂) inhibits the reduction of N₂O, although the mechanism of action is not fully known. For this reason, acetylene has been very useful in the study of denitrification (Zumft 1997, Shapleigh 2001). Therefore, nitrous oxide can be released or consumed during denitrification.

Because denitrifying bacteria belong to different phylogenetic groups (Zumft 1997), recent attempts to analyze denitrifying bacteria are based on the functional genes encoding the reductases enzymes. The genes involved in denitrification pathway contain highly conservative DNA regions, which can be successfully exploited for developing genes probes (Bothe *et al.* 2000).

The major prerequisite for denitrification is the availability of nitrate (including nitrite) in the environment. In addition, denitrification is strongly dependent on temperature, oxygen concentration and the availability of organic matter. There is also evidence that denitrification can be indirectly affected by high rates of sulfate reduction, since the presence of sulphides completely inhibits nitrification which in turn is necessary for denitrification (Seitzinger 1988) if additional sources are unavailable. Generally, the most suitable conditions to occur denitrification are intermediate levels of carbon availability but the reduction of sulfate is still low or absent (Hensel and Zabel 2000).

Coastal ecosystems such as salt marshes, estuaries and inshore coastal waters, which in recent years have been subject to increased anthropogenic inputs of nitrogen arising from diverse sources, are natural highly productive environments of nitrous oxide (N₂O) production through denitrification (Usui *et al.* 2001, Dong *et al.* 2002, Punshon and Moore 2004, Magalhães *et al.* 2005). An overview of studies conducted in coastal systems (Seitzinger 2000) revealed that the removal of inorganic nitrogen by denitrifying activity although highly variable between systems, can reach up to 100%.

1.3. Denitrification in sediments

It is generally considered that the nutrients (N and P) availability is one of the major factors regulating primary production in coastal marine environments. The availability of N

and P within the ecosystem is partly due to the rate of enrichment of the external system and the permanent removal within the system by biological, chemical and/or physical. Nitrogen can be a limiting nutrient in many estuaries, coastal systems, continental shelf, lakes and rivers (Seitzinger 1990, Cornwell *et al.* 1999). Being estuaries the boundary between land and sea, they are sites of major importance in biogeochemical processes occurring on a global scale including those associated to the nitrogen cycle.

Denitrification has been recognized as an important biological process that produces free nitrogen. Denitrification in sediments or anoxic water, is a key process in the nitrogen cycle since it decreases the amount of nitrogen available to the primary producers as the gaseous end products (N_2O and N_2) diffuse into atmosphere and therefore exerts a negative feedback on eutrophication (Nowicki *et al.* 2007).

Coastal sediments present an ideal environment for denitrification (Figure 3). They are a place of concentration of organic matter from the water column, which after decomposition releases NH_4^+ . The ammonium is then made available for subsequent nitrification and denitrification. In addition, the NO_3^- from overlying water can diffuse into the sediments, especially in relatively eutrophic systems where the concentration of NO_3^- in water is high. These characteristics, combined with the juxtaposition of tracks aerobic and anaerobic microenvironments in the interface sediment - water, lead to high capacity for denitrification in aquatic sediments (Seitzinger 1990, 2000).

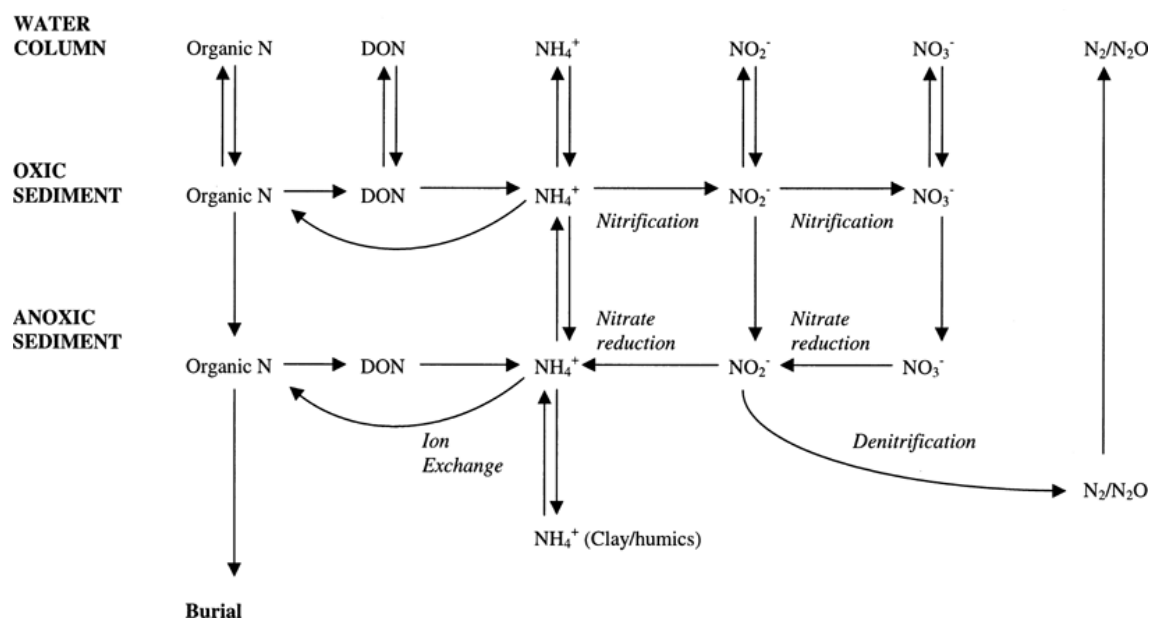


Figure 3: Schematic representation of nitrogen cycling in coastal marine sediments (Herbert 1999).

Different sediment systems show a wide range of denitrifying activity (Seitzinger 1988, 2000). The lower denitrification rates generally occur in deep-sea sediments (0.03 to $4 \text{ mmol N m}^{-2} \text{ h}^{-1}$), with rates in sediments of the continental shelf approximately one order of magnitude higher (up to $20 \text{ mol N m}^{-2} \text{ h}^{-1}$) (Seitzinger 1990, Herbert 1999). In oligotrophic to moderately eutrophic lakes, denitrification rates generally range between 20 to $60 \text{ mmol N m}^{-2} \text{ h}^{-1}$, with the highest rates found in eutrophic lakes (20 to $292 \text{ mmol N m}^{-2} \text{ h}^{-1}$). Some of the highest rates of denitrification occur in much polluted estuarine sediments ($> 500 \text{ mmol N m}^{-2} \text{ h}^{-1}$) (Seitzinger, 1990), however, rates in most estuaries vary from 5 to $250 \text{ mmol N m}^{-2} \text{ h}^{-1}$. In the estuary of the River Douro, denitrification values were measured between 9 and $360 \text{ mmol N m}^{-2} \text{ h}^{-1}$, in sandy sediments and rocky biofilms in the intertidal zone (Magalhães *et al.* 2005). Denitrification is also active in rivers where rates generally range from 40 to $2121 \text{ mmol N m}^{-2} \text{ h}^{-1}$ (Seitzinger 1990).

In many aquatic systems, sediments are an important source of recycled nitrogen (NH_4^+ and NO_2^-) to primary production. For example, in estuaries and coastal areas, the recycling of nitrogen from the sediment contributes between 20% and 80% for the N needs of the phytoplankton (Seitzinger 1990, Herber, 1999). However, a larger portion of water recirculated organic nitrogen does not return to the water column in the form of NH_4^+ or NO_3^- , being removed by denitrification. In this case, the removal of nitrogen by denitrification in the sediments in these systems may thus be important for regulating the production of algae and/or macrophytes.

1.4. Salt marshes

Estuarine salt marshes are intertidal wetlands vegetated by salt tolerant, non-woody, rooted, vascular plants. They are found in temperate, boreal and arctic biogeographic provinces worldwide and have an extent of $38,105 \text{ km}^2$ (Maltby 1988). Worldwide, over 600 species of plants grow in salt marshes (Chapman 1974), but although rich in flora, they are dominated by only a few species. *Puccinella maritime*, *Halimione portucaloides*, *Suaeda maritime*, and *Limonium vulgare* historically dominated salt marshes in Europe, however over the last decades the hybrid *Spartina anglica* has become more common and in some cases dominant in northern Europe (e.g. Morris and Jensen 1998). Salt marshes are important components of estuarine systems because they provide a food source to both estuarine and coastal ocean consumers, serve as habitat for numerous young and adult estuarine organisms, provide refuge for larval and juvenile organisms, and regulate important components of estuarine chemical cycles.

Salt marshes are among the most productive ecosystems in the world (Odum 1971). This high production is attributable to several factors, including nutrient enrichment from watershed runoff and tidal mixing (Day *et al.* 1989). Due to their physical location between coastal ocean and uplands, which are often heavily polluted and developed (NRC 1994) salt marshes can function as “buffer zones” by intercepting, stabilizing and removing pollutants (Smith and Hollibaugh 1993, Teal and Howes 2000) and excessive nutrients (Howes *et al.* 1996). The ever-increasing anthropogenic N loads from land, raising concern about their susceptibility to eutrophication and interest in their potential for removing the N before it enters estuarine and coastal ocean waters.

Marsh sediments differ fundamentally from soils and marine sediments in that salt marshes are exposed to a unique combination of environmental variables, including strong salinity gradients, fluctuating water levels and water tables, and anaerobic, waterlogged sediments with important effects in the sediment chemical environment. The flooding and porewater drainage affect sediment oxygen availability and redox potential, which in turn affect solubility of various (Patrick and DeLaune 1977).

The microbial community present in the rhizosphere is diverse, which may even be considered a separate compartment inside the sediment or soil where the plant grows. Currently, the plant-sediment interaction is not yet sufficiently known to allow the understanding of the role of the microbial community present there, its dynamics and influence of the presence of plants in their activity. However, the presence of plants can influence the bioavailability of metals (Almeida *et al.* 2004, 2006) and the bacterial response may also be altered. Plants act efficiently in retaining sediments and floating matter including associated metals and organic contaminants. At the interface of macrophytes root-sediment there is intense microbiological activity, liberation/uptake of O₂ and CO₂, organic compounds and metals. For instance, Caçador *et al.* (2000) and Sundby *et al.* (2005) have observed in the Tagus estuary that, in comparison with sediments without vegetation, the rhizosphere was richer in heavy metals, which are in chemical forms of relatively low availability (e.g. complexes with organic ligands, including exudates).

The few studies on the effect of heavy metals in the rates of denitrification and production of nitrous oxide (N₂O) revealed that the denitrification can be inhibited by the addition of heavy metals (Bardgett *et al.* 1994, Sakadevan *et al.* 1999, Holtan-Hartwig *et al.* 2002).

However, the different steps in the reduction of NO_3^- to N_2 appears to show variable tolerance to the addition of heavy metals (Holtan-Hartwig *et al.* 2002).

The perceived low susceptibility of salt marsh estuarine systems to N-enrichment and eutrophication is often attributed to high rates of denitrification (NRC 2000). It has been suggested that through denitrification and burial, fringing salt marshes also play an important role in intercepting land-derived nutrients and thereby helping to prevent eutrophication in downstream ecosystems, such as sea grass meadows (Valiela and Cole 2002).

N_2 is the primary form of N lost during denitrification in salt marshes (Cartaxana and Lloyd 1999, Smith *et al.* 1983). NO and N_2O losses are orders of magnitude smaller by comparison. Maximal rates of N_2O loss normally do not exceed $0.14 \text{ mg N m}^{-2} \text{ day}^{-1}$ (Smith *et al.* 1983). NH_3 volatilization, while not a component of denitrification, is another form of gaseous N loss in salt marsh systems. It too is found in orders of magnitude lower than rates of N_2 loss due to the fairly low sediment pH values (<8) in most marsh sediments (Koop-Jakobsen 2003, Smith *et al.* 1983). The published rates of denitrification in vegetated sediments range from 0 to more than $100 \text{ mg N m}^{-2} \text{ day}^{-1}$. Median values of $14 - 28 \text{ mg N m}^{-2} \text{ day}^{-1}$ are in general higher than those reported for other environments including estuaries and continental shelves (Boynton and Kemp 2008). Denitrification may also be important in marsh sediments that receive nitrate-rich groundwater inputs, being the estimated rates as high as $504 \text{ mg N m}^{-2} \text{ h}^{-1}$ with up to 90% removal of nitrate load to the marsh (Tobias *et al.* 2001). NO_3^- availability, labile organic matter and oxygen (required for nitrification) seem to be the primary factors controlling the rate of denitrification (e.g., Cornwell *et al.* 1999, Thompson *et al.* 1995).

1.5 The Cavado and Sado estuaries: brief description

Two different Portuguese estuarine systems were selected for the present study: one in the North of Portugal – Cavado (41.5228 N; 8.7846 W) and another more South – Sado. Two sampling sites were selected in the Sado estuary: Lisnave (38.4879 N; 8.7912 W) and Comporta (38.4879 N; 8.8312 W), located respectively in the north bank upstream Setúbal and in the south bank upstream Troia (Figure 4).

The Cavado River has $1,600 \text{ km}^2$ of watershed and 135 km of length with an estuary that occupies 2.56 km^2 . The average flow is about $66 \text{ m}^3 \text{ s}^{-1}$ and the residual volume and

residence time is low, as a consequence, the estuary has in average low salinity. The freshwater occupies most of the estuary in low tide and in high tide the saltwater penetrates to about half of the estuary. The southern part of the estuary is separated from the Atlantic sea by a long sandbank, upstream of which is the main area of salt marsh. The Cavado estuary suffers the impact of an area of port infrastructure, fisheries, shipbuilding, industry and domestic use.

The Sado is a 180 Km-long river with 7,640 km² of watershed and an estuary with approximately 160 km². The average annual flow of the river is about 40 m³ s⁻¹, showing strong seasonal variability. This is an estuary with a complex topography, a sharp curvature, and two channels (north and south) with different hydrodynamic characteristics separated by banks of sand. The salt marshes are more abundant in the south bank occupying about 1/3 of the estuary and are integrated in the Sado estuary Natural Reserve. In this area fishing, agriculture and aquaculture are important economic activities. The town of Setúbal, on the north bank, with about one hundred thousand inhabitants and intensive industrial, petrochemical, shipyards and port activities is responsible for a large anthropogenic pressure on the system. In a recent study from Caeiro *et al.* (2005) Lisnave site was classified as a highly polluted site with a high impact potential and high risk to cause adverse effects on the biota and Comporta site was presented as a low contaminated site with low to moderated impact potential.

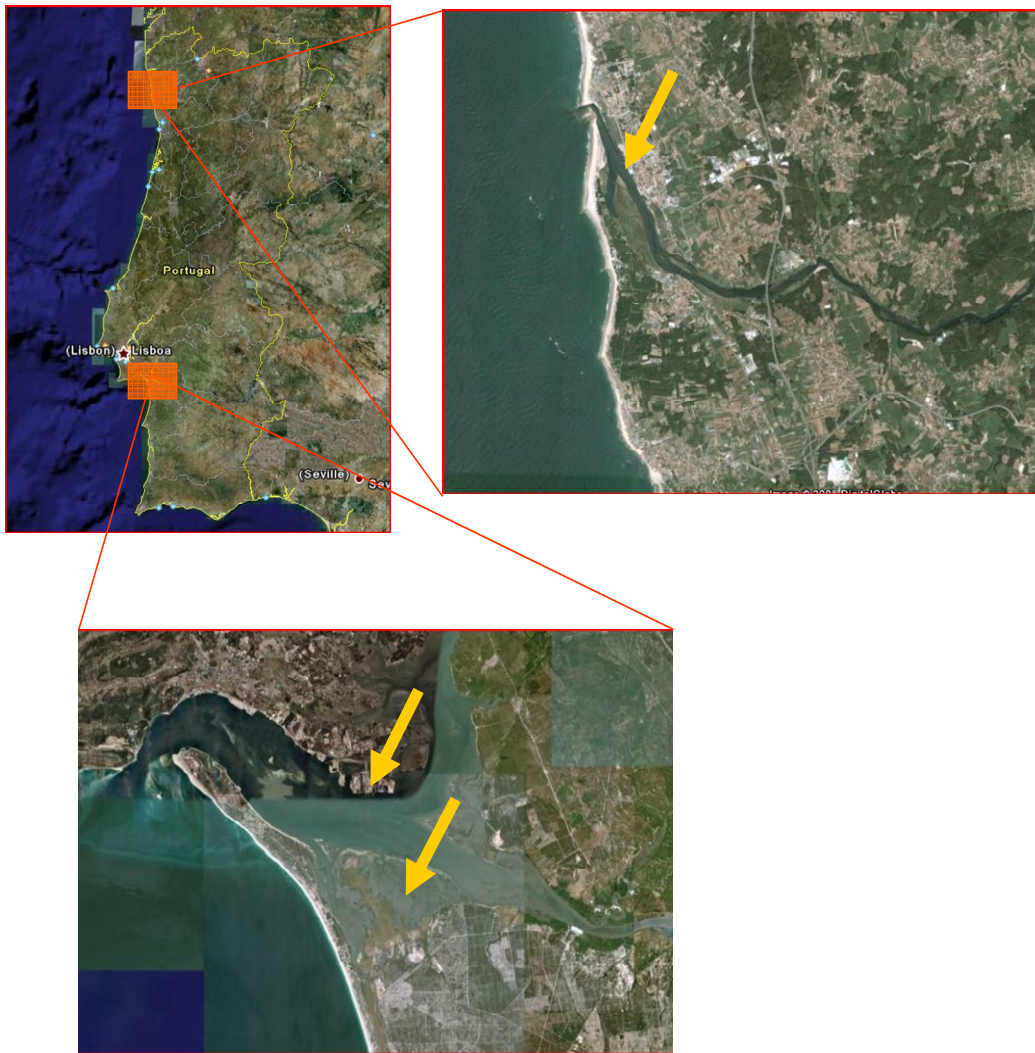


Figure 4: Cavado and Sado estuaries and location of sampling sites (source Google Earth).

1.6. Objectives

The present work aims to study the microbial communities, particularly denitrifiers and evaluate the effect of the presence of marsh plants in its structure, abundance and activity in two Portuguese estuaries. In order to achieve those objectives, research was carried out in order to:

- i. Characterize the microbial communities present in salt marshes sediments.
- ii. Identify possible interactions between measured environmental parameters (metal contamination) and the dynamics of the bacterial communities, investigating possible ecological roles of these communities.

- iii. Evaluate spatial and temporal variation of potential denitrification in colonized and uncolonized salt marsh sediments.
- iv. Evaluate the temporal dynamics of microbial communities evaluate the effect of the presence of marsh plants in the structure and abundance of denitrifying communities.
- v. Analyze the most representative phylotypes denitrifiers in the salt marsh studied.
- vi. Evaluate the effect of metal contamination in the shape of the bacterial communities, specifically the denitrifiers.

Chapter 2

Microbial communities within salt marsh sediments: composition, abundance and pollution constrains¹

2.1. Introduction

Temperate salt marshes are one of the typical estuarine ecosystems and are among the most productive environments on Earth (Constanza *et al.* 1997), harboring diverse communities implicated in multiple ecosystem functions. Estuaries can act as a buffer zone and final repositories for runoff pollutants (Teal and Howes 2000), including metals (Almeida *et al.* 2004, Reboreda and Caçador 2007), pathogens (Grant *et al.* 2001) and nutrients (Magalhães *et al.* 2002) that are introduced in the aquatic environment due to anthropogenic pressures from metropolitan and industrial areas (Rajendran *et al.* 1993).

Bacterial communities play essential roles in biogeochemical cycling of major nutrient (Bagwell *et al.* 1998, Cunha *et al.* 2005), turnover (transformation and mineralization) of organic matter (Pomeroy 1981, Cho and Azam 1990), and soil development processes (Lillebo *et al.* 1999, Kuske *et al.* 2002). The root exudates of marsh plants provide large amounts of organic carbon stimulating the growth of bacterial populations in vicinity of those roots (Rovira 1965). Therefore, structural and functional diversity of bacterial rhizosphere populations may reveal host specificities due to differences in root exudation and rhizodeposition (Jaeger *et al.* 1999), and therefore could reflect adaptation to distinct environments. The rizosphere is defined as the volume of soil adjacent to and influenced by the plant root (Sørensen 1997). Plants can change the characteristics of the surrounding sediments through the modification of pH and redox chemistry (Sundby *et al.* 2005), and by altering, for example, metal availability (Almeida *et al.* 2004, 2006). Salt marsh plants may play an important role in removing pollutants from the system, both directly by phytoremediation (e.g. accumulation of metals; Almeida *et al.* 2008) and indirectly by the improvement of the microorganisms' potential to bioremediation because they may lead to the selection of a well adapted pollutant-degrading microbial community (Johnson *et al.* 2004). Previous studies indicated that *H. portucaloides*, a commonly found plant in Portuguese temperate salt marshes, has the capability to accumulate metals and

¹ The content of this chapter is based on the following paper: Ana Machado A., Magalhães C., Mucha A.P., Almeida C.M.R., Bordalo A.A. Microbial communities within salt marsh sediments: composition, abundance and pollution constrains. Submitted to Estuarine Coastal and Shelf Science.

change the metal availability of the surrounding sediment (Caçador *et al.* 2000, Almeida *et al.* 2009).

Microbial rhizosphere diversity (e.g. Franklin *et al.* 2002;, Keith-Roach *et al.* 2002, Blum *et al.* 2004), and the impact of pollutants in those communities (e.g. Polymenakou *et al.* 2005, Cordova-Kreylos *et al.* 2006, Mucha *et al.* 2011) have been object of study through the years. However, a better understanding of the microbial communities involved in pollutant-degrading processes is needed to develop mitigation and recovery strategies. The aim of this study was to understand the role of salt marsh plant's (*H. portucaloides*) on microbial community distribution under different degrees of metal contamination. The study was carried out in three salt marshes systems in two contrasting seasonal conditions (winter and summer).

2.2. Material and Methods

2.2.1. Description of the study area

Two different Portuguese estuarine systems were selected for the present study: one in the North of Portugal – Cavado (41.5228 N; 8.7846 W) and another– Sado, southerly located. In the latter estuary, two sites were identified: Lisnave (38.4879 N; 8.7912 W) and Comporta (38.4425 N; 8.8312 W), located respectively in the north bank upstream of an urban – industrial area (Setúbal) and in the south bank upstream of Troia.

The Cavado River has 1,600 km² of watershed and 135 km of length with an estuary that occupies 2.56 km². The average flow is 66 m³ s⁻¹ with a short residence time fostering low salinity during low tide. Salt intrusion penetrates to about half of the estuary length. The southern part of the estuary is separated from the Atlantic sea by a long sand spit, upstream of which is the main area of salt marsh. The Cavado estuary suffers the impact of a small port infrastructure, fisheries, shipbuilding, industry and urban use.

The Sado is a 180 Km-long river with 7,640 km² of watershed and an estuary with approximately 160 km². The average annual flow of the river is 40 m³ s⁻¹, showing strong seasonal variability. The topography is complex with a sharp curvature S – N, and two channels (north and south) with different hydrodynamic characteristics separated by sand banks. The salt marshes are more abundant in the south bank occupying about one third of the estuary and are integrated in the Sado estuary Natural Reserve. In this area fishing, agriculture and aquaculture are important economic activities. The town of Setúbal, on the north bank, with about one hundred thousand inhabitants and intensive industrial,

petrochemical, shipyards and port activities is responsible for a large anthropogenic pressure on the system. In a recent study from Caeiro *et al.* (2005), the Lisnave area was classified as a highly polluted site with a high impact potential and high risk to cause adverse effects on the biota, whereas the Comporta site was presented as a low contaminated site with low to moderate impact potential.

2.2.2. Sample collection

Sediment from sites with *H. portucaloides* (colonized sediment or rhizosediment) and uncolonized sediment by any plant were collected during the 2006 winter and summer seasons at low tide, using plastic shovels. Nine different sediments and rhizosediments cores were retrieved between 5 and 20 cm depth to cover the sediment area representative of each salt marsh directly influenced by the plant's roots. Samples from each site were homogenized (composite sample), stored in sterile plastic bags and transported to the laboratory in the dark in refrigerated ice chests. The use of composite samples enables lower micro-site variations and therefore more liable global comparisons between marshes. For each composited sediment sample, three independent sub-samples were retrieved for total cell count, structure of microbial communities, and metals analysis. For microbial abundance analysis triplicate samples were fixed with formaldehyde (4 % v/v) whereas for microbial structure, samples were immediately frozen at -80 °C until further processing. For metal determination, sediment samples were dried at room temperature until constant weight.

2.2.3. Analytical procedures

Organic matter content in sediments was estimated by loss on ignition (4 h at 500 °C), in sediments previously dried at 60 °C. Grain size analysis (determination of the fraction < 0.063 mm) was performed by wet sieving samples previously treated with hydrogen peroxide (Mikutta *et al.* 2005). For metal analysis, ca. 0.25 g of dry sediment was digested by microwave (MLS-1200 Mega, Milestone, Bergamo, Italy) under high-pressure, in proper Teflon vessels with suitable amounts of concentrated nitric-acid as described elsewhere (Almeida *et al.* 2004). Total-recoverable levels of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn in the obtained solution were assayed either with flame (Philips PU 9200 X, Cambridge, UK) or with electrothermal atomization (Perkin–Elmer 4100 ZL, Norwalk, CT, USA) depending on the metal levels (Almeida *et al.* 2004, 2008). Metal concentrations were normalized to Fe content before further statistical analysis, an approach usually

used to establish the level of sediment contamination and to understand the potential different metal sources (Almeida *et al.* 2008).

2.2.4. Direct Microscopic Count (DMC) of Microbial Cells

Triplicates of 0.1 g of un-colonized sediments or rhizosediment were fixed with formaldehyde (4 % v/v). The amount of sample was optimized in order to achieve a maximum number of counts with the minimum of sample. Sub-samples (150 µl) of each replicate were stained with 4', 6'-diamidino -2-phenylindole (DAPI) and filtered onto black 0.2 µm Nucleopore polycarbonate membranes (Whatman, UK) (Porter and Feig 1980). Microbial cells were counted directly with an epifluorescence microscope (Labphot, Nikon, Japan) equipped with a 100 W high-pressure mercury lamp and a specific filter sets (UV-2B) at 1,875x magnification. A minimum of 10 random microscope fields for each replicate were counted in order to accumulate at least 300 cells per filter.

2.2.5. DNA extraction and PCR amplification

Total community DNA was extracted from 0.25 g of wet weight of rhizosediment or un-colonized sediment using the PowerSoil DNA Isolation Kit (MoBio laboratories Inc, Solana Beach, Calif.). For each sample, duplicate DNA extractions were performed with the purpose of accounting for variability between replicates. The 16S rDNA fragments of about 200 bp (positions 344 to 534 (*Escherichia coli* numbering)) were amplified using a primer set specific to Bacteria: 341F-GC (5'CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG -3') and 534R (5'-ATT ACCGCGGCTGCTGG-3') (Muyzer *et al.* 1993).

Amplification was done in 25 µl reaction mixture containing 1-5 ng DNA template, 10x Reaction Buffer (MgCl₂ free), 1.5 mM MgCl₂, 200 µM dNTP, 100 pmol of each primer and 1U Taq polymerase (STAB-VIDA, Lisbon, Portugal). A PCR reaction mixture with all reagents except template DNA served as a negative control. The temperature profile conditions was as follows: initial denaturation at 95°C for 5 min, 94 °C for 30 s, 65 °C for 30s decreased by 1 °C every second cycle until a touchdown at 55 °C, and 72 °C for 30 s; at each temperature 30 additional cycles were carried out and a final elongation step at 72 °C for 10 min (adapted from Muyzer *et al.* 1993). After each PCR the size of the expected amplified fragments were verified on a 1.5 % agarose gel electrophoresis.

2.2.6. DGGE

DGGE was performed using a CBS Scientific DGGE system (Del Mar, CA, USA). Samples containing approximately equal amounts (600 ng) of PCR product previously purified with the Qia-quick PCR purification kit (Qiagen, Valencia, CA, USA) were loaded onto 6.5 % (w/v) polyacrylamide gel in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) containing a gradient of denaturant from 40 % to 65 % (100 % denaturation conditions contains 7M urea and 40 % formamide). The electrophoresis was run for 18 h at a constant voltage of 57 V in 1x TAE buffer at 60 °C. PCR reactions containing genomic DNA from *Clostridium perfringens* and *Bacillus thuringiensis* (Sigma, USA) were used as a standard. Denaturing gradient gels were stained with 1x SYBR Green (1:10 000 dilution, Molecular Probes, USA) and photographed on a UV transillumination table using a gel documentation system equipped with a digital camera (Kodak EDAS100, USA).

2.2.7. Statistical analysis

Spatial and seasonal differences between sediment parameters were evaluated through analysis of variance (one-way ANOVA) followed by a post hoc Tukey honestly significant difference (HSD) multi-comparison test using the software STATISTICA 6.0 (StatSoft, Tulsa, USA). Images of DGGE profiles were analyzed with the GelComparII version 5.1 software (Applied Maths, Kortrijk, Belgium). Assuming that each different band in DGGE profile corresponded to a different OTU (Operational Taxonomic Unit), a presence or absence matrix was generated and used as input data to evaluate differences in Bacteria assemblage composition by multidimensional scaling (MDS) and hierarchical cluster analysis based on UPGMA ("Unweighted Pair Group Method with Arithmetic Mean"). Principal components analysis (PCA) was applied to the log (x+1) transformed environmental variables (sediment characteristics and metals concentrations) and microbial abundance. Dendograms were generated using the group average method and euclidean distances calculated for environmental variables and Bray-Curtis similarities to species data. ANOSIM analysis (Clarke 1999) was used to test the significance of the different clusters generated; the values of the R statistic were an absolute measure of how well the groups separated and ranged between 0 (indistinguishable) and 1 (well separated). The link between the biotic pattern and environmental variables was explored using the biological environmental gradients (BIO-ENV) analysis. Such procedure enabled the selection of the abiotic variable subset that maximized the rank correlation (ρ) between biotic and abiotic (dis)similarity matrices (Sokal and Rohlf 1995). Latter,

multivariate analyses were performed in PRIMER version 5 software (Primer-E Ltd, UK) (Clarke and Warwick 1994, Clarke 1999).

Relationships between microbial composition (presence/absence matrix of DGGE profiles) and environmental variables were analyzed by redundancy analysis (RDA) using the software package CANOCO for Windows 4.5 (Biometris, Wageningen, The Netherlands). Inflation factors were examined and highly correlated variables with little contribution to the total variation were removed (ter Braak and Smilauer 2002). Intrasite correlations were used to examine the relative contribution of each variable to the separate ordination axis. The unrestricted Monte Carlo permutation test (499 permutations) was used to test the statistical significance. The significance level used for all tests was 0.05.

2.3. Results

2.3.1. Sediment characterization

Sediment characteristics in terms of organic matter, grain size fraction < 0.063 mm (percentage of fines) and metal concentrations at each study site are presented in Table 1. The metal levels differed among the different marshes. Lisnave site (Sado estuary), showed the highest metal concentrations in sediments and rhizosediment (Table 1), as expected. On the other hand, Cavado samples were characterized by high content of organic matter and overall lower metal concentrations and percentage of fines (Table 1). Indeed, the levels of both, Zn/Fe and Cr/Fe, were lower in Cavado estuary (Figure 5), although statistical significance was only observed for Cr/Fe (Tukey HSD test results, $p < 0.05$). The Comporta salt marsh showed lower concentrations of Pb/Fe and Cu/Fe (Tukey HSD test results, $p < 0.05$; Figure 5). When looking into the organic matter content, a clear separation between sediment and rhizosediment emerged, the latter with higher values (Tukey HSD test results, $p < 0.05$).

PCA analysis applied to sediment characteristics (Figure 5) showed that PCA1 and PCA2 axis together explained 58.1 % of the total variability of the variables included in the analysis. With an additional PCA3 axis, the percentage increased to 76.6 %. While Zn/Fe and Cr/Fe concentrations were weighted heavily in PCA1 (with eigenvectors of -0.518 and -0.497 respectively), Cu/Fe and Pb/Fe concentrations, were weighted heavily in PCA2 (with eigenvectors of 0.655; and 0.612 respectively). ANOSIM test revealed that samples were primarily clustered according to the estuary with statistically significant differences between Cavado and Sado ($n = 12$; $R = 0.613$; $p = 0.05$).

Table 1: Percentages of organic matter content (OM) and grain size fraction < 0.063 mm (fines, % of total weight), as well as Cd, Cr, Cu, Pb, Mn, Ni, Zn and Fe concentrations (mean and standard deviation, n=3), observed in sediments colonized (Rhizo) by *H. portulacoides* and un-colonized (Sed).

	Winter										Summer													
	Cavado River Estuary				Sado River Estuary						Cavado River Estuary				Sado River Estuary									
					Lisnave				Comporta						Lisnave				Comporta					
	Rhizo ^a		Sed ^a		Rhizo ^a		Sed ^a		Rhizo ^a		Sed ^a		Rhizo		Sed		Rhizo		Sed		Rhizo		Sed	
OM (%)	19.2	± 0.6	11.4	± 0.7	13.4	± 0.1	9.2	± 0.2	10.7	± 0.4	12.1	± 0.4	14.5	± 0.1	7.9	± 0.4	12.2	± 0.2	9.9	± 0.1	12.7	± 0.2	9.6	± 0.1
< 63 µm (%)	67		67		97		99		98		97		89		49		98		96		91		92	
Cd (ng g-1)	179	± 23	76	± 19	40	± 14	416	± 22	41	± 21	295	± 13	65	± 1	63	± 13	43	± 8	375	± 33	57	± 6	157	± 18
Cr (µg g-1)	41	± 2	34	± 1	79	± 4	84	± 4	76	± 6	74	± 7	38	± 5	25	± 7	81	± 1	73	± 2	79	± 2	79	± 2
Cu (µg g-1)	66	± 2	57	± 3	111	± 7	136	± 5	63	± 3	59	± 2	82	± 6	50	± 11	127	± 2	187	± 7	79	± 2	77	± 1
Pb (µg g-1)	55	± 7	46	± 4	102	± 5	77	± 4	53	± 4	55.7	± 0.7	62	± 5	36	± 7	102	± 3	102	± 4	56	± 3	49	± 10
Mn (µg g-1)	160	± 42	184	± 25	872	± 49	148	± 20	448	± 35	122	± 4	160	± 5	144	± 32	238	± 12	136	± 5	202	± 10	165	± 2
Ni (µg g-1)	13.1	± 0.7	15	± 2	38	± 6	35	± 2	33	± 2	32	± 4	25	± 5	14	± 2	54	± 0	46	± 0	43	± 3	47	± 5
Zn (µg g-1)	127	± 1	104	± 2	324	± 15	370	± 46	270	± 20	391	± 16	135	± 2	104	± 21	250	± 2	347	± 27	288	± 4	318	± 13
Fe (%)	2.8	± 0.2	2.68	± 0.06	4.29	± 0.05	4.5	± 0.6	4.5	± 0.2	3.4	± 0.2	2.84	± 0.08	2.30	± 0.40	4.88	± 0.09	4.40	± 0.40	4.65	± 0.60	4.64	± 0.90
^a adapted from Almeida et al. (2008)																								

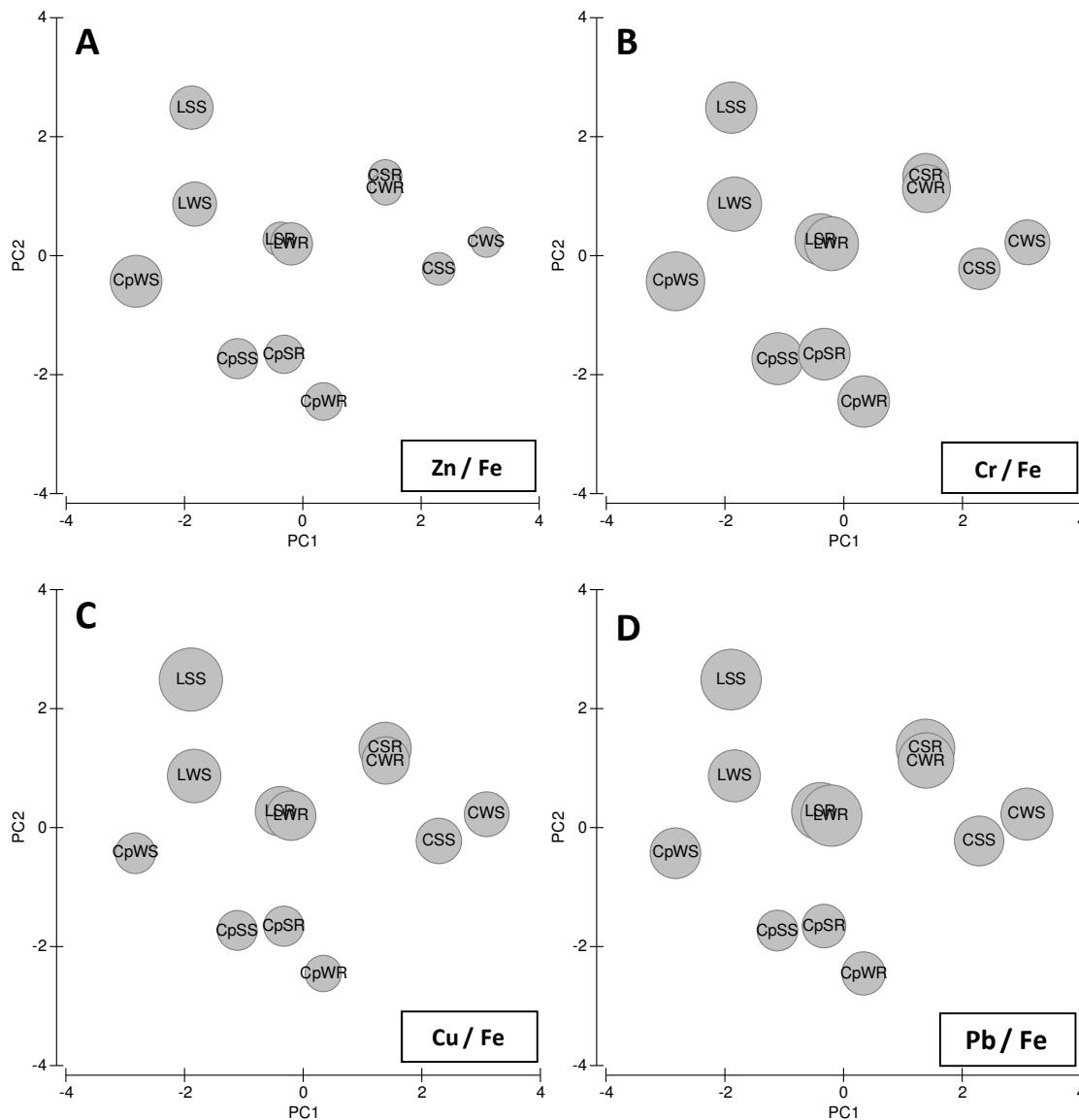


Figure 5: Two-dimensional PCA ordination of the sediment characteristics described in Table 1 (transformed and normalized) for each salt marsh (Cavado – C; Lisnave – L; Comporta – Cp), in the respective season (W-winter; S – Summer) for colonized (R) and un-colonized (S) sediments. Values of Zn/Fe (A), Cr/Fe (B), Cu/Fe (C) and Pb/Fe (D), for each sample represented as circles of a diameter proportional to the magnitude of the value.

2.3.2. Abundance of microbial populations

Total counts of microbial cells ranged $1.28 - 4.94 \times 10^8$ cells g wet sed⁻¹ (Figure 6). Cavado salt marsh had higher bacterial abundance compared to the salt marshes from Sado estuary (Tukey HSD test results, $p < 0.05$ and ANOSIM, $n = 12$; $R = 0.662$; $p = 0.05$).

Although the DAPI stained counts were higher in rhizosediments when compared to uncolonized sediments, the differences were not statistically significant. However, a significant negative correlation was observed between DAPI cells counts and the Ni/Fe, Zn/Fe and Cr/Fe concentrations ($r = -0.70, -0.74, -0.79$; $p < 0.05$; $n = 12$, respectively). In fact, these variables were selected by the BIO-ENV analysis ($\rho_s = 0.695$) to best match the microbial abundance distribution. Similarly, RDA analysis (Figure 8) showed that the variables that correlated most strongly with RDA 1 axis (Cr/Fe, Zn/Fe and Ni/Fe; intersect values of $-0.8044, -0.7813, -0.7295$ respectively) explained 90.6 % of the total cumulative microbial abundance data variance and 100 % of the cumulative variance of the microbial abundance-environment relationship. Monte Carlo permutation test confirmed that the contribution of combined variables of the first axis was significant ($F = 5.501$ and $p = 0.0420$).

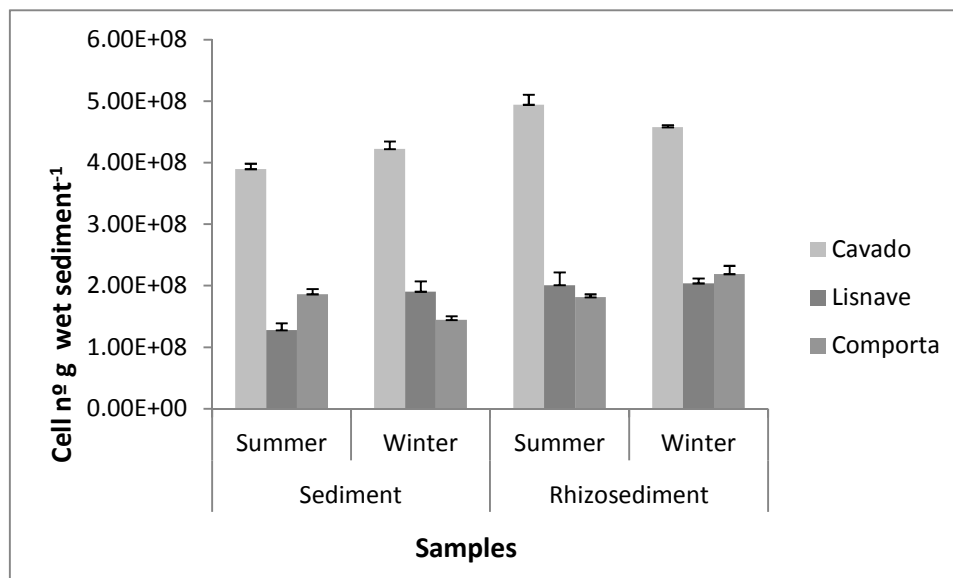


Figure 6: Microbial abundance estimated by total cell counts (mean and standard deviation, $n = 3$) in un-colonized sediments and rhizosediments, for each one of the salt marshes studied in the different sampling seasons.

2.3.3. Microbial community structure

In order to assess to the dynamics of microbial diversity in the different salt marshes, PCR-amplified 16S rRNA gene fragments were run in DGGE and hierarchical cluster analysis was applied based on the presence or absence of DGGE bands. A total of 19 distinct OTUs, defined as constrained above, and thus corresponding to different bacteria phylotypes, were identified in the DGGE profiles. For the study of the community structure

the distribution of phylotypes within the sample emerges as more relevant than the number of different phylotypes. Hierarchical cluster analysis for all DGGE profiles showed that replicates were grouped together (98 % - 100 % similarity), being more similar between each other than with any other sample suggesting good methodological replication. To facilitate interpretation, only one of the replicates was displayed in the cluster.

Hierarchical cluster analysis (Figure 7) showed that for each salt marsh, samples were grouped together, being more similar among each other than with samples from the other marshes. The only exception was for un-colonized sediments from Cavado collected during the winter. Between marshes, Comporta samples showed the most dissimilar bacteria community structure, clustering together in a different branch at a similarity level of 45 %. Within this cluster, the samples from un-colonized sediments were more similar between them forming a sub cluster at 67 % of similarity. The remaining samples (Cavado and Lisnave) clustered together at a similarity level of 35 %. With the exception of the above mentioned un-colonized sediment sample from Cavado, the remaining were separated according the salt marsh at a level of 55 %. Within those clusters, the samples from summer were more similar to each other with 83 % of similarity (Figure 7). The ANOSIM test revealed statistically significant differences between these groups generated by hierarchical cluster analysis ($n = 12$; $R = 0.628$; $p = 0.05$).

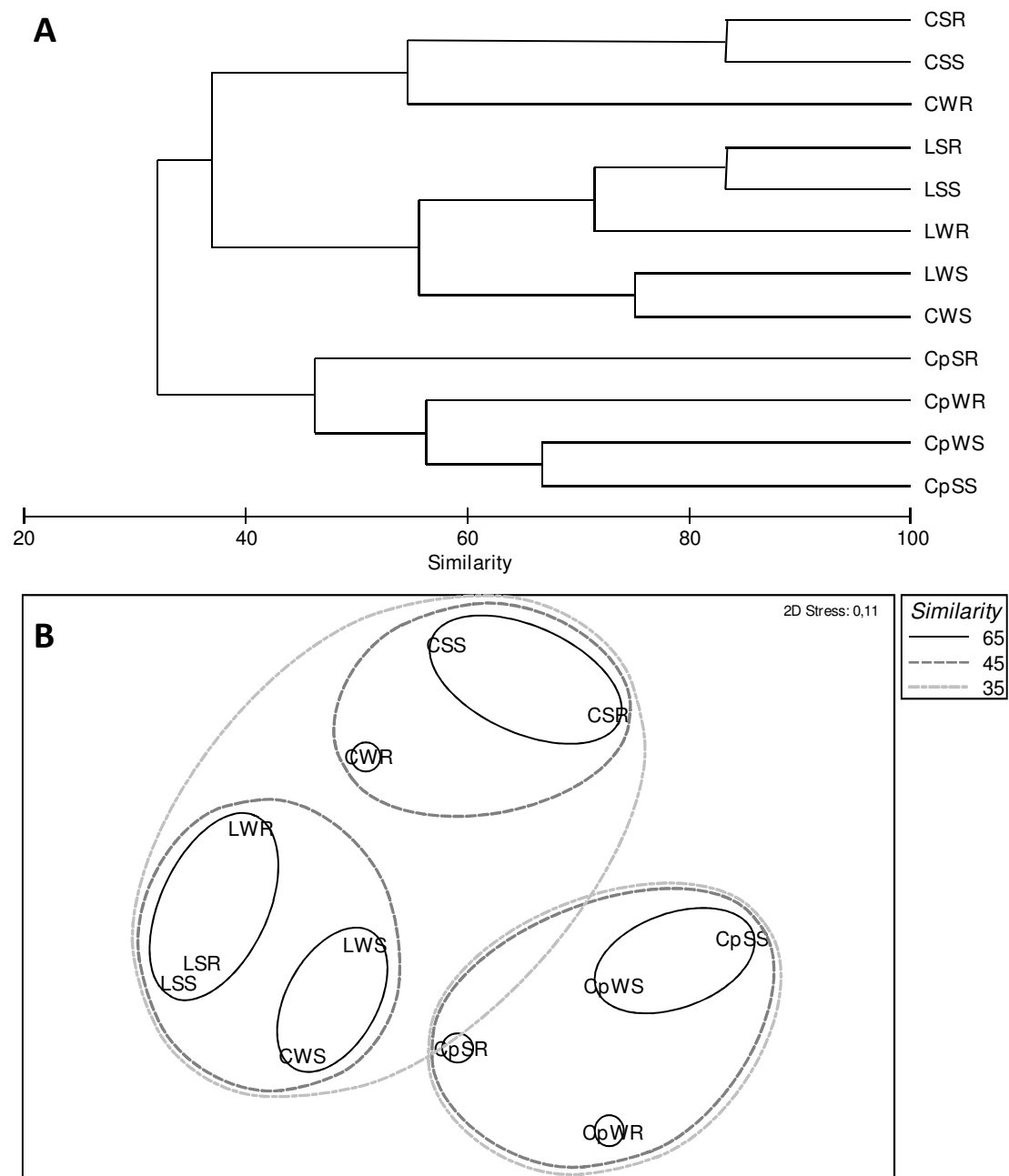


Figure 7: Cluster analysis (A) and non-metric multidimensional scaling (MDS) (B) ordination (with superimposition of hierarchical analysis) of the sampling sites, using Bray-Curtis similarities on presence/absence matrix obtained of the DGGE profiles. (Salt marsh: Cavado – C; Lisnave – L; Comporta – Cp; Season: Winter - W; Summer -S; presence/absence of plant: colonized sediment - R and un-colonized sediments – S)

2.3.4. Influence of sediment characteristics on microbial diversity

The BIO-ENV procedure was used to select the combinations of environmental variables that best correlated with the bacterial community structure obtained above. Indeed, the variables that best matched biota MDS were the combination of Pb and Cr concentrations normalized to Fe ($p_s = 0.46$). Although the BIO-ENV procedure was unable to give the direction of such trend, these variables may be pivotal to ascertain the differences in the community structure that emerged between the different salt marshes. Correlations between sediment characteristics and microbial composition assemblages were also examined using RDA (Figure 8). The first two RDA axes explained 36.2 % of the total cumulative species data variance and accounted for 67.5 % of the cumulative variance of the species-environment relationship. The unexplained fraction of variation that was explained by unknown (non-studied) factors represented 32.5 % of the total variation. Monte Carlo permutation test showed that the contribution of combined variables was significant ($F = 1.378$ and $p = 0.04$). The variable that correlated most strongly with RDA 1 was Pb/Fe concentration, whereas Cr/Fe and Zn/Fe correlated best with RDA 2 (Figure 8).

Furthermore, although some OTU's were commonly present in all the marshes, others were more related to a specific marsh due to the sediments characteristic present there. Therefore, while Lisnave bacterial assemblage appeared more related to high Pb/Fe concentration, explaining 41.5 % of the variance found for the microbial distribution, Comporta samples were more associated with Ni/Fe, Cr/Fe and Zn/Fe concentrations, responsible for the remaining 26 % of the variation and subsequent distribution of microbial population. RDA analysis also suggested that Cavado microbial populations were linked to the higher content of organic matter and the lower metal concentrations found in that environment (Figure 8).

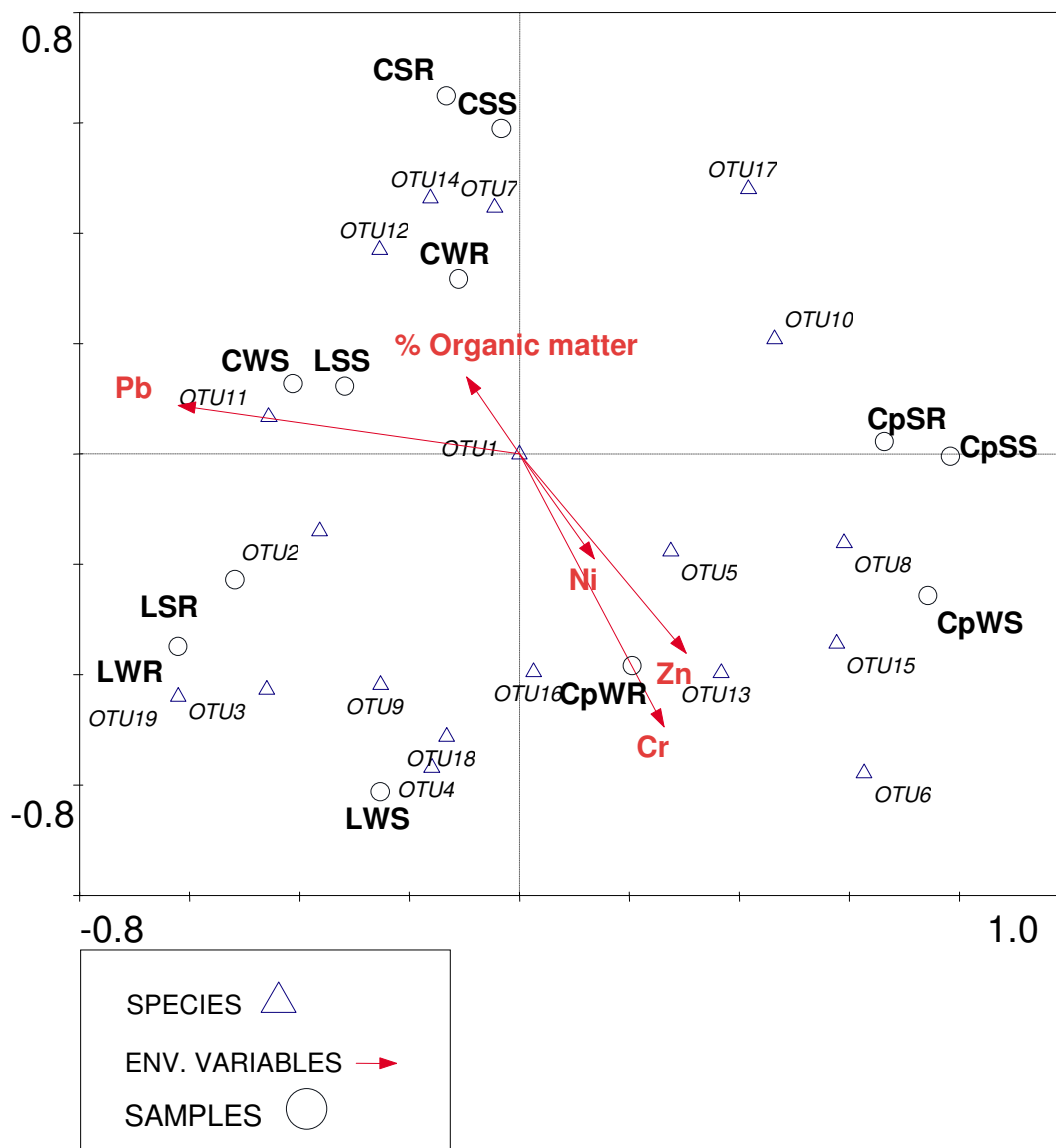


Figure 8: RDA ordination plot showing the relationship between the distribution of microbial composition and measured sediment characteristics (metals concentrations Fe normalized and organic matter content).

2.4. Discussion

Estuarine sediments are often considered sinks for pollutants (Teal and Howes 2000) in aquatic systems, but sediments with different characteristics have different capacities for accumulating contaminants (Wang *et al.* 2001). Several studies have showed that the presence of vegetation influences the metal availability by changing pH and redox chemistry in the vicinity of the roots (Sundby *et al.* 2005, Almeida *et al.* 2008). Moreover, bacterial abundance, structural and functional diversity of the microbial community present in the sediments can be affected by the presence of plants due to root exudates, rhizodeposition (Sørensen 1997, Jaeger *et al.* 1999), and by increase of the surface for

colonization (Kirk *et al.* 2005). Our results indicated that microbial community abundance and diversity from the different estuarine systems evaluated in this study, was affected by the level of metals concentration and by the presence or absence of salt marsh plants. We observed a significantly higher microbial abundance in Cavado compared to Sado estuary whose levels of metal concentrations were generally higher (Table 1). The adverse effects of different metals on soil microbial communities have also been established in other studies (Said and Lewis 1991, Khan and Scullion 2000, Turpeinen *et al.* 2004). While not significant, slightly higher microbial abundance was detected in plant-colonized sediments compared to the un-colonized sediments in both Cavado and Sado estuaries. This can be related to the higher organic matter content generally observed in colonized sediment. Other authors identified factors such as the aerobic, nutrient-rich environment created by the plants (Olson *et al.* 2003, Kuiper *et al.* 2004) as responsible for the higher microbial abundance in rhizosediment. Nevertheless, the lack of significance on these microbial relative abundances in colonized and un-colonized sediments that is in concordance with results obtained for nitrogen-fixing bacteria in salt marsh sediments (Burke *et al.* 2002), may suggest that bacterial communities do not depend exclusively on the plants as an organic matter source. Other factors such like turnover rates, other limiting nutrients, predation/grazing and differences in resource utilization may interfere with this relationship. Similarly, no significant changes in microbial abundance were observed between the winter and summer surveys at the different salt marshes. The correlation between bacterial abundance and organic carbon availability is well documented for soils, sediments and water for systems where the organic matter reached their maximum in summer (Alexander 1977, Palmborg *et al.* 1998, Espeland *et al.* 2001). Nevertheless the relative stability of bacterial communities between different sampling periods found in our study has also been observed in other salt marshes systems (Piceno and Lovell 2000a, b, Burke *et al.* 2002).

We applied 16S rDNA PCR-DGGE analysis to study the dynamics of bacterial community structure in colonized and un-colonized sediments in three salt marshes with different levels of metal contaminations in two seasonal contrasting situations (winter and summer). We generated clear DGGE profiles presumably representative of the dominant phylotypes present in the analyzed samples. Similarly to other studies (Heuer and Smalla 1997), we found several OTU's that were present across all samples collected in the different estuarine systems and seasons both in colonized and un-colonized sediments. Hierarchical cluster analysis based on the DGGE profiles revealed a clear division between the microbial assemblages that inhabited the three salt marshes. Comporta salt marsh was unique among them, being clustered in a separated branch. The RDA analysis

explained this separation due to OTU's in the community structure associated to higher Ni/Fe, Cr/Fe and Zn/Fe concentrations and lower Pb/Fe concentration. These data suggested that increased deposition of metals may select metal-tolerant communities (Feris *et al.* 2004, Becker *et al.* 2006). Furthermore, the dissimilarity between rhizosediment and sediment bacterial communities was also evident in Comporta salt marsh samples (Figure 7), which could be explained by the influence of plants in creating different niches in the roots surrounding environment (Bowen 1980) supporting higher abundant and diverse bacterial assemblage (Lovell *et al.* 2000). Also, the rhizosphere can influence the community structure since the salt marsh plants keep the roots neighborhood more stable while the un-colonized sediment is washed, especially in winter (increase of flow) in more hydrodynamic exposed marshes (Cavado and Lisnave).

Although samples from Lisnave did not show a marked discrimination between colonized and un-colonized sediment, samples from summer appeared to be more similar with each other. Lisnave sampling site is more exposed to the main river channel with more pronounced environmental shifts that can lead to populations more uniformly distributed. The presence of intensive industrial, petrochemical, shipyards and port activities can be responsible for the incidence of a bacterial composition that tolerates high levels of metals, especially Pb that according to the RDA analysis explained 41.5 % of the variation in the distribution of the microbial assemblages.

The microbial composition of Cavado estuary salt marsh appeared more similar to the one present in Lisnave salt marsh, although the levels of metal contamination and organic matter were clearly different. The community structure followed the same trend being the season responsible for the formation of a subcluster with samples belonging to summer that present higher similarity between colonized and un-colonized sediment. Through the RDA analysis it was possible to confirm the existence in Cavado salt marsh of OTU's related to the low pressure from metals and the higher organic matter content, except for the winter un-colonized sediment samples that seem to be associated to the presence of Pb. Although rather different, the community composition of the dominant microbial taxa, among salt marsh belonging to different estuaries (Cavado and Sado-Lisnave) clustered together in the same branch being more similar between each other than with Comporta microbial population what appeared to be structured by local environmental factors, as previously described for similar cases (Bowen *et al.* 2009). This hypothesis was here reinforced since in both cases there was a pronounced exposure to environmental changes inherent to the hydrodynamics of the main river channel at the vicinity of which the sites were located. On the contrary between marshes Comporta site, located in the

south channel, with much more stable hydrodynamic conditions, presented a more dissimilar community structure.

Although it is tempting to conclude that metal contamination influenced the shaping of the community composition, it is difficult to quantify the relative importance of the abiotic and the biotic environmental factors (Bagwell *et al.* 2001). Controlled contaminant exposure experiments in microcosms are essential to test this hypothesis and remove the background present in the field. Although, RDA demonstrated that metals contamination accounted for a significant amount of variability in the community composition (67.5 %) a large fraction of variation (32.5 %) was still unexplained. In fact, other environmental variables not quantified in the present study, like hydrodynamic characteristics, residence time, dissolved oxygen and nutrient concentrations have been described as possible factors contributing for the determination of the microbial composition (Bouvier and del Giorgio 2002, Crump *et al.* 2004).

The approach employed in this study allowed us to give some insights on the microbial community dynamics in colonized and un-colonized salt marsh sediments in response to different levels of contamination. However we acknowledge the limitations due to the DGGE technique (Heuer and Smalla 1997). As in all PCR-based tools, DGGE may have several associated biases related to DNA extraction efficiency, and selective amplification genes from mixed DNA communities (Kopczynsky *et al.* 1994, Suzuki *et al.* 1996). This technique captures only sequences that are present in at least 0.5-1% of the total cells in the sample (Muyzer *et al.* 1993), i.e. the dominant phylotypes. Moreover, one band may represent more than one species since phylogenetically related species share rather similar sequences in the fragment analyzed (Gomes *et al.* 2001). On the other hand, the same organism may produce more than one DGGE band due to multiple, heterogeneous rRNA operons (Cilia *et al.* 1996, Nubel *et al.* 1996, Rainey *et al.* 1996). Despite these shortcomings, PCR-DGGE approach proved to be a powerful method allowing a comprehensive picture of the community structure and constraints associated with it.

2.5. Conclusion

In this study, we can conclude that sediment characteristics, the presence of salt marsh plants and metal contamination fostered the selection and adaptation of different microbial populations to the anthropogenic pressures present in salt marsh ecosystems. Since salt marshes may constitute large areas in temperate and subtropical estuaries and are

important ecologically, this work represents an important contribution to the understanding of how and at which level pollutants like metals can interfere with the natural environmental variability and may influence the abundance and structure of microbial communities in those environments.

Chapter 3

Diversity and functionality of denitrifier communities from different salt marshes²

Denitrification, a stepwise microbial dissimilatory reduction of nitrate (NO_3^-) to nitrogen gases (NO , N_2O and N_2) under suboxic conditions has been shown to represent the main biological sink for fixed (biological available) nitrogen in estuarine and coastal ecosystems (Seitzinger and Nixon 1985, Seitzinger 1987, White and Howes 1994, Howes *et al.* 1996, Nowicki 1999). The global importance of denitrification lies also in its contribution to global warming (Houghton *et al.* 1992) and to the destruction of stratospheric ozone (Crutzen 1970, Dickinson and Cicerone 1986) through the production and accumulation of potent greenhouse gases, such as NO and N_2O (Braker *et al.* 2000).

Nitrite (NO_2^-) reduction to nitric oxide (NO) is the rate-limiting step in denitrification and is catalyzed by the metalloenzyme nitrite reductase (Nir), considered the key enzyme in the process (Zumft 1997). The NO_2^- reduction step is particularly important because distinguishes denitrifiers from other bacteria that use NO_3^- metabolism without being able to reduce NO_2^- to gas (Shapleigh 2006). Two structurally different but functionally and physiologically equivalent forms of NO_2^- reductases may occur: NirK, a Cu-containing enzyme encoded by *nirK*; and NirS, containing iron (cytochrome cd1) encoded by *nirS* (Glockner *et al.* 1993, Zumft 1997, Philippot 2002). Although, these enzymes are found in microorganism within a wide range of taxonomic distinct groups of Bacteria and Archaea (Zumft 1997, Philippot 2002) they have never been found to coexist in the same denitrifying organism (Coyne *et al.* 1989). Due to the high phylogenetic diversity among denitrifiers, including over 50 different genera (Zumft 1997), these NO_2^- reductase genes have been extensively used as functional molecular markers, rather than a 16 rRNA approach, to elucidate denitrifier communities structure in a variety of environments. These include soils (Avrahami *et al.* 2002, Priemé 2002, Wolsing and Priemé 2004, Throbäck *et al.* 2007), estuarine sediments (Santoro *et al.* 2006, Dang *et al.* 2009, Mosier and Francis 2010, Magalhães *et al.* 2011), marine sediments (Braker *et al.* 2000, Liu *et al.* 2003, Hannig *et al.* 2006, Falk *et al.* 2007), groundwater (Yan *et al.* 2003), lakes and brackish water (Junier *et al.* 2008, Kim *et al.* 2011), and seawater (Castro-González *et al.*

² The content of this chapter is based on the following paper: Ana Machado A., Magalhães C., Mucha A.P., Almeida C.M.R., Bordalo A.A. Microbial communities within salt marsh sediments: composition, abundance and pollution constrains. Submitted to Microbial Ecology.

2005, Hannig *et al.* 2006, Falk *et al.* 2007, Oakley *et al.* 2007). However, in what salt marsh systems are concerned, much less attention has been given to the activity and diversity of the denitrifier communities. Salt marshes are typical of temperate estuarine ecosystems and due to their location between upland and coastal waters can act as a buffer zones intercepting, stabilizing and removing runoff pollutants (Teal and Howes 2000), including metals (Almeida *et al.* 2004, Reboreda and Caçador 2007), pathogens (Grant *et al.* 2001), and nutrients (Magalhães *et al.* 2002, Davis *et al.* 2004), that are introduced in the estuarine environment due to an intense development pressure and human encroachment in the metropolitan and industrial vicinity areas (Rajendran *et al.* 1993). In addition, the tidal regime with associated redox potential, salinity, and inundation fluctuations (Montague and Odum 1997), renders the salt marshes sediments unique from soils and marine sediments.

In salt marshes, plants can change characteristics of the adjacent soil, defined as rhizosphere (Sørensen 1997), through the modification of pH and redox chemistry (Sundby *et al.* 2005), and by altering, for example, metal availability (Almeida *et al.* 2004, 2006). Previous studies indicated that *Halimione portucaloides*, a plant commonly found in Portuguese salt marshes, has the capability not only to accumulate metals, but also to change the metal availability of the surrounding sediment (Caçador *et al.* 2000; Almeida *et al.* 2009).

The diversity and activity of salt marsh microbial communities play essential roles in biogeochemical processes (Teal and Howes 2000, Keith-Roach *et al.* 2002), such as denitrification, being their magnitudes controlled by a multitude of environmental factors and anthropogenic pressures (Wallenstein *et al.* 2006). Several studies have demonstrated inhibitory effects of metals on aerobic and anaerobic microbial respiration, biomass, N-mineralization, nitrification and on the microbial community structure of soils, sediments and other aquatic habitats (Giller *et al.* 1998, Holtan-Hartwig *et al.* 2002, Granger and Ward 2003), and particularly on the denitrification enzymatic pathway (Sakadevan *et al.* 1999, Holtan-Hartwig *et al.* 2002, Magalhães *et al.* 2007, Magalhães *et al.* 2011).

Although recent reports are available on the microbial rhizosphere diversity (e.g. Franklin *et al.* 2002, Keith-Roach *et al.* 2002, Blum *et al.* 2004), impact of pollutants in those microbial communities (e.g. Polymenakou *et al.* 2005, Cordova-Kreylos *et al.* 2006, Mucha *et al.* 2011), and specifically on denitrifier communities (e.g. Priemé *et al.* 2002, Davis *et al.* 2004, Cao *et al.* 2006, 2008), a better knowledge of the underlying

composition and diversity of denitrifier communities is urgently required to develop mitigation and recovery strategies to manage the complex nitrogen cycling in sediments. In his study, we combined biogeochemical activity and functional genes abundance, community structure and diversity approaches to gain insights into the role of salt marsh plants (*H. portucaloides*) on the denitrifier community dynamics. The study was carried out in three salt marshes systems under different degrees of metal contamination and in contrasting seasonal conditions.

3.1 Material and Methods

3.1.1. Description of the study area

In the present study we examined three salt marshes: Cavado (41.5228 N; 8.7846 W), in the North of Portugal and southerly, in Sado estuary, Lisnave (38.4879 N; 8.7912W) and Comporta (38.4425 N; 8.8312W) located in the north bank upstream of an urban – industrial area (Setúbal) and in the south bank upstream of Troia, respectively. The Cavado estuary is described in the literature (Gonçalves *et al.* 1994, Moreira *et al.* 2006, Almeida *et al.* 2008) as a contaminated estuary owing to the impact of port infrastructures, fisheries, shipbuilding, industry and urban use. On the south bank, the Sado estuary supports fishing, agriculture and aquaculture activities, whereas on the north bank the town of Setúbal (100,000 inhabitants), intensive industrial, petrochemical, shipyards and port activities are responsible for an important anthropogenic pressure on the system. Within the Sado estuary, Lisnave and Comporta sites were classified by Caeiro *et al.* (2005), respectively, as a highly polluted site with a high impact potential and high risk to cause adverse effects on the biota and as a low contaminated site with low to moderated impact potential.

3.1.2. Sample collection

The sampling survey was performed seasonally during 2006, at low tide, using plastic shovels. Sediment from sites with *H. portucaloides* (colonized sediment or rhizosediment) and un-colonized by any plant were collected between 5 and 20 cm depth to cover the sediment area representative of each salt marsh directly influenced by the plant's roots. The samples were homogenized, stored in sterile individual plastic bags and transported to the laboratory in the dark in refrigerated ice chests. For microbial abundance analysis (qPCR), microbial diversity and structure (DGGE and cloning) samples were immediately frozen at -80 °C until further processing. Simultaneously, overlying estuarine water

samples were collected and stored in acid-cleaned polyethylene flasks. Sampling survey was performed seasonally in 2006, at low tide, using plastic shovels. Sediment from sites with *H. portucaloides* (colonized sediment or rhizosediment), and un-colonized by any plant (un-colonized sediment), were collected between 5 and 20 cm depth to cover the sediment area representative of each salt marsh directly influenced by the plant roots. Samples were homogenized, stored in sterile individual plastic bags and transported to the laboratory in the dark in refrigerated ice chests. For gene abundance analysis (qPCR) and for microbial diversity and structure analysis (DGGE and cloning) samples were immediately frozen at -80 °C until further processing. For metal determination, sediment samples were dried at room temperature until constant weight. Simultaneously, overlying estuarine water samples were collected and stored in acid-cleaned polyethylene flasks.

3.1.3. Analytical procedures

For metal analysis, 0.5 g of triplicate dry sediments of each site was digested in high-pressure Teflon vessels, using a microwave (MLS-1200 Mega, Milestone), with 6 ml of suprapure concentrated nitric acid (Merck). Total-recoverable levels of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn in the obtained solution were assayed either with flame (Philips PU 9200 X, Cambridge, UK) or with electrothermal atomization (Perkin–Elmer 4100 ZL, Norwalk, CT, USA) depending on the metal levels (Almeida *et al.* 2004, 2008). Metal concentrations were normalized to Fe content before further statistical analysis, an approach usually used to establish the level of sediment contamination and to understand the potential different metal sources (Almeida *et al.* 2008).

3.1.4. Denitrification activity measurements

Denitrification potential and N₂O accumulation rates were measured for each sample in three replicates using the acetylene inhibition technique according to Magalhães *et al.* (2005). Briefly, slurries comprised serum bottles with the homogenized sediment sample and 10 ml of incubation water (overlying site water amended with 300 µM KNO₃ and 2 mM glucose), were hermetically sealed with a butyl stopper and aluminum crimp and purged 15 min with helium to remove oxygen (O₂). Slurries with and without acetylene (C₂H₂) addition (20 % vol:vol) were incubation in parallel. A separate set of time zero samples was sacrificed immediately after acetylene addition, to quantify N₂O levels with and without C₂H₂ at 0 h. All samples were incubated in the dark for 4 h at constant temperature (20 °C) and stirring (100 rpm). The linearity of the processes during incubations was confirmed in previous experiments (Magalhães *et al.* 2005). At the end of

incubation, 12 ml of headspace sample were recovered from each serum bottle (after headspace equilibration) by displacement with 3MNaCl solution (Joye and Paerl 1993). Gas sample was injected into a Varian gas chromatograph (CP-3800) equipped with an electron-capture detector, two Hay Sep D columns and an automatic back flush system to prevent C₂H₂ from passing to the detector. Quantification was determined using standard curves generated from purified gas (N₂O in He, Scott Specialty Gas), and the detection limit of the method was approximately 20 nM N₂O. N₂O production rates were calculated based on the N₂O concentrations in the treatments without C₂H₂, and the potential denitrification rates (N₂ plus N₂O production) was calculated as the difference between the N₂O produced with and without C₂H₂ (Joye and Paerl 1993).

3.1.5. DNA extraction

Total community DNA was extracted, using a PowerSoil DNA Isolation Kit (MoBio laboratories Inc, Solana Beach, Calif.), from 0.25 g of wet weight rhizosphere and uncolonized sediment collected in winter and summer, based on the contrasting denitrification rates observed at these seasons. For each sample, duplicate DNA extractions were performed with the purpose of accounting for variability between replicates. The efficiency of DNA extraction was tested according to Okano *et al.* (2004) by adding a known number of *Ruegeria pomeroyi* cells to the sediment, following the protocol by Magalhães *et al.* (2009). The extraction efficiency of the PowerSoil DNA isolation kit was 27.5 ± 2.2 %, which is in agreement with DNA recovery efficiencies calculated in other studies (e.g. Mummy and Findlay 2004, Okano *et al.* 2004).

3.1.6. Quantitative real-time PCR

In order to determine the bacterial 16S rDNA, *nirS* and *nirK* genes copy numbers using a quantitative PCR was conducted using the previously described primer sets (Table 2). About 4 ng of each DNA extraction were added to a reaction mix containing 1× iQ SYBR Green Supermix (Bio-Rad) and 1 µl of each primer (10 µM), making a total volume of 25 µl per reaction. Reactions were performed in duplicate and no template controls were included for each run. All reactions were run in a 96-well plate in a real-time PCR detection system (iQ5, BioRad). PCR program was set with the following conditions: initial denaturation at 94 °C for 5 min, 94 °C for 30 s, 63 °C for 30s (decreased by 1 °C every cycle until 57 °C), 72 °C for 30 s, 80 °C for 18 s (data collection) for 30 cycles and a final elongation step at 72 °C for 10 min. A melting curve and agarose gels were generated for every run to confirm the specificity of the assays. qPCR efficiency was determined

through a standard curve of 6 serial dilutions of cloned DNA fragments containing the target region. Standards for each primer set were generated by cloning a DNA fragment from sediment of Cavado estuary as described below. DNA concentration of plasmids and samples were determined fluorometrically with PicoGreen ds DNA quantitation kit (Molecular Probes, Invitrogen, Eugene, Oreg.). Standard curve efficiency was close to 100 % with $R^2 = 0.993$. Target copy numbers for each reaction were calculated from the standard curves, assuming that the average molecular mass of a double-stranded DNA molecule is 618 g mol^{-1} , and data were presented in number of gene copies per gram of wet sediment, based on the DNA extraction efficiency calculated above for the PowerSoil DNA Isolation Kit (MoBio).

Table 2: Oligonucleotide probes used in this study

Bacterial target genes	Primers	Fragment size (bp)	Sequence (5' – 3')	Reference
	GC-Clamp	-----	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC C	Muyzer <i>et al.</i> 1993
<i>narG</i>	narG1960m2F narG2050m2R-	110	TAYGTSGGGCAGGARAACTG CGTAGAAGAAGCTGGTGCTGTT	López-Gutiérrez <i>et al.</i> 2004
<i>nirK</i>	FlaCuF R3CuR-GC	453	ATCATGGTCTGCCGCG GCCTCGATCAGRTTGTTGGTT	Throback <i>et al.</i> 2004
<i>nirS</i>	Cd3aF R3cdR-GC	406	G TSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	Throback <i>et al.</i> 2004
<i>nosZ</i>	nosZF2 nosZR2	267	CGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTCAGGAGAA	Henry <i>et al.</i> 2006

3.1.7. DGGE

DGGE was performed by using a CBS Scientific DGGE system (Del Mar, Calif.). PCR reactions targeting the genes that codifies the enzymes of denitrification pathway (*narG*, *nirS*, *nirK*, *nosZ*) and bacterial 16S rDNA gene were performed using previously described primer sets (Table 2) although a 40-bp GC clamp was added to the 5'-end of each forward primer. PCR reactions were carried out using Ready-to-Go PCR Beads (Amersham Biosciences, Buckinghamshire, UK) in 25 µl reactions containing 1-5 ng of DNA template and 20 pmol/µl of each primer. A PCR reaction mixture with all reagents except template DNA served as a negative control. PCR temperature profile conditions was as follows: initial denaturation at 95 °C for 5 min followed by 30 cycles consisting of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final elongation step at 72 °C for 10 min (Magalhães

et al. 2008). PCR products were separated by agarose gel electrophoresis (1.5 %) in 1x TAE buffer (0.04 Tris-acetate, 0.001 M EDTA [pH 8.0], stained with ethidium bromide and visualized with UV light.

PCR products containing approximately equal amounts (600 ng) were loaded onto 6.5 % (w/v) polyacrylamide gel in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4) containing a gradient of denaturant from 45 to 65 % for bacterial 16S rRNA gene and *narG* and 40-80 % for DNA *nirS*, *nirK* and *nosZ*. The electrophoresis was run for 15 h at a constant voltage of 100 V in 1X TAE buffer at 60 °C. PCR reactions containing genomic DNA from *Clostridium perfringens* and *Bacillus thuringiensis* (Sigma, USA) were used as a marker standard. Denaturing gradient gels were stained with 1 X SYBR Green (1:10 000 dilution, Molecular Probes, USA) and photographed on a UV transillumination table using a gel documentation system equipped with a digital camera (Kodak EDAS100, USA).

3.1.8. Cloning

Clone libraries were constructed for DNA *nirS* and *nirK* functional genes in all the salt marshes studied for samples collected in winter and summer colonized and un-colonized sediments (CWRhizo, CSRhizo, CSSed, LSRhizo, LSSed, CpSRhizo, CpSSed). PCR reactions were performed as described above for DGGE. The PCR product was electrophoresed on agarose gel (1.5 %), and bands of appropriate size excised. DNA was extracted from bands using Illustra GFX PCR DNA and Gel band purification (Amersham Biosciences, Buckinghamshire, UK), and cloned into TOPO-TA vector (Invitrogen Corp., Carlsband, CA, USA) following the protocol supplied by the manufacturer with the exception of a slight modification (vector DNA and salt solution was decreased to 0.5 µl and chemically competent cells decreased to 25 µl). Plasmids were isolated from *E. coli* host cells with a Plasmid Miniprep kit (Sigma). Insert size was verified by digestion with *EcoRI* and clones with the correct insert size were sequenced (STAB-VIDA, Portugal).

3.1.9. Phylogenetic analysis

All sequences generated were compared with known sequences using the Basic Local Alignment Search Tool BLAST (Altschul *et al.* 1990). Sequences were also checked for chimeras using the CHECK CHIMERA program from the Ribosomal Database Project. Sequences were manually aligned with gene sequences retrieved from the previously mentioned databases, using ClustalW program (Thompson *et al.* 1994) integrated in the BioEdit 7.0.5. software (Hall 1999). Regions of ambiguous alignments were excluded from analysis. Phylogenetic trees were constructed using Juke-Cantor distances and the

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method (Mega package, version 3.1, obtained from the company web site) (Kumar *et al.* 2004). Tree robustness was tested by bootstrap analysis (1 000 replicates). The clone sequences have been deposited in GenBank under the accession numbers (under submission).

3.1.10. Statistical analysis

Spatial and seasonal statistically significant differences among samples were evaluated through analysis of variance (one-way ANOVA) followed by a post hoc Tukey honestly significant difference (HSD) multi-comparison test using the software STATISTICA 6.0 (StatSoft, Tulsa, USA).

DGGE profiles were analyzed with GelComparII version 5.1 (Applied Maths, Kortrijk, Belgium). A presence or absence matrix was generated assuming that each different band in DGGE profile corresponded to a different OTU (Operational Taxonomic Unit). Hierarchical cluster analysis based on UPGMA ("Unweighted Pair Group Method with Arithmetic Mean") was used to evaluate differences in denitrifier assemblages composition. PRIMER version 5 software (Primer-E Ltd, UK) (Clarke and Warwick 1994, Clarke 1999) was used for the latter multivariate and cluster analysis. Principal components analysis (PCA) was applied to the log (x+1) transformed environmental variables and microbial abundance. Euclidean distances were calculated for sediment characteristics and Bray-Curtis similarities to species data. Microbial community structure was examined using multidimensional scaling (MDS) and hierarchical cluster analysis. Dendograms were generated using the group average method. ANOSIM analysis (Clarke and Warwick 1994) was used to test differences between clusters generated; the values of the R statistic were an absolute measure of how well the groups separated and ranged between 0 (indistinguishable) and 1 (well separated). Relationships between microbial composition (binary matrix of DGGE profiles) and environmental variables were analyzed by redundancy analysis (RDA) using the software package CANOCO for Windows 4.5 (Biometris, Wageningen, The Netherlands). Inflation factors were examined and highly correlated variables with little contribution to the total variation removed (ter Braak and Smilauer 2002). Intrasets correlations were used to examine the relative contribution of each variable to the separate ordination axis. The unrestricted Monte Carlo permutation test (499 permutations) was used to test the statistical significance of RDA analysis. The significance level used for all tests was 0.05.

3.2. Results

3.2.1 Denitrification and N₂O production

Denitrification potentials varied between 0.41 and 26 nmol N₂ g wet sed⁻¹ h⁻¹ (Figure 9) with higher rates registered in Cavado rhizosediments collected in fall and the lowest values observed at Comporta un-colonized sediments in winter. Overall, a strong temporal variation was detected, with higher mean denitrification rates in summer and fall seasons and significant lower rates in winter and spring (Tukey HSDtest, $p < 0.05$).

In what N₂O production rates are concerned, noticeable differences between sediments and rhizosediments were found at all salt marshes, with general higher N₂O production rates in rhizosediments (Tukey HSDtest, $p < 0.05$). In agreement, values for N₂O:N₂, ratios were also always higher in rhizosediment (0.5 to 251 %) than in un-colonized sediments (1.1 to 19 %).

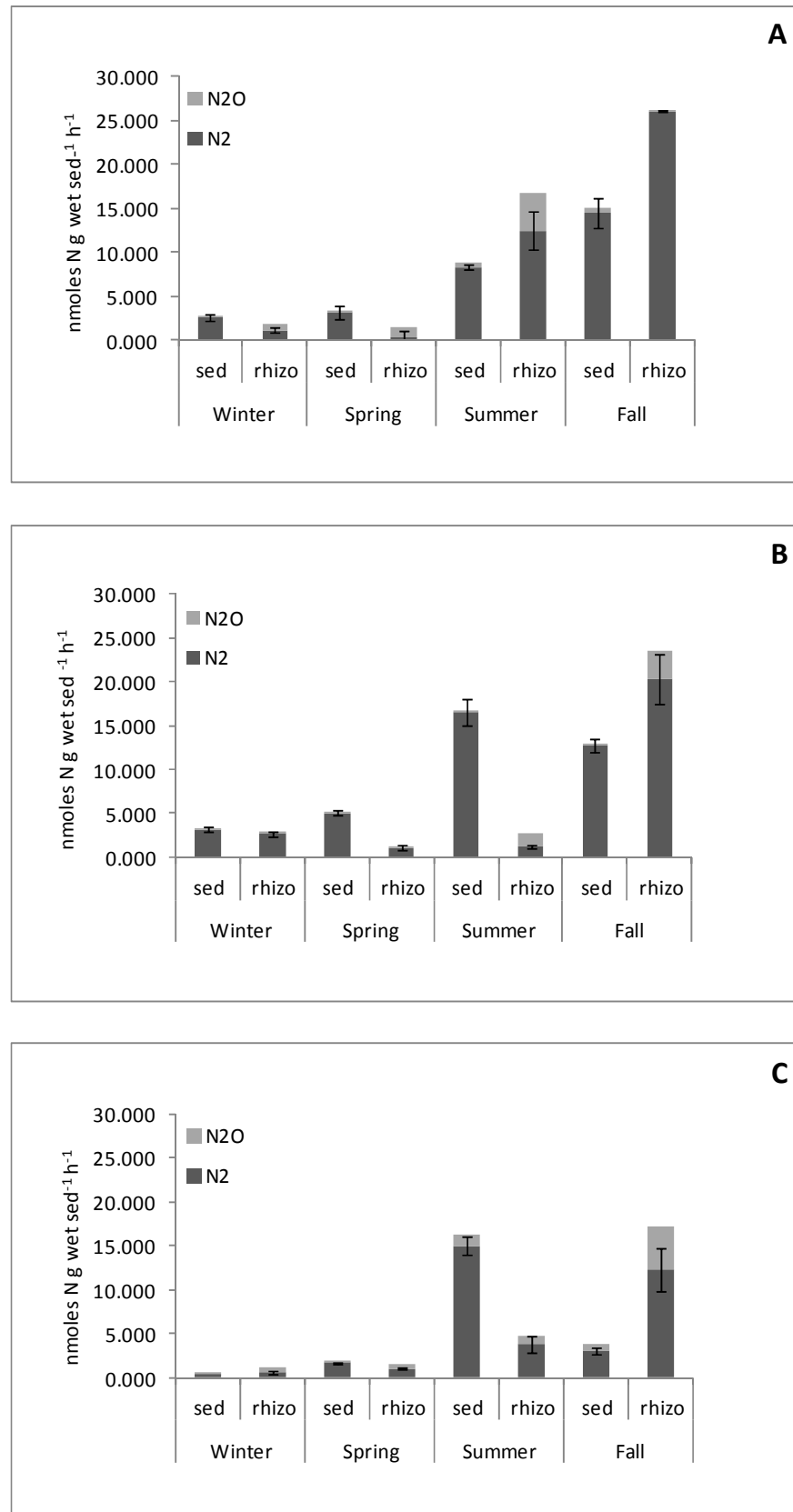


Figure 9: Denitrification rates (dark bar) and N₂O (light bar) production rates at each salt marsh (a) Cavado, (b) Lisnave, (c) Comporta, in the respective season for colonized (Rhizo) and uncolonized (Sed) sediments. Error bars represent SE of the mean (n=3).

3.2.2 Abundance of genes involved in the denitrification pathway (*nirS* and *nirK*)

Quantitative analyses (qPCR) of *nirS* and *nirK* were performed in order to evaluate the abundance of the genes involved in the NO_2^- reduction step of the denitrification process. Abundance of *nirS* and *nirK* genes ranged from 1.10×10^3 to 3.10×10^5 and from 2.98×10^3 to 4.70×10^4 copies per gram of sediment, respectively (Figure 10). Quantitative data clearly showed higher copy numbers of *nirS* relatively to *nirK* gene, the expression of *nirS* is above 90 % in 58 % of the samples and 50 % in all samples with the exception of winter un-colonized sediment from Comporta (Figure10).

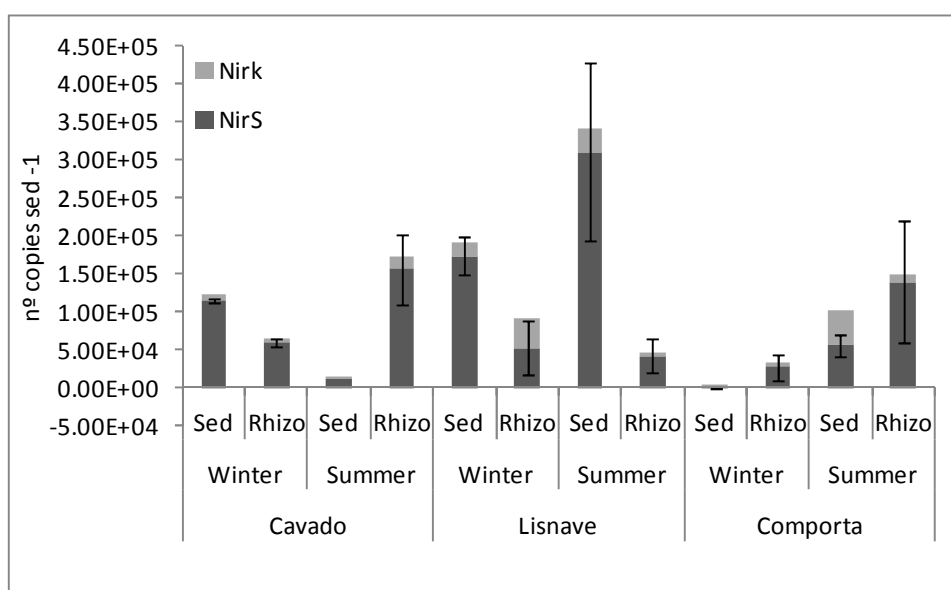


Figure 10: *NirS* (dark bar) and *nirK* (light bar) abundance found at each salt marsh, in the respective season for colonized (Rhizo) and un-colonized (Sed) sediments. Error bars represent SE of the mean (n=4).

Higher NO_2^- reductase genes (*nirS* + *nirK*) copy numbers were found in samples from summer surveys in all marshes studied. Within these, the highest values belong to colonized sediment in Cavado and Comporta salt marshes, and to un-colonized sediment in Lisnave salt marsh (Figure 10). In what individual genes were concerned, *nirS* followed the same pattern described for the total nitrite reductase genes, since it accounted for the most part of the abundance found, and *nirK* gene higher copy numbers were registered in summer un-colonized and the winter colonized sediment samples from Comporta and Lisnave salt marshes, respectively. The relative abundance of denitrifiers to total bacteria was estimated through the calculation of the ratios between *nirS* + *nirK* and 16S rDNA genes. Results revealed that the percentage of denitrifiers with the capability for nitrite reduction relatively to total bacteria varied between 0.007 and 0.302 %. While *nirS*

abundance was not related with denitrification rates, *nirK* gene copy numbers were found to be positively and significantly correlated with N₂ production rates ($r = 0.71$, $p < 0.05$).

3.2.3 Diversity of genes implicate in the denitrification process (*narG*, *nirS*, *nirK* and *nosZ*)

PCR-DGGE analyses of *narG*, *nirS*, *nirK* and *nosZ* were performed in replicate DNA samples in order to evaluate shifts in the diversity of the genes that catalyze the different steps of the denitrification process. Hierarchical cluster analysis, applied to the different DGGE profiles generated, revealed higher similarity between replicate DGGE banding patterns than between different samples, confirming high reproducibility between DNA extractions from the same site, DGGE gels and PCRs. To facilitate interpretation, only one of the replicates was displayed in the cluster.

Cluster analysis of DGGE profiles showed differences in denitrifier assemblages composition for all the genes studied, being the samples primarily clustered by sampling site. Moreover, while seasonal differences in the denitrifying community structure prevail in samples from Cavado estuary, in Sado estuary differences between rhizosediments and un-colonized sediments overlapped the seasonal variability (Figure 11).

In what individual genes were concerned, the *narG* PCR-DGGE profiles showed an evident separation between the different salt marshes communities (Figure.11a). Lisnave samples were grouped in a separated branch and Cavado samples clustered together at 68% of similarity (Figure.11a). In *narG* PCR-DGGE profiles samples were divided by season in the case of Cavado estuary and by colonization by plant in the Sado estuary. The ANOSIM test revealed statistically significant differences between these groups generated by hierarchical cluster analysis ($n = 12$; $R = 0.706$; $p = 0.05$). The number of bands in *narG* PCR-DGGE profiles was higher in the samples from Cavado estuary being rather stable between seasons and with the presence or absence of plant colonization. In *nirK* PCR-DGGE profiles (Figure.11c) was also clear the differentiation between salt marshes. Hierarchical cluster analysis revealed a separation of Comporta samples at 22 % similarity whereas Lisnave and Cavado samples separated at a higher similarity level (38 %) (ANOSIM, $n = 12$; $R = 0.703$; $p = 0.05$). The exception was a summer sample from Cavado un-colonized sediment (Figure.11c). Within each marsh the samples divided once more by season in Cavado (78 %) and by the presence of plant colonization in Sado (Lisnave – 77 %, Comporta – 58 %). The total number of different bands registered in *nirK* PCR-DGGE profiles was 38, and the number of DGGE bands per sample varied between 10 and 22, being higher in samples from Sado estuary.

For *nirS* gene, differences between DGGE profiles from the different salt marshes evaluated were not so evident (Figure.11b). Here, Comporta summer samples clustered in a different branch, while winter samples clustered together with samples from Cavado estuary. Lisnave samples for *nirS* gene diversity clustered together at 40 % of similarity, and the winter samples formed a subcluster at 73 % of similarity. The groups generated by hierarchical cluster analysis showed statistical significant differences (ANOSIM, $n = 12$; $R = 0.784$; $p = 0.05$). The total number of bands positions observed in *nirS* PCR-DGGE profiles was 29 being rather stable in all samples.

The hierarchical cluster analysis of the *nosZ* PCR-DGGE profiles (Figure.11d) revealed the same general trend as for the other genes involved in denitrification process. The difference between the salt marshes studied was very obvious; Comporta was the most dissimilar marsh clustering in a different branch (samples with 58 % of similarity) while the samples from Cavado and Lisnave created a cluster with 32 % of similarity (Figure.11d). Within this cluster a subdivision was observed with the samples from each marsh forming a different subcluster. Once more the samples from Cavado estuary were divided by seasonality whereas the samples from Sado estuary were separated according to the presence and absence of plant (ANOSIM, $n = 12$; $R = 0.806$; $p = 0.05$). In the DGGE profiles 30 different bands were identified within all samples.

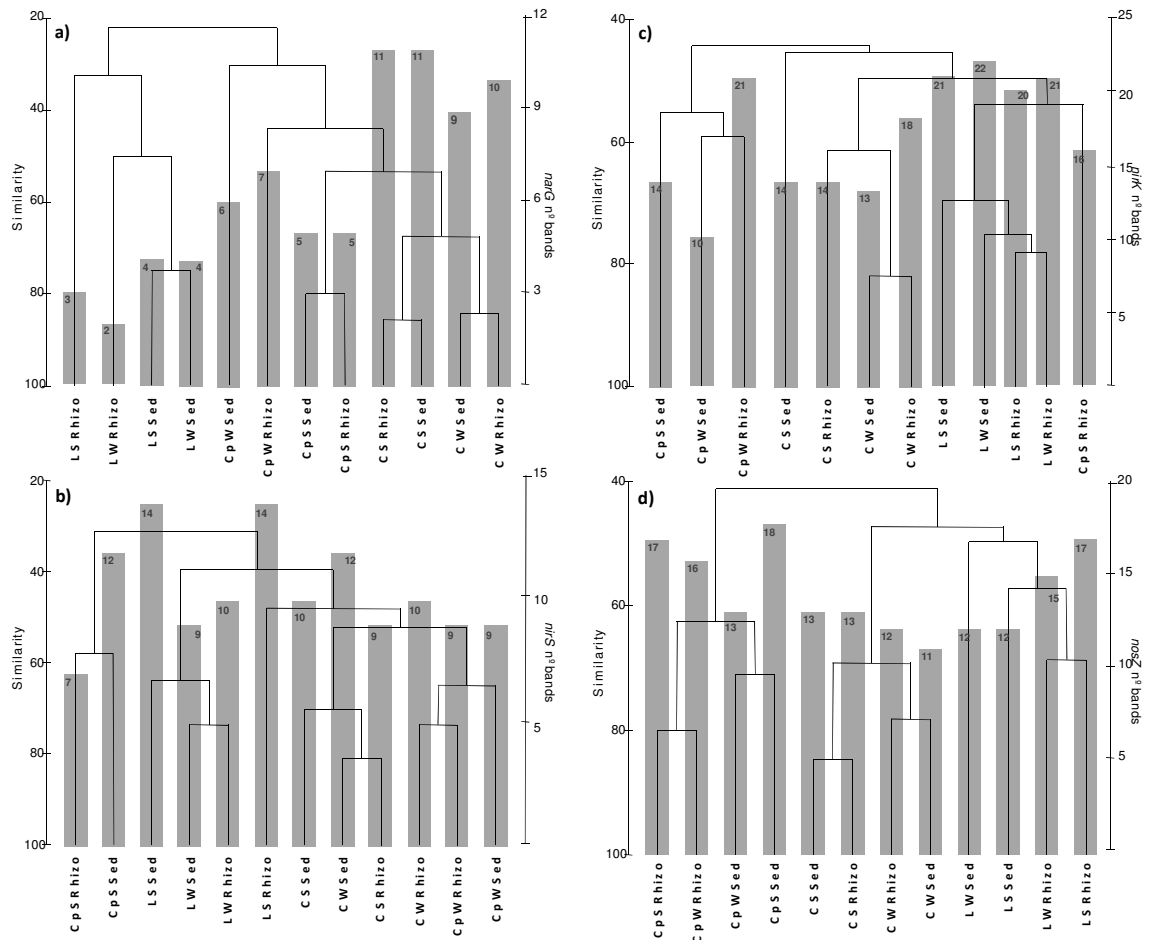


Figure 11: Hierarchical cluster analysis, based on average linkage of Bray–Curtis similarities for the presence or absence of *narG* (a), *nirS* (b), *nirK* (c) and *nosZ* (d) DGGE profiles and respective indication of the number of bands of each PCR-DGGE profile generated (grey bars). (Salt marsh: Cavado – C; Lisnave – L; Comporta – Cp; Season: Winter - W; Summer -S; presence/absence of plant: colonized sediment - Rhizo and un-colonized sediments – Sed).

3.2.4 Phylogeny of genes implicate in the denitrification process (*nirS* and *nirK*)

In order to identify the denitrifier community composition, both *nirK* and *nirS* gene fragments were cloned from sediment DNA extracts obtained from the three salt marshes studied. Clones generated were screened by RFLP and the clones representing all distinct enzyme digest patterns were sequenced including several clones with similar pattern. Phylogenetic trees were constructed with 42 *nirK* and 48 *nirS* sequences obtained from all stations and also with a selected published reference sequences (Figures. 12, 13).

For both, *nirK* and *nirS* genes, the majority of clones did not branched with any known cultured denitrifying bacteria, indicating that these salt marshes sediments have unique denitrifiers not known among cultivated microorganisms. Moreover, phylogenetic analysis did not grouped clones into subclusters associated with different habitats (different marshes or presence/absence of plant colonization) or seasons. Phylogenetically, the *nirK* sequences formed seven major clusters (Figure. 12) with 26.1 to 100 % similarity between each other. Similarities with uncultured denitrifying bacteria from other marine and coastal environments ranged from 76 to 98 %, and only one *nirK* sequence from colonized sediment (CSRhizo6) was related to *nirK* of the cultured denitrifier *Pseudomonas* sp. (83 % identity). The *nirS* sequences recovered shared 28.1 - 99.4 % identities among each other and 79 – 99 % identities with their closes-matched GenBank sequences being distributed by eight clusters within the phylogenetic tree (Figure. 13). The closes-matched *nirK* and *nirS* sequences detected were from a variety of marine environments, including from others marsh soils (Priemé *et al.* 2002), mangrove roots (Flores-Mireles *et al.* 2007), estuarine and coastal sediments (Santoro *et al.* 2006, Tiquia *et al.* 2006, Dang *et al.* 2009, Mosier and Francis 2010) and from different oceans (Jayakumar *et al.* 2004, Kim *et al.* 2011).

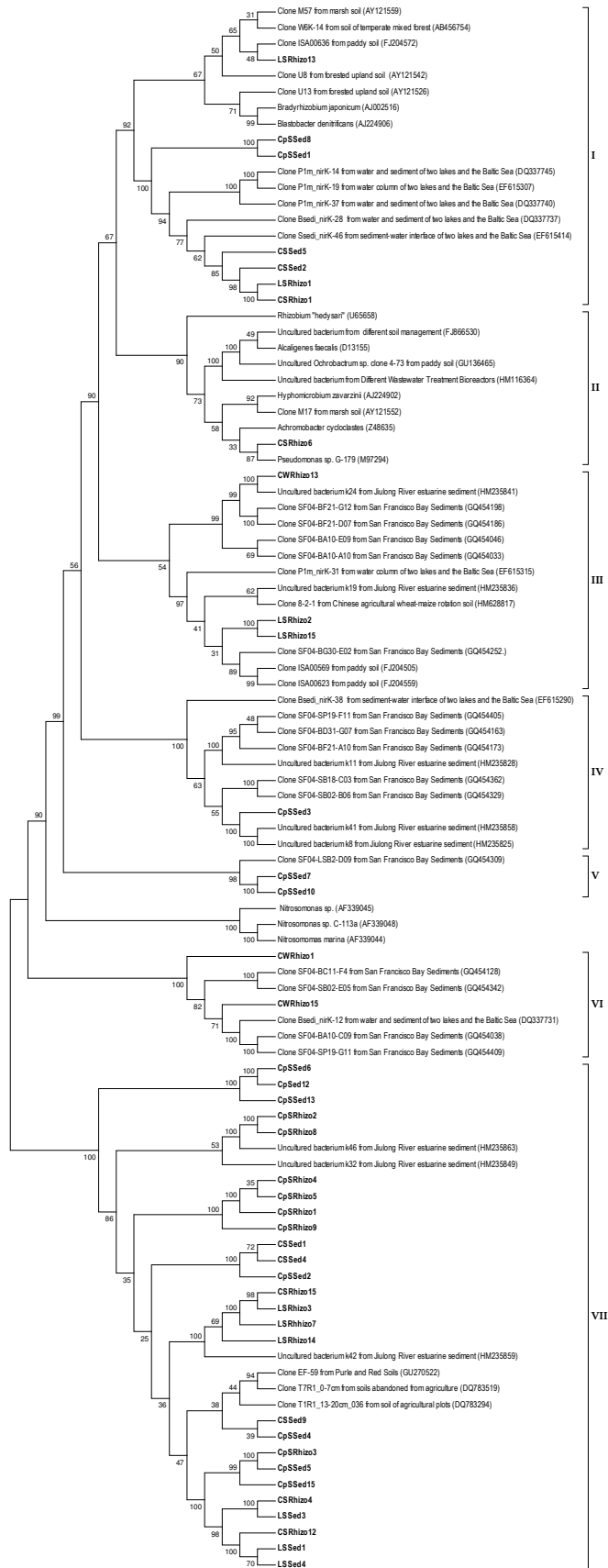


Figure 12: Phylogenetic analysis of partial sequences (379 bp) of *nirK* genes retrieved from the different salt marshes studied. The evolutionary history was inferred using the UPGMA method and the evolutionary distances computed using the Jukes-Cantor method. Clones obtained from this study are shown in boldface. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

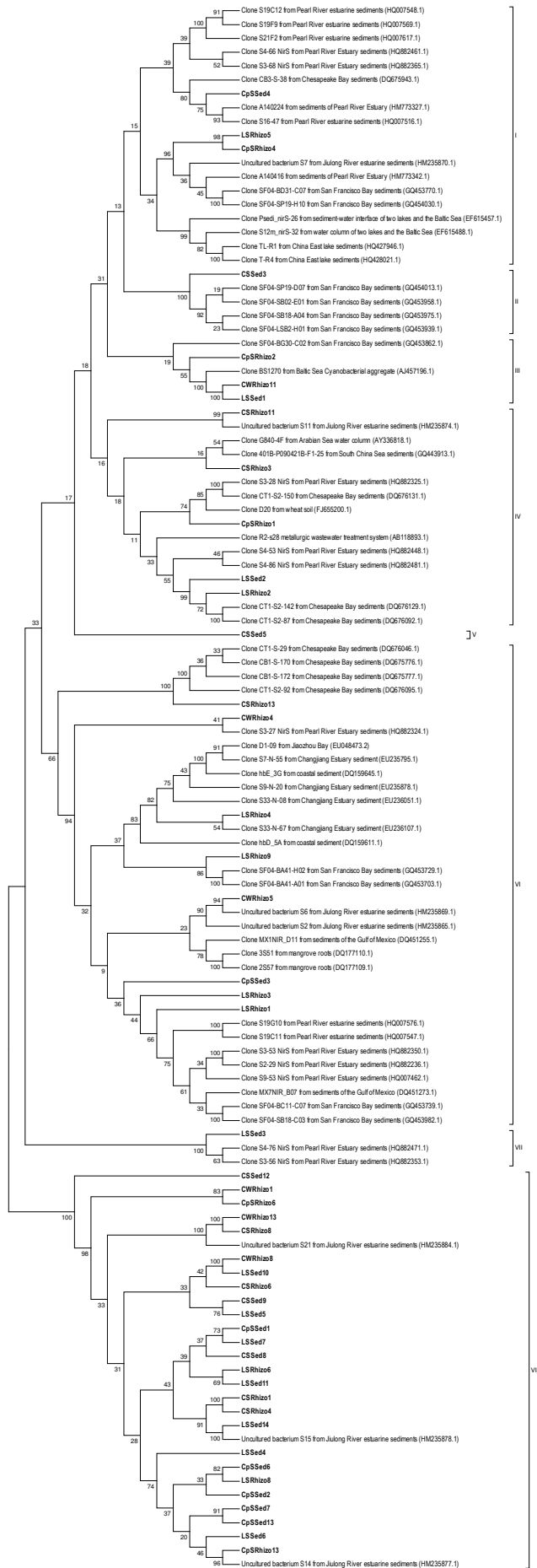


Figure 13: Phylogenetic analysis of partial sequences (326 bp) of *nirS* genes retrieved from the different salt marshes studied. The evolutionary history was inferred using the UPGMA method and the evolutionary distances computed using the Jukes-Cantor method. Clones obtained from this study are shown in boldface. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

3.2.5 Relationships between metals and denitrifiers abundance and activity

Correlations between metal concentrations found in the sediments (Table 1) and the rates of denitrification potential and N_2O accumulation were examined using RDA analysis (Figure.14). The first two RDA axes explained 94.1 % of the total cumulative species data variance and accounted for 100 % of the cumulative variance of the species-environment relationship. The unexplained fraction of variation that was explained by unknown (non-studied) factors represented 5.9 % of the total variation. Monte Carlo permutation test revealed that the contribution of combined variables was significant ($F = 6.019$ and $p = 0.038$). Results showed that rates of potential denitrification were positively associated with the Cu/Fe concentration (intersect value of 0.4562), whereas N_2O accumulation was negatively related to the metal concentration, particularly Cd/Fe and Zn/Fe (intersect values of -0.5649 and -0.5161 respectively) (Figure.14).

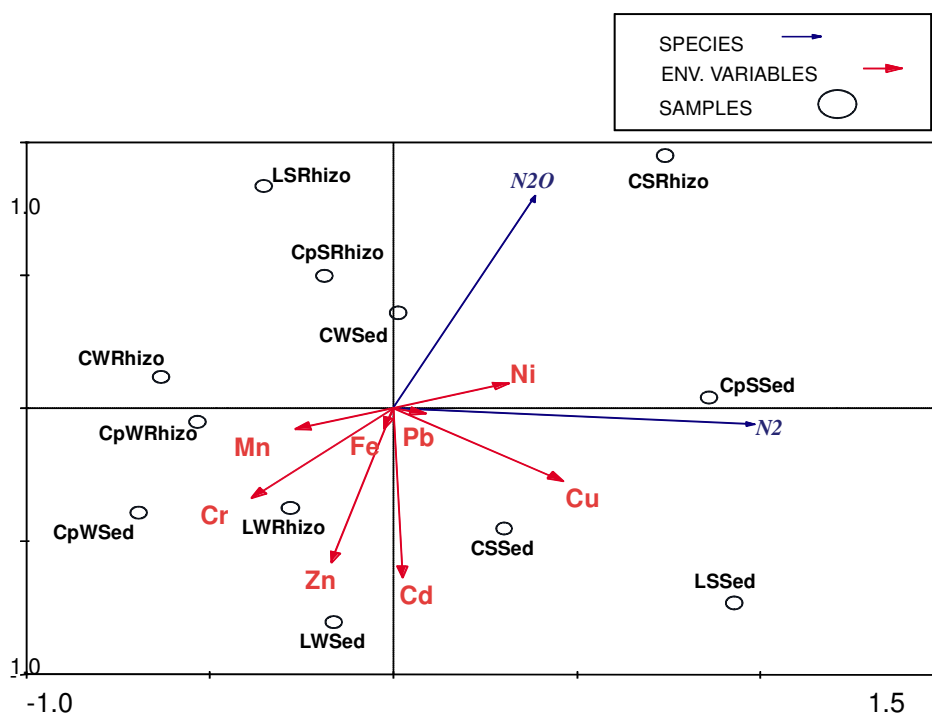


Figure 14: Redundancy analysis ordination (RDA) plot for denitrification activity (N_2 and N_2O production rates) and metals concentrations in sediments. (Salt marsh: Cavado – C; Lisnave – L; Comporta – Cp; Season: Winter - W; Summer -S; presence/absence of plant: colonized sediment - Rhizo and un-colonized sediments – Sed).

Furthermore, the diversity of genes implicated on denitrification processes were also examined using RDA (Figure. 15) and was found to be significantly correlated to the concentration of metals. Although 20.6 % of the total variation was not explained by the

factors included in this analysis, 69.4 % of the total cumulative species variance and 87.4 % of the cumulative variance of the species-environment relationship were explained by the first two RDA axes. The variables that correlated most strongly with RDA 1 were Ni/Fe and Cr/Fe concentrations (intersect values of -0.5849 and -0.5653 respectively), while Cu/Fe, and Pb/Fe correlated best with RDA 2 (intersect values of -0.5948 and -0.4701 respectively). The Monte Carlo permutation test showed that in this analysis the contribution of the combined variables was significant ($F = 3.210$ and $p = 0.012$).

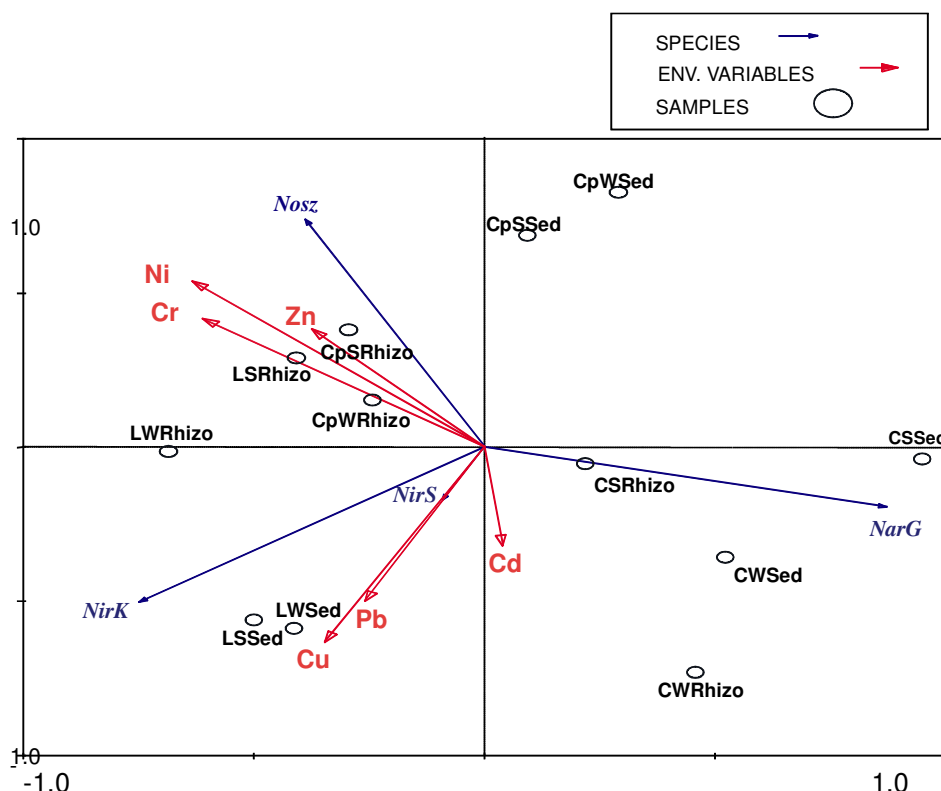


Figure 15: Redundancy analysis ordination (RDA) plot for the diversity of the different genes analyzed (*narG*, *nirS*, *nirK*, *nosZ*) and metals concentrations in sediments. (Salt marsh: Cavado – C; Lisnave – L; Comporta – Cp; Season: Winter - W; Summer -S; presence/absence of plant: colonized sediment - Rhizo and un-colonized sediments – Sed).

Therefore, RDA analysis suggested that while *narG* diversity was negatively affected by all metals. *NirS* and *nirK* diversity appear to be more related to high Cu/Fe and Pb/Fe concentrations, whereas *nosZ* diversity was positively related with Ni/Fe, Cr/Fe and Zn/Fe concentrations.

3.3. Discussion

3.3.1 Salt marsh denitrifier activity

Denitrification is of particular interest in estuaries because it represents the primary process of reducing the effects of N-enrichment from anthropogenic sources (Devol 2008). The important role of salt marshes in intercepting land-derived nutrients and thereby helping to prevent eutrophication in downstream ecosystems (Valiela and Cole 2002) is often attributed to the potential for high rates of denitrification (NRC 2000). Denitrification rates measured in our study ($0.41 - 26 \text{ nmol N g ww}^{-1} \text{ h}^{-1}$) fall in the range of values founded in other ecosystems such as river sediments $< 0.01 - 260 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (García-Ruiz *et al.* 1998) and $0 - 12 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Wall *et al.* 2005), coastal sediments, up to $240 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Aelion *et al.* 1997), and in estuarine sediments $20 - 100 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Magalhães *et al.* 2005), $0.4 - 38 \text{ nmol N g}^{-1} \text{ h}^{-1}$ (Teixeira *et al.* 2010). Seasonal denitrification rates on the salt marshes studied in this work were significantly ($p < 0.05$) higher during summer and fall when higher temperatures would potentially enhance microbial mediated NO_3^- reduction (Koch *et al.* 1992), suggesting temperature as a primary variable limiting microbial activity. In theory, the marsh plant rhizosphere should be a *hot spot* for nitrification and denitrification coupling due to plant roots, which provide oxygen and labile organic matter (Reddy *et al.* 1989, Caffrey and Kemp 1992). Indeed, the obtained results confirm the findings found in earlier reports for salt marshes (DeLaune *et al.* 1989, Smith *et al.* 1985), which found the highest denitrification rates in rhizosediments.

Moreover, at all sites noticeable differences of N_2O production were found between sediments and rhizosediments, with general higher N_2O accumulation rates and $\text{N}_2\text{O}:\text{N}_2$ ratios in rhizosediments. This is in agreement with some previous studies performed in other salt marshes (DeLaune *et al.* 1989), and in nitrogen-enriched rivers (García-Ruiz *et al.* 1998, McMahon and Dennehy 1999). Higher N_2O production rates have been linked to eutrophic environments with anaerobic conditions and high NO_3^- availability and denitrification rates (Seitzinger and Nixon 1985, Middelburg *et al.* 1995, Kenny *et al.* 2004), conditions that are commonly met in most salt marsh sediments. Increased N_2O production rates have also been reported to be associated to the presence of nitrite (Anderson and Levine, 1986), lower pH (Simek and Cooper, 2002; Liu *et al.*, 2010), fluctuating oxygen (Usui *et al.*, 2001), H_2S (Sørensen *et al.*, 1980; Senga *et al.*, 2006) and MeSH (Magalhães *et al.* 2011) concentrations in sediments. Furthermore, dissimilatory reduction of nitrate to ammonia (DNRA) that was showed to be fostered in reduced and C-

rich environments (Buresh and Patrick, 1981) such salt marshes might also contribute for the higher N₂O production rates founded.

Salt marshes are frequently the last barrier between the coastal ocean and upland, being critical in the maintenance of healthy coastal ecosystems. However N₂O emissions tend to increase as the rate of N loading to the system increase (Seitzinger *et al.* 2000). Since N₂O is recognized as a powerful greenhouse gas (Braker *et al.* 2000) implicated on the destruction of stratospheric ozone (Crutzen 1970, Dickinson and Cicerone 1986), N₂O:N₂ ratios should not be overlooked by controlling anthropogenic activities as an efficient way of reducing this ratio.

3.3.2 Salt marshes denitrifier abundance and diversity

The values found for the abundance of *nirS* and *nirK* genes (1.10×10^3 - 3.10×10^5 and 2.98×10^3 - 4.70×10^4 copies per gram of sediment, respectively) are within the range reported for other systems such as Chesapeake Bay (10^5 - 10^7 copies per gram of sediment, Bulow *et al.* 2008), Colne estuary (10^4 - 10^7 copies per gram of sediment, Smith *et al.* 2007) and San Francisco Bay (*nirS*: 10^5 - 10^7 , *nirK*: 10^3 - 10^6 copies per gram of sediment copies per gram of sediment, Mosier and Francis 2010). Quantitative data clearly showed a higher abundance of *nirS* relatively to *nirK* gene copies for all samples, however denitrification rates only correlate with *nirK* gene abundance suggesting that, despite the lower abundant of *nirK* copies, *nirK*-type, denitrifiers may be more biogeochemical actives in our salt marshes. The trend of abundance supremacy by *nirS* relatively to *nirK* was previous reported for other studies in a subtropical Fitzroy estuary (Abell *et al.* 2010) and in San Francisco Bay (Mosier and Francis 2010). The two NO₂⁻ reductases genes have different substrate requirements; *nirK* overcome in oxygen-exposed environments (Desnues *et al.* 2007, Knapp *et al.* 2009) whereas *nirS* diversity increases with moderate NO₃⁻ availability (Yan *et al.* 2003). These results were not surprising since *nirS* was found to be more widespread in the bacterial worldwide (Priemé *et al.* 2002, Liu *et al.* 2003, Throbäck *et al.* 2004, Oakley *et al.* 2007, Dang *et al.* 2009, Mosier and Francis 2010, Huang *et al.* 2011). *NirS* may also be important in anammox bacteria for providing the oxidant (NO) for anaerobic ammonium oxidation (Strous *et al.* 2006). The differences in the relative abundance of bacteria containing *nirS* and those containing *nirK* indicate that both types of denitrifiers apparently occupy different ecological niches like was showed in previous studies (Kim *et al.* 2011).

Cluster analysis of DGGE profiles showed differences in denitrifier assemblage composition for all the genes studied, being samples primarily clustered by sampling site. Similarly to previous investigations (Rich and Myrold 2004), in this study no direct correlation between denitrifier activity and community structure (*narG*, *nirS*, *nirK* and *nosZ* diversity) was found. However, has been previously demonstrated that the denitrifier community composition influence the denitrification process (Jayakumar *et al.* 2004, Rich and Myrold 2004) through its adaptation to environmental conditions (Cavigelli and Robertson 2000). This apparent paradox can be explained by the fact that the capacity to denitrify is widespread among diverse phylogenies (Zumft 1992), but nonetheless gene expression (Philippot and Hallin 2005) and enzymatic activity may vary greatly among species (Firestone 1982). So, shifts in the denitrifier community composition may not necessarily lead to changes in the magnitudes of denitrification rates. Moreover, within the denitrifier community different subcommunities become more active under different environmental conditions (Philippot and Hallin 2005) being functionally complementary.

NirK and *nirS* sequences from this study close-matched with uncultured sequences in the databases recovered from a widespread variety of marine environments, showing a high dispersion capacity of these denitrifiers, both for *nirK* and *nirS*. However, the majority of clones did not branch with any known denitrifying bacteria, indicating that the salt marshes sediments have unique denitrifiers not known among cultivated microorganisms. Moreover, phylogenetic analysis failed to group clones into subclusters associated to different habitats (different marshes or presence/absence of plant colonization) from which the clones were obtained. These findings suggest that the genetic information to denitrify is widespread within the microbial communities across the different locals, and thus, the structure and activity of the denitrifier communities must be shaped throughout environmental forces and anthropogenic pressures. Unfortunately, the study of genes at the DNA level method gave no insights on the viability of the organisms, but only on the diversity of the genes that were preserved in sediments, which is believed to be a limitation for this approach. Thus, in future studies functional genetic markers (targeting environmental RNA) in combination with biogeochemical processes and the environmental controls will provide a more accurate and comprehensive knowledge.

3.3.3 Metal contamination vs denitrification activity and diversity

Few studies have addressed the effects of metals on denitrification enzymatic pathway (Sakadevan *et al.* 1999, Holtan-Hartwig *et al.* 2002, Magalhães *et al.* 2007, 2011). In this study the rates of potential denitrification were positively associated with the Cu/Fe, and

N₂O accumulation was negatively related to all metals studied. In addition, RDA analysis suggested that while *narG* diversity was negatively affected by all metals, bacterial assemblages with *nirS* or *nirK* appeared more related to high Cu/Fe and Pb/Fe concentrations. In the case of *nosZ* diversity was positively related with Ni/Fe, Cr/Fe and Zn/Fe concentrations. These results seem to be in accordance with previous studies in which development of tolerance towards heavy metals by microbial communities was observed (Bååth 1998, Holtan-Hartwig *et al.* 2002) being the sensitivity different within the enzymatic cascade of denitrification pathway (Holtan-Hartwig *et al.* 2002, Magalhães *et al.* 2011, McKenney and Vriesacker 1985).

Although, trace metals can negatively affect aerobic and anaerobic microbial respiration, biomass, N-mineralization, nitrification and microbial community structure of soils, sediments and aquatic habitats (Giler *et al.* 1998, Holtan-Hartwig *et al.* 2002, Granger and Ward 2003), it is also known that micronutrient metals such Cu were essential to life (Granger and Ward 2003). Moreover, decreasing in the grow rates of denitrifying bacteria and denitrification activity has been observed in response to Cu limitation (Granger and Ward 2003). In agreement, a positive correlation between Cu/Fe concentrations and potential denitrification rates and *nirS* gene diversity was observed. This finding together with the fact that higher abundance of *nirS* was always observed suggested an important role of Cu in the denitrification pathway of the salt marshes studied, since this NO₂⁻ reductase enzyme contains Cu at it reaction center (Zumft 1997) .

Differences in the denitrification rates and N₂O accumulation between rhizosediment and un-colonized sediment could be also explain by the different levels of metal immobilization that can occur depending on the type of soil/sediment (Sundelin and Eriksson 2001, van van Griethuysen *et al.* 2003), and by the metal bioavailability that can be affect by the presence of plant colonization (Almeida *et al.* 2004, 2006). Higher N₂O accumulation rates observed in colonized sediments could indicate a selection of denitrifier community lacking the gene encoding the N₂O reductase (*nosZ*). However, these findings can also be explain by higher sensitive N₂O reductase from rhizosediments to metal concentration or even increasing metal bioavailability to toxic levels due to the presence of plant (Almeida *et al.* 2004, 2006).

3.4. Conclusion

In this study, it was found that although the denitrifier ability appeared to be widespread in the microbial world, the presence of salt marsh plants and metal contamination fostered the selection, adaptation and activity of different microbial denitrifying populations in salt marsh ecosystems. Moreover, the denitrification process in rhizosediments seems to have lower efficiency leading to higher levels of N₂O accumulation, a powerful greenhouse gas. Since salt marshes may constitute large areas in temperate and subtropical estuaries and feature important ecological and biochemical roles, this work represents a valuable contribution to the understanding of the impact of plant colonization and pollutants like metals in the abundance and structure of microbial communities implicated in the denitrification pathway essential in order to design recovery and mitigation strategies for those systems.

Chapter 4

General Conclusions and Future Directions

The results obtained in this study allowed the following considerations:

- The sediment characteristics, the presence of salt marsh plants and metal contamination fostered the selection and adaptation of different microbial populations to the anthropogenic pressures present in salt marsh ecosystems.
- A strong temporal variation was found, with higher denitrification rates during the summer and fall seasons and significant ($p < 0.05$) lower rates in winter and spring. Values of $N_2O:N_2$ ratios were always higher in rhizosediments (0.5 to 251 %) than in sediments (1.1 to 19 %). Since $N_2O:N_2$ evaluate the magnitude of the N_2O accumulation during denitrification, these results suggested lower efficiency of the denitrification process in rhizosediments than in sediments not colonized by plants.
- Quantitative data clearly showed a higher expression of *nirS* relatively to *nirK* gene.
- Differences in the composition of denitrifier assemblages for all the genes studied, being the samples primarily clustered by sampling site. While in Cavado estuary seasonal differences in the denitrifying community structure prevailed, in Sado estuary differences between rhizosediments and un-colonized sediments overlapped the importance of the seasonal effect.
- Rates of potential denitrification were positively associated with the Cu/Fe concentrations, whereas N_2O accumulation was negatively related to the metal concentration, particularly Cd/Fe and Zn/Fe.
- *NarG* microbial composition was negatively affected by all metals. *nirS* and *nirK* bacterial assemblages appeared more related to high Cu/Fe and Pb/Fe concentrations, whereas *nosZ* diversity was positively related with Ni/Fe, Cr/Fe and Zn/Fe concentrations.
- The majority of clones recovered did not branch with any known denitrifying bacteria, indicating that unique denitrifiers adapted to salt marsh conditions were present.

The set of results obtained represents an important contribution to the understanding of how and at which level pollutants like metals occurring in the environment may influence the abundance and structure of microbial communities, specifically denitrifiers. This work provided also some insights about the activity of denitrifying and nitrogen recycling in temperate salt marshes and the effect of plant colonization in the biogeochemical processes.

Because the role of salt marshes habitats for processing nutrients typically has been overlooked, more detailed studies along the line of the research presented in this thesis are needed in order to corroborate the findings presented here. Also, further research is necessary to improve our knowledge of the factors influencing denitrification in those environments, like the direct characterization of denitrifiers communities to different levels of resolution (DNA and RNA), since the presence of the functional genes responsible for the denitrification is not synonymous that those are expressed and active. Marshes have experienced increased input of anthropogenic N loads over the last century. There is a growing evidence that fixation, denitrification and even sedimentation rates are altered by increased external N inputs but these responses require better understanding and quantification.

Since salt marshes may constitute large areas in temperate and subtropical estuaries and are important ecologically through pollutant-degrading processes and contribution to the greenhouse effect (N₂O emissions), the research on the microbial communities present should be taken in account in the process of development of mitigation and recovery strategies.

Bibliography

- Abell, G.C., Revill, A.T., Smith, C., Bissett, A.P., Volkman, J.K., Robert, S.S., 2010. Archaeal ammonia oxidizers and *nirS*-type denitrifiers dominate sediment nitrifying and denitrifying populations in a subtropical macrotidal estuary. *ISME Journal* 4, 286 – 300.
- Abreu, C., Jurgens, G., De Marco, P., Saano, A., Bordalo, A.A., 2001. Crenarchaeota and Euryarchaeota in temperate estuarine sediments. *Journal of Applied Microbiology*. 90, 713 – 718.
- Aelion, C.M., Shaw, J.N., Wahl, M., 1997. Impact of suburbanization on ground water quality and denitrification in coastal aquifer sediments. *Journal of Experimental Marine Biology and Ecology* 213, 31 – 51.
- Alexander, M., 1977. "Introduction to Soil Microbiology", 2nd edition. Wiley, New York, 472 pp.
- Almeida, C.M.R., Mucha, A.P., Bordalo, A.A., Vasconcelos, M.T.S.D., 2008. Influence of a salt marsh plant (*Halimione portulacoides*) on the concentrations and potential mobility of metals in sediments. *Science of the Total Environment* 403, 188 – 195.
- Almeida, C.M.R., Mucha, A.P., Dias, A.C., Bordalo, A.A., Vasconcelos, M.T.S.D., 2009. Study of the influence of different organic pollutants on Cu accumulation by *Halimione portulacoides*. *Estuarine, Coastal and Shelf Science* 85, 627 – 632.
- Almeida, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D., 2004. Influence of the sea rush *Juncus maritimus* on metal concentration and speciation in estuarine sediment colonized by the plant. *Environmental Science Technology* 38, 3112 – 31128.
- Almeida, C.M.R., Mucha, A.P., Vasconcelos, M.T.S.D., 2006. Comparison of the role of the sea club-rush *Scirpus maritimus* and the sea rush *Juncus maritimus* in terms of concentration, speciation and bioaccumulation of metals in the estuarine sediment. *Environmental Pollution* 142, 151 – 159.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215, 403 – 410.
- Amann, R.L., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbial Molecular Biology* 59, 143 – 169.

- Anderson, I.C., Levine, J.S., 1986. Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. *Applied and Environmental Microbiology* 51, 938 – 945.
- Avrahami, S., Conrad, R., Braker G., 2002. Effect of soil ammonium concentration on N₂O release and on the community structure of ammonia oxidizer and denitrifiers. *Applied and Environmental Microbiology* 68, 5685 – 5692.
- Bååth, E., Díaz-Raviña, M., Frostegrd, Å., Campbell, C.D., 1998. Effect of metal-rich sludge amendments on the soil microbial community. *Applied and Environmental Microbiology* 64, 238 – 245.
- Bagwell, C.E., Dantzler, M., Bergholz, P.W., Lovell, C.R., 2001. Host-specific ecotype diversity of rhizoplane diazotrophs of the perennial glasswort *Salicornia virginica* and selected salt marsh grasses. *Aquatic Microbial Ecology* 23, 293 – 300.
- Bagwell, C.E., Piceno, Y.M., Ashburne-Lucas, A., Lovell, C.R., 1998. Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. *Applied and Environmental Microbiology* 64, 4276 – 4282.
- Bardgett, R.D., Speir, T.W., Ross, D.J., Yeates, G.W., Kettles, H.A., 1994. Impact of pasture contamination by copper, chromium, and arsenic timber preservative and soil microbial properties and nematodes. *Biology of Fertile Soils* 18, 71 – 79.
- Barnes, S.M., Fundyga, R.E., Jeffries, M.W., Pace, N.R., 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proceedings of the National Academy of Sciences* 91 (5), 1609 – 1613.
- Becker, J.M., Parkin, T., Nakatsu, C.H., Wilbur, J.D., Konopka, A., 2006. Bacterial activity, community structure, and centimeter-scale spatial heterogeneity in contaminated soil. *Microbial Ecology* 51, 220 – 231.
- Bernan, J.M., Francis, C.A., 2006. Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified subtropical estuary: Bahia del Tobari, Mexico. *Applied and Environmental Microbiology* 72, 7767 – 7777.
- Blum, L.K., Roberts, M.S., Garland, J.L., Mills, A.L., 2004. Distribution of microbial communities associated with the dominant high marsh plants and sediments of the United States east coast. *Microbiology Ecology* 48, 375 – 388.

- Bonin, P., Omnes, P., Chalamet, A., 1998. Simultaneous occurrence of denitrification and nitrate ammonification in sediments of the French Mediterranean Coast. *Hydrobiologia* 389, 169 – 182.
- Bothe, H., Jost, G., Schlöter, M., Ward, B.B., Witzel, K.-P., 2000. Molecular analysis of ammonia oxidation and denitrification in natural environments. *FEMS Microbiology Reviews* 24, 673 – 690.
- Bouvier, T.C., del Giorgio, P.A., 2002. Compositional changes in free-living bacterial communities along an estuarine salinity gradient in two temperate estuaries. *Limnology and Oceanography* 47, 453 – 470.
- Bowen, G.D., 1980. Misconceptions, concepts, and approaches in rhizosphere biology. In “Contemporary Microbial Ecology” (Ellwood, D.C., Latham, M.J., Hedger, J.N., Lynch, J.M., Slater, J.H., eds.). Academic Press, New York. pp. 283–304.
- Bowen, J.L., Crump, B.C., Deegan, L.A., Hobbie, J.E., 2009. Salt marsh sediment bacteria: their distribution and response to external nutrient inputs. *ISME Journal* 3, 924 – 934.
- Boynton, W.R., Kemp, W.M., 2008. Estuaries. In “Nitrogen in the Marine Environment” (Bronk, D.A., Mulholland, M.R., Carpenter, E.J., eds.). San Diego, California, USA. pp. 809 – 856.
- Braker, G., Zhou, J., Wu, L., Devol, A.H., Tiedje, J.M., 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Applied and Environmental Microbiology* 66, 2096 –2104.
- Bulow, S.E., Francis, C.A., Jackson, G.A., Ward, B.B., 2008. Sediment denitrifier community composition and *nirS* gene expression investigated with functional gene microarrays. *Environmental Microbiology* 10, 3057 – 3069.
- Buresh, R.J., Patrick Jr., W.H., 1981. Nitrate reduction to ammonium and organic nitrogen in an estuarine sediment. *Soil Biology and Biochemistry* 13 (4), 279 – 283.
- Burke, D.J., Hamerlynck, E.P., Hahn, D., 2002. Interactions among plant species and microorganisms in salt marsh sediments. *Applied and Environmental Microbiology* 68, 1157–1164.

Caçador, I., Vale, C., Catarino, F., 2000. Seasonal variation of Zn, Pb, Cu and Cd concentrations in the root-sediment system of *Spartina marítima* and *Halimione portucaloides* from Tagus estuary salt marshes. *Marine Environmental Research* 49, 279 – 290.

Caeiro, S., Costa, M.H., Ramos, T.B., Fernandes, F., Silveira, N., Coimbra, A., Medeiros, G., Painho M., 2005. Assessing heavy metal contamination in Sado Estuary sediment: an index analysis approach. *Ecological Indicators* 5, 151 – 169.

Caffrey, J.M., Kemp, W.M., 1992. Influence of the submersed plant *Potamogeton perfoliatus*, on nitrogen cycling in estuarine sediments. *Limnology and Oceanography* 37, 1483 – 1495.

Cao, Y., Cherr, G.N., Córdova-Kreylos, A.L., Fan, T.W.-M., Green, P.G., Higashi, R.M., LaMontagne, M.G., Scow, K.M., Vines, C.A., Yuan, J., 2006. Relationships between sediment microbial communities and pollutants in two California salt marshes. *Microbial Ecology* 52, 619 – 633.

Cao, Y., Green, P.G., Holden, P.A., 2008. Microbial community composition and denitrifying enzyme activities in salt marsh sediments. *Applied and Environmental Microbiology* 74 (24), 7585 – 7595.

Cartaxana, P., Lloyd, D., 1999. N₂, N₂O and O₂ profiles in a Tagus estuary salt marsh. *Estuarine, and Coastal Shelf Science* 48, 751 – 756.

Castro-González, M., Braker, G., Farías, L., Ulloa, O., 2005. Communities of *nirS*-type denitrifiers in the water column of the oxygen minimum zone in the eastern South Pacific. *Environmental Microbiology* 7, 1298 – 1306.

Cavigelli, M.A., Robertson, G.P., 2000. The functional significance of denitrifier community composition in a terrestrial ecosystem. *Ecology* 81, 1402 – 1414.

Chapman, V.J., 1974. "Salt Marshes and Salt Deserts of the World", 2nd edition. Verlag von J. Cramer, Bremeshaven, 392 pp.

Cho, B.C., Azam, F., 1990. Biogeochemical significance of bacterial biomass in the ocean's eutrophic zone. *Marine Ecology Progress Series* 63, 253 – 259.

Cilia, V., Lafay, B., Christen, R., 1996. Sequence heterogeneities among 16S ribosomal RNA sequences, and their effect on phylogenetic analyses at the species level. *Molecular Biology and Evolution* 13, 451 – 461.

Clarke, K.R., 1999. Nonmetric multivariate analysis in community-level ecotoxicology. *Environmental Toxicology and Chemistry* 18, 118 – 127.

Clarke, K.R., Warwick, R.M., 1994. "Change in marine communities: an approach to statistical analysis and interpretation". Plymouth, UK: Natural Environment Research Council, 144 pp.

Cole, J.A., 1978. The rapid accumulation of large quantities of ammonia during nitrite reduction by *Escherichia coli*. *FEMS Microbiology Letters* 4, 327 – 329.

Colwell, R.R., Hawksworth, D.L., 1991. Microbial Diversity 21: Action Statement, International Union of Biological Sciences and International Union of Microbiological Societies, U.K. Copies can be obtained from Professor D.L.Hawksworth, IMI, Kew Surrey, TW9 3AF.

Constanza, R., d'Arge, R., deGroot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P., VanDenBelt, M., 1997. The value of the world's ecosystem services and natural capital. *Nature* 387, 253 – 260.

Cordova-Kreylos, A.L., Cao, Y., Green, P.G., Hwang, H.-M., Kuivila, K.M., LaMontagne, M.G., Van De Werfhorst, L.C., Holden, P.A., Scow, K.M., 2006. Diversity, composition, and geographical distribution of microbial communities in California salt marsh sediments. *Applied and Environmental Microbiology* 72, 3357 – 3366.

Cornwell, J.F., Kemp, W.M., Kana T.M., 1999. Denitrification in coastal ecosystems: methods, and ecosystem level controls, a review. *Aquatic Ecology* 33, 41 – 54.

Coyne, M.S., Arunakumari, A., Averill, B.A., Tiedje, J.M., 1989. Immunological identification and distribution of dissimilatory heme cd1 and non-heme copper nitrite reductases in denitrifying bacteria. *Applied and Environmental Microbiology* 55, 2924 – 2931.

Crump, B.C., Armbrust, E.V., Baross, J.A., 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. *Applied and Environmental Microbiology* 65, 3192 – 3204.

Crump, B.C., Hopkinson, C.S., Sogin, M.L., Hobbie, J.E., 2004. Microbial Biogeography along an Estuarine Salinity Gradient: Combined Influences of Bacterial Growth and Residence Time. *Applied and Environmental Microbiology* 70, 1494 – 1505.

- Crutzen, P., 1970. The influence of nitrogen oxides on the atmospheric ozone content. Q. J. R. Meteorological Society 96, 320 – 325.
- Cunha, M.A., Pedro, R., Almeida, M.A., Silva, M.H., 2005. Activity and growth efficiency of heterotrophic bacteria in a salt marsh (Ria de Aveiro, Portugal). Microbiology Research 160, 279 – 290.
- Dalsgaard, T., Canfield, D.E., Petersen, J., Thamdrup, B., Acuna-Gonzalez, J., 2003. N₂ production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature 422, 606 – 608.
- Dang, H., Wang, C., Li, J., Li, T., Tian, F., Jin, W., Ding, Y., Zhang, Z., 2009. Diversity and distribution of sediment *NirS*-Encoding bacterial assemblages in response to environmental gradients in the eutrophied Jiaozhou Bay, China. Microbial Ecology 58, 161 – 169.
- Davis, J.L., Nowicki, B., Wigand, C., 2004. Denitrification in fringing salt marshes of Narragansett Bay, Rhode Island, USA. Wetlands 24 (4), 870 – 878.
- Day, J.W., Hall, C., Kemp, W., Yanez-Arancibia, A., 1989. "Estuarine Ecology". Wiley, New York, 557 pp.
- DeLaune, R., Feijtel, T., Patrick, W., 1989. Nitrogen flows in Louisiana Gulf Coast salt marsh: Spatial considerations. Biogeochemistry 8, 25 – 37.
- Delwiche, C.C., 1970. The nitrogen cycle. Scientific American 19, 331 – 369.
- Desnues, C., Michotey, V.D., Wieland, A., Zhizang, C., Fourcans, A., Duran, R., Bonin, P. C., 2007. Seasonal and diel distributions of denitrifying and bacterial communities in a hypersaline microbial mat (Camargue, France). Water Research 41, 3407 – 3419.
- Devol, A.H., 2008. Denitrification including Anammox. In "Nitrogen in the Marine Environment" (Bronk, D.A., Mulholland, M.R., Carpenter, E.J., eds.). San Diego, California, USA. pp. 263 – 292.
- Dickinson, R.E., Cicerone, R.J., 1986. Future global warming from atmospheric trace gases. Nature 319, 109 – 115.
- Dong, L.F., Nedwell, D.B., Underwood, G.J.C., Thornton, D.C.O, Rusmana, I., 2002. Nitrous oxide formation in the Colne estuary, England: the central role of nitrite. Applied and Environmental Microbiology 68 (3), 1240 – 1249.

- Einsle, O., Kroneck, P.M.H., 2004. Structural basis of denitrification. *Biological Chemistry* 385, 875 – 883.
- Espeland, E.M., Francoeur, S.N., Wetzel, R.G., 2001. Influence of algal photosynthesis on biofilm bacterial production and associated glucosidase and xylosidase activities. *Microbial Ecology* 42, 524 – 530.
- Falk, S., Hannig, M., Gliesche, C., Wardenga, R., Koster, M., Jürgens, K., Braker, G., 2007. *nirS*-containing denitrifiers communities in the water column and sediment of the Baltic Sea. *Biogeosciences* 4, 255 – 268.
- Feris, K.P., Ramsey, P.W., Frazar, C., Rillig, M., Moore, J.N., Gannon, J.E., Holben, W.E., 2004. Seasonal Dynamics of Shallow-Hyporheic-Zone Microbial Community Structure along a Heavy-Metal Contamination Gradient. *Applied and Environmental Microbiology* 70, 2323 – 2331.
- Ferrari, V.C., Hollibaugh, J.T., 1999. Distribution of microbial assemblages in the Central Arctic Ocean Basin studied by PCR/DGGE: analysis of a large data set. *Hydrobiology* 401, 55 – 68.
- Firestone, M.K., 1982. Biological denitrification. In “Nitrogen in agriculture soil” (Stevenson, F.J., ed.). American Society of Agronomy, Inc., Crop Science Society of America, Inc., and Soil Science Society of America, Inc., Madison, WI. pp. 289 – 326.
- Flores-Mireles, A.L., Stephen, C.W., Holguin, G., 2007. Molecular Characterization of Diazotrophic and Denitrifying Bacteria Associated with Mangrove Roots. *Applied and Environmental Microbiology* 73 (22), 7308 – 7321.
- Franklin, R.B., Blum, L.K., McComb, A.C., Mills, A.L., 2002. A geostatistical analysis of small-scale spatial variability in bacterial abundance and community structure in salt marsh creek bank sediments. *FEMS Microbiology Ecology* 42, 71 – 80.
- García-Ruiz, R., Pattinson, S.N., Whitton, B.A., 1998. Denitrification in river sediments: relationship between process rate and properties of water and sediment. *Freshwater Biology* 39, 467 – 476.
- Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. *Soil Biology and Biochemistry* 30, 1389 – 1414.

Glockner, A., Jungst, A., Zumft, W., 1993. Copper containing nitrite reductases from *Pseudomonas aureofaciens* is functional in mutationally cytochrome cd1-free background (*NirS*–) of *Pseudomonas stutzeri*. Archives of Microbiology 160, 2136 – 2141.

Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Hagler-Mendonca, L., Smalla, K., 2001. Bacterial diversity of the rhizosphere of maize (*Zea mays*) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant and Soil 232, 167 – 180.

Gonçalves, E.P.R., Soares, H.M.V.M., Boaventura, R.A.R., Machado, A.A.S.C., Silva, J.C.G.E., 1994. Seasonal variations of heavy metals in sediments and aquatic mosses from the Cavado river basin (Portugal). Science of the Total Environment 142, 143 – 56.

Granger, J., Ward, B.B., 2003. Accumulation of nitrogen oxides in copper-limited cultures of denitrifying bacteria. Limnology and Oceanography 48, 313 – 318.

Grant, S.B., Sanders, B.F., Boehm, A.B., Redman, J.A., Kim, J.H., Mrse, R.D., Chu, A.K., Gouldin, M., McGee, C.D., Gardiner, N.A., Jones, B.H., Svejksky, J., Leipzing, G.V., 2001. Generation of *enterococci* bacteria in a coastal saltwater marsh and its impact on surf zone water quality. Environmental Science and Technology 35, 2407 – 2416.

Gruber, N., 2008. The Marine Nitrogen Cycle: Overview and Challenges. In “Nitrogen in the Marine Environment” (Bronk, D.A., Mulholland, M.R., Carpenter, E.J., eds.). San Diego, California, USA. pp.1 – 43.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41, 95 – 98.

Hannig, M., Braker, G., Dippner, J., Jurgens, K., 2006. Linking denitrifier community structure and prevalent biogeochemical parameters in the pelagial of the central Baltic Proper (Baltic Sea). FEMS Microbiology Ecology 57, 260 – 271.

Henry, S., Bru, D., Stres, B., S. Hallet, S., Philippot, L., 2006. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. Applied and Environmental Microbiology 72(8), 5181 – 5189.

Hensen, C., Zabel, M., 2000. Early diagenesis at the benthic boundary layer: oxygen and nitrate in marine sediments. In: “Marine Geochemistry” (Schulz, H.D., Zabel, M., eds.). Springer-Verlag, Berlin. pp. 209-231.

Herbert, R.A., 1999. Nitrogen cycling in coastal marine ecosystems. *FEMS Microbiology Reviews* 23, 563 – 590.

Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In “Modern soil microbiology” (van Elsas, J.D., Trevors, J.T., Wellington, E.M.H., eds.). Marcel Dekker, Inc., New York, NY. pp. 353–373.

Holtan-Hartwig, L., Bechmann, M., Risnes Høyås, T., Linjordet, R., Reier Bakken, L., 2002. Heavy metals tolerance of soil denitrifying communities: N₂O dynamics. *Soil Biology and Biochemistry* 34, 1181 – 1190.

Houghton, J.T., Callander, B.A., Varney, S.K., 1992. Climate change 1992: the supplementary report to the IPCC scientific assessment. Cambridge University Press, New York, N.Y.

Howes, B.L., Weiskel, P.K., Goehring, D.D., Teal, J.M., 1996. Interception of freshwater and nitrogen transport from uplands to coastal waters: the role of saltmarshes. In “Estuarine Shores: Evolution, Environments and Human Alterations” (Nordstrom, K.F., Roman, C.T., eds.). John Wiley and Sons Ltd, New York, NY, USA. pp. 287–310.

Huang, S., Chen, C., Wu, Q., Zhang, R., Yang, X., 2011. Distribution of typical denitrifying functional genes and diversity of the *nirS*-encoding bacterial community related to environmental characteristics of river sediments. *Biogeosciences Discussions* 8, 5251 – 5280.

Jaeger, C.H. III, Lindow, S.E., Miller, W., Clark, E., Firestone, M.K., 1999. Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. *Applied and Environmental Microbiology* 65, 2685 – 2690.

Jayakumar, D.A., Francis, C.A., Naqvi, S.W.A., Ward, B.B., 2004. Diversity of nitrite reductase gene (*nirS*) in the denitrifying water column of the coastal Arabian Sea. *Aquatic Microbial Ecology* 34, 69 – 78.

Johnson, D.L., Maguire, K.L., Anderson, D.R., McGrath, S.P., 2004. Enhanced dissipation of chrysene in planted soil: the impact of a rhizobial inoculum. *Soil Biology and Biochemistry* 36, 33 – 38.

Joye, S.B., Paerl, H.W., 1993. Contemporaneous nitrogen fixation and denitrification in intertidal microbial mats: rapid response to runoff events. *Marine Ecology Progress Series* 94, 267 – 274.

- Junier, P., Kim, O.-S., Witzel, K.-P., Imhoff, J.F., Hadas, O., 2008. Habitat-partitioning of denitrifying bacterial communities carrying *nirS/nirK* genes in the stratified water column of Lake Kinneret, Israel. *Aquatic Microbial Ecology* 51, 129 – 140.
- Keith-Roach, M.J., Bryan, N.D., Bardgett, R.D., Livens, F.R., 2002. Seasonal changes in the microbial community of a salt marsh, measured by phospholipid fatty acid analysis. *Biogeochemistry* 60, 77 – 96.
- Kenny, C., Yamulki, S., Blackwell, M., Maltby, E., French, P., Birgand, F., 2004. The release of nitrous oxide from the intertidal zones of two European estuaries in response to increased ammonium and nitrate loading. *Water, Air, and Pollution* 4, 61 – 66.
- Khan, M., Scullion, J., 2000. Effect of soil on microbial responses to metal contamination. *Environmental Pollution* 110, 115 – 125.
- Kim, O.-S., Imhoff, J.F., Witzel, K.-P., Junier, P., 2011. Distribution of denitrifying bacterial communities in the stratified water column and sediment–water interface in two freshwater lakes and the Baltic Sea. *Aquatic Ecology* 45, 99 – 112.
- Kirk, J.L., Klironomos, J.N., Lee, H., Trevors, J.T., 2005. The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environmental Pollution* 133, 455 – 465.
- Knapp, C., Dodds, W., Wilson, K., O'Brien, J., Graham, D., 2009. Spatial heterogeneity of denitrification genes in a highly homogenous urban stream. *Environmental Science & Technology* 43, 4273 – 4279.
- Koch, M., Maltby, E., Oliver, G., Bakker, S., 1992. Factors controlling denitrification rates of tidal mudflats and fringing salt marshes in South-west England. *Estuarine and Coastal Shelf Science* 34, 471 – 485.
- Koike, I., Hattori, A., 1978. Simultaneous determination of nitrification and nitrate reduction in coastal sediments by ¹⁵N dilution techniques. *Applied and Environmental Microbiology* 35, 853 – 857.
- Koop-Jakobsen, K., 2003. “Processes controlling losses of ammonium in intertidal sediments”. MS Thesis, University of Roskilde.
- Kopczynski, E.D., Bateson, M.M., Ward, D.M., 1994. Recognition of chimeric small-subunit ribosomal DNAs composed of genes from uncultivated microorganisms. *Applied and Environmental Microbiology* 60, 746 – 748.

- Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., Lugtenberg, B.J.J., 2004. Rhizoremediation: a beneficial plant–microbe interaction. *Molecular Plant-Microbe Interaction* 17, 6 – 15.
- Kumar, S., Tamura, K., Nei, M., 2004. Mega3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics* 5, 156 – 163.
- Kuske, R.C., Ticknor, O.L., Miller, E.M., Dunbar, M.J., Davis, A.J., Barns, M.S., Belnap, J., 2002. Comparison of soil bacterial communities in rhizosphere of three plant species and the interspaces in an arid grassland. *Applied and Environmental Microbiology* 68, 1854 – 1863.
- Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jorgensen, B.B., Kuenen, J.G., Damste, J.S.S., Strous, M., Jetten, M.S.M., 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* 422, 608 – 610.
- Liesack, W., Stackebrandt, E., 1992. Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *Journal Bacteriology* 174, 5072 – 5078.
- Lillebo, A.I., Flindt, M.R., Pardal, M.A., Marques, J.C., 1999. The effect of macrofauna, meiofauna and microfauna on the degradation of *Spartina maritima* detritus from a salt marsh area. *Acta Oecologica* 20, 249 – 258.
- Liu, B., Mørkved, P.T., Frostegård, A., Bakken, L.R., 2010. Denitrification gene pools, transcription and kinetics of NO, N₂O and N₂ production as affected by soil pH. *FEMS Microbiology Ecology* 72, 407 – 417.
- Liu, X.D., Tiquia, S.M., Houlguin, G., Wu, L.Y., Nold, S.C., Devol, A.H., Luo, K., Palumbo, A.V., Tiedje, J.M., Zhou, J., 2003. Molecular diversity of denitrifying genes in continental margin sediments within the oxygen-deficient zone off the Pacific coast of Mexico. *Applied and Environmental Microbiology* 69, 3549 – 3560.
- López-Gutiérrez, J.C., Henry, S., Hallet, S., Matin-Laurent, F., Catroux, G., Philippot, L., 2004. Quantification of a novel group of nitrate-reducing bacterial in the environment by real-time PCR. *Journal of Microbiological Methods* 57, 399 – 407.
- Lovell, C.R., Piceno, Y.M., Quattro, J., Bagwell, C.E., 2000. Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora*. *Applied and Environmental Microbiology* 66, 3814 – 3822.

Luther, G.W., Sundby, B., Lewis, B.L., Brendel, P.J., Silverberg, N., 1997. Interactions of manganese with the nitrogen cycle: alternative pathways to dinitrogen. *Geochimica et Cosmochimica Acta* 61 (19), 4043 – 4052.

Madigan, M.T.; Martinko, J.M., Parker, J., 2003. “Brock biology of microorganisms”, 10th edition. Pearson Education Inc., NJ.

Maltby, E., 1988. Global wetlands-History, current status and future. In “The Ecology and Management of Wetlands” (Hook, D. D., ed.). Timber Press, Portland, Oregon. pp. 3 –14.

Magalhães, C., Bano, N., Wiebe, W.J., Bordalo, A.A., Hollibaugh, J.T., 2008. Dynamics of nitrous oxide reductase genes (*nosZ*) in intertidal rocky biofilms and sediments of the Douro River Estuary (Portugal), and their relation to N-biogeochemistry. *Microbial Ecology* 55, 259 – 269.

Magalhães, C., Bordalo, A.A., Wiebe, W.J., 2002. Temporal and spatial patterns of intertidal sediment-water nutrient and oxygen fluxes in Douro River estuary, Portugal. *Marine Ecology Progress Series* 233, 55 – 71.

Magalhães, C.M., Machado, A., Bordalo, A.A., 2009. Temporal variability in the abundance of ammonia-oxidizing bacteria vs. archaea in sandy sediments of the Douro River estuary, Portugal. *Aquatic Microbial Ecology* 56, 13 – 23.

Magalhães, C.M., Machado, A., Matos, P., Bordalo, A.A., 2011. Impact of copper on the diversity, abundance and transcription of nitrite and nitrous oxide reductase genes in an urban European estuary. *FEMS Microbiology Ecology* 77, 274 – 284.

Magalhães, C., Silva, J., Teixeira, C., Bordalo, A.A., 2007. Impact of trace metals on denitrification in estuarine sediments of the Douro River estuary, Portugal. *Marine Chemistry* 107, 332 – 341.

Magalhães, C.M., Wiebe, W.J., Joye, S.B., Bordalo, A.A., 2005. Inorganic nitrogen dynamics in intertidal rocky biofilms and sediments of the Douro River estuary (Portugal). *Estuaries* 28 (4), 592 – 607.

Master, Y., Shavit, U., Shaviv, A., 2005. Modified isotope pairing technique to study N transformations in polluted aquatic systems: theory. *Environmental Science & Technology* 39 (6), 149 – 1756.

McKenney, D.J., Vriesacker, J.R., 1985. Effect of cadmium contamination on denitrification processes in Brookston clay and Fox sandy loam. *Environmental Pollution* 38, 221 – 233.

- McMahon, P.B., Dennehy, K.F., 1999. N₂O emissions from a nitrogen-enriched river. *Environmental Science & Technology* 33 (1), 21 – 25.
- Middelburg, J.J., Klaver, G., Nieuwenhuize, J., Markusse, R.M., Vlug, T., van der Nat, F.J.W.A., 1995. Nitrous oxide emissions from estuarine intertidal sediments. *Hydrobiologia* 311, 43 – 55.
- Mikutta, R., Kleber, M., Kaiser, K., Jahn, R., 2005. Organic matter removal from soils using hydrogen peroxide, sodium hypochlorite, and disodium peroxodisulfate. *Soil Science Society of America Journal* 69, 120 – 135.
- Montague, C.L., Odum, H.T., 1997. Setting and functions. In “Ecology and management of tidal marshes: a model from the Gulf of Mexico” (Coultras, C.L., Hsieh, Y.-P., eds.). St. Lucie Press, Delray Beach, Florida. pp. 9 – 35.
- Moreira, S.M., Moreira-Santos, M., Guilhermino, L., Ribeiro, R., 2006. An *in situ* postexposure feeding assay with *Carcinus maenas* for estuarine sediment-overlying water toxicity evaluations. *Environmental Pollution* 139, 318 – 329.
- Morris, J.T., Jensen, A., 1998. The carbon balance of grazed and non-grazed *Spartina anglica* salt marshes at Skallingen Denmark. *Journal of Ecology* 86, 229 – 242.
- Mosier, A.C., Francis, C.A., 2010. Denitrifier abundance and activity across the San Francisco Bay estuary. *Environmental Microbiology Reports* 2 (5), 667 – 676.
- Mucha, A.P., Almeida, C.M.R., Magalhães, C.M., Vasconcelos, M.T.S.D., Bordalo, A.A., 2011. Salt marsh plant-microorganism interaction in the presence of mixed contamination. *International Biodeterioration & Biodegradation* 65, 326 – 333.
- Mulder, A., van de Graaf, A.A., Robertson, L.A., Kuenen, J.G., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology* 16, 177 – 184.
- Mumy, K.L., Findlay, R.H., 2004. Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. *Journal of Microbiological Methods* 57, 259 – 268.
- Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by Denaturing Gradient Gel Electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16 rRNA. *Applied and Environmental Microbiology* 59, 695 – 700.

Nixon, S.W., 1981. Remineralization and nutrient cycling in coastal marine ecosystems. In "Estuaries and Nutrients" (Neilson, B.J., Cronin, L.E., eds.). Clifton, NJ, Humana Press. pp. 111-138.

Nowicki, B., Requentina, E., Van Keuren, D., Portnoy, J., 1999. The role of sediment denitrification in reducing groundwater-derived nitrate inputs to Nauset Marsh Estuary, Cape Cod, Massachusetts. *Estuaries* 22, 245 – 259.

Nowicki, P., Weeger, C., van Meijl, H., 2007. Scenar 2020 - Scenario study on agriculture and the rural world: land use simulation tool. European Commision/Directorate-General for Agriculture and Rural Development. 232 pp.

NRC, 1994. "Priorities for Coastal Ecosystem Science." National Academy Press, Washington, DC, 106 pp.

NRC, 2000. "Clean Coastal Waters." National Academy Press, Washington, DC. 405 pp.

Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W., Backhaus, H., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology* 178, 5636 – 5643.

Oakley, B.B., Francis, C.A., Roberts, K.J., Fuchsman, C.A., Srinivasan, S., Staley, J.T., 2007. Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. *Environmental Microbiology* 9, 118 – 130.

Odum, E.P., 1971. "Fundamentals of Ecology", 3rd edition. Saunders, Philadelphia,. 574 pp.

Okano, Y., Hristova, K.R., Leutenegger, C.M., Jackson, L.E., Denison, R.F., Gebreyesus, B., Lebauer, D., Scow, K.M., 2004. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Applied and Environmental Microbiology* 70, 1008 – 1016.

Olson, P.E., Reardon, K.F., Pilon-Smits, E.A.H., 2003. Ecology of rhizosphere bioremediation. In "Phytoremediation transformation and control of contaminants" (McCutcheon, S.C., Schnoor, J.L., eds). Wiley, New York. pp. 317–353.

Pace, N.R., Stahl, D.A., Lane, D.J., Olsen, G.J., 1986. The analysis of natural microbial-populations by ribosomal-RNA sequences. *Advances in Microbial Ecology* 9, 1 – 55.

Palmborg, C., Nordgren, A., Bååth, E., 1998. Multivariate modelling of soil microbial variables in forest soil contaminated by heavy metals using wet chemical analyses and pyrolysis GC/MS. *Soil Biology & Biochemistry* 30, 345 – 357.

Patrick Jr., W.H., DeLaune, R.D., 1977. Chemical and biological redox systems affecting nutrient availability in coastal wetlands. *Geoscience and Man* 19, 131 – 137.

Philippot, L., 2002. Denitrifying genes in bacterial and Archaeal genomes. *Biochemical and Biophysical Acta* 1577, 355 – 376.

Philippot, L., Hallin, S., 2005. Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. *Current Opinion in Microbiology* 8, 234 – 239.

Piceno, Y., Lovell, C., 2000a. Stability in natural bacterial communities: I. Nutrient addition effects on rhizosphere diazotroph assemblage composition. *Microbial Ecology* 39, 32–40.

Piceno, Y.M., Lovell, C.R., 2000b. Stability in natural bacterial communities: II. Plant resource allocation effects on rhizosphere diazotroph assemblage composition. *Microbial Ecology* 39, 41–48.

Polymenakou, P.N., Bertilsson, S., Tselepidis, A., Stephanou, E.G., 2005. Links between geographic location, environmental location, environmental factors and microbial community composition in sediments of the eastern Mediterranean sea. *Microbiology Ecology* 49, 367 – 378.

Pomeroy, L.R., 1981. Status and future needs in protozoan ecology. In “Protozoan and their Role in Marine Process” (Reid, P.C., Turley, C.M., Burkhill, P.H., eds). NATO ASI Series G: Ecological Sciences, Vol. 25. Springer-Verlag, Heidelberg. pp. 475 – 492.

Porter, K.G., Feig, Y.S., 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography* 25, 943 – 948.

Postgate, J., 1987. “Nitrogen Fixation, New Studies in Biology”, 2nd edition. Edward Arnold Ltd., London.

Priemé, A., Braker, G., Tiedje, J.M., 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology* 68, 1893 – 1900.

Punshon, S., Moore, R.M., 2004. Nitrous oxide production and consumption in a eutrophic coastal embayment. *Marine Chemistry* 91 (1 - 4), 37 – 51.

Rainey, F.A., Ward-Rainey, N.L., Janssen, P.H., Hippe, H., Stackebrandt, E., 1996. *Clostridium paradoxum* DSM 7308(T) contains multiple 16S rRNA genes with heterogeneous intervening sequences. *Microbiology* 142, 2087 – 2095.

Rajendran, N., Suwa, Y., Urushigawa, Y., 1993. Distribution of phospholipid ester-linked fatty acid biomarkers for bacteria in the sediment of Ise Bay, Japan. *Marine Chemistry* 42, 39 – 56.

Reboreda, R., Caçador, I., 2007. Halophyte vegetation influences in salt marsh retention capacity for heavy metals. *Environmental Pollution* 146, 147 – 154.

Reddy, K., Patrick, W., Lindau, C., 1989. Nitrification-denitrification at the plant root-sediment interface in wetlands. *Limnology and Oceanography* 34, 1004 – 1013.

Rich, J.J., Myrold, D.D., 2004. Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. *Soil Biology & Biochemistry* 36, 1431 – 1441.

Rovira, A.D., 1965. Interactions between plant roots and soil micro-organisms. *Annual Review of Microbiology* 19, 241 – 266.

Rysgaard, S., Risgaard-Petersen, N., Sloth, N.P., 1996. Nitrification, denitrification and ammonification in sediments of two coastal lagoons in Southern France. *Hydrobiologia* 329 (1-3), 133 – 141.

Said, W.A., Lewis, D.L., 1991. Quantitative assessment of the effects of metals on microbial-degradation of organic chemicals. *Applied and Environmental Microbiology* 57, 1498 – 1503.

Sakadevan, K., Zheng, H., Bavor, H.J., 1999. Impact of heavy metals on denitrification in surface wetland sediments receiving wastewater. *Water Science Technology* 40, 349 – 355.

Santoro, A.E., Boehm, A.B., Francis, C.A., 2006. Denitrifier community composition along a nitrate and salinity gradient in a coastal aquifer. *Applied and Environmental Microbiology* 72, 2102 – 2109.

Schlesinger, W.H., 1997. "Biogeochemistry: An Analysis of Global Change", 2nd edition. Academic Press, San Diego.

Seitzinger, S.P., 1987. Nitrogen biogeochemistry in an unpolluted estuary: the importance of benthic denitrification. *Marine Ecology Progress Series* 41, 177 – 186.

Seitzinger, S.P., 1988. Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. *Limnology and Oceanography* 33 (4), 702 – 724.

Seitzinger, S.P., 1990. Denitrification in aquatic sediment. In “Denitrification in Soil and Sediment” (Revsbech, N.P., Sørensen, J., eds.). Plenum Press, New York. pp. 301 – 322.

Seitzinger, S.P., 2000. Scaling up: site-specific measurements to global-scale estimates of denitrification. In “Estuarine Science: A Synthetic Approach to Research and Practice” (Hobbie, J.E., ed.). Island Press, Washington, DC. p. 211 – 241.

Seitzinger, S.P., Nixon, S.W., 1985. Eutrophication and the rate of denitrification and N₂O production in coastal marine sediments. *Limnology and Oceanography* 30, 1332 – 1339.

Senga, Y., Mochida, K., Fukumori, R., Okamoto, N., Seike, Y., 2006. N₂O accumulation in estuarine and coastal sediments: the influence of H₂S on dissimilatory nitrate reduction. *Estuarine Coastal and Shelf Science* 67, 231 – 238.

Shapleigh, J.P., 2001. The Denitrifying Prokaryotes. In “The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community” (Dworkin, M. et al., eds.). 3rd edition, release 3.7, November 2, 2001. Springer-Verlag, New York. <http://link.springer-ny.com/link/service/books/10125/>.

Shapleigh, J.P., 2006. The denitrifying prokaryotes. In “The Prokaryotes” (Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., eds). Springer, New York, NY, USA. pp. 769 – 792.

Šimek, M., Cooper, J.E., 2002. The influence of soil pH on denitrification: progress towards the understanding of this interaction over the last 50 years. *European Journal of Soil Science* 53 (3), 345 – 354.

Smith, C.J., DeLaune, R.D., Patrick Jr., W.H., 1983. Carbon dioxide emission and carbon accumulation in coastal wetlands. *Estuarine, Coastal and Shelf Science* 17, 21 – 29.

Smith, C.J., DeLaune, R.D., Patrick Jr., W.H., 1985. Fate of riverine nitrate entering an estuary: 1. Denitrification and nitrogen burial. *Estuaries* 8(1), 15 – 21.

Smith, C.J., Nedwell, D.B., Dong, L.F., Osborn, A.M., 2007. Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. *Applied and Environmental Microbiology* 73, 3612 – 3622.

Smith, S.V., Hollibaugh, J.T., 1993. Coastal metabolism and the oceanic organic-carbon balance. *Reviews of Geophysics* 31, 75 – 89.

Sokal, R.R., Rohlf, F.J., 1995. “Biometry”, 3rd edition. WH Freeman and Company, New York, 887 pp.

Sørensen, J., 1997. The rizosphere as a habitat for soil microorganisms. In “Modern soil microbiology” (van Elsas, J.D., Trevors, J.T., Wellington, E.M.H., eds.). Marcel Dekker, Inc., New York, NY. pp. 21-45.

Sørensen, J., Tiedje, J.M., Firestone, R.B., 1980. Inhibition by sulfide of nitric and nitrous oxide reduction by denitrifying *Pseudomonas fluorescens*. *Applied and Environmental Microbiology* 39 (1), 105 – 108.

Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W., Horn, M., Daims, H., Bartol-Mavel, D., Wincker, P., Barbe, V., Fonknechten, N., Vallenet, D., Segurens, B., Schenowitz-Truong, C., Médigue, C., Collingro, A., Snel, B., Dutilh, B.E., Op den Camp, H.J., van der Drift, C., Cirpus, I., van de Pas-Schoonen, K.T., Harhangi, H.R., van Niftrik, L., Schmid, M., Keltjens, J., van de Vossenberg, J., Kartal, B., Meier, H., Frishman, D., Huynen, M.A., Mewes, H.W., Weissenbach, J., Jetten, M.S., Wagner, M., Le Paslier, D., 2006. Deciphering the evolution and metabolism of an anammox bacterium from a community genome. *Nature* 440, 790 – 794.

Sundby, B., Caetano, M., Vale, C., Gobeil, C., Luther, G., Nuzzio, D., 2005. Root-induced cycling of lead in salt marsh sediments. *Environmental Science and Technology* 39, 2080 – 2086.

Sundelin, B., Eriksson, A.-K., 2001. Mobility and bioavailability of trace metals in sulfidic coastal sediments. *Environmental Toxicology & Chemistry* 20, 748 – 756.

Suzuki, M.T., Giovannoni, S.J., 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied and Environmental Microbiology* 62, 625 – 630.

Systematics Agenda 2000, 1994. Charting the biosphere: A global initiative to discover, describe and classify the world's species. Technical report. American Society of Plant Taxonomy, Society of Systematic Biologists, and the Willi Hennig Society, New York.

Teal, J.M., Howes, B.L., 2000. Salt marshes values; retrospection from the end of the century. In “Concepts and controversies in tidal marsh ecology” (Weinstein, M.P., Kreeger, D.A., eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands. pp. 3 – 7.

- Teixeira, C., Magalhães, C., Boaventura, R.A.R., Bordalo, A.A., 2010. Potential rates and environmental controls of denitrification and nitrous oxide production in a temperate urbanized estuary. *Marine Environmental Research* 70, 336 – 342.
- ter Braak, C.J.F., Smilauer, P., 2002. *CANOCO Reference Manual and Canodraw for Windows User's Guide: Software for Canonical Community Ordination. Version 4.5.* Microcomputer Power, Ithaca, New York. 500 pp.
- Thamdrup, B., Dalsgaard, T., 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Applied and Environment Microbiology* 68, 1312 – 1318.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673 – 4680.
- Thompson, W., Paerl, H., Go, M., 1995. Seasonal patterns of nitrification and denitrification in a natural and a restored salt marsh. *Estuaries* 18, 399 – 408.
- Throbäck, I.N., Enwall, K., Jarvis, Å., Hallin, S., 2004. Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiology Ecology* 49, 401 – 417.
- Throbäck, I.N., Johansson, M., Rosenquist, M., Pell, M., Hansson, M., Hallin, S., 2007. Silver (Ag^+) reduces denitrification and induces enrichment of novel *nirK* genotypes in soil. *FEMS Microbiology Letters* 270, 189 – 194.
- Tiquia, S.M., Masson, S.A., Devol, A., 2006. Vertical distribution of nitrite reductase genes (*nirS*) in continental margin sediments of the Gulf of Mexico. *FEMS Microbiology Ecology* 58, 464 – 475.
- Tobias, C., Anderson, I.C., Canuel, E.A., Macko, S.A., 2001. Nitrogen cycling through a fringing marsh-aquifer ecotone. *Marine Ecology Progress Series* 210, 25 – 39.
- Turpeinen, R., Kairesalo, T., Häggblom, M., 2004. Microbial community structure and activity in arsenic, chromium and copper-contaminated soils. *FEMS Microbiology Ecology* 47, 39 – 50.
- Usui, T., Koike, I., Ogura, N., 2001. N_2O production, nitrification and denitrification in a estuarine sediment. *Estuarine, Coastal and Shelf Science* 52, 769 – 781.

- Valiela, I., Cole, M., 2002. Comparative evidence that salt marshes and mangroves may protect seagrass meadows from land-derived nitrogen loads. *Ecosystems* 5, 92 – 102.
- van de Graaf, A.A., Mulder, A., de Bruijn, P., Jetten, M.S.M., Robertson L.A., Kuenen, J.G., 1995. Anaerobic oxidation of ammonium is a biologically mediated process. *Applied and Environmental Microbiology* 61 (4), 1246 – 1251.
- van Griethuysen, C., Meijboom, E.W., Koelmans, A.A., 2003. Spatial variation of metals and acid volatile sulphide in floodplain lake sediment. *Environmental Toxicology & Chemistry* 22, 457 – 465.
- Wall, L.G., Tank, J.L., Royer, T.V., Bernot, M.J., 2005. Spatial and temporal variability in sediment denitrification within an agriculturally influenced reservoir. *Biogeochemistry* 76, 85 – 111.
- Wallenstein, M.D., Myrold, D.D., Firestone, M., Voytek, M., 2006. Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. *Ecological Applications* 16, 2143 – 2152.
- Wang, X.C., Zhang, Y.X., Chen, R.F., 2001. Distribution and partitioning of Polycyclic Aromatic Hydrocarbons (PAHs) in different size fractions in sediments from Boston Harbor, United States. *Marine Pollution Bulletin* 42, 1139 – 1149.
- Ward, B.B., 1992. Nitrogen cycle of the sea. In "Encyclopedia of Earth System Science" Academic Press, Inc.. Vol. 3, pp. 295 – 306.
- White, D.S., Howes, B.L., 1994. Long-term ¹⁵N-nitrogen retention in the vegetated sediments of a New England salt marsh. *Limnology and Oceanography* 39, 1878 – 1892.
- Woese, C.R., 1994. Microbiology in transition. *Proceedings of the National Academy of Sciences USA* 91 (5), 1601 – 1603.
- Wolsing, M., Priemé, A., 2004. Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of nir gene fragments. *FEMS Microbiology Ecology* 48, 261 – 271.
- Wu, Q., Zhao, X.H., Zhao, S.Y., 2006. Application of PCR-DGGE in research of bacterial diversity in drinking water. *Biomedical and Environmental Sciences* 19 (5), 371 – 374.
- Yan, T., Fields, M.W., Wu, L., Zu, Y., Tiedje, J.M., Zhou, J., 2003. Molecular diversity and characterization of nitrite reductase gene fragments (*nirK* and *nirS*) from nitrate- and uranium-contaminated groundwater. *Environmental Microbiology* 5, 13 – 24.

Ye, R.W., Averill, B.A., Tiedje, J.M., 1994 Denitrification: production and consumption of nitric oxide. *Applied and Environmental Microbiology* 60 (4), 1053 – 1058.

Zehr, J.P., Ward, B.B., 2002. Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Applied and Environmental Microbiology* 68 (3), 1015 – 1024.

Zhao, X., Yang, L., Chen, C., Xiao, L., Jiang, L., Ma, Z., Zhu, H., Yu, Z., Yin, D., 2008. Microbial diversity in lake sediments detected by PCR-DGGE. *Frontiers of Biology in China* 3 (3), 293 – 299.

Zumft, W.G. 1992. The denitrifying prokaryotes, In “The prokaryotes” (Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H., eds.). Springer, Heidelberg, Germany. p. 443 – 582.

Zumft, W.G., 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* 61 (4), 533 – 616.